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Silvio Pampiglione, maestro di molti (1925-2008)

M. Letizia Fioravanti

Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale, Università di Bologna.

Il 5 ottobre 2008 è scomparso il professor Silvio Attilio Pampiglione. In qualità di allieva e stretta collaboratrice, soprattutto nei suoi ultimi anni di attività, mi è stato chiesto di curarne il necrologio. Operazione non facile, considerata l'ecletticità ed il grande numero di esperienze scientifiche, didattiche e sociali che compongono la sua lunga e pienissima vita. Un altro motivo, molto più banale, è il ricorrere nella mia mente di una frase che egli stesso mi disse durante la commemorazione di un collega parassitologo: "*Quando muoio evitate necrologi noiosi e pomposi… perché quando qualcuno muore bisogna fare tutte queste storie?*". Questo era il suo modo di vedere le cose, senza falsi romanticismi e con un pragmatismo che gli permetteva di affrontare con semplicità e allo stesso tempo con grande rigore scientifico qualsiasi argomento delle scienze mediche e della parassitologia, senza trascurare mai quell'impegno politico che è sempre stato elemento basilare della sua vita privata e professionale.
Dopo aver conseguito la laurea in Medicina e Chirurgia presso l'Università di Roma nel 1950, discutendo una tesi di laurea sulla Spirochetosi discromica sotto la guida di Ettore Biocca, Silvio Pampiglione sceglie di praticare la professione medica in zone rurali del Lazio fino al 1956, avendo modo di fare esperienza nella medicina di base e di studiare al contempo le condizioni di vita e le tradizioni di un territorio al quale rimarrà sempre affettivamente legato.
Il forte interesse per la parassitologia lo porta a frequentare attivamente, a partire dal 1957, l'Istituto di Parassitologia dell'Università di Roma, dove collabora a ricerche in diversi settori. Dopo un primo lavoro pubblicato nel 1950 sull'argomento di laurea [1], nella seconda metà degli anni '50 produce diverse pubblicazioni sulle miasi dell'uomo, con particolare attenzione a quella da *Oestrus ovis* [3-12, 18], sulla diffusione dell'enterobiasi nel Viterbese [13], su alcuni casi di latrodectismo [14] osservati nella zona di Cerveteri fornendo strumenti utili alla diagnosi differenziale con il tarantolismo e sull'impiego di metodi tradizionali nella terapia di affezioni parassitarie [15, 16], pubblicando inoltre alcuni lavori inerenti



Fig. 1. Il professor Silvio Pampiglione in una vecchia foto.



Fig. 2. Silvio Pampiglione con suo figlio Guglielmo.

la storia della medicina [2, 17], argomento per il quale manterrà sempre un vivo interesse.
Nel frattempo consegue un diploma in Malariologia presso l'Istituto "Ettore Marchiafava" di Roma e, nel 1959, la libera docenza in Parassitologia.
Nel biennio 1958-59 il suo impegno civile e la sua professione di medico lo spingono a partecipare alle attività del Centro del Gruppo Danilo Dolci, conducendo ricerche sulle condizioni socio-sanitarie della Sicilia occidentale che gli valgono il riconoscimento di "cittadino onorario" di Palma di Montechiaro nel 1960 per il suo impegno sociale. Interessantissima e toccante è la sua "Inchiesta igienico-sanitaria a Palma di Montechiaro" [19] pubblicata da Einaudi nel 1960 all'interno del libro "Spreco" di Danilo Dolci, dove emergono il suo interesse verso le condizioni di vita dei disagiati e la sua convinzione che la medicina e la parassitologia rappresentino un mezzo con cui studiare, interpretare e migliorare la qualità di vita delle popolazioni. Successivamente altre pubblicazioni riporteranno i risultati delle indagini da lui condotte sulle elmintiasi umane nella Sicilia occidentale [20-22], sempre con attenzione a quei fattori sociali ed umani che rappresentano la chiave di comprensione del dato parassitologico.
Nel 1963 hanno inizio le sue numerose attività di educazione sanitaria, assistenza medica e ricerca nel settore della parassitologia umana nel continente africano. Dal 1963 al 1965 è in Algeria, dove dapprima dirige l'Ospedale di El Bayad e quindi diviene professore di Hygiene and Community Health presso l'Università di Algeri, operando nel contempo come Coordinatore del Servizio Nazionale di Parassitologia per il Ministero della Salute algerino. Dal 1964 cominciano ad apparire i suoi lavori scientifici inerenti argomenti di parassitologia africana, in gran parte in collaborazione con Paola Orecchia e Lia Paggi, comprendenti la descrizione di una nuova specie di digeneo, *Zonorchis elephantulus* [24], e numerose pubblicazioni sull'echinococcosi nel dromedario, nell'uomo e nel cane [25-28] e sulla diffusione delle elmintiasi in popolazioni algerine [29-36, 38]. Nel 1966 pubblica, con De Alencar e Ilardi, il suo primo lavoro sulla messa a punto della tecnica di fissazione del complemento per la diagnosi della leishmaniosi viscerale [37], argomento sul quale tornerà più volte negli anni successivi [40, 49, 91], e nel 1967 descrive una nuova specie di flagellato, *Leptomonas oestri*, in larve di *Oestrus ovis* [39].
Divenuto specialista in Igiene e Tecniche Ospedaliere nel 1967 presso l'Università di Roma, dal 1968 al 1971 è Professore incaricato di Parassitologia alla Facoltà di Medicina e Chirurgia dell'Università di Milano. In questo periodo la sua produzione scientifica tocca argomenti diversi, dalla botriocefalosi [41] all'echinococcosi [42] ed alla capillariosi intestinale [43] (prima segnalazione di caso umano in Italia).
Nello stesso periodo svolge numerose missioni in Africa, conducendo per la prima volta indagini parassitologiche presso popolazioni locali e comunità di pigmei del Camerun, dello Zaire, ora

Repubblica Democratica del Congo, e della Repubblica Centrafricana, in stretta collaborazione con Maria L. Ricciardi, delle quali pubblicherà i risultati negli anni successivi unitamente ad osservazioni di carattere antropologico-sanitario sulle abitudini di vita di queste popolazioni.
Nel 1969 risulta vincitore del concorso nazionale alla cattedra di Parassitologia e dal 1971 è professore ordinario di Parassitologia presso la Facoltà di Medicina Veterinaria di Bologna nell'Istituto di Malattie Infettive, Profilassi e Polizia Veterinaria (diretto da Adriano Mantovani), poi confluito nel Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale, dove svolgerà tutta la sua carriera accademica fino al 2000, anno del pensionamento.
Nei primi anni '70 si registra una produzione scientifica molto intensa, con la pubblicazione della prima rassegna sui molluschi di interesse parassitologico-medico in Italia [44], di descrizioni di casi umani di leishmaniosi [45], trichinellosi [46], enterobiasi [57], di indagini parassitologiche condotte in Africa [47, 69-71, 75, 77, 87, 88] e di numerosi lavori su *Strongyloides fülleborni* [48, 51-56, 59]. Di questo parassita studia la diffusione in Africa ed autosperimenta il ciclo biologico, individuando per la prima volta la possibilità di una sua trasmissione diretta da uomo a uomo [53]. Cominciano in questo periodo i lavori nel settore della parassitologia veterinaria, con numerose pubblicazioni su *Toxoplasma gondii*, di cui studia la diffusione nell'uomo e negli animali [58, 60, 61, 64, 68, 72] descrivendo con Giovanni Poglayen per la prima volta in Italia l'infezione naturale nel gatto [61], sulle parassitosi intestinali di cani e gatti [62, 63] e sulle coccidiosi di animali selvatici africani [65, 66].
A partire dal 1974 vengono pubblicati i risultati delle indagini condotte nel corso del focolaio di leishmaniosi viscerale verificatosi in Emilia-Romagna negli anni precedenti [73, 74, 76, 78-81, 83, 90]. A quest'ultimo argomento dedica grande interesse, collaborando a livello diagnostico con diversi medici ospedalieri della provincia di Bologna ed a livello di ricerca epidemiologica con diversi colleghi italiani e con Manson-Bahr e Killick-Kendrick, con il quale si instaura un rapporto duraturo di stima ed amicizia. In quegli anni conduce ricerche sulla leishmaniosi anche in Toscana [92], nella repubblica di Guinea [96, 98] e successivamente in Abruzzo [110], giungendo a compilare con Bettini nel 1981 una revisione della letteratura esistente su questa malattia parassitaria in Italia [114] e concludendo i suoi lavori sull'argomento nel 1982 con pubblicazioni in collaborazione con colleghi dell'Istituto Superiore di Sanità e dell'allora Istituto di Malattie Infettive, Profilassi e Polizia Veterinaria di Bologna [118-120]. Nello stesso periodo conduce osservazioni sulla trichinellosi umana ed animale, partecipando alle prime ricerche sperimentali effettuate sul cavallo [99, 103].
È nello stesso periodo che Silvio Pampiglione intraprende con Giorgio Canestri Trotti i primi studi sulla dirofilariosi umana da *Dirofilaria repens* [115, 116], zoonosi parassitaria fino ad allora poco conosciuta ma che rappresenterà l'oggetto principale della sua attività scientifica nei successivi decenni, producendo circa 80 lavori inerenti casi umani, con accurate descrizioni anamnestiche, cliniche ed istologiche grazie



Fig. 3. Silvio Pampiglione, il primo da destra, con colleghi e collaboratori.

alla continua collaborazione con Francesco Rivasi e diversi colleghi medici del settore ospedaliero, e revisioni della casistica nazionale e mondiale [172, 210, 214, 234, 287] comprensive di rivisitazioni critiche di segnalazioni pregresse di dirofilariosi umane la cui eziologia era stata attribuita erroneamente a *D. immitis* [353]. Di grande rilievo è il numero di collaborazioni che Pampiglione è riuscito a creare in questo ed in altri ambiti di ricerca, cooperando con medici, operatori sanitari e ricercatori di tutto il mondo e divenendo il punto di riferimento per le filariosi zoonosiche a livello internazionale, facilitato senz'altro anche dalla sua grande conoscenza delle lingue straniere.
Continua nel frattempo a pubblicare su argomenti molto diversi della parassitologia medica e veterinaria, con lavori su indagini epidemiologiche in Africa [96, 98, 102, 103, 111, 126, 146, 148], criptosporidiosi [134, 139, 182, 183], enterobiasi [161, 167, 217], check-list della malacofauna italiana di interesse veterinario [155], dermatiti da insetti [173, 192, 249] e miasi umane [174, 179, 194, 207, 220, 248].
La sua curiosità intellettuale lo spinge inoltre ad indagare i risvolti storici ed umani, oltre che i contenuti scientifici, della medicina e della parassitologia. Ne sono esempio i numerosi lavori pubblicati nella seconda metà degli anni '90 in collaborazione con Salvatore Giannetto sui controversi rapporti tra Battista Grassi e l'allievo Salvatore Calandruccio, lavori che riescono a rivalutare lo spessore scientifico di una figura trascurata della zoologia e della parassitologia italiana, Calandruccio, senza tuttavia sminuire l'indiscutibile valore del maestro Grassi [257, 262, 269, 281, 289].
Negli ultimi anni '90 si dedica allo studio della tungiasi da *Tunga penetrans* [266, 270, 302], ma è la scoperta di una nuova specie di pulce penetrante in Ecuador che dal 2000 lo impegna notevolmente. Gli studi di carattere sistematico, morfologico, biologico, epidemiologico e patologico condotti in collaborazione con Massimo Trentini, Andrea Gustinelli, Giovanni Onore, Francesco Rivasi e con la sottoscritta producono numerosi lavori che descrivono una nuova specie di interesse medico e veterinario in America Latina, *T. trimamillata* [305, 312, 314-316, 329, 333, 334, 347]. È attualmente in corso di stampa su una rivista internazionale una review sulle tungiasi animali e dell'uomo nella quale vengono condensati gli studi e le osservazioni condotti da Pampiglione e dal suo gruppo di ricerca sull'argomento e che lo aveva impegnato fino all'ultimo [354].
Contemporaneamente continua a studiare con energia e passione le zoonosi parassitarie, descrivendo il primo caso mondiale di infezione umana da *Macacanema formosana* [307], undici casi di anisakiasi umana in Italia [308] ed i primi casi umani di opistorchiasi da *Opistorchis felineus* con Daniele Crotti [318].
Oltre ai lavori scientifici, Silvio Pampiglione produce numerose pubblicazioni di educazione sanitaria fra le quali di grande valore appaiono i manuali "Guida sanitaria per i Tropici" (1974) [67], "As doenças infecciosas: o que são, como são transmitidas, como se devem combater" (1977) [93], "Guia veterinario ilustrado" (1977) [94], "Manuale di formazione di base per l'Operatore Sanitario in Africa (1984)[128], "Manual de parteira tradicional" (1986) [137], "Manual do promotor de saude" (1986) [138]. Rimane inoltre ancora di estrema utilità didattica il libro "Guida allo studio della Parassitologia" [113] scritto in collaborazione con Giorgio Canestri Trotti ed edito in prima edizione nel 1980 ed in seconda e terza edizione rispettivamente nel 1990 e 1999.
Parallelamente all'attività didattica condotta dal 1971 con continuità presso la Facoltà di Medicina Veterinaria di Bologna, svolge attività di docenza su argomenti di parassitologia e di medicina tropicale presso diverse scuole di specializzazione e perfezionamento in Italia ed all'estero (Somalia, Cuba ed Eritrea), continuando a condurre missioni e ricerche nei Paesi in via di sviluppo e soprattutto nel continente africano. Nel 1976 e 1977 è Direttore del Dipartimento di Salute della Comunità dell'Università di Maputo in Mozambico, dal cui Governo riceverà nel 1990 la "Bagamoyo Medal" per la sua attività a favore della popolazione mozambicana.
Dal 1968 al 1998 svolge numerose missioni volte alla valutazione di progetti di carattere sanitario in Paesi in via di sviluppo (Capo Verde, Etiopia, Guinea, Guinea Bissau, Mali, Burkina Faso, Senegal, São Tomé e Principe, Zaire, Tanzania, Bangladesh, Nicaragua, Albania, Angola, Eritrea, Palestina, Namibia, Algeria e Mozambico) per conto del Ministero degli Affari Esteri, Università, Comune di Bologna, ONG, Comunità Europea, nonché missioni scientifiche di campo per conto della Wenner Gren Foundation, OMS, CNR ed UNICEF. Nel 1974 riceve il premio "Città di Firenze", istituito da Giorgio La Pira, per la sua attività a sostegno del popolo africano, e nel 1986 il "Premio della Cultura" della Presidenza del Consiglio dei Ministri per le attività culturali svolte a favore dei Paesi in via di sviluppo.
Dal 1979 al 1995, dapprima come fondatore e quindi come vice-presidente, lavora attivamente nella ONG denominata CESTAS (Centro di Educazione Sanitaria e Tecnologie Appropriate) di Bologna. Particolarmente attivo è il suo impegno per il popolo Sahraui, a favore del quale svolge numerose azioni di solidarietà e sostegno civile in stretta collaborazione con la moglie Jacqueline. Di grande spessore è la lettera al re del Marocco Mohamed VI, pubblicata nel 2006 su *Review of African Political*

Economy [341], dove adduce stringenti motivazioni alla necessità di risolvere l'occupazione dei territori del Sahara occidentale e le azioni di oppressione nei confronti dei Sahraui utilizzando il linguaggio di un grande mediatore politico e di un profondo conoscitore della realtà del mondo africano e della sua storia.
È stato socio fondatore della Società Italiana di Parassitologia, di cui fu Presidente dal 1984 al 1988 e successivamente socio onorario, ricevendo nel 2002 la "medaglia Battista Grassi" per il suo impegno nel campo della parassitologia. Socio fondatore e membro dal 1983 al 1991 del Consiglio Direttivo della Società Italiana di Medicina Tropicale; membro e Segretario per l'Italia dal 1973 al 1982 della Royal Society of Tropical Medicine and Hygiene di Londra; membro della Società Italiana di Hansenologia, della World Association for the Advancement of Veterinary Parasitology, della Società Italiana di Malattie Infettive, della Società delle Scienze Veterinarie e del Comitato Scientifico dell'Istituto Italiano per l'Africa e l'Oriente, membro corrispondente della Belgian Society of Tropical Medicine, socio onorario della Società Italiana di Veterinaria e Zootecnia Tropicale per la Cooperazione internazionale.

Per non dover operare rinunce verso le sue convinzioni, il prof. Pampiglione ha fatto scelte che sono state talvolta criticate dal mondo accademico. Un appunto che molti gli facevano era quello di non "creare scuola" e di non impegnarsi a sufficienza per lasciare un'eredità stabile. Eredità. Se con questa parola intendiamo lasciare in mano ad allievi e collaboratori una continuità di argomenti, un ruolo istituzionale, una identità di obiettivi, allora forse non si può parlare di eredità. Se invece questa parola significa aver insegnato un metodo, aver dimostrato che la passione può rendere un lavoro una professione, aver indicato con quale rigore vada condotta la ricerca perché assuma valore scientifico, aver mostrato come la conoscenza debba essere consapevole delle proprie mancanze più che delle proprie conquiste, allora il prof. Pampiglione ha lasciato una grande e preziosa eredità ed è stato maestro non di pochi ma di molti.
La sua mancanza verrà sentita da tantissime persone, in Italia ed in tutto il mondo.

Da parte di tutti noi parassitologi, un caro saluto è rivolto alla moglie, Jacqueline Philippe, ed ai suoi figli.

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A cura di Andrea Gustinelli

Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale, Università di Bologna.

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Giuseppe Saccà, an example of rigour and coherence (1916-2008)

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Giuseppe Saccà was born in Palermo, Sicily, on January 31, 1916. His father was a physician and his maternal grandfather, Antonio Borzì, was the Director of the Botanical Garden of Palermo and founder of the Colonial Garden. Saccà had three sons.

Saccà began his scientific activity in 1939 when he was still a university student and assistant entomologist at the Istituto di Patologia del Libro (*Pathology of the Book Institute*) of Palermo[1, 3-5], where he studied book pests. In 1940, he earned a Medical Degree with thesis at the Parasitology Institute of Rome University. From 1939 to 1957, he performed research on the geographical distribution, biology, taxonomy and adult and larval morphology of phlebotomine sandflies and their role in the epidemiology of leishmaniasis [2, 6-10, 14, 17, 21, 27, 34]. During World War II, he was a medical officer in Sicily and was taken prisoner by the Allied Forces, who allowed him to work at the Hygiene Institute of Palermo University with Mario Mariani and Giuseppe D'Alessandro[11, 13].

In March 1945, Saccà returned to Rome and worked on malaria control in the Province of Latina (Central Italy). In 1947, he began to work as an assistant at the Laboratory of Parasitology of the Istituto Superiore di Sanità (ISS; Italy's National Health Institute), where he worked in applied entomology[16, 18, 20] and research in insect control[15, 19]. This is also the period in which he published first important research paper on insect control (Saccà, 1948) where he reports a case of resistance to DDT in *Musca domestica*[15, 16, 22, 26, 28-33, 35-42 ,53, 56, 62, 63]. This paper made him well recognized



Figure 1. Professor Giuseppe Saccà (January 31, 1916 - May 23, 2008) attending an international Congress in Moscow in 1968.

internationally. From 1947 to 1952, he was greatly involved in Italy's malaria eradication program, working in the provinces of Latina and Rome and on the Island of Sardinia, where he collaborated on the Rockefeller Foundation's ERLAS (Ente Regionale per la Lotta anti-anofelica in Sardegna) program. After malaria was successfully eradicated in Italy, he continued to work on maintaining the country's malaria-free status[43-46, 59-61].

In light of his vast experience in the field of vector control, in 1957 the World Health Organization (WHO) invited him to join the Expert Committee on insecticides in Geneva. Two years later, Professor Saccà, as a WHO expert, traveled to Morocco to investigate malaria. From 1960 to 1961, he spent one year in Jordan for the WHO Malaria Eradication Program. From 1961 to 1962, he worked at the WHO Office in Beirut, Lebanon, for the malaria eradication programs in the Middle East; during this period, he visited Iraq, Syria and Egypt. In 1962, WHO appointed Professor Saccà as Project Leader of the Insecticide Testing Unit in Lagos, Nigeria. In 1965, as a WHO expert, he visited Kuwait to support the local government in its fly control initiatives.

In his 25 years of scientific activity, Professor Saccà focused on disease vectors and their control, publishing 106 original papers on the following subjects: phlebotomine sandflies, flies, insecticides[59, 60, 63, 64], mosquitoes of the genus *Anopheles* [48, 49], speciation of the genus *Musca*, genetic control of vectors [47, 53, 56, 61, 62], and Arboviruses [51, 52, 54, 55, 57], among others[12, 50, 58, 64, 65]. In 1957, Saccà wrote some entries for the Italian Medical Encyclopedia. As an entomologist, he collected a great number of dipterans, and the collection, which is maintained at the ISS, is one of the largest in Italy. Professor Saccà was also actively involved in the study of arthropod fauna contaminating foodstuffs. He was an active member of the Italian Society of Parasitology of which he was also elected Honorary President, the Italian Society of Entomology, the Italian Society of Zoology and the Italian Society of Biogeography. He attended numerous national and international conferences and congresses, presenting original scientific works and acting as chairperson (Fig. 1). In 1973, Professor Saccà early retired from the ISS with the title of Research Director. Professor Saccà traveled again, as a WHO expert to Malta in 1973, to Tunisia for two years (1973-1974) and in Turkey in 1979 to investigate malaria. From that time until his death on May 23, 2008, he was devoted to his hobby of beekeeping and to his seven grandchildren.

On the part of the members of the Italian Society of Parasitology, warmest regards to Professor Saccà's wife, Maria Luisa, and his son and two daughters and seven grandchildren.

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First detection of *Babesia* EU1 and *Babesia divergens*-like in *Ixodes ricinus* ticks in north-eastern Italy

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Abstract. Babesiosis is a tick-transmitted disease of veterinary and medical importance. The first Italian case due to *Babesia* EU1 was recently recorded. In this study, we examined 1861 *Ixodes ricinus* ticks collected in an area of north-east Italy (closed to Slovenia) for the presence of *Babesia* spp. A nested PCR targeting the beta-tubulin gene and PCR using the 18S rRNA gene were used to detect positive samples: sequence alignment followed by the development of a phylogenetic tree was used to identify the genospecies of *Babesia*. The mean infection prevalence ranged from 0.8% to 1.1% in the two years of study. Twelve of the *Babesia* positive sequences were in the genospecies EU1, and the remaining two clustered in the *B. divergens*/*B. capreoli* group. This is the first report of *Babesia* EU1 sp. in Italian ticks.

Key words: *Babesia*, *Ixodes ricinus*, zoonosis, Italy.

Protozoa of the genus *Babesia* – intracellular parasites of red plasma cells – are transmitted by ticks, and are the agents of babesiosis. This disease is common among domestic animals such as cattle, dogs and horses, and occurs with fever, anaemia, jaundice and haemoglobinuria (Telford *et al.*, 1993). Recently the infection has gained attention as an emerging tick-borne zoonosis in humans (Herwaldt *et al.*, 2003), having over 300 cases reported (Genchi, 2007). In healthy individuals, babesiosis causes mild symptoms (Homer *et al.*, 2000) but can become fatal in immunocompromised subjects. The first well documented human case of babesiosis was reported in 1957 in an asplenic man in former Yugoslavia (Skrabalo and Deanovic, 1957). Since then, a hundred human cases have been reported in North America in contrast to sporadic cases (about 60) reported in Europe and other parts of the world (Meliani *et al.*, 2006).

In the United States, human babesiosis is caused by *B. microti* (Kjentrup and Conrad, 2000), while *B. divergens* is a predominant human infection in Europe (Gorenflot *et al.*, 1998). The development of molecular biology has revealed new species of *Babesia,* WAI-type and CA1-type, closely related to small babesial parasites of wild animals and dogs, as well as MOI-type, closely related to *B. divergens*. Recently in Europe, a non-*B. divergens* organism referred to as EU1 was found to cause two human cases of babesiosis in Italy and Austria (Herwaldt *et al.*, 2003); more recently, Haselbarth described a human *Babesia* isolate, named Babesia EU3, clustering with EU1 (Haselbarth *et al.*, 2007).

In Europe, *Babesia* is transmitted by *Ixodes ricinus*, which acts as a vector of other important pathogens, such as *Borrelia burgdorferi*, TBE virus, *Rickettsia* spp. and *Anaplasma phagocytophilum*. Knowing the infectivity of the tick population for a pathogen can be very useful for evaluating the risk to human and animal health. This approach was applied to a series of investigations in the Friuli Venezia Giulia region (north-eastern Italy), a territory endemic for Lyme borreliosis and cases of tick-borne encephalitis (TBE), which have constantly risen in the last few years (Cinco *et al.*, 2008). No human case of babesiosis has been reported in Italy, but the circulation of *Babesia* in ungulates and ticks near Slovenia has been described (Duh *et al.*, 2005b).

In this preliminary study, we aimed to locate *Babesia* in *I. ricinus* in a region of north-east Italy. Here, we report the first detection by molecular methods of different species of *Babesia* in *I. ricinus* ticks collected in a territory of north-eastern Italy. Species of tick positive for *Babesia* as determined by DNA sequencing indicated that the majority of *Babesiae* hosted by *I. ricinus* had significant homology with the species *Babesia* EU1 and some within the *B. divergens*/*B. capreoli* group.

Materials and methods

Study area and tick collection

The study area, known as Karst, consists of a rocky limestone plateau that rises steeply from the coast. The climate ranges from Mediterranean on the coast to pronounced continental features on the plateau, characterized by a gradient of Mediterranean plants near the coast to continental coenoses in the upper inland area, with predominate meso-Mediterranean extrazonal vegetation (*Ostryo-Quercetum ilicis*) and gariga (*Stipo-Salvietum officinalis*) (Poldini and Feoli, 2001).

Questing ticks were collected by flagging from five sampling sites during 2006 and 2007 in the

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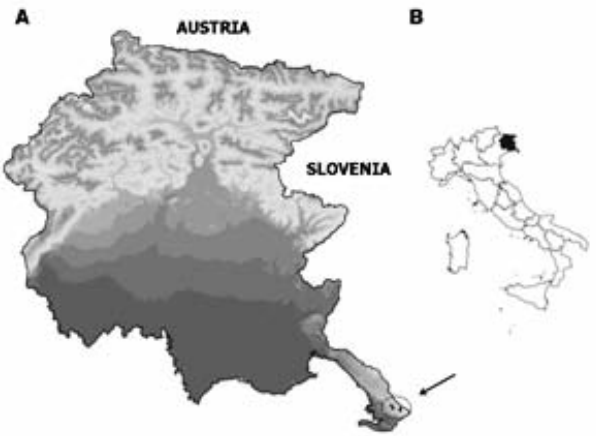


Figure 1. Study area: sampling sites (arrow) in the Friuli Venezia Giulia region (A) in Italy (B).

upper inland area of Karst, close to the western border of Italy with Slovenia (Fig. 1). Standard taxonomic keys (Manilla, 1998) were used to identify ticks as *I. ricinus*, classified as nymphs and adults (male and female) and stored at –80°C.

DNA extraction and detection of *Babesia* spp.

Total DNA was extracted from each homogenized single adult tick and pooled samples of three nymphs using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions.

The following isolates were used as positive controls and to set up amplification conditions: Italian isolate, BABBO (*B. divergens*), was kindly provided by Dr. Roberta Galuppi of the Department of Veterinary Public Health and Animal Pathology, Alma Mater Studiorum-University of Bologna, Italy; Slovenian isolates, SLO1 (*B. divergens*) and SLO3 (*Babesia* EU1), were kindly provided by Dr. Darja Duh of the Institute of Microbiology and Immunology, Medical Faculty of Ljubljana, Ljubljana, Slovenia; German strains, G1 and G2 (*B. microti*), were kindly provided by Prof. Klaus-Peter Hunfeld of the Institute of Medical Microbiology and Infection Control, University Hospital of Frankfurt, Frankfurt am Main, Germany.

Babesia was detected by nested PCR using specific primers for the β-tubulin gene (Cacciò *et al.*, 2000). The size of the amplicons varied from 169 to 319 bp, depending on the species. A total PCR reaction mix of 25 µL contained 5 µL of the isolated DNA, 2.5 µL of 10 fold PCR buffer (New England Biolabs Inc., USA), 0.25 µM of each primer (Sigma-Genosys Ltd, UK), 200 µM of each nucleotide (Eppendorf AG, Hamburg, Germany) and 0.5 U of Taq polymerase (New England Biolabs Inc., USA). The primary and nested PCR were performed in an automated DNA thermal cycler (PTC 200, Biozym, Hessisch Oldendorf, Germany) as described by Cacciò *et al.* (2000). PCR products were electrophoresed in a 3% SeaKem LE agarose gel (Cambrex Bio Science Rockland Inc., USA), stained with ethidium bromide at a final concentration of 0.5 mg/mL and imaged.

To confirm the presence of *Babesia* DNA in the tick samples, a specific PCR for the 18S rRNA was performed. The following primers, kindly provided by Dr. Norman J Pieniazek of the Centers for Disease Control and Prevention (CDC), Atlanta (Georgia, USA), were used: 5′-GYYTTGTAATTGGAATGATGG-3′ (forward) and 5′-CCAAAGACTTTGATTTCTCTC-3′ (reverse). The PCR reaction mixture (total volume of 25 µL) contained 5.0 µL of the isolated DNA, 2.5 µL of 10-fold PCR buffer (Promega Corporation, Madison, USA), 0.3 µM of each primer (Sigma-Genosys Ltd., UK), 200 µM of each nucleotide (Amersham Biosciences, UK), and 0.8 U of Taq polymerase (Taq DNA Polymerase in Storage Buffer B, Promega Corporation, Madison, USA). The reactions were run in an automated DNA thermal cycler (PTC 200, Biozym, Hessisch Oldendorf, Germany) and consisted of an initial denaturation step at 92°C for 2 min, followed by 40 cycles of 30 sec at 90°C, 30 sec at 50°C, 1 min at 72 °C and a last extension cycle at 72°C for 7 min. Amplicons of 560 bp were visualized as described for nested PCR.

All samples positive for *Babesia* were sequenced in an Applied Biosystems ABI PRISM automated DNA sequencer and were compared with sequences in GenBank using the Blast program and ChromasPro Version 1.41. Phylogenetic analysis used the Multialin program (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html).

Statistical analysis

The prevalence of *Babesia* in nymphal ticks was normalized to that of a single nymph using the formula of Cinco *et al.* (1998a), since the infection rate was determined from pooled samples derived from three nymphs:

$$p = 1 - \sqrt[k]{\frac{n}{N}}$$

where p = estimated probability that a single tick is infected, n = the number of uninfected ticks, N = the number of samples examined and k = the number of specimens in each pool.

Results

Ticks collected in 2006 and 2007 consisted of 1861 individuals, of which 85.8% were nymphs, and 12.6% were adults. Nymphs were examined in pools of three individuals, and adults were examined as a single sample. We analysed by nested PCR for the β-tubulin gene of *Babesia* using 541 pools of three nymphs each and 238 samples of single adults (151 males and 87 females) and detected positive bands in 10 pools of three nymphs (1.8%) and in 9 (3.8%) single adults (8 males and 1 female). The total infection rate was 0.8% in 2006 and 1.1% in

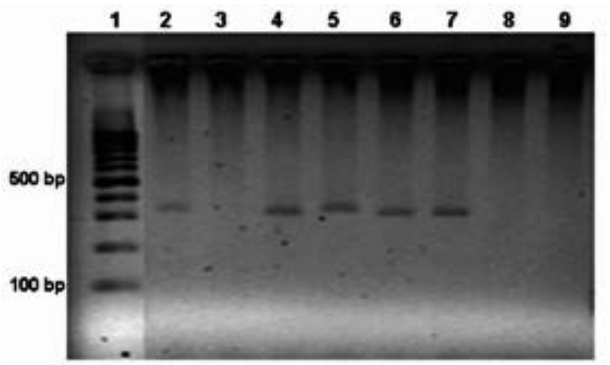


Figure 2. Gel electrophoresis of nested-PCR products obtained with primers for β-tubulin gene of *Babesia*. Lanes 1: 100 bp Molecular Ladder; lane 2: Slovenian isolate SLO1 (*B. divergens*); lane 3: negative control; lane 4: Slovenian isolate SLO3 (*Babesia* EU1); lanes 5-9: tick samples.

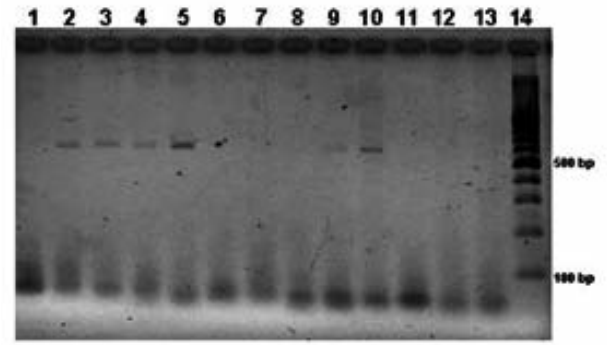


Figure 3. Gel electrophoresis of 650 bp products obtained with primers for 18S rRNA gene of *Babesia*. Lanes 1,6: negative controls ; lane 2: Slovenian isolate SLO1 (*B. divergens*); lane 3: Slovenian isolate SLO3 (*Babesia* EU1); lane 4: Slovenian isolate SLO2 (*B. microti*); lanes 5: German strains G1 (*B. microti*); lanes 7-13: tick samples; lane 14: 100 bp Molecular Ladder.

2007. *Babesiae* were present in *I. ricinus* in every sampling site of the study area except one.

The method described by Cacciò *et al.* (2000) did not include the species *Babesia* EU1. The amplification of the Slovenian isolate SLO 3, identified as *Babesia* EU1, resulted in an amplification product similar to that for SLO 1 (*B. divergens*). Figure 2 shows the amplification products of 310-320 bp obtained from tick samples and the positive controls, SLO1 (*B. divergens*) and SLO3 (*Babesia* EU1).

In order to identify the infecting species of *Babesia*, all positive samples were further sequenced and compared with sequences downloaded from the GenBank database. Sequence homology evidenced two distinct genetic groups: one consisted of two sequences related to *B. divergens*; the second included 12 identical sequences with a high homology to *Babesia* EU1. These results were confirmed by a phylogenetic tree constructed using the Multialin software. As shown in Figure 3, sample 36-01, representing the two identical sequences, clustered within the group of *B. divergens*, while sample 3-01, representing the group of 12 positives with the same sequence, was closely related to Slovenian isolate SLO3 identified as *Babesia* EU1 (with an identity of 96%); no sequence of the β-tubulin gene for *Babesia* EU1 was available in GenBank.

It was not possible to identify the remaining five samples positive for *Babesia*, owing to the difficulty in obtaining a clear electropherogram, but it was sufficient to confirm the presence of *Babesia* spp. in the PCR-positive ticks.

To confirm the presence of *Babesia* in the positive samples, the 18S rRNA was amplified. As shown in Figure 3, the positive samples were visualized as 560 bp. This second approach consisted of a single PCR, characterized by a lower sensitivity compared with a nested PCR. Consequently, the positive results were only partially confirmed. The homology of the 18S rRNA positive samples confirmed the classification of representative sample 3-01 as related to *Babesia* EU1 having a homology of 99.6% with sequences from GenBank. Sequence 36-01 had 99.4% homology with *B. divergens* and 99.8% with *B. capreoli*. These homologies are shown in Fig. 4. Sample 36-01 and the two positive controls, BAB-

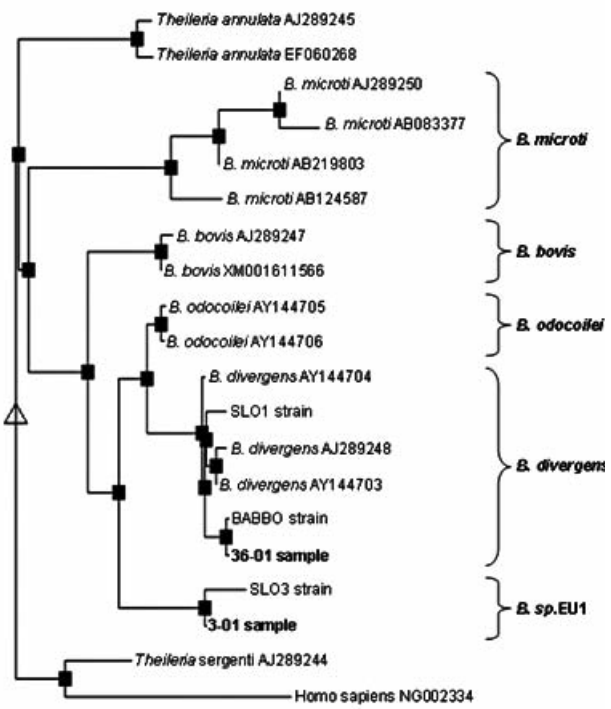


Figure 4. Phylogenetic tree with PAM (Percent Accepted Mutation) distances from the β-tubulin sequences deduced by the Multialin programme. The tree shows the phylogenetic relationship of the *Babesia* species detected in northeasthern Italy. The codes 36-01 and 3-01 represent the two different groups of sequences corresponding to *B. divergens*-like and *Babesia* EU1 respectively. Strains SLO1 and SLO3 from Slovenia and BABBO from Italy correspond to the isolates included in the study. *Theileria* spp. and *Homo sapiens* have been used as the outgroup. The scale bar represents 10 PAM of divergence.

BO and SLO1, classified as *B. divergens*, clustered with the sequences of *B. divergens* downloaded from GenBank and the sequence corresponding to *B. capreoli*, a species nearly identical to *B. divergens* (Duh *et al.*, 2005a).

Discussion

In the present survey, we provided molecular evidence that the etiologic agent of babesiosis is present in ticks collected in north-east Italy, namely Friuli Venezia Giulia. In this territory, no case of zoonotic babesiosis has been reported, though *Babesia* in ungulates and ticks has been documented in the nearby Slovenia (Duh *et al.*, 2005b). The prevalence of *Babesia* infection in *I. ricinus* observed in our survey (0.8% in 2006 and 1.1% in 2007) is quite low, but is similar to that reported in the Belluno province (1.6%-6%), which is close to our study area (Piccolin *et al.*, 2006). It is lower than that found in Slovenia (9.6%) (Duh *et al.*, 2001). Studies in other European countries have revealed similar rates of tick infection with *Babesia* spp., with values of 1% in Germany (Harteldt *et al.*, 2004), 1.5% in the Czech Republic (Rudolf *et al.*, 2005), 2% in Poland (Pleniazek *et al.*, 2006) and 3.7% in Switzerland (Hilpertshauser, 2006); in France, however, the prevalence reached 20.6% (Halos *et al.*, 2005).

Comparisons of the tick infection rate between the two years of study, and among the stations, are difficult because of the small number of sampling sites, but it is sufficient to confirm the circulation of the pathogen in the study area, and in every sampling site except one.

PCR amplification of the β-tubulin gene yielded a product of 310-320 bp of both positive samples and controls, which was sequenced in order to identify the *Babesia* species. The phylogenetic analysis revealed that the two sequences represented by sequence 36-01 form a cluster with *B. divergens*, and indicates that the group of 12 sequences represented by sequence 3-01 is closely related to the Slovenian isolate SLO3 classified as *Babesia* EU1, forming a sister group.

The phylogenetic analysis of sequences obtained by the amplification of the 18S rRNA gene with sequences available in GenBank reported in Fig. 5 confirmed classification of 12 positive samples to *Babesia* EU1, and the close relationship of the two sample sequences corresponding to *B. divergens* to both *B. divergens* and *B. capreoli*, distinct from *Babesia* EU1. Molecular genetic analyses showed that 18S rRNA of these samples have high, but not complete, identities with both *B. divergens* and *B. capreoli*, suggesting that a different genetic variant ascribed as *B. divergens*-like is circulating in the study area.

It is of note that *B. divergens* is the most prevalent species identified as the causative agent of human babesiosis in Europe (Genchi, 2007).

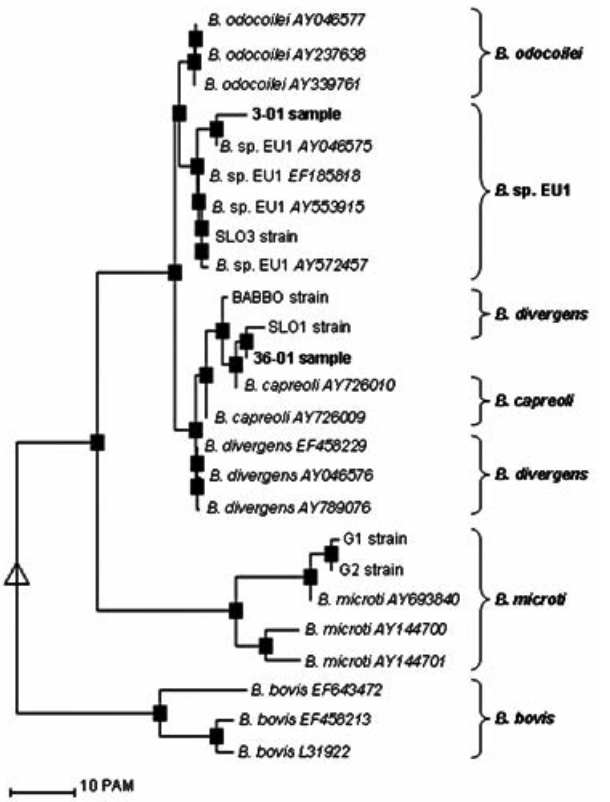


Figure 5. Phylogenetic tree with PAM (Percent Accepted Mutation) distances from the 18S rRNA gene sequences deduced by the Multialin programme. The tree shows the phylogenetic relationship of the *Babesia* species detected in north-easthern Italy. The codes 36-01 and 3-01 represent the two different groups of sequences corresponding to *B. divergens*-like and *Babesia* EU1 respectively. Strains SLO1 and SLO3 from Slovenia, G1 and G2 from Germany, BABBO from Italy correspond to the isolates included in the study. The scale bar represents 10 PAM of divergence.

Babesia EU1, described as the causative species of babesiosis in two Italian patients, one a resident in the Emilia Romagna region of Italy and the second in Austria (Herwaldt *et al.*, 2003), circulates in wild ungulates in Italy, as recently demonstrated by Pietrobelli *et al.* (2007). *Babesia* EU1 seems to be prevalent among the *Babesia*-positive ticks in northern Italy; likewise, other European authors note the higher prevalence of this species in *I. ricinus* in other countries (Hilpertshauser *et al.*, 2006; Casati *et al.*, 2006; Nijhof *et al.*, 2007; Schmid *et al.*, 2008) and in cervides (Bonnet *et al.*, 2007).

Babesia EU1 merits increased attention as an emerging agent of human babesiosis, because its vector, *I. ricinus*, is the most prevalent and widely distributed tick in Europe, and frequently bites humans. The reported evidence of transovarian and transtadial transmission of the parasite within this artropode suggests that it could also act as a reservoir of EU1 (Bonnet *et al.*, 2007). Maybe the lack of identification of EU1 species has been due to the

inability to distinguish this *Babesia* from *B. divergens* with molecular tools.

Another important topic is the presence of coinfections in ticks; a serological survey in France indicated that asymptomatic infection with *B. divergens* may coexist with *B. burgdorferi* (Gorenflot *et al.*, 1998). In Switzerland, Casati *et al.* (2006) reported two coinfections of *I. ricinus*, one tick hosting *B. burgdorferi* s.s. plus EU1 and the second containing EU1 and *B. afzelii*. The coinfections with *B. microti* and *B. burgdorferi* are well documented in North America and in Europe (Swanson *et al.*, 2006). Though no human case of coinfection has been documented in Europe, dual infection of ticks with *Babesia* and *B. burgdorferi* could cause simultaneous transmission to humans of both pathogens with a single bite. Data from American patients coinfected with *B. microti* and *B. burgdorferi* (Krause *et al.*, 1996) indicate that, though the initial symptoms overlap, they are more severe and persistent than those caused by a single pathogen. This necessitates a correct diagnosis and application of an appropriate therapy, which is different from that used for patients affected by Lyme disease only.

The study area here and the Friuli Venezia Giulia region are endemic for Lyme borreliosis (LB); coinfection occurs most commonly in patients with LB (Swanson, 2006). Since our findings document the circulation of *Babesia* in *Ixodes ricinus*, it is possible that this pathogen is cotransmitted with the tick bite. Therefore, molecular investigations targeting the sequences of *Babesia* should be performed in patients affected by borreliosis when the symptoms include haematologic abnormalities, marked influenza-like symptoms, unexplained splenomegaly and failure to respond to antimicrobial therapy directed against *B. burgdorferi*.

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Safety and efficacy of artesunate+sulfadoxine-pyrimethamine in the treatment of *Plasmodium falciparum* malaria in northeast India

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Abstract. *Background*. Northeast India is home to both *Plasmodium vivax* and *P. falciparum*, but P. falciparum is the majority parasite (>60%). Chloroquine resistance is widespread in this region, and is considered the corridor for spread of drug-resistant malaria to rest of India. For effective treatment, the therapeutic efficacy of artemisinin-based combination therapy (artesunate + sulfadoxine-pyrimethamine) was investigated against uncomplicated *Plasmodium falciparum* malaria in bordering ethnic population groups along Assam-Bhutan and that of Meghalaya-Bangladesh border reporting most cases and malaria-attributable deaths. *Methods*. It was an open label single arm prospective study undertaken in malaria endemic areas in the peak transmission season (May-October) during 2006-2007. The subjects confirmed positive for *P. falciparum* who met the inclusion criteria were treated with three day regimen of artesunate + sulfadoxine-pyrimethamine for follow up *in-vivo* investigation as per standard WHO protocol. Nested PCR assays were employed to distinguish recrudescent from that of re-infection to ascertain the true therapeutic efficacy. *Results*. Based on *in viv*o 28 day follow up study, the PCR corrected treatment success rate varied from 94.4 to 96.2% between sites. Inclusive of both study sites, of the 107 subjects evaluated, 102 (95.3±0.04%) were treatment success to the given drug-regimen, and almost all subjects (99.1%) except one were aparasitaemic by day 2. There was no early treatment failure case. However, 5/107 (4.7%) were late clinical failures reporting fever with parasitaemia on follow up day 14 (one case), day 21 (two cases) and day 28 (two cases). No adverse event reported by the subjects or observed. *Conclusion*. This drug combination resulted in rapid parasite clearance, and concluded to be safe and effective for treatment of uncomplicated *P. falciparum* malaria. We strongly advocate rolling out artemisinin-based combination therapy for every single case of *P. falciparum* to avert impending disease outbreaks, and help contain spread of drug-resistant malaria.

Key words: border malaria, *Plasmodium falciparum*, artemisinin-based combination therapy, drug-resistance, northeast India.

Malaria is major public health illness in northeastern states of India which contribute >10% *Plasmodium falciparum* and 20% death cases of those reported in the country annually[1]. Ever since detection of chloroquine-resistant *P. falciparum* malaria in Karbi Anglong district of Assam in 1970s[2], there has been steady increase in drug-resistant foci and consequent proportions of *P. falciparum* cases that have risen substantially from 13% in 1978 to presently constituting >45% of reported malaria cases in India [3-7]. In northeastern states, focal disease outbreaks are frequent and associated death toll is largely ascribed to drug-resistant *P. falciparum* malaria[8, 9]. The problem is more acute in marginalized populations living in impoverished conditions along inter-state and inter-country border area with little access to healthcare services[10]. Field evaluation of artemisinin derivatives alone for treatment of *P. falciparum* resulted in good treatment success reporting rapid parasite clearance[11, 12]. However, to obviate the development and spread of artemisinin-resistance that has already been reported in Thai-Cambodia border[13, 14], the National Vector Borne Disease Control Programme of India have adopted artemisinin-based combination therapy (artesunate+sulfadoxine-pyrimethamine) for treatment of every confirmed case of *P. falciparum* in high-risk districts that have been declared chloroquine-resistant beginning 2007[15]. In this study, we report the initial response of artesunate+sulfadoxine-pyrimethamine (AS+SP) therapy for treatment of uncomplicated *P. falciparum* in malaria in chloroquine-resistant pockets of northeastern Indian state of Assam (Baksa district) bordering Bhutan, and that of Meghalaya (West Garo Hills district) bordering Bangladesh, which could serve as baseline for monitoring drug response and development of drug treatment policy.

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Methods

Study sites and disease transmission

The northeastern states of India (22.4°, 29.31° N; 89.48°, 97.25° E) have a vast international border with China to the North, Bhutan to the West, Myanmar to the East and Bangladesh to the South (Figure 1). These border areas are highly porous permitting



Figure 1. Map of the northeastern region of India showing states of Arunachal Pradesh, Assam, Meghalya, Manipur, Mizoram, Nagaland and Tripura, and its international border with adjoining countries. The symbol (×)denote Kumarikata (Baksa district) of Assam along the international border with Bhutan located north of Brahmaputra river, and (▲) marks Dalu (West Gao Hill district) of Meghalaya located south of Brahmaputra river along Indo-Bangladesh border. Inset is a map of India showing location of the Northeastern region.

mixing of parasite strains due to movement of work force, and generally there is lack of coordinated vector control operations resulting in fulminating disease outbreaks. Both *P. falciparum* and *P. vivax* are prevalent, but *P. falciparum* is the most predominant parasite (>60%), and transmission is low-to-moderate largely maintained by *Anopheles minimus* (in the foothills) and *An. baimaii* (in the forest fringe). The other topographical features are detailed elsewhere[16]. The present study was undertaken in malaria endemic communities living in Kumarikata (district Baksa) of Assam bordering Bhutan, and in Dalu (district West Garo Hills) of Meghalaya bordering Bangladesh during August-October 2006 and May-June 2007 respectively. These areas are remote inhabited by indigenous tribes living in poverty with little access to healthcare services. Malaria cases are reported throughout the year with seasonal peak during May-September corresponding to months of rainfall. Both sites were declared chloroquine-resistant and presently under artemisinin-based combination therapy (artesunate + sulfadoxine-pyrimethamine) for treatment of *P. falciparum* cases. For control of malaria, besides radical treatment of confirmed cases, DDT is the mainstay for containment of mosquito vector populations.

Study design

It was an open label single arm prospective study to assess the therapeutic efficacy of artesunate + sulfadoxine-pyrimethamine (AS+SP) in the treatment of uncomplicated *P. falciparum* malaria. At each study site, besides malaria clinic as passive detection agency, fever surveillance were carried out by domiciliary visits on daily basis for case detection and enrollment. Villagers presenting with fever (>37.5°C) or with history of fever in the recent past were examined for malaria parasite in finger-prick peripheral blood-smear. Subjects confirmed positive for *P. falciparum* by microscopic examination of thick and thin smears (parasite density 1000-100,000/µl), who met the inclusion criteria and gave their own informed consent or their parents/guardians (subjects aged <15 years) were enrolled for follow-up investigations. The exclusion criteria included pregnancy, infants (aged <1 year), mixed infections and those presenting with severe malaria complications. The enrolled study subjects were administered artesunate 4 mg/kg daily for 3 consecutive days (day 0, 1 and 2) and a single dose of sulfadoxine-pyrimethamine (25 mg sulfadoxine + 1.25 mg pyrimethamine/kg) on day of enrollment (day 0).

Following initial dose on day 0, subjects were scheduled to visit the clinic on day 1, 2, 3, 7, 14, 21 and 28 for therapeutic assessment. On day 0, and on each scheduled day or any other time when subject presented in the clinic, axillary temperature was recorded, and finger-prick blood-smear was prepared (except on day 1) for asexual parasite density counts against 200 leucocytes multiplied by x 40 to obtain density per µl of blood assuming 8000 leucocytes/µl as standard mean. Based on the clinical outcome, the subjects were classified as early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF) and adequate clinical and parasitological response (ACPR) as per given WHO guidelines[17].

PCR assay

For distinguishing re-infection from recrudescent parasite strain infection, finger-prick blood was spotted on sterile filter paper (Whatman No. 3) strips on day 0 (before initiation of therapy) and post-treatment on any day of reappearance of parasitaemia during 28 day follow-up study period. Genomic DNA from *P. falciparum* parasitized blood spots was isolated using QiaAMP DNA minikit as per the manufacturer's protocol (Qiagen, Hilden, Germany). Individual DNA sample was subjected to PCR with *Taq-polymerase* (Genei, India) and with oligonucleotide primers (MWG, GmbH) under buffer conditions. Primers and nested PCR assays were carried out following procedures described by Snounou *et al.*[18] for paired samples (day 0 and day of reappearance of parasitaemia) using family specific allele analysis of *msp-1*, *msp-2* and *glurp*.

Statistical analysis

The data were subject to statistical analysis using chi-square test with Yates's correction and Student's t-test using SPSS software package (SPSS Inc.,

Chicago, IL, USA) for comparisons of proportions and treatments (p-value of <0.05 was considered significant).

Results

Of subjects screened for malaria parasite at each study site, blood-smear parasite rate varied from 18.2-30.7 per cent, but *P. falciparum* was the most predominant (Table 1). The baseline characteristics on day of enrollment (day 0) of subjects who each satisfied all the inclusion criteria are summarized in Table 2. The enrolled subjects included children and adults of both sexes; however, mean age (range) was significantly lower at Dalu, Meghalaya (P<0.05). The levels of parasitaemia ranged from 1040 to 99280 µl across enrolled subjects inclusive of all age groups. No subject died of malaria during follow up study period.

Clinical and parasitological responses

Of total 108 subjects enrolled at both study locations who received full course of treatment, 107 (99.1%) completed the stipulated follow up investigations except one (0.9%) that was lost to follow up. Of these subjects treated, 102/107 (95.3±0.04%) showed adequate clinical and parasitological response to the given drug regimen inclusive of PCR confirmed re-infections that were taken as treatment success (Table 3). The treatment success rate varied from 94.4 to 96.2 per cent between

Table1. Prevalence of malaria along international border with Bhutan and Bangladesh, and number of subjects enrolled in northeastern states of India

Study site (International border)	Study period	No. of blood-smears examined	No. and (%) of smears positive for malaria	No. and (%) of malaria cases positive for *P. falciparum*	No. of subjects enrolled
Kumarikata (Indo-Bhutan)	August-October 2006	918	282 (30.7)	160 (56.7)	53
Dalu (Indo-Bangladesh)	May-June 2007	1136	207 (18.2)	171 (82.6)	55

Table 2. Baseline characteristics of subjects on day of enrollment (day 0).

Enrollment characteristic	Study site	
	Kumarikata	Dalu
No. subjects enrolled	53	55
Mean age in years and (range)	24 (03-52)	10 (01-34)
Males (%) [a]	31 (58)	21 (38)
Geometric mean and (range) parasitaemia (parasites/ µl) of *P. falciparum* [b]	21463 (1040-99280)	12962 (1120-88400)

[a] Sex-ratio was statistically insignificant at both study sites (P >0.05).
[b] Mean parasite density was significantly different at given study sites (P<0.05).

Table 3. Therapeutic response to artesunate + sulfadoxine-pyrimethamine for treatment of uncomplicated *Plasmodium falciparum* malaria (PCR corrected) in northeastern states of India.

Study site	Number and (%) of cases in		
	Kumarikata	Dalu	All sites
No. subjects enrolled	53	55	108
No. completed study	53 (100)	54 (98.2)	107 (99.1)
Lost to follow up	0 (0.0)	1 (1.8)	1 (0.9)
ETF[a]	0 (0.0)	0 (0.0)	0
LCF[b]	2 (3.8)	3 (5.6)	5 (4.7)
LPF[c]	0 (0.0)	0 (0.0)	0 (0)
LTF[d] (LCF+LPF)	2 (3.8)	3 (5.6)	5 (4.7)
ACPR[e]	51 (96.2)	51 (94.4)	102 (95.3)

[a] ETF = Early treatment failure; [b]LCF = Late clinical failure; [c]LPF = Late parasitological failure; [d]LTF= Late treatment failure;
[e] ACPR = Adequate clinical and parasitological response.

Table 4. The re-appearance of *Plasmodium falciparum* parasitaemia post artesunate + sulfadoxine-pyrimethamine therapy.

Study site	No. and (%) of subjects parasitaemic on follow-up day of						
	Day 0	Day 2	Day 3	Day 7	Day 14	Day 21	Day 28
Kumarikata	53 (100)	1 (1.9)	0 (0)	0 (0)	0 (0)	4 (7.5)*	2 (3.8)
Dalu	54 (100)	0 (0)	0 (0)	0 (0)	1 (1.8)	2 (3.7)	0 (0)

*All four cases were characterized as re-infection based on PCR nested assays.

sites, but was statistically similar (P >0.05, 95% CI = 0.063-0.099). The parasite clearance was rapid and almost all subjects (107/108) except one were aparasitaemic by day 2 (Table 4). However, PCR uncorrected cure rate for Kumarikata and Dalu was 88.7 and 94.4% respectively. Inclusive of both study sites, there was no early treatment failure case; however, 5/107 (4.7%) were late treatment failure cases (PCR corrected) of which two were in Kumarikata and three in Dalu. Given the criteria, all five late treatment failure cases were late clinical failures reporting fever with parasitaemia on follow-up day 14 to 28. Among these, one (day 14), two of six (day 21), and two (day 28) were established to be recrudescent infections showing similar genotypes, and the remaining four were established to be re-infections with different genotypes. No adverse event reported by the subjects or observed.

Discussion

Based on the parasite prevalence among febrile villagers (Table 1), it was evident that the transmission intensities were variable at given study sites that could partly be attributed to variable risk factors, immune status and genetic differences to malaria receptivity of the ethnic tribes involved. The lower mean age of subjects enrolled at Dalu (Indo-Bangla border) could be attributed to high degree of herd immunity in adults due to inadequate interventions and repeated attacks where there is acute poverty, lack of awareness on disease prevention, and having poor access to healthcare services.

Based on 28 day *in vivo* follow up investigations, the present study revealed that among artemisinin-based combination therapies (ACTs) that have been recommended by WHO, this particular (AS+SP) combination was safe and effective in achieving rapid parasite clearance well within 48 hours of treatment initiation with cumulative cure rate of 95.3 ± 0.04% per cent. Nevertheless, given the extended half-life of SP, 42 day study would have more informative as many failures might have surfaced between 28 and 42 day of follow up giving true therapeutic assessment. Given the small sample size, there were just not enough data on gametocyte carriage which remained the limitation of the study. Similar research findings have been documented in Africa with same regimen reporting extended therapeutic efficacies by combining sulfadoxine-pyrimethamine with artesunate preventing treatment failures[19-21]. Owing to rapid parasite clearance, the large scale deployment of ACTs would prove to be an evidence-based intervention in reducing much needed disease transmission. As this study was undertaken during peak transmission season, the possibility of re-infection was not excluded. In the present study, PCR genotyping of alleles of *msp-1*, *msp-2* and glutamine-rich protein (*glurp*) for paired blood samples of those recrudesced, 4/6 LTF cases analyzed in Kumarikata revealed different genotypes, thus were established to be cases of re-infection and considered as treatment success. Hence, the true therapeutic efficacy of this combination is validated to be >95%, thus holds good potential for treatment of drug-resistant malaria in given areas with the variable transmission intensities. However, the late treatment failure case owing to the recrudescence of parasite on day 14 is suggestive of persistence of drug-resistance to SP component of the ACT which may threaten long-term use of this particular drug combination. The decreased therapeutic efficacy to SP alone has been documented in several study locations in the northeastern states of India[4, 6], and has been discontinued forthwith[15]. Furthermore, *P. falciparum* isolates of the study subjects revealed high degree of polymorphism in *Pfcrt, DHFR* and *DHPS* genes supportive of widespread resistance to chloroquine and SP in the given study locations (data unpublished). Fortunately, among other recommended combination therapies, mefloquine-artesunate regimen have been attempted in Assam with reported >93% sustained treatment response at 42 day[22], and several others are presently being subjected to evaluation (Neena Valecha, pers. commun.) before these be considered by the policy and programme managers.

It is strongly believed that drug-resistant strains of *P. falciparum* were carried into India via northeast corridor from neighboring Myanmar where these are widely prevalent[23-25]. The parasite strains of the northeast are reported genetically diverse and rich in point mutations that are known to confer drug-resistance[26-28]. The bordering population groups that bear the brunt of disease burden serve as foci for multiplication and spread of resistant parasite strains propagated by efficient mosquito vector species of *An. minimus* and *An. baimaii* that are widely abundant. There are confirmed reports of multi-drug resistant strains along Indo-Myanmar and Indo-Nepal international borders that

call for cross-border collaborative efforts to formulate appropriate drug policy to contain the spread of drug-resistant malaria[6, 29].

Nevertheless, it is the opportune time to roll out artemisinin-based combination therapy for every single case of *P. falciparum* to avert impending disease outbreaks, and saving lives. In conjunction with effective chemotherapy, it is just as important to strengthen healthcare services where there is need providing on-the-spot diagnosis that is affordable. In rolling back malaria initiative, we strongly advocate sustained political commitment for increased allocation of resources ensuring intensive disease surveillance and case management by monitoring therapeutic efficacy and upgrading drug policy in force to thwart the development and spread of drug-resistant malaria[30-33].

Conclusions

The study revealed that for treatment of uncomplicated *P. falciparum* malaria, this particular drug combination (artesunate + sulfadoxine-pyrimethamine) resulted in rapid parasite clearance well within day 2 with cumulative cure rate of 95.3 per cent. This drug regimen was well tolerated, and concluded to be safe and effective. It is strongly advocated to roll out artemisinin-based combination therapy for treatment of every single case of *P. falciparum* to contain the spread of drug-resistant malaria, and saving lives.

Competing interests. The authors declare no competing interests concerning the work reported in this paper.

Authors' contribution. VD collected and analyzed the data, and developed the first draft of the manuscript. SB, HJ, SKP did the molecular assays, data interpretation and wrote portions of the manuscript. NV, APD: planning, study protocol development, coordination and approval of the final version.

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Impact of placental malaria and HIV co-infection on congenital malaria and perinatal HIV transmission in sub-Saharan Africa: an overview

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Abstract. Malaria and human immunodeficiency virus (HIV) infection constitute serious public health challenge in sub-Saharan Africa and critically intersect in pregnancy producing adverse outcomes. Studies conducted in sub-Saharan Africa from 1996 to 2006 that investigated effects of placental malaria and HIV co-infection on perinatal transmission of malaria and HIV, were identified using the Medline search, and systematically reviewed. Maternal malaria and HIV co-infection prevalence ranged from 1.9% to 16.0% and rates of placental malaria were consistently higher in the HIV-positive than the HIV-negative mothers. In two of the three studies conducted in Malawi, the prevalence of parasitaemia in the umbilical cord blood was higher among the newborns delivered by HIV-positive than HIV-negative women, while in the third study the reverse was the case but parasite densities were generally higher in HIV infected women. Multivariate analysis conducted by one of the reviewed studies demonstrated that HIV infection was an important determinant of umbilical cord parasitaemia. Conflicting results were reported on the effect of placental parasitaemia on the risk of perinatal HIV transmission, and ranged from an increased risk in Uganda, to no effect in Mombasa, Kenya, and a significant protective effect in Kisumu, Kenya. In spite of the variations in the reports of the studies reviewed, all findings seemed to suggest that making motherhood safer is an urgent priority in sub-Saharan Africa. Therefore the provision of integrated preventive and curative health services for pregnant women that are sustainable and affordable in areas heavily affected by malaria and HIV is crucial for reducing the burden of the two diseases.

Key words: malaria, HIV, pregnancy, sub-Saharan Africa.

Infection by human *Plasmodium* parasites which cause malaria and human immunodeficiency virus (HIV) which causes acquired immunodeficiency syndrome (AIDS) represent major public health problems in many parts of the world. Both infections have been described as two of the greatest medical challenges facing developing nations today, particularly in the tropical and sub-tropical regions of the world, and yet the interaction between these two infections has been little studied (Chandramohan and Greenwood, 1998). Together, malaria and HIV infection cause more than 4 million deaths per year, and both are scourges of nations of Africa, India, Southeast Asia, and South America (WHO, 2005; Huff, 2000).

It has been estimated that between 270 and 480 million clinical malaria cases may occur and up to 3 million deaths every year (WHO, 1997). About 90% of all malaria deaths in the world today occur in the sub-Saharan Africa and this is because majority of infections are caused by *Plasmodium falciparum,* the most dangerous of the four human malaria parasites, accounting for an estimated 1.4 and 2.6 million deaths per year in this region (WHO, 1997; WHO, 2003). In addition, the most effective malaria vector, the mosquito *Anopheles gambiae*, is the most wide spread in the region and the most difficult to control (WHO, 2003). In areas of stable malaria transmission, very young children and pregnant women are the population groups at highest risk for malaria morbidity and mortality (WHO, 2005; WHO, 2003). Concerning the global HIV/AIDS epidemic, the sub-Saharan Africa remains by far the worst-affected region with 25.4 million people living with HIV (just under two thirds, i.e. 64% of all people living with HIV) (WHO, 2004). The HIV/AIDS epidemic is affecting the females most severely and because heterosexual transmission is predominant in the sub-region, women of reproductive age and girls make up almost 57% of adults living with HIV (WHO, 2004; Dabis and Ekpini, 2002).

Given the wide overlap between areas where HIV and malaria are each prevalent, the epidemic of HIV/AIDS in areas where *P. falciparum* is endemic has generated concern about potential interactions between the two infections (Steketee *et al.*, 1996a; Parise *et al.*, 1998; Verhoeff *et al.*, 1999). There are several ways that malaria and HIV could potentially interact, with effects upon transmission, clinical manifestations, and treatment outcomes of either disease. Findings from a number of studies had noted the existence of substantial uncertainty about the

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extent to which the two pathogens interact, describing it as no more than clinical coexistence and suggested that there is no significant or clearly demonstrable biological relation between HIV infection and *P. falciparum* malaria in adults and children (Nguyen-Dinh *et al.*, 1987; Muller and Moser, 1990; Kalyesubula *et al.*, 1997; Chandramohan and Greenwood, 1998; Uneke *et al.*, 2005). However, other reports including some very recent ones have clearly demonstrated that HIV infection is associated with an increased frequency of clinical malaria and parasitaemia (Whitworth *et al.*, 2000; Grimwade *et al.*, 2004; Kamya *et al.*, 2006; Mermin *et al.*, 2006). In a report of HIV infection as a cofactor for severe malaria, Grimwade *et al.* (2004) noted that the identification of any interaction is complicated by the nature of the immune response to malaria especially in endemic areas where repeated childhood exposure and disease episodes generate partial immunity, which ameliorates infection but does not fully prevent parasitaemia or clinical disease adding that in a sick patient with fever and who is also parasitaemic, the malaria infection can either be causal or coincidental. Nevertheless, malaria and HIV infection critically intersect in pregnancy and have been shown to have serious consequences in pregnant women, their fetuses, and infants (Ticconi *et al.*, 2003; ter Kuile *et al.*, 2004).

Malaria during pregnancy is a serious problem in sub-Saharan Africa, affecting an estimated 24 million pregnant women (Steketee *et al.*, 2001). The sub-Saharan Africa also accounts for 80% of the world's HIV-infected women, with HIV prevalence rates sometimes exceeding 40% among pregnant women (UNAIDS, 2001; DeCock and Weiss, 2000). HIV infection appears to impair malarial immunity among pregnant women, as pregnant women infected with HIV demonstrate more frequent and higher density parasitaemia than pregnant women not infected with HIV (Steketee *et al.*, 1996b; Parise *et al.*, 1998; Verhoeff *et al.*, 1999). *P. falciparum* malaria during pregnancy can lead to parasite sequestration in the maternal placental vascular space, with consequent maternal anaemia, abortion, stillbirth, foetal distress, prematurity, low birthweight, congenital malaria and neonatal or maternal death (WHO, 2005; Brabin, 1983; Guyatt and Snow, 2001).The risk of these adverse pregnancy outcomes is further increased with HIV co-infection (Ticconi *et al.*, 2003; Ayisi *et al.*, 2003, 2004). As a result, a considerable proportion of infants exposed *in utero* to both placental malaria and maternal HIV infection have an increased risk for postneonatal death three- to eightfold higher than infants born to mothers with either infection alone (Bloland *et al.*, 1995).

Whether the dual infection with placental malaria and HIV increases the risk of mother-to-child transmission (MTCT) of HIV (perinatal HIV transmission) or congenital malaria is yet to be unequivocally established, as studies examining these relationships have inconsistent findings and a wide range of unanswered questions. The implications of such findings for public health policy, research needs and health service delivery in the sub-Saharan Africa are yet to be fully evaluated. In this report, a review of scientific information and findings from studies conducted in the sub-Saharan Africa on the interaction of maternal malaria and HIV infection and its impact on MTCT and congenital malaria, is presented. The need for the development of appropriate public-health policy on the prevention, care, treatment and support activities in light of the epidemiological overlap and global importance of the two diseases especially with respect to sub-Saharan Africa, is discussed.

Materials and methods

Studies conducted in the sub-Saharan Africa reported in English from 1996-2006, that investigated the interaction between maternal malaria and HIV infection and its effects on MTCT and congenital malaria were identified using the Medline search. Combinations of key words such as placental malaria, HIV, pregnancy, perinatal HIV transmission, and congenital malaria were used in the search. References from selected publications were also used to identify additional relevant literature for the review. Particular attention was paid to key articles providing information on prevalences of both infections, the extent of their association, and the contributions to perinatal HIV transmission and congenital malaria. The various reports were systematically reviewed with respect to the population and sample size of each study, the period, setting and the type of study, to enhance comparison between studies.

Results

There is paucity of information on the synergism between placental malaria and HIV infection and its impact on congenital malaria and MTCT in sub-Saharan Africa. Eight studies providing sufficient information to enable meaningful and reasonable comparisons were however identified and used for this review. Five studies (Steketee *et al.*, 1996a, 1996b; Verhoeff *et al.*, 1999; van Eijk *et al.*, 2003; Villamor *et al.*, 2005) provided information on the effects of placental malaria/HIV co-infection and congenital malaria (Table 1). The remaining three studies provided key data on the effects of placental malaria and HIV co-infection on MTCT and these included reports by Inion *et al.* (2003), Brahmbhatt *et al.* (2003) and Ayisi *et al.* (2003). Two of the studies that investigated congenital malaria were chemoprophylaxis-intervention trials conducted in Malawi, another two were cross sectional studies from Kenya and Malawi while the fifth was a cohort study conducted in Tanzania. Although two of the studies provided incomplete information on umbilical cord and placental parasitaemia (van Eijk *et al.*,

Table 1. Summary of studies that investigated the relationship between placental malaria and HIV co-infection and congenital malaria.

Type of study	Location	Total studied	No. (%) HIV+	No. (%) malaria & HIV co-infection	Prevalence of parasitaemia						Reference
					Placental			Umbilical cord blood			
					HIV+ Mothers	HIV– Mothers	CRR[c] (95%CI)[d]	HIV+ mothers	HIV– mothers	CRR[c] (95%CI)[d]	
CPx[a] Intervention with CQ & MQ	Malawi	2781	152(5.5)	53(1.9)	38.2%	22.5%	1.70 (1.30-2.21)	25.5%	6.8%	3.76 (2.54-5.57)	Steketee *et al.* (1996a)
CPx[a] Intervention with CQ & MQ	Malawi	1767	140(7.9)	34(1.9)	28.3%	19.3%	1.47 (0.91-2.03)	17.6%	6.2%	2.84 (2.07-3.62)	Steketee *et al.* (1996b)
Cross-sectional	Malawi	1521	159(25.6)[e]	87(5.7)	29.2%	15.6%	1.90 (1.21-2.59)	3.8%	6.1%	0.62 (0.23-1.01)	Verhoeff *et al.* (1999)
Cross-sectional	Kenya	2502	612(24.5)	184(7.4)	30.7%	18.1%	1.70 (1.22-2.36)	NA[b]	NA[b]	NA[b]	van Eijk et al. (2003)
Cohort	Tanzania	275	275(100.0)	44(16.0)	NA[b]	NA[b]	NA[b]	15.0%	NA[b]	NA[b]	Villamor *et al.* (2005)

[a] Chemoprophylaxis intervention with chloroquine and mefloquine.
[b] NA=Data not assessed/available.
[c] CRR=Crude relative risk.
[d] (95%CI)=95% confidence interval.
[e] (25.6) =only 621 individuals were screened for HIV infection (i.e.159/621).

2003 and Villamor *et al.*, 2005 respectively), they were included in this review because of the vital information they provided on the maternal malaria and HIV co-infection. Cross-sectional studies were used to investigate the risk of MTCT in the two studies conducted in Kenya, while in Uganda the risk of MTCT was studied in a community-randomized trial of STD control.

Effects of placental malaria and HIV co-infection on congenital malaria

In studies that investigated congenital malaria, the prevalence of HIV among the pregnant women at delivery ranged from 5.5% to 25.6% while maternal malaria and HIV co-infection prevalence ranged from 1.93% to 16.0% (Table 1). The prevalence of placental malaria was consistently higher in the HIV-positive than the HIV-negative mothers as reported by Steketee *et al.* (1996a) (38.2% *vs* 22.5%, RR=1.70; 95% confidence interval (CI), 1.30-2.21), Steketee *et al.*(1996b) (28.3% *vs* 19.3%, RR=1.47; 95% CI, 0.91-2.03), Verhoeff *et al.* (1999) (29.2% *vs* 15.6%, RR=1.9; 95% CI, 1.21-2.59) and van Eijk *et al.* (2003) (30.7% *vs* 18.1%, RR=1.70; 95% CI, 1.22-2.36). Congenital malaria was described as the presence of malaria parasite in the umbilical cord blood. Among the newborns delivered, the prevalence of umbilical blood infection in those whose mothers were HIV-positive and HIV-negative according to Steketee *et al.* (1996a) was 25.5% and 6.8%, respectively (RR=3.76, 95% CI, 2.54-5.57), the corresponding result was 17.6% and 6.2% (RR = 2.84, 95% CI, 2.07-3.62), as reported by Steketee *et al.* (1996b). Verhoeff *et al.* (1999) reported the that prevalence of umbilical blood parasitaemia was higher in HIV-negative mothers than the HIV-positive mothers (3.8% and 6.1% respectively) (RR = 0.63, 95% CI, 0.23-1.01) (Table 1); they observed however that the parasite densities were generally higher in HIV infected women. Multivariate analysis conducted by Steketee *et al.* (1996b) demonstrated that HIV infection was an important determinant of umbilical cord parasitaemia (OR=6.8, 95% CI., 3.4-13.6).

Effects of placental malaria and HIV co-infection on mother-to-child HIV transmission

Results from the three studies that investigated MTCT showed that the prevalence of placental malaria ranged from 7.1%-25.0% (Table 2). The prevalence rates of placental malaria were consistently higher among HIV-positive than HIV-negative mothers as reported by Inion *et al.* (2003) (9.1% *vs* 4.3%) and Brahmbhatt *et al.* (2003) (13.6% *vs* 8.0%). The prevalence of MTCT (peripartal) ranged from 19.4% to 19.9%. Although Inion *et al.* (2003) noted a lower prevalence of MTCT from HIV-positive mothers with placental malaria (11.5%) compared with HIV-positive mothers without placental malaria (15.4%) (RR= 0.75, 95% CI, 0.29-1.86), no correlation was found between placental malaria and perinatal HIV transmission. On the contrary, Brahmbhatt *et al.* (2003) observed a higher prevalence of MTCT among HIV-positive mothers with placental malaria (40.0%) than HIV-positive mothers without placental malaria (15.4%) (RR= 2.60, 95% CI, 1.16-5.84), adding that univariate analysis indicated that MTCT was significantly associated with placental malaria infection (ARR= 2.89, 95% CI, 1.12-7.52) (Table 2). In their report, Ayisi *et al.*

Table 2. Summary of studies that investigated effect of placental malaria and HIV co-infection on mother-to-child transmission (MTCT) of HIV.

Type of study	Location	No. of placenta examined			Placental parasitaemia			Prevalence of MTCT (peripartal) from HIV+ mothers					Reference
		Total overall	HIV+ Mothers	HIV– Mothers	Total overall	HIV+ Mothers	HIV– Mothers	Total overall	with placental malaria	without placental malaria	CRR[b] (95%CI)[d]	ARR[c] (95%CI)[d]	
Cross-sectional	Kenya	649	372	277	7.1% (46/649)	9.1% (34/372)	4.3% (12/277)	19.6%	11.5%	15.4%	0.75 (0.29-1.86)	No correlation	Inion *et al.* (2003)
Randomized trial	Uganda	668	155	510	93% (62/668)	13.6% (21/155)	8.0% (41/510)	19.4%	40.4%	15.4%	2.60 (1.16-5.84)	2.89 (1.12-7.52)	Brahmbhatt *et al.* (2003)
Cross-sectional	Kenya	512	512	NA[a]	25.0% (128/512)	25.0% (128/512)	NA[a]	19.9%	14.1%	21.9%	0.64 (0.40-1.03)	0.44 (0.27-0.72)	Ayisi *et al.* (2004)

[a] NA = Data not assessed/available.
[b] CRR = Crude relative risk.
[c] ARR = Adjusted relative risk
[d] (95%CI) = 95% confidence interval.

(2004) noted a lower prevalence of MTCT among HIV-positive mothers with placental malaria (14.1%) than those without placental malaria (21.9%) (RR= 0.64, 95% CI, 0.40-1.03), and indicated that placental malaria especially at lower density parasitaemia (<10,000 parasites/μL) was significantly associated with a reduction in the risk for perinatal MTCT (ARR= 0.44, 95% CI., 0.27-0.72) (Table 2).

Discussion

This review of data on the interaction of placental malaria and HIV infection suggests that the synergism between both infections may to a great extent influence the occurrence of congenital malaria and perinatal HIV transmission in sub-Saharan Africa. Studies in this review indicate a prevalence of maternal malaria and HIV co-infection among pregnant women, ranging from 1.9% to 16.0%. This has important implications, since both HIV infection and malaria are among the leading causes of morbidity in pregnancy in sub-Saharan Africa, and even modest effects of one infection on the other could lead to a substantial negative impact on the health of pregnant women and their newborns (Whitworth *et al.*, 2000; Corbett *et al.*, 2002). Furthermore, the evidence for an interaction between HIV and malaria in pregnancy is convincing, with more peripheral and placental parasitaemia, higher parasite densities, more clinical malaria, more anaemia, and increased risks of adverse birth outcomes (ter Kuile *et al.*, 2004).

Studies conducted in Kenya (Parise *et al.*,1998) and Malawi (Steketee *et al.*, 1996a) demonstrate that HIV contributes to approximately 25% of maternal malaria infections and high levels of placental malaria. Data from the studies in this review indicate a consistent increased risk of placental malaria and higher density of umbilical cord parasitaemia in HIV-positive compared to HIV-negative women. Although infection with HIV causes progressive cellular immunosuppression, and any resulting impairment in immune response to malaria might be associated with failure to prevent infection or to suppress parasitaemia and clinical disease (Good and Doolan, 1999), the true clinical impact remains to be determined (Whitworth and Hewitt, 2005). However, the enhanced alteration of the placental integrity caused by HIV in women with placental malaria may increase the likelihood of mother-to-foetus passage of malaria-infected red blood cells, leading to congenital malaria infection (Steketee *et al.*, 1996a).

Before the advent of the global HIV/AIDS epidemic, studies from the sub-Saharan Africa, rarely identified clinical disease as a consequence of congenitally acquired malaria and only a few reports before 1970s documented detectable parasitaemia in infants younger than one month of age born in malaria-endemic areas (Covell, 1950; Bruce-Chwatt, 1952; Foll, 1968; Logic and McGregor, 1970). These studies have been interpreted as showing either that transplacental transmission of malaria occurs infrequently or that after transplacental transmission some elements of immunity acquired from the mother protects infants from becoming infected (Reed *et al.*, 1996). However, recent studies from Nigeria have reported the congenital malaria prevalence of up to 54.2% in Ile-ife (Obiajunwa *et al.*, 2005) and the incidence of 15.3% in Lagos (Mukhtar *et al.*, 2006), in both reports, there was a strong association between placental malaria and umbilical cord parasitaemia. Although HIV infection was not investigated, its influence on the umbilical cord parasitaemia observed in both studies may not be completely ruled out. Furthermore, Steketee *et al.* (1996b) had demonstrated via a multivariate analysis that HIV infection was an important determinant of umbilical cord parasitaemia. In addition, Reed *et al.* (1996) noted that HIV infection was associated with umbilical cord parasitaemia in uni-

variate analysis and was probably acting as surrogate marker for maternal malaria infection. Hence the combination of HIV infection and umbilical cord parasitaemia may have an important impact on prematurity and neonatal mortality as observed by Steketee *et al.* (1996b) who concluded by noting that HIV potentiates malaria infection in the umbilical blood of newborns especially in multigravidas.

Although a few other reports have suggested that there is no consistent effect of maternal HIV on congenital malaria (Verhoeff *et al.*, 1998; van Eijk *et al.*, 2002), reports from the 12th World AIDS Conference in Geneva provided additional information from various researchers indicating that the presence of HIV may reduce a pregnant woman's ability to control the perinatal transmission of malaria (Anderson, 1998). It has been demonstrated that maternal HIV infection induces pathological changes in the placenta that potentially could interfere with the materino-fetal transfer of antibodies; however, the mechanism of this process and whether a decreased transfer of antibodies to some malaria antigens has an impact on increased susceptibility to malaria in infants is not known (WHO, 2005). In a randomized trial in Tanzania, Villamor *et al.* (2005) noted that cord parasitaemia among HIV-infected women was associated with a large and significant increase in the risk of neonatal death (ARR = 8.75; P = 0.003), and suggested that successful treatment of HIV-infected women who present to the first prenatal visit with malaria parasitaemia and avoidance of reinfection are likely to decrease the risk of adverse outcomes during pregnancy and the early postpartum period.

The conflicting results reported by research investigating the impact of placental malaria infection on the risk of MTCT ranging from an increased risk in Uganda (Brahmbhatt *et al.*, 2003) to no effect in Mombasa, Kenya (Inion *et al.*, 2003), and a significant protective effect in Kisumu in western Kenya (Ayisi *et al.*, 2004), leaves the question of whether malaria during pregnancy enhances the risk of MTCT unanswered. During pregnancy, the presence of malaria parasites was associated with a higher HIV-1 load (Kapiga *et al.*, 2002), and placental HIV-1 viral load is increased in women with placental malaria especially those with high parasite densities (Mwapasa *et al.*, 2004). Therefore it can be hypothesized that an increased placental HIV-1 load, due to the presence of malaria parasites, might be associated with increased excretion of HIV-1 in the genital tract, thus increasing the risk of MTCT. However the apparent discrepancies in the studies reviewed (Brahmbhatt *et al.*, 2003; Inion *et al.*, 2003; Ayisi *et al.*, 2004), may reflect a complex relationship between maternal immune responses to malaria that on the one hand may stimulate HIV viral replication in the placenta thereby increasing the local viral load, and on the other hand may potentially control the severity of malarial infection and HIV replication, and may either have a protective effect or increase in the risk of MTCT (ter Kuile *et al.*, 2004; WHO, 2005), ter Kuile *et al.* (2004) had noted that the direction of the effect may depend on the degree of HIV related immunosuppression and on the severity of the malaria and thus the degree of placental monocyte infiltrates and proinflammatory cytokine and chemokine responses. Furthermore, differences between the studies in maternal immunological status, plasma viral load, HIV sub-type or mode of delivery may account for the inconsistent findings (WHO, 2005). The immunological bases for the increased susceptibility of HIV-infected mothers to malaria and for the effect of co-infection on mother-to-child transmission of HIV are areas of major importance in public health. Ned *et al.* (2005) reviewed current data about humoral and cellular responses to HIV-placental-malaria co-infection and presented an immunological hypothesis to explain the epidemiological findings.

The findings of studies reviewed here have potential public health relevance because making motherhood safer is an urgent priority in the sub-Saharan Africa. Providing integrated preventive and curative health services that are sustainable and affordable in areas heavily affected by malaria and HIV is crucial in order to reduce the burden of the two diseases. Although the measures to approach the complex interplay between the two diseases are not readily apparent, total eradication of both is of course the ultimate goal and as such any studied endeavour to reduce their transmission is desirable (Samak, 2004). The control of malaria and HIV in pregnant sub-Saharan African women has been particularly challenging (Brentlinger *et al.*, 2006). The challenge is to combine activities to control malaria and HIV at various levels of the health system, tailor responses to community needs, and optimize the use of scarce resources for integrated service delivery (WHO, 2005). The World Health Organization currently recommends a three-pronged approach for malaria control during pregnancy in sub-Saharan Africa: intermittent preventive treatment (IPT), insecticide-treated bed nets, and effective case management of malaria illness (WHO, 2003). However the need for the introduction of new medicines by malaria and HIV programmes cannot be overstated. The programs for pregnant women must incorporate surveillance systems to monitor for severe adverse drug reactions that occur as a result of the interactions between antimalarial and antiretroviral drugs (Greenberg *et al.*, 1991; Parise *et al.*, 1998; Brentlinger *et al.*, 2006). This is essential because the apparent dramatic increase in the risk of severe cutaneous reactions among HIV-infected women receiving both cotrimoxazole and sulfadoxine-pyrimethamine (SP) (Gimnig *et al.*, 2006), also points to the need for both a greater understanding of the efficacy of cotrimoxazole alone in preventing malaria during pregnancy, and a greater integration of HIV prevention

and ante natal clinic services (ter Kuile *et al.*, 2004; WHO, 2005).

In developing countries that have high birth rates, a high endemicity of malaria, and alarming rates of new cases of HIV, prophylaxis against both diseases with combination agents during pregnancy has been described as a great challenge (Okereke, 1999). Low adherence, poor-quality drugs and drug resistance decrease the effectiveness of both antiretroviral and anti-malarial drugs and may further hamper treatment outcome. This calls for more research on malaria and HIV infection treatment and prevention interventions strategies because only with sufficient evidence can appropriate public-health policy be devised on integrating the prevention, care, treatment and support activities for malaria and HIV.

In conclusion, it is worth noting that this review has provided additional insight on the burden of congenital malaria and perinatal HIV transmission occasioned by the placental malaria and HIV co-infection in the sub-Saharan Africa. Previous review articles on malaria and HIV co-infection (Steketee *et al.*, 2001; ter Kuile *et al.*, 2004) as well as the WHO Report of a Technical Consultation on Malaria and HIV (WHO, 2005) provided useful information included in this review. A major draw back of this review was the limited number of studies considered. Furthermore the studies were conducted in only a few countries of the sub-Saharan Africa (Malawi, Uganda, Kenya, Tanzania) and may not have been sufficient to make any general conclusion regarding the sub-Saharan Africa as a whole. However, the overwhelming evidence is that placental malaria and HIV co-infection represents a real and quantifiable risk for maternal, neonatal and infant mortality in the sub-region. This calls for a global effort in a multidimensional and inter-sectoral manner to tackle the HIV infection and malaria menace in vulnerable populations. The substantial but sustained international aid to the resource poor sub-Saharan African countries could aid local efforts towards achieving meaningful success in the nearest future.

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First report of *Eucoleus böhmi* (Nematoda: Trichuroidea) from Italy: parasitological findings and veterinary implications

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Abstract. During routine copro-parasitological examinations of dogs kennelled in Rome, trichinelloid eggs, presumably attributable to the genus *Eucoleus*, were recovered. The report was confirmed when two coprologically positive dogs were submitted for necropsy and adult worms were found in nasal cavities. Presence of free space between embryo and egg shell, number and length of stichocytes in adults and their localization allowed identification of our specimens as *Eucoleus böhmi*. Observed prevalence among 619 faecal samples was 6.78% (IC 95% 4.9-8.1). None of the infected dogs showed any specific clinical sign. Some dog confirmed positive at successive examinations, even after treatment with the more common anthelminthics. This is the first report of this species from Italy. Nevertheless, because of the similarity of *E. böhmi* eggs with those of both *T. vulpis* and *E. aerophilus*, the difficulty in recovering adult specimens and the lack of symptoms, it's assumable that this parasite has been frequently overlooked and/or misdiagnosed.

Key words: *Eucoleus*, *Eucoleus böhmi*, dog, kennel, Italy.

Two species of the genus *Eucoleus* Dujardin, 1845 are known from dogs: *E. aerophilus* (*Capillaria aerophila*) (Creplin, 1839) in tracheal and bronchial epithelium and *E. böhmi* (*Capillaria böhmi*) (Supperer, 1953) in nasal mucosa. *E. aerophilus* was also reported from nasal mucosa of canids (Christenson, 1935; Erlich, 1938; Evinger *et al.*, 1985; Radman *et al.*, 1986), but most of these identifications were anterior to *E. böhmi* description and could possibly be misidentifications (Schoning *et al.*, 1993). According to Campbell and Little (1991) most, if not all, the reports of nasal capillariasis in dogs should be attributed to *E. böhmi*, described for the first time in Austria from frontal sinuses of red foxes by Supperer (1953) and successively reported from foxes, dogs and wolves in Poland (Kozlowska, 1957; Zarnowski and Patyk, 1960; Malczewski, 1962) and from foxes in Hungary (Sreter *et al.*, 2003). While *E. aerophilus* is known to require an earthworm as intermediate host (King *et al.*, 1990), *E. böhmi* life cycle is unknown. In fact, other species of the same genus have direct life cycles.

At present, *E. aerophilus* has been recorded in Italy from foxes (Manfredi *et al.*, 2003), while no records of *E. böhmi* are reported. During routine parasitological examination of faecal samples from dogs kept in three kennels in Rome, trichinelloid eggs morphologically attributable to capillarid worms of the genus *Eucoleus* were detected. In the present paper identification of eggs and adult worms is described, leading to the first report of *E. böhmi* from Italy. Veterinary consequences due to this new finding are discussed.

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Materials and methods

Between April 2006 and February 2008, 619 dog faecal samples from three kennels in Rome were analysed for parasites in the Laboratory of Parasitology of the Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana (IZSLT). Public health veterinarians, responsible for sanitary status of kennelled dogs, use to send samples both for routine controls and for laboratory confirmation of endoparasitic diseases, in case of clinical suspect. Copro-diagnosis for the detection of the most common dog parasite eggs was performed with standard techniques: fresh smear and flotation in sugar-sodium nitrate solution (density 1.300) (Ambrosi, 1995). Eggs detected in faecal samples were examined at the microscope (40-100×) and measured with the aid of a micrometric ocular (400×).

In September 2006 and February 2008, two dogs previously found positive at coprological examination for trichinelloid eggs died in the kennel and were sent to IZSLT for necropsy. Frontal and nasal bones were incised in order to explore the nasal cavities. Turbinates and nasal mucosa were removed and observed under dissecting microscope looking for adult nematodes. Recovered worms were killed in 50% ethanol heated at 70°C to prevent contraction, stored in 70% ethanol and successively studied under the microscope as wet mounts in lactophenol. For specific identification of adults, number and length of stichocytes were considered, according to Supperer (1953).

Results

Of the 619 examined individual faecal samples, 42 (6.78% IC 95% 4.9-8.1) were positive for *E. böhmi* eggs at salt flotation. Six dogs confirmed positive at successive examinations, even one year and half after the first diagnosis and treatments with the most common anthelminthic drugs (based on one or more of the following molecules: febantel, pyrantel, praziquantel, oxantel), administered *per os* following firm instructions. Positive dogs were found in all the three sampled kennels. Eggs appeared ellipsoid-shaped, similar to those of *Trichuris vulpis*, very frequent among kennel dogs in Rome; differentiation from this parasites egg was possible because of the smaller dimensions (55-60 µm in length respect to 70-85 µm of *T. vulpis* eggs) and the marked asymmetry of most of them. Suspicion of not being in presence of *E. aerophilus* eggs, species already described from Italy, arose as embryo of this species entirely fills the shell, while in our samples free space between embryos and egg shells was observed, as described for *E. böhmi* (Campbell and Little, 1991).

The identification at species level was confirmed with the finding and examination of adult worms in the nasal cavities of two dogs positive at copro-diagnosis and sent to IZSLT for necropsy.

At necropsy, the first dog, more than 10 years old, showed chronic lesions interesting all the parenchyma, including severe hepatopathy, nephropathy and lung anthracosis. At the opening of nasal cavities, no macroscopic lesion was observed. The second dog was euthanized because of multiple metastases of prostatic adhenocarcinoma and heart failure. This animal presented a chronic rhinitis, with abundant mucopurulent exudates in the nasal cavities.

Nineteen capillarid nematode female specimens were recovered (4 in the first and 15 in the second dog, respectively), 1.3-4.5 cm long, whitish, tapering at both ends, narrower anteriorly, with cuticle transversely striated. In some specimen mounted in lactophenol, it was possible to observe two of the characters differentiating this species from *E. aerophilus*, i.e. number and length of stichocytes. As described by Supperer (1953), our specimens had 30-34 stichocytes (29-34 in the original description), 215-264 µm long (210-294 in the original description), both values compatible with *E. böhmi*, while *E. aerophilus* has an higher number (45-50) of smaller (180 µm) stichocytes.

Discussion

This is the first report of *E. böhmi* from Italy. Both localization in the host and eggs and adults morphological and morphometrical characters confirmed our identification. Nevertheless, due to the similarity of *E. böhmi* eggs with those of both *T. vulpis* and *E. aerophilus* and to the difficulty in recovering adult specimens, being opening of nasal cavities not usual during necropsies, it's possible to hypothesize that this parasite has been frequently misdiagnosed and could be more common and diffused than supposable in our country (cfr. Schoning *et al.*, 1993). The record of this parasite from three different public kennels in Rome strongly supports this hypothesis. Many dogs infected with *E. böhmi* could possibly have been considered in the past positive for *T. vulpis*.

E. böhmi is believed to cause light if any clinical symptom (Campbell and Little, 1991), even if in some case sneezing and nasal discharge are reported (Evinger et al., 1985). In our case, veterinarians didn't report any sign of disease of the upper respiratory tract in any of the positive dogs, even in presence of high eggs count in faecal examination, possibly indicating high worm burdens. However, the second dog positive for adult worms at necropsy presented abundant mucopurulent exudates in nasal cavities.

Due to the low pathogenic significance, from a management point of view the bigger relief of this report is the awareness of past and possible future misidentifications with *T. vulpis*, one of the most common parasites in kennelled dogs. Hence, the importance of making aware veterinarians and diagnosticians of the presence of this parasite and taking it into consideration in copro-parasitological differential diagnosis. Repeated positivities of the same dogs, even after treatments with anthelminthic drugs commonly used for intestinal worms, point out the un-effectiveness of these systemic drugs against this parasite, probably due to the low drug concentration reaching nasal mucosa when treatment is performed *per os*.

Finally, regarding kennels management, the possible direct life cycle makes this parasite a threat in structures characterized by high animal densities and consequent fecalization. Besides, it can't be ruled out a role of worms in carrying pathogenic bacteria, thus facilitating occurrence of respiratory syndromes in animal communities.

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Development of an *in vitro* test to compare natural and chemical products effectiveness against L3 gastrointestinal strongylids of sheep

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Abstract. Gastrointestinal nematodes are still one of the main constraints to sheep production. The rising resistance to anthelmintic drugs and the increasing interest for organic livestock products have drawn attention for the use of natural drugs. In this study we perform an *in vitro* method to evaluate the effectiveness of several natural products, compared to some chemicals, against the third larval stage (L_3) of gastrointestinal strongylids of ruminants. L_3 obtained from sheep faecal cultures were distributed in 24-wells plates with serial dilutions of the testing products. To evaluate the larvicidal activity of the candidate drugs, the number of surviving larvae was counted after 24, 48 hours and 7, 14, 21, 28 days. The mean number of surviving larvae was compared according to the different products tested at various concentrations and at different contact time, and statistical analyses were performed. In this study, a dry extract of leaves of Neem (*Azadirachta indica*), essential oils of Oregano (*Origanum vulgare*), Lemon (*Citrus limon*) and Rosemary (*Rosmarinus officinalis*), Levamisole and Calcium cyanamide nitrate were tested. After 24 hours, Levamisole ≥ 2,5 ppm, Calcium cyanamide ≥ 5000 ppm and Oregano essential oil ≥ 5000 ppm concentrations killed all the larvae. Neem caused total death of larvae at ≥ 10000 ppm after 7 days, but after 24 hours the number of surviving larvae was significantly lower than the control. Rosemary and Lemon essential oils were not effective. This method, easy to perform and replicable, can be used as screening to assess the effectiveness of different products against the L3 of gastrointestinal strongylids. Further experiments are necessary in order to better understand the real effectiveness on the field.

Key words: L3 gastrointestinal strongylid, *in vitro* test, chemicals, natural products, sheep.

Gastrointestinal strongylids (GIS) are the most widespread helminths of the grazing ruminants. The life cycle of these parasites is direct and the hosts become infected after the ingestion/skin penetration of the infective third-stage larvae (L_3).

The repetition of the cycle causes a permanent environmental contamination, so grazing management strategies, as preventive grazing (putting worm free animals in a parasite free pasture), evasive grazing (as pasture rotation) and delusive grazing (obtained by mixed grazing or reducing animal density or stocking rate), can help to reduce pasture infectivity (Barger, 1997). In areas with heavy concentration of strongylids, also chemical treatments of pasture were proposed. In the past, in Italy, caustic lime, iron sulphate and copper sulphate were used, while in 1970 calcium cyanamide, a good disinfectant and fertilizing, was introduced (Restani *et al.*, 1972). In the years the development of resistance to anthelmintic drugs is becoming a worldwide problem in ruminants industry (Waller, 1994; Kaplan, 2004). In Italy, the resistance to benzimidazoles in *Trichostrongylus colubriformis* from a goat flock (Cringoli *et al.*, 2007) and the multiple drug resistance in trichostrongyles affecting sheep (Traversa *et al.*, 2007) were reported. These problems, associated with the environmental impact of conventional anthelmintics and the increasing interest for organic livestock products have raised the attention to the use of natural products, both in animal therapy and environment. For example some studies were performed on biological control with natural enemies of nematodes such as earthworms, bacteria, nematophagous fungi, dung beetles and predatory mites that might be able to reduce the number of free-living stage of parasitic nematodes in dung and surrounding soil (Larsen, 1999). In this research an *in vitro* method to evaluate the effectiveness of several natural products, compared to some chemicals, against the third larval stage (L_3) of gastrointestinal strongylids of ruminants was performed.

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Material and methods

In vitro assay

Faecal cultures were prepared in Petri dishes with a pool of sheep faeces containing about 700 epg of Gastrointestinal strongylids (GIS). Vermiculite N.3 and water were added, gently mixed and incubated for 10 days at 26±2°C in a ventilated, humidified

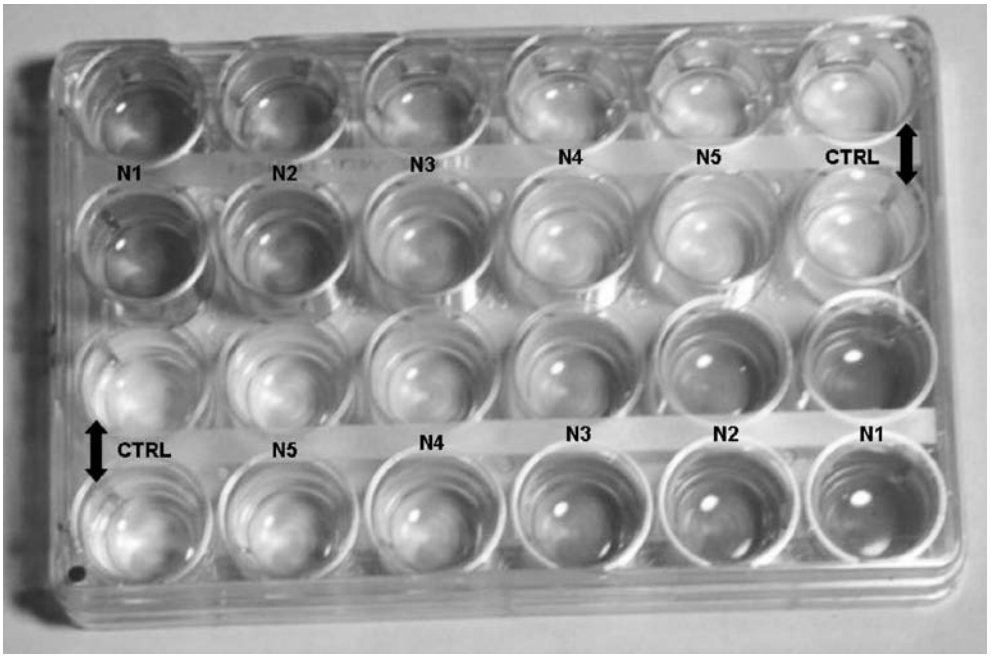


Figure 1. Example of a 24-well culture plates used to test 4 replicates for each Neem concentration (N1-N5); CTRL=control wells.

chamber. The L3 larvae were collected with a Baermann apparatus. After centrifugation, the supernatant was discharged by aspiration and the larvae presents in 50 ml of sediment were counted for three times in order to establish the average concentration. An amount of distilled water was added to achieve about 20-30 L3 larvae/50 µl of larval suspension. To improve the larval-survival rate, the suspension was prepared just before the use.

Twenty-four-well plates (Nunclon, Delta, Nunc-Inter Med.) were used; each well was filled with 50 ml of larval suspension and an amount of distilled water. The living larvae were counted (time 0) and different concentrations of the chemicals tested up to 1000 µl were added. On each plate 3 or 4 dilution series of a chemical were tested (example in Figure 1). Water or diluting was used as negative control. The plates were shaken in a micro-shaker (Dynatech, PBI) for 30-60 seconds and incubated at 26±2°C throughout the trial. The number of living larvae was checked after 24 and 48 hours and 7, 14, 21, 28 days, by stereomicroscope (Leica $MZ9_5$) and inverted microscope (Leitz Labovert). Immobile larvae during a 6 second observation were considered dead (Athanasiadou *et al.,* 2001).

Identification of the third-stage larvae used in the test

A drop of larval suspension with iodine was placed on a slide with coverslip and observed by light microscope at 200-400× magnification. A minimum of 100 larvae were identified from the culture, according to the key of Corticelli and Lai (1964), Euzeby (1981), Hansen and Perry (1994).

Substances tested

A dry extract powder obtained from the leaves of *Melia azadiracta* (*Azadiracta indica*) (Neem), prepared in India following the Ayurveda Medicine processing method and imported by Ambrosi sas, Florence, was used. The Neem's concentrations used were 20000 (N1), 15000 (N2), 10000 (N3), 4000 (N4) and 1000 ppm (N5). Water or dimethyl sulphoxide (DMSO) solution were used for dilution (200 µl of DMSO in 1000 µl of total volume in each well) to improve the homogeneity of the ingredients into the wells (Taylor, 1990). Sixteen replicates for each Neem concentration, of which 8 with DMSO (ND) (two plates) and 8 without DMSO (N) (two plates), and respective 16 control wells without neem were prepared.

Essential oils of Oregano (*Origanum vulgare*) (OR), Lemon (*Citrus limon*) (LM) and Rosemary (*Rosmarinus officinalis*) (RO) diluted in distilled water with 0,1% of Tween 20 were tested. The essential oils used were produced by Flora srl, Lorenzana, Pisa. Six concentrations in 6 replicates were used for each oil: 10000 (1), 5000 (2), 1000 (3), 100 (4), 10 (5) and 1 ppm (6). To evaluate the action of Tween 20 on larval survival, the controls were prepared with (control T) and without (control) Tween 20 addition.

Two plates were prepared with Levamisole: one with DMSO (100 µl of DMSO in 1000 µl of the total volume in each well) and one without DMSO. The chemical was diluted in water and the concentrations were adjusted at 10 (1), 7.5 (2), 5 (3), 2.5 (4) and 1 ppm (5) (Hubert and Kerboeuf, 1992). Eight replicates for each dilution and distilled water-

control were prepared: 4 with DMSO (LD) and 4 without DMSO (L).

Granulated Calcium Cyanamide Nitrate (CCA) (AL.FE. srl, Parma), a fertilizing product also used for parasitic larvae control (Restani *et al.*, 1972) was tested at 6 concentrations: 10000 (CCA1), 5000 (CCA2), 1000 (CCA3), 100 (CCA4), 10 (CCA5) and 1 ppm (CCA6). Two plates were used for 6 replicates of each concentration and 12 control-wells.

Statistical analysis

For the analysis of each product, non-parametric Wilcoxon and Kruskal-Wallis tests and comparison with Student's test for each pair were applied.

To compare Neem's and essential oils' best performance with the references drugs (Levamisole and CCA) the data were also analyzed by General Linear Model Procedure, with Analysis of Variance for repeated measures (SAS, 1998). Four products (Neem, Oreganum, Lemon and Rosemary essential oils), 2 drugs-reference (CCA and Levamisole), and 2 dilutions (with and without DMSO) at six levels of concentration in 7 different moments were compared. Manova test criteria and exact F statistics for the hypothesis of no-time effect, no time-product effect, and no time-level of product effect with Wilks' Lambda, Pillais's Trace, and Hotelling-Lawley Trace tests were performed.

Results

In the larval suspension used for the test, the genus *Cooperia* was prevalent (42%) followed by *Trichostrongylus* (32%), *Ostertagia* (12%), *Oesophagostomum* (9%), *Haemonchus* (3%), *Bunostomum* (1%) and *Nematodirus* (1%).

Differences in larvae survival (P< 0.0001), according to time, time-product and time-level of product and also between subjects (product and level of product) or within subject (time, time-product and time-level of product) were observed. For clear understanding of every interaction, each product was firstly analyzed into itself and then the best performance of each product was compared.

Neem

The mean of initial number of living larvae in Neem plates is summarized in Table 1 (Time 0). No statistical differences in the number of the larvae in each well have been noticed at the beginning of the test; this assures the compliance of the test.

After 24 hours, the mean number of surviving larvae for every Neem's concentration was different in

Table 1. Means and standard deviation for surviving larvae under Neem treatment by day (each concentration was performed in eight replicates).

Product	Time of observation						
	Day-0	Day-1	Day-2	Day-7	Day-14	Day-21	Day-28
Control	35 ± 7	28 ± 8a	25 ± 5a	21 ± 5a	11 ± 4a	10 ± 3a	08 ± 1a
N1	31 ± 4	14 ± 4b	5 ± 2b	0.1 ± 0.3b	0b	0b	0b
N2	32 ± 4	13 ± 3b	8 ± 3b	0.1 ± 0.3b	0b	0b	0b
N3	32 ± 4	12 ± 3b	7 ± 5b	0.1 ± 0.3b	0b	0b	0b
N4	34 ± 4	10 ± 2b	7 ± 3b	3 ± 2b	2 ± 2b	3 ± 2c	1.7 ± 1c
N5	35 ± 5	14 ± 5b	13 ± 7c	10 ± 4c	7 ± 3c	8 ± 4a	7 ± 3a

Different letters mean significant difference (P< 0.0001).

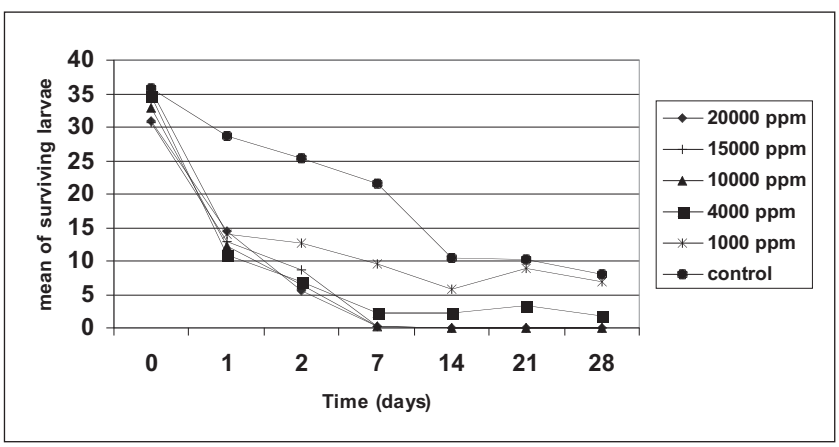


Figure 2. Trend of larval survival in various Neem concentrations.

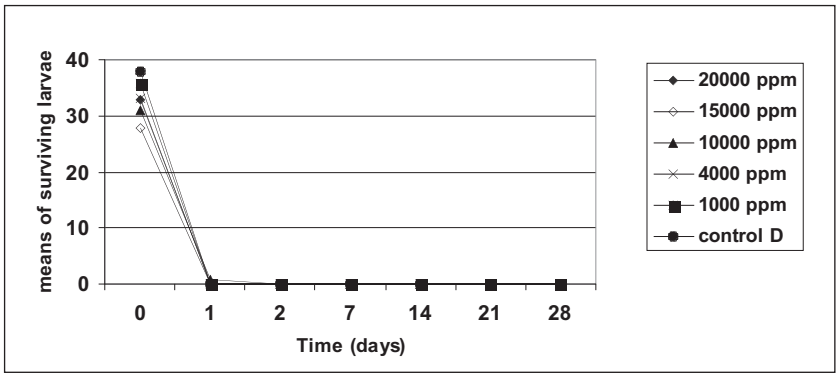


Figure 3. Trend of larval survival in various Neem with DMSO concentrations.

comparison with the control group in Wilcoxon and Kruskal-Wallis tests ($P<0.001$) and in Student's *t* test ($P<0.05$), while was equal between Neem's concentrations. The means of surviving larvae in the 2nd day at concentrations of 20000-4000 ppm (N1-N4) were statistically different from the control group ($P<0.0001$), while N5 concentration was different from the other, showing a higher number of surviving larvae. The trend present in the last observation was maintained and at 7th days in some wells with Neem's concentration ≥ 10000 ppm (N1-3), all larvae were dead. At 28 days, the best results for Neem's concentration were observed at 20000 ppm (N1), 15000 ppm (N2) and 10000 ppm (N3). Larval survival rate in the lowest Neem's concentration, 1000 ppm (N5), was equal to the control group (Table 1, Figure 2). The use of 20% DMSO interfered with Neem solubility and caused, after 24 hours, the dead of the most part of the larvae also in the control wells (Figure 3). The test was not considered in the statistical analysis.

Essential oils

Concerning the essential oils, no statistical differences have been observed from the analysis of the number of survivimg larvae at each Lemon and Rosemary essential oils concentration and controls, with (control T) and without (control) Tween 20, in non-parametric Wilcoxon and Kruskal-Wallis Tests and in Student's t Test (Figures 4 and 5).

The test shows best results for Oregano essential oils plates (Table 2, Figure 6), for which statistical differences in the number of surviving larvae have been observed after the first day. The number of larvae in Oregano plates at the beginning of the test showed no significant differences, even if the distribution was not ideal. After 24 hours, the concentrations of the Oregano essential oil at ≥ 5000 ppm (OR 1-2) showed high mortality of the larvae, and also the concentration at 1000 ppm (OR 3) showed significant lower number of larvae in comparison with control. Lower concentrations, ≤ 100 ppm (OR 4-6), showed the same surviving larvae mean value in comparison with control and control with Tween 20 groups. The situation was the same in the second day of observation. More variations were observed on day-7 at low concentrations (OR 4-6), which were equal to control group and different from Tween 20 control group. The tendency of the larvae means to decrease in the OR 3 and Tween 20 wells was clearly visible after 14 days of the test: it was equal to higher concentrations of the oil and different from control and lower concentration

Table 2. Means and standard deviation for surviving larvae under *Origanum vulgare* treatment by day (each concentration was performed in six replicates).

Product	Time of observation						
	Day-0	Day-1	Day-2	Day-7	Day-14	Day-21	Day-28
OR1	16 ± 4,6	0a	0a	0a	0a	0a	0a
OR2	19 ± 3,5	0.1 ± 0.4a	0.1 ± 0.4a	0.1 ± 0.4a	0a	0a	0a
OR3	10 ± 3	3.6 ± 1.8a	3.6 ± 1.5a	3.5 ± 2.9b	0.5 ± 0.5a	0a	0a
OR4	16 ± 5	9.5 ± 2.6b	9.1 ± 2.1b	7.6 ± 2.8c	2.5 ± 0.8b	1.6 ± 1.6bc	01 ± 1,6ab
OR5	16 ± 4	9.8 ± 5.5b	9.1 ± 4.7b	5.3 ± 2.2bc	2.8 ± 2.1b	1.8 ± 0.9bc	01 ± 1,3b
OR6	14 ± 3	7.6 ± 3.7b	7.8 ± 3.3b	8 ± 3.8c	4.5 ± 2.8b	2.3 ± 2c	1,5 ± 1,3b
Control	15 ± 2	8.8 ± 2.6b	9.1 ± 1.9b	6.5 ± 2.6c	3.3 ± 1.8b	0.6 ± 1.2ab	0,6 ± 1ab
Control Tween	14 ± 2	10 ± 1.5b	9.5 ± 1.8b	3.6 ± 2.3b	0a	0a	0a

Different letters mean significant difference ($P< 0.0001$).

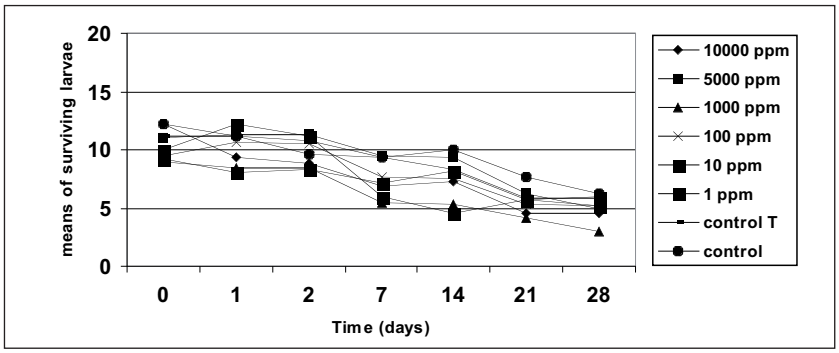


Figure 4. Trend of larval survival in various Lemon essential oil concentrations.

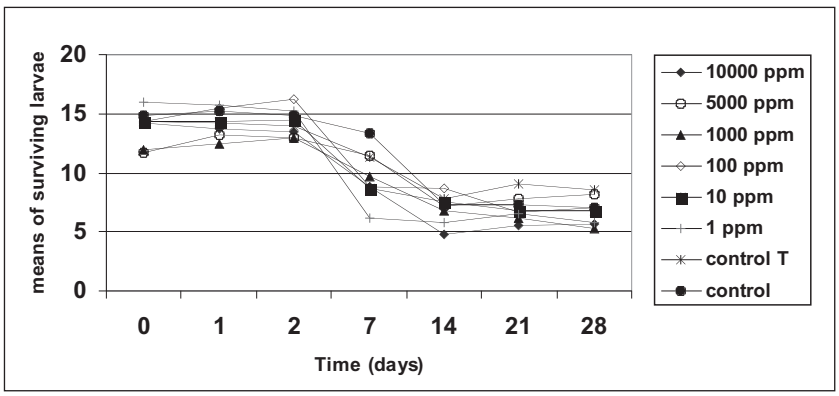


Figure 5. Trend of larval survival in various Rosemary essential oil concentrations.

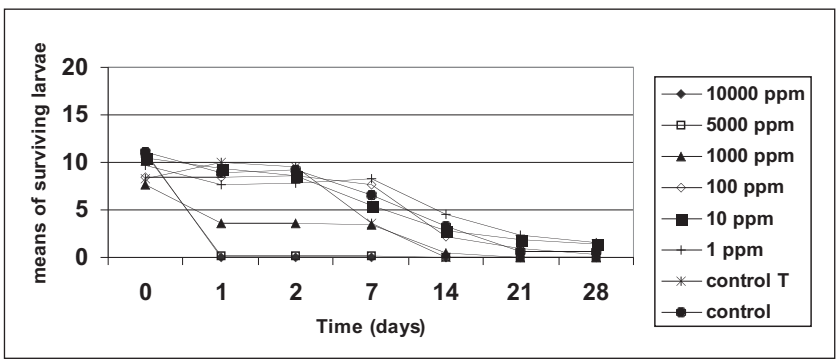


Figure 6. Trend of larval survival in various Oregano essential oil concentrations.

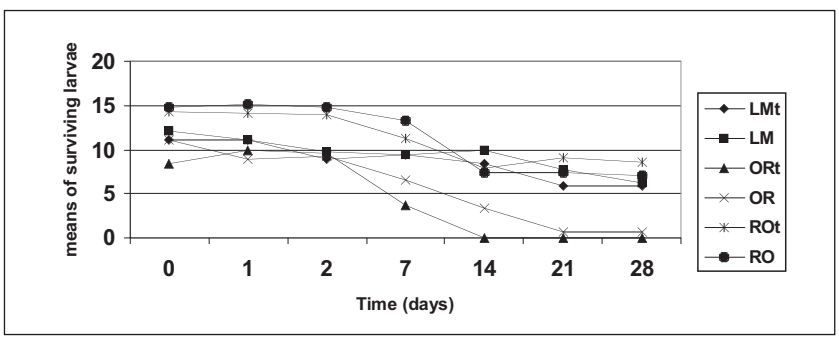


Figure 7. Trend of larval survival in control wells with (t) and without tween in essential oils tests (LM = Lemon, OR = Oregano, RO = Rosemary).

groups. After 21 days, the mean number of surviving larvae in control group decreased and was not differentiated by the highest concentration of Oregano oil and control with Tween 20 groups.

In all the plates with Oregano essential oil, the wells containing the Tween 20 control were located near to the wells with the highest concentration of the oil and showed high rate mortality of larvae, while in the control wells (with and without Tween 20) of the other essential oils' plates, the larval survival was higher (Figure 7). We hypothesized a larvicidal effect of volatile substances of O. vulgare.

Levamisole

The analyses of Levamisole plates are summarized in Table 3 and Figures 8 and 9. At the beginning of the test there were no differences between the number of larvae at each concentration with and without DMSO. High concentration of Levamisole, with (≥ 7.5 ppm - LD 1-2) or without DMSO (≥ 2.5 ppm - L 1-4), showed after 24 hours the death of most of the larvae. At the others concentrations, both with DMSO (LD 3-5) and without DMSO (L5) a statistically different result was observed when compared with control wells. The control wells with DMSO have low surviving larvae in comparison with the control group without DMSO (LC). On day 2, all concentrations with or without DMSO showed the same results. The mean number of surviving larvae were very low and the difference between control with or without DMSO was maintained. The presence of 10% DMSO influenced the larval survival: this is more evident in the test with Neem, where the DMSO was at 20% concentration (Figure 10). The DMSO at the tested concentrations had a toxic effect on L3.

Table 3. Means and standard deviation of surviving larvae under Levamisole treatment by day (Each concentration was performed in four replicates).

Product	Time of observation						
	Day-0	Day-1	Da-2	Day-7	Day-14	Day-21	Day-28
L1	31 ± 4	0a	0a	0a	0a	0a	0a
L2	21 ± 3	0a	0a	0a	0a	0a	0a
L3	30 ± 7	0.5 ± 1a	0.5 ± 1a	0a	0a	0a	0a
L4	27 ± 7	0.5 ± 0.5a	0.25 ± 0.5a	0a	0a	0a	0a
L5	31 ± 13	4.5 ± 2b	1.25 ± 0.9a	1.25 ± 0.9a	0.25 ± 0.5[a]	0a	0a
Control	25 ± 6	21 ± 6c	21 ± 6b	10 ± 2.5b	5 ± 3b	3.8 ± 1.9b	4 ± 0.9b
LD1	20 ± 3	0a	0a	3 ± 2.4c	0a	0a	0a
LD2	22 ± 5	0.25 ± 0.5a	0a	3.8 ± 0.9c	0a	0a	0a
LD3	25 ± 2	3.5 ± 2b	0a	1.5 ± 0.5a	0a	0a	0a
LD4	24 ± 3	4,8 ± 3b	1 ± 0,8a	0a	0a	0a	0a
LD5	25 ± 7	6 ± 3b	0,75 ± 0,9a	0,25 ± 0,5a	0a	0a	0a
Control DMSO	27 ± 10	14 ± 3d	15 ± 3c	14 ± 4d	4 ± 3b	2 ± 2c	0.75 ± 0.9c

Different letters mean statistical difference ($P < 0.05$).

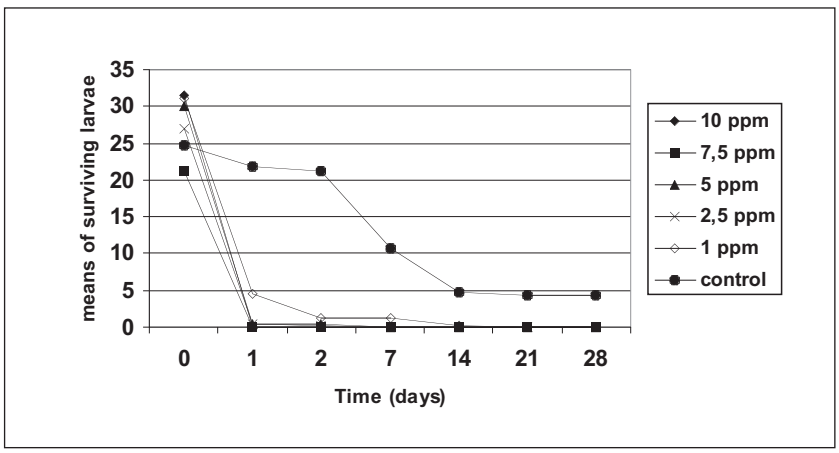


Figure 8. Trend of larval survival in various Levamisole concentrations.

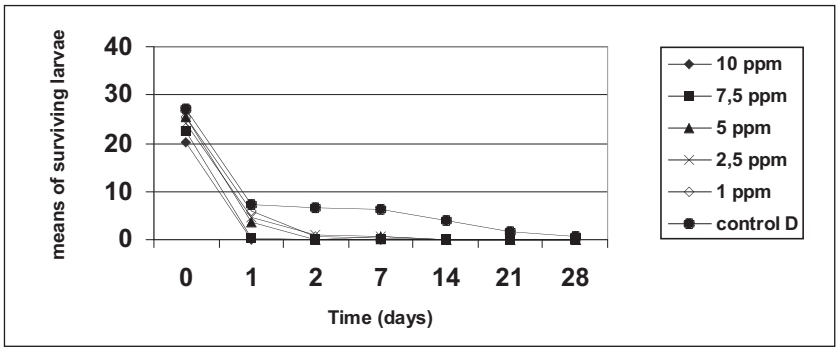


Figure 9. Trend of larval survival in various levamisole with DMSO concentrations.

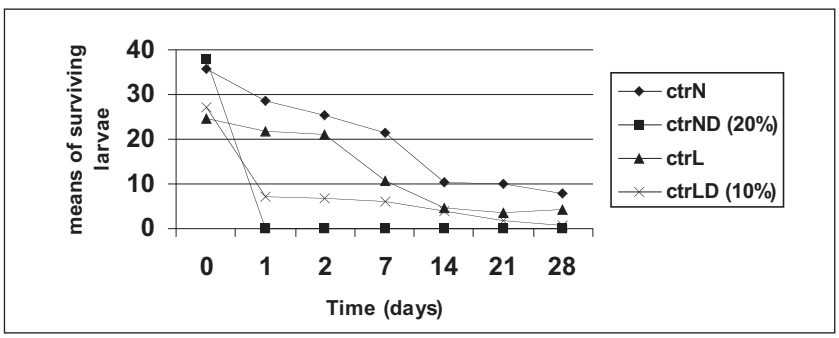


Figure 10. Trend of larval survival in control wells with and without DMSO in Neem (ctrND, ctrN) and levamisole (ctrLD, ctrL) tests.

Calciocianamide (CCA)

In the analysis of CCA, no statistical difference in number of larvae was present on day-0. After 24 hours CCA concentrations ≥ 5000 ppm (CCA 1 and 2) showed a few number of living larvae, while the other concentrations were similar to the control group. The number of surviving larvae was slightly reduced in CCA 3 and, from the second week (day 14), this was similar to the number into the wells with higher concentration, whereas at low concentrations (CCA 4-6) the means of surviving larvae did not differ from control wells during all the trial (Table 4; Figure 11).

Comparison to different substances tested

The best results obtained with Neem leaves' powder (N1-3) and essential oil of O. vulgare (OR 1-3) were compared with drug-reference CCA and Levamisole. As statistical differences in the initial number of larvae were observed, we have compared Neem with levamisole, and oregano essential oil with CCA, in which, even if the number of larvae present in the wells on day-0 was not the same, the differences were not significant ($P<0.0001$) in Wilcoxon and Kruskal-Wallis tests. No statistical difference was observed on the survival rate of larvae in all the control wells.

Table 4. Means and standard deviation for surviving larvae under CCA treatment by day. (Each concentration was performed in six replicates).

Product	Time of observation						
	Day-0	Day-1	Day-2	Day-7	Day-14	Day-21	Day-28
CCA1	10 ± 3	0a	0a	0a	0a	0a	0a
CCA2	11 ± 3	0a	0a	0a	0a	0a	0a
CCA3	9 ± 3	12 ± 2.5b	9 ± 1.5b	5 ± 4b	0,3 ± 0.8a	0.2 ± 0.4a	0a
CCA4	11 ± 5	11 ± 3b	10 ± 3b	9 ± 2b	6 ± 3b	7 ± 2.5b	4.5 ± 2.4b
CCA5	12 ± 3	10 ± 3b	10 ± 2b	9 ± 2b	8 ± 3b	8 ± 1.9b	6.7 ± 1.9b
CCA6	13 ± 4	12 ± 2.5b	11 ± 2.5b	10 ± 2b	9 ± 0.9b	8 ± 1.4b	5 ± 2b
CCAC	14 ± 5	12 ± 3b	12 ± 3b	11 ± 3b	9 ± 4b	7 ± 2b	6 ± 2.6b

Different letters mean statistical difference ($P < 0.0001$).

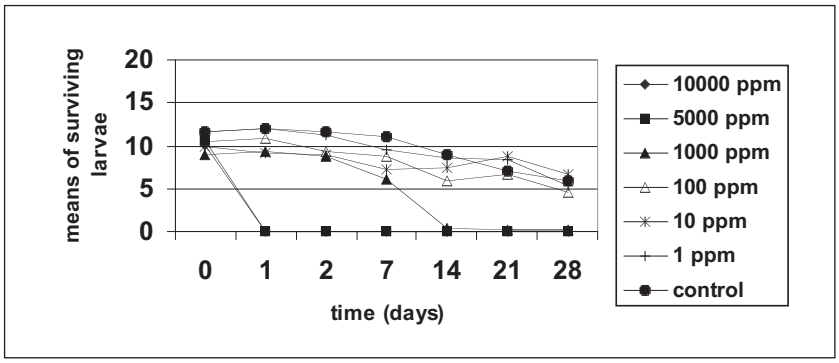


Figure 11. Trend of larval survival in various CCA concentrations.

Table 5. Means and standard deviation for surviving larvae under Neem treatment compared with levamisole treatment.

Product	Time of observation						
	Day-0	Day-1	Day-2	Day-7	Day-14	Day-21	Day-28
Control	35 ± 6	28 ± 8a	25 ± 4a	21 ± 5a	11 ± 3a	10 ± 3a	8 ± 2a
L1	31 ± 4	0b	0b	0b	0b	0b	0b
L2	21 ± 3	0b	0b	0b	0b	0b	0b
L3	30 ± 7	0.5 ± 1b	0.5 ± 1b	0b	0b	0b	0b
L4	27 ± 7	0.5 ± 0.5b	0.25 ± 0.5b	0b	0b	0b	0b
L5	31 ± 13	4.5 ± 2b	1.25 ± 0.9b	1.25 ± 0.9b	0.25 ± 0.5b	0b	0b
N1	31 ± 3	14 ± 4c	5.5 ± 2c	0.12 ± 0.3b	0b	0b	0b
N2	32 ± 4	13 ± 3c	8.6 ± 3c	0.12 ± 0.3b	0b	0b	0b
N3	33 ± 4	12 ± 2c	7 ± 4c	0.12 ± 0.3b	0b	0b	0b
N4	34 ± 4	10 ± 1c	7 ± 2c	2 ± 1b	1.75 ± 1.9b	2.8 ± 2b	1.75 ± 1b
N5	35 ± 4	14 ± 5c	13 ± 7d	10 ± 4c	7 ± 3.5a	8.8 ± 4a	7 ± 3a

Different letters mean statistical difference ($P < 0.0001$).

Table 6. Means and standard deviation for surviving larvae under *Origanum vulgare* treatment compared with CCA treatment.

Product	Time of observation						
	Day-0	Day-1	Day-2	Day-7	Day-14	Day-21	Day-28
Control	14 ± 4	12 ± 3b	11 ± 3b	11 ± 3b	9 ± 4b	7 ± 2b	6 ± 2b
CCA1	10 ± 3	0a	0a	0a	0a	0a	0a
CCA2	10 ± 3	0a	0a	0a	0a	0a	0a
CCA3	9 ± 3	12 ± 2.5b	9 ± 1.5b	5 ± 4b	0.3 ± 0.8a	0.2 ± 0.4a	0a
OR1	16 ± 4	0a	0a	0a	0a	0a	0a
OR2	19 ± 3	0.1 ± 0.4a	0.1 ± 0.4a	0.1 ± 0.4a	0a	0a	0a
OR3	10 ± 3	3 ± 2c	3 ± 1.5c	3.5 ± 3c	0.5 ± 0.5a	0a	0a

Different letters mean statistical difference ($P < 0.0001$).

The number of surviving larvae in Neem at concentrations of 20000 (N1), 15000 (N2) and 10000 (N3) ppm, in levamisole at concentration of 10 (L1), 7.5 (L2), 5 (L3), 2.5 (L4) and 1 (L5) ppm and in the control group (C) was compared from the first day until day-28 (Table 5). On day-1, each Neem concentration showed a number of surviving larvae statistically lower than the control, but higher than any levamisole concentration, in which many wells without any surviving larvae were observed. On the second day, the number of surviving larvae in N1-4 wells began to decrease but only

after a week it was almost equal to the number of levamisole larvae. N5 was different from all other Neem's concentrations.

The performances analysis of Oregano essential oil concentrations at 10000, 5000 and 1000 ppm (OR 1-3) and CCA concentration at 10000 and 5000 ppm (CCA 1-2), expressed as the number of surviving larvae, is summarized in Table 6. The differences observed in the number of larvae for each product at the beginning of the test were not significant. Twenty-four hours later, Oregano essential oil concentrations $\geqslant$ 1000 ppm and the CCA concentrations $\geqslant$ 5000 ppm influenced the number of living larvae respect to the control. After 14 days and until the end of the test, the number of surviving larvae in each oregano and CCA concentrations was statistically different from the control wells.

Discussion and conclusion

The method described in this paper is easy to perform and replicable. As the number of larvae, at the beginning of the test, should be as uniform as possible, a required condition is to maintain in agitation the larval suspension, when added in the wells. In this work, we observed no significant differences inside each plate, but some variations among different plates. A magnetic stirrer could uniform the larval suspension until the wells are filled. The levamisole, chosen for their solubility in water, was used as reference drug to evaluate the efficacy of the test. This drug was active in 24 hours at concentrations $\geqslant$ 2.5 ppm, according to the in vitro test performed by Taylor (1990) against the development eggs-L3 in non-resistant strongylid strains.

This method can therefore be used as screening to assess the effectiveness of different products against the L3 of GSI.

The emergence of resistance to anthelmintic drugs, which is now a world wide phenomenon, and the increased awareness of consumers about drug residues that potentially enter the food chain, have stimulated investigation into alternatives to commercially available anthelmintics, such as medicinal plants. However, not in all cases the evidence on the antiparasitic properties of plants is consistent with the expectation arising from traditional views. For example the Neem tree, known for its medicinal properties and widely used as biopesticide against a wide range of plant and animal ectoparasites, has been recommended for use against gastrointestinal nematodes and related problems in many parts of the world (Biswas *et al.*, 2002; Subapriya and Nagini, 2005). However not all the studies agree in the evaluation of the effectiveness of this plant (Athanasiadou *et al.*, 2007). The Neem product used in this study had a significant effect on larval survival at all concentrations tested in 24 hours, while only after 14 days it killed all the larvae at concentrations $\geq$ 10000 ppm. CCA, which is used in the pastures for its fertilizing and larvicidal effects (Restani *et al.* , 1972), was effective after 24 hours at concentrations $\geq$ 5000 ppm. Even if it was not possible to compare directly with statistical analysis these two products, because of the statistical difference in the initial number of larvae, the Neem tested seems to be less effective, but it should not be underestimate its possible use in the pasture. Neem derivates mixed with other fertilizers seem to be toxic to plant-parasitic nematodes while are not detrimental to beneficial free-living soil organisms (Akhtar & Alam, 1993; Akhtar and Mahmood, 1994; Akhtar and Mahmood, 1997); consequently, some Neem derivates could be active in pasture against L3 larvae of GIS of ruminants. Field experiments are necessary to better understand the real effectiveness of Neem products. In addition it is necessary to investigate the methods used for the extraction and the composition of Neem derivates, as some extracts need alcohol or ricinoleate to solubilise them, that influencing the larval survival (Tampieri *et al.*, 2002).

The results obtained with essential oils are interesting; in particular, Rosemary and Lemon essential oils do not shown any negative effect against L3 larvae, while Oregano essential oil revealed a significant larval lethality in 24 hours at concentrations $\geq$1000 ppm. Lethal effects have been observed also in the control wells near to the highest oregano concentrations, probably caused by volatile substances. Considering this eventuality it would be better, during the preparation of the test, to use separate plate for the control when volatile principles are tested. In the Oregano essential oil tested, gascromatographic analysis revealed as major components the charvacrol (55.08%), a phenolic monoterpenic compound (Tampieri *et al.*, 2003) well known for the bactericidal, fungicidal and toxic properties, mainly due to the denaturation activity on the proteins (Goodman and Gilman, 1963). The massive use of this essential oil on pasture is not suitable for the high cost, but the results obtained during this research allow some considerations about the aromatic plants. It has reported that lambs which grazed sward on different herbage species acquired differing level of gastrointestinal nematode infection (Scales *et al.*, 1995). This has recently been correlated to the presence of plant with high concentration of tannins, that seems to have direct and indirect (nutritional) anthelmintic activity (Genchi, 2006), but it is also been observed that the different pasture herbage species can play an important role in the sward microclimate, directly or indirectly affecting larval development and survival. In particular Niezen *et al.*, (1998) observed a significant effect on development and vertical migration of larvae connected to the pasture plant species. It not be excluded that also the presence of plants producing essential oils could affected the survival of the L3 on the grazing.

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Development of a single-round PCR method for the simultaneous detection of *Babesia caballi* and *Theileria equi*

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Abstract. The aim of this study was the development of a diagnostic system based on derivative melting curves analysis for simultaneous detection of *Babesia caballi* and *Theileria equi,* the agents of equine piroplasmosis. The study was performed only on these predominant and clinical relevant species and not on those whose importance is unknown. The target sequence of this study was a fragment within the gene coding for the 18S ribosomal RNA that allows to differentiate these two species. Therefore, a real-time PCR using LCGreen I fluorescent dye followed by comparison of derivative melting curves in post-amplification phase was developed. The dissociation curve analysis showed a 0.9°C (SD±0.36) mean melting temperature difference between, respectively *T. equi* and *B. caballi* DNA, allowing differentiation of the two species in an homogeneous assay.

Key words: real-time PCR, LCGreen, differentiation, *Babesia caballi*, *Theileria equi.*

Equine piroplasmosis, caused by haemoprotozoan parasites *Theileria equi* and *Babesia caballi,* is an important protozoan infection of horses, endemic in Europe, Africa, Middle East and continental Asia (Avarzed *et al.*, 1997; Kuttler, 1988; Schein, 1988). Some cases of horses, unexpectedly infected by piroplasmids with different host range as *B. bovis* (Criado-Fornelio *et al.*, 2006; Buling *et al.*, 2007), *B. bigemina* (Buling *et al.*, 2007), *B. canis canis* (Criado-Fornelio *et al.*, 2003a), and *B. microti* like(Pietrobelli *et al.*, 2006) were also described.

The detection and identification of *T. equi* and *B. caballi* were traditionally based on the light-microscopy examination of thin Giemsa or Wright-stained blood films (Saal, 1964; Bose *et al.*, 1995). This method is simple but insufficient for accurate identification of these parasites during mixed infections or low parasitemias and laborious when large numbers of blood smear samples need to be simultaneously examined (Rampersad *et al.*, 2003; Krause, 2003). In the last years molecular techniques have been used for the detection and identification of species belonging to *Theileria/Babesia* group. These methods are based upon the species-specific polymerase chain reaction (PCR) assays targeting either 18S rRNA gene sequences (Bashiruddin *et al.* 1999: Birkenheuer *et al.*, 2003; Cacciò *et al.*, 2000; Criado-Fornelio *et al.*, 2003b; Rampersad *et al.*, 2003) or Merozoite Antigen 1 (EMA-1) gene sequence (Nicolaiewsky *et al.*, 2001). Different strategies of PCR for a more rapid and sensitive identification have been proposed: nested PCR for *T. equi* (Rampersad *et al.*, 2003), multiplex PCR for simultaneous detection of the two species (Alhassan *et al.*, 2005; Alhassan *et al.*, 2007), and Taqman real time PCR for quantitative detection of *T. equi* (Kim *et al.*, 2008). However, these methods can be at risk of carryover-contamination like nested-PCR or have high cost like Taqman realtime PCR. The goal of this paper was to develop a rapid and inexpensive method to achieve an accurate differentiation between *T. equi* and *B. caballi*. Our attention was addressed on melting peaks analysis of the amplicons generated by the melting reaction after a LCGreen real-time PCR based on the 18S ribosomal RNA gene.

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Materials and methods

Biological samples and DNA extraction

Forty-five field blood samples were collected from horses living in Italy. Genomic DNAs were extracted from the blood samples by using Maxwell® 16 Blood DNA Purification Kit (Promega) with Maxwell® 16 Instrument (Promega) according to the manufacturer's instructions and then stored at 20°C until use.

In vitro-cultured parasites

An isolate of *T. equi* from a natural infected horse was grown in purified equine red blood cells (RBC). The infected RBC were subjected to DNA extraction as described above and then used for the determination of the detection limit of realtime PCR assay.

Primer design and PCR amplification

The forward primer babe 1-5 (5′-TCGAAGACGATCA GATACCG-3′) and the reverse primer babe 1-3 (5′-TTTCTCTCAAGGTGCTGAAGG-3′), corresponding

to a fragment within the *T. equi* and *B. caballi* 18S ribosomal RNA gene sequence (Criado-Fornelio *et al.*, 2003b), were designed by program Primer 3 (Rozen *et al.*, 2000). The accession numbers of the sequences used to design the primers for *T. equi* and *B. caballi* were respectively Z15105, AY150064, AY150062, AY150063, AY534882, DQ287951 and Z15104, AY534883, AY309955. The nucleotide sequence of the selected fragment from *T. equi* and *B. caballi* was compared by BLASTN software with those available in the GenBank database of other *Babesia* spp. which were detected in horses (*B. bigemina*, *B. canis canis*, *B. bovis* and *B. microti*). The *B. caballi* fragment showed 91%, 93%, 95% and 99% similarity with *B. bovis, B. canis canis, B. microti* and *B. bigemina* respectively. The *T. equi* fragment showed 90% similarity with *B. canis canis* and *B. bovis* and 94% and 98% similarity with *B. bigemina* and *B. microti* respectively. The real-time PCR reaction was performed in a reagent mixture containing 0,2 µM of each primer, PCR buffer 1x, $MgCl_2$ 3 mM, dNTPs 0.2 mM, 0.75 U of FastTaq (Roche), BSA Dnase free 0.01% (Roche), fluorescent LCGreen Plus 1x (Idaho Technology Inc.) in a final volume of 15 µl. The 45-cycle program of the real-time PCR was performed by LightCycler II Instrument (Roche Diagnostics, Mannheim, Germany). The optimized protocol for the assay was, after an initial heating at 94°C for 5 min, a denaturation at 94°C for 5 sec, annealing at 54°C for 10 sec and extension at 72°C for 15 sec. Amplification on the LightCycler II was immediately followed by melting analysis with an additional denaturation at 95°C with a 0 s hold, cooling at a programmed rate of 20°C/s to 40°C with a 0 s hold and a continuous melting curve acquisition during a 0.2°C/s ramp to 90°C. LightCycler software was used to calculate the derivative melting curves.

Real time PCR

With the aim of establishing the detection limit of the method eight 10-fold serial dilutions of *T. equi*-infected RBC with 4% parasitemia (8.8×10^5 infected cells/µl) in non-infected RBC were prepared. DNA was then extracted from each dilution and the real time PCR was carried out as described above. Moreover the real time PCR was tested both with 45 Italian horse blood samples, that were analyzed also using a PCR method (Bashiruddin *et al.* 1999), and with 3-fold serial dilutions of the DNA of *T. equi* and *B. caballi*, extracted from positive blood samples. In order to confirm the results obtained by the real time PCR, the positive samples were amplified by a standard PCR and the products of amplification were sequenced. PCR reaction was performed in a reagent mixture containing 0.2 µM of the primers babe1-5 and babe 1-3, buffer 1x, $MgCl_2$ 1,5 mM, dNTPs 0.2 mM, 0.75 U of Taq (Roche). The amplification, performed by DNA Engine DYAD (MJ Research), involved a hot start of 5 min at 94°C followed by 30 cycles of 30 sec at 94°C, 30 sec at 54°C and 1 min at 72°C and a final extension of 5 min at 72°C. The DNA amplified products were purified by the NucleoSpin Extract kit (Macheray-Nagel, GmbH, Germany), according to the manufacturer's recommendations. The purified products were then analysed by the Agilent 2100 Bioanalyzer (Agilent Technologies), a miniaturized platform for electrophoresis applications, that allows to estimate the size and the concentration of each fragment. The PCR amplicons were then sequenced by CEQ 8000 automatic DNA Analysis System (Beckman-Coulter, Fullerton, CA, USA) using a commercial Kit (GenomeLab™ DTCS-Quick Start Kit, Beckman-Coulter) according to the manufacturer instructions. The primers used for sequencing of the amplified fragment were babe1-5 and babe 1-3.

Results

Comparison of the 18S rRNA PCR of *T. equi* and *B. caballi* partial sequences (corresponding to the amplified fragment) showed a low variability. These differences consisted in 1 nucleotide (nt) insertion between *T. equi* (101 nt) and *B. caballi* (100 nt) and in 5 mismatches. After suitable primers had been selected, they were first tested with two DNA stock solutions obtained by blood of *T. equi* and *B. caballi* infected samples. The derivative melting curve plot obtained in the presence of LCGreen Plus produced two clearly defined melting peaks, one of 84,66 (SD±0.08)°C for *T. equi* and the other of 83,79 (SD±0.12)°C for *B. caballi* (Fig. 1). The lowest level of detection, was found to be corresponding to $8{,}8\times10^1$infected-RBC/µl. To evaluate the field usefulness of the *T. equi-B. caballi* real-time PCR assay, 45 field blood samples previously analyzed by PCR following the method described by Bashiruddin (1999), were tested. Of 45 analyzed field blood samples, the real-time PCR assay detected the parasites in 15 samples (33.3%), confirming the results obtained by PCR. Three of the positive samples were identified as *B. caballi* and the others as *T. equi* on the basis of Tm. Melting curve analysis of the various products showed an average melting temperature of 84.5 (SD±0.3)°C for *T. equi* and 83.6 (SD±0.06)°C for *B. caballi*, for a mean difference of 0.9 (SD±0.36)°C between the two targets. In order to confirm that the differences among the melting curves and the melting peaks correspond to different sequences, we first performed the sequencing of the amplicons of the positive samples obtained by a standard PCR, obtaining a full concordance with the results of the real time.

Discussion

In the past, detection and identification of *Babesia/Theileria* group members were based on the use of microscopic examination and immunologic and biological methods; recently more rapid and sensitive methods based on PCR technology have been proposed. The goal of the paper was to develop a novel

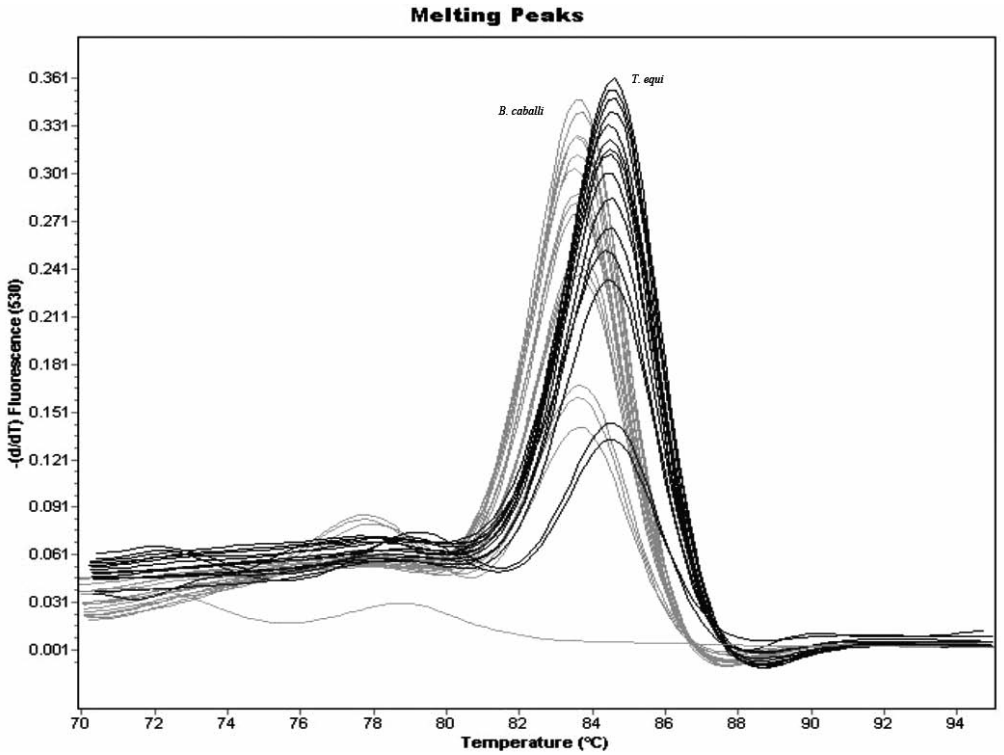


Figure 1. Analysis of amplicons obtained by testing in triplicate 3-fold serial dilution of the DNA of *T. equi* and *B. caballi.* Real-time PCR was done using the LCGreen as dye and the LightCycler II (Roche Applied Science). Following LCGreen real-time PCR amplification of the template, the products were analysed for the temperature at which the double-stranded DNA dissociates. This dissociation curve analysis revealed a mean melting temperature difference of 0.9°C between *T. equi* (84.66 SD±0,08°C) and *B.caballi* (83.79 SD±0.12°C).

real-time PCR for the rapid detection and differentiation of *T. equi* and *B. caballi* that does not rely on a second round of amplification or hybridization to achieve high sensitivity and accurate species identification. The 18S ribosomal RNA gene sequence was chosen as gene target for its low intraspecies variability and the presence of species-specific point substitutions that allow to differentiate the species by sequences analysis, specific probe or melting curves analysis. The differentiation between the two species is crucial because *T. equi* is more refractory to treatment than *B. caballi*, and higher dosages of imidocarb are required (Vial *et al.,* 2006). Moreover, species identification could give important information on the pathogenicity of the disease. Our strategy was addressed on the analysis of Tm melting curves obtained by a LCGreen real-time PCR because this method represents a just compromise among costs, rapidity and specificity. We choose LCGreen as dsDNA dye because it can be used at 90% saturation, whereas SYBR Green I completely inhibits PCR at 50% saturation. Therefore the melting results obtained with LCGreen are much more representative of the products present than results obtained with SYBR Green I, which is strongly biased against low-melting products. (Wittwer *et al.,* 2003).The results indicate that the assay developed in this study can detect with high sensitivity the presence of parasites in the samples; moreover the dissociation curve analysis revealed a mean melting temperature difference between the two targets, that allowed the simultaneous differentiation of *T. equi* and *B. caballi.* The existence of *B. bovis, B. canis canis* and *B. bigemina*-infected horses (Criado-Fornelio *et al.,* 2003a, 2006; Buling *et al.,* 2007) suggest a low host specificity of piroplasmids, but the epidemiological and pathological importance of these infectious agents in the horses is unknown. At the present *T. equi* and, with low frequency, *B. caballi* represent the predominant and clinical relevant piroplasmid infecting horses and this discriminatory method may be suitable for the routine screening of the infections.

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Contribution to the knowledge of the Nycteribiidae (Diptera) from Venetian Region

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Abstract. The flies belonging to the Nycteribiidae family are pupiparous, blood-sucking, obligatory ectoparasites of Chiroptera. Their biology and morphology are the result of the adaptation to an ectoparasitic life on their host: the bats. Nine species are reported from Italy, six from the region Veneto. Fly specimens, collected from bats belonging to the main colony (*Myotis myotis* and *Miniopterus schreibersii*, 200-250 individuals in 1975-1980) living into "La Bislonga" cave (1001 V/TV), were studied and identified as *Nycteribia latreillii*, new for Northern Italy, *N. schmidlii* and *Penicillidia dufourii*. These data confirm the host specificity of the parasite species, as previous quoted. The significant reduction in bat richness and abundance occurred in the last years has done direct effect on parasite distribution and in general on biodiversity.

Key words: *Nycteribia latreillii*, *Nycteribia schmidlii,* Chiroptera, cave, biodiversity.

The flies belonging to the Nycteribiidae family are pupiparous, blood-sucking, obligatory ectoparasites of Chiroptera. Their biology and morphology are the result of the adaptation to the ectoparasitic life on their host: the bats. The sternites of the thorax are fused into a broad plate, while the mesonotum is membranous, the opposite to the other flies. The pleurae are displaced dorsally and the legs are inserted on the dorsal surface. The head is kept folded back at rest, so that its dorsal surface rests on the mesonotum. It is rotated forward through 180° for feeding. Wings are absent in all Nycteribiidae, but halteres are present; they have a thin stalk and a spherical or ovoid head. The femora are thick and have a ring of weaker integument near the base, the tibiae vary in form in the different species, they are either laterally compressed or more or less cylindrical. The basitarsus is usually very long, sometime shorter, whereas others tarsal segments are short. The praetarsus triangular, broad, has two strong claws and pulvilli, whereas the empodium is absent (Theodor, 1975). The life cycle is rather uniform throughout the Nycteribiidae. The female ovaries produce one egg at time that descends into the uterus for developing after fertilisation. The larva feeds and growth within the female uterus, where it is nourished by the secretion of the "milk gland". The female leaves its host bat just before larviposition and usually deposits the larva on a vertical surface of the bat roost. The larva transforms into the pupal stage from which emerged after 20-40 days.

Three degrees of host-parasite specificity are present in the Nycteribiidae: (1) restriction to one species of host; (2) restriction to a genus or a family of host; (3) lack of specificity. The host specificity seems to be determined in some case by ecological factors and geographical isolation.

Today, twelve genera, with about two hundred fifty specie, are included in this family. The species are widely distributed in the world, mainly in the warmer regions. The family is believed to have had its centre of origin in the Malaysian subregion (Peterson and Wenzel, 1981). From the European region 15 species are reported (www.faunaeur.org). The knowledge about the flies parasites of bats present in the Italian territory (9 species of Nycteribiidae; 1 species of Streblidae) is still incomplete, although several data have been summarised by Stefanelli (1942) and Lanza (1999). The reports of Nycteribiidae from the Venetian region are scarce and occasional (Caoduro, 1994; Lanza, 1999; Ruffo, 1938; Vanin and Vernier, 2005) (Table 1).

The aims of this paper are (i) improve the knowledge on the Nycteribiidae-fauna in the Venetian region starting from new original data; (ii) point the "state of the art" on the knowledge of this family in Northern Italy, and (iii) propose a conservational reflection.

Material, methods and site description

Twenty wingless flies, kept in a vials containing also several acari, were studied. The specimens were collected on 30.07.1977, from bats belonging to the biggest colony of "La Bislonga" cave (1001 V/TV; Long 29 25 6, Lat 45 52 42 9) which is located in the Venetian Prealps, in Pederobba municipality (Province of Treviso). The cave is long 321 m, high 15, and ends with a small lake. The main bat colony, just reported since 1800, counted during the summer (reproductive period), in the years 1975-1980 about 200-250 individuals of the species *Myotis myotis* (Borkhausen, 1797), and *Miniopterus schreibersii* (Kuhl, 1817) (Vernier, 1977, 1996a, 1996b). Also few individuals of

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Table 1. Nycteribiidae reported from the Venetian region.

Species	Localities	Province	References
Nycteribia latreillii	Grotta La Bislonga (1001 V/TV) (Pederobba)	TV	Present study
Nycteribia pedicularia[1]	Grotta Regosse (Roverè)	VR	Ruffo, 1938; Lanza, 1999
Nycteribia schmidlii	Grotta La Bislonga (1001 V/TV) (Pederobba)	TV	Present study
	Covoli del Velo (44 V/VR)	VR	Ruffo, 1938; Lanza, 1999
Penicillidia dufourii	Grotta A del Ponte di Veja (117 V/VR)	VR	Caoduro *et al.*, 1994
	Buso del Pozzo Comune 19 (n.i. V/VR)	VR	Caoduro *et al.*, 1994
	Grotta della Guerra (127 V/VI)	VI	Vanin and Vernier 2005
	Grotta La Bislonga (1001 V/TV) (Pederobba)	TV	Present study
Penicillidia conspicua	Covoli del Velo (44 V/VR)	VR	Ruffo, 1938; Lanza, 1999
Phthiridium biarticulatum[2]	Grotta di Veja (117 V/VR)	VR	Ruffo, 1938; Lanza, 1999
	Grotta Regosse (Roverè)	VR	Ruffo, 1938; Lanza, 1999

[1] *Nycteribia kolenatii.*
[2] In the checklist of the Italian fauna (Minelli *et al.*, 1993-1995), this species is reported only from Southern Italy and Sardinia.

Myotis capaccinii (Bonaparte, 1837), and *Rhinolophus ferrumequinum* (Schreber, 1774) were reported (Vernier, 1996c). Since 1994 the main colony was drastically reduced to few individuals both for collapses and human disturb. The bat species recently observed in this cave (1994-2004), were few specimens of *Myotis daubentonii* (Kuhl, 1817) and *Myotis mystacinus/brandtii* (Vernier, unpublished data).

Results

The specimens of parasites, determined by one of the authors (S.V.), belong to *Nycteribia (N) latreillii* (Leach, 1817), 2♀♀, 1♂, *N. (N) schmidlii* (Schiner, 1853), 2♀♀, 4♂♂ and *Penicillidia dufourii* (Westwood, 1834) 9♀♀, 2♂♂. The specimens in alcohol 70% are in good conditions and are stored in the private collection of one of the authors in Padova (E.V.).

The specimens belonging to the seven species, known from the Palaearctic region, of the genus *Nycteribia* Latreille 1796, are small or medium size flies without eyes. The tibiae are laterally compressed and short. The genus is divided in two subgenus: *Nycteribia* and *Acrocholidia.* The first subgenus is present in the West-Palaearctic region with four species [*N. (N.) latreillii*, *N. (N.) kolenatii*, *N. (N.) pedicularia*,

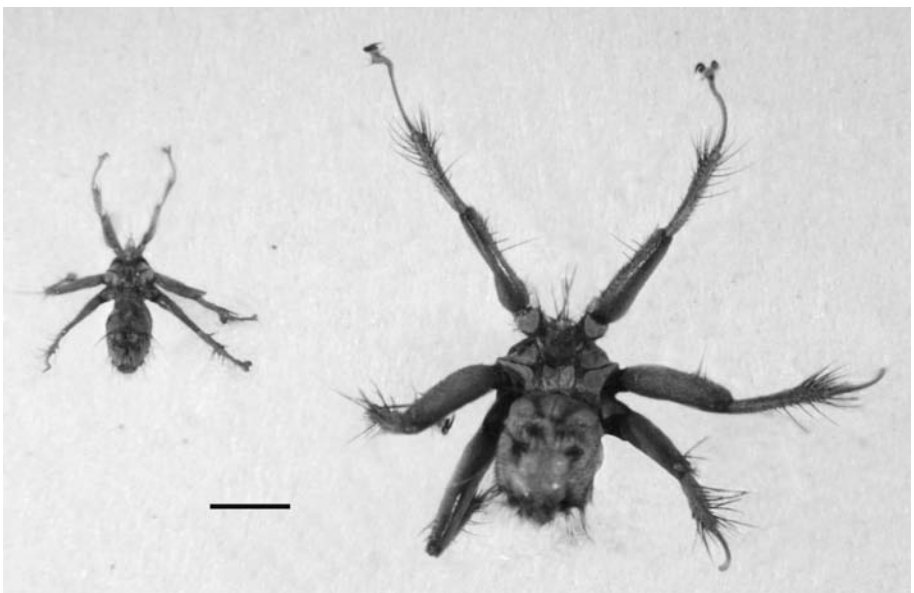


Figure 1. Male of *Nycteribia (Nycteribia) schmidlii* (left) and *Penicillidia dufourii* (right). Scale bar: 2 mm.

and *N. (N) schmidlii*] whereas the second one with only one species: *N. (A). vexata*.

The species of genus *Penicillidia* Kolenati, 1863, are big flies characterized by "unilobated" eyes, that in the dry specimens appear white. The genus is present in the Palearctic region with 4 species *P. conspicua* Speicer, 1901, *P. dufourii* (Westwood, 1835), *P. monoceros* Speiser, 1900 reported from Europe while *P. jenynsii* (Westwood, 1834) is reported from China, Taiwan and doubtfully from Japan (Soós and Hurka, 1986). Two species, *P. conspicua* and *P. dufourii*, are reported from Italy (Lanza, 1999; Vanin and Vernier, 2005).

Taxonomic account

Nycteribia (Nycteribia) latreillii (Leach, 1817)

Distribution: This species occurs from South Western Asia (Kazakistan, Kirghizistan, Asia) to Continental Europe and Northern Africa. In Italy it is reported from Abruzzo, Sicilia, Sardegna (Lanza, 1999).

Host: This species is reported specially on *Myotis myotis* and *M. blythii*. Moreover it was collected surely also from *Myotis capaccinii*, *M. emarginatus*, *Eptesicus serotinus*, *Miniopterus schreibersii*, *Rhinolophus euryale*, *R. ferrumequinum*, and *R. hipossideros* (Theodor, 1975; Lanza, 1999). Other dubious findings are not reported in this paper.

Nycteribia (Nycteribia) schmidlii Schiner, 1853

Distribution: This species shows the same distribution of the previous species, living from South Western Asia to Continental Europe and Northern Africa. In Italy it is reported from Lombardia, Trentino, Veneto, Emilia-Romagna, Toscana, Abruzzo, Lazio, Campania, Sardegna (Lanza, 1999).

Host: This species is reported specially on *Miniopterus schreibersii*, but it was found also on other cave bats (*M. blythii*, *M capaccinii*, *M. emarginatus*, *Myotis myotis*, *Rhinolophus euryale*, *R. ferrumequinum*, *R. hipossideros,* and *R. mehely*) and from bats living in crevice and tree clefts (*Barbastella barbastellus*, *Myotis bechsteinii*, *M. daubentonii*, *M. mystacinus*, *Plecotus auritus,* and *P. austriacus*) (Theodor, 1975; Lanza, 1999).

Penicillidia dufourii (Westwood, 1834)

Distribution: Species known from Europe, Central Asia and Northern Africa (Falcoz, 1926; Soós and Hůrka, 1986). In Italy individuals of this species are reported from Liguria, Piemonte, Lombardia, Trentino, Veneto (Table 1), Emilia, Toscana, Lazio, Puglia, Sicilia, Sardegna (Lanza, 1999).

Host: This species is reported on *Rhinolophus euryale* Blasius, 1853; *R. blasii* Peters, 1866; *R. hipposideros* (Bechstein, 1800); *R. ferrumequinum* (Schrebers, 1774); *Myotis capaccinii* (Bonaparte, 1837); *M. myotis* (Borkhausen, 1797); *M. blythii* (Tomes, 1857), *M. daubentonii*, *M. emarginatus*, *M. myotis*, and *Miniopterus schreibersii* (Falcoz, 1926; Lanza, 1999; Theodor, 1975).

Discussion and conclusion

The data, presented in this contribute, allow to count 6 species of Nycteribidae from North-Eastern Italy being *Nycteribia latreillii* a new record for this area. This species has been collected on bats belonging to the main colony of the studied cave, composed by *Myotis myotis* and *Miniopterus schreibersii.* This finding confirms the previous data about the hosts of this species. These two bat species, *M. myotis* and *M. schreibersii,* are typical cave-dwelling bats, and lives in caves also in large plurispecific colonies (Vernier, 1997). This fact allows the passage of the parasite from a species to an other. A same situation occurs also for the second fly species collected, *Nycteribia schmidlii,* that has been reported on *Miniopterus schreibersii* but also on other cave bats. It is worth mentioning that the caves "Grotta della Guerra" (127 V/VI) and "Grotta La Bislonga" (1001 V/TV) have a similar morphology and a comparable bat community (Vernier, 1977; 1998). In both these cave *Penicillidia dufourii* was collected.

The records of only 6 species is far to be exhaustive of the real composition of the Nycteribidae fauna in the Venetian region that shows an elevate heterogeneity of habitat and a high diversity of bats species. In the Venetian region 25 species of bats were reported (Bon *et al*., 1996) [the total number of Nycteribidae in the Italian territory is nine (Lanza, 1999), and the number of bats species is 30 (Vernier, 1997)].

The significant reduction in bat richness and abundance occurred in the last years, both for human and natural causes, in "La Bislonga" cave, as well as in other bat roosts, has direct effect also in their parasite and in general on the biodiversity. The loss of species, independently from their ecological role (predator, parasite, saprophage, etc.) has consequences on the biodiversity not only in a mathematical count as reduction in the species richness but also on the relationships within species with unpredictable consequences on the whole ecosystem. "The unpredictability of ecosystems is consequence of the particularity of the species that compose them. Each species is an entity with a unique evolutionary history and set of genes, and so each species responds to the rest of the community in a special way" (Wilson, 1992).

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Presence of renal disease in dogs with patent leishmaniasis

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Abstract. The aim of this study is to retrospectively evaluate the presence of renal disease in 116 dogs suffering from leishmaniasis and the evolution of renal function during a follow up of six months after the diagnosis. Renal disease was assessed based on three clinical criteria [urinary specific gravity (USG), urine protein-creatinine ratio (UP/C) and serum creatinine (SCr)]. According to these criteria, dogs were allocated in group A, B or C. Group A includes dogs without renal disease, group B includes non hyperazotemic dogs with USG <1025 and/or UP/C>0.5 and group C includes dogs with hyperazotemia (SCr≥1.4 mg/dL). Some degree of renal alteration was present in 46/116 (39.6%) of dogs at the time of diagnosis of leishmaniasis. During the follow up, dogs with leishmaniasis and renal disease without uremic signs had a long survival time and a partial recovery of renal function after specific treatment. On the other hand, dogs with leishmaniasis, renal failure and uremic signs had poor prognosis and it was the main cause of death. SCr, UP/C and USG, as easily assayed methods in practice, could offer the appropriate information to perform an early recognition of renal dysfunction in dogs with patent leishmaniasis.

Key words: dogs, leishmaniasis, proteinuria, hyperazotemia, urine specific gravity.

Canine leishmaniasis (CL) is a prevalent disease in the Mediterranean basin, with infection rates as high as 67% (Solano *et al.,* 2001). CL is manifested with a wide range of clinical signs involving several organs, especially the kidneys. According to previous reports, inmmune complex glomerulonephritis and tubulointerstitial nephritis have been described as the main causes of proteinuria, chronic renal failure and death of affected dogs (Slappendel and Ferrer, 1998; Koutinas *et al.,* 1999; Zatelli *et al.,* 2003). Several studies documented a variable prevalence of renal disease, which ranges from 16 to 52% of dogs naturally infected with *Leishmania* (Ciaramella *et al.,* 1997; Font, 1999; Cortadellas *et al.,* 2006). Moreover, histopathologic evidence of nephropathy has been described in 100% of dogs with leishmaniasis (Costa *et al.,* 2003). Infected dogs can initially suffer from a moderate to severe proteinuria in the absence of hyperazotemia. As glomerular disease progresses, tubulointerstitial lesions, hyperazotemia and end-stage renal failure may appear (López *et al.,* 1996; Zatelli and Bonfanti, 2004). Due to the severe nature of renal damage and the elevated percentage of renal disease in dogs with leishmaniasis, it is important to perform an early recognition of kidney dysfunction. Diagnostic methods to evaluate renal function include clearance methods to evaluate GFR, serum creatinine levels, UP/C ratio and urine concentrating ability (USG).

Abbreviations: **SCr**: serum creatinine; **UP/C**: urine protein/creatinine ratio; **USG**: urine specific gravity; **GFR**: glomerular filtration rate; **CL**: canine leishmaniasis.

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Urinary clearance of inulin is a cumbersome method to evaluate GFR but requires an accurate collection of timed urine samples (Finco, 2005). Other clearance methods, such as plasma exogenous creatinine clearance, is a reliable indicator of GFR in dogs and an interesting method to use in the future but need to be more evaluated for its routine application in clinical practice (Cortadellas *et al.,* 2008).

The aim of this study is to retrospectively evaluate the presence and evolution, during a follow up of six months, of renal disease in dogs naturally infected by *L. infantum* using methods easily assayed in practice, such as SCr, UP/C and USG.

Material and methods

The medical records of 116 dogs with patent leishmaniasis and admitted to the Veterinary Teaching Hospital-UAB between 2003 and 2007 were retrospectively reviewed. CL diagnosis was confirmed by direct observation of the parasite in hematoxilin-eosin stained aspiration smears taken from lymph nodes or bone marrow and detection of anti-*Leishmania* antibodies using an ELISA test. According the ELISA technique described by Riera et al (1999), dogs with a positive titre were those with an antibody percentage superior to 21%. Dogs with confirmed diagnosis of leishmaniasis but presenting signs of other systemic disease were excluded from the study.

Information evaluated included historical and physical examination findings, CBC, serum biochemistry results, urine protein/creatinine ratio (UP/C), and complete urinalysis.

Dogs were allocated into 3 groups on the basis of the results of UP/C, USG and SCr, according to IRIS classification of chronic renal failure. Group A included dogs without renal disease (UP/C<0.5,

Table 1. Mean, median, range and limit values of SCr, UP/C and USG of dogs from group A, B and C.

	Group A	Group B			Group C		
		mean	median	range	mean	median	range
SCr (mg/dL)	< 1.4	0.97	1	0.67-1.4	3.67	2.82	1.6-7.55
UP/C	< 0.5	2.47	1.15	0.22-11.8	6.8	2.1	0.4-22
USG	> 1025	1021	1020	1005-1040	1019	1020	1014-1025

USG >1025 and SCr<1.4 mg/dL). Group B included dogs without hyperazotemia but with proteinuria (UP/C≥0.5) and/or inadequate concentrating ability (USG<1025) without identifiable non-renal cause. Group C included dogs with hyperazotemia (SCr≥1.4 mg/dL).

A six month follow up period was obtained in 87/116 dogs. Renal function response to treatment was categorized in three groups: improvement of renal function (reduction in UP/C with stable or reduced SCr), stable renal function (stable UP/C and SCr) and progressive renal disease (increase in UP/C and/or increase in SCr or euthanasia due to renal failure).

All dogs included in the study were treated with antimoniate meglumine (100 mg/kg SC q 24 h for 30 days) and allopurinol (10 mg/kg PO q12 h minimum six months). Dogs included in group B and C were also treated with renal prescription diet and angiotensin converting enzyme inhibitor (ACEI) when UP/C was >2. Some dogs included in group C also needed intensive care treatment (fluid therapy, anti-H2, antiemetic).

Serum samples were stored at –20ºC until used for biochemical analysis and the detection and quantification of *L. infantum* specific antibodies (ELISA) (Riera *et al.,* 1999). Urine was collected by sterile urethral catheterization or cystocentesis, and a complete urinalysis was performed (USG, dipstick chemistry, sediment examination and UP/C). Inactive urinary sediment was a prerequisite to evaluate UP/C in urine. Protein and creatinine were measured in urine by PRM method using Urinary/CSF Protein Olympus® and Jaffé method, respectively, both performed in the Olympus AU400 analyzer. Bacterial culture was done in all urine samples with low USG (<1025).

Basic descriptive statistics (mean and median) were performed using Microsoft Excel.

Results

Of the 116 dogs included in this study, 26 (22.4%) were males and 90 (77.6%) were females. Eighty nine (76.7%) were purebreds and 27 (23.3%) were crossbreds. The most frequently breeds represented were Boxer (n=14), German shepherd (n=12), Rottweiler (n=7), Husky (n=7), Labrador (n=5) and Cocker Spaniel (n=5).The age of the dogs ranged from 1 to 14 years, and body weight ranged from 4 to 60 kilograms. Mean, median and range values of SCr, UP/C and USG are described in Table 1.

Seventy out of 116 (60.35 %) dogs were classified in group A, 25/116 (21.55%) dogs were classified in group B and 21/116 (18.1%) dogs were classified in group C. At the time of diagnosis a total of 46/116 (39.65%) dogs with leishmaniasis presented some evidence of renal involvement (group B and C). From these dogs an 84.7 % (39/46) presented proteinuria (UP/C>0.5) and were considered to suffer from glomerular disease and a 45.6% (21/46) suffered from hyperazotemia. In group C, 10 out of 21 dogs had uremic signs at the moment of diagnosis and were treated with intensive therapy.

No correlation was found between ELISA titres and renal function at the moment of the diagnosis and at six month's follow up.

Renal function response to treatment was evaluated by measurement of USG, UP/C and SCr six months after diagnosis in 53 out of 70 dogs of group A, 16 out of 25 dogs of group B and 18 out of 21 dogs of group C. Only one out of 53 dogs included in group A (53/70) developed renal disease six months after diagnosis of leishmaniasis. According to the evolution of renal function in dogs from group B (16/25), seven dogs were categorised as dogs with improvement of renal function, four dogs as stable renal function and five dogs as progressive renal disease (three dogs had an increased UP/C and two dogs finally developed hyperazotemia). After intensive treatment, nine out of 18 dogs from group C presented progression of renal disease and died in less than two weeks. Four dogs maintained stable renal failure and five dogs presented improvement of renal disease six months after diagnosis. From these five dogs, two showed a complete resolution of hyperazotemia whereas three dogs had a reduction in their creatinine value (Table 2).

Table 2. Evolution of renal function from each group of dogs included in the study in a six months period after diagnosis of leishmaniasis. RF (renal function); RD (renal disease).

	Dogs with follow up	Improvement RF	Stable RF	Progressive RD
Group A (n=70)	53	–	52	1
Group B (n=25)	16	7	4	5
Group C (n=21)	18	5	4	9

Discussion

This study was designed to perform a retrospective evaluation of the prevalence of renal disease at diagnosis and after 6 months of follow up in dogs suffering from leishmaniasis.

There are different studies of CL that evaluate the prevalence of renal disease based in the presence of hyperazotemia and/or proteinuria, and reported results varied from 16 to 52% (Ciaramella *et al.,* 1997; Font, 1999; Koutinas *et al.,* 1999; Cortadellas *et al.,* 2006). The present study reported a prevalence of 39.65%, included in the same range of previous publications, using three useful, simple, rapid and inexpensive procedures (USG, UP/C and serum creatinine) to evaluate renal function in veterinary practice.

Dogs with SCr greater than 1.4 mg/dL with a correct hydration status were considered hyperazotemic-based in the International Renal Interest Society (IRIS) staging scheme of canine chronic kidney disease. The prevalence of hyperazotemia obtained was 18.1% (25/116), very similar to the 16% described by Ciaramella *et al.* (1997) although their inclusion criteria were different (SCr<1.8 mg/dL).

According to the consensus statement of proteinuria (Lees *et al*., 2005), dogs with a UP/C>0.5 without signs of haemorrhage or inflammation in the urinary sediment were considered proteinuric. Glomerular disease without hyperazotemia was present in 18.9% (22/116) of dogs naturally infected with leishmaniasis. Previous studies reported similar results, 18.1% (Font, 1999) and 16.2% (Cortadellas *et al.,* 2006). Costa *et al.* (2003) reported that all dogs with naturally acquired leishmaniasis, with or without laboratorial findings of renal disease, have some degree of glomerular lesion on histopathology. As UP/C evaluation may not be enough sensitive to detect all levels and types of proteinuria, the present study can not completely exclude that the hyperazotemic dogs with UP/C in the normal range had mild glomerular lesions. Although to better define renal lesions histopathology is required, determination of microalbuminuria or a qualitative study of urinary protein could be helpful to detect an initial glomerular lesion in dogs with leishmaniasis (Zatelli *et al.,* 2003; Roura *et al.,* 2005).

There is limited data about evaluation of USG in dogs suffering from CL. USG value of 1.025 or higher has been considered an evidence of adequate renal concentrating ability (Osborne *et al.,* 1972). In this study USG <1025 in dogs with leishmaniasis without any sign of other systemic diseases and normal urinary sediment was considered suggestive of abnormal renal function. To confirm that low USG is due to renal lesion, all other causes of poliuria-polidipsia should be ruled out. Due to the retrospective nature of our study, endocrine test had not been done in all cases, but clinical and laboratorial signs and negative urine culture were not suggestive of other diseases but CL.

Those results could suggest that low USG was associated with an initial renal lesion. However, other methods of GFR estimation and renal histopathology should have been evaluated in those dogs in order to confirm a renal lesion. Low USG as the unique renal abnormality is not common in dogs suffering leishmaniasis because the most frequent initial renal lesion is glomerular although tubulointerstitial lesions may also be present (Nieto *et al.,* 1992; Koutinas *et al* 1999). Therefore, USG may add more information to renal function in dogs with leishmaniasis, mainly when there is a slight glomerular lesion (UPC<0.5) without hyperazotemia.

Follow up results showed that dogs with leishmaniasis suffering from renal disease without uremic signs can have a long survival time and a partial recovery of renal function after specific treatment against *Leishmania* and symptomatic treatment for renal disease. Only one dog without renal involvement at the moment of diagnosis developed renal disease in a 6 months period. Those results may point to the fact that dogs without renal disease at the moment of diagnosis of leishmaniasis have better prognosis and a low percentage of them develop renal disease. On the other hand dogs with renal failure and uremic signs secondary to leishmaniasis have poor prognosis and is the main cause of death. These results coincide with a report from Plevraki *et al.* (2006) that described that most dogs suffering from glomerular disease due to leishmaniasis under specific treatment did not experience further deterioration of the renal function and had even a decrease or disappearance of proteinuria.

There were several limitations in the study presented here. It was a retrospective study and accuracy of the data relies on case records being reliably maintained. Additional UP/C, serum creatinine and USG values in two weeks apart would have contributed to confirm the results obtained. Also the follow up period was relatively short to evaluate the progression of the disease.

The present study shows that CL is associated with an elevated prevalence of renal disease and highlights the importance of performing an early diagnosis. Easy diagnostic tests such as UP/C, USG and serum creatinine should be considered as important part of clinical examination in dogs with leishmaniasis at the moment of diagnosis and during follow-up.

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Thaparocleidus siluri, monogenoidean parasite of *Silurus glanis*: a new record from Italy

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Abstract. During spring 2006 several specimens of *Silurus glanis* from Comabbio Lake (Italy) were screened for monogenoideans, revealing the presence of the two congeneric *Thaparocleidus vistulensis* (Siwak, 1932) Lim, 1996 and *T. siluri* (Zandt, 1924) Lim, 1996. This is the first Italian record for the latter.

Key words: alien species, invasive species, enemy release, biodiversity.

Silurus glanis is a Siluriformes native of East Europe where it is widely diffuse, especially in the danubian basin. However, through reiterate introductions, it has now reached a widespread distribution also in western Europe. It is a large predator feeding mainly on fish, crayfish and ducks, and it is considered an invasive species, capable to induce heavy modifications in the aquatic ecosystems of introduction. As regarding for Italian freshwater, it has been probably introduced in 1956, and had already reached a common distribution in the Po river basin since the 80s (Manfredi, 1957; Piccinini and Pattini, 1996).

Similar situations of ecological success are quite common when considering introduced species, and are usually explained as a consequence of three main factors: (i) better environmental attributes of the new habitat, (ii) fewer/poorer competitors, and (iii) paucity of natural enemies (predators and parasites).

Considering in particular parasites, they are usually lost during larval migration, or as a consequence of colonization by a limited number of adult individuals, which reduces the probability of introducing parasitized hosts and creates a starting population characterised by low density, and so not favourable to the spread of a directly-transmitted infective agent (Torchin *et al*., 2002).

Loss of native parasites in introduced species has been significantly demonstrated both for fish and other living taxa, in terms of number of parasite species per host and prevalence, while the acquisition of local parasites by alien host species is episodic, and requires long time to became a stable symbiotic relationship (Goren and Galil, 2005).

Among the three monogenoidean species known to parasitize *S. glanis* in its native areal (*Thaparocleidus vistulensis* (Siwak, 1932) Lim, 1996, *T. siluri* (Zandt, 1924) Lim, 1996 and *T. magnus* (Bychowsky and Nagibina, 1957) Lim, 1996, just one (*T. vistulensis*) has been recorded also from Italian freshwater (Lim *et al.*, 2001; Gussev, 1985; Galli *et al*., 2003).

In this work we provide new contributions to this knowledge, in order to make clear if the lack of parasite species records from *S. glanis* is simply due to poorness of information, or if it effectively subsists as a consequence of native parasites lost connected to the introduction processes.

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Material and methods

During spring 2006, eleven specimens of *S. glanis* were collected from Comabbio Lake, Varese, Italy (45° 45′ 52″ N-8° 41′ 27″ E). Fish were taken using professional nets.

Gill baskets were removed at the site of collection and placed in containers of hot (50°C) 5% formalin to relax and fix the attached monogenoideans. Specimens were subsequently stained with Gomori's Trichrome (Kritsky *et al.*, 1978) and mounted in Euparal. This procedure furnished relaxed and well-preserved specimens for taxonomic purposes. Some specimens were mounted with ammonium picrate and glycerine (Malmberg, 1957) for study of sclerotyzed parts. Morphological and morphometrical analysis of parasites was performed under a differential interference contrast compound microscope model Zeiss 25 (objectivies: 20x, 40x and 100x oil immersion). Drawings were made with the aid of a drawing tube. Taxonomical identification was based on Gussev (1985).

To obtain three-dimensional reconstruction of the sclerotized parts of haptor, male reproductive organs and of the entire body, some specimens were prepared according to Galli *et al*. 2006, and processed under a laser scanning confocal fluorescence microscope (LSCFM) Leica TCS SP2 coupled to an inverted Leica DMIRE2 microscope equipped with a PL APO 63X oil immersion objective (NA = 1.4). The sample was excited with the argon laser at 515 nm and fluorescence emission was collected through a band-pass filter between 525 nm and 730 nm. Images (8-bit) with 1024×1024 pixels per frame were obtained. Z-series were collected with a step size of 0.115 nm to maximize axial resolution of 3-D images.

Results

Parasite prevalence on examined hosts was of 100%. The totality of parasites recovered from gills belong to two different species, *Thaparocleidus vistulensis*

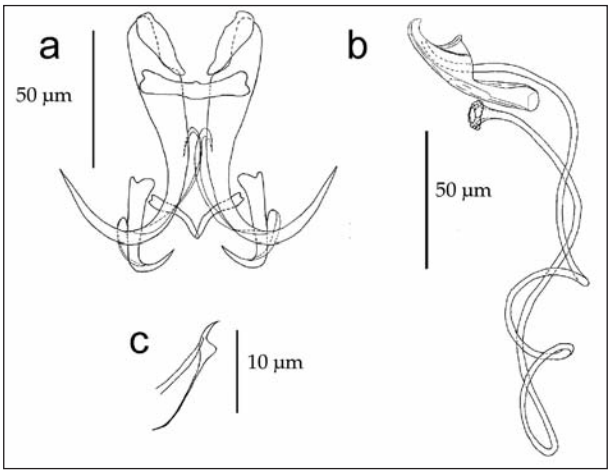


Figure 1. Sclerotised parts of *Thaparocleidus siluri*. a: haptor; b: male reproductive organs; c: marginal hooks.

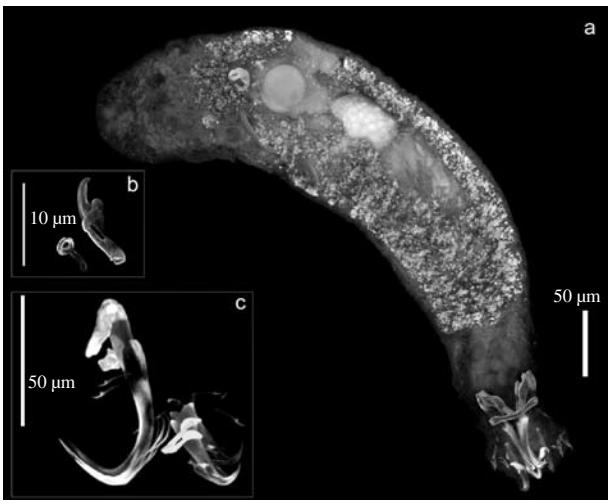


Figure 2. Tridimensional reconstructions of *Thaparocleidus siluri* made by confocal microscope. a: body; b: particulars of male reproductive organs; c: haptor.

and *T. siluri*. *T. siluri* is a new locality record for Italy. Measurements resulting from morphometrical analysis of 20 specimens of *T. siluri* are here resumed (in micron): body: 550±110.3; ventral anchor length: 92.2±3.7; ventral anchor accessory piece: 24.0±2.3; dorsal anchor length: 39.4±2.8; ventral bar: 43.7±2.9; dorsal bar: 31.8±2.2; hooks: 15±0.9.

Drawings of its haptorial and copulative sclerites are shown in Figure 1, while their LSCFM three dimensional reconstruction, and that of the entire parasite body, are reported in Figure 2.

Discussion

Italian freshwater fish communities are in rapid evolution as a consequence of the introduction of alien species. Considering an alien fish as a potential threat to native fish fauna in terms of competition is correct, but does not represent a complete view on the problem. Further than a competitor, an alien fish should be considered as a potential Trojan horse, as moving it may also mean moving its parasites. This has been confirmed for the monogenoideans hosted by *Silurus glanis*. Among the three monogenoid species known to parasitize *S.glanis* in its native countries (*Thaparocleidus vistulensis*, *T. siluri* and *T. magnus*), just two of them (*T. vistulensis* and *T. siluri*), have taken part in the translocation process up to this moment.

T. siluri is then added to the extant number of Italian alien species, demonstrating once more what already stated by Galli *et al*. (2005): studying introduction of organisms without considering their related parasitofauna leads inevitably to an underestimation of their contribute to the new habitats biodiversity.

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Ex-vivo effects of methanol extracts of *Chenopodium ambrosioides* and *Mallotus philippinensis* on some phosphatases of *Stilesia* species

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Abstract. *Ex-vivo* effects of methanol extracts of leaves of *Chenopodium ambrosioides* and fruit powder of *Mallotus philippinensis* were tested against some phosphatases – alkaline phosphatase (ALP), acid phosphatase (ACP) and adenosine triphosphatase (ATPase) – of *Stilesia* sp. Activity of ALP, ACP and ATPase were found to be 0.228±0.005, 0.064±0.003 and 0.22±0.006 respectively. The methanol extract of *M. philippinensis* decreased the activity of ALP, ACP and ATPase by 52.77%, 82.81% and 70.35% respectively. On the other hand, methanol extracts of *C. ambrosioides* decreased the activity of these enzymes by 43.05%, 75% and 77% respectively. Both the extracts need further investigation for their anthelmintic activity.

Key words: anthelmintic, *Chenopodium ambrosioides, Mallotus philippinensis.*

India has largest livestock population in the world which contributes nearly 7% towards its national income. Helminthiasis is one of the world's most prevalent and economically important parasitosis of domestic animals. It is estimated that more than 300 species of helminths, including tapeworms, parasitize livestock in India. A number of benzimidazole drugs e.g., albendazole, cambendazole and mebendazole have been successfully used to control tapeworms (Meleney, 1982). Studies have shown, however, that with the passage of time the helminths develop resistance towards these chemical anthelmintics. Due to their high cost and tendency to delay or interfere with natural host immune mechanism and due to the problem of anthelmintic resistance, use of chemical anthelmintics may not be most desirable method of managing helminths. Several herbal extracts have been found to have antibacterial, anti-inflammatory, ascaricidal and anthelmintic activity. In this paper the effect of methanol extracts of leaves of *Chenopodium ambrosioides* and fruit powder of *Mallotus philippinensis* on selected phosphatases of *Stilesia* sp. namely alkaline phosphatase (ALP), acid phosphatase (ACP) and adenosine triphosphatase (ATPase) is being described.

Materials and methods

Live specimens of the tapeworm *Stilesia* sp. were collected from the small intestine of sheep at the Chandigarh slaughter house. One g of blot dried cestodes was taken in 5 ml each of citrate buffer for ALP and ACP and of 0.25 M sucrose for ATPase and homogenized using an electric homogenizer. Homogenate was then centrifuge at 179 g for 30 minutes. The pallet was discarded and the supernatant was used for the biochemical investigations.

The leaves of *Chenopodium ambrosioides* and fruit powder (present on the surface of fruit) of *Mallotus philippinensis* were shade dried and extracted in methanol using Soxhlet extractor. The extract was filtered and the filtrate evaporated at 40°C. The semisolid mass left was used for the studies. Distilled water was used for dilution and in the controls.

Activities of the enzyme have been calculated and expressed as micromole/min per mg of proteins. The amount of protein was estimated by Lowry's method (1951). Acid phosphatase (ACP), alkaline phosphatase (ALP) and adenosine triphosphatase (ATPase) were estimated by the method of Bergmeyer (1963) and Kieley (1972).

Results and discussion

In the present study, the effect of methanol extracts of the leaves of *C. ambrosioides* and fruit powder of *M. philippinensis* on the phosphatases (ACP, ALP and ATPase) of *Stilesia* sp. has been studied.

All the three phosphatases were found to be present in the parasite studied. The presence of enzymes ACP, ALP and ATPase has been detected both histochemically and biochemically in a number of helminth parasites. These enzymes have been associated with tegument, sub tegument and somatic musculature (Kwak and Kim, 1996; Buchman, 1998). However, Fischer and Starling (1975) had reported ACP but no ALP activity in the cuticular layer (*sic*) and absorptive surface of several species of cestodes.

Specific activity of ALP in the homogenate of *Stilesia* sp. was found to be 0.288±0.005 which was inhibited by the addition of methanol extract of fruit powder of *C. ambrosioide*s. As we increased the volume of the extract added (from 0.2 ml to 1.0 ml)

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Table 1. The effect of methanol extracts of leaves powder of *Chenopodium ambrosioides* and fruit powder of *Mallotus philippinensis* on the specific activity (activity/mg of protein) of alkaline phosphatase (ALP), acid phosphatase (ACP) and adenosine triphosphatase (ATPase) of *Stilesia* sp.

Enzyme	ALP				ACP				ATPase			
Vol. of extract (in ml)	*Chenopodium ambrosioides*		*Mallotus philippinensis*		*Chenopodium ambrosioides*		*Mallotus philippinensis*		*Chenopodium ambrosioides*		*Mallotus philippinensis*	
	Specific activity	% Change	Specific activity	% Change	Specific activity	% Change	Specific activity	% Change	Specific activity	% Change	Specific activity	% Change
Control	0.288±0.005		0.288±0.011		0.064±0.003		0.064±0.003		0.22±0.006		0.22±0.006	
0.2	0.239±0.005*	17.01	0.208±0.011	27.77	0.036±0.003$	39.06	0.030±0.004	53.12	0.182±0.007*	19.46	0.154±0.009	31.85
0.4	0.210±0.006#	27.08	0.190±0.008	34.02	0.0.33±0.001*	48.43	0.023±0.002	64.06	0.146±0.006	35.39	0.138±0.005	38.93
0.6	0.206±0.015#	28.40	0.177±0.005	38.54	0.031±0.001*	51.56	0.019±0.003	70.31	0.124±0.005	46.90	0.127±0.002	43.80
0.8	0.179±0.005$	37.84	0.165±0.008	42.70	0.023±0.002*	64.06	0.012±0.003	81.25	0.079±0.006	65.04	0.089±0.008	60.60
1.0	0.164±0.009*	43.05	0.136±0.006	52.77	0.016±0.003$	75.00	0.011±0.002	82.81	0.051±0.009$	77.43	0.067±0.007	70.35

p value = Specific activity of ALP, ACP and ATPase of *Stilesia* sp. for *Chenopodium ambrosioides* vs *Mallotus philippinensis*; * = p < 0.001; # = p < 0.01; $ = p < 0.05.

the specific activity of ALP decreased from 0.222±0.005 to 0.164±0.009. The decrease in specific activity of ALP with the increasing volume of herbal extract added was gradual (Table 1; Fig. 1). When methanol extract of *M. philippinensis* was added, the specific activity of ALP decreased from 0.208±0.011 to 0.136±0.006 as the volume of extract added was increased from 0.2 ml to 1.0 ml (Table 1; Fig. 1).

Specific activity of ACP was found to be 0.064±0.003 (Table 1). Methanol extract of *C. ambrosioides* decreased the specific activity of ACP from 0.064±0.003 to 0.016±0.003 as the volume of herbal extract added was increased from 0.2 ml to 1.0 ml. There was a steady decrease in specific activity of ACP (Table 1; Fig. 2). Methanol extract of *M. philippinensis* decreased the specific activity of ACP from 0.064±0.003 to 0.011±0.002 as the volume of herbal extract was increased from 0.2 ml to 1.0 ml. There was a steady decrease in specific activity (Table 1; Fig. 2).

The specific activity of ATPase decreased from 0.22±0.006 to 0.051±0.009 with increasing volume of extract of *C. ambrosioides* from 0.2 ml to 1.0 ml (Table 1; Fig 3). The methanol extract of *M. philippinensis* decreased the specific activity of ATPase from 0.22±0.006 to 0.067±0.012 activity per mg of protein as the volume of extract added was increased from 0.2 ml to 1.0 ml (Table 1; Fig 3).

On comparing the *ex-vivo* effect of the two plant extracts, it has been found that both of them inhib-

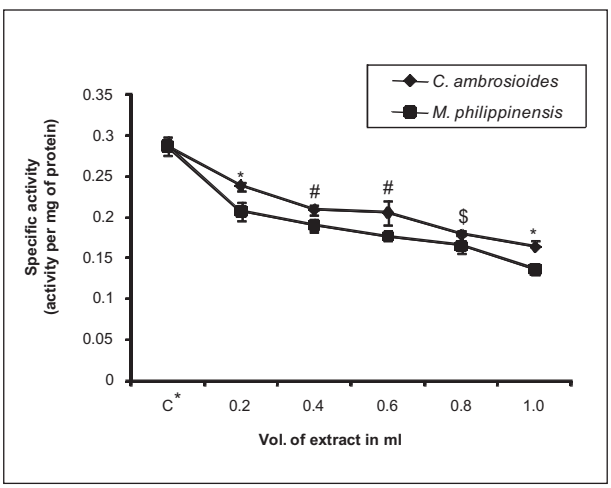


Figure 1. Effect of methanol extracts of *Chenopodium ambrosioides* and *Mallotus philippinensis* on the ALP of *Stilesia* sp. C* = Control (pure solvent); p value = Specific activity of ALP of *Stilesia* sp. for *Chenopodium ambrosioides* vs *Mallotus philippinensis*; * = p < 0.001; # = p < 0.01; $ = p < 0.05.

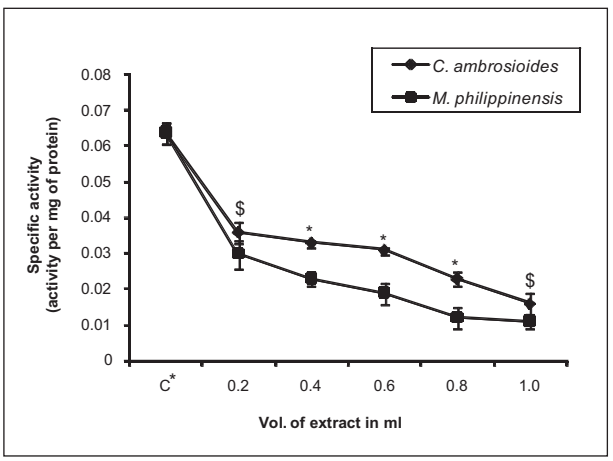


Figure 2. Effect of methanol extracts of *Chenopodium ambrosioides* and *Mallotus philippinensis* on the ACP of *Stilesia* sp. C* = Control (pure solvent); p value = Specific activity of ACP of *Stilesia* sp. for *Chenopodium ambrosioides* vs *Mallotus philippinensis;* * = p < 0.001; # = p < 0.01; $ = p < 0.05.

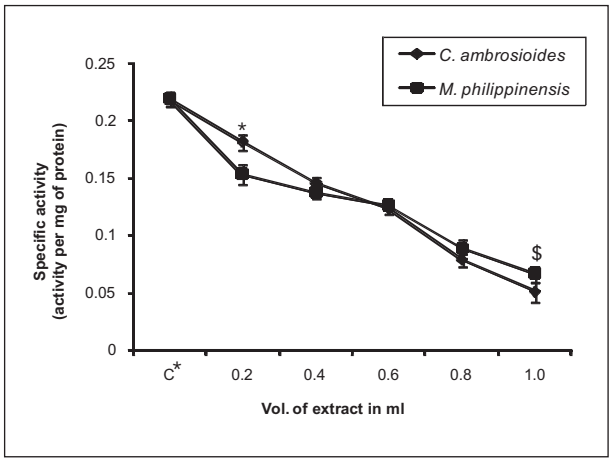


Figure 3. Effect of methanol extracts of *Chenopodium ambrosioides* and *Mallotus philippinensis* on the ATPase of *Stilesia* sp. C* = Control (pure solvent); p value = Specific activity of ATPase of *Stilesia* sp. for *Chenopodium ambrosioides* vs *Mallotus philippinensis*; * = $p < 0.001$; # = $p < 0.01$; $ = $p < 0.05$.

it the phosphatases which are important enzymes for carbohydrate metabolism of the parasite. Although both the extracts have shown similar effect on these enzymes, the extract of *M. philippinensis* is little more effective than that of *C. ambrosioides* on ACP and ALP, whereas the extract of *C. ambrosioides* is slightly more inhibitory for ATPase than that of *M. philippinensis.*

Therefore, it can be concluded that both the extracts are good candidates for their evaluation as anthelmintic substances and should be further investigated.

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Miasi cutanea umana da *Oestrus ovis*: segnalazione di un caso in Piemonte (Diptera, Oestridae)

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Abstract. *Human cutaneous myiasis by* Oestrus ovis*: a case recorded in Piedmont (Diptera, Oestridae).* It is recorded a case of cutaneous myiasis in a 46 years old man breeder of oxen caused by *Oestrus ovis* (Diptera, Oestridae) who had an infestation in a ragged wound.

Key words: *Oestrus ovis*, human myiasis, North Italy, Oestridae.

Oestrus ovis è un dittero brachicero appartenente alla famiglia degli Oestridae. Gli adulti hanno un aspetto tozzo tipico di un moscone con tegumenti grigio-giallastri con maculatura di tonalità più scura. Sull'addome le chiazze scure sono più numerose ed estese in alternanza a pollinosità grigio-biancastra. Zampe giallastre, ali trasparenti con aree brune in prossimità dell'attaccatura al torace. Ambedue i sessi allo stadio adulto non si nutrono per via dell'apparato boccale atrofico. Le femmine depongono, generalmente in volo, centinia di larve (L1) in corrispondenza delle narici, degli occhi e, più raramente, in prossimità dell'apertura boccale di ovini e caprini, che rapprsentano gli ospiti tipici della specie (Tremblay, 1997).

Le larve migrano immediatamente verso i seni etmoidali e frontali, dove si nutrono di muco e di tessuti epiteliali (Bolchi Serini *et al*., 2000; Jacquiet *et al*., 2002). Le larve di terza età misurano all'incirca 2,5-3 cm e presentano i tegumenti giallo-scuri; quando esse raggiungono la maturità si lasciano espellere dai frequenti starnuti degli animali. La metamorfosi in pupa avviene nel terreno e richiede da 30 a 60 giorni o anche di più a seconda delle condizioni termo-igrometriche ambientali.

Il bestiame infestato, generalmente ovino o caprino, presenta emissioni purulente dal naso, scolo, difficoltà respiratoria e vertigini. *Oestrus ovis* (Linné) può infestare anche il cane (Lujan *et al*., 1998; Bolchi Serini *et al*., 2000) specialmente quando è a stretto contatto con il gregge (es.: cani da pastore).

Nell'uomo la specie può determinare rinomiasi (Pampiglione *et al*., 1991; Petrarca *et al*., 2004), oftalmomiasi (Chandra *et al*., 1981; Bolchi Serini *et al*., 2000; Crotti *et al*., 2004) e miasi orali (Hakimi *et al*., 2002), mentre non sono noti casi di infestazioni di ferite o comunque di sviluppo a carico di tessuti necrotici.

Caso clinico

A luglio del 2006 un uomo di 46 anni, allevatore di bovini di professione, in ottime condizioni di salute, consegna all'entomologo dell'Ospedale di Cuneo delle larve raccolte, all'atto della medicazione, da una ferita lacero-contusa al fine di comprendere di cosa si trattasse e dell'implicazione clinica della presenza parassitaria.

La ferita, verificatasi da 12 giorni circa, interessa il polpaccio destro e consiste in un taglio poco profondo a bordi slabbrati lungo circa 15 cm e suppurante ma senza tessuti necrotici. La lesione viene immediatamente automedicata dal soggetto e nei primi giorni viene protetta da bendaggio, che poi, per facilitare la guarigione, viene rimosso e la ferita viene medicata solo di sera con soluzioni a base di sali quaternari di ammonio. Il soggetto sottolinea che in tutto il periodo da quando si è procurato la ferita a quando è ricorso alla visita medica ha continuato la sua attività lavorativa in continuo contatto con gli animali.

Il materiale, portato in vita all'interno di un'albarella, consiste in 6 larve di dittero lunghe all'incirca 10 mm e 3 larve lunghe all'incirca 2-3 mm.

Il materiale viene portato in laboratorio, mentre al soggetto viene praticata una pulizia più accurata della ferita e gli viene prescritta, dal medico di guardia, una terapia antibiotica con amoxicillina.

Risultati

Da un primo screening, effettuato allo stereomicroscopio, è stato possibile isolare subito 3 larve di *Oestrus ovis* per la particolare conformazione degli spiracoli posteriori e per la conformazione dello scheletro cefalofaringeo armato di due forti uncini. Le altre 6 larve sono risultate appartenere alla famiglia dei Sarcophagidae e quattro larve L2 sono state poste in allevamento su fegato di maiale. Dopo circa 12 giorni è avvenuta la metamorfosi in pupa e dopo altri 8 giorni è avvenuto lo sfarfallamento degli adulti che ha permesso di poterli ascrivere a *Sarcophaga haemorroidalis* (=*Bercaea cruentata* Meig). Due larve di *Oestrus* sono state poste anch'esse in allevamento su carne, ma il giorno successivo le larve avevano abbandonato il substrato ed erano decedute. Il materiale non posto in allevamento è stato fissato in etanolo al 70%.

Conclusioni

Mentre molte specie appartenenti al genere *Sarcophaga* (es.: *Sarcophaga haemorroidalis* Fallen) sono assai note

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per il loro sviluppo allo stadio larvale su substrati costituiti da materiale organico in putrefazione (es.: cadaveri, ferite necrotiche, etc.), così non si può dire per le larve di *Oestrus ovis*, che invece, da specie miasigena obbligata, è specializzata nella patogenesi di oftalmomiasi e miasi rino-faringee (Pampiglione, 1957, 1958 a,b,c; Pampiglione e Canestri Trotti, 1991), nonostante siano segnalati anche casi di miasi oculare esterna (Weinand *et al.*, 2001) e infestazioni della mucosa orale (Hakimi *et al.*, 2002).

In conclusione, resta rilevante segnalare come *O. ovis* possa essere riscontrato allo stadio larvale anche nelle ferite traumatiche. È comunque molto probabile che nel caso analizzato si tratti di una localizzazione parassitaria accidentale che potrebbe essere occorsa nei due giorni precedenti in occasione di una visita del soggetto presso un'area adibita a pascolo di ovini.

Dati i diversi stadi di sviluppo che hanno infestato la ferita, si può supporre che la colonizzazione sia avvenuta in due luoghi e momenti diversi. Sicuramente *Sarcophaga haemorroidalis* può aver dato avvio alla parassitosi durante le attività lavorative in allevamento dove la specie è particolarmente frequente e lo stesso allevatore in più occasioni ha osservato casi di infestazione da larve sui bovini successivamente a ferite, traumatiche o chirurgiche, o successivamente al taglio delle corna.

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ISTITUTO D'IGIENE SPERIMENTALE E DI PARASSITOLOGIA
DELL'UNIVERSITÀ DI LOSANNA

Studi e ricerche sui Culicidi

V MEMORIA

per BRUNO GALLI-VALERIO e JEANNE ROCHAZ-DE JONGH.

Aderendo con piacere al gentile invito del prof. Celli, esporremo in questa quinta memoria un insieme di osservazioni e di ricerche sui culicidi fatte nelle stesse zone (Valtellina, Cantone di Vaud) in cui abbiamo fatto quelle riferite nelle memorie precedenti (1) e per di più, da uno di noi, in Tunisia, nel periodo di tempo che va dal novembre del 1905 al novembre del 1907 (2).

1. — *Osservazioni sull'habitat dei culicidi.*

Le nostre osservazioni dimostrano sempre più, come piccolissime raccolte d'acqua, abbiano spesso un'importanza più grande di estesi paludi, per lo sviluppo di numerose zanzare. Abbiamo infatti trovato in una serra larve di *Culex*, in una scodella con pochissima aqua. Ne trovammo moltissime nello scolo dell'acquaio d'una casa infestata da zanzare e le trovammo a milioni in parecchi serbatoi di acqua per inaffiare e in una fossa a letame dell'ospedale cantonale. In Tunisia, mentre le larve mancavano nei grandi serbatoi d'acqua, erano numerose nelle pozzanghere vicine a questi serbatoi. Durante l'estate eccessivamente secca del 1906, abbiamo trovato in campagna molte larve di *Culex* entro poca acqua contenuta in cocci,

(1) Atti della Società it. per gli studi della malaria, vol. IV, V, VI, VII, pag. 1.

(2) Vedi Centralblatt für Backt., I, Abt. orig. vol. 43, 1907 p. 468 e vol. 46, 1908 p. 130. Bull. de la Soc. Vaudoise des Sc. nat., vol. 43, 1907 p. 201.

bottiglie rotte e vecchie scatole di carne conservata. La mancanza di acqua fece, ancor più che d'ordinario, deporre le uova a *A. bifurcatus* e *A. maculipennis* in acque putride: così ad esempio abbiamo raccolto molte larve di queste due specie in barili contenenti acqua carica di materie organiche, e una fossa a letame ci fornì durante tutto l'estate numerose larve di *A. maculipennis*. Tutto questo conferma quanto abbiamo sempre sostenuto, e cioè, che acque putride possono divenire focolai di anofeli e quindi non devono essere dimenticate nella lotta contro la malaria. È interessante notare, che presso Sondrio, appunto là ove stava la fossa a letame ricca in larve di *A. maculipennis* c'erano alcuni fossati ad acqua stagnante completamente coperta da *Lemna palustris*. Ora, nonostante che in tale anno le zanzare avessero una sì scarsa scelta di acqua per deporre le uova, non trovammo larve di culicidi in questi fossati. *Lemna palustris*, quindi protegge realmente i fossati contro lo sviluppo dei culicidi. Notammo invece ancora, come le larve di *Culex*, o sopratutto di *Anopheles*, si tengan numerose fra le foglie di *Nasturtium officinale* e di *Ranunculus aquaticus*.

Anche in Tunisia, quest'ultima pianta è molto ricercata dalle larve dei culicidi. In Tunisia si trovano larve di culicine in pozzi, la cui acqua contiene il 2 ‰-3 ‰ di cloruro di sodio, e ninfe di *Theobaldia spathipalpis* si rinvennero in un fossato col 2.35 % di cloruro di sodio.

Abbiamo portato di nuovo la nostra attenzione sull'habitat delle larve e ninfe dei culicidi sulle Alpi: così abbiamo potuto notare, che nel lago di Rogneda a 2390 m. sulle alpi valtellinesi, lago ricchissimo in larve e ninfe di *Culex nemorosus*, pur non presentando vegetaziene alcuna, le larve si nutrono del fino straterello di alghe aderenti alla superficie dei blocchi di schisto. A 1500 m., sempre sulle stesse Alpi, abbiamo constatato che *Culex nemorosus* si sviluppa nella poca acqua contenuta nelle escavazioni delle roccie a picco che rinserrano il fiume. Le modificazioni che sopravvengono nei fossati e nelle raccolte d'acqua in generale, possono modificare il tipo dei culicidi che d'ordinario vi si trova: così ad esempio, in un barile affondato nel suolo nella pianura dell'Orbe, barile ove negli anni precedenti si trovavano larve di *Anhopeles* e di *Theobaldia annulata*, nel 1907 non v'eran più che larve di *C. nemorosus* e sopratutto di *C. pipiens*, l'acqua essendo stata inquinata da molto letame.

In un fossato della stessa pianura, ove si trovavan molte larve di *A. maculipennis*, queste scomparvero quasi completamente nel 1907,

il fossato essendo stato sbarazzato dalle piante acquatiche, cosa che dimostra sempre più l'importanza di mantenere ben puliti i canali di scolo per impedire che diventino focolai di anofeli.

2. — *Osservazioni sulle larve e ninfe dei culicidi in inverno.*

La quantità di larve che può svernare qualche volta sotto il ghiaccio, può essere straordinaria. In una fossa che conteneva circa 3000 centimetri cubi di acqua e coperta da uno strato di ghiaccio, abbiamo calcolato potessero esservi all'incirca 3,750,000 larve di *Culex*. Queste larve che rimangono nello stesso stato durante l'inverno, si trasformano in ninfe nei primi giorni della primavera e le immagini che ne escono costituiscono l'origine di una buona parte dei culicidi che tormentano nella buona stagione. Di qui la necessità, sulla quale insistemmo già altre volte (1), di petrolizzare le acque al primo inizio della primavera. Abbiamo trovato pure larve di *Culex* e di *Anopheles* nella piccola quantità di acqua rinchiusa fra due strati di ghiaccio sopra un fossato. Al momento della fusione delle nevi, abbiamo visto schiudere molte larve di *Culex* nelle escavazioni del suolo di un bosco, riempite di foglie morte, foglie fra le quali avevan svernato le uova di *Culex*. Per la prima volta il 29 dicembre 1905, trovammo in un fossato presso Sondrio larve di *A. maculipennis;* esse stavano fra le foglie di *N. officinale* con una temperatura dell'acqua di + 0. 5° (temp. aria — 1° C.).

Negli inverni miti adunque, anche le larve di *A. maculipennis* possono svernare. Nell'inverno 1905-1906 trovammo dal 2 novembre 1905 al 7 gennaio 1906, con una temperatura media di + 4. 6 (nov.) + 1. 3 (dic.) + 1. 39 (gen.) ninfe di *T. annulata*. Vi fu dunque formazione di ninfe di questa specie, durante tutto l'inverno. Queste ninfe, tenute a temperature medie di + 0. 75 (dic.) + 1. 13° (genn.) vi sono sviluppate in immagini che resistettero a queste stesse temperature 20-23 giorni senza cibo. Ciò dimostra, come d'inverno, le immagini possano resistere più a lungo al digiuno che in estate. (Resistenza massima d'estate, 17 giorni).

Noteremo pure che il 2 novembre 1905 trovammo ancora una ninfa di *A. maculipennis* nella pianura dell'Orbe, e il 20 ottobre 1907 (temp. aria + 13. 15°, acqua + 13°) molte uova di questa specie.

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(1) Manuel pour la lutte contre les moustiques. Lausanne, 1906.

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Durante l'inverno 1906-1907, le larve di *Anopheles* furono rarissime, tanto nella pianura dell'Orbe che a Sondrio. Un fatto analogo fu notato da uno di noi in Tunisia.

3. — *Osservazioni sulle uova dei culicidi.*

Abbiamo fatto numerose esperienze con immagini di *C. pipiens*, *C. nemorosus* e *C. vexans*, nutrite con aranci e ciliegie, per vedere se fossero capaci di deporre uova con un nutrimento esclusivamente vegetale, ma il risultato fu negativo.

Invece, femmine di *C. cantans*, nutrite con sangue umano, deposero uova presentanti caratteri analoghi a quelli di *Stegomyia*. Queste uova erano deposte alla superficie dell'acqua, separate le une dalle altre, irregolarmente disposte o a corti nastrini (fig. 1). Erano lunghe $^1/_2$-$^3/_4$ di millimetro, e esaminate al microscopio, apparivano leggermente fusiformi, con un lato un po' più convesso dell'altro e involte da una membranella a cellule d'aria, permettente all'uovo di rimanere alla superficie dell'acqua. Questo aspetto affatto diverso da quello delle uova ordinarie di *Culex* è importante a essere ricordato, per non far confusioni colle uova di *St. fasciata*.

4. — *Osservazioni sulle punture dei culicidi.*

Attireremo ancora l'attenzione sul fatto, che se i culicidi hanno una importanza grandissima come agenti di trasmissione di malattie, ne hanno uno non meno grande, pei disturbi diretti che provocano colle loro punture. Stazioni balneari e alpine, importantissime, sono rese d'un soggiorno sgradevole per la presenza di questi parassiti.

Nel 1907, ad asempio, le zanzare furono sulle Alpi una vera piaga fino a 2000 metri di altitudine. Abbiamo avuto di nuovo la occasione di vedere lattanti completamente sfigurati dalle punture delle zanzare, e messi nell'impossibilità di dormire e di poppare. Per questi fatti, si dovrebbe estendere di più in più il sistema delle reticelle metalliche, anche nelle zone ove la malaria manca ma abbondano le zanzare.

L'influenza del colore degli abiti per attrarre o allontanare le zanzare, la verificammo ancora, per il fatto che uno di noi, vestito di scuro, era più attaccato dell'altro che portava abiti chiari. Ab-

biamo constatato come in certi casi i culicidi, abituati a succhiare sangue di animali, possano preferirlo a quello degli uomini; così sulle Alpi valtellinesi, uno di noi era punto da numerosi *C. nemorosus*, accompagnato da un cane, le zanzare si portavano tutte su quest'ultimo, benchè il suo mantello chiaro avesse dovuto proteggerlo, più che l'abito scuro di chi l'accompagnava. Ebbimo del resto già altra volta l'occasione di notare come il cane sia fra gli animali preferiti dalle zanzare. Abbiamo fatto nuove esperienze per constatare se vi siano sostanze capaci di tener lontano i culicidi dalla superficie del corpo. Allo scopo, ci siamo serviti del manicotto di Rees, nel quale stavano *C. pipiens*, *C. vexans*, *C. nemorosus*, *A. bifurcatus*, *A. maculipennis*. Il braccio nudo, con una parte spalmata dalla sostanza da sperimentare, era introdotto nel manicotto o appoggiato esternamente contro la garza dello stesso, e il braccio era tenuto in posto da 10 a 15 minuti. I risultati ottenuti furono i seguenti:

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Numero	Sostanze sperimentate	Risultati
1	Formalina 40 %.....	Non pungono la parte spalmata.
2	Saprolo	Id. id.
3	Essenza di garofani.....	Non pungono anche vicino alla parte spalmata finchè l'essenza non è evaporata.
4	Id. trementina.....	Id. id.
5	Id. pino pumilione.....	Id. id.
6	Id. rosmarino	Id. id.
7	Id. bergamotto	Id. id.
8	Id. lavanda.....	Id. id.
9	Id. cannella.....	Id. id.
10	Id. fiori d'arancio	Id. id.
11	Id. sabina	Id. id.
12	Id. patchouli.....	Id. id.
13	Id. valeriana	Id. id.
14	Id. menta piperita.....	Id. id.
15	Id. luppolo	Id. id.
16	Id. timo.....	Id. id.
17	Id. limone	Id. id.
18	Id. calamo aromatico ...	Id. id.
19	Id. cajeput rett.....	Id. id.
20	Id. origano	Id. id.
21	Id. anice moscato.....	Id. id.
22	Id. ruta	Id. id.
23	Id. eucalyptus	Id. id.
24	Id. carvi.....	Id. id.
25	Id. lauro.....	Pungono la parte spalmata dopo 5 minuti, a volte anche immediatamente.
26	Vasellina canforata 10 %.....	Non pungono la parte spalmata nè vicino a questa.
27	Polvere di Piretro di Dalmazia .	Nella maggioranza dei casi pungono la parte spalmata.
28	Id. id. del Caucaso..	Id. id.
29	Soluzione alcoolica di timolo 2 %.	Non pungono la parte spalmata.
30	Tintura di muschio.....	Id. id.
31	Balsamo peruviano	Id. id.

Questa tabella ci dimostra che la maggioranza delle sostanze sperimentate tien lontano i culicidi dal punto spalmato e dalle sue vicinanze immediate, ma la maggior parte di queste sostanze non può essere impiegata pel suo prezzo e per la sua azione irritante, e se si mescolano con olio, Otto e Neumann (1) hanno constatato che danno risultati poco sicuri. La sola sostanza che può avere una certa applicazione è sempre la vaselina canforata al 10 per cento, che emana per un tempo abbastanza lungo, odore di canfora, non è irritante e di un prezzo relativamente poco elevato (50 centesimi per ogni 100 grammi).

Quanto alle sostanze che possono diminuire il dolore e la tumefazione dopo la puntura delle zanzare, segnaleremo:

a) Una mescolanza di formalina (40 %): 15 gm. xilolo: 5 gm. acido acetico: 5 gm. balsamo del Canadà: 1 gm. (Joly).

b) Formalina (40 %).

c) Ammoniaca.

d) Tintura di iodio.

e) Guaiacolo 1 %. Acido fenico 3-5 %.

La formalina a 40 %, è una delle sostanze più pratiche per chi non abbia idiosincrasia speciale.

5. — *Osservazione sulla disseminazione delle zanzare per mezzo del vento.*

Confermiamo che i venti non hanno una grande azione nella disseminazione dei culicidi. In zone ove eravamo attaccati da *C. pipiens*, *C. vexans* e *C. nemorosus* a tempo calmo, non se ne vedevano volare quando il vento soffiava. Ma scuotendo fortemente le cannelle e gli arbusti, se ne facevano partire molte zanzare che si posavano subito su cannelle e arbusti vicini. Nella pianura dell'Orbe, c'è una pozza d'acqua in una escavazione del suolo e circondata da boscaglie: nei giorni tranquilli, vi eravamo attaccati da *C. pipiens*, *C. vexans* e *C. cantans*, mentre nei giorni di vento, anche con temperature di + 19°, non volavano che poche zanzare appena sopra il pelo dell'acqua, in modo che per farci pungere, dovevamo abbassarci sotto il livello delle boscaglie. Le immagini non mancavano, ma si tenevano fissate sotto le foglie e sulle cannelle. Abbiamo constatato

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(1) Zeitschr. für Hyg., vol. LI, 1905, p. 357.

ancora, e questa volta a Orbe, come un brusco abbassamento della temperatura nel mese di settembre, abbia fatto penetrare molte zanzare negli appartamenti.

6. — *Osservazioni sui mezzi di distruzione dei culicidi.*

Parecchie nuove sostanze furono da noi sperimentate per la distruzione delle larve e ninfe di culicidi nelle acque. I risultati ottenuti possono venire così riassunti:

1. *Olio verde di schisto.* È ottenuto dalla distillazione degli schisti carboniferi della Francia centrale, e ci fu gentilmente favorito dal signor Barillet di Alfort (Senna). L'abbiamo steso alla superficie dell'acqua cogli stessi metodi impiegati per il petrolio e per il saprolo, e le esperienze fatte in laboratorio e in campagna ci hanno dimostrato che può occupare un posto accanto a queste due sostanze. A proposito di olî, osserveremo che uno di noi constatò in Tunisia come fossati a cui arrivano scoli di oleifici, non contengono nè larve nè ninfe di culicidi.

2. *Carburo di calce.* Ha un'azione abbastanza energica sulle larve dei culicidi, anche a dose di 0.001 %. Le soluzioni più forti (1.6 - 5 %) hanno un'azione energica anche sulle ninfe. Queste soluzioni conservano il loro potere nocivo, in ogni caso per un mese.

3. *Soluzione del Rabot.* È composta da solfato di ferro gm. 0,175; calce viva gm. 0.25; acqua gm. 150. Questa soluzione impedisce la vita delle larve, ma non delle ninfe.

4. *Solfato di ferro.* Le soluzioni a 0.05 % hanno già azione nociva sulle larve, ma le ninfe possono svilupparsi ancora in soluzioni al 5 %.

5. *Bordolese.* L'abbiamo preparata, sui consigli del prof. Faës, disciogliendo una parte di solfato di rame in 50 parti di acqua e aggiungendovi acqua di calce a neutralizzazione, poi portando a 100 parti. Le larve soccombono, e quasi sempre anche le ninfe.

6. *Acetato di rame neutro.*

Le soluzioni dal 0.25 all'1 % uccidono le larve, ma delle ninfe alcune giungono allo sviluppo.

Nelle soluzioni di solfato di ferro, nella bordolese e sopratutto nelle soluzioni d'acetato di rame, le larve presentano spesso l'espulsione dell'intestino, che resta aderente al corpo come un filamento giallastro o verdastro e che al microscopio si presenta riempito di granulazioni di solfato di ferro, di solfato o di acetato di rame. È una lesione analoga a quella che abbiamo descritto nelle larve

dei culicidi in seguito a infezione coll'*Aspergillus niger* (1), e ciò conferma la nostra ipotesi, che le spore dell'Aspergillo agiscano meccanicamente nel determinare la lesione.

Dalle nostre esperienze risulta che i barili contenenti bordolese o soluzioni di acetato di rame, barili lasciati nelle vigne ove si pratica la cura della peronospora, non rappresentano un pericolo di trasformarsi in focolai a culicidi.

7. *Melioform.* È un liquido contenente il 25 % di formalina e il 15 % di acetato di allumina, liquido impiegato come antisettico (2).

Avendo già sperimentato un liquido analogo, il lisoform (3), abbiamo voluto vedere quale fosse l'azione del melioform sulle larve e le ninfe dei culicidi, e abbiamo constatato che è affatto analoga a quella del Lisoform. Anche soluzioni al 0.05 % uccidono le larve. Quanto alle ninfe, possono qualche volta dar immagini che spesso però, muoiono subito dopo esser schiuse.

Esperienze fatte con animali capaci di divorare larve e ninfe di culicidi, ci han dimostrato che, contrariamente a quanto verificammo sempre per *Rana esculenta* e *Rana temporaria*, due anuri di Tunisia, *Discoglossus pictus* Dies. e *Bufo variabilis*. Pall. distruggono gran numero di larve e di ninfe, comportandosi in ciò come i tritoni.

In un'esperienza di laboratorio, abbiamo constatato che un ragno, accidentalmente introdotto in una scatola con immagini di culicidi, ne accalappiava molte nelle sue tele e le succhiava. Ora, O' Connell (4) attira appunto l'attenzione sull'azione che può essere esercitata dai ragni nella distruzione di un certo numero di culicidi.

Quanto all'azione distruttiva di acari, parassiti sulle immagini dei culicidi, contrariamente a Mankowsky (5) non l'abbiamo osservata affatto. Infatti, su immagini di *C. vexans*, *C. nemorosus* e *C. ornatus* abbiam trovato fissati al torace, uno a due acari (Trombididae) già da noi precedentemente segnalati (6); ma queste immagini volavano e pungevano assolutamente come le altre e quindi non sembravano soffrire per la presenza di questi parassiti.

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(1) Centralblatt für Bakt. Abt. I, orig. vol. 38 p. 174 e vol. 40 p. 630.
(2) GALLI-VALERIO: Therap. monatsh. 1906, giugno.
(3) Atti della Società ital. ecc., vol. V e VI, p. 1.
(4) Journal of Trop. med. 1907, p. 392.
(5) Centralblatt für Bakt. Abt. I, orig., vol. 39, 1905, p. 27.
(6) Atti della Società ital. ecc., vol. V. p. 1.

ERRATA CORRIGE

This “errata” concerns the article by Y. Cambefort “Knowledge of Diptera in France from the beginning to the early twentieth century” published in Parassitologia 50(3-4): 173-185.

The useless note on page 173 first column, added during the editing of the manuscript, contains two mistakes as follows: 1) not all “Diptera” have biting mouthparts; 2) the word “dipterist” exists in English language as shown by Webster's Third International Dictionary, 1966 edition, volume I, page 639 column 3.

This note was added during the editorial work without any control from the author.