The Associated Microflora to the Larvae of Human Bot Fly *Dermatobia hominis* L. Jr. (Diptera: Cuterebridae) and its Furuncular Lesions in Cattle

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The microflora associated to furuncular lesions, larvae and pupae of Dermatobia hominis, *as well as the relationships between parasite, host and microflora associated, as a comprehensive microsystem, has been studied. One hundred and two furuncular myiasis due to* D. hominis *larvae in several breeds of cattle were studied and the following bacterial species were significant:* Staphylococcus aureus, S. epidermidis, S. warneri, Bacillus subtilis *and* Escherichia coli. *Closely related, the microflora associated to 141 samples from first, second, third instar larva and both external surface and larval cavities has been studied. The representative associated microflora to the larvae were:* S. aureus, B. subtilis, S. hycus *and* Moraxella phenylpiruvica, Moerella wisconsiensis, Proteus mirabilis *and* P. vulgaris, M. phenylpiruvica, M. wisconsiensis, P. mirabilis *and* P. rettgeri *were the representative microflora associated to 64 pupae of* D. hominis.

Key words: *Dermatobia hominis* - associated bacterial microflora - larvae - pupae - furuncular myiasis

Dermatobia hominis L. Jr. is a Neotropical fly responsible for most prevalent myiasis in Central and South America (from Mexico to Argentina) (Guimarães et al. 1983). Myiasis due to *D. hominis* or dermatobiasis (Kasai et al. 1988) is easily identified by its characteristic furuncular cutaneous lesions, characterized by a tumoration which involves both epidermis and dermis. The larva is situated in a cavity screened by a non-keratin flat epithelium that has a communication orifice with the exterior (Neel et al. 1955). This orifice is produced when the larva penetrates the host and is maintained during the entire parasitic phase, about 35 to 47 days (Sancho & Boschini 1985).

Their effects on the wild fauna are unknown, however the bot fly maggots are a seriuos pest to livestock, specially on cattle and humans (Steelman 1976). Though the literature on its epidemiology is extensive (Guimarães & Papavero 1966), little information about the *D. hominis* biology is available (Neiva & Gomes 1917, Catts 1982, Sancho 1988).

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The importance of the studies on the associated microflora to myiasis-producing flies has been showed in Australian sheep blowfly *Lucilia cuprina* Wied (Diptera: Calliphoridae) (Guerrini et al. 1988), the new world screwworm *Cochliomyia hominivorax* Coquerel (Diptera: Calliphoridae) (Bromel et al. 1983) and in the palearctic screwworm *Wohlfahrtia magnifica* Schin (Diptera: Sarcophagidae) (Ruíz-Martínez & Villa-Real 1995). The role of associated microflora in the attraction, oviposition, larval culture and in the new tendences of trapping flies are being intensely studied.

Adult bot fly *D. hominis* does not frequently visit the hosts (Catts 1982) but, instead, they oviposit on a carrier, usually a zoophilic fly or mosquito (Neiva & Gomes 1917, Sancho 1988). The carrier behaviour allows the larvae to reach the host's skin. In another paper, we consider the possible attractive factor for several primarily and secondarily myiasis (Ruíz-Martínez & Villa-Real 1995). However, the possible role and implications of bacteria associated to dermatobiasis for *in vitro* culture of parasitic stage -larvae- (Zeledón & Silva 1987) is clear and it is obviously important in the knowledge of the pathogenicity or pathomorphology of the furuncular lesions (Sancho 1988).

On the associated bacterial microflora of *D. hominis* short reports are available (Sancho et al. 1986, Coronado 1989), but they are not extensive

and do not stablish correlations between furuncular lesion and larvae or pupae. Theoretically, the exposure of the wound to field environment and, mainly, bacterial contamination resulting from numerous flies feeding on lesions, could explain some associated bacterial microflora to larvae and foruncular myiasis, but not all of them. There are some interactions larvae-microflora, host body-microflora and furuncle microflora-exogenous microflora that must be intensely studied.

The main objective of this study is to isolate, identify and analyze the bacterial microflora in the furuncular lesion, larvae (first, second and third instars) and pupae of *D. hominis*, and to discuss the role of these bacteria.

MATERIALS AND METHODS

The study has been carried out between April and October 1993. Hides from slaughterhouse containing active furuncular myiasis due to first, second and third instar larvae of *D. hominis* were recovered as follows: the exterior surface of the wound was shaved with alcohol-solution and a hot spatula was used to eliminate any remaining bacterial contamination from surrounding hide. With sterile forceps pressure was applied to the base of each tumoration for extracting the larvae and removed from the cavity with a cotton-swab in triplicate and were introduced in vials Portagerm (41996 BioMerieux) with resazurine as ox-red indicator (Barry et al. 1972, Yrios et al. 1975).

Larvae were placed in sterile vials and classified in instars according with Jobsen and Mourier (1976). All samples were kept in thermic plastic bags at 26-28°C, 80% RH at darkness for analysis, within 24 to 48 hr, being the samples refrigerated at 4°C.

From six 1st instar larva, 43 2nd instar larva and 92 3rd instar larva for associated bacterial microflora were studied. For to obtain pupae, the mature 3rd instars larva were cultured to pupae stage on a sterile filter paper in a climatic chamber at 25±1°C, 60-65% of RH and photoperiode 12:12 hr. The pupae (n= 64) were analyzed 20 days after (yellow pharate adult; according to Sivasubramanian & Biagi 1983). Larvae and pupae were washed with 5 ml of sterile saline solution and shaked in Mixo-Tub during 5 min. The resulting fluid was analyzed for external microflora. Then, the samples were homogenized in a Potter (glass homogenizer) and a centrifugation was made. With the supernatant as a sample for internal microflora, 0.2 ml aliquots of each dilution were made and spread in triplicate on identification media for statystical analysis.

Samples were processed following the technics recorded in Sonnenwirth (1987). The plates were incubated at 30°C from 48 hr to 5 days, with or without CO_2 and extend to 7 d for anaerobic bacteria. Gram-negative proofs were made following the system Pasco/mic/ID (Difco Lab., Detroit) with 30 substrates and 19 antibiotics. For Gram-positive and anaerobic bacteria the criteria of Krieg and Holt (1987) was followed, using 10 substrates and antibiotics of Oxoid (Ltd., London), BioMerieux (BM Lab, Paris) and Difco. The results were analyzed by API 20 E, Octals number of Difco and Micro-Scann computerized systems. The criteria employed in bacterial taxonomy followed the classification of Bergey's Manual of Systematic Bacteriology (Krieg & Holt 1987). The percentages shown in the Tables were referred to mean bacterial counts that were found in each wound ($3x10^6$) and 100% is the maximum colony forming units (CFU). All data were recorded on DB3 files and proccessed by Microstad Prog. and BMDP statistical-packet.

RESULTS AND DISCUSSION

Of 102 furuncular lesions studied, on 97 bacteria were isolated (95.1% of positive results, on five furuncular lesions none bacterial species were isolated). Ten bacterial species were identified and in decreasing order of importance (% from total) were: *S. aureus* (41.00%), *E. coli* (10.33%), *S. warneri* (9.00%), *E. aerogens* (8.66%), *S. epidermidis* (8.50%), *B. subtilis* (6.00%), *C. freundii* (2.50%), *S. liquefaciens* (1.50%), *E. cloacae* (1.00%) and *E. agglomerans* (1.00%) (Table I).

S. aureus is common in piogenic proccesses but also on animal skins, as well as *S. warneri, S. epidermidis* and *E. coli* (Jansen & Hayes 1987). The other bacteria species with enteric characteristics, probably appeared by contamination due to flies visiting-wounds, as we can usually see on furuncular lesions of *Dermatobia*, looking for nutritive sources (such as *Stomoxys calcitrans, Musca domestica, Muscina stabulans*, personal observations). It may explain the lower isolation percentages for these species obtained in our study.

Whereas only in 66.7% samples of L-I bacteria were recorded (4 bacteria species) (Table II), in the 79% samples of L-II (8 bacteria species) and 97.8% of L-III (19 bacteria species) samples positive isolations were recorded. The associated microflora to larvae (L-I to L-III) in the external surface were: *S. aureus, S. epidermidis, B. subtilis* (all of these corresponding with frequent microflora isolated in the furuncular myiasis), *S. hycus, S. sciuri* and *M. phenylpiruvica*. On the other hand, the main associated bacteria microflora to larvae in the internal cavities were: *M. phenylpiruvica, M. wisconsiensis, P. mirabilis, P.vulgaris* and *B. subtilis* (Table II).

TABLE I

Microflora associated to furuncular lesions due to *Dermatobia hominis* in cattle

Bacterial species	Presence / sample	% / total	Mean % of isolation	Relative importance index
Staphylococcus aureus	85	83.33	41.00	34.16
Staphylococcus epidermidis	43	42.16	8.50	3.58
Staphylococcus warneri	32	31.37	9.00	2.82
Bacillus subtilis	50	49.02	6.00	2.94
Escherichia coli	60	58.82	10.33	6.08
Enterobacter agglomerans	5	4.90	1.00	0.05
Enterobacter aerogens	17	16.67	8.66	1.44
Enterobacter cloacae	10	9.80	1.00	0.10
Citrobacter freundii	9	8.82	2.50	0.22
Serratia liquefaciens	6	5.88	1.50	0.09
# isolated species= 10				
Bacterial absence	5	4.90		

The 'relative importance index' is defined as the result of the mean percentage of isolation x the presence by sample and / by the total observations (in this case 102 furuncular lesions).

TABLE II

Discriminative analysis of microflora associated to furuncular lesions, larvae and pupae of *Dermatobia hominis*

Type of sample	Furuncular	Larvae - I		Larvae - II		Larvae - III		Pupa
Bact. species/	lesions	Ext.	Int.	Ext.	Int.	Ext.	Int.	
Staphylococcus aureus	34.16	6.00	1.25	14.88	0.84	30.44	0.72	-
Staphylococcus epidermidis	3.58	-	-	-	-	2.17	0.04	-
Staphylococcus hycus	-	-	3.39	5.21	-	1.60	0.44	-
Staphylococcus hominis	-	-	-	-	-	0.16	-	-
Streptococcus haemolyticus	-	-	-	-	-	0.26	-	-
Streptococcus sciuri	-	-	-	-	-	2.20	-	0.11
Streptococcus similaris	-	-	-	-	-	-	0.08	-
Streptococcus warneri	2.82	-	-	-	1.77	-	-	-
Streptococcus xylosus	-	-	1.10	-	-	-	-	-
Bacillus subtilis	2.94	-	-	3.06	3.72	4.96	1.56	0.51
Escherichia coli	6.08	-	-	-	0.51	0.69	0.11	-
Aeromonas salmonicida	-	-	-	-	-	-	0.18	-
Proteus mirabilis	-	-	-	-	8.62	-	4.45	4.22
Proteus vulgaris	-	-	-	-	-	-	4.21	-
Proteus rettgeri	-	-	-	-	-	-	0.90	2.25
Providencia stuartii	-	-	-	-	-	-	0.11	-
Enterobacter aerogens	1.44	-	-	-	-	-	-	-
Enterobacter agglomerans	0.05	-	-	-	-	-	-	-
Enterobacter cloacae	0.10	-	-	-	-	-	-	-
Moerella wisconsiensis	-	-	-	-	13.92	-	4.79	9.38
Moraxella phenylpyruvica	-	-	11.80	0.66	10.05	-	9.54	10.00
Citrobacter freundii	0.22	-	-	-	-	0.08	-	-
Serratia sp.	-	-	-	-	-	-	-	0.07
Serratia liquefaciens	0.09	-	-	-	-	-	0.07	-
Serratia marscescens	-	-	-	-	-	0.08	-	-
Total = 25	10	1	4	4	7	10	14	7

(#): positive isolation reffering to relative importance index in each sample-kit; (-): negative isolation

Only bacteria species belonging to wounds were isolated on the external surface of larvae, increasing the number and relative importance rate of isolation from second to third instar larvae (from only one bacteria species isolated -*S. aureus*- in L-I, to four bacteria isolated in L-II and ten bacteria species isolated in L-III) (Table II). When we tried to measure the statistical similitude within samples from wounds and from external surface of larvae by Sorensen's coeficient, the percentages obtained were 85.0% (for L-II) and 96.0% (For L-III) respectively. These facts show that the microflora associated with larval surface was only a draging product from wounds.

On the contrary, the results obtained from internal body (digestive and respiratory tracts) of larvae indicates that these bacteria were not significant and *S. aureus* was never isolated in significant levels from the internal body of the larvae, whereas other bacteria such as *M. phenylpiruvica, M. winconsiensis, P. mirabilis, P. vulgaris* and *B. subtilis* are the dominant ones (Table II) and were never isolated significantly from wounds. It is very probable that these bacteria species stablish a competence against those from wounds (Greemberg et al.1970) and they may constitute endosymbionts of the larvae of *D. hominis*, that contain a bactericide or bacteriostatic substance against foreign microflora (Baba et al. 1987). Our results seem to point out in this direction: the larvae contains their proper bacteria microflora (this phenomenon is clear in L-II and L-III) and these bacteria reject the foreign one ingested by the larval nutrition.

Probably, this phenomenon will be common in myiasis-producing flies: similar results have been obtained on Australian sheep blowfly *Lucilia cuprina* (Emmens & Murray 1982), in new world screwworm *Cochliomyia hominivorax* (De Vaney et al. 1973) and in palearctic screwworm fly *Wohlfahrtia magnifica* (Ruíz-Martínez & Villa-Real 1995).

As a whole, the number of bacteria species isolated increased from instar I to instar III (from 4 to 19) (Table II) and decreased from instar III to pupae (from 19 to 7) (Table II). Obviously, the increasing size of the furuncular hole (due to larval growth) and its exposed surface to the environment produces a correlative increase of microflora associated or imported (from environment). Moreover, the infestation itself increased the microflora coming from feeding fly visiting-wounds. In this way, the high number of flies feeding on wound exudates may be the major cause of foreing contamination of the furuncles under natural conditions (Hawley et al. 1951). The larger development time of third instar larvae (Sancho & Boschini 1985) could favour the continuous contamination throughout the field environment and wound-visiting flies.

On the other hand, although one bacteria capable of producing piodermitis as *S. aureus* was isolated from the wound-samples, no secondary bacterial infection was recorded. Noticeably there have been no cases of acute secondary bacterial infection in human skin parasitized by warble flies (Brenes & Maezerville 1963). Nevertheless, when the parasite attacks other host tissues a secondary bacterial infection is recorded (such as a meningoencephalitis, case reported by Céspedes et al. 1962). These may suggests that the skin has an immunological function with an inhibitory effect on bacteria present in the wound (Patterson & Edelson 1982). Another possibility is the larval substance with bactericide properties in *D. hominis* (as suggested the data of Picado 1935) and in other warble flies (Bennett 1955, Beesley 1968) as Baba et al. (1987) showed with the antibacterial effect of 'sarcotoxins I, II and III' from flesh flies. In this way, Coronado (1989) observed the bactericide effect of larval extracts from *D. hominis* against *S. aureus, S. epidermidis, S. caprae* and *Streptococcus* spp. Independently or together, showed the absence of acute secondary bacteria infection on human skin and bovine cattle hides as facts for intensive studies in the future, but with one noteworthy difference: the hypothesis on antibacterial properties of *D. hominis*'s larval extracts working on living tissues (not in carcases, decayed or dead tissues as flesh-flies and 'sarcotoxins').

A group of 64 pupae of *D. hominis* was studied (Table II) and in 44.75% positive results were obtained. This relative sterility was observed by Greemberg et al. (1970) for pupae of several flies. Seven bacteria species were isolated and only four with relative importance: *M. winconsiensis, M. phenylpiruvica, P. mirabilis* and *P. rettgeri*. These bacteria species were isolated from internal body of first, second and third instar larvae (Table II) and never isolated in the furuncular myiasis.

Observing the bacteria microflora associated to different studied samples (Table II), we conclude that *M. phenylpiruvica, M. wisconsensis* and *P. mirabilis*, plus *B. subtilis* constitute the microflora proper of larvae and pupae of *Dermatobia*, and secondarily *P. vulgaris* and *P. rettgeri*. Curiously, these bacterial species are commonly found in other myiasis producing-flies (Emmens & Murray 1982, Bromel et al. 1983, Ruíz-Martínez & Villa-Real 1995) with different life-habits. This is the first record about the presence of *M. wisconsensis* (Hickman et al. 1984) in animal tissues.

As stated above, we assumed that the antibacterial properties of some bacteria species (Baba et

al. 1987, Coronado 1989), alone or added to the skin immunological factors (Patterson & Endelson 1982) for explain the results of Tables I and II. Nevertheless, another idea suggests the role of specific bacteria associated to the larvae of *D. hominis* (Table II). Recently Guerrini et al. (1988) showed that some bacteria create a double effect. First, to produce smells attractive to gravid females (perhaps for the carriers?), that explain the prevalence of furuncular myiasis (Thomas, 1988). Moreover, faty acids coming from wounded tissues and other decomposed tissues would justify the attraction for this wounds (Mulla et al. 1977). Second, to maintain a pH coinciding with the maximum rates of larval survival. Certainly, the pH rates observed over 419 foruncular lesions due to *D. hominis* were very constant (6.88±0.05) and its variations for samples of second and third instars larvae and pH from the outer or inner part of the cattle hides were very small (personal observations) and coincides with the optimal pH for *S. aureus* (Tables I, II).

In our opinion the furuncular lesion, the larvae and its associated microflora must be considered as a whole micro-habitat. It is possible that the external microflora would be essential for maintaining the pH. The optimal pH and bacteria would be neccesary for an adequate larval development. The wound and larvae metabolism would be probably adequate for attractions of new carriers (with egg mass adhered). The larvae associated microflora would help this process and provide an adequate inner-habitat to larvae. The understanding of this complex situation may lead to succes for *in vitro* culture of larval instars, to better undestand of the larval development, the development of traps and to comprehensive studies about biology of *D. hominis.*

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