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EDITORIAL OFFICE

Dipartimento di Scienze di Sanità Pubblica
Sezione di Parassitologia "Ettore Biocca"
Università di Roma "La Sapienza"
Piazzale Aldo Moro 5, I-00185 Roma, Italy
Tel ++39 06 4455780
Fax ++39 06 49914653
e-mail: mario.coluzzi@uniromal.it

PUBLISHER

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Via Centrale 89 (Lama),
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Tel ++39 075 8583860
Fax ++39 075 8610415
e-mail: infolombardo@lombardoeditore.it

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SOCIETÀ ITALIANA
DI PARASSITOLOGIA

UNIVERSITÀ DI PISA

SOCIETÀ ITALIANA DI PARASSITOLOGIA

XXV Congresso Nazionale

Letture Magistrali
Relazioni dei Simposi

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Pisa, 18-21 Giugno 2008

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The individual authors take responsability for linguistic quality of the articles and presentations.

NOTA EDITORIALE

Il Congresso Nazionale della Società Italiana di Parassitologia ritorna a Pisa dopo ventidue anni con la sua 25ª edizione, organizzato dalle componenti mediche e veterinarie presenti nel nostro Ateneo.
Esso giunge in un momento particolare della nostra Società perché se da un lato il livello della produzione scientifica è notevolmente aumentato, vedendo molti nostri colleghi firmare articoli sulle riviste internazionali piu' prestigiose, non soltanto del settore parassitologico, la componente universitaria che costituisce la maggioranza degli appartenenti vive uno stato di profonda incertezza sul destino del proprio settore disciplinare.
Problematiche emergenti quali l'aumento dei flussi migratori nella nostra Nazione, la diffusione di potenziali vettori di agenti virali (Chikungunia e dengue) infezioni opportuniste nei soggetti immunodepressi, soprattutto i trapiantati d'organo solido e di cellule staminali e l'emergere ed il ri-emegere di zoonosi parassitarie costituiscono delle sfide importanti per i Parassitologi italiani. Quest'ultimo problema di salute pubblica offre una formidabile opportunità di stretta collaborazione tra la componente medica e quella veterinaria della nostra Società.
Il Congresso si articola in quattro Simposi a cui hanno partecipato esperti italiani e stranieri del settore, dedicati a:

1) **Zoonosi protozoarie: Toxoplasmosi** patrocinato dalla Società Italiana di Malattie Infettive e Tropicali (SIMIT),
2) **Il pianeta *Malassezia:* storia, epidemiologia, clinica, diagnostica e trattamento di una zoonosi emergente**, patrocinato dalla Federazione Italiana di Micologia Umana ed Animale (FIMUA),
3) ***Aedes albopictus* in Italia: da insopportabile parassita a vettore di virus Chikungunya**
4) **Il ruolo della ricerca nella lotta alla Malaria**, sotto l'egida del'Italian malaria Network, da poco costituito.

Le sessioni scientifiche, ben 14, comprendono 87 comunicazioni orali e 119 poster sui seguenti argomenti: Biologia molecolare, Parassiti e fauna acquatica, Immunologia, Patologia e Clinica, Parassiti e fauna selvatica, Entomologia medica e veterinaria, Protozoi di interesse sanitario, Epidemiologia e diagnosi delle malattie parassitarie, Micologia, Terapia e farmacoresistenza.
In particolare quest'anno sono stati molto numerosi i contributi di argomento entomologico, sia in campo medico che veterinario.
Abbiamo comunque cercato di dare anche agli autori di poster la possibilità di intervenire brevemente, alla fine delle sessioni orali.
Dalle proposte suggerite sono scaturiti un Workshop intitolato *Pig Parasites in Italy from History to Epidemiology* ed una sessione di case reports parassitologici, illustrati da infettivologi dei vari ospedali toscani.
Questo fascicolo (Volume 50, No.1 ed il suo supplemmento) della rivista *Parassitologia*, giornale ufficiale della Società Italiana di Parassitologia, raccoglie le letture magistrali, le relazioni dei Simposi e gli *abstracts* delle comunicazioni scientifiche e dei poster.
Il fascicolo inizia con un ricordo del Prof. Lisimaco Casarosa, un Maestro della Parassitologia italiana, che viene ricordato con affetto e profonda gratitudine da chi di noi ha qualche hanno di più.
Mi è gradito ringraziare tutti coloro che, in varia maniera, hanno contribuito all'organizzazione di questo importante evento e rivolgo un caloroso benvenuto nella città della Torre, con l'augurio di un proficuo lavoro.

Pisa, Giugno 2008

Il Presidente del Comitato Organizzatore
Prof. Fabrizio Bruschi

RICORDO DEL PROF. LISIMACO CASAROSA

Con profonda e sincera commozione mi trovo oggi a commemorare il professor Lisimaco Casarosa, venuto a mancare il 27 agosto 2006 a Pisa.

La prima volta che ho incontrato il professor Casarosa risale al 1975, quando ancora studentessa seguivo il corso di Parassitologia veterinaria da Lui tenuto. Mantengo tuttora vivido il ricordo dell'interesse che sapeva suscitare nello studio di una materia che altrimenti sarebbe potuta risultare arida e nozionistica. La nostra collaborazione scientifica ha avuto inizio dopo che, nel 1980, sono risultata vincitrice di un concorso per ricercatore per l'allora settore scientifico disciplinare V32B di cui il professor Casarosa presiedeva la commissione. Da quel momento è cominciato un rapporto di profonda stima e di affetto reciproci che si è concluso solo con la Sua morte.

Nato il 22 marzo 1920 e laureatosi in Medicina Veterinaria a Pisa nel 1943, rivestì il ruolo di assistente incaricato presso l'Istituto di Anatomia nel 1945 e fu quindi nominato assistente ordinario presso la Cattedra di Patologia Generale ed Anatomia Patologica della nostra Facoltà. Dal 1948 al 1950 fu assistente incaricato e successivamente assistente ordinario nell'Istituto di Anatomia Patologica dell'Università di Pisa. Fu abilitato alla libera docenza in Patologia Generale ed Anatomia Patologica nel 1954. Dal 1948-49 fino al 1951-52 e 1952-53 fu incaricato degli insegnamenti di Parassitologia e di Ispezione degli Alimenti di Origine Animale, rispettivamente. Dopo una parentesi che lo volle a Messina richiamato dal suo maestro, il professor Bruno Romboli, e dove per alcuni anni tenne la direzione dell'Istituto di Anatomia Patologica, fece ritorno a Pisa come professore ordinario. Direttore dell'Istituto di Parassitologia dalla sua costituzione fino al 1982, quando confluì nell'attuale Dipartimento di Patologia Animale, Profilassi ed Igiene degli Alimenti, fu insignito dell'Ordine del Cherubino nel 1967. Le sue pubblicazioni hanno trattato argomenti inerenti l'anatomia e l'istologia patologica applicate alla parassitologia e quindi temi parassitologici in generale. La Parassitologia è stato il suo interesse scientifico precipuo. Egli fu appassionato cultore di questa nuova disciplina cui si dedicò con competenza ed entusiasmo. A partire dagli anni '90, in collaborazione con la sua equipe, ha promosso e coordinato ricerche che riguardavano diversi aspetti della microascaridiosi da larve di ascaridi propri degli animali selvatici, svolte presso il Dipartimento di Patologia Animale, Profilassi e Igiene degli Alimenti dell'Università di Pisa. In particolare, l'attività di ricerca del Prof. Lisimaco Casarosa in questo periodo ha riguardato:

- La descrizione morfologica di parassiti adulti e delle loro larve infestanti;
- L'efficacia terapeutica sia nei confronti di parassiti adulti che di forme larvali;
- La sensibilità di diverse specie animali alle microascaridiosi;
- Il pattern migratorio di larve ascaridiche in seguito a diverse vie di somministrazione;
- I quadri atomo-patologici macroscopici e microscopici indotti dalla migrazione di larve ascaridiche;
- La sintomatologia clinica osservabile in corso di tale migrazione;
- La possibilità di trasmissione per via verticale della microascaridiosi.

I risultati di tali ricerche, basate in gran parte su modelli sperimentali, hanno trovato diffusione su riviste scientifiche di carattere internazionale e hanno contribuito alla delucidazione del possibile ruolo che larve ascaridiche diverse da quelle classicamente coinvolte, possono avere nella sindrome da larva migrante viscerale e oculare dell'uomo e degli animali, sia domestici che selvatici. Ricordo ancora la sua estrema precisione e l'approfondimento accurato nella ricerca bibliografica e nell'archiviazione dei dati, che hanno portato alla stesura del libro "Parassitologia degli animali domestici", testo tuttora fondamentale nella didattica parassitologica e nella consultazione. Il professor Casarosa ha prestato particolare cura nell'espletamento dell'attività didattica della Parassitologia ed è ancora ricordato da generazioni di veterinari per le sue spiegazioni chiare ed esaurienti, per le sue doti di umanità, per la sua disponibilità nei confronti dei giovani ricercatori e dei suoi allievi, cui metteva a disposizione i laboratori dell'Istituto e la sua vasta esperienza scientifica.

Ma, cosa più importante, il Prof. Casarosa è stato per alcuni di noi un maestro e una figura paterna, a cui ci si poteva rivolgere per un consiglio, un parere, un aiuto, con la sicurezza di averlo sempre disponibile, paziente, attento, partecipe.

Il suo amore e il suo entusiasmo per il proprio lavoro di docente e studioso non sono mai venuti meno. Pur collocato a riposo dal 1996, continuava comunque la sua attività di ricerca e di consultazione in ambito parassitologico, con lo stesso entusiasmo e la stessa passione che lo hanno accompagnato in tutta la sua lunga e proficua carriera. Con la scomparsa del professor Lisimaco Casarosa si chiude una generazione di studiosi che tanto ha contribuito alla nascita della scuola di parassitologia italiana, da cui hanno preso l'avvio le attuali linee di ricerca. La Sua profonda umanità, la Sua vasta cultura che spaziava in campi molto diversi e distanti da quelli più strettamente professionali, la Sua onestà di uomo e di scienziato rimarranno sempre vive nel ricordo di quanti hanno avuto la fortuna di conoscerlo. Chi di noi ha strettamente collaborato con lui per molti anni, in particolare la dottoressa Marta Magi, il dottor Roberto Papini e la sottoscritta, suoi stretti collaboratori Lo ricorda con rimpianto.

Francesca Mancianti

LETTURE MAGISTRALI

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The complexity of the CD4 T-cell responses: old and new T-cell subsets

G. Del Prete

Department of Internal Medicine, University of Florence, Viale Morgagni 85 – 50134 Florence,Italy; phone +39-055-4378103, Fax 055-417289 e-mail: gdelprete@unifi.it

Abbreviations used in the text: Antigen-presenting cell, APC; cytotoxic T lymphocyte antigen-4, CTLA-4; Dendritic cells, DCs; *Helicobacter pylori* Neutrophil Activating Protein, HP-NAP; inducible costimulator ligand, ICOS-L; Interferon, IFN; Interleukin, IL; Janus kinase, JAK; major histocompatibility complex, MHC; natural killer T cells, NKT; pathogen-associated molecular patterns, PAMPs; pattern recognition receptors, PRRs; programmed death-1 ligand, PD-L1; receptor, R; signal transducers and activators of transcription, STAT; T-cell receptor, TCR; Toll-like receptor, TLR; Transforming growth factor, TGF; Tumour necrosis factor, TNF.

Abstract.The T-cell compartment of the immune system reacts to an enormous variety of antigens, including self antigens, due to its a wide repertoire of T-cell clones. Self-reactive T cells undergo a negative selection process resulting in apoptosis of T cells with high affinity for self-peptides. Self-reactive T cells escaped to negative selection are then controlled by natural T regulatory (Treg) cells. Regulation also controls excessive effector T-cell responses. Three types of effector T cells are recognized: T helper 1 (Th1) cells, which protect against intracellular bacteria; Th2 cells, which play a role against parasites; Th17 cells, which would face extracellular bacteria, but also are involved in autoimmunity. Effector T-cell polarization is determined by the complex interaction of antigen-presenting cells with naïve T cells and involves a multitude of factors, including the dominant cytokine environment, costimulatory molecules, type and load of antigen presented and signaling cascades. The decision for the immune response to go in a certain direction is based not onto one signal alone, rather onto many different elements acting synergistically, antagonistically and through feedback loops leading to activation of Th1, Th2, or Th17 responses. Both Th1 and Th2 can be suppressed by adaptive Treg cells through contact-dependent mechanisms and/or cytokines.

Key words: T-cell polarization, T-cell regulation, Th1, Th2, Th17, Treg

Introduction

Cells and products of the innate immunity, the B cell-mediated antibody responses and cytotoxic T lymphocytes are fundamental for protection from pathoges. However, T helper (Th) cells are the central elements of the effector branch of the immune system.

Naive T helper (Th) cells are activated by recognition by their T-cell receptor (TCR) of a peptide antigen in the context of MHC class II molecules of antigen-presenting cells (APCs). After activation, Th cells begin to divide, giving rise to clones of effector cells.

In the last 20 years, $CD4^+$ effector Th cells had been divided into two main functional subsets, with distinct cytokine-secretion profiles and unique functional characteristics for each type. In both mice and humans, these cells were referred to as Th1 or Th2 cells. A third subset with a mixed panel of Th1 and Th2 cytokine secretion and intermediate functional properties was referred to as Th0 (Mosmann 1986, Del Prete 1991a, 1991b). Th1 cells secrete interferon (IFN)-γ, and tumour necrosis factor (TNF)-α and TNF-β, which make these cells particularly effective in protection against intracellular pathogens, as well as in elimination of cancer cells (Kidd 2003). Th2 cells secrete interleukin (IL)-4, IL-5, IL-10 and IL-13, which up-regulate antibody production and target a number of parasites. Th2-derived IL-4 and IL-13 activate B cells to IgE production, IL-5-induces eosinophilia, and IL-3- and IL-4-stimulate mast cell proliferation and degranulation. Th2-dominated responses against common environmental allergens are responsible for allergic disorders (Romagnani 1997).

Until recently the CD4 effector responses were defined according to the so-called "Th1/Th2 paradigm". However, a third subset of effector CD4 cells, known as Th17 cells, was recently discovered. Th17 cells secrete IL-17, IL-6, IL-22 and TNF-α and seem to play a role in tissue inflammation and activation of neutrophils to face extracellular bacteria.

The existence of T suppressor (Ts) cells had been suggested in the past (Gershon 1972; Green 1983). However, since neither the cells nor their postulated soluble factors were ever characterized, the entire concept was underscored for many years. In the last decade, however, their existence has definitively been demonstrated and Ts cells have been re-named as T reg-

Correspondence: Gianfranco Del Prete
Department of Internal Medicine, University of Florence,
Viale Morgagni 85 – 50134 Florence,Italy;
Tel +39-055-4378103; Fax 055-417289,
e-mail: gdelprete@unifi.it

ulatory (Treg) cells. Treg cells devoted to control immune responses to self-antigens were defined as "natural Treg cells" including natural killer T (NKT) and $CD4^+CD25^+Foxp3^+$ T cells. NKT cells represent a distinct population of T cells showing properties of NK cells, but expressing α/β TCR, which specifically recognize glycolipids often expressed by pathogens and tumour cells (Bendelac 1997). NKT secrete large amounts of IL-4, IL-10, IFN-γ and transforming growth factor-β (TGF-β). It is generally accepted that Foxp3 is a master control gene for the development and function of natural $CD4^+CD25^+$ Tregs, and there is no doubt that $CD4^+CD25^+Foxp3^+$ T cells originate from the thymus as a distinct T cell subset (Sakaguchi 2000, Holm 2004), mainly devoted to control self-reactive T cells escaped to negative selection, thus ensuring peripheral tolerance to autoantigens and protecting from autoimmunity. However, the mechanism by which natural Tregs exert their suppressive activity is still elusive.

Mechanisms of T-cell polarization

Differentiation of naive Th cells into Th1, Th2 or Th17 effector cells occurs upon direct contact with APCs. A number of factors are involved in determining the nature of the effector phenotype that will develop, including the nature and affinity of the antigen, the type of TCR signaling, the nature of the coreceptor signals, and, more importantly, the predominant cytokine environment. Th cells respond to the products of many signaling cascades from a wide range of membrane-bound receptors, including cytokine receptors, and undergo four steps of development: (i) activation of particular cytokine genes; (ii) commitment to a certain effector phenotype (Th1, Th2, or Th17); (iii) inhibition of the opposing cytokine genes, and (iv) stabilization and potentiation of the phenotype (Grogan 2001). The developmental stages of effector T cells are mediated by different mechanisms, including control of gene expression by intracellular signaling cascades from cell-surface receptors and chromatin remodelling.

APCs initiate the first step in the development of adaptive immunity and tune the T-cell response according to the nature of the invading pathogens. Pathogen-associated molecular patterns (PAMPs) of APCs through ligation of pattern recognition receptors (PRRs) start APC activation, in particular of DCs (Janeway 2002, Kapsenberg 2003). The most common receptors involved are the Toll-like receptors (TLRs), members of the IL-1R superfamily (Akira 2001), which discriminate between different types of pathogens. Receptors on DCs bind inflammation-associated tissue-specific factors, which are characteristic for the type of tissue and the pathogen-specific response pattern of that tissue. The binding of tissue factors also activates DCs and constitutes an indirect method of pathogen-induced maturation.Tissue factors include cytokines, chemokines, eicosanoids, heat-shock proteins and necrotic cell lipids (Beg 2002). Bone marrow DCs migrate as immature cells to sites of pathogen invasion, where pathogens activate their maturation into immunostimulatory effector cells. Mature DCs migrate into lymphoid organs and provide naive T cells with antigen that stimulates specific TCRs, costimulatory molecules that prevent tolerance, and polarizing factors that determine which phenotype of effector T-cell will be produced. DC maturation results in the production of functionally different effector DC subsets that release polarizing signals (the most important are cytokines), which selectively promote the development of Th1, Th2, or Th17 cells (Reis e Sousa 2001). Mature effector DCs, which are referred to as DC1, DC2 and regulatory DC, selectively express cytokines, coreceptors and other polarizing signals that promote the development of Th1, Th2 or regulatory T cells, respectively.

The majority of TLRs mediate the development of Th1-promoting DCs (DC1), whereas most of the PRRs that mediate Th2-cell-inducing DCs (DC2) remain undefined. However, it has recently been shown that the synthetic TLR2 ligand Pam3Cys can elicit a Th2-inducing phenotype in DCs (Redecke 2004). Therefore, according to the nature of the foreign antigen, DCs determine the intensity and the functional phenotype of the immune response generated. The next step in the development of a Th1 or a Th2 immune response is the physical interaction between the different activated DCs and the naive Th cell. An immunological synapse is formed between T cells and APCs with a dynamic process occurring when a T-cell comes into physical contact with the DC (Boes 2002). The activated naive Th cell then matures into a cell able to perform Th1 or Th2 functions (Table 1). Whether a Th1 or a Th2 response is induced is determined when TCRs recognize the specific antigen peptide and induce intracellular signals, such as protein kinase C (PKC), calcium ions, nuclear factor-κB, that help generate the appropriate immune response (Constant 1997, Rogers 1999 , Noble 2000).

Other signals are required for the activation of Th cells and these are dependent on cell interactions involving coreceptor molecules on the DC and the T-cell. For example, CD40, CD80, CD86, the programmed death-1 ligand (PD-L1), programmed death-2 ligand (PD-L2) and inducible costimulator ligand (ICOS-L) are expressed on APCs and their respective binding partners CD40L, CD28/cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death-receptor 1 (PD-1), and ICOS are expressed on T cells. In order to achieve proper differentiation of the activated naive T cells, T-cell polarizing cytokines are required. For secretion of these factors to occur at the optimal time, i.e. when the DC comes into contact with the T-cell, the DC must be restimulated by the binding of CD40 to its ligand (CD40L) on the T-cell membrane (Cella 1996).

Cytokines are the most influential factors that modulate the T-cell phenotype, and their action involves intracellular signals transmitted through cytokine receptors expressed on the T-cell surface. When cytokines bind to their receptors on naive Th cells, the

Table 1. Summary of the main activities of the different CD4 T-cell subsets

T-cell subset	Protection against	Possible mechanisms
Th1[a]	Intracellular bacteria and some viruses	Macrophage activation, cytotoxic activity, increased cytoxity of NK and CD8 cells
Th2[b]	Helminth parasites	Activity of IL-4, IL-5 & IL-13, mediator release by activated mast cells and eosinophils
Th17[c]	Extracellular bacteria, some fungi	Granulocyte recruitment, chemokines
Treg[d]	Tolerance to self Control of excessive responses to non-self	Inhibition of anti-self effector responses Th3, T1R, CD4+CD25+Foxp3+ through the release of IL-10 & TGF-β or other

[a] Involved in organ-specific autoimmunity (Hashimoto's thyroiditis, atrophic gastritis, Crohn's disease, granulomatous disorders.
[b] Responsible for allergic diseases, mucus hyper-secretion induced by IL-9.
[c] Involved in chronic inflammatory and autoimmune diseases (animal models), inflammatory bowel disease, multiple sclerosis, rheumatoid and Lyme arthritis, chronic obstructive pulmonary disease, other?
[d] Compliant to autoimmunity when deficient or inactive; compliant to cancer when they inhibit tumor-specific effector T cells; responsible for immunodeficiency when hyper-active.

receptor subunits move closer together and Janus kinase and signal transducers and activators of transcription (JAK-STAT) pathways are activated and induce tyrosine phosphorylation of the cytokine receptor. The STAT proteins bind to the phosphorylated tyrosines on the receptor and also become phosphorylated. Phosphorylated STAT proteins translocate to the nucleus where they act as DNA transcription factors. The development of Th1 cells is regulated by transcription factors, such as STAT-4 and T-bet, which are different and antagonistic to those controlling the Th2 development, which are STAT-6, GATA-3 and c-maf (Szabo 2003). STAT-4 and T-bet are activated when IL-12 is produced by DCs (Hsieh 1993), often in association with IFN-γ produced by NK cells, in response to the interaction of PAMPs with TLRs present on the surface of cells of the innate immunity (Iwasaki 2004). In contrast, Th2 transcription factors are activated when IL-4 production occurs (Le Gros 1990). The source of early IL-4 production remained unknown for many years. Two possibilities are now suggested: (a) IL-4 is produced by the naïve T cell following the interaction of its Notch receptors with the Jagged ligand on DCs (Amsen 2004); (b) at least in some parasitic infections, IL-4 is produced by a still-undefined cell type (non-T/non-B, c-kit$^+$, FcεR1$^-$) in response to stimulation by IL-25/IL-17E produced by macrophages or mast cells (Fallon 2006).

Cytokine-induced Th1 polarizing signals

Th1-cell development starts with the secretion of IL-12 and type 1 IFNs (IFN-α and IFN-β) released by macrophages and DCs upon activation by intracellular pathogens (Farrar 2002). IL-12 acts in an autocrine manner to generate a positive feedback loop, producing further IL-12. The IL-12 production induces NK cells to release IFN-γ, which also reinforces the macrophage and DC production of IL-12 in another amplifying positive feedback loop. While IFN-γ, IL-12 and type-1 IFNs directly induce T cells to differentiate into Th1 cells, it is the IFN-γ from APCs and NK cells that also acts as an inhibitor of the Th2 pathway by preventing Th2 cell expansion (Murphy 2000). IFN-γ interaction with naive Th cells leads to the activation of STAT1, which then induces the expression of T-bet. T-bet production initiates the remodeling of the IFN-γ gene locus, the production of IFN-γ, the expression of the IL-12R and the stabilization of its own expression through the autocrine activity of IFN-γ (Mullen 2001). Once the IL-12R is expressed, this cytokine further reinforces the Th1 differentiation. IL-12 signalling activates STAT3, STAT4 and NF-κB to promote the production of cytokines associated with the Th1 phenotype and chromatin remodelling. The IFN-γ secreted by Th1 cells as they develop stimulates surrounding naive Th cells to polarize into Th1 cells, in a self-renewing paracrine loop (Kidd 2003). IL-12 also up-regulates IL-18R expression, and DC-derived IL-18 potentiates the functions of IL-12 at a later stage in the development of Th1 cells (Stoll 1998, Yoshimoto 1998). However, the role of IL-18 in promoting Th1 cell development is less crucial than that of IL-12 because partially redundant.

Cytokine-induced Th2 polarizing signals

The differentiation of Th2 effector cells primarily involves the action of IL-4, IL-6, IL-10 and IL-11. IL-4 induces the production of STAT6 in naive T cells, which in turn activates the expression of the zinc finger transcription factor GATA-3 (Kaplan 1996, Ouyang 1998). GATA-3 and T-bet are mutually antagonistic. When IFN-γ, IL-12 and T-bet levels are high, GATA-3 production is inhibited, whereas when IL-4 and GATA-3 levels increase, T-bet release is repressed. Both IL-4 and TCR signaling are required to up-regulate GATA-3 transcription, which induce remodeling of the Th2 cytokine gene cluster (Zheng 1997), resulting in the release of IL-3, IL-4, IL-5, IL-9, IL-10 and IL-13 and in the inhibition of the expression of the IL-12R and

therefore of the Th1 development (Farrar 2002). Another transcription factor that is specific for Th2 cells is c-MAF, which is also responsible for regulating IL-4 synthesis through the activation of the IL-4 promoter (Ho 1996). Once GATA-3 production reaches a certain threshold, its own gene expression is auto-activated, hence stabilizing the Th2 phenotype through an intrinsic positive-feedback loop (Farrar 2002). As Th2 cells mature, they produce increasing levels of IL-4, which generates a paracrine loop and induces neighboring naive T cells to develop into Th2 cells.

IL-6, another cytokine released by macrophages, mast cells and pulmonary DCs during the early stages of a Th2 response, induces the Th2 phenotype through the up-regulation of IL-4 and inhibition of STAT1 phosphorylation, thereby preventing IFN-γ gene expression (Dodge 2003, Detournay 2005). In humans, IL-11 released by myeloid cells acts directly on T cells to stimulate IL-4 and IL-5 expression, while simultaneously inhibits IFN-γ production. IL-11 also suppresses IL-12 secretion and therefore also contributes to Th2 differentiation through this indirect mechanism (Curti 2001). Activated DC2 may induce Th2 differentiation indirectly via the secretion of IL-10, which then inhibits IL-12 synthesis at mRNA level and thus the Th1 pathway (Koch 1996). IL-10 also down-regulates IL-12β2R expression (Romano 2005), suggesting that the development of the Th2 phenotype would be the default pathway, occurring spontaneously in the absence of IL-12, but this is a point of controversy (Langenkamp 2000, Maldonado-López 2001). Whether DC2 secrete other soluble factors that promote Th2 cell development, remains unknown. Mast cell degranulation and mediator release can reduce the capacity of DCs to induce Th1 cells and promote the development of IL-4-secreting T cells (Mazzoni 2006) suggesting that mast cells may have a role in the development of the antigen-specific Th2 cells in mast cell-related disorders, such as atopy. Several coreceptors are implicated in the activation and the reinforcement of the Th2 phenotype. Following Th cell activation, the costimulatory molecule ICOS is up-regulated and is retained on both effector and memory cells (Hutloff 1999). Its ligand (ICOS-L), is expressed on most APCs, including DCs, B cells, activated monocytes, fibroblasts and endothelial cells (Wassink 2004). ICOS participates in the regulation of T-cell activation by supporting the release of many cytokines (Okamoto 2004). $ICOS^{-/-}$ T cells are selectively deficient in IL-4 production (Nurieva 2005), and inhibition of ICOS activity results in the arrest of Th2 cell-mediated allergic airway responses without change of Th1-mediated IFN-γ secretion (Coyle 2000).

Regulation of Th1/Th2 responses

An impressive series of *in vitro* and *in vivo* data obtained in both experimental animals and humans, have shown that Th1 and Th2 responses are mutually regulated in a process known as re-direction or immune deviation. IL-12, IL-18, IFN-γ and IFN-α not only favour the development of Th1 cells, but also inhibit the development of Th2 cells. Likewise, a number of pathogen products or even synthetic adjuvants, which are agonists of TLRs present on DCs and/or NK cells and are able to induce the production of IL-12 and/or IFNs by cells of the innate immunity, promote the shifting of Th2 responses to the less polarized Th0, or even to the Th1 polarized profile and effector functions (Erb 1998, Klinman 2004, Mohamadzadeh 2005, Revets 2005, Amedei 2006, Filì 2006). For example, the Neutrophil Activating Protein (HP-NAP) of *Helicobacter pylori*, a TLR2 agonist, is able to redirect to Th1 allergen-specific Th2 responses (Amedei 2006) (Figure 1). Even established human Th2 responses can be shifted, at least *in vitro*, to a Th1 profile by antigen-stimulation in the presence of IL-12 (Annunziato 2001) and this phenomenon seems to be due to the IL-12-induced long-term persistence of the β2 chain of the IL-12R, which is transiently expressed by Th2 cells after TCR stimulation alone (Smits 2001). Conversely, IL-4 inhibits Th1 cell development and shifts Th1 responses to a less-polarized phenotype, even if established Th1 responses seem to be less susceptible to re-direction than Th2 responses (Ghoreschi 2003, Skapenko 2004).

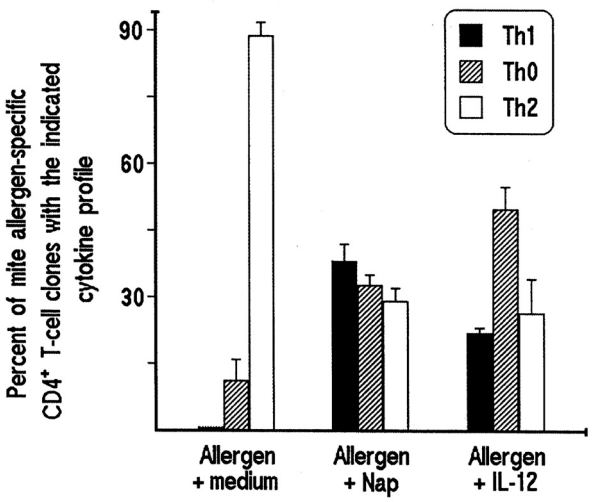

Fig. 1. Re-direction to Th1 of mite allergen-specific CD4 Th2 cells induced by *H. pylori* Neutrophil Activating Protein (HP-NAP). Mite allergen-induced T-cell lines were generated in the presence of medium alone, HP-NAP or IL-12. T-cell blasts of each line were then cloned and allergen-specific T cell clones were stimulated for 48 h with medium or the allergen in the presence of irradiated autologous APC. Culture supernatants were then collected and assayed for their IFN-γ and IL-4 content. Clones able to produce IFN-γ, but not IL-4, were categorized as Th1, clones producing IL-4 but not IFN-γ were coded as Th2, whereas clones producing both IFN-γ and IL-4 were categorized as Th0. Results represent mean percent proportions (± SD) of clones with the indicated cytokine profile obtained from series of three T-cell lines for each condition.

Chemokines have several functions and exert chemotactic activity on several cell types, including Th1 and Th2 cells (Zlotnik 2000). The selective recruitment of Th1 cells at the site of tissue inflammation usually excludes Th2 cells and *vice versa*. However, some chemokines can influence also the polarization of Th1 or Th2 responses by directly interacting with these cells or their precursors and/or favouring the production of Th1- or Th2-promoting cytokines. It has been shown that IP-10/CXCL10 promotes the production of Th1- and inhibits the production of Th2-cytokines, whereas PF-4/CXCL4 inhibits the production of Th1- and promotes the development of Th2-cytokines (Fliescher 2002; Romagnani 2005).

While cytokines or chemokines can re-direct Th1/Th2 responses to a less polarized or even to the opposite phenotype, both types of effector cells are regulated by a heterogenous family of cells referred to as adaptive Tregs. Adaptive Tregs include: i) Th3 cells that are induced by oral antigen administration and exert their suppressive activity *via* the production of TGF-β (Weiner 2001), ii) T regulatory Tr1 cells, which are induced in the presence of IL-10 and exert their suppressive activity through IL-10 production (Roncarolo 2001), and (iii) a subset of $CD4^+CD25^-$ cells that under certain condition can become $CD4^+CD25^+$ and acquire the expression of Foxp3 (Chen 2003, Walker 2003). Neither Th3 nor Tr1 cells express Foxp3 and their suppressive effect is mediated by their TGF-β and IL-10 on both Th1 and Th2 responses through a MHC- and antigen-unrestricted mechanism (Vieira 2004). The functional mechanisms and even the origin of adaptive $CD4^+CD25^+Foxp3^+$ cells are still unclear. TGF-β1 can convert $CD4^+CD25^-$ T cells into Tregs *in vitro* (Chen 2003, Walker 2003, Fantini 2004, Zheng 2004).

The Th17 lineage

In recent years it has become evident that the diversity of CD4 effector T-cell responses has been underestimated. A lineage of IL-17-producing CD4 T helper (Th17) cells, which are distinct from Th1 and Th2 cells, was recently discovered and shown to be crucial in autoimmune diseases and defence against extracellular bacteria (Murphy 2003, Nakae 2003, Harrington 2005, Langrish 2005, Park 2005). Th17 cells represent a distinct subset of effector T cells induced as a consequence of IL-23 production by DCs (Aggarwal 2003). IL-23 is a heterodimer that shares the p40 chain with IL-12, but differs in the presence of a p19 instead of the p35 chain. Similar subunits sharing occurs for the IL-12R and the IL-23R: the IL-12R is a heterodimer composed of β1 and β2 chains, whereas the IL-23R contains the β1 chain but in combination with a specific receptor known as IL-23R (Kolls 2004). Th17 cells produce IL-17 or IL-17A and IL-17F, two of several members of the IL-17 family (Tato 2006), as well as IL-22 (a member of the IL-10 family) (Liang 2006). These cytokines take part in inflammation by stimulating fibroblasts, endothelial cells, epithelial cells and macrophages to produce chemokines, as well as granulocyte- and granulocyte-macrophage colony-stimulating factors (G-CSF, GM-CSF), with recruitment of polymorphonuclear leucocytes (Ye 2001) that may play an important role in the protection against extracellular bacteria (Cua 2003). In some conditions, however, IL-17-induced inflammation is dominated by macrophages, with production of IL-1, IL-6, metalloproteinase (MMPP3) and inducible nitric oxide synthase (NOS2) (Park 2005) (Table 2). Th17 cells represent a distinct subset which does not express T-bet, GATA-3, or Foxp3 that are characteristic of Th1, Th2 and Treg cells, respectively, but the transcription factors involved in their development are still unknown (Dong 2006). Th17 cells are predominantly found in the lung and gut mucosa, suggesting a homeostatic role in these tissues (Kryczek 2007). Both *in vitro* differentiation of Th17 cells and *in vivo* Th17-mediated inflammation are dependent on the transcription factor retinoic acid receptor-related orphan receptor γ-t (RORγt) (Ivanov 2006). The generation of Th17 cells is inhibited by IL-4 and IFN-γ, possibly via down-regulation of the IL-23R (Iwakura 2006). Furthermore, IL-2, IL-25 and IL-27 play a role in abrogating Th17 cell development and in the suppression of inflammation in murine models of autoimmune disease; however, their

Table 2. Induction and effects of CD4 Th17 cells

Inducing elements	Target cells	Main effects
Klebsiella pneumoniae, Bacterioides fragilis, Borrelia burgdorferi, Bordetella pertussis, Candida albicans	Dendritic cells	Production of IL-23, IL-6, TGF-β
DCs + antigens + IL-23, IL-6, TGF-β	Naïve T cell	Differentiation into Th17 cells, production of IL-17A, IL-17F, IL-22, IL-6, TNF-α
IL-17, IL-6, IL-22, TNF-α	Endothelial cells, Fibroblasts, Epithelial cells, Macrophages	Production of IL-1, IL-6, TNF-α, G-CSF, GM-CSF, MMPP3, NOS-2, CXCL1, CXCL2, CXCL5, CCL2, CCL5
Cytokines & chemokines	Granulocytes, Macrophages	Granulocyte recruitment, chronic inflammation, autoimmunity

mechanisms of action remain undefined (Batten 2006, Kleinschek 2007). More recently, a functional antagonism between Th17 and Foxp3$^+$ Treg cells has been reported (Bettelli 2006). Th17 seems to originate not only as a consequence of the production of IL-23 by DCs, but mainly because of the combined activity of IL-6 and TGF-β. Since TGF-β is also involved in the generation of Treg cells (Chen 2003, Walker 2003, Vieira 2004, Allan 2005), the fact that IL-6 inhibits their development suggests the existence of a dichotomy in the generation of pathogenic Th17 cells that can induce autoimmunity and that of Foxp3$^+$ Treg cells that inhibit autoimmunity. Intrestingly, both IL-4 and IFN-γ inhibit the development of Th17 cells (Iwakura 2006) (Figure 2), whereas it is still unclear whether Th17 cells exert any inhibitory effect on the development of Th1 and Th2 cells. Likewise, the precise effects of Tregs on Th17 cells are as yet unknown.

In conclusion, the development of a polarized Th cell from a naïve T-cell is a complex process involving stimulation of TLRs, and activation and maturation of DCs, which leads to TCR engagement and cytokine release that triggers distinct signaling cascades. The phenotype of the T-cell that is generated is influenced by the tissue microenvironment, the dominant cytokines, costimulatory molecules and the nature and dose of antigen presented. In any case, it is becoming clear that the pathway of both effector and regulatory activities is much more complex and tightly regulated than so far thought.

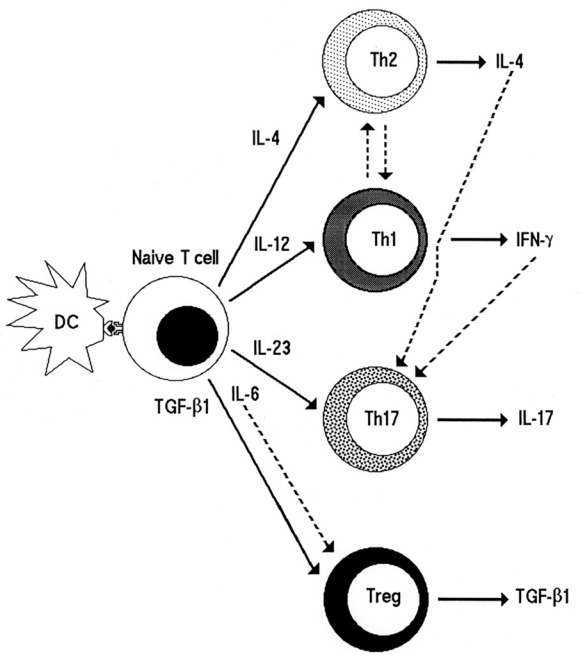

Fig. 2. The complexity of the effector and regulatory network of the CD4 T-cells. The availability of IL-4 in the absence of IL-12 or IFN-γ results in the preferential development of Th2 effector cells, whereas the production of IL-12 by DCs favours the differentiation of antigen-activated naïve T cells into Th1 effectors. Th1 and Th2 cells differentiation tends to be mutually exclusive through the secretion of IFN-γ and IL-4, respectively. If a combination of IL-23, IL-6 and TGF-β is produced by DCs, then the Th17 development is favoured. However, both Th2 and Th1 cells can inhibit Th17 cells through their secretion of IL-4 and IFN-γ, respectively. Interestingly, TGF-β is important also for the development of Treg cells, but IL-6 is inhibitory on this T-cell subset. Dashed arrows indicate inhibitory circuits.

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Epidemiology and control prospects of foodborne parasitic zoonoses in the European Union

E. Pozio

Community Reference Laboratory for Parasites, Istituto Superiore di Sanità, viale Regina Elena 299, 00161 Rome, Italy;

Abstract. In the 27 Member States of the European Union, zoonotic parasites transmitted by food are circulating with different prevalence according to the country, the environmental conditions, the human behaviour, and the socio-economic level. Foodborne parasites can be divided in two main groups according to the way of transmission to humans. These foodborne parasites reach the human beings through the consumption of raw infected food such as muscle tissues of different animal species (*Toxoplasma gondii*, *Sarcocystis hominis*, *Sarcocystis suishominis*, *Diphyllobotrium latum*, *Taenia solium, Taenia saginata, Opisthorchis felineus, Anisakis* spp., *Pseudoterranova* spp., *Trichinella* spp.), or vegetables (*Fasciola hepatica*), and contaminated food and water resources (*Giardia duodenalis*, *Cryptosporidium* spp., *T. gondii*, *Echinococcus granulosus sensu latu*, *Echinococcus multilocularis*, *T. solium*, *Taenia multiceps*). As a general role, the control strategies should be based on the education of the consumers, farmers and shepherds, the improvement of farming conditions, the improvement or the development of more sensitive methods to detect these parasites in slaughtered animals and in foodstuff, a control of sewage sludge on pastures and of drinking water resources, and the reduction of contacts between livestock and wild animals which frequently represent the most important reservoir of these pathogens.

Keywords: Foodborne parasites; zoonoses; European Union; epidemiology; control.

Introduction

In the European Union, the Health and Consumer Protection Directorate General (DG SANCO) of the European Commission and the European Food Safety Authority (EFSA) have made special efforts (including the designation of economic resources) to improve the microbiological and chemical quality of food in the European Union (European Community regulations EC 178/2002, 852/2004, 853/2004, 854/2004, 882/2004, and 2075/2005). To this end, the DG SANCO has created a network of National Reference Laboratories (NRL), co-ordinated by Community Reference Laboratories (CRL), responsible for establishing EU-wide standards for testing, routine procedures and reliable testing methods. On this purpose, 40 European laboratories have been appointed as CRLs (12 for biological risks, 13 for animal health, 13 for chemical risks, 1 for GMOs, and 1 for feed additives). In 2006, the Division of Gastroenteric and Tissue Parasitic Diseases of the *Istituto Superiore di Sanità* has been designated as the CRL for foodborne parasites (European Community EC 776/2006). The coordination of NRLs for parasites has allowed to acquire information on what is known on the epidemiology of the most important foodborne parasitic zoonoses and to know the control prospects in the Member States.

Correspondence: Edoardo Pozio,
Community Reference Laboratory for Parasites, Istituto Superiore di Sanità, viale Regina Elena 299,
00161 Rome, Italy
Tel +39 06 49902304; Fax +39 06 49903561,
e-mail: edoardo.pozio@iss.it

In the 27 Member States, there is a wide panel of foodborne parasitic zoonoses circulating between animals and humans, yet the attention spent greatly varies by individual country, depending on the impact on animal husbandry, human health and related risk perception, and to available economical resources. Herein, the epidemiology of foodborne parasites in Europe is described, followed by general indications for the control of these parasites.

Epidemiology of foodborne parasites

Giardia duodenalis

Although the zoonotic transmission of *G. duodenalis* has been described (Traub et al., 2004; Savioli et al., 2006), its role as a zoonotic agent is still under debate (Cacciò et al., in press). Furthermore, there has been relatively little interest in this pathogen because the economic loss in terms of livestock seems to be limited.

Cryptosporidium spp.

The amount of information available in Europe on *Cryptosporidium* parasites in humans and animals greatly varies by specific country. Regarding human cryptosporidiosis, in Denmark and other Nordic countries, the annual incidence is estimated to be 3,340 symptomatic cases per 100,000 population (Hörman et al., 2004). In France, among HIV-infected persons, the annual incidence of cryptosporidiosis has been reduced to 12 per 10,000 population since the introduction of highly active retroviral therapy (HAART). Among children with diarrhoea in France, the prevalence ranges from 2.1% to 5.0% (Lacroix et al., 1987; Bretagne et al., 1990). In Germany, among children aged 1-14 years, a prevalence of nearly 2.0% has been reported

(Krause et al., 1995). In Italy, there is only limited information on the prevalence of cryptosporidiosis (Giangaspero et al., 2007). In children, it has been reported to range from 1.9% to 7.2% across the country (Caprioli et al., 1989; Brandonisio et al., 1996). Since the introduction of HAART in 1995, the prevalence among HIV-positive people has decreased dramatically (Pozio and Gomez-Morales, 2005). In the Netherlands, case-control studies have reported a prevalence of 2.2 % among persons with gastroenteritis, whereas the prevalence was only 0.3% in controls (Mank et al., 1997, de Wit et al., 2001). In this country, there was a seasonal peak in the late summer. In Spain, according to the Microbiological Information System, which reports cases on a voluntary basis and covers about 25% of the Spanish population, from 1995 to 2002 there were 823 cases of cryptosporidiosis, with a yearly average of 103 cases.
With regard to *Cryptosporidium* infection in livestock in Denmark, the herd prevalence has been reported to be age-specific; it is 16% among sows, 31% among piglets, and 100% among weaners; in cattle, it is 14% among cows, 96% among young calves, and 84% for older calves (Maddox-Hytell, et al., 2006). In France, the prevalence has been reported to be 43.4% among calves (Lefay et al., 2000); in goats, it is 16-58% among very young animals, decreasing to 2.5% in older animals (Castro-Harmida et al., 2005). In Germany, a prevalence as high as 50% has been reported in young calves and lambs with diarrhoea. In five German state veterinary laboratories, *Cryptosporidium* infection was diagnosed yearly in 19-36% of bovine faecal samples (Joachim et al., 2003); in pigs, horses, dogs and cats, the reported prevalence is 1-2% (Epe et al., 2004). In Italy, a high prevalence (up to 75%) has been detected in young calves, kids, and lambs, yet only on farms with a low level of hygiene such as the backyard livestock, whereas this parasite is practically non-existent on modern industrialised farms (Giangaspero et al., 2007). In the Netherlands, a one-year study performed at a dairy farm reported a prevalence of up to 39% in calves that were 1-3 weeks old; seasonal variation was also observed, with the lowest prevalence in June (2.4%) and the highest prevalence in December (22%) (Huetink et al., 2001). Moreover, in the Netherlands, studies in veal calves showed high prevalences on herd bases from 99% herd prevalence at a age of 1-6 weeks to 70% at an age of 35 weeks (Medema and Schijven, 2001). In Spain, *C. parvum* oocysts were identified in 59% of lambs aged from 1 day to 3 months and in 7.8% of ewes older than 1 year. In pigs, the prevalence was significantly higher in weaned 1-2-month-old piglets (59.2%) than in fattening 2-6-month-old pigs (34.3%) (Quilez et al., 1996). A prevalence of 7.4% was found in dogs (Causape et al., 1996). In Sweden, *C. parvum* was the second most common pathogen in diarrhoeic calves younger than 3 months (Björkman et al., 2003).

Sarcocystis spp.
Sarcocystis spp. are very common in domestic animals, and although two species are zoonotic (i.e., *Sarcocystis hominis*, related to cattle, and *Sarcocystis suishominis*, related to pigs), the knowledge on the prevalence of these two protozoa in humans is very limited. Fewer than 10 cases of muscular sarcocystosis have been documented in Europe and fewer than 100 cases have been reported worldwide. Intestinal sarcocystosis was documented in 10.4% and 7.3% of faecal samples of asymptomatic children in Poland and Germany, respectively. In Germany, *S. hominis* has been detected in up to 63% of cattle, yet the identification of the parasite at the species level is questionable. In Germany and Austria, the prevalence of *S. suihominis* in pigs is lower than that of *S. hominis* in cattle (Fayer, 2004).

Toxoplasma gondii
This zoonotic parasite is widespread in humans, though its prevalence greatly varies by country. In the UK, it is estimated that 16-40% of the general population is infected, whereas in continental Europe the estimates range from 50 to 80% (Dubey and Beattie, 1988). In some European countries (e.g., France and Austria), testing for *T. gondii* infection is compulsory for all pregnant women, whereas in many other countries the cost-benefit ratio of such mass screening is currently being debated (Remington et al., 1995). Because the epidemiological studies on the prevalence of *T. gondii* antibodies in humans have been carried out using different tests, among different groups, and at different times, it is very difficult to compare the results. In Italy, in the 1980s, the sero-prevalence was 17.9% for paediatric ages, whereas it was as high as 48.5% in the general adult population. In pregnant women, the seroprevalence ranges from 10.9% in Norway to 67.4% in France, with a prevalence of 21.1% in Sweden, 25.7% in Spain, 40.0% in Italy, and 50.0% in Belgium (Hall, et al., 2001).
In livestock, the prevalence greatly varies depending on the animal species, age, and type of breeding, with the highest prevalence in sheep and goats, in which the parasite can cause embryonic death and resorption, foetal death and mummification, abortion, stillbirth, and neonatal death. The disease is more severe in goats than in sheep. Outbreaks of toxoplasmosis have been reported in pigs with mortality in piglets. In Poland, the seroprevalence has been found to be high in cattle (53.8%) and pigs (15%) (Sroka, 2001). In the Netherlands, the seroprevalence was very low in finishing pigs (1.8%) and in fattening calves (1.2%), whereas it was 30.9% among sows and 27.9% among dairy cattle (van Knapen et al., 1995). In Denmark, the seroprevalence was 0.5% in fattening pigs bred indoors, whereas it was higher among sows (29%) because they have outdoor access. In Portugal, the seroprevalence was 15.6% in fattening pigs with outdoor access and 27.1% in free-range poultry. In Greece, the seroprevalence in cattle and sheep grazing in the same pasture was 39.7% and 26.2%, respectively (www.iss.it/crlp/).

Diphyllobotrium latum
There are three types of epidemiological situations in Europe: countries where human infection has been documented relatively frequently; countries where spo-

radic or imported cases have been observed; and countries where this infection is unknown (Dupouy-Camet and Peduzzi, 2004). Specific diphyllobothriasis surveillance only exists in Estonia, Lithuania, and Poland. In Finland, at least 20 cases are reported each year. In Sweden, 10-50 cases are observed each year. In Estonia, between 1990 and 1997, the number of cases decreased from 715 to 440. In the region of the French, Italian, and Swiss Alps, more than 35 cases have been documented in humans. In Romania, although the historical foci of the Danube delta were subjected to massive treatment campaigns, cases continue to be reported. In Poland and Lithuania, a few cases are reported each year. Five cases were observed in Vienna between 1991 and 2003. Two cases were reported in Spain, one of which was caused by salmon imported from an unidentified country. Three cases were reported in Greece. Cases, though rare, have also been reported in Norway and the Slovak Republic. No autochthonous human infection has been reported in the other EU countries. The species of fish that are most commonly infected with *D. latum* are perch, pike, burbot, big white fish, charr, lake trout, and rainbow trout. The reservoir hosts are fish-eating wild carnivorous mammals (e.g., bears, foxes, raccoon dogs, cats, and mustelids) and domestic and stray cats and dogs.

Taenia solium
Swine-related taenia has been eradicated from almost all EU countries; however, some foci continue to be reported in Bulgaria, Latvia, Lithuania, Poland, Portugal, and Romania. In these countries, this parasite is probably circulating among free-range and backyard pigs and wild boars, yet the only available data that I managed to obtain was on the incidence of cysticercosis in Romania, where about 15 cases are diagnosed annually, most of which are neurocysticercosis (C. Cretu, personal communication). Sporadic cases of neurocysticercosis have also been documented in other EU countries, yet it is not known if transmission occurred recently or in the past. In the past ten years, nobody has succeeded in obtaining cysticerci from pigs reared in the EU, which strongly suggests that this parasite is rare in the EU, if it exists at all.

Taenia saginata
Given that taeniosis is not subject to mandatory notification, its incidence is usually estimated based on the sale of drugs for treating it. In Europe, prevalence rates between 0.01% and 10% have been reported, with the highest rates in Slovakia (Cabaret et al., 2002). However, it is very difficult to compare the prevalence rates because the studies conducted to estimate it have had very different designs.
The prevalence of bovine cysticercosis, which is usually estimated based on meat-inspection reports, ranges from 0.007 to 6.8%, with great variation among countries, regions and abattoirs (Cabaret et al., 2002). However, these data are grossly defective, since efforts on diagnostics are not identical from one site to another, and data are recorded over a long period of time. Bovine cysticercosis appears to be more common in Eastern Europe, compared to the rest of the continent. However, few EU countries report their data to the OIE, these data are rather fragmentary and reliable conclusions are difficult to make. Moreover, very few studies report the age or breeding type of infected animals. Dorny et al. (2000) has demonstrated that the seroprevalence of bovine cysticercosis is positively correlated with age, which can be explained by the fact that infection is accidental and that the risk of exposure increases with the age of the animals. The simultaneous investigation of larval and adult stages in cattle and humans, respectively, has been investigated and a good relationship between the infections in the intermediate and final hosts has been detected (Spearman coefficient = 0.81, $p < 0.05$), suggesting that attention must be paid to cattle infection and human faeces dispersion in the environment (Dorny and Praet, 2007).

Taenia multiceps
In the EU, the number of documented infections in humans is a few dozen, yet underdiagnosis exists. In Europe, *T. multiceps* coenuri have been detected in sheep in Bulgaria, France, Great Britain, Ireland, Italy, and Romania, yet it is very likely that infection is underreported in other countries, given that adult worms have been detected in canides (domestic dog and/or wolves) of Estonia, Italy, and Spain (Lloyd, 1998; Scala et al., 2007).

Echinococcus granulosus sensu latu
This is one of the most prevalent foodborne parasites in the EU, with different species/genotypes in different geographical regions of Europe. In countries of the Mediterranean basin (i.e., Bulgaria, France, Italy, Portugal, Spain, and Romania) and in Ireland and the United Kingdom, where sheep farming prevails, the genotypes G1, G2, and G3, which are considered as *E. granulosus* s.s., are prevalent. The presence of these genotypes also coincides with the high prevalence of hydatidosis in humans (Romig et al., 2006). For example, in Bulgaria, there is an average incidence of 6.3 cases per 100,000 population, with a 0.8% mortality rate, yet the incidence can reach 27.5 cases per 100,000 population in endemic areas (i.e., in Gypsy villages) (www.iss.it/crlp/). In Italy, the national level incidence is 1.3 cases per 100,000 population, yet the incidence reaches 4-8 cases per 100,000 population on the island of Sardinia. *Echinococcus equinus* (G4 genotype) is known from parts of Great Britain, Ireland, Belgium, Switzerland, Italy and Spain (Eckert and Thompson, 1988), and epidemiological evidence from Great Britain suggests that it may not be infective to humans (Thompson, 1995). *Echinococcus ortleppi* (G5 genotype) is adapted to transmission by cattle. The previous cattle-based lifecycles in central Europe are attributed to this species. Frequent records from slaughtered animals occurred until as late as the 1980s (Eckert and Thompson, 1988), yet in many regions the taxon is now considered to be extinct; in Switzerland, the Netherlands and Italy, occurrence is sporadic (Casulli et

al., 2008). A single patient in the Netherlands has been the only proven case of human cystic echinococcosis caused by this species. The pig genotype G7, considered also as *Echinococcus canadensis* with the genetically close related genotype G10 (European cervid strain), has been detected in domestic pigs from Spain, Poland, Lithuania, Slovakia, Romania, Italy, and the Ukraine (Bart et al., 2004; Romig et al., 2006; Varcasia et al., 2006). The presence of G7 is expected in most countries in Eastern and Southeastern Europe (Eckert and Thompson, 1988), where pigs are often kept by small holders and where home slaughter is a common practise. The presence of cystic echinococcosis in pigs is in itself not diagnostic, since other genotypes can develop in this intermediate host. Apart from domestic pigs, the wild boar (*Sus scrofa*) is involved in transmission of G7 in Spain and Ukraine (Romig et al., 2006) and probably elsewhere. Even where G7 is frequently found in pigs and dogs, human cases are rare, suggesting a low infectivity of this taxon for humans (Pawlowski et al., 1993). A variant of the pig strain (known as "G9") has only been found in humans of Poland (Scott et al., 1997), and the identity and host range of this genotype are still under debate. The presence of the newly described European cervid strain (G10) has been confirmed in northeastern Finland and in Sweden (Lavikainen et al., 2003). Transmission currently seems to be based on wolves (*Canis lupus*) and semi-domesticated reindeer (*Rangifer tarandus*). It is suspected that the wild forest reindeer and moose (*Alces alces*) populations are also involved in transmission, since they are the main prey of wolves in this region. Before domestic sleigh dogs were replaced by snowmobiles, the typical transmission pattern in this area was between dogs and reindeer. G10 appears also to have low infectivity for humans. In the past (when dogs were the main definitive hosts), sporadic cases were reported, yet currently no human patient is on record from the endemic area.

Echinococcus multilocularis

In Europe, this parasite is mainly transmitted in a wildlife cycle involving the red fox (*Vulpes vulpes*) and the raccoon dog (*Nyctereutes procyonoides*), as final hosts, and rodents (e.g., *Microtus arvalis*, *Arvicola terrestris*, *Ondatra zibethicus*), as intermediate hosts. Most of the parasites biomass occurs in these hosts, although domestic dogs, in particular, may play a key role in transmission to humans due to close contact (Gottstein et al., 2001). Whether or not the geographical range of *E. multilocularis* has expanded in recent years cannot be proven because of the lack of adequate retrospective studies in newly recognized endemic areas (Romig et al., 2006). Today the parasite is spread with different prevalence rates in Denmark, Belgium, France, Germany, Hungary, northern Italy, Lithuania, Poland, Slovakia, and the Netherlands (Romig et al., 2006; Casulli et al., 2005). Drastic increases of fox and raccoon dog population densities resulted in an increase of the parasite biomass. Alveolar echinococcosis in humans shows a very low prevalence level (Eckert et al., 2001). The long asymptomatic period, which varies among patients, also makes it difficult to determine the time and place of infection. The prevalence in highly endemic areas ranges from 2 to 40 per 100,000 population, with peaks of 152/100,000 in recognised risk groups of France (Eckert et al., 2001). According to a review of 210 cases from central Europe, 61.4% of patients were engaged in professional or part-time farming, gardening, or other outdoor activities, whereas 70.5% owned dogs (Kern et al., 2003). The adaptation of foxes to urban environments is known for several towns and cities with infection rates of 44% in Zurich, 43% in Geneva, 17% in Stuttgart, and 1% in Vienna. Since the prevalence of human alveolar echinococcosis can be high where people live in close contact with infected definitive hosts, the increasingly close association between fox and man in urban areas is cause for concern.

Opisthorchis felineus

This trematode has been sporadically documented in humans in Germany (Bernhard, 1985; Sanger et al., 1991) and in red foxes and cats in Germany and Poland (Schuster et al., 2003). The only other EU country in which this pathogen has been reported is Italy, where it has been detected in cats and dogs of Pisa (Rivolta, 1884), and in cats of Turin (Perroncito, 1901). Afterwards, this parasite sank into oblivion until 2003, when two cases were documented in humans for the consumption of carpaccio of a tench (*Tinca tinca*) from the Trasimeno lake (Crotti et al., 2007). In 2006 and 2007, three outbreaks involving 30 persons occurred for the consumption of carpaccio of tenches from the Trasimeno and Bolsena lakes (Central Italy) (Crotti et al., 2007; Armignacco et al., 2008). Epidemiological investigations have shown that 30-80% of tenches from the Bolsena, Bracciano, Trasimeno, and Vico lakes are infected (Bossu et al., 2008). No other fish species from these lakes harboured *O. felineus* metacercariae. Moreover, this parasite has been detected in faecal samples of stray cats from the Trasimeno and Bolsena lakes (Crotti and Crotti, 2007; Bossu et al., 2008).

Fasciola hepatica

Because fascioliasis is not subject to mandatory notification, the prevalence in humans can only be estimated based on the retrospective analysis carried out at the laboratory of hospitals or health centres. In Europe, France is considered to be an important endemic area, with more than 9,000 cases between 1950 and 1983 (Anonymous, 1988; Danis et al., 1985; Gaillet et al., 1983). Most cases were documented in the areas of Lyon, Bretagne Nord, and South West. The disease is also documented in Portugal, where more than 1,600 cases were diagnosed in the northern part of the country between 1970 and 1992 (Sampaio-Silva et al., 1996). In Spain, human fascioliasis appears to be underestimated and mainly distributed in northern regions (autonomous communities of the Pais Vasco, Castilla-Leòn, Cantabria, Navarra and Rioja) (Sorribes et al., 1990). Sporadic cases are diagnosed in many

other EU countries, yet the impact of this pathogen on human health in Europe is unknown. In animals, which are the only reservoir of this parasite in Europe, the infection is widespread in cattle, sheep and goats, yet the prevalence greatly varies by type of breeding, characteristics of the habitat, and climate.

Anisakidae

In Europe, anisakiasis mainly occurs in Western countries, where there is a higher consumption of sea fish. Approximately 2,000 cases have been documented in Europe, mostly in France, the Netherlands, and Spain (Bouree et al., 1995; Smith, 1999; Audicata et al., 2002), although dozens of cases have been also documented in Belgium, Italy, and the United Kingdom (Mattiucci et al., 2007). In the last years, there has been a marked increase in prevalence, probably because of the use of new diagnostic techniques, in particular, endoscopy. However, the increase in prevalence is probably also related to two other factors: i) the growing preference for raw or lightly cooked seafood; and ii) the increasing population size of potential definitive hosts, although this second factor should be proved with more convincing data, since the published results are contrasting. A very large number of fish and cephalopod species act as hosts for *Anisakis* spp. (200 fish and 25 cephalopod species) and *Pseudoterranova* (75 fish species in the North Atlantic only), and the global market easily allows that infected fish originating from far sea are consumed in EU few hours after fishing.

Trichinella

In Europe, the source of human trichinellosis varies by country. In the original 15 EU countries, in the past 30 years only in France, Germany, Italy and Spain, human infections for the consumption of autochthonous domestic and/or wild animals have been reported (Pozio, 2007). In the new Member States, outbreaks of trichinellosis for the consumption of local animals have occurred in Bulgaria, Estonia, Latvia, Lithuania, Poland, Romania, and the Slovak Republic (Pozio, 2007). Today in Western Europe, there are increasingly reports of trichinellosis among immigrants from Eastern countries, who acquire the infection in the country of origin. The most prevalent species is *Trichinella britovi*, which has been detected in all countries but Cyprus, Denmark, Ireland, Luxembourg, Malta, and the UK (Pozio, 2007). The second most prevalent species is *T. spiralis*, which circulates prevalently among wild boars and domestic pigs. *T. britovi* is more widespread than *T. spiralis* in sylvatic carnivores (89% vs. 11%), whereas *T. spiralis* is more widespread than *T. britovi* in both sylvatic swine (62% vs. 38%) and domestic swine (82% vs. 18%) and in rodents (75% vs. 25%). *Trichinella nativa* is restricted to carnivorous mammals of countries of the Scandinavian peninsula and Estonia, and seldom it has been documented in Latvia and Lithuania (Pozio, 2007). *Trichinella pseudospiralis* shows a sporadic distribution in sylvatic animals of Bulgaria, Denmark, France, Finland, Germany, Hungary, Italy, and Sweden, though infections have also been documented in domestic pigs and synanthropic rats of the Slovak Republic (Pozio and Murrell, 2006; www.iss.it/site/Trichinella/index.asp).

Control prospects

This brief review shows the broad spectrum of foodborne parasitic zoonoses circulating in EU countries and the prevalence of these zoonoses in humans. However, from a practical standpoint, the control strategies that have been developed in Europe are limited and only concern a few of the pathogens, whereas most of them are not subject to any form or control or there are only control regulations in some Member States. Furthermore, most of the diseases induced by these pathogens are not subject to mandatory notification or are subject to mandatory notification in some countries only.

In general, very little attention has been placed on the epidemiology of foodborne parasitic zoonoses caused by protozoa. One of the main causes of this indifference is probably the lack of an inexpensive, rapid, and simple method for detecting animals infected with these pathogens which can be used at the slaughterhouse.

None of the zoonotic protozoa is subject to controls in livestock, at the farm or slaughterhouse, or in derived food products during their processing. Only in some EU countries (e.g., Denmark, Germany, the Netherlands, Sweden, and the UK), the detection of *Cryptosporidium* sp. in humans or in water resources should be notified to regional health authorities, yet never if the parasite is detected in animals. However, there has been increasing interest in *T. gondii*, because of its pathogenicity to both humans and animals and for the economic loss resulting from abortions in sheep, goats and pigs. To this regard, initial attempts have been made to produce *Toxoplasma*-free pigs (e.g., in Denmark and the Netherlands), although the type of pigsty that should be built to avoid contamination of the pigsty environment with *T. gondii* oocysts shed by cats entails breeding the pigs indoors for the entire breeding period, which is in contrast with the new regulations on animal welfare, which require that pigs have outdoor access.

The control of zoonotic parasitic infections in both saltwater and freshwater fish only consists of visually examining few fish from each stock at the fish market by the veterinary services. Only in few cases, the muscle tissues of fish are tested by candling. Evidently, this control approach cannot prevent infected fish from reaching the consumer. Thus preventive measures can only be based on consumer education and to appropriately frozen fish which should be consumed raw (at least -20 in the core of the fish product for at least 52 hours); smoking and marination are insufficient. Dry salting can be successfully used, providing that the salt reaches all of the edible parts of the fish in concentrated form, but it is difficult to establish a protocol.

In livestock, most of the zoonotic parasites (e.g.,

Taenia spp., *Echinococcus granulosus*, *Fasciola hepatica*) are detected only by veterinary visual inspection at the slaughterhouse. The only pathogens that are searched for using a specific test (digestion) are nematodes belonging to the genus *Trichinella*, for which there exists a well-defined regulation for all susceptible animals used for human consumption (EC regulation 2075/2005). Unfortunately, the digestion test is one of the few diagnostic test which does not use positive and negative control samples; therefore, it is very important the validation of the test before its use in the routine.

Taenia solium has been successfully controlled in Europe through consumer education, improved pig farming, and the appropriate treatment of wastewater, and we can currently affirm that the eradication of this zoonosis is at hand. By contrast, the same success was not obtained for *T. saginata* as above reported. In most Member States, the consumption of beef *carpaccio* is very common, and the use of improperly treated wastewater for agriculture purposes is probably one of the main causes of its circulation, together with poor human hygiene, favouring the transmission of this cestode. The low sensitivity of serological tests for detecting cysticerchi in cattle prevent the control of this zoonosis. In fact, these tests can detect only infections with more than 50 cysticerchi (Dorny et al., 2000), which are quite unusual in low endemic regions, such as those in Europe.

The circulation of *Taenia multiceps* and *Echinococcus gralulosus* is almost exclusively due to the low socio-economic level of shepherds, who continue to illegally slaughter animals in the field. As long as the rearing of sheep and goats will be a prerogative of people with a low socio-economic level, all efforts to control these parasites will fail. The best control strategies will be the education of shepherds and the need for authorization to rear animals based on objective evaluations.

In most EU countries, improvements in pig-breeding practices have resulted in the eradication of *Trichinella* spp. infections in pigs reared on industrial farms. However, these parasites continue to circulate among backyard and free-ranging pigs reared in poor sanitary conditions, which survive in areas where there is a low socio-economic level. Moreover, given that all *Trichinella* species have a natural reservoir among wildlife, the eradication of these nematodes is impossible. It is extremely important to educate hunters, whose common habit of leaving animal carcasses in the field after skinning or removing and discarding the entrails increases the probability of transmission to new hosts (Pozio and Murrell, 2006).

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Artemisinins from Folklore to Modern Medicine - Transforming an Herbal Extract to Life-Saving Drugs

P.J. Weina

Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, Maryland 20910

Abstract. The history of the artemisinins from Ge Hong in China during the 4th century, to the re-discovery of the *qing hao* derivatives in the 1970s, to the explosion of artemisinin derivatives and combinations throughout the world today is a fascinating story. The central and underappreciated role of the United States Army's 'drug company' known as the Division of Experimental Therapeutics at the Walter Reed Army Institute of Research is a story worth relating. From being the first group outside China to extract the active component of *qing hao*, to leading the work on neurotoxicity of the class in animals, to bringing a Good Manufacturing Practices intravenous formulation to the worldwide market is traced.

Keywords: Malaria, Artemisinin, Artesunate, *Qing hao.*

The Walter Reed Army Institute of Research (WRAIR) has a group within it called the Division of Experimental Therapeutics. This small of group of physicians and scientists comprise one of the most influential, successful, but relatively unknown anti-malarial drug programs in the world. Since World War II, this group has filed over sixty Investigational New Drug Applications with the U.S. Food and Drug Administration for antimalarials and have had a hand in virtually every antimalarial licensed in the developed world. And yet, with all of this history, most of the older researchers and military officers at the WRAIR can still remember the first time they heard about artemisinin. At the time, few people knew it by this name though. Artemisinin was the name used by only a very few scientists and the name used by most researchers "*Qing hao su*", had a certain exotic and lyrical ring to it that caught everyone's attention. Not only its name, but also the remarkable promise of the activity of this new compound captured people's attention like few other compounds had done in many, many years. The WRAIR always had teams looking for new leads in the field and the WRAIR teams quickly learned of the evolving miracle from the Far East. Dr. Wilbur Milhous, considered by many at the WRAIR as the "village elder" for the Division of Experimental Therapeutics, recalls, "It was 1982 and I was a fellow at Burroughs Wellcome. We received a report from the WHO (World Health Organization) malaria steering group in Geneva saying there was a promising new Chinese plant called *qing hao*." Rumors of a miraculous new Chinese cure for malaria had been circulating for years in the very small circle of 'science geeks' who specialized in the search for new chemotherapeutic drugs for malaria at the time. The Chinese had first published their findings in the Chinese Medical Journal in 1979, but being in Chinese and having limited distribution before the days of the internet explosion, only a few people outside of China knew of their work. When discovered by scientists at the WHO, Chinese scientists were approached for samples of the plant so they could conduct their own assays, but they were rebuffed. Clearly in retrospect, it can appreciated that as this was just after the Nixon era and Mao Tse-tung was still in power, the Chinese were very skeptical about sharing information with the outside world. Their greatest fear was that it would be utilized by large western commercial pharmaceutical companies for monetary gain.

Ironically, *qing hao* had been known to Chinese herbalists for more than 2,000 years. Although there is some confusion regarding the difference between *Artemisia annua* L. and *A. apaicea,* known in the 1st century BC as *cao hao* (or herbaceous hao) and in the 2nd century BC as *qing hao* (or blue-green hao), both were in common use in Chinese herbal medicine and both contain the antimalarial substance artemisinin. Elisabeth Hsu in the Transactions of the Royal Society of Tropical Medicine and Hygiene describes and dissects the sometimes confusing differentiation between the two plants and their use in the Chinese *materia medica*. This relatively common weed found in many parts of the world is better known to gardeners by its common name, sweet wormwood. Like cinchona, and many other members of the Compositae family to which it is related (sagebrush, tarragon, absinth), sweet wormwood is particularly noted and prized for its aromatic bitterness. For as long as this herb has been known and used though, its soon to be central role in antimalarial usage was only truly appreciated at the height of the Cultural Revolution when Mao ordered his scientists to solve the problem of malaria that was spreading through China's southern provinces and ravishing the Vietnamese military. Even though well known to people like Ge Hong, who in the 4th century was the first to write about recommending the drug *qing hao* for the

Correspondence: Peter J. Weina
PhD, MD, FACP Colonel, Medical Corps, USA
Walter Reed Army Institute of Research
503 Robert Grant Avenue
Silver Spring, Maryland 20910
Tel: 301-319-9956; fax: 301-319-7360,
e-mail: peter.weina@amedd.army.mil

treatment of 'intermittent fevers', it is Mao and the malaria problem the Vietnamese were having that is credited with providing the primary push needed for *qing hao* to be rediscovered by the Chinese scientific community. Working diligently on the issue, by 1972 the Chinese succeeded in crystallizing the active principle – *qinghaosu*, or artemisinin – and Mao's scientists quickly moved to begin testing it on humans. Seven years later they reported their findings to the world. According to the Chinese, artemisinin cured falciparum malaria more rapidly and with less toxicity than either chloroquine or quinine, and it was even effective against strains that were chloroquine-resistant. Much of this work has been extensively documented in the literature of late and can be found in various well written and engaging writings. Only a portion of this fascinating story will be recounted below.

What is little known though is the role of the U.S. Army's Division of Experimental Therapeutics in the development of the artemisinins. The reports of artemisinin's success came as somewhat of a surprise to the U.S. military drug development community. Current drug developers within the WRAIR doubted the Chinese claims about artemisinin and resisted the idea that a natural product worked so well. Their skepticism was reminiscent in many ways of classic European physicians who knew all about the treatment of malaria but were resistant and reluctant to accept the claims of the "Jesuit bark" 300 years before. The Chinese, many of these skeptics pointed out, had made similar claims in the past, most conspicuously when they said they could cure malaria with acupuncture, only to be proven wrong later when this was clinically tested. Many believed that these studies, some dating back nearly ten years, offered proof that the Chinese could not be trusted in this case either.

What the skeptics in the U.S. Army did not know at the time was that the discovery of *qing hao* came as a direct result of the Vietnam War. Ho Chi Min asked Mao Tse-tung to assist Vietnam in its war with the Americans by providing new treatments for malaria. Unknown to most of the world, malaria was responsible for exceedingly high mortality in Vietnamese troops. By May 1969, the China Science Institute set up "Office 523" where the initial re-discovery of artemisinin was first made. It is reported that it was Zhenxing Wei, a professor of Chinese Traditional Medicine at the Research Institute of Shandon Province in China, who laid claim to having made this re-discovery. Apparently, it first appeared in a Chinese recipe book dated 168 BC as a treatment for hemorrhoids. Later it appears in *The Handbook for Emergency Treatments*, a book written by Ge Hong in 340 AD, where he recommends using it as a treatment for 'intermittent fevers', although some claim the translation is a recommendation specifically for chills and fevers. Regardless, Ge Hong in his writing instructs fever-sufferers to soak *qing hao* in one sheng (about 1 liter) of tea, squeeze out the juice and drink the remaining liquid. Following Hang's recipe, scientists prepared the concoction described and fed it to mice infected with *Plasmodium berghei*, a lethal rodent malaria parasite. Incredibly, they found that *qing hao* was as good as chloroquine and quinine at clearing the parasite and also cured chloroquine-resistant strains of the rodent malaria. Wei, with his knowledge of general traditional medicine and his previous knowledge of Hong's book describing anti-fever prescriptions, most of which contained artemisina leaves, had an advantage in finding the active compound. He had made two prior attempts during his career at trying to find these active compounds from these preparations, in 1958, and again in 1963, but failed in both of these attempts. Finally, working in Office 523, he succeeded in October of 1970 in obtaining 30mg of a pure crystalline product that was not toxic. There are detractors from the Wei claim that assert the re-discovery of the properties of *qing hao* is properly attributed to Professor Tu Youyou working at the same Office 523 in the early 1970s. They contend that it was Youyou's group that showed the efficacy of this product in mice had a cure rate in the 95-100% range. What is known though in spite of the initial re-discovery of Ge Hong's work is that in August of 1972, this extract was eventually shown to have remarkable efficacy in 21 patients suffering with malaria in Beijing.

In another part of the world, back on the campus of the WRAIR, it was Dr. Dan Klayman, Chief of the Department of Medicinal Chemistry in WRAIR's Division of Experimental Therapeutics, who decided to take a closer look at what was known of the Chinese study. The more he looked at the available data, the more his interest grew. Artemisinin was an endoperoxide, a molecule consisting of two oxygen atoms, but in a form he had never seen before in a drug. On exposure to air, endoperoxides normally become unstable and fall apart, yet the Chinese were claiming that artemisinin could be crystallized into a compound that would persist in the body long enough to destroy the parasite. China's refusal to share their techniques left others with nothing to compare to or to even attempt to reproduce their work. The Chinese refusal to assist even the WHO was understandable in retrospect given the presence of multiple WRAIR representatives on the WHO chemotherapy and malaria steering groups. Rightly or wrongly, the Chinese feared that if they handed over the formula for artemisinin, WRAIR would patent it for the benefit of "capitalist" pharmaceutical firms. Under these circumstances, Klayman really had no choice but to gather his own samples of the plant if he wished to pursue this promising lead.

Klayman recruited botanists from Experimental Therapeutics and the Smithsonian Institute in Washington to determine whether *A. annua* existed in North America. Astonishingly, they found it growing right in the back yard of the Nation's Capital, near a little town steeped in Civil War history called Harper's

Ferry along the Potomac River. The site was virtually 'just down the road' from the WRAIR headquarters within commuting distant from the main research lab. Klayman solicited the help of his colleague, Dr. Willis Reid, Chief of the Department of Parasitology in Experimental Therapeutics, who enlisted boy scouts from his Scout troop to harvest the plant. Klayman then set about the difficult process of trying to duplicate the Chinese extraction process without the benefit of any roadmap of the proper processes to use. It took Klayman almost two years, but in 1984 he cracked it, and was featured on the May cover of one of the most prestigious U.S. journals, *Science*, with his announcement that artemisinin was a poorly-water-soluble crystalline compound. He revealed in his manuscript in Science that these two oxygen atoms were like a small nuclear weapon waiting to explode if given the right trigger. This trigger turned out to be free iron, and malaria-infected red blood cells are full of this free iron. Malaria pigment, or haem, that the parasite produces from the breakdown of hemoglobin served as the perfect trigger for this bomb. When artemisinin enters the free iron-rich haem in these red blood cells, the two oxygen atom molecule falls apart violently and triggers a cascade of free radicals that are toxic to the parasite. The more free iron in a parasitized red blood cell, the more artemisinin enters the cell, and the greater the killing effect of the drug. This reaction is so rapid, and artemisinin is so explosive, Klayman speculated the parasite would have little time to recognize its structure and develop resistance.

With Klayman's discovery, work began rapidly on the various extracts of the parent artemisinin structure. Klayman's work on the lipid soluble forms of artemisinin, artemether and arteether, was rapidly followed by work within the Parasitology and Pharmacology groups at Experimental Therapeutics. This work started to verify some of the Chinese claims of efficacy of this remarkable group of drugs. Arteether was selected by the U.S. Army and the WHO for development as an intramuscular sesame oil solution principally for the emergency treatment of severe malaria. Unfortunately, the promising work and optimism about this drug class was about to change. Dr. Thomas Brewer was the principal investigator working on the toxicology of this class and whose teams started to see some disturbing results in work with rats and dogs treated with both arteether and artemether. All animals given high doses of these compounds developed a progressive neurologic defect, with eventual cardiorespiratory collapse and death in five of six animals studied. These neurologic findings included gait disturbances, loss of spinal and pain response reflexes, and prominent loss of brain stem and eye reflexes. Pathologic examination of rat brain sections showed a dose-related, region-specific pattern of injury. The microscopic examination even showed complete loss of some critical brain cell bodies in these rats, but worse for the progress in developing these drugs, the changes were also seen in a second animal model, dogs. The publication of this work in 1994 effectively silenced the work on the artemisinin drug development program for the U.S. Army.

Development of the artemisinins continued in many countries and along many fronts throughout the world despite the red flag of neurotoxicity raised by the U.S Army's program. And despite this setback, even the U.S. Army's drug development program had believers who refused to give up on this very promising group of antimalarial drugs. Chemists like Dr. A.J. Lin in Medicinal Chemistry at Experimental Therapeutics worked tirelessly to try to find other artemisinin derivatives that would not have this toxicity. It was his team's work that found water soluble extracts of artemisinin that preserved the active site endoperoxidase bridge but with some very different properties than the lipid soluble forms. The lion's share of the work with the pharmacodynamics and pharmacokinetics with virtually all of the artemisinin derivatives was done either personally or under the direction of Dr. Qigui Li. It was Dr. Li's diligent and steadfast work with the precise and exhaustive preclinical animal work for most of the artemisinin derivatives that laid the groundwork for future successful efforts in making these important compounds available to those who needed these drugs the most. It was quickly discovered that the water soluble forms, while relatively unstable compared to the lipid soluble forms, had little of the toxicity noted in arteether and artemether.

The faith of those visionaries throughout the world who did not give up on the artemisinins has since been justified many times over. Today artemisinin drugs are the main line of defense against drug-resistant malaria virtually everywhere in the world. The most rapidly acting of all is artesunate, a water-soluble derivative of artemisinin originally isolated by the Chinese. But although oral and intravenous forms of artesunate are manufactured in China and Vietnam, no Western pharmaceutical company were willing to make it. The reasons are all too predictable. "Because the Chinese isolated it first, artesunate is not patentable," says Milhous. "And without a patent no pharmaceutical firm is willing to pick up the co-development costs." Much of this has changed recently with the predominance of public-private partnerships and large grants of money from donors such as the Bill and Melinda Gates Foundation. This change has made these products available in many parts of the world, but licensure in the Western world, including the U.S. and the European Union in general, has been lagging severely behind the rest of the world. WRAIR took up the cause and concentrated first on two other derivatives: artemotil and artelinic acid, both of which were patentable. In March 2000, the Dutch company ARTECEF registered artemotil in Holland with work done by the WRAIR on this compound. The significance of the artemisinin drugs to parts of the world most heavily

affected by malaria cannot be overstated or ignored. The WHO even published its guidelines in December of 2005, for the first time stating that artemisinins are first line therapy for much of malaria. These recommendations did not take long to catch on in most of the world most severely affected by malaria. Table 1 shows the governmentally accepted first line artemisinin therapy recommendations as of 2007.

Table 1. Governmental Policy for 1st Line Artemisinin Recommendations as of 2007

	AS + AQ	AS + SP	AS + MQ	AL	DHA/Pip
Africa	Burundi, Cameroon, Congo, Côte d'Ivoire, Democratic Republic of Congo, Equatorial Guinea, Gabon, Ghana, Guinea, Liberia, Madagascar, Malawi, Mauritania, Senegal, Sao Tomé & Principe, Sierra Leone, Sudan (S), Tanzania (Zanzibar)	Mozambique, Djibouti, Somalia, South Africa (Mpumalanga), Sudan (N)		Angola, Benin, Burkina Faso, Central African Republic, Chad, Comoros, Ethiopia, Gambia, Guinea Bissau, Kenya, Mali, Namibia, Niger, Nigeria, Rwanda, Uganda, South Africa (Kwa Zulu Natal), Tanzania (Mainland), Togo, Zambia, Zimbabwe	
Asia	Indonesia	Afghanistan, India (5 Provinces), Iran, Tajikistan, Yemen	Cambodia, Malaysia, Myanmar, Thailand	Bangladesh, Bhutan, Laos, Saudi Arabia	Viet Nam
South America		Ecuador, Peru	Bolivia, Peru, Venezuela	Brazil, Guyana, Suriname	

AS – artesunate; AQ – amodiaquine; SP – sulfadoxine/pryimethamine; MQ – mefloquine; AL – artemether/lumefantrine; DHA/Pip – dihydroartemisinin/piperaquine

The U.S. military's most recent saga in the development of artemisinin compounds started in late 2000 when the drug development group at the WRAIR decided that it was time to replace the only currently licensed drug to treat severe and complicated malaria in the United States. In a committee first known as the "Severe and Complicated Malaria Working Group" and later as the "Intravenous Artesunate Integrated Product Team," there was a momentous decision to be made, and that was that quinidine must be replaced. To understand the importance of this decision, one must first understand a little about quinidine. While this drug has saved the lives of people with severe malaria in the United States over the time it has been available, it has toxicities that require admission to an intensive care unit to safely give the drug. Like its cousin quinine, quinidine has several side effects associated with its use. Quinidine happens to be "better" for the treatment of malaria, as far as how rapidly it works, but it is also much more toxic than its cousin quinine. Among the numerous side effects common to both drugs is prolongation of the "Q-T" interval. This action of the drug is why quinidine was originally licensed in the United States. It was a pro-arrhythmic drug used to cause changes in the heart's electrical impulses and correct unstable electrical rhythms in cardiology patients. The fact that quinidine was already licensed for cardiac reasons in the U.S., and the fact that it was more effective for treating malaria than even quinine, made it a perfect candidate for being the drug of choice for severe malaria. But this ease in licensing came at a price, and this price was more difficult and costly treatment of severe malaria in intensive care units.

The artemisinin compounds, especially the water soluble forms, seemed the perfect possible replacement for this potentially highly toxic drug. The WRAIR drug development group began developing artesunic acid (artesunate) and artelinic acid side-by-side to determine which drug to carry forward into intensive advanced clinical development. Artelinic acid was certainly favored by the Experimental Therapeutic team in this side-by-side comparison. Artelinic acid was developed by chemists at the WRAIR, was much more stable than artesunate, and most importantly for finding a commercial partner, it was patentable. Much of the development effort in the early days focused on artelinic acid as the "odds on favorite" in this unusual race for predominance in development to licensure. On October 7, 2002, the entire WRAIR team gathered together with a broad collection of scientists, medical professionals, and advisors from the throughout the Army's drug development program. All eyes were on artelinic acid as the most probable winner of the race between these two compounds. Certainly a quick vote of those entering the room that day would have resulted in artelinic acid as the choice for further development. Over the course of that day fifteen different presentations were given that compared the pros and cons

for each of these chemical entities. These presentations covered the spectrum of necessary features of a successful drug candidate, including chemistry, pharmacology, toxicology, efficacy, and clinical parameters. In what would later be viewed as a singular triumphant moment for the Army's drug development program, a subtle shift started happening in the audience's opinion during the course of that day and before the team left that day, the home town favorite had been voted down unanimously in favor of continuing development of artesunate over artelinic acid. Rather than bowing to pressures to protect what was the team's favored candidate, the team courageously decided to develop the drug that had the best chance of licensure and acceptance at a time when a replacement for quinidine was so urgently needed.

Within two years, the team completed the necessary preclinical work for artesunate, and by November of 2004, an Investigational New Drug Application was successfully filed with the U.S. Food and Drug Administration (FDA). This paved the way for rapid progress of this compound to be the first ever Good Clinical Practices Phase 1 trial done with Good Clinical Manufacturing (GMP) Practices Artesunate in both single dose administration and multiple dose administrations. The drug proved to be remarkably safe in these trials with normal healthy volunteers, even at levels that approached four times the anticipated dose to treat severe malaria in critically ill malaria patients. The drug was granted Orphan Drug Status due to the relatively few cases that are seen in the U.S. as well as Fast Track Status due to the pressing need to replace the rapidly dwindling availability of quinidine. Phase 2 clinical trials in Kenya and Thailand were planned, executed, and the first of these completed in Kenya confirmed the remarkable efficacy of this drug in rapidly treating malaria in sick malaria patients.

In August 2005, the South East Asian Quinine Artesunate Malaria Trial (SEAQUAMAT) Group published results of almost 1500 patients with severe malaria showing that artesunate had superiority over quinine using mortality as an endpoint for the trial. This remarkable study added fuel to the fire that quinine, and in the U.S., quinidine, should be replaced by artesunate in the treatment of severe malaria. This study lead to the WHO's guidelines at the end of 2005 and confirmed to the WRAIR development team that they had indeed made the right choice of drug candidate for development three years earlier. These results also provided encouragement for the team to redouble their efforts in getting this product to market as quickly as possible. The problem to date had been that of getting a commercial partner. This drug, as noted earlier, had little commercial interest because it was not patentable. Few pharmaceutical companies would be knocking at the door to share co-development costs with the Army in getting this product to market, much less take the chance of providing this drug over a sustained period after licensure. Luckily, there are new pharmaceutical companies emerging that specialize in niche markets such as orphan drugs. It was while the Army's program was within a year or two of approaching its goal of filing a New Drug Application with the FDA that WRAIR researchers met the Italian subsidiary in the U.S., Sigma-Tau Pharmaceuticals, an Italian subsidiary in the U.S. that specializes in rare diseases and orphan drugs. This partnership was sealed in early 2007 and should bring this critically needed product not only to the U.S. market, but also hopefully to the European and broader world market.

As noted in Table 1, there certainly are other agents already on the market, bringing artemisinins to the people who need them the most, saving lives, and several are being made to International Commission on Harmonization (ICH) standards for GMP. Most of these other artemisinin preparations are produced for the larger market out there, uncomplicated malaria, which by some estimates affects several hundred million people in the world each year. Most all of these drugs are oral formulations that can be taken before patients with malaria become so sick that they cannot tolerate oral medicines. Among these include combinations of the artemisinins and older antimalarial agents that follow the WHO's insistence that we combine the artemisinins with other agents to preserve the efficacy of this class of compounds and delay what some believe is the inevitability of resistance. The history of the artemisinins is most certainly still being written and likely will be as exciting as its long and celebrated history to date.

RELAZIONI DEI SIMPOSI

SIMPOSIO 1

ZOONOSI PROTOZOARIE: TOXOPLASMOSI

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Ocular impaiment of toxoplasmosis

E. Antoniazzi, R. Guagliano, V. Meroni, S. Pezzotta, P.E. Bianchi

Clinica Oculistica-Università di Pavia-Fondazione I.R.C.C.S. Policlinico S. Matteo; Clinica di Malattie Infettive- Università di Pavia- Fondazione I.R.C.C.S. Policlinico S. Matteo.

Abstract. The purpose of this review is to update the latest information about ocular toxoplasmosis. The infection can be congenital or acquired, but also depends about the immune condition of the patient and can affect the eye. Ocular symptoms are variable according to the age of the subject. Retinochoroiditis is the most common manifestation of toxoplasmic infection. Toxoplasmic retinochoroiditis typically affects the posterior pole, and the lesions can be solitary or multiple. Active lesions present as grey-white focus of retinal necrosis with adjacent choroiditis, vasculitis, hemorrhage and vitreitis. Anterior uveitis is a common finding. Atypical presentations include punctate outer retinitis, neuroretinitis and papillitis. Depending on the patient's age and the localization of the lesion, ocular symptoms vary usually presenting with reduced visual acuity or without symptoms.

The laboratory diagnosis of toxoplasmosis is based on detection of antibodies and T. gondii DNA using polymerase chain reaction (PCR) which fulfillis clinical findings.

Toxoplasmosis therapy includes antimicrobial drugs and corticosteroids. There are several regimens with different drug combinations including, among others, pyrimethamine, sulfadiazine, clindamycin, and trimethoprim-sulfamethoxazol.

Introduction

Toxoplasmosis in humans may be conveniently considered under four general headings:
(i) acquired
(ii) congenital
(iii) toxoplasmosis in immunocompromised host
(iv) ocular (Ryan)

Ocular toxoplasmosis in one of the most common types of infectious uveitis affecting the posterior pole worldwide. The infection is prevalent throughut the world, affecting a large proportion of young adults, who usually have no symptoms.

For many years ocular toxoplasmosis was thought to be a local reactivation in the eye of a systemic congenital infection.

Contents

Acquired toxoplasmosis

Acquired toxoplasmosis is generally a subclinical and asintomatic infection; only in 10-20% the acute infection is syntomatic. Clinically these patients have fever, lynphoadenopathy, myalgias, maculopapular skin rush and less often epatosplenomegaly and linfocitosis. In the immunocompetent host the desease is self-limited and benign. However in immunocompromised host (e.g. AIDS) a life-treatening encephalitis, pneumonitis or myocarditis may develop.

Actually is not clear if the acquired infection is only a reactivation of congenital disease or a new first infection.

Correspondence: Dr. Elena Antoniazzi
Clinica Oculistica-Università di Pavia-Fondazione I.R.C.C.S. Policlinico S. Matteo,
Tel +39 0382 503732,
e-mail: e.antoniazzi@smatteo.pv.it

Literature explain that toxoplasmic chorioretinitis is a postnatally acquired disease but clinical presentation is quite different from a congenital infection.

Usually the acquired form has the first manifestation in the 2^{nd}-4^{th} decade with or without symptoms and signs. Acquired ocular toxoplasmosis may present with primary ocular lesions in the absence of an old scar.

Atypical clinical presentation has to be expected in elder individuals or in the context of immunosuppression and immune defects.

Anyway laboratory means is necessary to do a differential diagnosis.

Congenital toxoplasmosis

Congenital toxoplasmosis results from transplacental transmission of *Toxoplasma gondii* infection. The prevalence is very different between countries.

Cronic maternal infection is not associated with congenital disease; only maternal infection acquired before or during gestation endangers the foetus.

The incidence and the severity of congenital infection vary with the time of infection (incidence in 1^{st} trimester: 15-20%; incidence in 3^{rd} trimester: 40%).

Most patients (80%) who has contracted the infection develop evidence of ocular disease in adolescence, with bilateral manifestation. When the manifestation is bilateral visual acuity is heavily compromised.

Clinical manifestation of ocular toxoplasmosis are: retinocoroiditis, hydrocephalus, mycrocephaly, cerebral calcifications, seizures and psychomotor retardation, organomegaly, rush, fever.

Bilateral retinochoroiditis is the most frequent manifestation, presenting in 80% of cases.

The most common finding are chorioretinal scars in the peripheral retina instead of the acute lesions which are predominantly in the posterior pole of the eye. The congenital form tends to be a bilateral disease with multiple satellite lesions located particularly in the macula.

Many children with congenital toxoplasmosis have retinal damage at birth and associated loss of vision. Active lesions become quiescent with treatment.

Immunocompromised host

Toxoplasmosis is a mayor cause of morbidity and mortality in immunocompromised host (AIDS, Hodgkin's disease, haematologic malignancy, collagen-vascular disorders, organ transplants).

They have retinochoroiditis by toxoplamosis with an atypical and sever necrotizing form of retinochoroiditis. Is important to define a differential diagnosis with CMV retinochoroiditis which is similar to toxoplasmic disease. Other diseases that present chorioretinitis or retinal necrosis are: tuberculosis, sarcoidosis, lues, borreliosis, viral retinal necrosis, fungal and autoimmune retinal disease (Behçet, Lupus).

Ocular toxoplasmosis

The age of the first attack of ocular toxoplasmosis is tipically on the second decade and 75% of cases occur between 10 and 35 years of age.

Ocular toxoplasmosis most often presents as a focal necrotizing retinitis. It is generally associated with a vitreitis and often a granolomatus anterior uveitis less commonly ocular infection may present as a papillitis. Tipical finding of toxoplasmic chorioretinitis include white focal lesions and intense vitreal inflammatory reaction. Montoya describes the active retinal lesion like "headlight in the fog" . Recurrent lesion are usually are recorded on the borders of the chorioretinal scars in multiple or single lesions.

The most frequent localization is the posterior pole and in particular the macula

There are three morfological variants of retinal toxoplasma:

(i) large destructive lesions
(ii) punctate inner retinal lesions
(iii) punctate outer or deep retinal lesions

The lesion s are yellowish-white, dense, elevated, sorrounded of a ring of retinal edema and associated witha severe vitreous inflammation.

Symptoms are present in over 90% of patients with active retinitis. If the acute lesion is located near a major retinal vessel, a branch retinal artery or branch retinal vein occlusion can results.

Patients complain a reduced central vision when lesion involve the fovea or posterior pole. If lesions are in the peripheral retinal patients are asymptomatic.

A minority of patients develop a foci of inflammation near the optic disc and in this case the diagnosis is very difficult if there aren't other retinal scars. The optic nerve head lesion present a white inflammatory mass associated or not with disc edema or adiacent retinal edema. There are dense visual fields defects corresponding to the site of the lesion.

Secondary complication of ocular toxoplasmosis include cataract, galaucoma, posterior sinchiae, cystoid macular edema, retinal perivasculitis and anc chorioretinal vascular anastomoses.

With long term follow up the 5 year recurrence rate is 79% and some patients may have multiple recurrences.

Diagnosis

The diagnosis of *Toxoplasma* retinitis is made on base of the appearance of characteristic lesion which fulfil the laboratory tests. Serologic test which demonstrate the presence of antibodies are: Sabin Feldman dye test, indirect fluorescent antibody test, indirect hemoagglutination test, complement fixation test and ELISA test.The interpretation of these test is often difficult. We can also compare the concentration or gammaglubulin in the acqueus and serum Actually PCR is the gold standard disgnosis base on specific IgG, IgM and IgA. The In adults differential diagnosis with sarcoidosis, syphilis, tubercolosis and viral or fungal infection is mandatory. In congenital toxoplasmosis the differential diagnosis includes congenital herpes simplex virus, CMV and foci of retinoblastoma.

Treatment

No treatment is generally given in an immunocompetent host with benign and selflimited illness. In immunocopromised hosts or in case of congenital toxoplasmosis treatment is generally given.

We can only observe small peripheral lesion without sequelae; if lesions are in the posterior pole and they are large and destructive with visual loss we usually treat them.

Drugs clinically used include pyrimethamine, sulfadiazine, trimetrthoprim-sulfametazole, clindamycin, and azithromycin. Oral corticosteroids are added to antibiotics (we can't give them alone) to minimize the damage to the ocular structures caused by inflammatory disease.

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Congenital Toxoplasmosis: The State of the Art

W. Buffolano

Aggregate Professor in Clinical Paediatrics, Heading The Regional Register and The Special Unit for Perinatal Infection, Federico II University, Naples Medical School- Italy

Abstract. A century after the first description the best preventative strategy against CT is still matter of debate. Over Europe, where prenatal and newborn screening are overspread, effectiveness/ cost ratio remains undemonstrated.

Key words: congenital toxoplasmosis, prevention strategies, health promotion, risl factors, screening, treatment

The Toxoplasmosis in Humans

Infection with the protozoan parasite *Toxoplasma gondii* occurs worldwide. Cats and other felids are the only definitive hosts in which sexual reproduction occurs to produce infective oocysts. Warm- blooded animals, including humans, are intermediate hosts that harbour tissue cysts in their bodies. Active infection is usually acquired by oral route and presumably results in lifelong parasite colonisation and specific antibodies production. Consequently, serological IgG testing offers the opportunity to measure specific individual protection and public health impact of toxoplasmosis (cross-sectional study). In general, seroprevalence displays highest in the southern America and Europe, in central America and sub-Saharian Africa, lowest in the far East. In the last decades a negative trend in infection rate has been demonstrated in many European countries and U.S.A. and attributed to improvement in general lifestyle hygiene and in food chain storage and transport, thus potentially biasing health impact evaluation (Forsgren M *et al.*, 1991).

As, with few exceptions, acute phase of toxoplasmosis in otherwise healthy humans occurs in a self- limited subclinical or mild form it can only be detected by serological screening for *T. gondii* antibodies. The most common clinical picture consists of isolated cervical or occipital lymphadenopathy staying for 4 to 6 weeks. Chorioretinitis leading to permanent visual loss in nearly 25% of patients can complicate congenital and postnatally acquired disease as a result of acute infection or reactivation, with differences in prevalence according to different settings (Jones JL *et al.*, 2006; McLeod RM *et al.*, 2006). In immunocompromised host complications such as myocarditis, polymiositis, hepatitis, or encephalitis may arise. Primary toxoplasmosis on gestation can be transmitted through the placenta to overall 30% of the foetus with serious permanent, even fatal consequences, such as death *in utero*, hydrocephalus, microcephalus, chorioretinitis, and intracranial calcifications. Non specific symptoms mimicking congenital toxoplasmosis (CT) with other pathogens, such as hepato-splenomegaly, purpura, jaundice and intra-uterine growth retardation, have been described (McAuley J *et al.*, 1994). Furthermore, over Europe preterm birth, low birth weight and small for gestational age were not confirmed significantly different in infected babies compared with uninfected, and severe generalised onset was found uncommon in a screening care setting (Freeman K *et al.*, 2005; Gras L *et al.*, 2005).

Which Prevention Strategy

Starting eighties there has been pressure for specific policy against CT (Mc Cabe R *et al.*, 1988), and different strategies have been adopted. The first approach, namely prenatal screening (i.e. monthly or 3 monthly testing of pregnant women without detectable antibodies at their first prenatal testing), was launched in Austria and France in the 1970s, and in Italy in the nineties. The second approach, namely newborn screening, was included in the New England Neonatal Screening Program, since 1988 and conducted for a limited period of time in Denmark, Sweden, Poland and Brazil. On demand, prenatal testing is over-spread in EU countries and was advocated in Finland and Norway. Effectiveness/cost ratio of prenatal screening is considered acceptable in medium-high prevalence setting (≥40% seroprevalence in childbearing age women), while in low prevalence setting newborn screening could be considered (Ades AE *et al.*, 2005). Prenatal screening is aimed to decrease incidence and onset severity of CT by early identification and treatment of infected pregnant women. Early identification of unprotected mother may allows for health education on how to avoid primary infection with *T. gondii* on gestation. The main drugs used for treatment of toxoplasmosis (spiramycine, pyrimethamine-sulphonamide combination) either on gestation, either congenital, and either symptomatic in otherwise healthy or immunocompromised patients were included in medical practice decades ago. None of these can eradicate *T. gondii* tissue cysts; as a consequence, many immunocompromised or congenitally infected patients still remain at risk of repeated ocular disease reactivation.

Correspondence: Wilma Buffolano
Tel +39 081 7462914, Fax +39 081 7463004;
e-mail: wilma.buffolano@unina.it

***Health promotion* (Primary Prevention)**

The key issue in primary prevention is how to break the chain of transmission from food. Depending on the presumed low cost, health promotion is the more attractive policy. Unfortunately, although printed material including leaflets, poster and individual and group counselling has been proposed, no proof are available of the message uptake and effectiveness (Gollub EL *et al.*, 2005). In a setting of prenatal screening, a study directly bearing on the question of primary prevention effectiveness showed differences among 3 temporal phases (the first without specific counselling, the second with instruction leaflet and full medical explanation of the risk of CT at first prenatal visit, the third with an oral reiteration of recommendations at mid-gestation), with a 63% and 92% reduction in seroconversion rate when comparing the 1st and 2nd, and the 1st and 3rd phases, respectively (Foulon *et al.*, 2000; Breugelmans M *et al.*, 2004). Unfortunately, comparison group were historical controls and during the same period the risk of *T. gondii* infection declined. In the context of a European multicentre case-control study, the proportion of women who cannot cite any risk factor ranged from 2% in Brussels to 51% in Naples (Cook AJ *et al.*, 2000). Moreover, knowledge not always leads to avoidance of exposure, as lower exposure rate was observed among women who mentioned raw meat as a risk factor but not among those who mentioned soil. The recommendations for CT prevention in pregnant women were CDC issued (2004) and recently reviewed (EUROTOXO PREVENTION Project, 2005).

Risk factors for toxoplasmosis

Identification of locally prevalent risk factors is critic for health education, and more in general for policy. Depending on lack of tests for distinguishing infection from environmentally robust stages (oocysts transmitted by soil contamination with cat faeces) from tissue stage (cysts ingested by infected meat), knowledge on the relative importance of different sources were derived from epidemiological surveys comparing risk factors distribution in infected and uninfected individuals. Unfortunately, questionnaire survey are biased by recall bias and results must be adjusted for main counfounders, such as age, education level, parity, gestational age at testing and at interviewing, making the analysis of results and conclusions quite complicated. An Italian prospective risk factor study on pregnant women found that eating cured pork or raw meat at least once a month was associated with a threefold higher risk of *T. gondii* infection (Buffolano W *et al.*, 1996). A European multicentre case-control study showed that contact with raw or undercooked meat, as well as contact with soil were independent risk factors for *T. gondii* seroconversion on gestation (Cook AJ *et al.*, 2000). The population attributable fraction demonstrated that 30-63% of seroconversions were due to the consumption of undercooked or cured beef, lamb, or other source meat products and 6-17% were a result of soil contact. None of multiple different cat exposures (specifically, having a cat or kitten at home, cleaning the litter box, and owning a cat that hunts) were found to be significant risk factors. Therefore, control of *T. gondii* infections should include provision of *T. gondii*-free meat products. The organotropism of *T. gondii* and the number of tissue cysts produced in a certain organ vary with the intermediate host species. Although *T. gondii* has never been isolated from edible beef in Europe or North America, beef has been found source of human infection in questionnaire surveys. Adulteration of beef by different cheaper meat species and non-skeletal muscle (heart, diaphragm, tongue) in grocery stores can't be excluded, especially in the case of minced meat such as in hamburger and sausages. Surprisingly, pork previously identified as a main risk factor in Norway and Italy was not reported as a route for infection in this study, possibly because the presence of tissue cyst in pork has decreased, and/ or pregnant women are most aware of this specific risk. Question arose on type of cooking, and freezing and/or curing methods safety. Sporulated oocysts are very resistant to environmental conditions and to disinfectants; however, they are killed within 1-2 min by heating to 55-60ºC and the risk of infection is reduced by deep-freezing meat (-12°C or lower) before cooking (Hill DE *et al.*, 2006) Tissue cysts are also killed by gamma irradiation at a dose of 1.0 kGy, but irradiation of meat has not been approved in the EU. Recently, high pressure processing at 300 MPa or higher has been shown to inactivate tissue cysts of *T. gondii* under laboratory conditions. Travel outside Europe, USA and Canada was also a risk factor for infection. In Cook's study (Cook AJ, 2000) no risk factor was identified in a third of the cases. Access for cat to outdoor environment, and feeding cats with leftovers or with raw viscera were shown risk factors for human infection in Mexico and Brazil (Galvan Ramirez ML *et al.*, 1999). Rain and surface water may transport infectious oocysts into drinking water supplies and irrigation waters. Climate play an indirect role in allowing the more (in the case of moist and hot climate) or less (in the case of dry and cold climate) survival of oocysts in the environment. In Brazil, drinking unfiltered water was demonstrated a risk factor (de Moura L *et al.*, 2006). The largest and best documented water associated outbreak of acute toxoplasmosis in humans occurred in 110 individuals in Vancouver, Canada, in 1995 (Aramini JJ *et al.*, 1999). *T. gondii* infection and agents thereof have to be reported by EU Member States according to their epidemiological situation (Directive 2003/99/EC); furthermore, nor humans nor animal nor food– related representative data were available on 2005. A recent questionnaire survey on programmes for the epidemiological surveillance of CT has shown 12 out of 28 responding countries did not have a surveillance system. Only four of them operate a specific surveillance (Denmark, France, Germany and Italy) (Benard A *et al.*, 2008).

Prenatal screening **(secondary prevention)**

Conversion from seronegative to IgM/ IgG positive forms a solid basis for indirect diagnosis of primary toxoplasmosis on gestation. Further testing for high or rising IgG level, low IgG avidity, positive IgA antibodies, or combinations of these tests are suggested in women displaying IgG and IgM positive at their first prenatal test. None of these tests reliably determine the timing of parasitaemia; furthermore, sequential use of highly sensitive IgM assays and methods examining IgG avidity or stage specificity in the first 12 weeks GA (gestational age) could reasonably exclude post- conceptional infection in a mother-to-be (Roberts A *et al.*, 2001). Once maternal infection is confirmed, secondary prevention is actuated up to delivery and the woman referred for amniocentesis (after 14 weeks GA) for foetal diagnosis. Polymerase Chain Reaction (PCR) is the gold standard procedure for detection of *T. gondii* DNA to avoid more invasive procedures and unjustified termination, and to inform change in treatment, such as stoppage of spiramycine and introduction of pyrimethamine-sulphonamide combination in the case of positive result, and immediate postnatal assessment and treatment. A French study of 2000 consecutive amniotic fluid samples confirmed that a positive PCR correlates with disease and that PCR is more sensitive than any other available test (Thalib L *et al.*, 2005). The test is highly specific but a negative PCR result does not rule out foetal infection. Sensitivity varies according to the gene target and GA at infection (sensitivity 64%, 95% Confidence Interval 53% to 75%). Unfortunately, the last multicentre proficiency study carried out on 33 EU laboratories displayed the need for improvement in both sensitivity and specificity, and for the development of international reference materials to help laboratories with the development and validation of their assays (Kaiser K *et al.*, 2007). In fact, the percentage of data set achieving all panel correct results was only 42.1% and in 84.2% of data sets an in- house test had been used. Transmission risk of mother to child rises steeply with GA at maternal seroconversion; it is estimated 15% at 13 weeks, 44% at 26 weeks, and 71% at 36 weeks GA, the odds of transmission increasing by 12% per week of maternal gestation at seroconversion (SYROCOT, 2007). Overall risk of clinical signs in infected child is 19%, 14% ocular lesions, 8% intracranial lesions. The odds of intracranial lesions shows a marked decreases with older GA at seroconversion whereas ocular lesions decline less significantly. Given the relationship between the risk of infection and clinical signs, the risk of giving birth to a child with clinical signs is greatest (10%) for women who seroconvert between 24 and 30 weeks GA (Gilbert R *et al.*, 2001).

There are nor European nor national guidelines on the management of seroconverters and there is therefore great variability between specialised centres in France, Austria, and Italy with regard to indications for therapeutic abortion and amniocentesis, treatment protocols or chemoprophylaxis, as well in the frequency of sonographical monitoring in the pregnant women as well as in their infants. As the use of pyrimethamine and sulphonamides rests on animal studies carried out during the 1950s, both prenatal and postnatal prophylaxis are given on the assumption that treatment is beneficial on either acute symptoms or in preventing later reactivation by killing the tachyzoites, thus limiting the number of actively dividing parasites in the lesions. Although the combination of pyrimethamine and sulphadiazine (p-s) is considered the treatment of choice, treatment regimens differs from 3 months continuous p-s in Denmark, over continuous p-s treatment for 1 year (US) to up to 2 years weekly treatment with pyrimethamine-sulphadoxine (Reims and Lyon Group, France).

Newborn Screening

Newborn screening is aimed to decrease long-term ocular sequelae by early treatment of asymptomatic infected newborn displaying positive IgM (and in some centres IgA) on Guthrie cards taken on day 3 to 5 post-partum at a cost about one-tenth as much as antenatal screening. Although attractive, practical and ethically acceptable for economic and psychological reasons in countries with low incidence, the opportunity to treat the children *in utero* is not guaranteed. IgM positivity was considered to detect 78% of infants with CT (Lebech M *et al.*, 1999). More recently, it has been demonstrated that only 52-55% of newborn with CT showed positive IgM, with variations according to trimester of pregnancy in which the mother seroconverted (29%, 34% and 71% in the first, the second, and the third trimester, respectively) (Gilbert RE *et al.*, 2007). Taking into account pre-test probability of 3, 27 and 59% in the first, second and third trimesters, respectively, a positive IgM result increases the post-test probabilities to 20% in the first trimester, 79% in the second trimester and 90% in the third trimester. A negative IgM result would reduce post-test probabilities to 2%, 20% and 32% in the first, second and third trimester. The largest changes would be a positive IgM test in the second trimester (change from 27% to 79%) and a negative test after third trimester seroconversion (change from 59% to 32%). In term of efficacy/cost of newborn screening, more than two-thirds of infants detected by IgM were born to mothers who seroconverted in the third trimester and, therefore, have a very low risk of neurological and visual impairment. A promising improvement in detection rate of newborn with CT seems possible through new not yet standardised nor fully marketed technologies, such as Western- Blot and recombinant antigens (rec-Ag). In 97% of 35 infected infants IgM were shown positive against at least one of the following recombinant antigens: MIC2, MIC3, MIC4, M2AP, AMA1, and SAG1 (Buffolano W *et al.*, 2005). Interestingly, infected infants recognized a more diverse repertoire of antigens than sera transferred over the placenta from the mothers. In fact, in 13 out of 20

infants with CT newly synthesised anti-MIC2 and SAG1 IgG, mainly of the IgG2 subtype, were demonstrated within 2 months of age, thus opening the door to an IgG-based, easy-to-perform, standardisable, marketable test for early postnatal diagnosis.

Effectiveness of secondary and tertiary prophylaxis

At the end of the second millennium, effectiveness of prenatal screening has been questioned. A retrospective European multicentre study found a 70% reduction in the relative risk of clinical signs in the first year of life in CT children born to treated mothers (the better with sulphonamide-pyrimethamine combination) versus untreated mothers with primary toxoplasmosis on gestation (Foulon W *et al.*, 1999). Furthermore, a large prospective European observational study (EMSCOT) evaluating the effect of treatment delay on transmission rate failed to confirm prenatal treatment effectiveness (with either spiramycine or sulphonamide-pyrimethamine) (Gilbert RE and Gras L, 2003). Finally, a metanalysis on an individual patient basis displayed weak evidence for an increased risk of transmission according to the later prenatal treatment was started (SYROCOT, 2007). The critically short time for starting prenatal prophylaxis (within 3 weeks) could open a new debate on the possibility of applying early prophylaxis. In fact, the principle on which secondary prevention was based is that there is a delay between maternal contamination and actual foetal transmission. The metanalytic study argued that only a prospective randomised clinical trial with a placebo arm can untie the question of secondary prophylaxis effectiveness. However, several difficulties can be faced when planning such a trial. First of all, ethical justification of the trial in countries where prenatal treatment has been prescribed for decades. Secondly, according to an estimated incidence of CT, between 1 and 10/10,000 live birth, proper sample size achievement which must involve several countries and will be extremely costly (Gilbert RE, 2000). In the US, on a large sample of 120 infants referred for CT to The National Collaborative Chicago-Based Congenital Toxoplasmosis Study and treated with 1 of 2 doses of p-s within 2 months after birth and continued for 12 months, in the group of infant without substantial neurological disease at birth the treatment resulted in normal cognitive, neurological, and auditory outcomes for all patients. In the group of infant who had moderate to severe neurological disease at birth it resulted in normal neurological and/ or cognitive outcomes for >72% of the patients, and none had sensorineural hearing loss. Ninety one percent of children without neurological disease and 64% of those with moderate or severe neurological disease at birth did not develop new eye lesions. Although uncontrolled, these outcomes were considered better than outcomes reported for untreated or treated for 1 month patients (McLeod R *et al.*, 2006). In a series of 20 infants with CT identified by screening one infant showed symptoms of disseminated infection at birth and 15-20% have healed inflammatory lesions in the brain and/ or in the eyes, which can only be detected by imaging or ophtalmoscopy (Binquet C *et al.*, 2003). In the 23 out of 79 CT cases new ocular lesions were found by 5 years of age. A parents filled questionnaire survey on development and behaviour in 3 years olds children recruited in the EMSCOT Study showed on average comparable development and behaviour in infected compared to the uninfected children and parental anxiety and concerns (Freeman K *et al.*, 2005). These conclusions have to be cautiously regarded. First of all, it must be taken into account the peculiar situation in which they have been drawn (Salt A, 2005). All the mother-child couples were enrolled in the setting of prenatal screening care. Secondly, subtle differences can be addressed only by clinicians. Thirdly, attrition bias (including lost to follow up) cannot be excluded. In fact, overall 67% of parents completed the questionnaire with differences between parents of infected versus uninfected children (80% vs. 64%). Fourthly, there were significant differences of response among centres, according to organisational attribute of the study centre, with direct involvement in follow up and access to an address register as main determinants (Salt A *et al.*, 2005). In the centre with the highest response rate of 94% and the highest organizational attribute, more than 75% of the children were admitted, regular contacts established, regular address updated and regular connections with paediatricians established while the most of the other centres were only central reference laboratories. Finally, there were not fully investigated outcomes of the screening, such as compliance with the screening programme. In the EMSCOT study on the effect of treatment delay, in which a prospective sequential sampling has been generated in each study centre, prenatal screening performed differently in France compared with Austria and Italy. In France, cases were spread all over gestational period. In Austria, and even more in Italy, the most of cases felt in the first half of the gestation, with very few cases enrolled late on gestation (Buffolano W, 2003). This distribution by GA found in the local sample may reflect local screening performance, thus introducing a selection bias for lower transmission rate and more severe onset. Additionally, the preference found in EMSCOT study centres for ultrasonography (USG), instead of CT-scan, may have introduced a selection bias causing underestimation of treatment effect. Poor reliability and accuracy of cerebral USG in detecting mild abnormalities, such as cerebral microcalcification are, has recently been demonstrated (Hintz SR *et al.*, 2007). Another unexplored issue has been outcomes measurement according to individual differences in pharmacokinetics, and compliance to treatment options. Due to bone marrow toxicity, combination treatment is to be modified or stopped in as much as 10% to 50% of infant with CT (Guerina NG *et al.*, 1994). An intriguing field of research could be to investigate the possibility to adjust treatment according to host and/or parasite-linked risk. Very recently, differ-

ences have been shown in the incidence of visual sequelae of CT between EU and South American patients (Gilbert RE *et al.*, 2008); moreover, polymorphisms at COL2A1 and ABCA4 locus have been associated with brain and ocular disease (Jamieson SE *et al.*, 2008).

The Future

Health demand for strategies against CT remains strong while determination of the proportion of the decline attributable to the current strategies remain difficult to evaluate, mainly depending on lack of appropriate unscreened control group, lack of cost figures precluding cost-effectiveness analysis and not fully explored side effects. Despite these concerns, screening remains the only way to diagnose infection in neonate with subclinical onset, in whom crippling late sequelae should be thwarted. Table 1 suggest possible actions and more research field for the next future.

Table 1. Hypothesis for more Research fields and Actions:

Prevention		More Research	Actions (countries with screening)
Primary	Health education	Assessment and improvement of adherence and efficacy	Education of pregnant women to high standard of food hygiene and personal cleanliness
			Training of healthcare providers to appropriate medical management in peer- led educational activities
			Care and after care instruction of sufferers from congenital or acquired toxoplasmosis complications
	Vaccine	Animal and human preventative or curative vaccines	
	Public Health (Food Safety)	Standardisation of tests for diagnosis in meat animals	Surveillance and monitoring on local risk factors
		Surveys on water employed in vegetable, fruits and animal- meat producing industry	
		Surveys on public drinking water for humans	
		Surveys on viable cysts in meat – store retails and on processes	Monitoring of meat products from sheep, goat and pigs processed at less than 67°C (at slaughtering and on processing)
Secondary	Diagnosis	New test for maternal infection timing and early postnatal diagnosis	Monitoring of compliance with screening programme and with treatment, of secular incidence on gestation and in newborn and of negative impact
	Treatment	Clinical Trials on impact and effects (countries without systematic screening)	National Register of CT with notification of pregnant women submitted to treatment and of children diagnosed at birth (including the obligation to long-term follow up)
		Comparative Clinical Trial on effectiveness of candidate new drugs (atovaquone), and of different regimen and schedules, including alternative regimen for intolerant to traditional combinations	Drug levels in infected pregnant women and infant according to outcomes
		Identification of genetic marker for increased risk of sequelae	Monitoring on serious side-effects of treatment
		Considering the hypothesis of a therapeutic vaccine for prophylaxis of recurrences	

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Clinical and Diagnostic Management of Toxoplasmosis in the Immunocompromised Patient

C. Contini

Section of Infectious Diseases, Department of Clinical and Experimental Medicine, University of Ferrara, via Fossato di Mortara 23, 44100 Ferrara, Italy

Abstract. With the advent of the highly active antiretroviral therapy (HAART), the natural course of HIV infection has markedly changed and opportunistic infections including toxoplasmosis have declined and modified in presentation, outcome and incidence. However, TE is a major cause of morbidity and mortality especially in resource-poor settings but also a common neurological complication in some countries despite the availability of HAART and effective prophylaxis. In most cases toxoplasmosis occurs in brain and toxoplasmic encephalitis (TE) is the most common presentation of toxoplasmosis in immunocompromised patients with or without AIDS. The need of a definitive diagnosis is substantial because other brain diseases could share similar findings. Rapid and specific diagnosis is thus crucial as early treatment may improve the clinical outcome. Classical serological diagnosis is often inconclusive as immunodeficient individuals fail to produce significant titres of specific antibodies. Polymerase chain reaction (PCR) has a high diagnostic value in the acute disease, but like many 'in-house' PCR assays, suffers from lack of standardization and variable performance according to the laboratory. Molecular diagnosis of toxoplasmosis can be improved by performing real-time PCR protocols. This article summarises the clinical manifestations, diagnostic procedures and management strategies for this condition.

Key words: toxoplasmosis, immunocompromised patients, AIDS, transplantation

Introduction and background

Toxoplasma gondii infects up to 80% of some European populations and 20% of people in the United States (Hall S *et al.*, 2001). Normally, infection is generally benign as immune system keeps the parasite in check and parasitaemia is self-limited resulting in an asymptomatic clinical form in most cases. In developing foetus and in immunocompromised individuals especially those with deficient cellular immunity, it can cause significant morbidity and mortality.

The first reports of *T. gondii* in immunocompromised patients involved persons with leukaemia and myeloma and cerebral toxoplasmosis was recognized early as a particular problem. With the advancements made in transplantation of different organs, especially of the heart and bone marrow (BMT), allogeneic haematopoietic stem cell transplants patients (HSCT) and, less often, after kidney transplants, toxoplasmosis in these subjects has become a well-recognized and serious clinical problem (Slavin MA *et al.*, 1994, Martino R *et al.*, 2005). The frequency of parasitic infections as complications of organ transplantation is unknown; however, these are much less prevalent than bacterial and viral infections. Only 5% of human pathogenic parasites have been reported to cause significant illness in heart transplant recipients (Montoya JG *et al.*, 2001).

With the advent of human immunodeficiency virus (HIV) pandemic, toxoplasmic encephalitis (TE) has become one of the most common cerebral opportunistic infection complicating the course of AIDS, often fatal, if untreated (Luft JB *et al.*, 1993; Richards F *et al.*, 1995) and the most frequent cause of focal intracerebral lesions in this group. TE is also the most common presentation of toxoplasmosis in immunocompromised patients other than AIDS (Israelski DM *et al.*, 1993).

Although the epidemiology of the central nervous system (CNS) opportunistic diseases has changed after the introduction of the highly active antiretroviral therapy (HAART), TE is still a common neurological complication in AIDS patients from Brazil, despite the availability of HAART and effective prophylaxis (Vidal JE *et al.*, 2005).

The peak incidence of TE is highest among patients between 25 and 35 years old, whereas the incidence is directly proportional to the prevalence of latent *Toxoplasma* infection which increases with age (Holliman RE *et al.*, 1990). Differences in genotypes of *T. gondii* specific strain, races and ethnicities and the mode of transmission seem also to account in influencing the occurrence of TE (Sarciron ME *et al.*, 2000; Khan A *et al.*, 2005).

Cellular immunity is one of the main mechanisms of defence in the control of toxoplasmosis. The variety of immune system defects found in individuals with AIDS, such as $CD4^+$ T lymphocyte deficiency, reduced activity of cytotoxic T and NK cells, and the low production of immunoregulatory lymphokines such as

Correspondence: Prof. Carlo Contini
MD Section of Infectious Diseases, Department of Clinical and Experimental Medicine, University of Ferrara, via Fossato di Mortara, 23, 44100 Ferrara, Italy.
Tel: +39 532 455490; Fax: +39 532 455495
e-mail: cnc@unife.it

IFN- g, may explain the high frequency of reactivated *T. gondii* infection (Fauci AS, 1984), the risk of which is increased especially when the CD4 T-cell count is less than 100/mm^3 and often accompanied by corresponding reduction in CD8 T-cells.
Patients with AIDS and recipients of bone-marrow transplants are also at risk to develop pulmonary toxoplasmosis presenting with acute respiratory failure which is associated with a high mortality. Moreover, patients with or without AIDS including those undergoing cancer chemotherapy can develop ocular toxoplasmosis, which accounts for 1-3% of ocular infections (Rodgers CA., 1996) and can be confused with other ocular diseases. In these patients, coexistent cerebral lesions, appearing as contrast enhancing on CT or MRI scans and which cause cranial nerve palsies and papilloedema may be found. Ocular toxoplasmosis also continues to be frequent in patients with CNS toxoplasmosis despite HAART.
In immunocompromised patients, the infection may disseminate rapidly with non specific symptoms like fever and malaise, and may affect a number of organs including the brain, eye, liver, lungs and skeletal muscle. Cardiac toxoplasmosis may occur during the course of multivisceral dissemination (Dixit PG *et al.*, 2007).

Clinical and Diagnostic Management of Toxoplasmosis in Patients with or without AIDS

In immunocompromised hosts and in AIDS patients, the reactivation of toxoplasmosis culminates in the conversion of the bradyzoite stage to the active and rapidly replicating tachyzoite form that may result in the often fatal tissue injury. In both group patients, the CNS is the site most typically affected by infection.

Clinical, laboratory and radiological findings

The diagnosis of CNS toxoplasmosis is difficult to make since the clinical signs of infection are often non specific. The clinical picture may show a wide range of findings including altered mental status, weakness, seizures, sensory abnormalities, cerebellar signs and neuropsychiatric manifestations. Cerebrospinal fluid (CSF) findings are normal or show non specific alterations such as lymphocytic pleocytosis and discrete CSF hyperproteinorraquia. In most cases, the diagnosis of TE in immunocompromised and AIDS patients is presumptive and mainly based on clinical presentation, computed tomography or magnetic resonance image (MRI) findings (isodense or hypodense, single or multiple lesions with a mass effect, and taking up the contrast dye in a ring-like or nodular manner in more than 90% of cases) and the presence of serum specific *T. gondii* IgG antibodies.
The need of a definitive diagnosis is substantial because other brain diseases such as lymphoma, abscess, progressive multifocal leucoencephalopathy, viral or fungal encephalitis, neurotuberculosis, could share similar clinical and computed tomographic CT scan signs. Rapid diagnosis is crucial in patients with severely impaired immune function, as early treatment may improve the clinical outcome.
The diagnosis is usually confirmed on resolution after empiric specific treatment, which generally occurs by more than 50% between 7 and 14 days (Luft BJ *et al.*, 1993). The most typically used and effective regimen is the combination of pyrimethamine/sulfadiazine and folinic acid, usually recommended for 4–6 weeks after resolution of all signs and symptoms. This should be given to patients with multiple ring enhancing brain lesions demonstrated by MRI. The combination of Atovaquone with either pyrimethamine or sulfadiazine has demonstrated to be useful for treatment of acute TE in patients with a Karnofsky performance status score (Chirgwin K *et al.*, 2002).
Definitive diagnosis of TE in immunocompromised people is mostly undertaken by direct demonstration of the parasite by brain biopsy. Direct demonstration of parasites by tissue culture or mouse inoculation, although specific, is time consuming and needs animal facilities. However, in addition to be an invasive method which is subject to complications, histopathological analysis confirms the diagnosis in only 50% of clinically diagnosed cases. Brain biopsy should be considered in immunocompromised patients with presumed CNS toxoplasmosis who have a single lesion on MRI, a negative IgG antibody serological test, or an inadequate clinical response to an optimal specific anti-*T. gondii* therapeutic regimen (Montoya JG *et al.*, 2004).
In general, the occurrence of low CD4 T-cell count (less than 150-200/mm^3) and the presence of *T. gondii* IgG antibody is generally accepted as being a good predictor of TE reactivation, although less than 6% of TE patients show negative tests (Skiest DJ *et al.*, 2002). The estimated risk of TE reactivation in AIDS patients who present *T. gondii* IgG antibodies in serum, ranges from 12 to 47% (Garly M *et al.*, 1997).
Diagnosing toxoplasmosis in transplant recipients may be often difficult because of the non-specific signs and symptoms of the infection and because immunosuppressive therapy or irradiation impairs the recipient's immune response. Routine PCR testing of peripheral blood mononuclear cells (PBMC) specimens has been shown to be an appropriate tool for guiding preventive therapy in HSTC patients at very high risk of developing invasive disease (Martino R *et al.*, 2005).
Patients with neoplastic diseases should be also periodically screened for *T. gondii* to prevent the possibility of severe toxoplasmosis.

Serological testing

Indirect serological methods widely used in immunocompetent patients, may be unreliable in immunodeficient individuals especially in allogeneic bone marrow transplant recipients and in patients with AIDS, as they fail to produce significant titres of specific antibodies.

The serological pattern found in these patients is similar to that observed for the general population with inactive infection. Since in basically all cases the disease results in the reactivation of a latent and non-acute infection, IgM antibodies are not habitually found and IgG antibodies do not discriminate between latent and active infection. Baseline *Toxoplasma* IgG detection level may however be a key component of the monitoring strategy in different patients including those with HIV-infection and transplant recipients, since it could allow to evaluate the risk of toxoplasmosis and to recommend appropriate prophylactic measures (Montoya JG *et al.*, 1992). Serological screening, in particular is helpful in identifying transplant patients at risk, especially seronegative recipients with seropositive donors, and can help in establishing the diagnosis by showing seroconversion.

IgA antibodies have shown conflicting results (Darcy F et al., 1991) whereas the determination of IgG avidity has revealed to be of no help in the diagnosis of cerebral or extracerebral toxoplasmosis in immunocompromised patients (Mechain B *et al*., 2000).

Several previous studies have demonstrated the usefulness of recombinant antigens for the serological diagnosis of *T. gondii* infection (Pfrepper KI *et al*., 2005; Beghetto E *et al*., 2006). However, the exact composition of a recombinant protein cocktail representative of the antigenic pattern present in the tachyzoite soluble extract to detect both IgG and IgM antibodies in an immunoassay, remains an open question.

Intrathecal Humoral Immune Response

The demonstration of an intrathecal antibody production based on detection of oligoclonal bands in CSF by the combination of antibody specific index (ASI) and affinity mediated immunoblot (AMI) technique has demonstrated to support diagnosis in a number of ongoing infections of the CNS (Zeman A *et al.*,1993). Reports concerning the detection of intrathecal *Toxoplasma* IgG antibodies are limited. Isolated case reports suggest an increased intrathecal production of *T. gondii* antibodies in the CNS of AIDS patients with TE (Potasman I *et al*., 1988). However, the calculation of ASI should be performed according to Reiber's formula; this may account for the accurately assessment of intrathecal antibody synthesis (Reiber H *et al*., 1991).

An intrathecal immune response (calculation of the ASI according Reiber's formula) characterized by the presence of specific *T. gondii* oligoclonal IgG bands restricted to the CSF indicative of acute disease, has been demonstrated by us in 54.5% of patients with TE (Contini C *et al.*, 1998). Determination of intrathecal synthesis of oligoclonal antibodies by AMI revealed *T. gondii* related IgG in all TE patients, compared to those without TE. By contrast, AMI failed to detect an intrathecal immune response in AIDS patients without TE, thus supporting the utility of this technique to discriminate TE from other opportunistic CNS infections in course of AIDS (Contini C *et al*., 1998).

Molecular diagnostic procedures. State of art and perspectives

Over the past decade, molecular techniques including PCR assays have allowed the sensitive detection of *T. gondii* DNA in clinical specimens. Their use is particularly appropriate for immunocompromised patients, as these techniques are not affected by the immunological status of the host.

PCR has shown to be rapid, sensitive and specific enough to be used as a front-line test for the detection of CSF *Toxoplasma gondii* DNA, thus avoiding invasive and expensive brain biopsy specimen procedures. Currently, the search for parasite presence in clinical specimens by PCR has overtaken direct inoculation into mice. However, considering that large diagnostic companies have shown little interest in making tests available commercially, most laboratories work largely with "home made protocols" (Remington J *et al*., 2004).

In this setting, several biological fluids have been tested by PCR based on amplification of different *T. gondii* gene targets such as the B1, P30, TGR1A, TGR1E, TGR2, TGR4, repetitive regions and ribosomal DNA genes (Burg JL *et al*., 1989, Nicoll S *et al*., 1996, Cazenave J *et al*., 1991, Pelloux H *et al*., 1997, Khouri H *et al*., 1999, Lamoril J *et al*., 1996). PCR assays targeting the multi-copy B1 gene of *T. gondii* are the most widely performed tests in molecular diagnosis of toxoplasmosis, as B1 appears to be conserved in different parasite strains and it is repeated 35 times in the *T. gondii* genome, thus being an adequate target for detection by PCR in clinical specimens. In most of the CSF samples from AIDS with TE, the sensitivities of n-PCR with B1 gene vary between 40% and 50% with specificities usually nearly 100% (Novati R *et al*., 1994, Schoondermark van de Ven E *et al*., 1993, Kalifa KES *et al*., 1993, Odberg-Ferragut C *et al*. 1996, Eggers C *et al*., 1995). Nested-PCR with B1 gene however, has not shown reproducible usefulness in monitoring therapy and has been less effective in detecting DNA released from cysts in course of recrudescence of disease (Contini C *et al*., 2002). Although the phenomenon of reactivation is far from being synchronous, during immunosuppression *in vivo*, the mechanisms that regulate conversion into bradyzoites are predominant and more bradyzoite surface molecules are expressed (Odaert C *et al.*, 1996). In this setting, several bradyzoite genes encoding for specific antigens have been identified and cloned including SAG4, BAG1/hsp30, LDH2 and MAG1 (Bohne W *et al*., 1996, Contini *et al*., 1999, Odberg-Ferragut C *et al*., 1996). The detection of these genes as well as their mRNA transcripts has also been shown to be useful in AIDS patients with relapse of TE (Cultrera R *et al*., 2002). Moreover, the cloning and characterisation of these stage specifically expressed genes has further contributed to the molecular analysis of this developmental differentiation (Weiss LM *et al*., 1996, Gross U *et al*., 1996).

The sensitivity and specificity of PCR do however

depend on several factors including i) the lack of standardized reagents and protocols for DNA extraction, ii) the amplification of DNA fragments of different size, iii) the inadequate storage of the clinical sample (*i.e* CSF), and iv) the time elapsing between the start of specific therapy and CSF collection which often affects PCR reproducibility and makes comparison of results difficult. External and internal amplification quality controls have shown to be helpful tools during development and validation of PCR assays in diagnosis of toxoplasmosis.

An interesting fragment, the 529-bp sequence, which has over 300 copies in the genome, has demonstrated to be specific for *T. gondii* (Homan WL *et al.*, 2000) and to improve also sensitivity and accuracy of real-time PCR (Edvinsson B *et al.*, 2006). A comparison of methods using the B1 and 529-bp sequences with real-time PCR revealed a ten-fold improvement in sensitivity when the 529-bp sequence was used (Reischl U *et al.*, 2003).

Repetitive sequences including mobile genetic elements (MGEs) and other single-copy sequences, including the SAG1, SAG2, SAG3, SAG4 and GRA4 genes (Terry RS *et al.*, 2001, Rinder H *et al.*, 1995, Meisel R *et al.*, 1996), have been used as PCR targets in research laboratories. Their efficacy in the clinical setting needs however to be further verified in large size studies.

In more recent years, Real-time PCR-based techniques have been employed for quantitative *T. gondii* DNA detection in clinical specimens. Several variations of the assay have improved sensitivity or specificity and seem to account for the usefulness of this technique for laboratory diagnosis. Targets such as the B1 and P-30 (SAG-1) and ribosomal DNA, have shown to be potential candidates to assure quality for clinical diagnosis of toxoplasmosis (Bretagne S *et al.*, 2000; Kupferschmidt O *et al.*, 2001, Hierl H *et al.*, 2004). *T. gondii* has been detected by various sequence detection systems including Light-Cycler (LC) in different human body fluids or tissue samples from transplant patients, with probes targeting different genomic fragments. The results varied in sensitivity (detection limit from 100 to 10 parasites/ml) and reproducibility (Costa JM *et al.*, 2000; Lin MH *et al.*, 2000; Kupferschmidt O *et al.*, 2001; Jones CD *et al.*, 2000; Botterel F *et al.*, 2002; Buchbinder S *et al.*, 2003).

Although real-Time PCR has been developed since 2000, like many molecular "in-house" assays, this technique suffers from the absence of an accepted standard method that allows an optimal comparison of sensitivity and specificity among the various laboratories.

Recently, we developed a highly sensitive Real-time PCR which employs LC to detect and quantify *T. gondii* B1 and SAG-4, MAG-1 genes in PBMC specimens from subjects with clinically suspected toxoplasmic retinochoroiditis, demonstrating that the combination of blood-real-time PCR with specific genes could assume an important value to accurately measure the parasite DNA load and its levels in different moments of infection, particularly during the course of therapy (Contini C *et al.*, 2005). The combination of blood-LC-PCR with these specific genes has demonstrated to be also useful in transplant patients (data not published).

An important step in the diagnosis of toxoplasmosis also involves the identification of the genetic group of *T. gondii.* In this setting, pyrosequencing has emerged a suitable technique to discriminate between *T. gondii* genotypes in clinical samples (Ahamadian A et al, 2006). Three main clonal lineages known as types I, II and III, which differ in virulence and epidemiological pattern of occurrence *T. gondii* have been genetically typed (Sibley LD *et al.*, 1992; Howe DK *et al.*, 1995, Genot JCM *et al.*, 2007). This suggests an influence of the parasite genotype on disease expression in immunocompromised patients. The potential correlation between genotype and disease pattern may thus have an important impact for the clinicians.

Molecular techniques have undoubtedly become a major tool in toxoplasmosis diagnosis. Further studies with large numbers of patients, based on direct analysis of genetic material by precise methods such as real-time PCR and more systematic external quality control and DNA sequencing, are required for a greater understanding of this important pathogen and also to optimize the diagnostic approach before they are implemented as routine methods.

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Toxoplasmosis in pregnancy: evaluation of diagnostic methods

V. Meroni, F. Genco

Infectious Diseases Department University of Pavia, Foundation IRCCS Policlinico San Matteo Pavia

Abstract. Toxoplasmosis in pregnancy is usually subclinic or associated with non specific symptoms. Diagnosis and timing of infection are usually based on serological tests. In this short review we tried to summarize the serological patterns we can encounter and to discuss the interpretation of test results

Key words: toxoplasmosis in pregnancy, serological tests, prenatal diagnosis

Introduction

Toxoplasma gondii is an obligate intracellular protozoan that infects almost a third of the world's population. Primary infection with *T. gondii* in pregnant women may result in congenital toxoplamosis via transplacental transmission. The extent of damage depends mainly on when the mother gets infected, with consequences being more severe during the early phases of gestation. However, transmission is more frequent during late pregnancy (Dunn *et al.,* 1999).
As parasitaemia lasts only few days and infection is often asymptomatic, the diagnosis relies mainly on serology (Montoya and Liesenfeld, 2004).
Diagnosis of toxoplasmosis in pregnancy has two goals: to evaluate the immune status of the woman, and in case of acute infection, to date the time of infection. It is therefore advisable to perform serological tests before or at the beginning of pregnancy.

Serological Tests

Given the kinetics of antibody production, the screening tests for toxoplasmosis are based on measurement of specific anti-*Toxoplasma* IgG and IgM antibodies with automated tests, which usually have a good sensitivity and specificity.
Anti–*Toxoplasma* IgG antibodies are produced throughout life after infection. Detection in a single sample at any titre with any test is a marker of previous infection.
IgM antibodies are detected in recently acquired infections but may persist for more than a year. Furthermore false positive, aspecific reaction may be recorded in IgG negative patients.

Four different serological patterns may be seen (Montoya and Liesenfeld, 2004; Sensini 2006).

Correspondence: Valeria Meroni
Infectious Diseases Department University of Pavia,
Foundation IRCCS Policlinico San Matteo Pavia,
e-mail: v.meroni@smatteo.pv.it

1-IgG negative IgM negative
No immunity. Hygienic alimentary prophylaxis and monthly follow up (if possible until one month after delivery) in order to avoid seroconversion are advised. Indeed there is some evidence that health education may reduce the risk of seroconversion and consequently of congenital toxoplasmosis. (Gollub *et al.*, 2008).
In Italy, the screening test in pregnancy is not mandatory but National Health System pays for a preconceptional test and for the follow-up of negative women (DPR245 10/09/98) so most of pregnant women undergo the monthly controls.

2-IgG positive IgM negative.
Previous immunity, if the tests have been done before or at the beginning of pregnancy.
No further sampling including further pregnancies is required.
In the third trimester, a negative IgM test cannot exclude an infection in the first trimester; in these cases it is necessary to perform other tests such as IgG avidity and further testing one month later to evaluate serological stability.

3-IgG negative IgM positive.
Early seroconversion or false positive result (Gussetti *et al.*, 1990).
In any seroconversion, IgG must must be produced, so it is mandatory to repeat a weekly sampling to detect IgG. If the patient has been treated, however, IgG production might be delayed and decreased. If this is the case, it is preferable to employ different tests (IgG-IgM Immunoblot, cellular immunity tests) to obtain a diagnosis as early as possible.
If a seroconversion has been proved, by using additional tests ,the clinician should prescribe the correct therapy. This also allows to advice the woman for prenatal diagnosis.
If seroconversion is not confirmed ,treatment can be safely discontinued..
Immunoblot for IgG and IgM is a very specific test when purified antigens (not commercially available) are employed.
The presence of 3 bands for IgG and two bands for IgM against 30-40 kD proteins can confirm seroconversion earlier than any other test (Sharma *et al.,* 1983).

Once therapy has been started it is very difficult to measure antibodies production. Tests to evaluate cellular immunity can then be employed in reference laboratories.
A positive Stimulation Index, the expression of CD25 marker on lymphocytes, γ interferon production after stimulation with purified *Toxoplasma* antigen, all confirm the infection and are not affected by therapy administration. (Fatoohi *et al.*, 2003; Ciardelli *et al.*, 2008).

4-IgG positive IgM negative
Recent infection. IgM can persist for many months but we need to date the time of infection.
If the infection was acquired before conception the mother must be reassured.
If the infection occurred during pregnancy the woman must be treated (spiramycin or pyrimethamine plus sulfadiazine according with the trimester) and advised for prenatal diagnosis. Immunoenzymatic tests for titration of specific IgA are not recommended as these antibodies may persist for many months after the acute phase and sometimes they are not produced after therapy. These antibodies may be diagnostic in newborns sera (like IgM they do not cross the placenta) and during reactivation in immunocompromised patients.

IgG Avidity test
This test measures the affinity of specific IgG antibodies for toxoplasmic antigens. (Hedman, *et al.*, 1989).
The avidity value increases with time and the time required for its maturation depends on the method employed. This test, is very useful to better define the timing of infection in IgG and IgM positive patients, provided some conditions are met:
1-only sera with a sufficient amount of IgG can be analyzed for avidity
2- the patient must not be treated at the time of testing (any therapy modifies the kinetics of antibodies and of avidity too) (Sensini *et al.*, 1996; Petersen *et al.*, 2005)
3- the test must be done in the first trimester of pregnancy.
The Avidity test can be fully or partially automated or manual, but whatever their features, a high avidity index excludes an acute infection in the previous 4 months. No information can be inferred from a low avidity index: in some cases a low avidity may persist for many months.
If acute infection is suspected, diagnosis cannot rely upon only one test but must consider also the different antibody kinetics and clinical evaluation.

Prenatal diagnosis

If an acute infection is diagnosed, different approaches should be taken depending on whether the onset was during the first part of pregnancy (before 22w -24w) or afterwards. For late infection the patient should be treated with pyrimetamine plus sulfadiazine and folinic acid until 15 days before the delivery. This time period was chosen based on the possibility of therapeutical interruption of pregnancy and varies across countries. In all other cases of acute infection the woman is counselled for prenatal diagnosis.
Prenatal diagnosis consists of a PCR performed on amniotic fluid drawn not earlier than the 18th week of gestation and 4-6 weeks after the presumable seroconversion.
Previous studies showed a good specificity and positive predictive value for PCR but sensitivity resulted quite low (64 %) . Many infected newborns, mainly with maternal infection in the first trimester had a negative PCR on amniotic fluid. (Thalib *et al.*, 2005).
At present , a larger amount of amniotic fluid (10 ml), a new sequence as target (gene bank accession number AF146527) that is repeated 200 -300 times instead of B1gene (Genebank accession number AF 179871) repeated only 20 times in toxoplasmic genome and the use of real time PCR improved the performance of the test. (Cassaing *et al.*, 2006) .
In our hands a nested PCR with the high repetitive gene target AF14 performed on 140 amniotic fluids showed a sensitivity and specificity of 100 %, evaluated as neonatal outcome independently from the period of maternal infection. (Meroni, unpublished)

Conclusions

One hundred years from the discovery of *T. gondii,* much progress has been made in the diagnosis of toxoplasmosis in pregnancy, and new tests are now available that give us more precise information on the infection. By using all the means in our possession the right diagnosis is possible in many cases. However, many unsolved questions such as the efficacy of therapy, its effect on the immune response, the strain virulence and the host/parasite relationship await further investigation.

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New insights in toxoplasmosis immunology during pregnancy. Perspective for vaccine prevention.

A.W. Pfaff, E. Candolfi

Institut de Parasitologie et de Pathologie Tropicale, EA 3950, Université Louis Pasteur, 3 rue Koeberlé, 67000 Strasbourg, France

Abstract. *Toxoplasma gondii* is one of the few pathogens that can cross the placenta. Frequency and severity of transmission vary with gestational age. While acquired toxoplasmosis is already well explored, the control of maternal-foetal transmission of the parasite remains almost unknown. This is partly due to inherent inadequacies of animal models. This review summarises the studies which have been undertaken and shows that the mouse is a valuable model despite obvious differences to the human case. The paramount role of the cellular immune response during primary infection has been consistently shown. Surprisingly, IFN-g has a dual role in this process. While its beneficial effects in the control of toxoplasmosis are well known, it also seems to have transmission-enhancing effects within the placenta and can also directly harm the developing foetus. This shows the importance of designing vaccines which protects both mother and foetus. Therefore, it is useful to study the mechanisms of natural resistance against transmission during a secondary infection. In this setting, the process is more complicated, involving cellular, but also humoral components of the immune system. In summary, even if the whole process is far from being elucidated, important insights have been gained so far which will help us to undertake rational vaccine research.

Key words: *Toxoplasma gondii*, pregnancy, placenta, foetus, immunity

Introduction

Toxoplasma gondii is a protozoan parasite with a global distribution. About 25% of the human population is estimated to be infected, placing *Toxoplasma* as the most successful parasite, along with *Plasmodium*. Infection is usually asymptomatic in immunocompetent individuals. The parasite, in its rapidly dividing tachyzoite form, disseminate into deep tissues and traverses biological barriers in placenta, brain, and retina (Barragan *et al.*, 2003). Under the influence of the developing immune response, *T. gondii* undergoes conversion to the slowly dividing bradyzoites, which organise themselves to tissue cysts and which are surprisingly resistant to external attack. Lifelong persistence of bradyzoites confers protective immunity against subsequent infections. Therefore, only primary infection with *T. gondii* can lead to foetal infection, leading to severe pathology, mostly retinochoroiditis which develop during the childhood or adolescence. In case of early transmission in pregnancy, neurological abnormalities may lead to severe malformation or stillbirth.

The development of a vaccine which could prevent such maternal-foetal transmission is hampered by our limited knowledge of protective mechanisms against infection. If the protective role of Th1 type immune responses, and especially the production of IFN-γ, is well established for acquired toxoplasmosis, its role is totally unknown during pregnancy.

Correspondence: Ermanno Candolfi
Institut de Parasitologie et de Pathologie Tropicale, EA 3950, Université Louis de Strasbourg, 3 rue Koeberlé, 67000 Strasbourg, France
Tel: +33-(0)390243679; fax: +33-(0)390243693
e-mail: ermanno.candolfi@medecine.u-strasbg.fr

Immune regulation of maternal-foetal infection

It is important to note that primary maternal toxoplasmosis does not necessarily result in foetal infection. Of the 200,000 to 300,000 cases of primary infection, which are estimated each year in France, 2,700 occur in pregnant women. These result in 600 cases of congenital transmission. About 150 of these patients show or will ultimately show clinical sequels, essentially ocular toxoplasmosis (Derouin *et al.*, 2005). As for the *Toxoplasma* strains found in congenital toxoplasmosis, at least in a French study, about 85% were due to infection with a type II (avirulent) strain. Type I strains, albeit reputedly more virulent, were rarely (8%) found (Ajzenberg *et al.*, 2002). Several mechanisms have been implicated in this protection. Most of the studies focused on cell mediated mechanisms, but recent work shows that antibodies also play a protective role. Clearly, this is difficult to investigate in humans. The importance of cell-mediated protective mechanisms can be deducted from a study which showed a limited, but visible risk of HIV infected women to pass on their *T gondii* infection to their offspring (Minkoff *et al.*, 1997). Despite the obvious discrepancies, animal studies gave some insights into the mechanisms at play. The mouse strain BALB/c shares central features with human congenital toxoplasmosis. In that rodent model, primary infection during pregnancy also results in about 50% of transmission and confers resistance to maternal-foetal infection during subsequent infections (Roberts and Alexander, 1992). Vaccination with soluble *T. gondii* antigen conferred a certain degree of protection to the foetus (Roberts *et al.*, 1994). This was

associated with an enhanced Th1-type immune response. The importance of such a Th1-type immune response, especially mediated by CD8+ cells and IFN-γ production, has been extensively proven (Denkers and Gazzinelli, 1998). However, data obtained in non-pregnant mice have to be extrapolated carefully to congenital models, since pregnancy modifies the balance between Th1 and Th2 immune responses by generating a Th2-type environment essential to maintain pregnancy (Ng *et al.*, 2002).

In pregnant BALB/c mice, we investigated the mechanisms of transmission control. Foetal infection is an early event occurring mostly during the first week of infection (Pfaff *et al*, 2007). Therefore, in the absence of immunologic memory to *T. gondii* infection, mechanisms of the innate immune response occupy a central place. To our surprise, RAG-2$^{-/-}$BALB/c mice, which are unable to produce T- and B-cells, showed a significantly lower transmission rate than the wild-type controls. This was associated with an enhanced production of IFN-γ in response to *T. gondii* infection thought to be due to greater NK cell production relative to wild-type mice (Abou-Bacar *et al.*, 2004). Cell enumeration revealed considerably enhanced numbers of circulating NK cells. Other studies have shown that this cell type is very important for a quick reaction to *T. gondii* infection, through IFN-γ production (Sher *et al.*, 2003) and, by inference from *in vitro* studies, cytotoxic activity (Hauser and Tsai, 1986). This role of NK cells was confirmed by a considerable increase of *T. gondii* transmission by depletion of NK cells in the RAG-2$^{-/-}$ mice. However, when IFN-γ was completely neutralized, a considerable increase in parasite numbers in the mothers' peripheral blood was observed, whereas the materno-foetal transmission rate was diminished. This indicates a transmission-enhancing effect of IFN-γ production which is effective within the placenta. Having in mind that infection of the placenta occurred very early, and was immediately followed by infection of the first foetuses, any protective responses have to act very quickly. This explains the importance of fast-acting NK cells specifically for the control of maternal-foetal transmission. This finding also shows that the placental barrier can, at least in some cases, be rapidly overcome. *T. gondii* can easily enter and survive in immune cells such as dendritic cells (Channon *et al.*, 2000), which migratory capacities allows *Toxoplasma* to disseminate throughout the body (Courret *et al.*, 2005) and enter in contact with trophoblast cell barrier. *In vitro* studies on the human trophoblast cell line BeWo suggest that IFN-γ is necessary for adhesion of *T. gondii* infected monocytes, thereby facilitating maternal-foetal transmission (Pfaff *et al.*, 2005a). Following infection, trophoblast cells are not able to limit *T. gondii* multiplication when stimulated by IFN-γ, in contrast to most other cell types (Pfaff *et al.*, 2005b). On the cellular level, *Toxoplasma* infected trophoblast cells stop proliferation and intrinsic suicide. This leads, at least partially, to a host cell cycle arrest and to an inhibition of their natural apoptotic capacities (Brunet *et al.*, 2008), resulting in parasite persistence in placental tissues. This shows, once again, the delicate balance between infection control and pregnancy maintenance.

Combining *in vivo* and *in vitro* results, the parasite therefore depends on the immune system and its production of IFN-γ to facilitate its transmission to the foetus.

Development of vaccines against congenital toxoplasmosis

The results we presented show that IFN-γ production is indispensable for host protection from uncontrolled parasite multiplication. On the other hand, uncontrolled IFN-γ production causes death by exaggerated immunopathological reactions, as already observed in the intestinal tract of *T. gondii* susceptible mice (Liesenfeld *et al.*, 1996) (Pawlowski *et al.*, 2007). Inflammatory cytokines and chemokines are responsible for chemo-attraction of macrophages, PMNs and CD11c+ and CD11b+ monocytes and dendritic cells to the lamina propria at day 7, responsible of the observed massive necrosis. As to foetal tissues, a different picture emerges. We demonstrated that *T. gondii* infection, via IFN-γ production, can lead to abortion in early gestation, however it is mainly due to a large apoptotic process, involving uterine NK cells and not to *Toxoplasma* cytolytic effect, nor to necrosis (Senegas *et al.*, unpublished data). On the other hand, we also demonstrated that IFN-gamma-induced indoleamine 2,3-dioxygenase (IDO) is abundantly produced at the maternal-foetal interface and one may hypothesise that it could protect the foetus from pathological consequences of Th1 related immune attack (Pfaff *et al.*, 2008). In the light of these new discoveries, we appreciate that recent publications include the outcome of gestation in their vaccine studies (Mevelec *et al.*, 2005; Ismael *et al.*, 2006).

At this point, it is important to keep in mind that transmission of *T. gondii* occurs only during primary infection of the mother. In subsequent infections, the mother's immune system is able to eliminate the parasites before they reach the maternal-foetal barrier. The ultimate goal of any vaccine should consequently be to imitate this ideal natural protection. Therefore, it is useful to study the mechanisms of protection against re-infection. Protection against maternal-foetal transmission during secondary infection is diminished when CD8+ cells are depleted or IFN-γ is neutralised (Abou-Bacar *et al.*, 2004). This underlines the importance of CD8+ cells as IFN-γ producing cells for protection of congenital toxoplasmosis, which has been demonstrated previously in vaccination studies of non-pregnant mice (Denkers 1999). CD4+ cells, while not completely redundant, seem to play a minor role in such recall responses.

Apparently contradictory results of vaccine studies reveal the subtleties of foetal protection. Whereas one study (Couper *et al.*, 2003), which used SAG1 DNA as vaccine, found protection for the mother, but not the foetuses, another study, using SAG1 protein, but the same mouse strain, did find a protective effect for the

offspring (Letscher-Bru *et al.*, 2003). This study showed also the interaction between genetic background, pregnancy and stimulation of the immune system by comparing the results of two mouse strains (Letscher-Bru *et al.*, 2003). Vaccination with SAG1, the immunodominant major surface protein, in BALB/c mice resulted in a mixed Th1 - Th2 response and conferred partial protection to congenital infection. In contrast, the same vaccination protocol induced in another mouse strain, CBA/J, caused a biased Th2 response to SAG1, and resulted in an even increased maternal-foetal transmission of *T. gondii*. Consequently, a finely tuned balance between the antiparasitic Th1 response and the pregnancy induced Th2 response has to be achieved when developing vaccines. Further studies in our laboratory showed that antibody production, probably in close interaction with CD8+ cells, do indeed have a role in the case of re-infection or vaccination (Letscher-Bru, unpublished results). This involvement of antibodies in a secondary response might also prevent a pronounced inflammatory response, which is characteristic of primary responses. As antibodies neutralise most of the invading parasites, a limited IFN-γ activated response is sufficient to completely eliminate the parasite and to prevent maternal-foetal transmission. In the light of recent work, it seems clear that sterile immunity might not be an absolute requirement for a vaccine. For example, some studies, using different strategies show that maternal-foetal transmission can be drastically reduced without completely eliminating maternal infection (Letscher-Bru *et al.*, 2003; Ismael *et al.*, 2006). Multiple novel delivery techniques have now been tested against toxoplasmosis: *T. gondii* RNA (Dimier-Poisson *et al.,* 2006), adenoviruses (Caetano *et al.*, 2006), dendritic cells (Ruiz *et al.*, 2005), and many others. When we combine these tools with the rationale extracted from the more fundamental research, considerable advances should be possible in the near future. The involvement of antibodies and their possible contribution to dampen the IFN-γ dependent cellular response during secondary responses are not without consequences for vaccine development. It is well known that infectious diseases of different aetiology can severely harm the foetus, or even lead to its expulsion (Entrican, 2002). Ongoing studies in our laboratory show also the effect of a strong IFN-γ production in response to a primary *T. gondii* infection to the early conceptus in a mouse model (Senegas and Villard, pers. comm.). This shows that the mouse model can also reproduce this immunopathological aspect of vaccine studies. In conclusion, it seems clear that the best protection is not given by the strongest Th1 response, but by an equilibrated reaction, which takes into account both the dual role of IFN-γ for transmission, and its potentially harmful effects on the foetus itself.

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Toxoplasmosis in livestock in Italy: an epidemiological update

L. Rinaldi[1], A. Scala[2]

[1]Dipartimento di Patologia e Sanità Animale, Università degli Studi di Napoli "Federico II"; [2]Dipartimento di Biologia Animale, Università degli Studi di Sassari

Abstract. Infection with *Toxoplasma gondii* is one of the most common parasitic infections of human being and other warm-blooded animals. It has been found worldwide from Alaska to Australia. Public health organizations repeatedly encourage the collection of accurate data about *T. gondii* in animals and humans due to its medical importance as a major source of parasitic zoonosis. For these reasons, epidemiological updates on toxoplasmosis in livestock are strongly advised also to plan control strategies. In the present paper, seroprevalence data on *T. gondii* that have been recorded in livestock from different Italian regions over the last 3 decades are reviewed, showing the high level of exposure of livestock to this parasite.

Key words: *Toxoplasma gondii*, livestock, epidemiology, Italy.

Infection by *Toxoplasma gondii* is a cosmopolitan zoonotic disease caused by a coccidian intracellular protozoan capable of infecting all warm-blooded animals, including mammals, birds and humans (Fayer, 1981). The parasite has a worldwide distribution - from Alaska to Australia - and it is mainly transmitted by food/water contaminated with oocysts dispersed by cats and other felines (definitive hosts), raw/undercooked meat containing tissue cysts or un-pasteurized milk containing tachyzoites, and transplacentally (Tenter *et al.*, 2000). *T. gondii* infection has important implications for public health, since it affects one-third of the world's population. In addition, it also has important veterinary implications because it causes disease, miscarriage or congenital malformations in the definitive and intermediate hosts. Cats, sheep, goat and pig are the domestic animal species most seriously affected by the protozoan. Since the 1950s, *T. gondii* has been recognized as a significant and common cause of endemic and epidemic abortions on sheep farms (Dubey, 2004).

Because of the great importance of *T. gondii* as a causative agent of a zoonosis, public health organisations, such as the World Health Organisation, have repeatedly advised the collection of accurate epidemiological data on this parasite. Such data are essential to elucidate the relative importance of the various sources of infection for humans, to control disease, and to prevent reduction in quality of human life caused by this parasite. However, only few countries of the world regularly monitor toxoplasmosis in humans, and even less countries monitor *T. gondii* infection in animals (Tenter *et al.*, 2000). For these reasons, we believed useful to report the seroprevalence data on *T. gondii* that have been recorded in livestock from different Italian regions over the last 3 decades (from 1980; see table 1).

This is an attempt to summarize the information on *T. gondii* in livestock in Italy based on published data; we apologize if we missed any other data. It should be noted, however, that when comparing seroprevalence data for infections with *T. gondii*, the different serological methods used should be taken into account (Piergili Fioretti, 2004). In addition, prevalence values might vary over time and with the age of animals (Tenter *et al.*, 2000). Besides serological surveys, also molecular studies have been conducted on ovine tissue and milk samples; however, these PCR-based techniques seems to be not so effective in showing the real distribution of the parasite. Indeed, the presence of *T. gondii* DNA was found only in 3.3% of the examined hearts from Sardinian sheep, whereas the other organs resulted negative (Purqueddu *et al.*, 2006). Similarly, *T. gondii* DNA was found only in 3.4% of the ovine bulk milk samples from Campania (Fusco *et al.*, 2007). The high seropositivity rates in livestock from the different Italian regions indicate that the consumption of raw or undercooked meat from livestock is still the main risk factor for human to contract toxoplasmosis as showed by Cook *et al.* (2000). Pigs, goats, sheep, horses and poultry are the major meat sources of human infection (Tenter *et al.*, 2000; Tassi, 2006). Of epidemiological interest is the fact that cattle and presumably water buffaloes are not an important source of human infection although they can become infected by *T. gondii* (Kijlstra *et al.*, 2006). The prevalence of *T. gondii* in free-ranging chickens is a good indicator of the prevalence of the parasite's oocysts in soil because chicken feed from the ground (Dubey *et al.*, 2008).

The prevalence values reported in livestock are noteworthy not only for the transmission of the protozoan from animals to human as food-borne zoonosis, but also because it causes substantial health problems in farm animals. Pigs, sheep and goats are the livestock species most seriously affected by the protozoan whereas cattle, water buffaloes and horses are considered less sensitive species to the pathogenic effects of *T. gondii*

Correspondence: Antonio Scala
Dipartimento di Biologia Animale, Sezione di Parassitologia e Malattie Parassitarie, Università degli Studi di Sassari,
Via Vienna 2, 07100 Sassari, Italia
Tel +39 079 229465, Fax +39 079 229464,
e-mail: scala@uniss.it

Table 1. Seroprevalence data on *T. gondii* recorded in livestock from different Italian regions over the last 3 decades

Livestock species	Region	No. examined animals	Seroprevalence (%)	Diagnostic method	References
Cattle	North	255	92.0	DAT	Avezza *et al.*, 1993
	Sicilia	317	11.3	IFAT	Vesco *et al.*, 2005
Buffaloes	Campania	187	94.0	MAT	Persechino *et al.*, 1980
Sheep	Lombardia	352	78.0	LAT	Gaffuri *et al.*, 2006
	Emilia Romagna	374	69.0	IFAT	Baldelli and Pietrobelli, 1985
	Campania	1,170	28.5	IFAT	Fusco *et al.*, 2007
	Puglia and	306	88.6	IFAT	Puccini *et al.*, 1981
	Basilicata	321	56.1	IFAT	Puccini *et al.*, 1983
	Sicilia	1,390	0.1	IFAT	Balbo *et al.*, 1980
		1,876	49.9	ELISA	Vesco *et al.*, 2007
	Sardegna	7,149	28.4	IFAT IgG	Masala *et al.*, 2003
			9.9	IFAT IgM	
		29,886	19.2	IFAT IgG	Tola *et al.*, 2006
			5.4	IFAT IgM	
		1,043	51.3	ELISA	Natale *et al.*, 2006
Goats	Lazio	198	95.0	MAT	De Capraris and Gravino, 1981
	Puglia and Basilicata	244	68.9	IFAT	Puccini *et al.*, 1983
	Sardinia	2,445	12.3	IFAT IgG	Masala *et al.*, 2003
			5.6	IFAT IgM	
		4,562	11.7	IFAT IgG	Tola *et al.*, 2006
			4.0	IFAT IgM	
Pigs	North	90	64	IFAT	Genchi *et al.*, 1991
	Emilia Romagna	1,521	9.0	LAT	Soldati *et al.*, 1986
	Umbria	576	16.7	IFAT	Piergili Fioretti *et al.*, 2008
	Sicilia	1,035	21.3/20.0	ELISA/IFAT	Vesco *et al.*, 2006
	Sardegna	408	15.2	ELISA	Scala *et al.*, 2008
Horses (human consumption)	Various	163	30.7	MAT	Tassi, 2006
Chickens	Various	80	12.5	MAT	Dubey *et al.*, 2008

Legend: DAT = direct agglutination test; IFAT = indirect fluorescent antibody tests; MAT = microscopic agglutination test; LAT = latex agglutination test; ELISA = enzyme-linked immunosorbent assay.

(Kijlstra *et al.*, 2006). Regarding pigs, the majority of the infections are sub-clinical or latent and few clinical cases of toxoplasmosis have been reported. However, four outbreaks of disease with mortality in different pig herds have been recently reported in the Lombardia and Emilia-Romagna regions of northern Italy (Gelmetti *et al.*, 1999).

Concerning small ruminants, *T. gondii* is recognized as one of major cause of infectious reproductive failure in several countries of the world causing fetal resorption, abortion at any stage of pregnancy, fetal mummification, stillbirth or birth of live but weak offspring. The important role played by *T. gondii* in ovine and caprine abortion as been documented in the Sardinia region (Tola *et al.*, 2002; Masala *et al.*, 2003); out of a total of 366 ovine aborted samples from Sardinia, *T. gondii* DNA was detected in 18.1% of fetuses and 13.1% of placentae (Masala *et al.*, 2007).

In conclusion, the present paper shows that seroprevalence data on toxoplasmosis in livestock are not uniformly distributed along the whole Italy. In addition, these data, although revealing a *scenario* at risk from a zootechnical and sanitary point of views, represent a situation highly heterogeneous, probably due to the different sampling and laboratory techniques utilized.

Thus, a coordinated national-scale survey on toxoplasmosis in livestock - based on homogeneous sampling and laboratory techniques - is strongly needed, in order to better assess the actual epidemiological situation of this under-estimated zoonosis in livestock and to clarify factors that influence its presence and distribution.

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SIMPOSIO 2

IL PIANETA *MALASSEZIA*

Parassitologia 50: 65-67, 2008

The Pathogenesis of *Malassezia* Yeasts

C. Cafarchia, D. Otranto

Dipartimento di Sanità Pubblica e Zootecnia, Facoltà di Medicina Veterinaria, Università di Bari, Bari, Italy.

Abstract. The genus *Malassezia* includes twelve species of yeast, many of which have been mainly associated with human and canine diseases. *Malassezia pachydermatis* colonizes the skin and mucosal sites of healthy dogs and cats. Despite being part of the normal cutaneous microflora, *Malassezia* spp. yeast may become pathogenic under certain circumstances. This article reviews the factors related to both host and yeast which affect the pathogenical or commensal phenotypes of *Malassezia* yeasts.

Key words: *Malassezia pachydermatis*, pathogenicity, host defence, virulence factors.

Introduction

Malassezia spp. are lipophilic yeasts belonging to the normal cutaneous microflora of most warm-blooded animals and sometimes act as opportunistic pathogens (Batra *et al*, 2005). The lipid-dependent species are frequently associated with human skin disorders, while the non-lipid dependent *Malassezia pachydermatis* is considered to be an opportunistic pathogen growing on skin surface and ear canal of dogs and cats (Guillot and Bond, 1999; Chen and Hill, 2005). *Malassezia* dermatitis may presents with pruritus, inflammation and epidermal hyperplasia (Chen and Hill, 2005) and the pathogenic role of *Malassezia* yeasts in the occurrence of lesions may be related to host immune system as well to yeast virulence factors. The aim of this article was to review and discuss the scientific literature available on the pathogenesis of *Malassezia* spp.

Host predisposing factors

The proliferation of *Malassezia* yeasts is likely to be a preliminary step toward dermatitis and/or otitis (Nardoni *et al*, 2005; Cafarchia *et al*, 2005a; Cafarchia *et al*, 2005b). The yeast overgrowth may be caused by changes in the cutaneous microenvironment and/or alterations in host defence mechanisms (Chen and Hill, 2005). Under the above circumstances, hypersensitivity diseases (e.g. atopic dermatitis) (Nardoni *et al*, 2007), parasitic infestation (e.g. *Otodectes, Sarcoptes, Demodex* mites -Radi, 2004; Anane *et al*, 2007), keratinization disorders (e.g. seborrheic dermatitis), alterations in host immune system by endocrine diseases (diabetes mellitus in dogs), FIV, Felv infections (Sierra *et al*, 2000; Peikes *et al*, 2001) and antibiotic and/or glucocorticoid treatment restricting microbial colonization of the skin (Chen and Hill, 2005), predispose to *Malassezia* overgrowth. The value of pH and humidity of the skin surface also may bias the growth of *Malassezia* yeasts: the higher is the cutaneous pH level the higher the release of *Malassezia sympodialis* allergens leading to inflammation (Selander *et al*, 2006) while a low pH inhibits *M. pachydermatis* growth (Matousek *et al*, 2003). Furthermore, *Malassezia* yeast growth may be also affected by skin humidity being this infection more common in warm, humid climates and seasons, and in certain anatomic sites such as skin folds (Bergbrant, 1995).

In animals with otitis a larger population size of *Malassezia* yeasts was observed in male cats and dogs, in cats over one year of age and in dogs under one year of age, in autumn for cats and in winter for dogs (Cafarchia *et al*, 2005a; Nardoni *et al*, 2005).

The chemical composition of the skin may play a role in selection of *Malassezia* genetic population (Cafarchia *et al*, 2008b). In fact, the distribution pattern of a particular genotypes (Cafarchia *et al*, 2007c, Cafarchia *et al*, 2007d) of *M. pachydermatis* on the skin of dogs is related to the affinity of the yeast to a particular skin site and to the presence of lesions (Cafarchia *et al*, 2008b).

In hosts *Malassezia* yeasts produce antigens that could penetrate the animal skin being captured by epidermal Langerhans' cells and stimulating Th1cells and/or Th2 cells to produce IgG and/or IgE immunoglobulin (in Chen and Hill, 2005). Consequently, the production of *Malassezia*-specific IgG antibodies might activate the complement system causing epidermal damage and inflammation and allergen-specific IgE antibodies could lead to a Type I hypersensitivity reactions (Chen and Hill, 2005).

Malassezia yeast virulence factors

Malassezia yeasts are also per se pathogenic to animals. For example, the zymogen pro-enzyme of the yeast cell wall may activate the complement system resulting in damage of keratinocyte integrity and thus epidermal spongiosis, inflammation and pruritus (Belew *et al*, 1980). *Malassezia* yeasts produce esterase, lipase, phosphatase acid, lipossigenase and protease

Correspondence: Domenico Otranto
Department of Veterinary Public Health,
Faculty of Veterinary Medicine, University of Bari,
Str. prov.le per Casamassima Km 3, 70010,Valenzano, Bari (Italy)
Tel/Fax: +39 080 467 9839,
e-mail: d.otranto@veterinaria.uniba.it

(Continuho and Paula, 2000; Mancianti *et al*, 2001) of which, the latter, could contribute to pruritus as mediators of itch (in Chen and Hill, 2005). Lipases, contribute to produce fatty acids on the skin which can be used by yeasts for nutrition providing protection by other inhibiting organisms (in Chen and Hill, 2005). *M. pachydermatis* also produces phospholipase and a higher phospholipase activity was found in isolates from skin lesion than in strains from healthy skin (Cafarchia and Otranto, 2004). Further studies demonstrated that β-endorphin (a class of endogenous opioid peptides) induces *M. pachydermatis* cell differentiation towards the production or non -production of phospholipase (Cafarchia *et al*, 2007a). The presence of mu-opioid receptors on the *M. pachydermatis* cells and the effect of naloxon (i.e. an opioid antagonist receptor), on the phospholipase activity has been investigated (Cafarchia *et al*, 2007b) and results indicated that mu opioid receptors are expressed in *M. pachydermatis* cell walls. The above results suggested that this receptor may be involved in mediating the effects of both opioid agonist (β-endorphin) and antagonist (Naloxon) on phospholipase production of *M. pachydermatis* thus opening new avenues for topical control of *Malassezia* lesions.

The host, the *Malassezia* and the *Leishmania infantum* protozoa.

The relationship among the frequency, population size and phospholipase activity of *M. pachydermatis* was investigated for dogs with (Li^+) and without (Li^-) *Leishmania infantum* infection. A significantly higher mean population size of *M. pachydermatis* was cultured from the skin of L^+ compared with L^- dogs. For *M. pachydermatis*, most phospolipase-producing cultures and the highest phospholipase activity were recorded for L^- dogs with lesions and L^+ dogs without lesions. Although *M. pachydermatis* was a common commensal on dogs with or without *L. infantum* infection, *L. infantum* infection in dogs without skin lesions are associated with increased growth of *M. pachydermatis* and production of phospholipase *in vitro* (Cafarchia *et al*, 2008a).

Conclusive remarks

The pathogenic role of *Malassezia* yeasts is related to changes in the normal physical, chemical or immunological mechanisms of the skin which may enhance or down regulate the molecular production of yeast virulence factors or antigens. As an example the chemical composition of *Malassezia* cell wall (i.e. the expression of mu -opioid receptors) may be strictly related to the chemical composition of the skin (presence of β-endorphin) and may play a fundamental role in influencing the pathogenic or commensal phenotype of *Malassezia* yeasts. Without any doubt further studies and researches are needed in this field in order to better understand the complex interactions between *Malassezia* and host immune system by investigating genomic and proteomic aspects of this relationship.

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Skin diseases associated with *Malassezia species* in humans. Clinical features and diagnostic criteria

E.M. Difonzo[1], E. Faggi[2]

[1]Dipartimento di Scienze Dermatologiche, Università di Firenze; [2]Dipartimento di Sanità Pubblica, Università di Firenze

Abstract. *Malassezia* yeasts not only cause the well known pityriasis versicolor and folliculitis, but also play an important role in other skin diseases, including seborrheic dermatitis and atopic dermatitis.
The presence of *Malassezia* yeasts may be confirmed by direct microscopic examination and cultures of skin scrapings.
In pityriasis versicolor the direct microscopic examination is the rapidest and surest test for confirming the clinical diagnosis. The preparation will show a cluster of globose budding spores with thick or double wall and short hyphae. For detecting *Malassezia* in the other diseases the cultures is preferable.
Culture is useful both for confirming the clinical diagnosis and for epidemiological investigations. The identification of the *Malassezia* species is not easy. The microscopic observation of the colony direct towards the identification of *Malassezia* species, but it is not enough to identify the colonies definitely. Several biochemical tests are necessary for a precise identification, such as catalase reaction, growth on media without lipid sources, ability to utilize hydrophilic emulsifiers as sole lipid source, esculin test, tryptophan test.

Key words: *Malassezia* species, pityriasis versicolor, seborrheic dermatitis, biochemical tests.

The yeasts of the genus *Malassezia* are normal skin commensals and lie in the stratum corneum and acroinfundibulum of the sebaceous follicle, particularly in sebaceous –rich areas, as face, scalp , head and upper back (Faergemann J, 1983; Faggi E, 1994).
Malassezia not only causes the well known pityriasis versicolor and folliculitis, but also play an important role in other skin diseases, including seborrheic dermatitis and atopic dermatitis (Gupta AK, 2004; Aspres N, 2004; Sugita T, 2003).

Pityriasis versicolor (PV)

PV is a worldwide chronic or relapsing mycosis, predominant in young adults and localized on the upper trunk, chest, back and shoulders. The disease is characterized by round or oval, hypo-or hyperpigmented macules covered with thin scales, which are easily removable. Margins of the lesions are sharply delineated. Lesions are 1 to 5 mm in diameter in the beginning, but may coalesce to form variously shaped patches. Their color is variable from whitish to pink, salmon or brown according to several factors, such as the age of the lesions, inflammatory response of the host, exposure to sunlight, as well as normal pigmentation of the patient. The conditions allow the overgrowth of *Malasssezia* in certain people and cause PV are not clearly defined. Numerous factors seem to favour the change from a yeast form to an invasive hyphal one. Important factors are hyperhidrosis , occlusive clothing, and genetic predisposition.

Correspondence: Elisa M. Difonzo
Dipartimento di Scienze Dermatologiche, via della Pergola 64, 50121 Firenze, Italy
Tel: +39 055 27588935; fax: +39 055 2758381;
e-mail: elisa.difonzo@asf.toscana.it

Malassezia spp cultured from lesions of PV are *Malassezia globosa* and *Malassezia sympodialis* (Gupta AK, 2004; Gupta AK, 2001; Gaitanis G, 2006; Tarazooie B, 2004; Hernandez F, 2003; Crespo-Erchiga V, 2006; Morishita N, 2006).

Malassezia folliculitis (MF)

MF is a chronic disease characterized by small erythematous follicular papules and/or pustules localized on upper trunk. Patients develop moderate or even severe pruritus. The lesions heal with an easily removable crust. Most frequently MF affects young oil skinned people with acne vulgaris or seborrheic dermatitis.
The development of MF is favoured by hot and humid climates, use of antibiotics, above all tetracyclines. Histological findings show invasion of the central and deep follicle with large number of *Malassezia* yeasts and inflammatory infiltrate consisting of lymphocytes and histiocytes, along with focal rupture of the follicular wall.

Seborrheic dermatitis (SD)

DS is a common chronic inflammatory disorder localized in sebaceous-rich areas occurring in 3% to 5% of the general population. The incidence is much higher in patients who are immunocompromised, especially those with AIDS, ranging from 30% to 80%. The disease, which is more common in male patients, tends to occur most frequently in adolescents and young adults. The characteristic presentation shows erythematous patches covered by greasy scales. Pityriasis simplex capitis (so-called “dandruff”) is a mild form of scalp SD. On the face the disease affects forehead, eyebrows, glabella, and nasolabial folds. On the sternal and interscapular regions DS is characterized by figurated circi-

nate patches (petaloid DS) . The cause of DS is unknown. Many factors have been cited as possible contributors to the development of this disorder. Despite the lack of a clear correlation between sebum levels and the development of SD, there still appears to be some connection between the disease and sebum levels. In the sebum cholesterol, triglycerides and paraffin are increased.
Several investigations suggest that psychological factors play an effective role in inducing lesions. Other important factors are seasonal and microenvironmental climate climate, namely temperature, humidity, and variation of sweating. The causative role of *Malassezia* yeasts has not yet been entirely explained. It has been suggested that the lesions are caused more likely by the host's anomalous inflammatory response, than by an overgrowth of the yeast. The inflammatory response may follow the toxin production or the lipase activity of *Malassezia*. The enzyme lipase splits triglycerides into irritant fatty acids, which may induce scaling, or releases arachidonic acid, which is involved in the inflammation of skin. Frequent observations of SD in AIDS patients have suggested a role of impaired cell-mediated immunity; but specific investigations do not confirm this hypothesis. The species that have been shown to be most closely associated with SD are *Malassezia globosa* and *Malassezia restricta.*

Atopic dermatitis (AD)

AD is a chronic inflammatory disorder marked by intense pruritus and eczematous lesions. The pathogenesis of AD, which is often associated with allergic rhinitis and/or asthma, is highly complex. Genetic factors are known to play an important role. Other factors are humidity, cold climates, environmental allergies, and alterations of the normal skin barrier. *Staphylococcus aureus*, *Candida* and other micro-organisms have been correlated with AD. *Malassezia* yeasts appear to play an important role, particularly in atopic adults with head and neck dermatitis. Several reports have documented that patients with this kind of AD have higher levels of specific *Malassezia* IgE antibodies. It has also been shown that these patients respond to antimycotic therapies. Great importance is attached to *Malassezia sympodialis* on the basis of a hypothized cross-reaction between the mycelial manganese superoxide dismutase and the human one.

Diagnosis

The presence of *Malassezia* yeasts may be confirmed by direct microscopic examination and cultures of skin scrapings. Skin biopsy is necessary for diagnosing only the folliculitis. In this case the histological findings show large number of yeasts in the hair follicles and in the inflammatory perifollicular infiltrate. For diagnosing pityriasis versicolor Woood's light examination is less and less frequently used, because the yellow-green fluorescence is positive in only about one-third of cases.
In pityriasis versicolor the direct microscopic examination of infected scales is the rapidest and surest test for confirming the clinical diagnosis. Scales can be scraped with a scalpel and collected on a glass slide. A drop or two of 20% potassium hydroxide (KOH) should be placed on the collected scales, covered with a coverslip. The slide is then examined under a phase-contrast microscope, using a low-power object (10x) in order to detect fungal cells followed by higher-power object (25 to 40x) for studying the morphology of cells. The preparation will show a cluster of globose budding spores , 2 to 5 ?m in diameter, with thick wall and short hyphae, 3-8 ?m in diameter, with sharp or round ends. The budding cells and hyphae have been described as having the appearance of "spaghetti and meat balls". These findings can be improved by adding Blue Parker ink to KOH.
In other skin diseases associated with *Malassezia* the microscopic examination reveals only some spores, while hyphae are not present. As fungal elements are present in small number the examination might show false negative results. For detecting *Malassezia* in these cases the culture is preferable.
Culture is useful both for confirming the clinical diagnosis and for epidemiological investigations. The genus *Malassezia* includes lipophilic species (*M. pachydermatis*) and lipid-dependent species (*M. furfur*, *M. sympodialis*, *M. globosa*, *M. restricta*, *M. sloffiae*, *M. obtusa*). Consequently in order to isolate these yeasts from skin scales it is necessary to use media containing medium- or long-chain fatty acids. The most used isolation media are modified Dixon agar (Guillot J, 1996) and the Leeming-Notman agar (Leeming JP, 1987). Colonies develop in 7-14 days at 32°C.
The isolates are to be stained with Blue Parker ink (Xiong L, 2004) , which is suitable to detect those that might belong to *Malassezia* genus. The Blue Parker ink positive colonies will be identified according to their morphological and biochemical features.
The morphology of yeasts direct towards the identification of *Malassezia* species, but it is not enough to identify the colonies definitely. Several biochemical tests are necessary for a precise identification. In our experience we have used the followings methods, available in literature: catalase reaction (Guillot J, 1996), growth on media without lipid sources, ability to utilize hydrophilic emulsifiers (Tween 20, Tween 40, Tween 60, Tween 80, Cremophor EL) as sole lipid source (Guillot J, 1996; Mayser P, 1997), esculin test to reveal β-glucosidase activity (Mayser P, 1997), tryptophan test to detect pigment production (Mayser P, 2004).
The execution and interpretation of these tests are non particularly difficult, except the evaluation of the hydrophilic emulsifiers utilization test. Frequently Tween hydrolysis may produce precipitates, which may be misinterpreted as a fungal growth with a consequent misunderstanding of lipid source use.
In these cases referring strains are useful for the exact evaluation of the findings.

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Epidemiology and variability of *Malassezia* spp.

R. Galuppi, M.P. Tampieri

Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale - Alma Mater Studiorum Università di Bologna

Abstract. A short review on *Malassezia* spp., completed with our experience, is made. The main epidemiological characteristics with particular regard to the diffusion in several animal species, the characteristics of skin colonization (in particular of the dog) and the distribution of the different *Malassezia* spp. in some hosts are discussed. Lastly the main phenotypic and genotypic characteristics, referred to *M. pachydermatis* especially, were described, showing their high variability and differentiation.

Key words: *Malassezia* spp., epidemiology, variability

Epidemiological aspects of the genus *Malassezia*.

The genus *Malassezia* includes lipophylic yeasts that are usual components of human and most mammals and avian species skin micro flora. After the first isolation in Indian rhinoceros (Weidman, 1925) *Malassezia* spp. (*Pityrosporum* spp) was afterward found both on wild (other species of rhinoceros, bears, wolf, coyote, fox, seal and eared seal, llama, porcupine, elephant, opossum, monkey, ferret, fennec, cheetah) and domestic mammals (dog, cat, horse, cattle, sheep, goat, swine). Some studies on rodents and lagomorphs skin did not found these yeasts, probably for the poor presence of sebum and cerumen (Guillot *et al*, 1994). We too, on 147 laboratory rats used for studies about carcinogenetic factors, did not found *Malassezia* spp. On the other hand, Dufait (1985) isolated *M. pachydermatis* in guinea pig and rabbit and Drouhet *et al* (1980) reproduced experimentally a seborroic dermatitis in mouse and rats by contact with *P. ovale* and *P. orbicolare* (*M. furfur* and *M. globosa* respectively). Various avian species also were found as host of *Malassezia* spp. (Midgley and Clayton, 1969; Dufait, 1985; Breuer-Strosberg *et al*., 1990); it is therefore supposable that these yeasts can be present in all homoeothermic vertebrates. As it is commonly accepted that *Malassezia* spp. is not found in the environment, their finding from sand (Marcelou-Kinti *et al*, 1973) and from environmental free-living nematodes (*M. restricta* associated to *Malenchus* sp. and *Tylolaimophorus typicus* and *M. globosa* associated to *Malenchus* sp. - Renker *et al,* 2002) is therefore a curiosity. The same authors point out that these yeasts could colonize also invertebrate hosts that might play a vector role. Several epidemiological studies in domestic mammals reported differences related to diffusion, localization of the yeast in the different sites of the body host (especially in dog and cat) and the species of *Malassezia* involved. In our experience on 178 dogs and 70 cats from 8 provinces of northern Italy, *Malassezia* spp. was found more frequently in dogs (62,9%) instead the cats (11,4%) (χ^2 51,30 p< 0,0001). Yeasts were often found at least in two different body sites, either in animal with and without otitis or dermatitis. It doesn't seem to be predisposition in sex or age for colonization and we don't found a breed predisposition in dogs, because of dispersion of the data, while some Authors assert that West Highland White Terrier, Basset Hound, Dachshund, Cocker Spaniel, Poodle, German Shepherd, Collies, Shetland, Jack Russell Terrier, Silky Terrier, Australian Terrier, Springer Spaniel and Shar Pei are predisposed to *Malassezia* dermatitis (Carlotti, 2001). Other Authors showed in Basset Hound a high number of yeasts in skin and mucous membrane in healthy dogs (Bond e Lloyd, 1997). Kennis *et al* (1996) reported a common colonization in perioral and chin skin in healthy dogs. Cafarchia *et al* (2005), in agreement with Bond *et al*., (1995), showed the perianal region was the most frequently colonized area (60.6%) while the inguinal region (3%) was the least, and the highest number of *Malassezia* yeasts was recovered from the perioral area and external ear canal. Dufait (1985) showed a comparable number of yeasts on the skin both in healthy and with dermatitis dogs, while Cafarchia *et al*. (2005), observed a higher yeast density in dogs with skin lesions compared to healthy dogs even in sites where there was no skin disorder. In our current experience, *Malassezia pachydermatis* was isolated from conjunctiva and oral mucous in 16 of 92 dogs examined (17.4%); Raabe *et al*, (1998) found these yeasts in dogs' faeces: this assume they can run through gastrointestinal apparatus surviving to the acid pH after lick of the skin.

Until 25 years ago, only two species of the genus *Malassezia* were known: *M. pachydermatis* and *M. furfur*, the first one in domestic carnivores, particularly in the dog, while in other domestic animals as swine, bovine and avian species, there was also the only lipid dependent species since than knew, *M. furfur* (Guillot *et al*., 1994). Afterwards the description of new lipid dependent species, the range of animals' hosts was enriched with many reports. Bond *et al* (1996) report-

Correspondence: Roberta Galuppi
Department of Veterinary Public Health and Animal Pathology,
Alma Mater Studiorum - University of Bologna,
Via Tolara di Sopra, 50 - 40064 Ozzano Emilia, Bologna, Italy
Tel. +39 051 2097059; Fax +39 051 2097039
e-mail roberta.galuppi@unibo.it

ed for the first time M*alassezia sympodialis*, a lipid dependent species, in cats. Subsequently other studies detected *M. furfur* and *M. globosa* on skin, mucous and external ear canal of healthy cats (Bond *et al*, 1997; Crespo *et al*, 1999; Corazza et al, 2001). *M. nana* was described in cats and in bovines (Hirai *et al*, 2004). *M. pachydermatis, M. obtusa M. globosa*, *M. sloffiae*, *M. furfur* and *M. sympodialis* were found in cattle as well (Duarte *et al*, 1999). *M. pachydermatis* (Didier, 2004) and *M. sloffiae* (Guillot *et al*, 1998; Uzal *et al*, 2007) were found in goats and recently a new lipid dependent species, *M. caprae* was also described (Cabañes *et al*., 2007). In swine *M. sloffiae* was mainly described (Guého *et al*, 1996; Matousek and Campbell, 2002). We reported the isolation of *M. sloffiae* associated to *M. sympodialis* from skin and external ear canal in healthy pigs (Galuppi *et al*, 2004). *M. sympodialis* (Senczek *et al*, 1999; Matousek e Campbell, 2002) and recently *M. equina* (Cabañes *et al*, 2007) were described in horse. *M. equi*, a new strain reported by Nell *et al*, (2002), is not recognized by various Authors because it is not reported a valid description or a type specimen. Lipid dependent species are commonly present on human skin, but also *M. pachydermatis* was found and the event of neonatal septicaemia in an intensive care unit (Marcon and Powell, 1992) is worthy to be mentioned. The infection was correlated to a carrier role played by the nurses and their own dog (Chang *et al,* 1998). This give rise to great interest about the zoonotic potential of the yeast, but until now, none extensive study concerning this question was carried out. *M. pachydermatis,* despite the isolation of lipid dependent species, is certainly the prevalent strain in dogs. Bonoli *et al* (2004) found this species more frequently (59.73%) than the lipid dependent species (4.03%) (χ^2 106.45; p<0,0001) while in cats (9.23% and 4.61% respectively) the difference was not significative. From dog skin it is possible to isolate strains that apparently behaved as lipid dependent yeasts, but belong to the species *M. pachydermatis* by kariotyping and they can grown on regular media after transfer (Bond and Antony, 1995). That induced heated debates between various Authors in the past (Raabe *et al,* 1998; Gueho and Guillot, 1999) and it leads us to the second point of this work.

Variability of *M. pachydermatis*

Among lipid dependent species of *Malassezia* several new species were described in the latest years unlike *Malassezia pachydermatis* is the only non-lipid-dependent species.

Many studies based on molecular, morphological and biochemical profiles show yet the heavy lack of homogeneity in this genus. For example two morphological microscopic types of cells are described: the former are small and ovoid (2.5-4.8 x 2.6-5.0 µm) and the second greater and club shaped (3.8-6.0 x 4.8-7 µm) (Sloff 1970; Kiss *et al*, 1996). In our studies we found a third cellular type, with round small cells (1.66 x 1.67 µm). In addition there are distinct colonial morphologies, on Sabouraud Dextrose Agar (SDA), with small or large colonies, not related with microscopic aspect (Huang *et al*, 1993). This is probably connected with the ability of growth in a medium without lipid supplement. We found various strains that did not growth on SDA at the first isolation but, after one or more passages on modified Dixon's agar, they had a good growth on SDA too (according to Bond and Antony, 1995). The 27.8% of strains isolated maintained a poor growth on SDA, with small, punctiform colonies, while the others had a good growth on SDA with large colonies according to Huang *et al* (1993) and Guillot *et al* (1997). Also most of the parameters used as biochemical profile for the differentiation of the lipid dependent species, are instead extremely variable in *M. pachydermatis*. For example it is described that *M.pachydermatis* growth at 40°C (Sloff, 1970; Guého *et al*, 1996; Mayser *et al*, 1997; Hammer e Riley, 2000; Hirai *et al*, 2004) while, in our study, only the 55.6% of the strains, isolated from dogs and cats of various provinces of Italy, had an abundant growth at this temperature, but 11 strains (13.9%) had more difficulty to growth and 24 (30.3%) did not growth absolutely, showing a greater variability than the literature. We found also very variability in the catalase reactions, depending on the different strains, in accord with many Authors: negative or weak (Guillot *et al*, 1996), poor (Hirai *et al*, 2004), markedly positive (Kiss *et al*., 1996). Using Tween test, according to Guillot *et al* (1996), *M. pachydermatis* should be able to appear on the entire surface of the agar; in some isolates the growth could be slightly inhibited directly around the four Tween and, in few isolates, to be even frankly inhibited by high concentration of Tween 20. In the tests performed we can observe that the strains with a good growth on SDA evenly grew on the whole plate, with different inhibition rings or halos around the different Tween wells. Unlike Guillot *et al* (1996), we found that the strains with low growth on SDA, had poor growth in the medium away from the lipid source, with pattern like to the lipid dependent strains. We also tested similarly the cremophor El and the wide variability of behaviour obtained doesn't agree with Mayser *et al* (1997), that claim this compound assimilated only by *M. furfur,* but concurs with Hirai *et al* (2004).

The presence of precipitates on mDixon agar was also variable and it was not present in 27.9% of the strains examined, in disagreement with Bond and Anthony (1995) that observed this phenomenon in all the strains they tested. The hydrolysis of esculin, according to Guého *et al*, (1996), Mayser *et al*,(1997), Hammer and Riley, (2000) and Hirai *et al*, (2004), was variable. The 83.6% of strains we tested, showed a black zone around the colonies, the 11.3% showed only a poor variation in colour, while 5.1% did not change colour

An evolution in the studies on the characterization of *M. pachydermatis* was due to molecular analysis. Anthony *et al* (1994), using RFLP (Restriction Fragment Length Polymorphism Analysis), showed 10 different patterns on 33 isolates of *M. pachydermatis*. Guillot (1997) investigated the diversity of *M.pachydermatis* isolates from a wide range of hosts by partial sequencing of the large subunit (LSU) of ribosomal

RNA. He discriminated seven type (Ia-Ig): the type Ia seems to be ubiquitous and was observed on domestic and wild carnivora, on monkey and human, while the sequences type Ic, Id and Ig seem to be more host-specific. The study indicated that the skin of an animal may be colonized by more than one type of *M pachydermatis* too. The type Id formed small colonies on SDA. Sugita *et al*., (2005) indicate that the sequence of the IGS region of *M. pachydermatis* has remarkable intra-specific diversity. Aizawa *et al* (1999; 2001) and Castellà *et al* (2005) with RAPD analysis using the same primer (FM1), found 4 genetic profiles, but the highlighted patterns are different each other.

In our RAPD experience with the same primer, we found a higher number of genotypes, according with Hossain *et al*. (2007) that observed more than four genotypes and indicate a high variability among strains of *M. pachydermatis*. Cafarchia *et al* (2007) using three distinct genetic markers in nuclear DNA reveal, on dog, multiple genetic variants of *M. pachydermatis*. This yeast could be in a state of differentiation and/or adaptation to a specific host, associated with an increasing dependency on exogenous lipid supplementation, explaining as the physiological features used to characterize the different *Malassezia* species are inaccurate.

The large genetics and biochemistry heterogeneity confirm the observation of Kurtzmann and Robnett (1994) that a high rate of nucleotide substitution exists among *Malassezia* spp. which far exceeds the levels of other yeast genera. These finding support the hypothesis of a rapid "molecular clock" resulting in enormous polymorphism in the genus.

Probably we are running after a fungus that runs more quickly than us.

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The genus *Malassezia*: old facts and new concepts

J. Guillot[1], S. Hadina[2], E. Guého[3]

[1]INRA, AFSSA, ENVA, UMR 956, Ecole Nationale Vétérinaire d'Alfort, Maisons-Alfort, France; [2]Faculty of Veterinary Medicine, University of Zagreb, Croatia; [3]Mauves sur Huisne, France

Abstract. Lipophilic yeasts are being considered as major opportunistic pathogens for a very long time. Most of the yeasts show an absolute requirement for long fatty acid chains and specific procedures are required for their isolation, conservation and identification. For that reason, the history of the nomenclature used for the *Malassezia* genus is quite complex. Before 1996, only 3 species were recognized: *Malassezia furfur*, *M. pachydermatis* and *M. sympodialis*. To date, the genus is composed of one non lipid-dependent species (*M. pachydermatis*) and 12 lipid-dependent species. No doubt that additional new taxa will be described in close future. Very recently the genome and secretory proteome of two *Malassezia* species was described. This analysis demonstrated the presence of multiple secreted lipases to aid in harvesting host lipids. It also revealed the presence of mating-type genes, providing an indication that *Malassezia* yeasts may be capable of sex.

Key words: *Malassezia*, yeast, species, taxonomy, nomenclature

For more than 150 years, *Malassezia* yeasts have been associated with skin diseases in humans and different animal species. In recent years these fungi have also been recognized as opportunistic pathogens causing invasive infections in neonates.

Malassezia species have an affinity for lipids as substrates and the term "lipophilic yeasts" has frequently been used to characterize the genus. In fact, most of the species show an absolute requirement for long fatty acid chains and they are therefore seldom isolated in the laboratory unless specific nutrients are provided in the medium. The cells of all *Malassezia* species have in common a monopolar and repetitive budding process (leaving a thick scar on the mother cell) and a multilayered cell wall with a corrugate innermost layer to which corresponds a helicoidal translucent band. However specific identification is not straightforward and history of the nomenclature used for the *Malassezia* genus is quite complex.

First descriptions

Malassezia yeasts were first recognized in 1846 when Eichstedt realized the fungal nature of pityriasis versicolor, a very common skin disease in humans. He reported the presence of round yeasts and filaments in scales of patients. The agent of pityriasis versicolor was named *Malassezia furfur* by Baillon in 1889. In subsequent years, lipophilic yeasts alone were observed by many authors in samples of healthy skin and also in conditions such as seborrheic dermatitis and pityriasis capitis (dandruff). In 1904, Sabouraud assigned these yeasts to the genus *Pityrosporum*. In 1913, Castellani and Chalmers described the species *P. ovale* by acknowledging an earlier name, *Saccharomyces ovalis* Bizzozero 1884 (Slooff 1970). In 1951, Gordon described a new *Pityrosporum* species, *P.orbiculare*. Although this was associated with healthy skin, he also identified the yeast with the organism *M. furfur*, the agent of pityriasis versicolor (Gordon 1951).

Weidman provided the first description of lipophilic yeasts from an animal species in 1925. He isolated yeasts with a similar morphology to that of *P. ovale* from skin lesions of an Indian rhinoceros. Weidman described the new species *P. pachydermatis*. In 1955, Gustafson isolated the same type of yeast from otitis externa in dogs. Gustafson wrongly concluded from early descriptions that the yeasts recovered from rhinoceros skin grew poorly and were very difficult to maintain. As a consequence, Gustafson created the new species *P. canis*. The synonymy between *P. pachydermatis* and *P. canis* was proved by Guillot & Guého in 1996.

The need to assign all lipophilic yeasts to a single genus was finally recognized by taxonomists. The genus *Malassezia* created by Baillon predates Sabouraud's *Pityrosporum*. As a consequence, the species mentioned above were all assigned to the genus *Malassezia* in recent reviews (Ahearn and Simmons 1998, de Hoog *et al*. 2000).

An increasing number of species

Before 1996, only 3 species were recognized within the genus *Malassezia*: *M. furfur* (Robin) Baillon 1889, *M. pachydermatis* (Weidman) Dodge 1935 and *M. sympodialis* Simmons and Guého 1990. In addition to the above species, stable variants identified by morphological and immunological features had been documented in the literature (Midgley 1989, Cunningham *et al*. 1990). However, these variants were proposed without a valid description or a type specimen. Genetic studies,

Correspondence: Jacques Guillot
Ecole Nationale Vétérinaire d'Alfort, Maisons-Alfort, France,
Tel 33 1 43 96 71 57; Fax 01 43 96 71 90
e-mail: jguillot@vet-alfort.fr

Table 1. Current composition of the genus *Malassezia*.

Species	Description	Isolated from humans	Isolated from animals
M. furfur	Baillon, 1889	• Healthy skin • Cutaneous lesions (mainly pityriasis versicolor) • Systemic infections	• Healthy skin
M. pachydermatis	(Weidman) Dodge, 1925	• Systemic infections	• Healthy skin (dogs, cats, many other animals) • Cutaneous lesions (mainly otitis and seborrheic dermatitis in dogs)
M. sympodialis	Simmons & Guého, 1990	• Healthy skin • Cutaneous lesions (mainly atopic dermatitis)	• Healthy skin • Cutaneous lesions (otitis in cats)
M. globosa	Midgley, Guého & Guillot, 1996	• Healthy skin • Cutaneous lesions (mainly seborrheic dermatitis or pityriasis versicolor)	• Healthy skin • Cutaneous lesions (otitis in cats)
M. obtusa	Midgley, Guillot & Guého, 1996	• Healthy skin • Cutaneous lesions	
M. restricta	Guého, Guillot & Midgley, 1996	• Healthy skin • Cutaneous lesions (mainly seborrheic dermatitis)	
M. slooffiae	Guillot, Midgley & Guého, 1996	• Healthy skin • Cutaneous lesions	• Healthy skin (in pigs) • Cutaneous lesions (otitis)
M. dermatis	Sugita *et al*., 2002	• Healthy skin • Cutaneous lesions	
M. nana	Hirai *et al*., 2004		• Cutaneous lesions (otitis in cat and cattle)
M. japonica	Sugita *et al*., 2003	• Healthy skin • Cutaneous lesions	
M. yamatoensis	Sugita *et al*., 2004	• Healthy skin • Cutaneous lesions	
M. caprae	Cabanes *et al.*, 2007		• Healthy skin (mainly in goats)
M. equina	Cabanes *et al*., 2007		• Healthy skin (mainly in horses) • Cutaneous lesions (in horses)

which included DNA/DNA relatedness and comparison of 26S rRNA sequences from a large number of isolates, confirmed the presence of phenotypical variants and clearly indicated the need to create new *Malassezia* species (Guillot and Guého 1995). Consequently, lipid-dependent species *M. globosa, M. slooffiae, M. restricta* and *M. obtusa* were described by Guého *et al*. in 1996.

More recently, the number of lipid-dependent *Malassezia* species increased. New species were isolated from human skin: *M. dermatis* (Sugita *et al*., 2002), *M. japonica* (Sugita *et al*., 2003) and *M. yamatoensis* (Sugita *et al*., 2004). Others were isolated from animals: *M. nana* (from cat and cattle) (Hirai *et al*., 2004), *M. caprae* (mainly from goats) and *M. equina* (mainly from horses) (Cabanes *et al*. 2007). To date, the genus *Malassezia* is composed of one non lipid-dependent species (*M. pachydermatis*) and 12 lipid-dependent species (Table 1). No doubt that additional new species will be described in close future.

New concepts

In a recent paper, Xu *et al*. (2007) described the genome and secretory proteome of two *Malassezia species (M. globosa* and *M. restricta*). They suggested that the lipid dependence of these species can be explained by the apparent absence of a fatty acid synthase gene. In parallel, the presence of multiple secreted lipases to aid in harvesting host lipids was demonstrated. The genome of *M. globosa* revealed the presence of mating-type genes, providing an indication that *Malassezia*

may be capable of sex. The genus *Malassezia* has been placed among the *Basidiomycota* (Ahearn and Simmons 1998), forming a monophyletic lineage, classified as Malasseziales, related to the Exobasidiomycetes (Ustilaginomycotina, Basidiomycota) (Begerow *et al*., 2000). Although no teleomorph has been described for any of the *Malassezia* species, their affinities have been indicated by the possession of characteristics that are found in other basidiomycetous yeasts. The genomic and proteomic analysis provided by Xu *et al*. (2007) highlighted an apparent conflict between phylogeny and host-specific adaptation: genome- and proteome-based data proved that basidiomycetous *Malassezia* species share similar sets of extracellular hydrolases with the phylogenetically distant *Candida albicans* that occupies an overlapping skin-related niche. In contrast, *Malassezia* species appear closely related to the plant pathogen *Ustilago maydis*. However, all these species are easily distinguished by differences in their sets of extracellular hydrolases and their host preferences.

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Diagnostic and clinical features of animal malasseziosis

S. Nardoni[1], M. Corazza[2], F. Mancianti[1]

[1]Dipartimento di Patologia Animale, Profilassi ed Igiene degli Alimenti, Università di Pisa. Viale delle Piagge, 2 56124 Pisa; [2]Dipartimento di Clinica Veterinaria, Università di Pisa. Via Livornese lato monte, San Piero a Grado Pisa.

Abstract. *Malassezia* yeasts infection represents a common clinical concern with a special regard to canine dermatology. The Authors review the main clinical features of malasseziosis in canine and feline medicine, summarizing predisposing factors and aetiopathogenesis of the yeasts' infection. A special reference was given to clinical and microscopical diagnosis.

Key words: *Malassezia*, diagnosis, otitis, dermatitis, dog.

The genus *Malassezia* includes 13 species of lipophilic, nonmycelial, unipolar budding yeasts characterized by a thick cell wall. *Malassezia* spp. are component of the cutaneous mycoflora of many warm-blooded animals included man. *Malassezia pachydermatis*, which is the sole not lipidodependent species, in dogs is considered as a commensal yeast and probably adheres to the skin corneocytes by tripsin-sensitive protein adhesion molecules. It is frequently isolated from the external ear canal, from the skin, particularly the anal area which could be a carriage zone, oral mucosa, vagina and eye of healthy dogs (Chen T and Hill PB, 2005; Prado MR et al., 2008; Scott DW *et al*., 2004). *M. pachydermatis* is also recovered from the distal hair in healthy dogs, but hair follicle carriage is infrequent and at that site yeast burden is low. In healthy dogs, *Malassezia* yeasts were most frequently isolated in the perianal and perioral areas, and in a study it has been established that *Malassezia* spp. are present in 15.7% of dogs in anal sac content, with no difference between healthy dogs and dogs with *Malassezia* dermatitis associated with atopic dermatitis.

Despite *Malassezia* spp. are part of normal skin mycoflora, the yeast may become pathogen in certain circumstances. In dogs with atopic dermatitis there is indirect evidence of transepidermal penetration of antigens and subsequent phagocytosis by Langherans cell that present antigen to T-cells initiating the cascade of immunologic responses. This leads to the destruction of the yeasts or to their mechanical removal via scaling. The pathogenic role of *Malassezia* spp. yeasts is unknown and it seems to be mainly related to a disturbance of the normal physical, chemical or immunological mechanisms that allow *Malassezia pachydermatis* to multiply and to become pathogenic. Variation of antigenic expression in different growth phases of *M. pachydermatis* could explain discrepancies among studies about immune response to the yeasts.

Correspondence: Simona Nardoni
Dip Patologia Animale, Profilassi ed Igiene degli Alimenti, Università di Pisa, Viale delle Piagge, 2 56124 Pisa (Italy)
Tel + 39 050 2216952; fax + 39 050 2216941
e-mail: snardoni@vet.unipi.it

Skin abnormalities enhancing *Malassezia* overgrowth are: excessive moisture and amount of sebum or cerumen, disruption of the epidermal barrier and intertrigo. The most common diseases acting as underlying causes of *Malassezia* dermatitis are allergies, pyoderma, demodicosis, keratinization disorders and endocrine disturbancies. Otitis are commonly complicated by yeasts overgrowth. *Malassezia* spp. were isolated from 41.2%-72.9% of cats and from 57.3%-62.2%of dogs with otitis externa. *M. pachydermatis*, either as a pure culture or in association with lipid-dependent species, was identified in most of all specimens (97%). (Nardoni S *et al*., 2004; Cafarchia C *et al*., 2005; Nardoni S *et al*., 2007)

Immunological dysfunction could also promote growth of the *Malassezia* population on the skin. For instance, epidermal dysplasia of the West Highland White Terrier could be associated with a genetic predisposition to a poor response of T-cells towards the yeast, despite the inability of yeast to stimulate keratinopoiesis.

Malassezia spp. produce enzymes, such as phospholipases, that alter the cutaneous lipidic film, pH and proteases that induce inflammation and pruritus through proteolysis and complement activation. In facts, the frequency of isolation and population size of *Malassezia* species were higher in dogs with localized dermatitis, especially in affected areas, indicating a role of *Malassezia* in the occurrence of skin lesions.

Dogs with *Malassezia* dermatitis have greater concentration of specific IgG than normal subjects, whereas atopic dogs, with or without concurrent *Malassezia* dermatitis, have higher levels of specific IgG and IgE than non-atopic dogs with *Malassezia* dermatitis or normal dogs. Skin testing with a *Malassezia* extract shows immediate hypersensitivity reactions and atopic dogs with cytologic evidence of *Malassezia* dermatitis had an increased lymphocyte blastogenic response to crude *M. pachydermatis* extracts, compared with clinically normal dogs and dogs with *Malassezia* otitis. In a study on atopic dogs, no statistical correlation between the presence of cutaneous alterations and *Malassezia* isolation was detected. Highest scores were not exclusively found on affected areas, but also on lesion-free sites, demonstrating that atopic animals can be heavily colonized also in apparently healthy areas.

There is no age or sex predilection, but *Malassezia* dermatitis or otitis are more often diagnosed in dogs between 1 to 3 years of age. Many breeds seem to be predisposed to *Malassezia* dermatitis: West Highland White Terrier, Basset Hound, Dachshund, Cocker Spaniel, Poodle, German Shepherd, Collies, Shetland, Jack Russell Terrier, Silky Terrier, Australian Terrier, Springer Spaniel, Newfoundland and Shar Pei. *Malassezia* dermatitis is often recognized during warm period at the time at which allergic dermatites are generally diagnosed. *Malassezia* overgrowth or dermatitis is not demonstrated to be contagious for companion animals or man (Morris DO, 2005). In certain breeds, for example Shar Pei and Newfoundland, clinical signs may be particularly severe and epidermal dysplasia in WHWT might be an inflammatory or hypersensitivity reaction to the *Malassezia* infection or a result of excessive self-trauma, rather than a congenital keratinization disorder.

Pruritus is always present but its severity is related to the importance of predisposing factors. Diffuse, regional or localized alopecia, lichenification, hyperpigmentation, erythema, erythematous macules, excessive scaling, and oily skin and hair are the clinical features of canine *Malassezia* dermatitis. The classical areas involved are neck, axillae, abdomen, pinnae and external ear canal, lips, and peri-anal area. Dogs with generalized lesions have a rancid odour. In allergic cats multifocal alopecia, erythema, crusting and greasy adherent brownish scales are signs of *Malassezia* overgrowth (Toma S *et al.*, 2006). The distribution is depending from the concurrent diseases, but face, ventral neck, pinnae and ear canal, chin, interdigital and claw fold skin are the most common site of infection. Especially in Devon Rex cats, high number of yeasts in cytology is associated with an abundant brown, greasy material in this natural intertrigo area (Colombo S *et al.*, 2007).

Malassezia dermatitis is suspected upon clinical evidence and diagnostic methods used to identify overgrowth of *Malassezia* organisms. The response to treatment with specific antifungal therapy is considered the best tool for a definitive diagnosis. Cytological examination allows to rapidly observe yeasts and to "quantify" their number. Many techniques are available to obtain material from the skin: 1) direct impression smear; 2) acetate tape (Scotch test); 3) scrape smear; and 4) swab smear. Impression smear and Scotch test seem to be the best techniques if the skin surface is flat or greasy, whereas swab smears should be more useful for cytological examination of the external ear canal. Heat-fixing does not seem to increase numbers of *Malassezia* on cytology of ear swab samples for cytologic evaluation, and a 1-step dip in the blue reagent alone as a rapid method of staining samples from canine ear canals has been proposed (10). Slides or acetate tape may be stained with Diff-Quick or other rapid methods, May Grünwald-Giemsa, Giemsa or new methylene blue. Microscopic examination with an oil immersion lens (100X) reveals free or adhered to keratinocytes yeasts appearing as oval or elongated cells of 3 to 5 µm in diameter, with a typical single polar budding. The minimum number of yeasts that indicates the possibility of a true *Malassezia* dermatitis is not really known. Variations between breeds and body sites have to be considered. Several criteria has been proposed to establish *Malassezia* overgrowth; as a general guide, 1-2 organisms per field (100X) in several fields in the presence of typical clinical signs are suggestive of *Malassezia* dermatitis. Methods to sample *Malassezia* from the skin for culturing are cotton swabs, acetate tapes, detergent scrubs and contact plates. Appropriate media for the growth of all *Malassezia* species are mDixon agar and modified Leeming and Notman agar, while selective medium for dermatophytes allows the growth of *M.pachydermatis* only. As the yeast is a normal component of the cutaneous flora of the dog, by itself a positive culturing has no or little value. However, as for all opportunistic agents, the number of colonies is perhaps an indication (this is comparable to the number of yeasts demonstrated by cytological examination). Dermohistopathology may sometimes show the yeasts on the surface of the epidermis and occasionally in the infundibula, particularly in PAS stained sections (although they are occasionally visible on HE stained sections). However, if they are not seen on biopsy, this does not exclude their presence. False negative results could be caused by the sampling of a non-infected area, removal of the stratum corneum during processing, etc. Cutaneous histopathology is a less sensitive technique than cytology. In contrast, the finding of *Malassezia* inside hair follicles could indicate a real pathogenicity. The common findings in biopsies from dogs with *Malassezia* dermatitis, include: orthokeratotic hyperkeratosis with prominent foyers of parakeratosis; spongiosis with irregular ridges; lymphocytic exocytosis of the epidermis; focal accumulation of neutrophils; subepidermal linear alignment of mast cells and moderate superficial perivascular to interstitial dermal inflammation with lymphocyte exocitosis. Clinical signs of *Malassezia* dermatitis are variable and may mimic many dermatoses, then differential diagnosis includes many pruritic dermatoses characterized by erythema, hyperpigmentation and seborrheoa together with all underlying dermatological diseases of the yeasts overgrowth.

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Management of *Malassezia*-related diseases in the dog

A. Peano, M.G. Gallo

Dipartimento Produzioni Animali, Epidemiologia ed Ecologia Università degli Studi di Torino

Abstract. Most cases of *Malassezia* dermatitis/otitis in the dog are associated with concurrent dermatoses or systemic diseases and recurrences are not uncommon. Recognition and control of the predisposing factors are therefore key factors for successful therapy and prevention of recurrent infections. Currently, *Malassezia* dermatitis/otitis is managed by the use of antifungal drugs. Systemic therapy is often necessary, in particular when clinical signs are severe and widespread. Ketoconazole and Itraconazole are the most commonly used drugs. Topical therapy is an alternative in case of localized lesions and external ear localizations. Different commercial formulations, available in clinical practice in form of creams, gels, lotions, sprays and ear drops are often used as adiuvants to systemic therapy. Topicals more frequently used are represented by imidazolic antifungals, chlorhexydine and lime sulphur. The presentation deals with more recent advances about the protocols for treatment of *Malassezia*-related diseases in the dog. New perspectives, as the use of natural compounds, immunotherapy and inhibitors of yeast adherence factors, are also discussed.

Key words: *Malassezia pachydermatis*; treatment; antifungals; dermatitis; otitis

Overgrowth of *Malassezia pachydermatis* organisms on canine epidermidis does not appear to be a self-resolving condition as it is usually secondary to a skin or systemic disorder. Changes in the cutaneous microenvironment, such as increased humidity and changes in lipids and sebum, and failure of topical and systemic immune mechanisms to protect the host against the yeast proliferation can lead to pathogenicity and various disease states with, in some cases, hypersensitivity reactions to the yeast itself. By causing these changes, various diseases have been suggested as underlying causes of *Malassezia* dermatitis with or without ear external canal involvement: hypersensitivity diseases, especially atopic dermatitis, parasite infestations, keratinization disorders, endocrine diseases, bacterial infections (Plant *et al*. 1992; Scott *et al*. 2001; Chen *et al*. 2002; Greene 2007).

Therapeutic approaches therefore rely on the treatment of yeast infections and management of the underlying problems. The hypersensitivity nature of this disease in some patients is emphasized by the response to antifungal therapy described in dogs with classical clinical findings and various surface sampling techniques demonstrating little or no yeast. This leads also to consider that the diagnosis of *Malassezia* dermatitis, based on cytological and cultural examination, ultimately rests on the response to antiyeast treatment (Scott *et al*. 2001).

Variations in the in vitro susceptibility to antifungal drugs have been reported for the different *Malassezia* species (Hammer *et al*. 1999; Velegraki *et al*. 2004). Most *M. pachydermatis* strains isolated from humans or dogs have been found susceptible to the antifungals amphotericin B, albaconazole, bifonazole, ciclopiroxolamin, econazole, ketoconazole, itraconazole, clotrimazole, miconazole, voriconazole, nystatin, pimaricin, terbinafine (Gupta *et al*. 2000; Nakamura *et al*. 2000; Garau *et al*. 2003; Cole *et al*. 2007; Lyskova *et al*. 2007) while few strains seem to be resistant to fluconazole (Lyskova *et al*. 2007) and most strains to flucytosine (Garau *et al*. 2003). Ketoconazole seems to be more active than clotrimazole and miconazole and equivalent to itraconazole (Cole *et al* 2007). In a recent survey some strains isolated from dogs with otitis externa were considered resistant to clotrimazole and miconazole, but the interpretation of in-vitro susceptibility/resistance was not achieved following a NCCLS guideline (Rougier *et al*. 2005). Among antiseptics chlorhexydine has been proved as an antiyeast compound (Lloyd and Lamport 1999; Lloyd and Lamport 2000; Nebbia *et al*. 2008). Also miscellaneous topical agents like selenium sulphide and lime sulphur possess anti-*Malassezia* properties (Scott *et al*. 2001).

Formulations of the commercial products have been shown to play a role in the final efficacy as they contain other substances that may, for example, act in synergy or permit a better in-vivo diffusion of the antifungal principles (Lloyd *et al*. 1999; Nebbia *et al*. 2008).

Studies on alternative therapeutic agents to the commonly used antimycotic and antiseptic synthetic substances have demonstrated an in-vitro anti-*Malassezia* activity for the essential oil of *Melaleuca alternifolia* (Weseler *et al*. 2002), for β-Thujaplicin, chemical substance obtained from the trunks/branches or roots of the conifer *Thujopsis dolabrata* (Nakano *et al*. 2005) and for xanthorrhizol, a bioactive compound isolated from the edible plant *Curcuma xanthorrhiza* (Rukayadi and Hwang 2007). *M. pachydermatis*-related dermatitis and otitis are often very difficult to control, with

Correspondence: Andrea Peano
Dip. Produzioni Animali, Epidemiologia ed Ecologia
Università degli Studi di Torino, Via Leonardo da Vinci 44,
10095 Grugliasco (To), Italy
Tel +39 011 6709001, Fax +39 011 6709000,
e-mail: andrea.peano@unito.it

repeated recurrences, and often require long-term antifungal agent therapy. Therefore these "natural" compounds showing in-vitro inhibition of the yeast growth may be studied as "maintenance" topicals in case of recurrent *Malassezia* infections. Moreover the appearance of resistant strains of *M.pachydermatis* to available antifungal agents is a concern (Nakano *et al.* 2005).

The clinical relevance of the in-vitro data is indirect because many pharmacokinetic factors participate in the final efficacy. For example penetration and accumulation of the antifungals in the stratum corneum, as well as synergism or counteracting effects mediated by components of the stratum corneum (Piérard *et al.* 2004). An ex-vivo bioassay, based on studying yeast growth on stratum corneum harvested by cyanoacrilate skin surface stripping, has been developed to complement the classic in-vitro tests (Rurangirwa *et al.* 1989). In human medicine corneofungimetry has been used to test antifungal compounds after applying them topically (Arrese *et al.* 1995) or after oral intake (Piérard *et al.* 2004) by health volunteers. By this technique it was possible to test the final formulations of the antifungals as they are used in clinical practice and the fungal strains tested, among which there were several *Malassezia* species, were allowed to grow on human stratum corneum, natural substratum for superficial dermatomycoses (Rurangirwa *et al.* 1989; Piérard *et al.* 2004). Concerning *Malassezia* dermatitis of dogs similar studies would permit to overcome in-vitro testing drawbacks by testing the final product formulations that, as above reported, have been shown to play a role in the final efficacy of commercially available products (Lloyd *et al.* 1999; Nebbia *et al.* 2008). In addition *Malassezia* strains may be cultivated directly on their natural substratum. Unlike many bacteria and other fungi *Malassezia* yeasts are rarely found in the environment, their habitat being primarily the skin and mucosae of mammals and birds (Chen and Hill 2005). Most in-vivo studies have been assessed on dogs with spontaneous *Malassezia* dermatitis and/or otitis, while a few have been carried out on dogs with induced infection (Uchida *et al.* 1992; Nascente *et al.* 2005). Some of the compounds showing in-vitro efficacy against *Malassezia* are successfully used in clinical practice either using systemic or topical approach. Different treatment protocols are effective. The commonly used systemic agents for *Malassezia* dermatitis in dogs are the azoles. Ketoconazole and itraconazole are given at 5-10 mg/kg^{-1} per day per os for 3 or 4 weeks (Morris *et al.* 1999; Matousek and Campbell 2002). Itraconazole is also effective with a pulse regimen: 5 mg/kg^{-1} on 2 consecutive days per week for 3 weeks (Pinchbeck *et al.* 2002). Recently oral terbinafine at 30 mg/kg^{-1} every 24 h has been shown to be a possible alternative to azole derivatives (Guillot *et al.* 2003; Rosales *et al.* 2005). The main advantages of this compound are represented by its good oral tolerability with no side effects demonstrated in man rodents, cats and dogs (Gupta *et al.* 1994; Mancianti *et al.* 1999; Castanon *et al.* 2001; Rosales *et al.* 2005) and its persistent clinical effect after cessation of the treatment due to residual tissue levels (Gupta *et al.* 1994). Azoles, chlorhexydine, natural (*Melaleluca alternifolia* oil) and miscellanous (selenium sulphide, lime sulphur) compunds are used in various forms of topical commercial products (shampoos, sprays, ointments, foams, gels, ear drops), often as coadiuvants to systemic therapy (Lloyd and Lamport 1999; Scott *et al.* 2001; Nebbia *et al.* 2008). Topical therapy is sometimes used prophylactically for recurring cases due to uncontrollable underlying factors (Morris et. al 1999).

Although in-vitro anti-*Malassezia* activity of different antifungals has repetitively been proved, commonly used commercial formulations containing the same principles often demonstrate lower in-vivo efficacy, with maintenance of yeast abnormal overgrowth or rapid recurrences. This is true especially for otologic formulations. Actually in the dog in-vitro results on strains isolated from clinical practice are not standardized tools for prediction of in-vivo response to drugs of *M.pachyermatis.* Poor responses to therapy and recurrences confirm that many important factors which mediate the initiation of inflammation or the developing of histological changes depend on dog/yeast immune relationship. Different evidences support, in atopic dogs, the role of hypersensitivity to the yeast in the pathogenesis of canine dermatitis/otitis by *Malassezia* (Farver *et al.* 2005). Secondary microbial infections can both initiate and perpetuate episodes of atopic dermatitis in dogs and humans, and could even participate in promotion of proallergic immunologic responses (DeBoer and Marsella 2001). Atopic dogs with *Malassezia* dermatitis show immediate skin test reactivity to the yeast antigens whereas atopic dogs without *Malassezia* overgrowth have generally negative skin test results (Morris *et al.*1998). Some dogs with typical cutaneous signs and low number of yeast at cytological examination show a good clinical response to antifungal therapy (Scott *et al.* 2001). Significantly higher concentrations of *Malassezia*-specific IgE have been detected in atopic dogs with or without yeast overgrowth than either healthy dogs or non atopic dogs with *Malassezia* dermatitis (Nuttall and Halliwell 2001). Hyposensitization to *Malassezia* by immunotherapy has then been proposed as an alternative to extended or repeated administration of antifungals (Morris *et al.*1998; Morris *et al.* 2002; Morris *et al.* 2003). Actually *Malassezia* antigens are often either included in skin test or IgE in-vitro panels for diagnosis of canine atopy and consequently in "vaccines" used to hyposensitize atopic dogs. Further investigations are required to determine whether immunotherapy for *Malassezia* type-1 hypersensitivity is really beneficial. Actually cases for which immunotherapy appear to be successful often receive concomitant other allergens and/or topical and systemic antifungal compunds. It is therefore questionable whether the positive response is due to hyposensitization to *Malassezia* or either to successful immunotherapy against other allergens and/or

elimination of yeasts by antifungals (Nett at al. 2001). The precise immunologic alterations occurring during yeast dermatitis in the dog remain to be determined with further studies.

Adherence is thought to play an important role in colonization and infection of dog by *M.pachydermatis* (Bond and Lloyd 1998a; Masuda *et al*. 2001). Yeast cell adherence to canine corneocytes seem to be mediated by trypsin-sensitive proteins or glycoproteins (Bond and Lloyd 1996) and lipids (Masuda *et al*. 2001). Use of inhibitors of the in vivo adherence factors of *M.pachydermatis* has recently been hypothesized as an "ecologically" form of therapy and prophylaxis (Bond and Lloyd 1998a; Masuda *et al*. 2001). Studies about this issue have not yet confirmed this hypothesis. For example enhanced adherence of the yeast to corneocytes does not appear to be important in the pathogenesis of canine seborreic dermatitis (Bond and Lloyd 1998b). Moreover, given the apparent variability between strains, it may be prove difficult to identify a universal inhibitor (Bond and Lloyd 1998a).

The data above discussed indicate that for management of canine *Malassezia* dermatitis/otitis the effectiveness of novel approaches alternative to antifungal therapy requires further confirmations.

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Essential oils in medicine: principles of therapy

F. Pisseri[1], A. Bertoli[2], L. Pistelli[2]

[1]Scuola CIMI-Koinè; [2]Dipartimento di Chimica Bioorganica and Biofarmacia, Università degli Studi di Pisa

Abstract. Essential oils (EOs) are extremely complex mixtures containing compounds of several different functional-group classes. A specific aromatic profile should be determined by gas-chromatography-mass detection methods, to define standards for their safety and efficacy. The chemical constituents of the essential oils, their flavour and their taste act both alone and in synergy, always determining a global psychosomatic action. The main therapeutic activities of the EOs are reported as spasmolythic, revulsive, anti-inflammatory and decongestant, immunomodulant, antimicrobial, antimycotic, expectorant, mucolythic, antioxidant, psychotrope, analgesic and acaricide. The use, posology, route of administration as well as toxicity and adverse effects are reviewed.

Key words: EOs, aromatherapy, therapeutic activity, adverse effects

Introduction

Essential oils (EOs) are extremely complex mixtures containing volatile aroma compounds of several different functional-group classes, extracted from plants. Nowadays, many International and European guidelines pertaining to therapeutic profiles for several market valuable EOs have been set. In fact it is extremely important to distinguish between the "therapeutic grade" and "fragrance grade" of EOs. A "fragrance grade" may show a very high quality (grade A quality) but, not necessarily, it can be considered for therapeutic purpose.

The EOs' quality must be defined before using them as herbal remedy in order to warrant the safety for customers. True therapeutic-grade oils must contain neither synthetic ingredients nor can be diluted. Furthermore balsamic period and way of distillation should be known, without any further addition of synthetic additives. The EOs should not show residual agricultural pesticides, herbicides nor other chemicals and cannot be extracted by solvents. Significant chemical differences in the EOs' composition depend on geographical area where the plant material was wild or cultivated, on the balsamic period, on the plant organ considered and on the extraction's technique. Therefore, it is important to define the best chemotype for therapeutic uses in order to fix the percentage of active constituents and reduce as least as possible the risk of toxic plant derivatives. For all these reasons, each lot production of EO should be related to a specific aromatic profile determined by gas-chromatography-mass detection methods. Only in this way, the safety and efficancy of EO therapy is warranted.

Correspondence: Francesca Pisseri
Dipartimento di Clinica Veterinaria,
V.le delle Piagge 2, 56125 Pisa
Tel +39 338 1406770,
e-mail: pisseri@vet.unipi.it

Therapeutical activities

EOs yield a polyvalent therapeutic activity whose mechanisms of action are not completely clarified, due to their complexity, and to a lack of clinical studies, with an adequate follow-up.

Nevertheless EOs' activity can enhance vital functions in many ways and some of these products show a specific tropism to influence different organs and/or apparatus.

The chemical constituents of the essential oils, their flavour and their taste act both alone and in synergy, always determining a global psychosomatic action.

The physician should make a critical evaluation of the available products for sale, considering that the composition and the quality of the EOs strongly influence both their therapeutic potentialities and toxicological risks.

The EOs' activity on the organism derives from the complex and associated action as well as for every phytocomplex.

The principal therapeutic activities of the EOs with some references to specific oils are listed below:

(i) Spasmolythic properties: *Citrus lemon, Pimpinella anisum, Rosmarinus officinalis, Foeniculum vulgaris, Mentha piperita, Thymus vulgaris*

(ii) Revulsive properties: *Melaleuca alternifolia, Citrus lemon, Mentha piperita*

(iii) Analgesic action: *Cymbopogon* spp*., Mentha piperita*

(iv) Anti-inflammatory and decongestant properties: *Salvia sclarea, Matricaria chamomilla, Citrus sinensis, Rosmarinus officinalis, Citrus lemon*

(v) Immunomodulant properties: *Citrus lemon, Thymus vulgaris, Rosmarinus officinalis, Origanum vulgaris*

(vi) Antimicrobial properties: *Melaleuca alternifolia, Thymus* spp*., Satureja* spp*., Citrus bergamia, Origanum vulgaris, Illicium verum, Ocimum basilicum, Matricaria recutita, Salvia officinalis, Satureja montana, Origanum majorana*

(vii) Antimycotic properties, proven both in vitro and in vivo: *Melaleuca alternifolia, Thymus vulgaris, Origanum vulgaris, Citrus lemon, Ocimum basilicum, Origanum majorana*
(viii) Expectorant action, mucolythic: *Eucalyptus* spp., *Thuja spp. , Pinus* spp.
(ix) Antioxidant action: *Rosmarinus officinalis, Citrus lemon*
(x) Psychotrope properties, with evident effects on both human and animal behaviour (anxiety, dementia), olfactory action, with psychological and neurological effects, especially in brain areas codifying for emotions: *Lavandula vera, Citrus aurantium, Anthemis nobilis, Melissa officinalis*
(xi) Acaricide properties: *Eugenia caryophyllata, Lavandula officinalis*

Clinical uses

EOs can be used internally or externally. It's important to notice the route of administration considering that in any case, being the oils highly volatile and fat-soluble substances, they are easily absorbed through the skin, mucous membranes or when inhaled as well as administered per os.
The internal use should be very carefully monitored since some EOs have a very low therapeutic index. The quality and the chemotype of the oils should be carefully considered.
The dermatology essential oils therapy can be based on aetiology (bacterial or fungal infections), symptoms (itching, flogosis, etc.) and used as a way to balance the skin. In fact EOs could stimulate both the immune system and the cheratocytes for improving local defences, the horny layer and lipidic intercellular material.
Their use can be advised as an alternative or together with conventional therapies for diseases such localized or widespread pyodermitis, seborrhoic dermatitis, mycoses, acne, dry and furfuraceous dermatitis.
The use of *Melaleuca* spp. and *Lemon* spp EOs in case of allergic dermatitis should be very carefully evaluated to avoid allergic reactions, considering also that some oils of the Labiatae family show crossed hypersensitivity. *Eucalyptus* spp. and *Pinus* spp. administered by inhalation act on the respiratory system for their espectorant and mucolythic effects, while *Rosmarinus officinalis* and *Foeniculum vulgaris* can be used on the digestive system for their spasmolythic action both on the intestinal tract and biliary ducts.
EOs should be mixed into a suitable excipient for both local and internal prescriptions. Almond oil seems to be a particularly suitable carrier for dermatological preparations applied to the skin or in the external ear, since it is well tolerated, easily absorbed and spreads to the dermic layer allowing the EOs to have a good absorption.
Almond oil is obtained through the cold pressing of *Prunus dulcis'*s seeds and is a well known eudermic, with a marked hydrating, emollient, lenitive activity. Some polyphenols with an antioxidant activity have been recently identified (Takeoka and Dao, 2003; Amico *et al*., 2006) in its composition and its polysaccaridic fraction has shown a both *in vivo* and *in vitro* stimulating activity of lymphocytes of rat. These reasons make it not a simple carrier only, since it actively contributes to improve the skin balance altered in dermatological diseases.

Prescription

The prescriptive technique of the essential oils can follow two different ways, either sectorial prescribing an EO according to its components related to the clinical evidences from literature and to patient's clinical status either a more complex and holistic way where all the oils' activities are considered, including the actions on the psychological and emotional level. Therefore the prescription in the second way considers the patient's psychological and physical characteristics.
We believe that the two ways can be integrated successfully.
For the antimicrobial and antimycotic prescription it would be appropriate to carry out an *in vitro* evaluation of such activities, because the sensitivity to EOs of different mycotic or bacterial strains may be variable.

Posology

For cutaneous administration, EO's amounts vary according to the oil, and to patient's clinical status. For human use the concentration is ranging from 1% to 10%, for veterinary use from 5% to 50%, due to the different thickness of the skin and the presence of the hair. A careful evaluation should be performed for the systemic use, considering the risks of toxicity, with a posology varying from 0,1 to 0,3 mg/kg/day.

Toxicology and adverse reactions

Acute, subacute and chronic toxicity following to EOs' administration have been reported. In dermatological prescriptions local reactions as direct irritation, from the first application, is frequently observed. The reaction is early developed, and its severity depends on the amounts of the irritant substances. EOs with a high ratio of phenols such as *Citrus lemon, Origanum vulgaris, Thymus vulgaris* can yield a skin irritation. Hypersensitivity, immunitary reaction, both type 1, including anaphylaxis and atopia, and cell-mediate reactions or delayed hypersensitivity (inflammatory reaction after 12-24h) with eczema and dermatitis has been referred as a consequence of EOs' use.
Several components of the oils have a dose-dependent general toxicity. The phenolic terpenes can be caustic, give renal irritation and lesions to the intestinal mucous membrane; some ketones have a strong neurotoxicity and tend to be accumulated in the organism; the non-oxigenated terpenes are blistening for skin and mucous membranes.
In case of inappropriate cutaneous applications at very high dosages (pure Tea Tree Oil had been used, 20 ml/adult cat) symptoms as depression, incoordination and muscular tremors are found (Bischoff *et al*., 1998).

An ototoxic effect of tea tree oil (*Melaleuca alternifolia*) has been reported, which counter-indicates the otological administration.

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Uncommon cases of pityriasis versicolor

C. Romano

Dermatologic Section of the Department of Clinical Medicine and Immunological Science. University of Siena, Italy

Abstract. *Malassezia* may play a role in several dermatoses. It is responsible for foliculitis and mainly for pityriasis versicolor. Pityriasis versicolor is the most known dermatitis because of its clinical aspects and frequently for its poor response to the therapy, mainly in chronic forms. The clinical aspects of uncommon and rare forms of pityriasis versicolor have been reported. The data related to the patients observed in the last thirty years in Siena are reported. In addition, a study was carried out in Pisa by Professor F. Mancianti to identify species of *Malassezia* isolated in 37 patients.

Key words: Pityriasis versicolor, mycoses, *Malassezia*

Malassezia is responsible for pityriasis versicolor (PV) and folliculitis, and it is hypothesized that play a role in the pathogenesis of seborrhoeic dermatitis (SD), and atopic dermatitis (AD). Nevertheless the most frequent dermatological disease caused by this mycete is certainly the pityriasis versicolor, which commonly manifests with hyperpigmented or achromic macules, according to the season. Lesions are most frequent on the seborrhoeic areas, such as the upper trunk, neck and upperarms. Besides the typical manifestations, there are those ones which are uncommon due to their clinical aspects (pityriasis versicolor atrophicans and pityriasis versicolor rubra) or due to their sites (pytiriasis versicolor areolar e periareolar, penile, in the groins, perianal, palmar and plantar areas). There are even forms thet affect almost all body surface.

Crowson and coll. reported twelve clinical cases in which lesions characterized as patches, macules, and atrophic plaques prompted clinical differential diagnosis with atrophy due to intralesional steroid therapy, collagen diseases or parapsoriasis. However, histologic examination showed hyphae and spores in the corneum layer, variable epidermal and dermal atrophies, reteridge effacement, subepidermal fibroplasia, pigment incontinence and elastolysis.This form was defined atrophying pitiriasis versicolor (1, 2).

Pitiriasis versicolor rubra clinically presented as soybean-sized, red colored macule. At first it was primarily reported by Horiuchi, and recently six cases attributed to *Malassezia sympodialis* have been described.

Rudolph and coll. described two patients who had some lightly pigmented, scaling, mildly pruritic eruptions in the groins, axillae, and perianal regions. The differential diagnosis included erythrasma, SD and dermatophyte infections. The authors have stressed a new nomenclature, “inverse tinea versicolor”, due to the highly atypical site.

Anthony reported circular areas roughly hypopigmented, unilateral or bilateral with thin scales localized in the areolae or in the periareolae.

Schosser and coll. reported the case of man who had a brownish, scaly lesion on the nipple microscopically diagnosed as areolar and periareolar PV.

Kamalam showed similar lesions in the penis, in the groins, in the perianal, palmar and plantar areas. Blumenthal described multiple hypopigmented and scaly macular lesions over the penis of a patient who has also been treated during the last five years with triamcinolone acetonide and prednisolone acetate for neurodermatitis, which was not the correct diagnosis.

Unusual extensive forms affecting almost the entire body surface are generally the prerogative of the immunodepressed patients: long-term systemic corticosteroid or estrogen-progestin use, chemotherapy or disorders affecting endocrine systems (fig. 1).

The rare cases of pityriasis versicolor observed in Siena in the last thirty years are briefly documented. There are two cases of pityriasis versicolor atrophycans and many cases of unusual extensive forms or in uncommon sites (Tab.1 and Tab. 2). In all reported cases the diagnosis was based on microscopic examination (fig. 2).

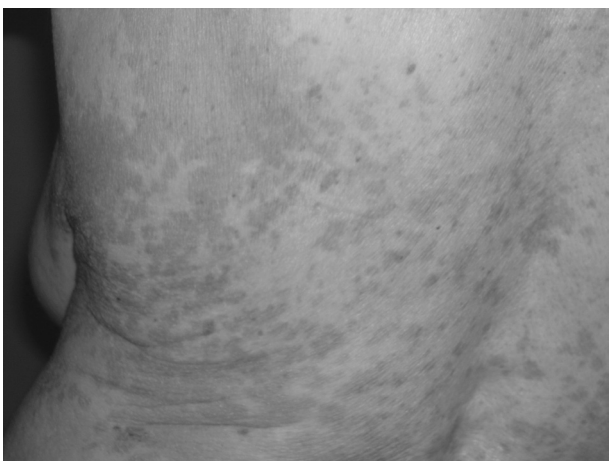

Fig. 1. Many hyperpigmented macules on the trunk in patient with hypothyroidism.

Correspondence: Clara Romano
Dept. of Clinical Medicine and Immunological Science,
Section of Dermatology, University of Siena,
Policlinico le Scotte, Viale Bracci 53100 Siena, Italy.
e-mail: romanoclara@unisi.it

Table 1. Uncommon forms of PV: 22 cases

FOR CLINICAL ASPECTS		
CLINICAL ASPECT	SEX	AGE
PV atrophycans	2 females	49,50
PV rubra	2 females	36,41
PV rubra	1 male	29
FOR SITES		
PV areolar and periareolar	2 females	33,45
PV areolar and periareolar	2 males	27,47
Penile PV	1 male	41
PV in the groin	2 females	39,43
PV in the groin	3 males	36,48,50
Perianal PV	1 female	42
Perianal PV	2 males	27,30
PV in the buttocks	2 females	35,40
PV in the buttocks	2 males	37,57

Table 2. Uncommon forms of PV

Forms that affect almost completely the body surface: 350cases (M 178, F 172)	
PREVALENT* CLINICAL INVOLVEMENT	
Back	180
Trunk	170
Face and neck	31

*some of the patients were affected in more than one site and we consider prevalent the site with more lesions

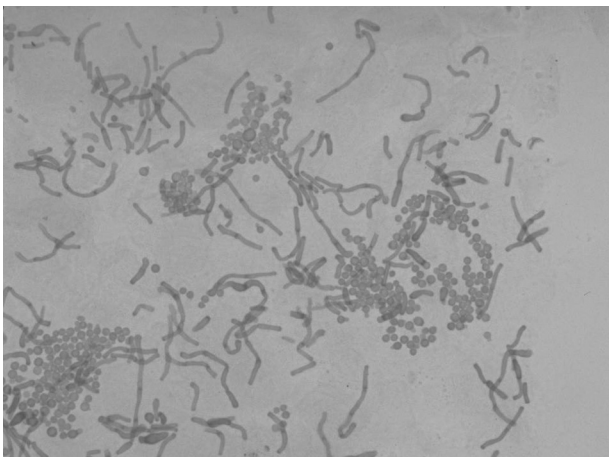

Fig. 2. Typical hyphae and spores of *Malassezia* (25X - Potassium hydroxide mixed with Blue Parker Ink).

From October 2001 to January 2004 Prof. Francesca Mancianti (Department of Animal Pathology, Faculty of Veterinary Medicine, University of Pisa, Italy) identified colonies of the species of *Malassezia* isolated in 37 patients. All those, affected by extensive, resistant to therapy, and long standing (from 1 to 15 years) forms of PV were from Siena.

Malassezia globosa was the form most frequently isolated. It was identified in 66.6% (54.5% in pure culture and in 9% associated with *Malassezia sympodialis*, in 3% with *Malassezia furfur*). *Malassezia sympodialis* was identified in 12.1%, *Malassezia furfur* and *restricta* in 9% and *Malassezia sloffiae* in 3%. We will carry out further studies to demonstrate the prevalent species of *Malassezia,* isolated for example on acute forms of PV.

A prior knowledge about its formes is absolutely important for the right dermatologic diagnosis. Normally, these forms are frequently misjudged for other dermatoses, therefore receiving inappropriate long-term treatment.

Concerning the other dermatoses related to *Malassezia*, one of the most inquired is the AD, whereas the role of the mycete has not been completely explained yet. Takahata and coll. suggests that the decrease of the fungal colonies due to antifungal drugs used on the neck and in the head can reduce the gravity of the AD manifestations. Besides, in order to verify if there are significant differences related to the different species of *Malassezia* present on the skin according to the age, they have assayed the level of anti-Malassezia IgE antibodies in vivo. As said by Takahata, in children with AD the *Malassezia restricta* was frequently found, while in adults with AD, *Malassezia restricta* and *Malassezia globosa* were normally found (3).

Further studies are needed to clarify the role of *Malassezia* in AD and in DS.

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SIMPOSIO 3

AEDES ALBOPICTUS IN ITALIA

Parassitologia 50: 97-98, 2008

Chikungunya epidemic outbreak in Emilia-Romagna (Italy) during summer 2007

P. Angelini[1], P. Macini[1], A.C. Finarelli[1], C. Po[1], C. Venturelli[2], R. Bellini[3], M. Dottori[4]

[1]Public Health Service of Emilia-Romagna Region; [2]Venturelli C. Public Health Department Ausl Cesena; [3]Bellini R. CAA Crevalcore (Bo), [4]Dottori M. Zooprophilactic Institute of Lombardia and Emilia-Romagna

Abstract. During summer 2007, an outbreak due to the local transmission of CHIKV by *Aedes albopictus* mosquitoes occurred moreover in Italy, Emilia-Romagna Region, in the areas of Ravenna, Forlì-Cesena, Rimini and Bologna cities.The original outbreak developed in Castiglione di Cervia and Castiglione di Ravenna, two small villages divided by a river. The first case was recorded on August 9th the epidemic outbreak then spread out, thus giving rise to smaller secondary outbreaks and further sporadic cases in the same area, for a total of 337 suspected cases, 217 of which confirmed by blood analysis. CHIKV has been isolated and characterized on both blood and mosquito samples.

Key words: Aedes albopictus, Arboviruses., Vector Borne Diseases, Europe, Italy

Introduction

Chikungunya virus (CHIKV) is an arthropod-borne virus transmitted to human beings by *Aedes* spp. mosquitoes; it has been suggested that the strain of CHIKV isolated on islands in the Indian Ocean and in India, during the epidemic of 2005-06, has better adapted to *Ae. albopictus* than it has to other *Aedes* spp. This is particularly worrying because *Ae. albopictus* is widespread in almost whole Italy with an especially high population density. In fact in Emilia-Romagna, during summer 2007, an outbreak due to the local transmission of CHIKV occurred moreover in the areas of Ravenna, Forlì-Cesena, Rimini and Bologna.

The first case was reported to the Public Health Department of the Local Health District of Ravenna on August 9th. During the following days an epidemiological investigation was immediately started. It was detected an unusuallly high number of cases of febrile illness in Castiglione di Cervia and Castiglione di Ravenna, two small villages divided by a river. A first list of 47 cases was already available from August 14th. First analysis of data suggested the hypothesis of an arborviral fever so there were located in the area some entomological traps and many specimens of *Ae. albopictus* were collected. Waiting for the laboratory confirmation of etiologic agent a first extraordinary pest control treatment with insecticides was conducted against adults of tiger mosquitoes in the whole centre of Castiglione di Cervia during the night between August 18th and 19th. Successively, from August 23th to 28th, pest control activities were systematically extended, covering the whole epidemic area and carried out in three stages, with the following synergy: adulticidal treatment, larvicidal treatment, removal of larval breeding grounds.

At the end of August serological testing and PCR by the National Health Institute confirmed the diagnosis of Chikungunya fever. Finally, on August 31st, the Chikungunya virus was isolated by the laboratory of the Experimental Zooprophylactic Institute of Lombardy and Emilia-Romagna, on a sample of *Ae. albopictus* collected by traps in the area. On August 29th the Emilia-Romagna Regional Authority passed the first regional directive addressed to all the Regional Local Health Districts to implement a surveillance system throughout the whole regional territory.

Results

The original outbreak developed in Castiglione di Cervia and Castiglione di Ravenna, where 142 confirmed cases were recorded; the epidemic outbreak then spread out, thus giving rise to smaller secondary outbreaks (Cervia with 19 cases, Ravenna with 9 cases, Cesena with 15 cases, Bologna with 5 cases and Rimini with 6 cases); further sporadic cases were recorded in various spots in the same area (figure 2). The distribution of positive confirmed cases by sex is rather homogeneous (45.6% males, 54.4% females). Cases are mainly concentrated in the more elderly population age bracket: as a matter of fact, 42% was older than 65, with an average age of about 57 years. As for the symptoms, 94.5% of cases reported fever, 93.6% arthralgia, 53.5% skin rash, in a few cases itching and in 94.5% of cases asthenia, 49.8% myalgia and, finally 50.2% cephalalgia. If the index case coming from a journey to India (region of Kerala) is ruled out, the first case dates from July 4th, whereas in the last case the onset of symptoms dates from September 28th. 337 suspected cases were reported, 217 out of which were confirmed as positive by laboratory test, 30 were classified as probable since patients refused to receive the blood

Correspondence: Paola Angelini
Servizio Sanità Pubblica, Regione Emilia-Romagna
V.le A. Moro, 21 - 40127 Bologna
Tel +39 0516397024, Fax +39 0516397065;
e-mail: PAngelini@regione.emilia-romagna.it

test, whereas for the remaining 89 patients, tests proved to be negative. Figure 1 shows the epidemic curve of confirmed and probable cases, thus showing the time distribution of cases since the onset date of symptoms.

Discusssion

The described event has proven that vector-borne diseases can spread not only in tropical areas but also in all those sites where the vector (in this case *Ae. albopictus*) is present. This new scenario is due to the massive presence of carrier insects that are responsible for the transmission of this type of diseases in the Emilia-Romagna Region. It requires an overhaul and timely adoption of effective and sensitive pest control measures as well as health surveillance systems.
These measures are not only required by international health authorities, but they have become absolutely necessary to avoid the recurrence of epidemic outbreaks, like the one that emerged last summer in the Emilia-Romagna Region, which is likely to cause serious public health problems. At this aim a "Regional Plan of the Emilia-Romagna Regional Authority for the fight against the Asian Tiger Mosquito and the prevention of Chikungunya and Dengue Fever" for the year 2008 has been adopted. The objectives of the plan are:
(i) optimization of the fight against the Asian Tiger Mosquito to reduce the pest population rate as much as possible, (ii) early detection of the presence of potentially viremic patients in view of an immediate and coordinated implementation of health protection measures.
The Regional Plan, which has been designed taking into account the specific situation of the Emilia-Romagna region, complies with the national rules and regulations in the field, with special reference to the compulsory transmissible disease notification scheme, surveillance and control system, international prophylaxis measures and international movement control of goods, blood donations and organ and tissue sampling.

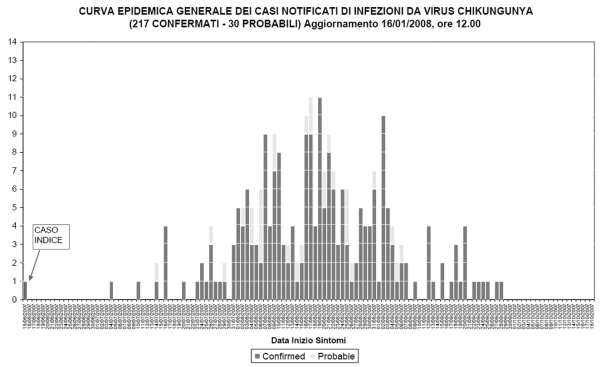

Fig. 1. CHICV outbreak, Emilia Romagna Region, Italy, August, 2008. Epidemic curve of confirmed and probable cases,s showing the time distribution of cases since the onset date of symptoms.

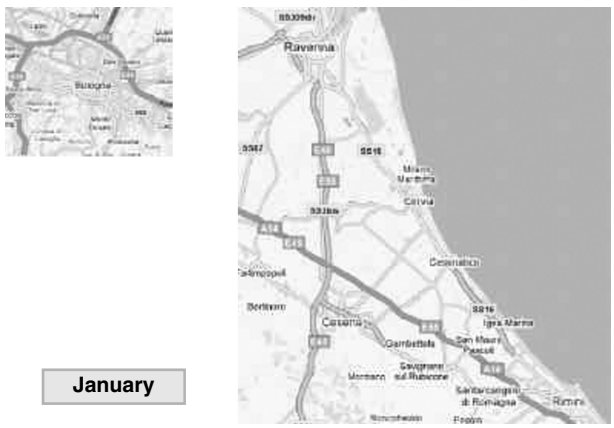

Fig. 2. CHICV outbreak, Emilia Romagna Region, Italy, August, 2008. Provinces of Ravenna, Forli-Cesena and Rimini. Distribution of the 217 confirmed cases

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Availability of insecticidal molecules to control *Aedes albopictus* (Skuse)

A. Baseggio
I.N.D.I.A. INDUSTRIE CHIMICHE S.p.A, Technical Assistance

Abstract. Following the implementation of the Directive 98/8/CE a few changes in the availability of insecticidal molecules to control Ae. albopictus have been outlined. Available products for larvicidal treatments will predominantly be based upon two growth regulators (diflubenzuron and pyriproxyfen). For the control of the adult forms there will mostly be active ingredients belonging to the pyrethroid group. Importance of surveillance for the onset of tolerance or resistance phenomena.

Keywords: Ae. Albopictus, Italy, insecticides, availability.

Introduction

The current availability of insecticidal molecules to control *Aedes albopictus* in Italy and the other European Community member states will undergo a reduction in both the number of active ingredients and commercial formulations that can be used. The reduction will affect both professional pest controllers as well as private consumers. The reason for this reduction is the implementation of the review enforced by the Directive 98/8/EC known as the "Biocidal Products Directive".The first "practical" consequences of this directive, with regards to the availability of insecticidal active ingredients, are included in the Commission Regulation (EC) N° 2032/2003 and have brought about, amongst other things, the ban on the use of triclorphon active ingredient, used in Italy for the production of slow-release tablets registered for the treatment of stagnant water for mosquito larvae in general. A more important further effect is the ban on the use of temephos active ingredient as of the 31st March 2008 (Ministry of Health decree 24/10/2007). Over the last 20 years temephos has been the most widely used active ingredient for the production of larvicidal formulations. This active ingredient was available in a wide variety of formulations: emulsifiable concentrates, water-based microemulsions and slow-release tablets.From the 22th August 2008 (G.U.U.E. L 216/17 of the 21.08.2007) malathion-based products can no longer be used (from this date the use of chlorpyrifos-ethyl and chlorpyrifos-methyl will also cease but this active ingredient is thought to have only been used for adulticidal treatments against *Ae. albopictus* in a few areas of central and southern Italy due to its high resistance to thermal degradation). The ban on malathion use could have a greater impact than that of chlorpyrifos-ethyl and methyl, particularly for the use made by professional pest controllers.

Correspondence: Alberto Baseggio,
I.N.D.I.A. Industrie Chimiche S.p.A. - Via Nona Strada, 55
35129 Padova, Italy
Tel + 39 049 8076144, Fax + 39 049 8076146,
e-mail: a.baseggio@indiaprodotti.com

The amount of malathion sold by I.N.D.I.A. S.p.A. during the 2007 season alone to professional pest controllers and public pest control services against mosquitoes amounts to approximately 15,000 litres of commercial formulations.The insecticidal molecules that are very likely to be authorised for use (but confirmation will only come once the dossiers submitted for review have been approved) following the review enforced by the BPD are:

1) **For larvicidal treatments**:
   (i) Bacillus thuringiensis var. Israelensis serotype H 14: (ii)Bacillus sphaericus (currently unavailable in Italy); (iii) Diflubenzuron (chitin synthesis inhibitor); (iv) Pyriproxyfen (juvenile hormone analogue):

In the summer of 2008 the experimentation of Spinosad-based formulations will also begin in Italy. Commercial products are expected to become available, for mosquito larvae control, from 2010-2011.
At the moment the availability of S-methoprene growth regulator doesn't appear to be certain. The dossier required by the Biocidal Products Directive will be submitted but probably without envisaging use in waterways.By examining the applicative conditions that characterise the larvae-control campaign treatments against *Ae. albopictus*, and based upon a few experiences collected from the public services who coordinate the *Ae. albopictus* control treatments in their territory, a few simple conclusions can be drawn.The use of *B. thur. var. israelensis*-based formulations can be helpful for citizens who wish to carry out their own larvicidal treatments, but the larval control only becomes significant if the commercial formulation is applied at least weekly and if, at the same time, the larval development breeding grounds on public land are subject to careful control.For the public services who need to plan the treatment of thousands (or tens of thousands) of micro breeding grounds present on public land (mostly road drainage grates of various shapes and sizes) the use of products marked by reduced persistence is not economically sustainable, not due to the cost of the product but due to the cost of the manpower necessary for its application. Diflubenzuron and pyriproxyfen are currently extremely efficient against *Ae. albopictus*. In particular,

pyriproxyfen is twice as biologically active as diflubenzuron, and therefore commercial formulations containing pyriproxyfen can be used that release smaller amounts of active ingredient into the environment. Usually pyriproxyfen-based commercial products contain a quarter of the active ingredient contained in diflubenzuron-based products whilst providing similar persistence.Spreading the larvicidal treatments over intervals longer than 3-4 weeks results in a risk of underestimating the operative structure (i.e. the number of operators occupied in the territory) and therefore in the inability to manage unplanned treatments, for example after heavy rainfall. In Japan it has been observed that *Ae. albopictus* can develop resistance to pyriproxyfen and diflubenzuron but it is a long process (Kasai S. *et al*, 2007, J. Med. Entomol. 44 (5): 822-829).

2) **For adulticidal treatments**

Table 1. comparison between the usage rates of common liquid larvicides containing IGR active ingredients

Composition	Composition	Usage rates taken from technical data sheets and referring to a street manhole with 40 litres water
Pyriproxyfen 4%	Water microemulsion	0.25 ml of product per manhole
Diflubenzuron 15%	Suspension concentrate	0.28 ml of product per manhole

The the list of insecticidal active ingredients for which companies are currently producing the review dossiers required by the BPD shows that pyrethroids will mainly be available in the future (Table 2)

Etofenprox has interesting toxicological characteristics;

Table 2. list of pyrethroids whose dossiers will most likely be submitted for review; synthetic data on acute toxicity.

Active ingredient	LD50 acute oral rat mg/kg	LD50 acute dermal mg/kg	LC50 Inhalation toxicity (4h)	Properties
d-Phenotrin	> 10000	> 10000	> 2100 mg/l	Non photostable
Permethrin	6000	> 2500	> 685 mg/l	Photostable
Deltamethrin (in water)	> 5000	> 2000	> 22 mg/l	Photostable I
Tetramethrin	> 5000	> 5000	2,73 mg/l	Non photostable
d-Tetramethrin	> 5000	> 5000	> 1,18 mg/l	Non photostable
Transfluthrin	> 5000	> 5000	> 0,513 mg/l	Non photostable
Cypermethrin	250 – 4150	> 4820	> 25 mg/l	Photostable, I
Deltamethrin (in naphta)	800	> 2000	> 22 mg/l	Photostable, I
Alpha-cypermethrin	474	> 2000	> 0,32 mg/l	Photostable
Esbiothrin	> 432	> 2000	2,63 mg/l	Non photostable
Natural pyrethrin	570-150	1500	3,4 mg/l	Non photostable
Cyphenothrin	318	> 5000	> 1,85 mg/l	Photostable
Esfenvalerate	88,5	> 5000	> 0,48 – 0,57 mg/l	Photostable, I
Lambda-cyalothrin	79	696	0,06 mg/l	Photostable, I
Bifenthrin	> 53,4	> 2000	> 0,8 mg/l	Photostable

I = irritating

Etofenprox is a phenoxyderivative substance marked by a mode of action similar to that of pyrethroids but with a particularly reduced acute toxicity (LD50 acute oral rat = 42880 mg/kg; acute dermal > 2140 mg/kg; Inhalation toxicity (4h) =5900 mg/m^3)

Average usage rates of a few insecticides available on the market:

Two OP active ingredients will also be available in addi-

Composition (%)	Formulation type	Average rates for adult mosquitocontrol
Cypermethrin 10 Tetramethrin 2 Piperonyl Butoxide 5	Water microemulsion	0.5%
Permethrin 15 Tetramethrin 2.5 Piperonyl Butoxide 5	Water microemulsion	0.8%
Deltamethrin 1 Esbiothrin 2.5 Piperonyl Butoxide5	Suspension concentrate	0.75%
Etofenprox 10 Piperonyl Butoxide 20	Water microemulsion	0.5%

tion to those listed: fenitrothion and naled, but probably for a limited period of time. The use of fenitrothion, in regions marked by high temperatures and in places where its use is acceptable, would secure longer persistence of the insecticidal activity. The decrease in biological activity as temperature rises is a widely known characteristic of most pyrethroids. With regards to product choice, personal communications received by the technical service managers from the main Italian formulation companies (Colkim, Copyr, Blue Line – Leica, VE.BI.) highlight that despite the availability of a wide range of active ingredients and formulated products, a few product types attract the interest of professional pest controllers (and private citizens).

Amongst these are:

(i) water based permethrin and tetramethrin microemulsions synergised with piperonyl butoxide;
(ii) water based cypermethrin and tetramethrin microemulsions synergised with piperonyl butoxide;
(iii) water based deltamethrin with natural pyrethrins;
(iv) deltamethrin suspension concentrate.

Information collected from a few professional pest controllers has highlighted that greater control of *Ae. albopictus* adults can be obtained by paying particular attention to application techniques. In particular, to treat gardens and other small to medium sized open areas the use of pressurised nozzles allows greater control of mosquitoes than using turbo atomisers. A number of professional pest controllers have begun to have doubts about the efficacy of insecticides when applied by means of equipment that creates too small particles (less than 100 microns approx.). It has been observed that vegetation treated to dripping turns into a barrier having a repelling or insecticidal effect lasting for several weeks (up to 6 if bifenthrin or lambdacyalothrin products are used, Trout R.T., *et al*. 2007, J. Med. Entomol 44 (3): 470-477). It is interesting to observe that the use of products containing solvents (naphta solvent, xylene, normal decane) is decreasing while the use of odourless, non phytotoxic, and non-flammable products such as suspension concentrates, water-based microemulsions or emulsions containing vegetable-origin solvents is increasing.

Conclusion

For the next 5 to 6 years current preferences are not expected to change a lot, at least until the Biocidal Products Directive imposes the re-registration of commercial formulations, following re-registration of the active ingredient. Starting from the summer of 2008 the adulticidal campaign against *Ae. Albopictus* might be slightly more complicated in those Italian areas characterised by periods of high temperatures, following the ban of malathion-based products. In these areas the thorough enforcement of larvicidal campaigns will become even more important. Finally, the future use of active ingredients belonging almost exclusively to the pyrethroid group makes the periodical monitoring of tiger mosquitoes' sensitivity strongly advisable.

Acknowledgements

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Blood-feeding preferences of *Aedes albopictus* (Diptera: Culicidae) in urban and rural settings within the Province of Rome, Italy.

L. Valerio[1], F. Marini[1], G. Bongiorno[2], L. Facchinelli[1], M. Pombi[1], B. Caputo[1], M. Maroli[2], A. della Torre[1]

[1]Parasitology Unit, Department of Public Health Sciences, University of Rome "Sapienza", Italy; [2]Section of Vector-Borne Diseases and International Health, MIPI Department, Istituto Superiore di Sanità, Rome, Italy.

Abstract. We here report the results of field trials carried out in Rome with the aim to obtain data on the feeding behaviour of *Aedes albopictus*, in relation to different availability and abundance of putative hosts. Human Blood Index values were found higher than 75% in urban areas, where humans represented the most abundant hosts, and lower than 60% in rural areas, where host alternative to humans were frequent. The overall results confirm the generalist feeding-behaviour shown by this species in its original range of distribution and highlighting its high potential as vector of human pathogens in urban areas of Italy.

Keywords: mosquitoes, human-blood index, host-feeding patterns, arbovirus.

Introduction

One of the most important parameters in vector-borne pathogen (e.g. arbovirus) transmission is the blood-feeding behaviour. In fact, this parameter can influence vector potential depending on the vertebrate host groups with which the mosquito interacts. Obviously, the likelihood of pathogen transmission by a vector species greatly increases if reservoir and amplification hosts are the primary targets of the vector's feeding activity. Consequently, knowledge of the frequency of human-mosquito contact is essential for understanding the role of each vector species in disease transmission to humans. Although *Aedes albopictus* is thought to be a generalist feeder[1], with a predilection to feed on mammals[2], it is not yet clear how frequently it feeds on humans and how its feeding pattern is influenced by the abundance and availability of alternative hosts.

Aim

During 2006 and 2007 *Ae. albopictus* reproductive seasons (May-October), we carried out field trials in urban and rural sites within the Province of Rome, with the aim to obtain data on its host-feeding patterns, in relation to different availability and abundance of putative hosts. In fact, so far, no studies on feeding preferences of *Ae. albopictus* have been conducted in recently colonised temperate regions of Europe, despite the great relevance of this parameter.

Correspondence: Alessandra della Torre
Department of Public Health Sciences, Parasitology Unit, "Sapienza" - University of Rome, P.le Aldo Moro, 5, 00185, Rome, Italy
Tel +39 06 4969 4268; Fax +39 06 4991 4653
e-mail: ale.dellatorre@uniroma1.it

Methods

The study was carried out in 4 sites, two of which (Site 1: "La Sapienza" University and Site 2: "Verano Cemetery") were located in urban settings close to the town's centre, while the other two (Site 3: a horse-breeding farm/riding school in "Acilia" and Site 4: a cattle-breeding farm in "Palestrina") were located in rural settings in the town's outskirts. Mosquitoes were collected weekly using the sticky-trap (ST) developed by Facchinelli and colleagues[3]. The mosquitoes collected were observed under a dissecting microscope, morphologically subdivided by species, gender and physiological stage. All blood-fed females were classified on the basis of the amount and colour of the blood in the abdomen and stored individually at -20°C. Blood meal origin was determined by direct ELISA on nitro-cellulose membrane according to Bongiorno *et al*[4]. Eight peroxidase-labelled anti-animal IgG antibodies (Sigma) were tested, namely anti-human, anti-dog, anti-cat, anti-bird, anti-rabbit, anti-bovine, anti-rat, anti-horse.

Results and conclusions

Cumulative collections from urban and rural areas yielded 518 *Ae. albopictus* blood-fed females, of which 303 (58.4%) produced results at the direct dot-ELISA: the relatively high frequency of unidentified blood-meals is to be attributed to a limited amount and/or bad conservation of the blood in the specimens collected on the ST. The Human Blood Index (i.e. the propor-

tion of blood meals carried out on humans) was significantly different among sites (Chisquare=87.8; df=3; p<0.0001): 95.7% in Site 1 [C.I.= 91.5-97.9], 78.5% in Site 2 [C.I.=68.2-86.1], 55.3% in Site 3 [C.I.=39.7-69.9] and 22.7% in Site 4 [C.I.=10.1-43.4]. Interestingly, in the rural sites a high percentage of blood-meals was observed on horses (Site 3: 47.4%) and on bovines (Site 4: 54.5%), consistently with the fact that they are abundant hosts in these sites. Moreover, it is interesting to note that, in Site 2, 26.6% of the meal have been carried out on cats, accordingly with the presence of a cat colony in this Site. Few blood meals were carried out on the other hosts tested: 6.6% of the total from all collecting sites on dogs, 3% on birds and <1% on rats and rabbits. The percentage of blood-meals carried out on two different host species was 4.9% in Sites 1, 11.4% in Site 2, 44.7% in Site 3 and 4.5% in Site 4; almost all included humans and, in Site 3, human-horse and human-dog combination were detected more frequently than other combinations. A single female was shown to have fed on 3 host species. The obtained results confirm the generalist feeding-behaviour shown for *Ae. albopictus* in its original range of distribution and highlights the high potential of this species as vector of human pathogens in urban areas of Italy, where both humans and the mosquito itself may reach very high density, suggesting that the recent Chikungunya epidemics in northern Italy may not remain an isolated episode.

Acknowledgements

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Decennial experience of the Municipality of Rome in the fight against Asian Tiger Mosquito

B. Cignini, R. Di Domenicantonio, M Chionne and A. Scirocchi

Municipality of Rome Department X, Environmental and Agricultural Policies

Abstract. Since September 1997 was detected the presence of the Asian Tiger Mosquito (*Aedes albopictus*) in the peripheral areas of the city of Rome, the Environment Department has put in a strategy to combat and control the spread of this insect throughout the city, collaboration with the Istituto Superiore di Sanità (ISS) to aspects of study and monitoring of the phenomenon and with the Azienda Municipale Ambiente (AMA) for actions in the urban environment. In 1998 began the first contrast campaign in the town territory. The data coming from ISS are processed through a geographical territorial system (GIS) that allows real-time locating the degree of infestation and effectiveness of interventions, allowing the display of trends over time and the development of plans of action in urban territory. In parallel to this methodology operational, the Municipality of Rome has put in an information campaign designed to involve citizens in the fight against this insect. Today the situation in the city is under control, in case of emergency due to the spread of the virus Chikungunya is possible identify in advance the areas at greatest risk of infestation. Using this methodology work has enabled to contain operating costs and minimize the environmental impact by limiting interventions only to areas found positive.

Keywords: Rome, Italy, Municipality, Dept of Environment, Vector control

Control methodologies

The control of this insect is immediately appeared extremely complex because of the particular adaptability of this mosquito, which has found in small focus a development environment virgin and totally devoid of competitors. In relatively large focus, such as manholes and machicolations road, where it is found in association with a likely competitor as the mosquito *Culex pipiens* this was underdog in the competition. A territory as the city of Rome, rich in agricultural areas devices, ancient villas, terraces and gardens subject to continuous irrigation, has encouraged the survival of adult, therefore it was not possible to prevent the establishment of this insect, that now can be considered part of the local fauna. In the urban area of the city of Rome, as recent studies have shown, this species is able to maintain its trophic activity in the winter as evidenced by the continued laying of eggs. Since 1999 the Municipality of Rome to control the infestation now present throughout the urban territory has prepared a plan of action based on the study and monitoring of a urban level, on the focusing of the interventions in the territory in high-density areas of infestation and on public awareness in contrast to the spread of this insect.

The Municipality of Rome in collaboration with ISS check the weekly 650 ovitrappole placed in the territory of the city; these containers are dark with water on the bottom with a rod placed inside masonite upright, which are replaced weekly and sent to ISS for the counting of the eggs (Fig. 1).

The monitoring carried out so allows for a framework up to date on the presence, distribution and density in the 19 City Districts. Since 2003 over monitoring of the summer (April to December) has been extended to the winter (January to March), the control of this additional period of the year is found that the Asian Tiger Mosquito has a trophic activity also in the winter period. Having reached the goal of localizing the phenomenon of the Asian Tiger Mosquito in the city territory since 2000 have "georiferito" the ovitrappole located in Zone and operating Sectors using a geographical territorial system (GIS). Monitoring data coming from the

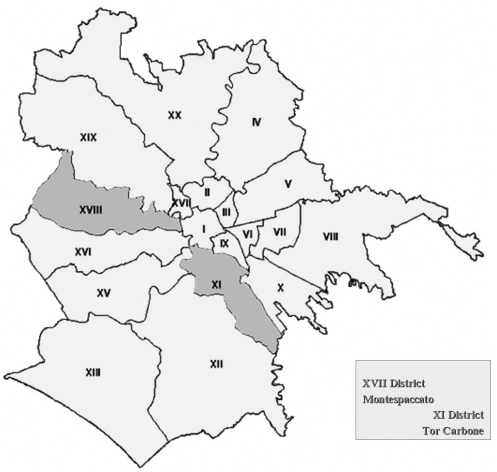

Fig. 1. Periferal Districts of Rome where foci of *Aedes albopictus* were firsr recorded in 1997.

Correspondence: Dr. Rita Didomenicantonio,
Dipartimento di Politiche ambientali e risorse agricole,
IV U.O. Tutela e gestione aree verdi e biodiversità
Comune di Roma
e-mail: r.didomenicantonio@comune.roma.it

Fig. 2. Periferal Districts of Rome where foci of Aedes albopictus were firsr recorded in 1997.

ISS are processed weekly from the system, creating thematic maps to be used for the planning of subsequent interventions. Currently stored data on seven years of management (2001-2007), allowing the study of trends, evaluating the effectiveness of interventions carried out across the city and at any time. The application of this methodology allows work to display in real time the performance of an individual in the various sectors and identification of areas to make treatments to prevent the onset of. Interventions carried out by AMA go in the period from April to October, are antilarval treatments and are developed at a frequency that affect public areas such as manholes roads, schools, cemeteries, senior citizens centres and other contexts identified as subjects of intervention. The Municipality of Rome, by 2000 launched a widespread information campaign (through leaflets, brochures, posters, web pages and press releases), and issue annually a Mayor Ordinance contains provisions designed to prevent the spread of Asian Tiger Mosquito. The effectiveness of the contrast action to the spread of this insect is closely related to the cooperation of citizens, it is now understood the importance of shifting their attention to the control of its territory housing, because only through their intervention was possible reach and neutralise the millions of small domestic focus. Through AMA, the Municipality of Rome provides citizens with a call center which collects reports of citizens and provides suggestions for the adoption of correct behaviour to counter the spread of this insect. Through AMA, the Municipality of Rome provides citizens with a call center which collects reports of citizens and provides suggestions for the adoption of correct behaviour to counter the spread of this insect. The methodology of control over territory are taken into consideration two indices of a direct (results of the monitoring) and an indirect (number of reports received the call center). The first with the egg counts indicates the numerical presence of female mosquito and their reproductive potential, the second indicates the presence of this insect through the discomfort felt by the citizens.

Evolution of the Asian Tiger Mosquito in the Municipality of Rome

The Asian Tiger Mosquito (*Aedes albopictus*) was first reported in Rome in late August 1997 in remote areas of northwest quadrant (Montespaccato) in the south-east quadrant (Tor Carbone) thanks to the numerous reports from citizens (Figure 2). In 1998 began the first campaign to combat the spread of this insect entrusting monitoring activity at the ISS and the interventions at the AMA. Despite the timely intervention by the Municipality of Rome, in the following 3 years, the Asian Tiger Mosquito is spreading as leopard spot in the urban territory, until in 2002 to spread through all the city territory. In 2003 the infestation is falling from north-east of the city, recording the lowest values in VII and VIII District, from 2006 there has been a gradual and slow decline of the urban territory until to stabilise in 2007 (Figures 3 and 4).

Conclusions

The experience in ten years of activity to control the spread of Asian Tiger Mosquito leads us to infer that in the city of Rome this insect develops in a myriad of micro focus present particularly in private areas, while in communal areas they are consisting almost exclusively from machicolations. As machicolations with the presence of water represent a small percentage of all those present in the city, is in course their census to limit treatments to those potentially positive. Another development will be the integration into Geographical Informatic System, of the census of several kinds of manholes roads and green areas in different public and private as well as all the features of the area that may be related to the presence of the Asian Tiger Mosquito. As highlighted by the decline in population density of the Asian Tiger Mosquito the currently situation in Rome can be considered under control, and this positive result can be attributed to good organization of the annual programme conducted by the Municipality of Rome, which allows targeted interventions in hazardous or highly infested and the information campaign that has responsible citizens transforming from spectators to players who helped themselves to the containment of this phenomenon. This methodology of work as well as allowing the display of real-time trends, allows mainly to identify in advance areas at risk of infestation higher is not just on account of the importance that health care could be the presence of this insect as a potential carrier of diseases transmitted by Arbovirus. The presence in a multiethnic city like Rome, by peoples from countries where these diseases are endemic, makes it theoretically possible arise epidemical episodes of Dengue or Fever by Chikungunya, and is for this reason that the Municipality of Rome considers this insect a “special watched.

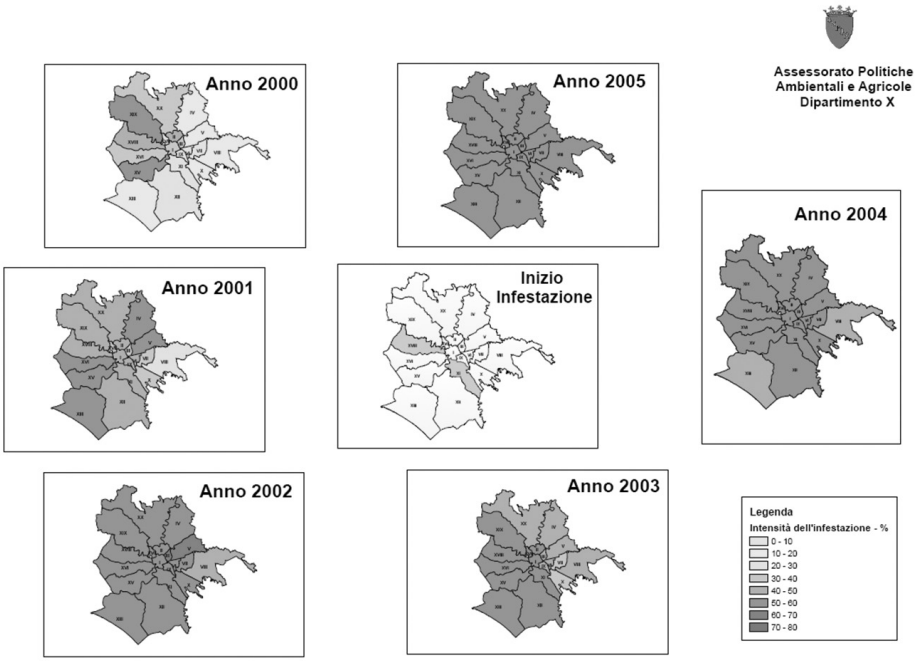

Fig. 3. Development density of infestation of the Asian Tiger Mosquito (*Aedes albopictus*) in the Municipality of Rome (from year 1997- 2005).

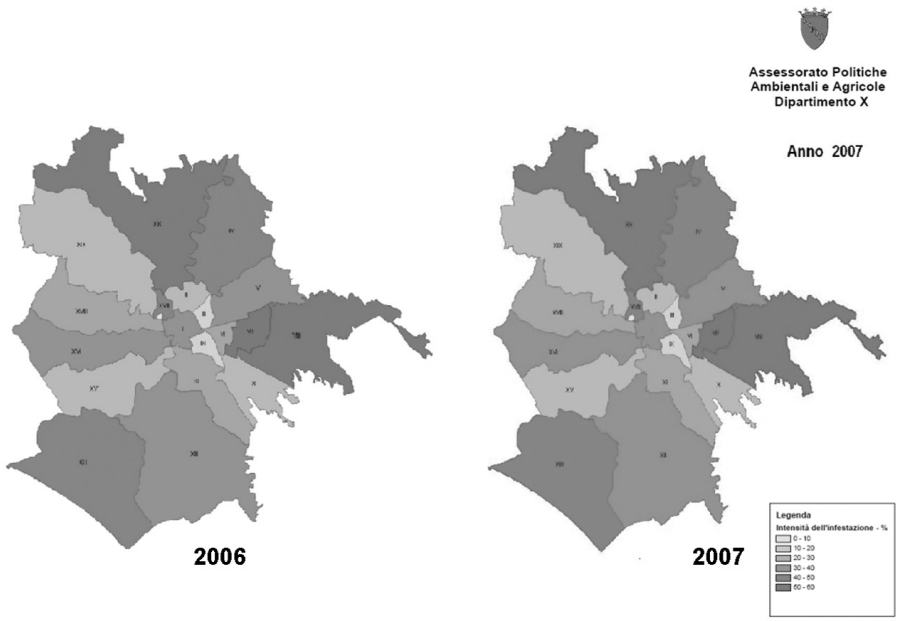

Fig. 4. Development density of infestation of the Asian Tiger Mosquito (*Aedes albopictus*) in the Municipality of Rome (from year 2006 -2007).

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Arboviruses in Italy

L. Nicoletti, M.G. Ciufolini, C. Fortuna, F. Magurano, C. Fiorentini, A. Marchi, E. Benedetti, P. Bucci

Department of Infectious, Parasitic and Immune mediated diseases, Istituto Superiore di Sanità, Rome,

Abstract. A brief review of the arboviruses isolated In Italy is presented and discussed. Some of the Arboviruses considered in this paper are endemic in the country and are transmitted by arthropods that play actually a role of vectors, (sand flyes, hard ticks and mosquitoes); other arboviruses, sporadically isolated, are potential agents of emerging human or zoonotic diseases

Keywords: Vector Borne Diseases, Sandfly, Ticks, Mosquitoes

Introduction

Italy extends approximately between latitudes 36 and 47°N. It is almost completely surrounded by sea and bordered in the north by mountains. There is a north-south central mountain range (Appennines) with highest altitudes of over 2600 m. Consequently, climatic conditions vary considerably within Italy: from mountainous, to continental, and to coastal. The southern extremity of Italy is greatly influenced by its proximity to North Africa, sometimes producing subtropical climatic conditions. Furthermore, the formation of an abundant and varied arthropod population, the presence of domestic and small wild animals in fairly large numbers in certain parts of the country, and a usually very temperate climate could maintain natural foci of arboviruses. The periodic migration of several species of birds between Europe and Africa across Italy can also contribute to the introduction and dissemination of arboviruses in the country.

The presence of arboviruses in Italy was first documented during World War II in epidemiological studies on the American troops by Sabin: two viruses of the Phlebotomus fever group were isolated, Sandfly Fever Sicilian and Sandfly Fever Neapolitan. For 20 years after these isolations, however, only limited amount of data on the activity of arboviruses in Italy was recorded. Then, beginning in 1965, our Department started a surveillance program for arboviruses in selected Italian regions. The program's studies centred on the collection of virologic and serologic data related to the presence of arboviruses as well as on determination of their importance in the public health of the population.

Mosquito-borne viruses:

A periodical mosquito collection was regularly performed during the years 1966 to 1968 in Northern Italy and in 1980-1987 in Central Italy. The dominant mosquito species were *Culex pipiens*, *Aedes vexans* and *Ae. caspius*. More than 70,000 mosquitoes were processed in 1967-1968 and more than 13,000 in 1980-1987. Two strains of Tahyna virus (California group) were isolated in 1967 from mixed pools of *Ae. caspius* and *Ae. vexans*, which were collected in Northern Italy.

Neutralizing antibodies (by mouse neutralization test) against Tahyna virus have been found in high percentage (70%) among people living near the areas where mosquitoes were trapped. (Balducci et al, 1968)

Antibodies against other mosquito-borne viruses have also been found. Human sera from Northern and Central Italy reacted with West Nile virus in HI. The virus caused and outbreak of meningoencephalitis in horses in 1999 in Tuscany Region. (Autorino et al, 1999). Retrospective studies have shown that during this episode some humans could have been infected by the virus with only minor symptoms of disease.

In July and August 2007, the local health unit of the province of Ravenna (region of Emilia Romagna, north-eastern Italy) detected an unusually high number of cases of febrile illness. Early in the outbreak investigation, infection with Chikungunya was suspected because of clinical symptoms and the fact that the first patient with febrile illness was a man from a country affected by an outbreak. Furthermore, the presence of *A. albopictus* in the area was known. Of the 334 suspected or probable Chikungunya cases involved in the outbreak, samples were examined of 281 and 204 were laboratory-confirmed. The virus was isolated from the serum of patients and from a pool of *A. albopictus* collected in the affected area. (Rezza et al, 2007)

Viruses isolated from birds

Bahig and Matruh viruses (Tete group) were isolated from the blood of birds captured by nets in the autumns of 1968 and 1969 (23). Each virus was isolated in both years and both from *Fringilla coelebs* (chaffinch) and *Fringilla montifringilla* (brambling finch). The birds were captured in North-eastern Italy during their fall migration. The same viruses had been previously isolated in Egypt from autumn migrants, suggesting that the site of initial infection was probably somewhere in Eastern Europe or Western Asia. The cir-

Correspondence: Loredana Nicoletti
Department of Infectious, Parasitic and Immune mediated disease, Istituto Superiore di Sanità, Viale Regina Elena 299
00161 Rome, Italy
Tel +39 06 49903242/3235, Fax Fax: +39 06 49902082,
e-mail: loredana.nicoletti@iss.it

cumstances of isolation of Bahig and Matruh viruses in Italy seemed to confirm this hypothesis.

Sandfly-borne virus

During World War Il, an outbreak of febrile illness occurred among USA military troops in Southern ltaly. From the blood of some patients, Sabin isolated two strains of viruses, the Sandfly Fever Naples virus and the Sandfly Fever Sicilian virus (Sabin et al., 1944). Since then, the two agents have been isolated from the sand fly *Phlebotomus papatasi* in several Mediterranean countries. Their circulation in Italy was probably terminated after the antimalaric campaign conducted in the late forties.

In 1973, a new virus (Toscana virus), serologically related to the Sandfly Fever Naples virus, was isolated from a pool of *P. perniciosus* sandflies collected in the Tuscany region (Verani et al., 1980). The Toscana virus has been associated with human acute CNS disease occurring during the period June-October with a peak in August (in the same month its vector sandflies have their peak of activity).

The virus is present in at least eight different regions of Central and Northern Italy (Tuscany, Marche, Abruzzo, Emilia Romagna, Umbria, Piedmont, Campania and Sardinia). The virus is largely diffuse: high levels of antibodies against Toscana virus have been found in healthy populations of endemic areas, suggesting that the virus can also be the cause of an infection with only minor symptoms of disease.

More than 100 different strains have been isolated from wild-caught sand flies of the *P. perniciosus* and *P. perfiliewi* species, and several strains have also been isolated from the cerebrospinal fluid of patients. (Nicoletti et al, 1991)

Two more viruses have been isolated from the same sand flies in the same areas: the *Phlebovirus* Arbia and the *Vesiculovirus* Radi. The Arbia virus is largely diffuse in the sand fly populations but only low levels of antibodies have been found in humans and no disease could be associated with its infection (Verani et al., 1988).

Little work has been done with the Radi virus. The virus has been isolated several times from sand flies but no antibodies have been found in humans and no human disease has been associated with it (Ciufolini et al., 1990).

Tick-borne viruses

TBE-virus. The first evidence of the presence of the TBE virus in Italy was obtained in 1967 on the basis of serologic surveys in Northern (Gorizia province) and Central Italy (Latina province) (Balducci et al., 1967; Verani et al., 1967). Subsequently, extensive studies were performed in Central and Northern Italy. Acute infection of central nervous system (CNS) disease were diagnosed in the Florence province of Toscana Region and in Trentino-Alto Adige, Friuli and Veneto where the most active focus is present. Field studies in these areas allowed for isolation of strains of TBE virus from pools of *Ixodes ricinus* ticks and from *Apodemus sylvaticus* mouse (Verani et al, 1991).

Bhanja virus. Bhanja virus (an ungrouped tick-borne virus) was repeatedly isolated from pools of *Haemaphysalis punctata* collected collected in Central Italy (in 1967 and 1973 in the Latina province, in 1977 in the Florence province). Serologic evidence of the circulation of this virus has been obtained in many areas of Northern, Central and Southern Italy. The highest prevalence was found in goats and sheep, but low levels of antibodies were also found in cattle, humans, wild rodents and birds. No human disease has been associated with this virus in our country. (Verani et al, 1970)

Tribec virus. The Orbivirus Tribec was isolated from *Rhipicephalus bursa* ticks collected in 1972 in Northern Italy (Gorizia province) and in 1977 in CentraI Italy (Siena province). No serologic survey on its distribution has been performed (Verani et al., 1978).

Thogoto virus. The Orthomyxovirus Thogoto was isolated from *R. bursa* ticks collected in 1969 in Sicily. The occurrence of antibodies to this virus in sera of cattle and sheep from different zones of Western Sicily has been reported (Albanese et al., 1971; 1972).

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Importance of *Aedes albopictus* in Veterinary Medicine

M. Pietrobelli
Department of Experimental Veterinary Science, Faculty of Veterinary Medicine, University of Padua, Italy.

Abstract. To assess the role of *Aedes albopictus* in transmission of filarial nematodes of veterinary importance, researches were carried out in different geographical areas. In Italy a first research was performed to study the susceptibility of *Ae. albopictus* to *Dirofilaria repens*, *D. immitis* and *Setaria labiatopapillosa*. The development of L3 larvae was longer than in other species of mosquitoes but *Ae. albopictus* could be a suitable vector of filariae. To understand the role of *Ae. albopictus* in the natural transmission of *Dirofilaria* and to assess the risk for animal and human health, in 2000, 2001 and 2002 another study was carried out in the town of Padua. A total of 2,534 *Ae. albopictus* were caught on human-attracted mosquitoes. Specific primers and sequencing identified filarial DNA as *D. immitis*; *Ae. albopictus* was proved a natural vector of *D. immitis*. Similar results were confirmed in Central Italy also for *D. repens*. The presence of *Ae. albopictus* increased the probability of transmission of canine and human dirofilariosis in urban environment and it could change the epidemiology of dirofilariosis, in particular for what concern the time of biting and the risk season. These aspects must be considered to outline a correct prophylaxis.

Keywords: *Aedes albopictus*, cattle, dog, *Dirofilaria* spp., *Setaria labiatopapillosa*

Introduction

Aedes albopictus (Skuse)(Diptera: Culicidae), the "tiger mosquito" which was imported in Italy in 1990 by shipping trade of used tires, is a very aggressive biting nuisance mosquito species. It received international attention for its importance in public health as a possible vector of arboviruses (for at least 22), causing infectious diseases like Dengue, Yellow Fever, Encephalites (Mitchell, 1995; Gratz, 2004) and Chikungunya, recently diagnosed in Italy (Emilia-Romagna Region)(Dottori *et al*, 2008).
What is the importance of *Ae. albopictus* in Veterinary Medicine? Is this mosquito a potential vector for parasites of veterinary importance? To assess its role in transmission of filarial nematodes, in the past some researches were carried out in different geographical areas. Concerning *Dirofilaria immitis*, it has been demonstrated a high degree of variability ranging from complete refractoriness to the infection (Apperson *et al*, 1989) to partial susceptibility (Konishi, 1989). In 1995 Comiskey and Wesson found first-stage *Dirofilaria* larvae infecting the Malpighian tubules in 3 of 163 *Ae. albopictus* collected from New Orleans (USA) and in 1999 Nayar and Knight found, 15 days after infection, the infective L3 stage in 10.9% of *Ae. albopictus* (number of L3 larvae ranged from 1 to 37) and demonstrated that *Ae. albopictus* is a potential vector of *D. immitis* in Florida, U.S.A.The spreading of *Ae. albopictus* in Italy raises questions on its possible role as a vector for indigenous filarial species, common in dogs (genus *Dirofilaria*), cattle, equines, pigs and wild ruminants (genus *Setaria*). Cancrini *et al* (1995) studied the susceptibility of an *Ae. albopictus* colony (collected in Civitavecchia, Central Italy) to *D. repens*, *D. immitis* and *Setaria labiatopapillosa*. Females of *Ae. albopictus* were artificially infected with a blood meal either on dog or on artificial membrane feeders. Mosquitoes were killed and frozen at various day-interval after feeding and then dissected. The development of L3 larvae (about 18 days after infection) was longer than in other species of mosquitoes but it was demonstrated that *Ae. albopictus* can be a suitable vector of *D. repens*, *D. immitis* and *S. labiatopapillosa*. Few data are available on the involvement of the "tiger mosquito" in the natural transmission of filariae. Ahid and Lourenco-De-Olivera (1999) reported the absence of *D. immitis* in specimens collected in an endemic area of Brazil, whereas Lai *et al*. (2001) found *D. immitis* infected mosquitoes in a Taiwan endemic area.

Materials and methods

To understand the role of *Ae. albopictus* in the natural transmission of *Dirofilaria* and to assess the risk that its presence might represent for animal and human health, a study was carried out in the town of Padova by Cancrini *et al* (2003) where there is an high mosquito density and the presence of *Dirofilaria* nematodes (Capelli *et al,* 1996). Three areas of the town were checked for the presence of *Ae. albopictus*: the garden of the Psychiatric Hospital (located in the outskirts), an Urban Park and the Botanical Garden (both located in the centre of the city). Mosquito sampling was carried out during summer 2000 (10 sampling-days), 2001 (12 sampling-days) and 2002 (5 sampling-days) by two humans used as bait to attract mosquitoes. Collections were made from 9.00 to 11.00 am and/or from 5.00 to 7.00 pm by aspirating females landed on the baits with a paper cup aspirator (Coluzzi

Correspondence: Mario Pietrobelli
Department of Experimental Veterinary Science,
University of Padua, Viale dell'Università 16
35020 Legnaro (PD)
e-mail: mario.pietrobelli@unipd.it

and Petrarca, 1973). The female mosquitoes sampled in 2000 were immediately killed and fixed in 70% ethanol, while in 2001 and 2002 were kept under controlled conditions (25-27°C, ~90% r.h.) for 5 days and then killed, identified in accordance with keys proposed by Snow (1987) and fixed in 70% ethanol. The presence of filarial parasites in mosquitoes (grouped for species and sampling area) was evaluated by PCR examination of the specimens in pools (usually 10 specimens each for insects collected in 2000 and 2001, and 5 each for those caught in 2002). DNA extraction was performed separately on the insect abdomen and thorax-head to discriminate between *Dirofilaria* infected/infective specimens. Pooled samples were analysed with specific ribosomal primers named S2-S16. DNA sequencing confirmed species assessment (MWG-Biotech.) and sequence comparison was achieved by CLUSTAL W analysis (Thompson *et al*., 1997). Minimum infection rates (MIRs) were calculated by the standard formula: number of positive mosquito pools/total number of mosquitoes tested x 100. According to a binomial distribution of the parasites, expected infection rates (P) were also evaluated and calculated as follows: $P=1-\sqrt[k]{n/N}$, where n is the number of negative pools, N is the number of tested pools and k is the average number of specimens in each pool.

Results

A total of 2,721 specimens were caught in the whole sampling period; as expected, human-attracted mosquitoes were almost all (97.1%) *Ae. albopictus* (2,534). Results concerning *Ae. albopictus* sampling are reported in table 1.
Results of PCR analyses on pools of all collected *Ae. albopictus*, MIRs observed and P values are shown in table 2.

Table 1. *Aedes albopictus* females collected in summer 2000, 2001 and 2002 in three sampling sites of Padova town (Veneto Region, Italy) on human bait, and exposition time to bites.

Year	Psychiatric Hospital		Urban Park		Botanical Garden		Total	
	Mosquit. (no.)	Exposition to bites (hours)	Mosquit. (no.)	Exposition to bites (hours)	Mosquit. (no.)	Exposition to bites (hours)	Mosquit. (no.)	Exposition to bites (hours)
2000	125	4	283	16	305	10	713	30
2001	60	4	8	2	1,148	24	1,216	30
2002	-	-	-	-	605	14	605	14
Total	185	8	291	18	2,058	48	2,534	74

Table 2. Minimum infection rates (MIRs) of *Dirofilaria immitis* evidenced by PCR and expected infection rates (P) in pools of Aedes albopictus females caught while landing on man (Padova town, Veneto Region, Italy).

Year	Specimens (no.)	Pool size (min. – max.)	Positive / tested pools	MIRs	P (95% C.I.)
2000	713	8 - 12	19/69 (A = 19)	2.67	3.07 (1.99 – 4.71)
2001	1,216	1 - 11	40/144 (A=24; ATH=8; TH=8)	3.29	3.78 (2.81 – 5.06)
2002	605	4 - 5	22/123 (A=16; ATH=2; TH=4)	3.64	3.93 (2.61 – 5.93)

A = abdomen; ATH = abdomen + thorax-head ; TH = thorax-head

Assuming the presence of only one positive mosquito/positive pool, MIRs resulted of 2.67% (19/713) in summer 2000, 3.29% (40/1,216) in summer 2001 and 3.64% (22/605) in summer 2002. All studied areas harboured infected mosquitoes. Specific primers and sequencing identified all filarial DNA as belonging to *D. immitis*.

Discussion

Aedes albopictus was the most abundant species in controlled sites. In summer 2000 PCR-based technologies of 713 specimens, allowed the detection of 27.5% pools infected by *D. immitis* but lacking of positive thorax-heads didn't allow defining the actual value of this mosquito as a natural vector for *D. immitis*. Specimens collected in 2001 and 2002 were kept for 5 days to allow that the development is overcome or, in case of incompetent host, the microfilariae are expelled yielding a negative PCR. In fact it has been shown that the insect defensive mechanisms against dirofilariae are efficient only on microfilariae recently ingested or penetrated in primary cells of the Malpighian tubules. Similar results were confirmed in Central Italy also for *D. repens* (Cancrini *et al*, 2003 and 2007). Results prove the risk for heartworm disease in the town of Padova and support the hypothesis that the stable presence of *Ae. albopictus* should increase the probability of transmission of canine and human dirofilariosis in urban environment. The circulation of filarial nematodes among animals might be improved and enhanced and, considering the aggressive anthropophylic behaviour of the species (30-48 bites/hour) proven in Padova town, transmission from animals to humans, enhanced.
The role of *Ae. albopictus* as an efficient vector could change the epidemiology of Dirofilariosis, in particular

for what concern the time of biting and the risk season. In fact this mosquito is more active during the day and, in some towns (for example Rome), all over the year. These aspects must be considered in order to plan a correct prophylaxis.

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An overview of the lesson learned in almost 20 years of fight against the "Tiger" mosquito

R. Romi, G. Majori
Reparto di Malattie Trasmesse da vettori e Sanità Internazionale, dipartimento M.I.P.I., Istituto Superiore di Sanità, Roma

Abstract. Since its introduction in Italy in 1990, *Aedes albopictus* has spread quickly across the country, being at present reported in scattered foci in all regions below 600 m of altitude. The most important items of the lesson learned in almost 20 years of fight against the "Tiger" in Italy are here reported and discussed.

Keywords: Aedes albopictus, Vector Borne diseases, Italy, Strategies of survival, Overwintering

Introduction

Establishment and spread of the Asian tiger mosquito *Aedes albopictus* in Italy and, at present also in other European countries, as well as the increase of imported cases of *Aedes*-borne viruses such as Chikungunya and Dengue, raise the question of risk that also these tropical diseases become stable in Europe.
As a matter of fact Chikungunya and Dengue arboviral diseases are in expansion throughout the world. *Aedes aegypti,* the principal vector of Dengue and Yellow Fever in from disappeared in Europe since the late 1940's while populations of *Ae. albopictus*, established in Europe recently and are spreading to most southern regions. In laboratory experiments, this species has proven competent for transmitting various viruses. At the same time an increase of virus importation is observed due to increased international travel. The Chikungunya fever outbreak which occurred in July-October 2007 in Emilia-Romagna confirmed that Europe is definitely at risk for *Aedes* borne diseases. Since its introduction In Italy, *Ae. albopictus* has represented the major human biting pest throughout much of its range. But in Ravenna the species has been proved to be responsible of the first outbreak of a tropical arbovirus in Europe, shifting its role of simple pest mosquito into of a dangerous vector. Despite that the presence of the mosquito in our country it continues to be considered by the Health Authorities as an environmental problem no plan of emergence is going to be implemented.

Aedes albopictus in Italy: background.

Since its introduction in Italy in 1990, *Aedes albopictus* spread quickly across the country. Although the first record of adults of the species has occurred in Genova (Liguria Region, North Western side), the most important foci of colonization quickly developed in the North Eastern Regions (Veneto, Friuli Venezia Giulia), along the Adriatic Coast as well as in the inner lands (i.e. Garda Lake or Euganei Hills), where the climate is quite mild also during the winter. There the species has found ideal environmental conditions for proliferating and extending its seasonal activity.
Different populations of the tiger mosquito have been probably introduced in Italy in separate periods and from different areas, but we certainly demonstrated its origin from the South of the USA (Atlanta, Georgia) in containers of used tires. Most of these imported populations of *Ae. albopictus,* although arrived in Italy after a long period of "acclimation" in the USA, certainly originated from areas located at the northern limit of the natural distribution area where the species survives in temperate countries through egg diapause induced by the short photoperiod and by low temperatures.
In Italy, two major tire rethreading companies, located in the outskirts of Padova and Bologna (Veneto and Emilia Romagna Regions respectively), that imported scrap tires directly from the USA, allowed the quick spread of the mosquito across the country throughout the internal trade of the tires sent to smaller companies. During the first 4 years since the first entry, almost all the new foci of colonization of *Ae. albopictus* were recorded in areas close to tire deposits. Nevertheless other kind of passive transportation certainly contributed to the spread (the species enter spontaneously cars, trucks and trains). At present scattered foci of the Tiger mosquito are reported in all the Regions of the country, with the exception of Valle d'Aosta, and in 82 out 107 Provinces, from coastal plains to inner lands, up to 600 meters of altitude.
When in 1997, *Ae. albopictus* was detected in Rome, we started to evaluate the length of the favorable season to the species and the factors that might induce diapausing egg production. At time, adults of the species were reported to be active from February-March to December (peaking in August-September) but most of the eggs ceased to hatch since mid October. In the following years, this 8-month full activity showed a constant increase up to the winter 2003-04, when about 30% of the ovitraps of our monitoring network where constantly egg-positive for the whole winter season. Nevertheless, eggs laid between mid November and mid February did not hatch up to the next March. Just

Correspondence: Roberto Romi
Vector Borne Diseases and International Health Section, Department of Infectious, Parasitic and Immunomediated Diseases; Istituto Superiore di Sanità, Viale Regina Elena, 299 00161 Rome, Italy
Tel + 39 06 49902301, Fax + 39 06 49903561,
e-mail: roberto.romi@iss.it

during the past winter season we observed the weekly hatching of few batches of eggs also during the coldest months. Even if the larvae originated from these eggs in outdoor breeding sites did not survive the low temperatures (but larvae from indoor sites were able to conclude the whole cycle in about 3 weeks) this event represents a clear signal that the characters that may determine the production of winter eggs are going to be deselected or, at least, not favored by the mild temperature of our country, and that the species is going to be active for the whole year round, also due to the predicted global rise of the mean temperature.

Factors that favoured the adaptation of *Aedes albopictus* to new environments, dynamics of the spread and Chikungunya virus

Italy is a long and narrow peninsula extending over the Mediterranean sea for about 8 degree of latitude. As a consequence of this fact, the seasonal dynamics of the species varies greatly, from 4-5 months of activity recorded in the pre-mountainous northern limits of the Pianura Padana to Rome, where it is quickly going to be active all year round. South of Rome the scarcity of rainfall during the 4 warmest months of the year (usually <200mm rain between June and September) contribute to limit the massive reproduction of the species as seasonally recorded in most of the infested sites north of Rome, where in presence of a mean temperature of 25°C the development cycle of *Ae. albopictus*, from the egg to the adult, it may lasts about 1 week.
Usually, when people become aware of the biting activity of the "Tiger" mosquito, the species is already strongly "rooted" in the area and the eradication of the focus is almost impossible. Usually the colonization of a new area starts from the outskirts of a city or town where industrial or adapted commercial "at risk" enterprises are located (i.e. tyre deposits, plant nurseries, car-crashed deposit, dumps, etc.) moving to colonize the street drains, that are peculiar of our country. Then this "container breeding" species colonize every small , clean fresh water collection. Nevertheless the species may breed also in water collections rich in organic matter that share mainly with *Culex pipiens* and/or *Culiseta sp.*
In this phase of spread, the invasive population of the "Tiger" mosquito reach the maximum of the abundance, that will be self-reduced after colonization according to the trophic resources available in the area.
Two main factors allowed to this species the quick and strong establishment in our country: 1) the above cited geographical origin of the imported populations able to survive to the cold season of the northern emisphere thanks to the egg diapause. Nevertheless has been proved that these populations are polymorphic for this character, some of them being able to lay "summer" eggs also during the winter when established in the regions of Italy where climate is mild also during the winter season. 2) the great biological plasticity to different environmental conditions other than temperature (kind of breeding sites, housing, different hosts suitable for the blood meal) and the ability to adapt the size and the behaviours of a well established colony to the resources of the new colonised area. This adaptability may be expressed also throughout behaviours that may be considered as anomalous: i.e. the nocturnal trophic activity, indoor biting and resting, different survival strategies. Up to now no breeding *Ae. albopictus* larvae have been recorded in natural breeding sites, with the exception of tree holes.
Ae. albopictus is able to adapt and to survive in different, often unfavorable, new environments thanks to strategies of survival other than winter diapause. These strategies are in part common to other species belonging to the same genera and may be summarized as follows:

(i) The ability to complete a full blood meal taking very quickly, few blood from different hosts;
(ii) females do not lie all the eggs of the same brood in a single breeding site, leaving few eggs in different containers;
(iii) part of the eggs laid during the summer do not hatch at the first wetting stimulus, sometime even at a second wet and dry shock different; the amount of summer eggs that do not hatch, rising progressively up to about mid October when this amount will represent the whole sample that will survive the winter; last but not least the ability to use indoor breeding sites (as pots for idroponic plant coltures), strictly related to a surprising endophagy/endophily.

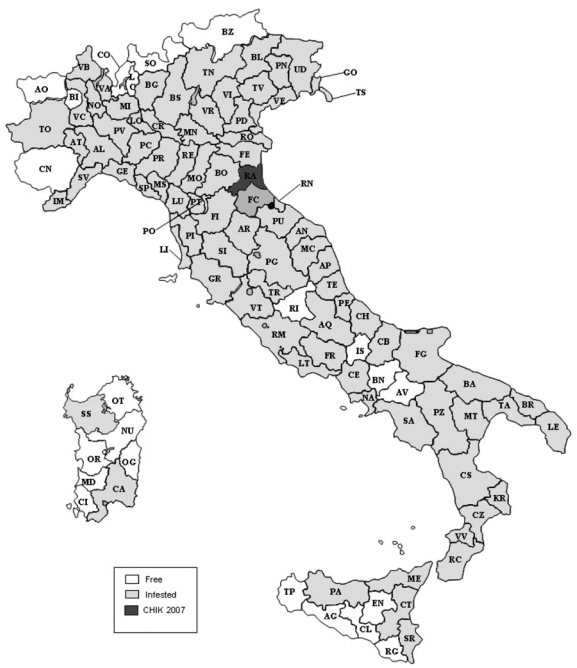

Fig. 1. Distribution of *Aedes albopictus* in Italy by provinces, 2007. (Based on an unofficial reporting net – last updating Dec. 2007). The yellow color indicates the administrative provinces where at least one stable focus of *Ae. albopictus* has been recorded and reported at the CNRAA at the I.S.S.: in red the Province of Ravenna were the CHIK outbreak occurred in the summer 2007; in pink the Province of Forlì-Cesena interested by some secondary small foci of the CHIK outbreak.

Conclusions and recommendations

The deep knowledge of vector's bionomics and behaviors and an efficient system of monitoring the presence and the abundance of the species are two necessary tools for facing *Ae. albopictus* in case of emergence. Emergency plans to adopt in case of outbreaks should be set up at regional level, following national guidelines and selecting personnel to be prepared to play the relative role in the alert system (Who dose that!). Part of this task may be performed by the ISS where, since 1991 a centre of reference for the surveillance and control of *Aedes albopictus* in Italy (CNRAA) has been set up by two official administrative acts of the Ministry of Health.

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Aedes albopictus in Rome: results and perspectives after 10 years of monitoring

F. Severini, M. Di Luca, L. Toma, R. Romi

Istituto Superiore di Sanità, Dip. M.I.P.I., Reparto di Malattie Trasmesse da Vettori e Sanità

Abstract. In 1997, *Aedes albopictus* (Skuse 1894) was detected in Rome in two opposite areas of the city. In the following 2 years, the species quickly spread. In 2000, scattered foci of the species were reported in the whole urban area and in the outskirts of the capital city. In Rome, *Ae. albopictus* seems to have found optimal environmental conditions to proliferate and to overwinter through and without diapausing eggs. In ten years *Ae. albopictus* has colonized the whole urban area through three phases: first massive spread, following maintenance of infestation, and colonization of alternative winter breeding sites with favorable climatic conditions. Data collected during the 2007 show that rainfall is no longer the most important factor for the development of the species, with respect to the past. In fact *Ae. albopictus* probably has found new alternative larval breeding sites through the colonization of small water collections refilled periodically by human activities. During 2007-2008 winter season, in order to evaluate the species adaptability, a study of eggs hatching and length of larval cycle at low temperatures, was carried out in laboratory and in simulated field conditions. Data and results are showed and discussed also by the light of existing literature.

Keywords: *Rome, Aedes albopictus, monitoring, ovitraps, overwintering eggs*

Introduction

Since its discovery in Italy in 1990, the "tiger mosquito" *Ae. albopictus* has spread quickly across the northern and central regions of the country, causing considerable concern among public health authorities. This species, which easily colonizes container habitats in the peridomestic environment, was accidentally introduced in Italy by importation of used tires from USA. In 1997, *Ae. albopictus* was first detected in Rome. In the following years the species has spread more quickly from the initial foci to 2 heavily populated suburbs of the town. At present, scattered foci of *Ae. albopictus* are presents throughout the whole urban area and in the majority of the towns that belong to the province of Rome. In Rome, the "tiger mosquito" seems to have found suitable environmental conditions for proliferating and its quick spread and establishment represent the 1st example in Europe of extensive colonization of an urban area, with involvement of hundreds of thousands people. Founded by the City Council of Roma, and coordinated by the Istituto Superiore di Sanità, a program of surveillance and control was launched since 1998.

Materials and metods.

The monitoring was carried out yearly, with a network of 650 ovitraps on the entire urban area (41.9°N Lat. - 12.4° Long. with about 350 km^2 of surface). The study area was divided into about 300 zones, with a different number of ovitraps (from 1 to 25), depending on the extension and the features of each area. Black plastic pots (500 ml capacity) were employed as ovitraps. A strip of masonite® (3x15 cm) was suspended vertically in the middle of the pot to provide a suitable surface for oviposition. Pots were filled with 350 ml of water. Every week, pots were rinsed and refilled, strips were changed and checked for egg presence. The number of eggs was counted by observing the strips under a dissecting microscope. In order to evaluate the distribution and the abundance of the species in the study area, two parameters were considered: 1) number of positive ovitraps by total working ovitraps and 2) mean number of eggs by positive ovitraps (Fig.1). Eggs number by positive ovitraps was divided in 5 classes of abundance (1-10; 11-50; 51-100; 101-300; more than 301 eggs). Data were collected weekly, entered in a database (Microsoft Access) besides to basic meteorological data (temperature, relative humidity and mm of rainfall; fig. 2) and analyzed using an adapted Arcview G.I.S software.

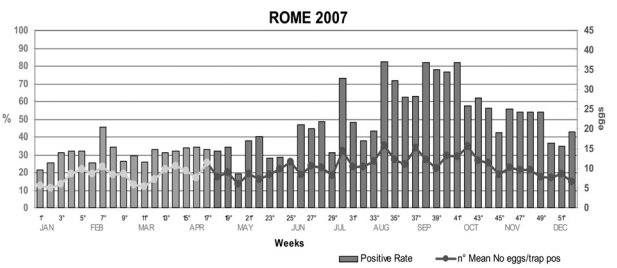

Fig. 1. Weekly rate of positive ovitraps for *Aedes albopictus* and mean number of eggs/ovitrap recorded in Rome during the 2007.

Correspondence: Francesco Severini
Vector Borne Diseases and International Health Section, Department of Infectious, Parasitic and Immunomediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena, 299 00161 Rome, Italy
Tel +39 06 4990 2301, Fax +39 06 4990 3561,
e-mail: francesco.severini@iss.it

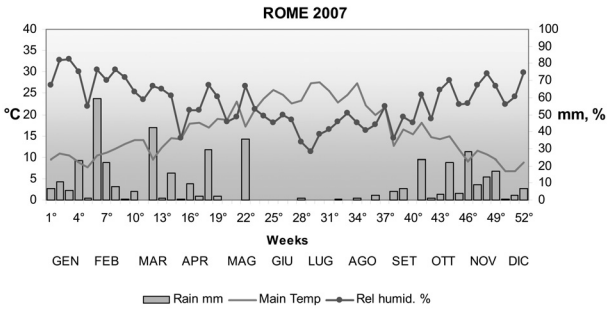

Fig. 2. Meteorological data collected in Rome during the 2007 (UCEA).

Study on winter eggs.

As implementation of the winter surveillance, a further study was carried out from mid December 2007 to the end of February 2008. Twenty strips from positive ovitraps were selected weekly in order to evaluate a possible hatching and, in case of positive response, the mean rate of hatching. Selected strips were placed individually for a maximum of 7 days into plastic trays (15 by 10 by 6 cm) containing 350 ml of dechlorinated tap water at room temperature (18±1°C), and with a natural photoperiod. In order to estimate the length of the larval cycle (indoor and outdoor temperatures), a combinated lab/semi-field study was performed. Batches of first instar emerged larvae were placed in trays commonly used in our insectary for mosquito rearing (15 x 25 x 8 cm), containing one litre of dechlorinated tap water). Half of the total trays were maintained in laboratory at the same conditions above described, and the remaining 50% into an open greenhouse, where temperature was 1-2 °C higher than outdoor. The bioassay was followed up to the development of larvae to adults or to the death of all the larvae used for the test.

Results and Discussion

From the analysis of the data showed in Tab. 1 it is noteworthy that the population of the species reached its maximum level of abundance during the first phase of invasion/colonization of a new area. As showed in column 3, the monitoring net (column 2) revealed rates of positivity that increased almost constantly during the first four years of colonization, reaching the maximum level of abundance (peak) in 2002. After that, a marked decrease was observed since 2003, corresponding to a phase of stabilization, related to trophic resources and/or breeding sites available. The number of positive zone (column 4) followed the same trend showing a marked decrease in the records belonging to the forth and the fifth classes of eggs abundance (101-300 and >301 eggs, respectively), from 220 to 8 records and from 14 to 1 respectively. In columns 3 and 6 is underscored the relationship between yearly mean rate of positivity and mm of rain-

Table 1. Data from ten years of monitoring in Rome (1998-2007). Data come from a net of ovitraps that was implemented in 1998 (50 ovitraps) and gradually improved (650 ovitraps at present). Ovitraps are distributed on to the whole surface of the urban area, on the base of the surface of the 19 Administrative Districts and about 300 further areas in which the town is divided. Data were collected weekly. In this table are reported the mean values of the already discussed parameters, along 24 weeks per year, corresponding to the majority of the season favorable to the massive reproduction of *Aedes albopictus* in Rome (June-November).

	Rome, whole urban area (data referred to the period of June-November)						Housing An example of single district mean rate of positive ovitrap (mean No. eggs/ pos.trap)		
1	2	3	4	5	6		7	8	9
Year	Monitoring net No. ovitraps	Mean rate of positivity (Seasonal peak)	No. zones with at least one weekly record to the IV or V class of abundance		Max No eggs/pos. trap	Rainfall mm (mean No eggs/pos.trap)	Central District (Downtown)	Residential District (Low hills)	Outskirts (Commercial Centre)
1997	Late summer, the first foci of *Ae. albopictus* were recorded in two opposite sites (NE-SW) of the urban area Beginning of the phase of invasion								
1998	~ 50	16,5 (34,4)	-	-	359	267 (16,1)	-	-	-
1999	~ 300	31,2 (63,5)	-	-	632	387,8 (43)	-	-	-
2000	500	25 (47,1)	220	14	572	305,4 (28,2)	38 (21)	45 (27)	36,5 (22)
2001	500	45,9 (86,2)	236	12	453	183,4 (27,7)	42 (24)	44,5 (19)	54,5 (23)
2002	500	62,8 (87,6)	320	15	429	513,2 (22)	62,3 (30)	63,1 (39)	68 (25)
	Foci of the species are reported in all 19 Districts Beginning of the phase of stabilization								
2003	650	48,9 (74,2)	185	10	612	233 (25)	52,7 (26)	57,8 (25)	52,2 (20)
2004	650	41,7 (81,1)	133	1	550	306,8 (12)	52 (22)	59 (26)	59,8 (23)
2005	650	50,2 (73,2)	75	8	506	451,4 (17)	53,5 (16)	54,4 (19)	57 (17)
2006	650	44,5 (69,4)	34	0	144	239,7 (13)	50,5 (13)	52 (14)	52,5 (15)
2007	650	44 (82,4)	8	1	337	139 (11)	55 (10)	60,32 (11)	57,1 (10)

fall recorded in the considered period. As previously observed and expected from existing literature, the amount of rainfall appeared to be mainly linked to mean rate of positive ovitraps with the exception of 2006-2007 summer seasons. The huge abundance of the species in these two seasons, despite the scarcity of rainfall, appeared to be no longer related to this parameter, with respect to the past. Probably the species adapted to new breeding sites, refilled continuously by human activities, where favourable microclimatic conditions may have determined the inversion in decreasing trend of seasonal abundance (columns 3,5,6). Results of the winter egg study are reported in the Tab.2.

Table 2. Data from *winter eggs study* (December 2007-February 2008).

Weekly stick sample				Larval-pupal development			
		Hatching rate		Outdoor		Indoor	
Wetting date	Mean T ° C	sticks/sample	eggs/stick	% mortality	instar and time	% mortality	instar and time
17/12/07	6,8	33,00	8,00	100	up to IV instar in 4 weeks	70,00	up to adult in 2.5 weeks
7/1/08	11,0	-	-	-	-	-	-
14/1/08	8,9	25,00	42,00	100	up to IV instar in 4 weeks	25,00	up to adult in 2.5 weeks
21/1/08	5,6	22,00	90,00	100	up to pupa in 6 weeks	35,00	up to adult in 3 weeks
28/1/08	10,4	-	-	-	-	-	-
4/2/08	7,6	55,00	88,00	100	up to pupa in 4 weeks	18,00	up to adult in 2 weeks
11/2/08	5,5	-	-	-	-	-	-
18/2/08	8,2	68,00	91,00	74	up to adult in 3 weeks	15,00	up to adult in 2 weeks
25/2/08	12,1	74,00	95,00	74	up to adult in 3 weeks	5,00	up to adult in 2 weeks

Conclusions

As previously reported the presence of *Ae. albopictus* in Rome may be considered stable. The mild climate of the city has probably contributed to deselect the characters that determine the ability of overwintering. In alternative, the climate may have simply favoured the development of those fractions of the population that in Rome, as previously reported, are probably polymorphic for the characters that allow them to survive the cold season. Moreover, for the first time, some winter eggs laid from December 2007 to February 2008 hatched: larvae did not survive to the low temperatures (greenhouse), while they were able to survive and reach adult stage in indoor sites (lab conditions). Further studies have been planned for the coming years.

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Parassitologia 50: 125-126, 2008

Tiger mosquito Control: new approaches to the issue in local context.

A. Talbalaghi[1,2]

[1]Freelance entomologist, consultant for Mosquito Control District-Alessandria; [2]National Director of European Mosquito Control Association (www.zanzare.eu)

Abstract. Until recently, the control of mosquitoes has primarily focused on them as a nuisance due to their biting behaviour. This has now evolved into a significant health problem. To deal with this serious issue, a rational approach to vector control should be adopted, with clear, technically sound guidelines enforceable by legislation.

The extensive outbreak of Chikungunya in the Indian Ocean during 2005-6 and the subsequent outbreak in the Emilia Romagna region of Italy in August 2007, should prompt a number of actions which must occur without delay in order to prevent any future recurrence of outbreaks.

An International Symposium on Chikungunya was held in Alessandria, Italy on February 27th 2008. A number of experts from various disciplines were in attendance, the sole aim to assess the risk of this disease and other mosquito borne diseases occurring in Europe. The meeting culminated in the signing of a declaration called the "Alessandria Resolution" by the experts in attendance and members of several local authorities (see www.zanzare.eu). This act signified joint commitment of an national and international standing, to tackle the spread of the Asian Tiger mosquito and raise awareness among the general public. This paper will share the experiences of the mosquito control programmes in the Italian regions and in Alessandria and Piedmont emphasising key lessons learned.

Keywords: *Aedes albopictus*, Chikungunya, outbreak, *Ochlerotatus caspius*, vector control, mosquito control, vector competence, monitoring, Alessandria resolution, Emca Italy, mosquito, Emilia Romagna, Piedmonte

From a global perspective, scientists have gathered a multitude of evidence that in recent decades, the world has experienced an accelerated change in ecological, climatic, demographic and economical variables which have been conducive to the development and spread of various vector borne diseases. Examples of these include West Nile Virus (WNV), Dengue Fever and Chikungunya to name a few. The expansion in the geographical range of medically important arthropods has been notable. With knowledge of this and the associated health risks , it is incumbent upon Public Health Agencies and Vector Ecologists to undertake initiatives to address this problem.

Vector control is a key weapon in tackling these arboviral diseases. However, historically, of the many vector control operations that have been carried out, most have been of limited success. There are several reasons for this lack of effectiveness:

(i) Insufficient knowledge of the vector, its ecology and management;
(ii) Little research into the pathogenic agents;
(iii) Inappropriate and effective control strategies;
(iv) Increased insecticide resistance;
(v) Vector biology.

We urgently need to know the vector competence of other mosquito species that are present in abundance in the territory as they could be potential vectors of Chikungunya and other diseases. A recent study (*A. Failoux* et *al*, 2008) highlighted this pressing need. Key to the fight against arboviral disease is also the determination of potential zoonotic reservoirs and the possibility of vertical transmission, that is, adult maternal females passing on the disease to their offspring. Other important areas to investigate include:

(i) The interaction between vector, pathogen and host;
(ii) Analysis of "wild populations" and reservoirs in their natural environment;
(iii) An evaluation of the modes of action of various insecticides currently on the market;
(iv) An increase in evaluation control strategies is needed as well as encouragement of individual countries and the relevant authorities to undertake these measures proposed by the scientific community.

In Italy, there is a distinct lack of awareness regarding the risk of vector borne disease and its associated vectors. Contributions to the knowledge and understanding of a preventative approach and also of care are also missing. We must not forget that in an open minded society with progress and vision, " considering all of the measures of undertaken that deal with the health of man and the environment the preventative ones will produce results that are more long lasting.

Correspondence: Asghar Talbalaghi
Entomologist Freelance Consultant for Vector Control, Via Martiri delle Foibe, 21 40068 San Lazzaro di Savena (BO)-Italy
Tel Fax +39 051 6272064
e-mail: talbalaghi@libero.it
www.zanzare.eu
National Director of European Mosquito Control Association (www.emca.asso.fr)

Remarks

Surprisingly, except for a few isolated cases where mosquito control is effectively managed, the agenda for most mosquito control programmes falls within the commercial sector, dictated by insecticide companies and by PCOs. There are many obvious flaws with this approach, namely the inability of the insecticide companies to guarantee impartiality and initiatives to be taken. There are approximately more than 900 companies' and many producing insecticides and they have become almost the main authority on how mosquito control is managed in many areas. This figure does not include sanitation companies which would invariably inflate the number of commercial organisations involved.

Clearly a more permanent, reliable and unbiased approach is needed for vector control.

In Piedmont, the problem of mosquitoes is a significant one, yet the approach to mosquito control is less than desirable. Vector control activities are not properly coordinated, without of permanent dedicated personnel and facilities. Across the whole of Italy, the prevailing system is one where disinfestations companies (PCOs) formulate, manage and execute vector control activities, as opposed the appropriate professionals.

This lack of accountability is further facilitated by the absence of national and local legislation. An example of the failings of this is illustrated by the situation in Piedmont. In this locality some mosquito control is carried out, but in the neighbouring area of Lombardy practically no mosquito control is employed, rendering the efforts in Piedmont ineffective in the long term. The strong flyer ricefield mosquito, *Ochlerotatus caspius* generated in almost 100.000 untreated hectares are able to travel great distances, thus migrating easily to Piedmont, confounding efforts made to eliminate them. So the question of whether current assumptions about disease risk in this locality are correct.

In regard to Chikungunya, national coordination of regional activities by the Ministry of Health is not clear. Fortunately, Emilia Romagna, equipped with the appropriately skilled staff, was able to effectively manage the recent Chikungunya outbreak. This situation is one that is not typical across Italy, and is an issue of great concern.

The following actions are recommended:

(i) Independent, competent professionals working on prevention of the sanitation risks
(ii) Dedicated scientists focusing on increasing knowledge of vectors
(iii) A contribution to the knowledge base of vector-pathogens interactions by skilled epidemiologists
(iv) The formulation of vector control guidelines by Entomologists

Rather than a sporadic response in an emergency situation, the scourge of mosquitoes should be met with long term, sustainable evidence based efforts.

Source reduction is a highly effective, environmentally friendly method of vector control and experience strongly suggests that this should be the main mode of intervention employed to fight the increasing spread of vector borne disease in Italy and worldwide.

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Control of the Asian tiger mosquito: technical and administrative aspects

A. Tamburro

U.F. Zoology Department of Environmental Prevention,Az. USL N° 9 Grosseto

Abstract. The Community legislator, through Directives 2004/17/CE and 2004/18/CE, wanted dictate to the Member States some "guidelines" to be used in the field of public procurement of services, in order to promote, through an inner market growth, developing appropriate operational protocols to document in the race; that, has the aim at testify the consolidated technical capacity of Company which conferred the health reclamation provided by the "Plans to put public health emergencies". By its nature, the legislative regulatory act which is capable of directing the gradual harmonization of national laws, giving also each state full autonomy on the form and means to be taken. Therefore, the objectives of the Community directives must be properly incorporated and interpreted, including the legislative adaptation about the regulation of the disinfestations Enterprises.

Keywords: Public services, Plans to put public health emergencies, Directives 2004/17/CE and 2004/18/CE

Introduction

With regard to public services, the Italian legislation highlights many administrative and technical difficulties for developing appropriate capitulated of competition aimed at the achievement of specific objectives, among which, in presence of vector-transmitted diseases "Plans to put public health emergencies" management it is introduced, and it could give back indispensable to improve the epidemiological surveillance and entomological, especially in the speed even through the activation of a pest control service.
Furthermore, in public health facilities, if to the lack of legislative clarity we add a shortage of trained in a complex and constantly evolving business sector technicians and managers, we are aware of the urgency of filling this "professional vacuum" indispensable for the standardization of operating procedure for interventions, planned gradual response to depending on the level of risk in respect of public health and the environment.
The Legislative Decree on April 12, 2006, n. 163 "Code of contracts related to public works, services and supplies in implementing Directives 2004/17/CE and 2004/18/CE", establishes rules for the proper application of rules for the award by the government, procurement whose estimated value of the service, excluding IVA, is equal to or greater than the equivalent euro 200.000,00. This amount, however, is rarely made available to the contracting stations, especially for disinfection services; so most procurement sector escapes to the D.Lgs. dictates, with obvious difficulties in the formulation of notices and application of procedures for the award.
In this way the contracting station is not only unable to secure a service whose requirements really match the quality criteria, but it is not even able to ensure the performance accuracy and timeliness. In practice, if we consider the quality as "all service characteristics that meets requirements customer (expressed or implied)", in the current public procurement management services for the control of communicable diseases from potential carriers don't ensure "a priori" the economic quality and appropriateness of the service to offer. Indeed, it is important to underline that transparency and credibility, in the relationship between public and private are on the subject of contracts, elements of administrative fairness.
Likewise, the recruitment by Companies as a quality system, circumscribed and limited to the application of ISO 9001 (UNI EN 9001), can not constitute guarantee element of technical-professional capacity of Companies, but is simply an "organization indicator", whose value in the award can not constitute evidence of preference.
Now, the Community legislature through Directives 2004/17/CE and 2004/18/CE, wanted dictate to the Member States "guidelines" to be used in the public services procurement, in order to promote, through an inner market growth, developing appropriate operational protocols to document in the race, testifying the consolidated technical capacity of Company which conferred the health reclamations provided by the "Plans to put public health emergencies". The legislative, regulatory act which by its nature is capable of directing the gradual harmonization of national laws giving to each state full autonomy on the form and means to be taken. Therefore, if the Community directives objectives were properly received and interpreted, the regulation of disinfestation Companies would be more adapted to the needs of domestic market.

Correspondence: Angelo Tamburro
AUSL9 (Local Public Health Services)
(AUSL 9) Grosseto Province,
U.O. Environmental Zoology
e-mail: a.tamburro@usl9.toscana.it

Conclusion

Unlike the current legislation in force in Italy (L. January 25, 1994 No 82 "Discipline of cleaning, disinfection, disinfection, rodent extermination and sanitation" Decree on July 7, 1997 No 274 "Rules of implementation of Articles 1 and 4 of the Act on January 25, 1994, n. 82"), offers no quality guarantee in the definition of technical and operational requirements that a Company must have for the award of services targeted to the management of "Plans to put public health emergencies".

Therefore, professional training, designed as an indispensable tool for improving emergency response capacity, must cover both government and private sectors, where the Technical Manager of Company, in a cooperation model, should prove to be able to manage organizational and operational solutions for health plans implementations in problems theme of "Pest Control". Mostly in this area could make disinfection Companies Associations, for example by requiring, at the time of the application for the specific documentation concerning quality management systems, including technical-operational and organizational procedures to be applied to individual intervention fields.

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A Regional plan of the Emilia-romagna regional bureau for *Aedes albopictus* control - year 2008

C. Venturelli, S. Mascali Zeo, P. Macini, P. Angelini, R. Bellini, R. Veronesi, M. Montanari

Venturelli C., Mascali Zeo S. Public Healt Department Ausl Cesena; Macini P., Angelini P. Public Healt Service of Region Emilia-Romagna; Bellini R., Veronesi R.. Centro Agricoltura Ambiente – Crevalcore (BO), Montanari M, Area Asset - Ravenna

Abstract. Following the outbreak of Chikungunya virus fever occurred in the summer 2007 in Emila Romagna (an administrative region located along the Adriatic (East) coast of Italy) a regional plan for Aedes albopicus control has been implemented. The major items of the plan are here reported and discussed.

Keywords: vector control, Italy, Emergence,CHYK outbreak

Among the objectives of the "Regional Plan of the Emilia-Romagna Regional Authority for the fight against the Asian Tiger Mosquito and the prevention of Chikungunya and Dengue Fever" there is optimization of the control methods against the Asian Tiger Mosquito to reduce the vector population density as much as possible. Control, intervention programmes, and actions decided upon at a regional level must be implemented locally. The local co-ordination unit must receive the technical support of the Public Health Departments of the Local Health Districts and of the technical Services and Departments of the Local authorities. The first detection of the Asian Tiger Mosquito was reported in Emilia-Romagna Region in 1994, close to a large deposit of second-hand tyres that had been imported by a company trading with non-EU Countries, such as the U.S.A. and Japan. Initially, only a few Municipalities were involved. Starting from the summer of 2003, the presence of the Asian Tiger Mosquito was massively recorded in all the Municipalities, except for those located in the hills or in the mountains, thus becoming a serious nuisance for the resident population. In many cases, the infestation took many local authorities by surprise, especially those that had never experienced mosquito problems in the past. At present, in the Emilia-Romagna Region, most Municipalities that are located in areas below 500 m above sea level are infested between April and October. In a few sites located on the plain and along the coast the infestation period often extends until late November. The maximum density of the mosquito adult population is normally reached between mid August and mid September and is anyway related to weather and climate conditions (temperature, rainfall, wind), to area characteristics (urban, rural, coastal or hill location) and to the microhabitat characteristics (scope and volume of the outbreak, insolation degree, etc). The Emilia-Romagna Region has since long implemented a surveillance system against the Asian Tiger Mosquito infestation based mainly on the use of oviposition traps and on the active search of adult mosquitoes and larvae.

The objective of the monitoring network that has been arranged for 2008 in the Emilia-Romagna region is to assess the level of Asian Tiger Mosquito infestation in all provinces and in major urban centers, through a quantitative definition of the number of eggs.

With reference to the control measures against the proliferation of te Asian Tiger Mosquito the plan envisages 4 types of actions: ordinary measures in all the Municipalities with the presence of the vector extraordinary measures in those areas where indigenous cases occurred in 2007 and around certain or suspected cases; other measures are envisaged on all the regional territory, also in areas that are not directly concerned with breeding grounds, in case of presence of several large-sized outbreaks of autochthonous cases with a high attack rate outbreak.

Ordinary control measures that are carried out in the framework of the control plan include the periodical larvicidal treatment in public road drains and education and awareness-raising activities addressed to citizens in the management of private areas; adulticiding in areas concerned with especially intense infestation and in sensitive sites such as schools, hospitals, nursing homes, etc. A specific and targeted strategy in those areas where indigenous breeding grounds of Chikungunya fever transmission were present in the past, envisages the following extraordinary "door to door" pest control in private properties with larvicidal treatment of the breeding grounds that cannot be eliminated and removal of all potential larval breeding grounds that can be eliminated, to be carried out in two stages. Measures to be carried out in case of presence of large-sized clusters or high attack rate outbreaks. The plan is therefore intended to avoid the possible resurgence of the virus transmission in the 2008 season.

Correspondence: Claudio Venturelli
Local Health Services (AUSL),
Dept of public Health, Province of Forlì- Cesena
e-mail: cventurelli@ausl-cesena.emr.it

SIMPOSIO 4

IL RUOLO DELLA RICERCA NELLA LOTTA ALLA MALARIA

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Old and new targets for innovative antimalarial compounds: the different strategies of the Italian Malaria Network.

N. Basilico[1,3], E. Bosisio[2], F. Buelli[6], G. Campiani[3,4], M. Casagrande[5], F. Castelli[6], P. Coghi[7], Y. Corbett[1], L. Cortelezzi[8], S. D'Alessandro[1], M. Dell'Agli[2], F. Esposito[9], C. Fattorusso[10], E. Fattorusso[3,10], S. Finaurini[1], G.V. Galli[2], S. Gemma[3,4], A. Habluetzel[9], L. Lucantoni[9], S. Melato[7], D. Monti[3,7], P. Olliaro[11], F. Omodeo-Salè[8], S. Parapini[1], M. Persico[10], M. Rizzi[12], S. Romeo[5], F. Rossi[12], C. Rusconi[5], A. Sparatore[5], O. Taglialatela Scafati[10], E. van den Bogaart[1], D. Taramelli[1,3], N. Vaiana[5] , S. Yerbanga[9]

[1]Dipartimento Salute Pubblica-Microbiologia-Virologia; [2]Dipartimento Scienze Farmacologiche, Università di Milano; [3]Centro di Ricerca Interuniversitario NatSynDrugs; [4]Dipartimento Farmaco Chimico Tecnologico, Università di Siena; [5]Ist. Chimica Farmaceutica e Tossicologica, Università di Milano; [6]Istituto di Malattie Infettive e Tropicali, Università di Brescia; [7]ISTM – CNR Milano; [8]Istituto di Fisiologia Generale e Chimica Biologica "G Esposito,Università di Milano; [9]Dipartimento di Medicina Sperimentale e Sanità Pubblica, Università di Camerino; [10]Dipartimento di Chimica delle Sostanze Naturali, Universita' di Napoli "Federico II"; [11]UNICEF-UNDP-World Bank-WHO-TDR, Geneva, CH; [12]DiSCAFF, Università del Piemonte Orientale "A. Avogadro".

Abstract. Clinical treatment-failures to affordable drugs encouraged new investigation for discovery and development of new prophylactic and therapeutic interventions against malaria. The Drug Discovery Cluster (DDcl) of the Italian Malaria Network gathers several highly integrated and complementary laboratories from different Italian Institutions to identify, synthesise, screen *in vitro* and *in vivo* new antimalarial molecules directed against the intraerythrocytic stage of *P. falciparum* parasites and/or with transmission blocking activity to select lead compounds for further development. Complementary research activities, both *in vitro* and in the clinics, aim at investigating the pathogenetic mechanisms of severe malaria anaemia and the different manifestations of the disease in malaria-HIV co-infected patients to identify new therapies and improve survival.

Key words: Malaria, *P. falciparum*, antimalarial drugs, trasmission blocking agents, severe anaemia.

Clinical treatment-failures to affordable drugs contributed to the global increase in the number of deaths arising from malaria infection (Breman, Alilio et al. 2007).There is an absolute requirement for new prophylactic and therapeutic interventions at all levels, as acknowledged by the G8 summits in 2006 and 2007, and by the new Strategy of TDR-WHO, that proposes **"to foster innovation for product discovery and development** as part of **an effective global research effort on infectious diseases of poverty in which disease endemic countries play a pivotal role"**(WHO-TDR 2007). In particular, the clinical and epidemiological features of falciparum malaria require new approaches targeting the parasite, its transmission and the most severe malaria complication, namely cerebral malaria or severe anaemia. Novel drugs should be cheap and safe, free from cross-resistance with existing ones and be adapted to use in combination chemotherapy. New combinations should include transmission-blocking compounds.

On this background, the principal aim of the **Drug Discovery Cluster (DDcl) of the Italian Malaria Network** is to identify, synthesise, screen *in vitro* and *in vivo* new antimalarial molecules directed against the intraerythrocytic stage of *P. falciparum* parasites and/or with transmission blocking activity to select lead compounds. Optimization of leads through iterative medicinal chemistry and pharmacological profiling will allow the selection of drug candidates for preclinical tests. Adjunctive therapies are under study for those patients, usually children, that develop severe anaemia.

To achieve these objectives **DDcl** has been organised in the last few years as a multidisciplinary network that gathers several highly integrated and complementary laboratories and clinicians from different Italian Universities and from the CNR. **DDcl** has the expertise for isolation and synthesis of new compounds (chemical labs), for *in vitro* and *in vivo* screening for efficacy (biological labs), for the characterization of the molecular targets and mechanism of action, for optimization of lead compounds through a "molecular modelling"

Correspondence: Donatella Taramelli
Dipartimento di Sanità Pubblica-Microbiologia-Virologia,
Università di Milano, Via Pascal 36, 20133 Milano, Italy
Tel: +39 02 50315071; fax: +39 02 505068;
e-mail: donatella.taramelli@unimi.it

approach and SAR studies. **DDcl** can also count on the expertise of Dr Piero Olliaro from UNICEF-UNDP-World Bank-WHO-TDR. Several research units within the cluster are collaborating with pharmaceutical industries for the completion of the preclinical work on some of the most promising molecules. A summary of the overall strategy and recent achievements of DDcl are reported in the following sections, which recapitulate the drug discovery process.

Target identification and validation; synthesis and collection of new molecular entities.

Different approaches are followed by DDcl researchers to discover new antimalarials: 1- a genomic-proteomic approach to identify specific drug targets inside metabolic pathways that are characteristic either of the parasite or the invertebrate vector and not of the human host; 2- isolation and identification of natural products from marine or plant origin; 3- modification of existing pharmacophore to increase efficacy and overcome resistance; 4- rational design of new chemical entities based on the characteristics of the microenvironment of the parasites.

Genomic-proteomic approach.

The Kynurenine Pathway (KP) of *Anopheles gambiae* is a promising target for novel transmission-blocking compounds or insecticides (Han, Beerntsen et al. 2007) The approach of the group of M. Rizzi of the University of Piemonte Orientale is focused on the structural and functional characterization of *A. gambiae* KP enzymes controlling kynurenines synthesis and homeostasis in the mosquito and of *Pf* kynases essential for parasite gametogenesis within the arthropod host (Rossi, Lombardo et al. 2005; Rossi, Garavaglia et al. 2006). The selected enzymes, produced in recombinant forms, will be subjected to biochemical characterization and X-ray crystallography to determine their 3D structure for the *in silico* identification and the structure-based rational design of selective inhibitors.
Highly active inhibitors of the aspartic proteases, plasmepsins II and IV, responsible of haemoglobin digestion in the parasite food vacuole during the intraerythrocytic stage have been synthesised as "double drugs" by S. Romeo and tested on recombinant enzymes by E. Bosisio, both groups at the University of Milan. The double-drugs consist of primaquine joined with a peptidic linker to a statine-based inhibitor. Some of them are among the most active plasmepsins inhibitors ever reported. They inhibited the recombinant PLM enzymes with a Ki in the low nM and were toxic against CQ-R, *Pf* parasites *in vitro* with IC_{50} of 200-500 nM (Romeo, Dell'Agli et al. 2004). Optimisation of the intraerythrocytic activity of these inhibitors has been achieved by a systematic change of peptide's aminoacids, also with non-peptidic structures (Dell'Agli, Parapini et al. 2006). One of the most recent compounds also significantly inhibited parasitaemia when given intraperitoneally in the rodent *P. berghei* model.

Natural products from vegetable and marine sources.

Plants and traditional medicine in third world countries are an important source of compounds with potential antimalarial activity. The group of E. Bosisio in Milan has been working on the antiplasmodial activity of *Ailathus excelsa* (Dell'agli, Galli et al. 2008) and is presently involved in the extraction, bio-guided purification and identification of the active principle of a vegetable remedy used in the ORISSA region, in India. Promising results have also been obtained by the group of A. Habluetzel in Camerino with neem (*Azadirachta indica*) extracts on the murine *P. berghei* model. A methanolic leaf extract reduced parasitaemia in treated mice after oral administration, and a commercial seed extract, NeemAzal®, exerted a complete block in parasite development in the vector. NeemAzal® also, had an impact on *A. stephensi* fitness, reducing blood feeding, oviposition and survival of treated females (Lucantoni, Giusti et al. 2006).
The researchers of the University of Naples Federico II, directed by E. Fattorusso recently defined the absolute configuration of plakortin, an endoperoxide extracted from the sponge *Plakortis simplex* with relevant antimalarial activity (Campagnuolo, Fattorusso et al. 2005). SAR studies have been conducted in a series of natural products related to plakortin and its semi-synthetic derivative to improve efficacy (Fattorusso, Campiani et al. 2006). These results are particularly interesting for understanding the mechanism of action of endoperoxides, since the artemisinin derivatives, which are the most potent antimalarial drugs presently available, are also characterized by a trioxane pharmacophore, crucial for activity (Haynes 2006).

Modification of existing pharmacophores to increase efficacy and overcome resistance

Aminoquinoline type drugs (i.e. chloroquine) have been the mainstay of antimalarial therapy until the emergence and spread of resistance hampered their usage. However, the 4 aminoquinolines are still interesting compounds since their mechanism of action and resistance seems to be unrelated (Egan and Kaschula 2007). The group of A. Sparatore at the University of Milan recently synthesised new quinolizidine derivatives of 4-aminoquinolines, characterised by the presence of bulky bicyclic, highly basic and lipophilic lateral chain, derived from lupinine (extracted from *Lupinus luteus*) (Sparatore, Basilico et al. 2005). Two of these compounds are extremely active against drug resistant strains of *P. falciparum in vitro* and orally *in vivo* in murine models at doses comparable and lower than CQ. They are not toxic with good pharmacokinetic profile and are presently under study for further preclinical development in collaboration with NeED Pharmaceutical and the financial support of the EU-

FP6 project ANTIMAL. At the same time, the group of D. Monti of CNR-ISTM-Milano is involved in the development of novel synthetic approaches and combinatorial synthesis for a rapid, cheap, clean and scalable route to 4-aminoquinolines. They first employed the microwave assisted reactions and subsequently they applied "click chemistry" for the efficient conversion of the commercially available 4,7-dichloroquinoline into a library of aminoquinolines in high yields and purities, in a very short time with no need for further purification steps. They also investigated the trichlorotriazine as core scaffold for the synthesis of multivalent antimalarial agents: they obtained a series of compounds that exibit good reactivity *in vitro* against CQ-R strains (Melato, Coghi et al. 2007; Melato, Prosperi et al. 2008).

Rational design of new chemical entities.

The research activity of the NatSynDrugs unit (www.natsyndrugs.org, G. Campiani, University of Siena and C. Fattorusso University Federico II, Naploli) is focused on the identification of new low cost, safe drugs for the treatment of drug resistant parasites. The synthetic strategy is based on the rational design of new compounds able to coordinate metals, haemoglobin iron in particular, in the reducing milieu of *Pf* (redox milieu -250 mV), thus producing radical species selectively toxic for the parasite (altering *Pf* fragile redox equilibrium). Several classes of new compounds with a common metal-mediated mechanism of action have been synthesised and some of them are characterized by (i) potent antimalarial activity *in vitro* against CQ-R strains of *Pf*; (ii) lack of *in vitro* cytotoxicity against normal cells; (iii) a promising *in vivo* activity against both *P. berghei* and *P. chabaudi* in mice (Gemma, Campiani et al. 2007; Gemma, Kukreja et al. 2007; Fattorusso, Campiani et al. 2008; Gemma, Campiani et al. 2008; Gemma, Kukreja et al. 2008). Some of these molecules have been selected as leads compounds for further *in vivo* studies and have been patent protected. (Campiani G., Gemma S. et al. (2007) US No. 60/890,862 EP2007/052174, EP2007/052176)

Evaluation *in vitro* against whole parasites (hit selection) and *in vivo* in animal models (lead selection).

All the synthetic compounds and isolated natural products have been assayed *in vitro* against *P. f.* strains with a different resistance phenotype by D. Taramelli group, University of Milan. A spectrophotometric chemosensitivity assay based on the evaluation of *P.f* pLDH has been used. Molecules with an IC50 <1μM against *Pf* are then assayed in association with "in use" antimalarials (such as the artemisinin derivatives) to verify the potential use in combination therapy. Toxicity tests against a panel of normal human or murine cells allows the selection of compounds with a good therapeutic index and suitable for *in vivo* testing.
The molecules active *in vitro* are tested *in vivo* using the murine model of *P. berghei - Anopheles stephensi* - Balb/c mice by the group of A. Habluetzel- F. Esposito at the University of Camerino. Standard protocols are applied to assess curative, prophylactic and transmission blocking activity with potential to be integrated in combination therapies designed for malaria control at the community level.

Studies on the mechanism of pathogenicity and toxicity in relation to host immunity and co-infections

The scientific activity of **DDcl** is not restricted to the discovery and screening of potential new antimalarial drugs, but is integrated by a series of biochemical, immunological and clinical studies. Part of the activity is focused on the understanding the pathogenetic mechanism of severe malaria anaemia in children (F. Omodeo Salè- D. Taramelli, University of Milan). There is experimental and clinical evidence that severe anaemia is related to a fast elimination of normal, non-infected erythrocytes, that are rendered prematurely senescent by parasite products. The attempt is to identify the mechanisms for the development of adjunct therapies (Omodeo-Salè, Motti et al. 2003; Omodeo-Salè, Motti et al. 2005; Nuchsongsin, Chotivanich et al. 2007).
The group of F. Castelli, University of Brescia is coordinating a series of clinical-epidemiological studies conducted both in endemic countries (i.e. Burkina Faso) and in Italy among immigrants. In Burkina Faso the attention is focused on understanding how the severity of malaria is influenced by a concomitant infection with HIV: in agreement with epidemiological data from other countries, it appears that there is a negative interaction between the two infections and new therapeutic regimens need to be employed. The studies performed in Italy are mainly related to investigate the anti-malarial immunity in migrants from endemic areas to Italy and to define the epidemiological characteristic of imported malaria cases (Castelli, Capone et al. 2007; Calleri, Beherens et al. 2008; De Iaco, Saleri et al. 2008).
In conclusion, **DDcl** appears as very dynamic network of researchers focused on the rational discovery of new antimalarial drugs from different sources and/or on the identification of new combination chemotherapies based on the pathogenetic mechanism of the disease and the epidemiological context. The strength of **DDcl** is confirmed by the number of publications and patents, and the extent of collaborations established with European and non European research groups and Developing Countries.

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The role of research in molecular entomology in the fight against malaria vectors

A. della Torre[1], B. Arca[2], G. Favia[3], V. Petrarca[4] , M. Coluzzi[1]

[1]Sezione di Parassitologia, Dipartimento di Scienze di Sanità Pubblica, Università "La Sapienza", Italy; [2]Dipartimento di Biologia Strutturale e Funzionale, Università Federico II, Napoli, Italy; [3]Dipartimento di Medicina Sperimentale e Sanità Pubblica, Università di Camerino, Italy; [4]Dipartimento di Genetica e Biologia Molecolare, Università "La Sapienza", Italy.

Abstract. The text summarizes the principal current fields of investigation and the recent achievements of the research groups presently contributing to the Molecular Entomology Cluster of the Italian Malaria Network. Particular emphasis is given to the researches with a more direct impact on the fight against malaria vectors.

Key words: *Anopheles gambiae*, genomics, vector control, malaria epidemiology.

In 1990, the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), together with the John D. and Catherine T. MacArthur Foundation and the University of Arizona, convened a meeting in Tucson, Arizona, where specialists in entomology, genetics and biochemistry were brought together to discuss the prospects for malaria control by genetic modification of the vector competence of natural vector populations. That meeting can be considered as the birth of modern medical entomology and, since then, an extraordinary amount of research has been carried out on malaria vectors, with particular focus on *Anopheles gambiae*, the principal vector of malaria in sub-Saharan Africa. Studies carried out in the last two decades led to major scientific advances, such as the genome sequencing and the stable germ-line transformation of *A. gambiae*, the engineering of laboratory strains refractory to *Plasmodium* infection and, overall, to a deeper understanding of *Plasmodium/Anopheles* interactions. Moreover, beside these achievements - which are directed towards the development of novel malaria control strategies that are probably many years from reaching implementation - several progress have been made in areas of molecular entomology that are likely to have a more near-term impact on malaria control. These include, among others, the understanding, detection and monitoring of some of the mosquito resistance mechanism against insecticides, the determination of the genetic structure of vector populations, the development of molecular tools for the identification of cryptic taxonomic units and a preliminary understanding of the molecular bases of their ecology and behaviours.

Molecular entomology in Italy stemmed from the pioneering studies by Mario Coluzzi and his first collaborators in the University of Roma "La Sapienza" who, since the years '60, dedicated a large part of their scientific career to the study of the genetic structure of natural *A. gambiae* populations through the analysis of the banding patterns of polytene chromosomes of the nuclei of the ovarian nurse cells of half-gravid females. The polytene chromosome map developed by Coluzzi's group has represented the basis for the international effort leading to the *A. gambiae* genome sequencing (Holt *et al.*, 2002). Moreover, Coluzzi's work has provided the first molecular method for identifying the members of the *A. gambiae* complex, differentially involved in the malaria vector system, as well as revealed the existence of intra-specific genetic variations based on paracentric inversion polymorphisms (reviewed in Coluzzi *et al.*, 2002). In the last 15 years, the group in Rome has been involved in a large variety of molecular studies on *A. gambiae*, ranging from the identification of sub-structuring within the major vector species, *A. gambiae* sensu stricto (s.s.), the development of molecular methods for the identification of novel Operational Taxonomic Units (OTUs), the study of their genetic and ecological divergence, the monitoring of insecticide resistance spreading and the molecular basis of *Plasmodium/Anopheles* interactions. The groups presently contributing to the Molecular Entomology Cluster of the Italian Malaria Network all originated from the group in Rome, although had differentiated their scientific targets. A brief description of their principal current fields of investigation and of their recent achievements is provided below, with particular reference to those with a more direct impact on the fight against malaria vectors.

The group of the University of Roma "La Sapienza" has continued the studies initiated by Coluzzi by integrating the cytogenetic approach with the molecular one. The main result coming out from these combined approaches has been the description of two "molecular forms" (namely, M- and S-form) within *A. gambiae* s.s. (della Torre *et al.*, 2001; 2002). These forms have been shown to be largely sympatric throughout west-Africa and to be characterised by a high level of gene-flow restriction and by a low degree of inter-form genetic differentiation; moreover, paracentric inversions on

Correspondence: Alessandra della Torre
Department of Public Health Sciences, Parasitology Unit,
"Sapienza" - University of Rome, P.le Aldo Moro, 5,
00185, Rome, Italy
Tel +39 06 4969 4268; Fax +39 06 4991 4653
e-mail: ale.dellatorre@uniroma1.it

chromosome-2 have been shown to be shared by the two forms, although often with very different frequencies of alternative arrangements, leading to the suggestion of a role of these inversions in ecotypic adaptation (della Torre *et al.*, 2005). From the biomedical perspective, the two forms do not appear to show obvious differences in their efficiency in transmitting malaria; however, their different temporal and spatial dynamics and, in particular, the strong association of the M-form with larval environment created by human activities such as rice cultivation, is expected to alter the malaria transmission dynamics, which have shifted from seasonal and rain-dependent to permanent and dependent-from-irrigation. Moreover, the two forms show different resistance mechanisms to insecticides used to control vector densities, as shown by the different distribution in the two OTUs of the *knock-down* (*kdr*) alleles conferring resistance to pyrethroids and DDT. Since the first description in 2001, the molecular forms have been the focus of several studies by the international malaria entomology community, aimed at understanding the process of genetic and ecological differentiation between them and to unravel the mechanisms of their speciation process, which seems to be strictly associated to and triggered by man-made modifications of the environment. In the last years the group in Rome has continued to participate to the international effort by contributing to studies on the genetic differentiation between M and S (see also Mancini E *et al.*, Santolamazza F *et al.* and Pombi *et al.*, this abstract book). One of the most direct contributions of these studies to the current fight against malaria is the development of molecular tools for the identification of *A. gambiae* species and forms, some of which have become the methods routinely used by the field entomologists (Fanello *et al.*, 2002; Santolamazza *et al.*, 2004; Mancini E *et al.*, this abstract book). The application of these tools has also allowed to provide a comprehensive picture of the M and S geographic distribution (della Torre *et al.*, 2005) and of the differential spreading of *kdr* alleles in the two forms (Santolamazza *et al.*, 2008). Moreover, preliminary information have been obtained on their feeding habits and sporozoite infection frequencies in Angola (Calzetta *et al.*, 2007), Cameroon (Wondji *et al.*, 2005), The Gambia and eastern Senegal (Caputo *et al.*, in preparation).

The group in the University of Napoli has mainly focused its research interests on the *A. gambiae* salivary glands, an organ that gets into multiple interactions with both the parasite and the host (Arcà *et al.*, 1999). In fact, recognition and invasion of salivary glands by *Plasmodium* sporozoites is a prerequisite for a successful transmission, making this organ a very interesting target of strategies aimed at interrupting the transmission in the mosquito vector. Moreover, the salivary secretions carry a large number of factors whose pharmacological activities affect crucial host responses such as hemostasis and immunity. The salivary transcriptome of *An. gambiae* has been largely worked out and more than 70 salivary proteins have been identified to date (Arcà *et al.*, 2005); surprisingly, so far no functions could be assigned to almost half of these proteins, pointing out how much we still have to learn on the salivary secretions of blood sucking Arthropods. These studies well conjugate basic research interests on salivary functions and evolution of blood feeding to potential applications finalized to the development of novel tools for mosquito and malaria control. In this respect there are at least two aspects that came to light recently and may have relevant implications for malaria control. First, mosquito salivary proteins may play an important, underestimated role in the vertebrate immune response to parasite infection. Indeed, two independent studies in murine malaria models reported that pre-immunization of mice by exposure to bites of uninfected mosquitoes has a protective effect on a following *Plasmodium* infection. Actually, this phenomenon is the result of a shift of the immune response toward a Th1-type, with induction of IFNg and iNOS, which in turn brings about lower levels of parasitemia (Donovan *et al.*, 2007; Fonseca *et al.*, 2007). Moreover, lymph nodes close to the cutaneous infection site seem to play an unexpected crucial role in protective antisporozoite response (Chakravarty *et al.*, 2007). Based on these experimental evidences it has been suggested that *Anopheles* salivary proteins may be useful components of malaria vaccines. Moreover, these observations have another important implication. In endemic areas, even with very high levels of transmission, the number of bites received by exposed individuals from non-infected anopheline mosquitoes is much higher than the bites from infected mosquitoes. Therefore, mosquito saliva may play an important, so far undervaluated, role in the acquirement of natural immunity in endemic areas. Second, human antibody response to *A. gambiae* saliva has been suggested as a possible indicator of the exposure to bites of Anopheline mosquitoes and, therefore, a potential marker of malaria risk (Remoue *et al.*, 2006). The detailed molecular knowledge of the *A. gambiae* salivary gland protein repertoire is of crucial importance for the development of both the aspects mentioned above. For example, comparative analysis of salivary transcriptomes of different mosquito species allowed to identify a relatively large group of salivary proteins that seem to be specific of *Anopheles* since they are not found in *Aedes* or *Culex* mosquitoes (Lombardo *et al.*, 2006). Some of these proteins, if immunogenic, could be very useful as epidemiological markers of exposure to *Anopheles* mosquitoes (see also Ronca R *et al*, this abstract book). For example, they could allow the evaluation of the efficacy of vector control measures or the potential malaria risk also in those cases in which the use of classical entomological methods are difficult or even impossible.

The main research focus of group in the University of Camerino is represented by the study of the microbiota associated to different mosquito vectors, in view of the possible development of paratransgenic protocols

to control the transmission of diseases. Paratransgenesis is in fact considered a promising approach through which exogenous genes with a parasiticidal effect can spread into natural mosquito populations through the genetic transformation of symbiontic bacteria. Obviously, an essential prerequisite for this approach is the identification of mosquito symbionts. The group in Camerino has recently discovered a strong and ubiquitous association of an acetic acid bacterium of the genus *Asaia* with some *Anopheles* species (Favia *et al*., 2007; Favia *et al*., 2008). *Asaia* bacteria appear to be have characteristics which make them a good possible target for the development of paratransgenesis protocols, i.e. they i) can be easily cultured and re-introduced into mosquitoes, thus allowing manipulation experiments, ii) appear to be associated to multiple mosquito tissues, such as midgut, reproductive tracts and salivary glands, iii) appear to be vertically transmitted from an infected female mosquito to its progeny. Further studies have allowed the development of a genetic transformation system for *Asaia* strains, implying Green Fluorescent Protein (GFP) *in situ* expression after transformation and infection. Further studies are in progress with the aim to obtain: i) a better definition of *Asaia* transmission routes; ii) the assessment of its functional role(s); iii) the determination of a possible microbiological competition with other potential symbionts; iv) the expression of effector molecules in *Anopheles* after challenge with *Plasmodium*; and v) the determination of *Asaia* distribution in different mosquito species. Overall, these studies are aimed to evaluate the potentiality of *Asaia* as a gene delivery system in malaria vectors, which, if confirmed, would open new perspectives for the control of malaria as well as of others vector borne diseases.

To summarise, the molecular studies carried out in Italy on malaria vectors directly contribute to several crucial aspects of the global fight against malaria vectors. In fact, thanks to these studies, new methods have been, or are being, developed for the characterization of parameters relevant for malaria epidemiology, such as the identification of new OTUs whose existence complicates the epidemiological analysis of the vectorial system, and the measurements of malaria risk through the evaluation of human antibody response to *A. gambiae* saliva. Moreover, both the group in Rome and in Camerino are developing new approaches for age-grading *Anopheles* populations, by means of the analysis of the epicuticular hydrocarbon profiles of single mosquitoes (Caputo *et al*., 2005) and of the analysis of transcriptional data of four genes whose abundance varies with age (Ricci and Favia, manuscript in preparation). The possibly to precisely assess this parameter would represent a crucial advance to the correct evaluation of the vectorial capacity. From the perspective of vector control, the study and monitoring of the spread of insecticide resistance mechanisms within the mentioned above different taxonomic units is providing relevant information for the design of insecticide-based control programmes, while new perspectives are opened in the genetic control of malaria vectors by the studies on *Asaia* symbionts and in the optimisation of future malaria vaccines by the studies on *Anopheles* salivary proteins.

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The role of research in the fight against malaria: the Italian contribution to malaria research in the frame of north-south cooperation in the last 25 years

F. Esposito[1], G. Majori[2], M. Coluzzi[3]

[1]Department of Experimental Medicine and Public Health, University of Camerino, 62032 Camerino (MC), Italy; [2]Department of Infectious, Parasitic and Immunomediated Diseases, Istituto Superiore di Sanità, Roma, Italy; [3]Department of Public Health Sciences, University of Rome "La Sapienza", Italy

Italy has an extraordinary tradition in malaria research, from the Grassi discovery of the parasite life cycle, to the Coluzzi breakthrough into the species problem of the anopheline vectors. Furthermore, Italy was a malaria endemic country until the first half of the XXth century and therefore has been the theatre of large scale 'experiments' aimed at controlling the disease and its transmission. Undoubtedly, these experiments and their successful outcomes guided, sometimes with exceedingly optimistic foresights, control programmes in various areas of the world. A deeper insight into the determinants of the successful malaria eradication in Italy allowed the roots of that success to be tracked and wiped off many illusions. Thus, over the years, Italy has remained a reservoir of expertise, and Italian scientists have been involved in malaria research and control programmes funded by international agencies, from the 'Garki Project' in Nigeria, to the most recent programmes devoted to counteract malaria resurgence in problem areas bordering the south-eastern fringe of Europe.

Interestingly, in the late 70's of last century, when the big draught hit the sahel area south of Sahara, there was in Italy a renewed rise of interest for tropical diseases, especially malaria. Then, the Italian Department for Co-operation in Development supported the idea of a malaria control programme in Burkina Faso, which actually started in 1983. Although specific results in terms of reduction of the malaria burden are just in the process of becoming reality 25 years later, it has to be recognised that the programme gave enormous contributions to a better understanding of malaria under extremely high transmission conditions. New and innovative methods for malaria epidemiology were established, which allowed previously unthinkable levels of transmission to be detected. A complexity within the vector populations which was before only guessed was definitely demonstrated. The very special characteristics of the immune response against the malaria parasites - of paramount importance in view of a malaria vaccine development - were elucidated, together with an unexpected heterogeneity in the susceptibility to malaria in individuals belonging to different ethnic groups. Various vector control tools, including insecticide treated nets, were challenged with the most unfavourable conditions which can be imagined, and the obtained results led to the adoption of the strategy within the national control programmes in Africa. Home management of malaria cases was extensively tested, and also in this case the positive results induced its incorporation within control programmes. In conclusion, the Italian Programme in Burkina Faso, with the participation of various research groups from Italian and international institutions represented an exceptional blend of curiosity driven and translational research which paradigmatically demonstrated the need for both components for a significant advancement of knowledge. The empowerment of the National scientists constitutes the most relevant strength of the Programme. The Italian research institutions provided a similar contribution in other African countries and in particular in Madagascar when, at the end of the '80s, malaria transmission reappeared on the Central Highlands with deadly epidemics. In that dramatic situation, the Italian malariologists, with funds made available by the Italian health cooperation, were able to assure technical assistance and leading operational research to the National Malaria Control Program since the resurgence of malaria. The international funding agencies strengthened in the following years the fundamental role of the applied research to the success of malaria control campaigns.

The most recent years showed unfortunately a decrease of interest by the Italian public donors, a surprising event, in view of the opposite attitude by public and private donors worldwide. Hopefully, the Italian Malaria Network may be instrumental to reverse the situation and renew the brilliant tradition of the Italian School of Malariology.

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Hemozoin and the human monocyte-A brief review of their interactions

E. Schwarzer, O.A. Skorokhod, V. Barrera, P. Arese

Department of Genetics, Biology and Biochemistry, University of Torino, Via Santena 5 bis, 10126 Torino, Italy

Abstract. In vitro, human monocytes avidly ingest hemozoin (HZ) that modifies a number of monocyte functions. Inhibitory effects: inhibition of: PMA-elicited respiratory burst, ability to killing and repeat phagocytosis, activity of NADPH-oxidase and PKC, expression of ICAM-1, integrin-CD11c, MHC-class-II (IFN-gamma-mediated), differentiation to functional, antigen-presenting dendritic cells. Stimulatory effects: increase in phagocytosis-related respiratory burst and accumulation of lipoperoxidation products; induction of metalloproteinase-9 and pro-inflammatory cytokines and chemokines. Mechanism of action: HZ generates by non-enzymatic catalysis large amounts of lipoperoxidation products, such as monohydroxy derivatives of arachidonic (HETE) and linoleic (HODE) acid, and 4-hydroxynonenal (HNE). Several HZ effects were reproduced by supplementation with plausible concentrations of HETE or HNE, the first most likely via interaction with PPAR-receptors, the second via adduct or crosslinks formation with critical targets.

Key words: Malaria, malaria pigment, hemozoin, monocytes, HETE, 4-hydroxynonenal

Human phagocytic cells avidly ingest hemozoin (HZ) and HZ-containing trophozoites and schizonts. *In vitro*, approx. 9-10 trophozoites/schizonts, or corresponding amounts of HZ were taken up per monocyte. Three hours after start of phagocytosis 79±30% of monocytes were extensively HZ-laden, and approximately 30% of cell volume was occupied by HZ HZ (Schwarzer *et al.*, 2001; Arese and Schwarzer, 1997).

Inhibitory effects of HZ

1. Role of HZ phagocytosis in malaria immunodepression

Altered cellular responses to blood-stage *Plasmodium* antigens, reduced induction of immunity to vaccines, reduced T cell proliferation, and short-lived antibody responses are common observations in malaria. It has been shown by us that induction of MHC class II in response to IFN-gamma stimulation was defective in HZ-laden monocytes (Schwarzer *et al.*, 1998). Abrogation of MHC class II expression was present at protein and mRNA expression level, providing a possible link between HZ loading, suppression of IFN-gamma responsiveness, failure of MHC class II upregulation and disturbances in antigen presentation and immunodepression in malaria (Schwarzer *et al.*, 1998; Scorza *et al.*, 1999). 4-hydroxynonenal (HNE), a potent aldehyde originating from lipoperoxidation of unsaturated fatty acids (Schwarzer *et al.*, 2003), accumulates in membranes and may be causally involved in the effect. Indeed, unpublished experiments (Schwarzer, unpublished) show that low-micromolar HNE inhibited IFN-gamma mediated MHC class II expression and mimicked HZ action. The same studies indicated that HZ-laden monocytes had reduced spontaneous upregulation of CD54 (ICAM-1), an adhesion molecule that contributes considerably to the capacity of monocytes to adhere and stimulate T-cell proliferation (Schwarzer *et al.*, 1998). Thus, our data may contribute to explain defective T-cell response in malaria.

2. Inhibition of differentiation/maturation to DC

Monocytes are a prime source of dendritic cells (DC) *in vivo* and *in vitro*, that play pivotal roles in adaptive immune responses and innate immunity. We have challenged human monocytes before the initial induction/final maturation to mature DC with HZ. Blunted expression of MHC class II and costimulatory molecules indicated that both differentiation and maturation of HZ-loaded monocytes to DC were severely impaired (Skorokhod *et al.*, 2004). These effect were reproduced dose-dependently by HNE supplementation, possibily via stimulation of PPAR-gamma receptor or interaction with CD14/LPS-receptor. Those studies may be significant in malaria immunodepression to explain inhibited response of T and B lymphocytes; reduction in expression of MHC class II; and insufficient antibody production. Recently in confirmatory studies HZ was found to induce failure of DC function in vivo and in vitro in a *P. chabaudi* murine model (Millington *et al.*, 2006). Contrasting results were obtained with highly purified HZ, though, shown to induce DC maturation and activation of murine DC via Toll-like receptor 9 (Coban *et al.*, 2005; Coban *et al.*, 2002).

3. Inhibition of erythropoiesis and thrombopoiesis

Severe malarial anemia, an important cause of mortality, is the result of destruction of parasitized and non-parasitized RBC, and impaired erythropoiesis. Bone-marrow (BM) macrophages produce a variety of

Correspondence: Paolo Arese
Department of Genetics, Biology and Biochemistry, University of Torino, Via Santena 5 bis, 10126 Torino, Italy.
Tel: +390116705846; fax: +390116705845;
e-mail: paolo.arese@unito.it

hematopoietic regulatory or suppressive factors, such as IL-1, TNF, TGF-beta and macrophageinhibitory proteins. Free HZ and HZ-containing trophozoites/schizonts, and HZ-laden macrophages are abundantly present in BM of malaria patients (Arese and Schwarzer, 1997). We have shown that HZ supernatants equivalent to 12.5 trophozoites/progenitor inhibited erythroid growth. Supernatant of delipidized HZ was significantly less effective. Supernatants of HZ-fed monocytes also inhibited BFUE growth whereas supernatants of latex-fed or RBC-fed monocytes had no effect (Giribaldi *et al.*, 2004). Inhibition of erythroid growth and thrombopoiesis was reproduced dose-dependently by HNE supplementation, found to generate adducts with crucial GM-CSF-receptor (Skorokhod *et al.*, 2004).

Stimulatory effects of HZ

1. Stimulation of production of pro-inflammatory molecules

Elevated serum concentrations of pro-inflammatory cytokines, MIP-1alpha and macrophage migration inhibitory factor (MIF) have been found in malaria patients, correlated with disease severity. Several *in vitro* studies have shown that phagocytosis of HZ by human monocytes induced release of several of the above factors. Those data confirm the importance of HZ as a stimulatory factor of monocytes in malaria. Preliminary data by our group (Giribaldi G, unpublished) have shown cytokine and MIP-1alpha upregulation by 15-HETE.

2. Activation of metallo-proteinase 9

It has been recently shown in our group (Prato *et al.*, 2005) that HZ-fed human monocytes displayed increased metalloproteinase-9 (MMP-9) activity and protein/mRNA expression. MMP-9 functions by proteolytically shedding pro-forms of cytokines such as TNF-alpha and IL-1beta in the blood, by disrupting the subendothelial matrix and enhancing extravasation of blood cells. Activation and induction of MMP-9 were reproduced dose-dependently by 15-HETE (Prato M, unpublished).

Mechanism of HZ action

In HZ and parasitized RBC a complex mixture of monohydroxy derivatives of arachidonic (HETE) and linoleic (HODE) acid, and large amounts of the terminal aldehyde HNE have been determined by our group (Schwarzer *et al.*, 2003). No evidence of lipoxygenase activity was found in parasites, while the large number of isomers, their racemic structure and generation by incubation of arachidonic acid with HZ indicated their non-enzymatic origin *via* hemecatalysis (Schwarzer *et al.*, 2003). Phagocytosed HZ ferries those lipid derivatives into the phagocyte, while ingested HZ further produces the same compounds (Schwarzer *et al.*, 2003). Mechanistically, we have provided evidence that specific HETE, HODE or HNE generated by HZ were responsible for the abrogation of oxidative burst and other inhibitory effects mediated by HZ phagocytosis (see above). HNE, which avidly reacts with thiols and amino groups of proteins to form stable Michael adducts or Schiff base crosslinks (Skorokhod *et al.*, 2005), seems to play an important mechanistic role. Work in progress will determine in detail localization of protein-HNE adducts in the various HZ-affected systems.

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Genetic epidemiology of susceptibility to malaria: not only academic exercises

F. Verra, P. Avellino, G. Bancone, V. Mangano, D. Modiano

Malaria Epidemiology Group, Department of Public Health Sciences, University of Rome "La Sapienza"

Abstract. Descriptive genetic epidemiology represents the initial step of a logical procedure of linked and consequential phases spanning from the identification of genes involved in the resistance/susceptibility to diseases, to the determination of the underlying mechanisms and finally to the possible translation of the acquired knowledge in new control tools. In malaria, the rational development and potential of this pathway is based on complementary interactions of heterogeneus disciplines going from epidemiology (the transmission, the infection, the disease) to vaccinology passing through genetics, pathogenesis, and immunology. Several epidemiological approaches can be applied in the study of the genetic susceptibility to *Plasmodium falciparum* malaria: intra-ethnic case-control studies comparing genetic candidates of resistance/susceptibility between subjects with different presentation of malaria (from severe disease to asymptomatic infection) and the general healthy population is the classic approach; inter-ethnic comparative analyses among populations with different genetic backgrounds, exposed to the same epidemiological context and showing different susceptibility to the disease is a further, complementary, strategy.

Key words: Malaria, hemoglobin, T regulatory cells, IRF-1

Plasmodium falciparum malaria represents one of the most selective forces for the human genome (Kwiatkowski DP, 2005). Even today in rural areas of sub-Saharan Africa entomological inoculation rates are often in the order of hundreds infective bites per person per year (Rogers DJ *et al.*, 2002) meaning that each individual in these areas is exposed to a potentially lethal infection more than once per day. This stable and perennial degree of selective pressure has contributed to the progressive accumulation of genetic conditions that decrease the susceptibility of human populations to malaria (Kwiatkowski DP, 2005). The study of this field of evolutionary biology, aims in the initial phase at the identification of genes involved in the resistance/susceptibility to the disease, then at the determination of the underlying protective mechanisms and finally at the possible translation of the acquired knowledge in new control tools.
A brief overview of recent achievements in this field by our group is presented.

Hemoglobin C and S role in acquired immunity against *Plasmodium falciparum* malaria (Verra *et al.*, 2007)

Conclusive evidence exists on the protective role of Haemoglobin C (HbC; β6Glu→Lys) against clinical *Plasmodium falciparum* malaria as well as of HbS (β6Glu→Val), both occurring in sub-Saharan Africa (Allison, A.C. 1954; Modiano *et al.*, 2001a). However, the mechanism/s of the protection exerted remain/s debated for both haemoglobin variants, HbC and HbS. Recently, an abnormal display of *Pf*EMP1, an antigen involved in malaria pathogenesis, was reported on HbAC and HbCC infected erythrocytes that showed reduced cytoadhesion and impaired rosetting *in vitro* (Fairhurst *et al.*, 2005). On this basis it has been proposed that HbC protection might be attributed to the reduced *Pf*EMP1-mediated adherence of parasitized erythrocytes in the microvasculature. Furthermore, impaired cytoadherence was observed in HbS carriers suggesting for the first time a convergence in the protection mechanism of these two haemoglobin variants (Cholera R., *et al.*, 2008). We investigated the impact of this hypothesis on the development of acquired immunity against *Plasmodium falciparum* variant surface antigens (VSA) encoding *Pf*EMP1 in HbC and HbS carriers in comparison with HbA of Burkina Faso. Higher immune response against a VSA panel and several malaria antigens were observed in all adaptive genotypes containing at least one allelic variant HbC or HbS in the low transmission urban area whereas no differences were detected in the high transmission rural area. In both contexts the response against tetanus toxoid was not influenced by the β-globin genotype. Thus, these findings suggested that both HbC and HbS affect the early development of naturally acquired immunity against malaria.

Quick but "costly" versus "slow but gratis" genetic adaptations to *Plasmodium falciparum* malaria (Modiano *et al.*, 2008)

Hemoglobin S (HbS; β6Glu→Val) and Hemoglobin C (HbC; β6Glu→Lys) strongly protect against clinical *Plasmodium falciparum* malaria (Allison, A.C. 1954; Modiano *et al.*, 2001a). HbS, which is lethal in homozygosity, has a multi-foci origin and a widespread

Correspondence: David Modiano, Dipartimento di Scienze di Sanità Pubblica, Università "La Sapienza", Piazzale Aldo Moro 5, 00185 Rome, Italy
e-mail: david.modiano@uniroma1.it

geographic distribution in sub-Saharan Africa and Asia (Pagnier *et al.*, 1984), whereas HbC, which has no obvious CC segregational load, occurs only in a small area of central West-Africa. To address this apparent paradox, we adopted two partially independent haplotypic approaches in the Mossi population of Burkina Faso where both the local S (SBenin) and the C alleles are common (0.05 and 0.13). We showed that C has accumulated a 4-fold higher recombinational and DNA slippage haplotypic variability than the SBenin allele (P = 0.003) implying higher antiquity. These results, consistently with epidemiological evidences, imply that the C allele has been accumulated mainly through a recessive rather than a semidominant mechanism of selection. This evidence explains the apparent paradox of the uni-epicentric geographic distribution of HbC, representing a 'slow but gratis' genetic adaptation to malaria through a transient and long-lagged polymorphism, compared to the polycentric 'quick but costly' adaptation through balanced polymorphism of HbS. The demonstration that the selection of the C allele occurred mainly through homozygosis has obvious implications when studying the possible protective mechanisms (Fairhurst *et al.*, 2005).

Interethnic differences in the susceptibility to malaria: the role of T regulatory cells (Torcia *et al.*, 2008)

Previous inter-ethnic comparative studies on the susceptibility to malaria performed in West Africa showed that Fulani are more resistant to *Plasmodium falciparum* malaria than sympatric ethnic groups (Modiano *et al.*, 1996; Dolo *et al.*, 2006) with different genetic background Modiano *et al.*, 2001b). This resistance was not associated to classic malaria resistance genes (Modiano *et al.*, 2001c) and the analysis of the antibody response to *P. falciparum* antigens revealed higher immune reactivity in the Fulani (Modiano *et al.*, 1996; 1998; 1999). The hypothesis of a stronger activation of the immune system in this ethnic group is also suggested by the higher frequency of the tropical splenomegaly syndrome reported in this population (Greenwood *et al.*, 1987). In this context, the analysis of the expression, in selected cellular populations of the immune system, of a large panel of genes involved in the immune response by Microarray and Real-Time PCR techniques might be helpful in the identification of genes for genetic susceptibility studies. We analyzed, in peripheral blood mononuclear cells (PBMC) from Fulani and sympatric Mossi, the expression profile of a large panel of genes involved in the immune response and obtained evidences suggesting a functional deficit of the mechanisms of immune regulation in Fulani: they showed an increased expression of genes related to TH1 or TH2 function, together with a reduced expression of CTLA4 and FOXP3, two genes involved in the immune modulation operated by T cells. Microarray analysis on RNA purified from peripheral blood CD4+CD25+ T-regulatory cells (Treg), showed great differences between the two ethnic groups, with important genes such as TGFβ, TGFβRs, CTLA4, and FOXP3, being less expressed in Fulani compared to Mossi, as well as to European donors not living in malaria endemic areas. The reduced expression of genes related to suppressive activity seriously affected the ability of T reg cells to suppress *P. falciparum*-induced cell proliferation in Fulani. In fact depletion of these cells did not significantly increase the proliferation of PBMC to *P. falciparum* antigens in this group, while it restored an optimal response to the same antigens in the sympatric Mossi.

Overall, these results suggested that a functional deficit of T-regulatory cells in Fulani could be involved in the lower susceptibility to malaria of this ethnic group.

This study highligthed the existence of clear-cut differences in strategic pathways of the immunoregulatory network between sympatric populations differing in their genetic background and degree of susceptibility to malaria. The functional deficit of Treg here reported in Fulani is consistent with their higher susceptibility to diseases with autoimmune pathogenesis such as diabete mellitus (Fisch *et al.*, 1987), pemphigus (Mahe *et al.*, 1996) and onchocercal skin disease (Brieger *et al.*, 1997). A higher resistance against infectious diseases like *P. falciparum* malaria could have been the driving selective force of this disorder. The definition of the genes involved could have important implications in the understanding of host-parasite relationships and in the development of anti-malaria vaccines.

***Interferon Regulator Factor-1* polymorphisms are associated with the control of *Plasmodium falciparum* infection (Mangano *et al.*, 2008).**

Interferon Regulatory Factor 1 (IRF-1) is a transcription factor that regulates the expression of a number of genes whose products play crucial roles in innate as well as adaptive immunity (Kroger *et al.*, 2002). IFN-gamma is the strongest IRF-1 inducer known and IRF-1 promotes transcription of a number of genes, acting as an important mediator of IFN-gamma activity. IRF-1 activity is essential for recognition of micro-organisms and antigen presentation, as it regulates the expression of genes such as *Toll-like Receptor 9*, MHC class I and class II genes. *IRF-1* locus lies in the 5q31 human genome region previously shown to be linked to *Plasmodium falciparum* infection (Rihet *et al.*, 1998). To determine whether genetic variation at the *IRF-1* locus affects resistance to malaria infection in humans, and might therefore underlie the *P. falciparum infection-level locus*, we studied *IRF-1* genetic diversity in two West African ethnic groups that show striking differences in their susceptibility and immune response to malaria, and conducted a candidate gene association study with carriage of *P. falciparum* infection. In order to evaluate the effect of *IRF-1* polymorphisms on disease severity, we also conducted a classical intra-ethnic case-control study where we compared the allele frequency of three haplotype-tagging SNPs (htSNPs) between healthy population controls, mild malaria

cases and severe malaria cases of Mossi ethnicity. We showed that *IRF-1* polymorphisms entail different abilities to control *P. falciparum* infection, both in healthy adult subjects and in children with uncomplicated and severe malaria. This study provided the first evidence on the role of a specific locus within the 5q31 region, in the control of *P. falciparum* infection.

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