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Mortality caused by experimental infection with the yeast *Candida haemulonii* in the adults of *Ornithodoros moubata* (Acarina: Argasidae)

Gabriela Loosová, **Libor Jindrák** and **Petr Kopáček**

Institute of Parasitology, Academy of Sciences of the Czech Republic and Faculty of Biological Sciences, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic

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Abstract. A relatively high rate of mortality among engorged females of *Ornithodoros moubata* (Murray, 1877) was observed in our laboratory colony. The general aim of the study was to identify the causative agent responsible for this mortality. The diagnostic tests were performed by Yeast Identification Service (CBS-Delft, Netherlands) and the pathogen was identified as the yeast *Candida haemulonii* (van Uden et Kolipinski, 1962) Meyer et Yarrovi, 1978. The artificial infection study was performed by intrahaemocoelic inoculation of yeast suspension, resulting in a mortality of 37%. The maximum mortality of ticks infected *per os* by contaminated blood meal was 13%. Re-isolated yeast cells from haemolymph of dead and paralysed ticks were apparently identical with primary yeast cells, without loosing reproductive abilities. An occasional formation of elongated chains of yeast cells (pseudomycelium) was recorded. The majority of ticks infected in both experiments mentioned above survived and displayed no evident symptoms of the infection. The presence of yeast cells in the haemolymph of surviving ticks was not detected. The *in vitro* phagocytosis assay performed with FITC-labelled yeast cells showed that about 4% of tick haemocytes were phagocytically active against the pathogenic yeast cells. Thus phagocytosis seems to be a potent defence reaction against spreading and multiplying of the yeast *C. haemulonii* within the tick haemocoel.

Ticks, blood-sucking arthropods, are important ecto-parasites of humans and animals. They are responsible for severe economic losses through the direct effects of blood sucking on livestock, and indirectly as vectors of pathogens. Feeding by large numbers of ticks causes reduction in live weight gain and anaemia among domestic animals. However, the major losses caused by ticks are due to their ability to transmit protozoan, rickettsial and viral diseases (Jongejan and Uilenberg 1994). The soft tick *Ornithodoros moubata* (Murray, 1877) is one of the most serious vectors of pathogens that attack human health in tropical and subtropical areas. It is the main vector of African tick-borne relapsing fever, caused by spirochaetes *Borrelia duttoni*.

One approach that may limit tick populations is the use of organisms such as pathogens, parasitoids and predators. These alternative biological control methods may avoid some of the problems associated with chemical acaricides (Samish and Glazer 1991). In spite of their importance, we still know very little about the application of biological control agents.

The present paper describes isolation of a fungal agent responsible for high mortality among engorged females of *O. moubata* in our laboratory colony. An additional aim was to determine the nature of the infection and discover the possible route of pathogen transmission.

MATERIALS AND METHODS

Ticks. Adult *O. moubata* were kept separately in polyethylene cages in a room maintained at 25-27°C and 80-90% relative humidity. The ticks were fed artificially through a Parafilm^{TM} membrane on pre-warmed (40°C) whole-citrated bovine blood until repletion. Excess blood was washed from fed ticks. Ticks remained on filter paper until coxal fluid excretion was completed and then maintained as described above.

Haemolymph collection and examination. Haemolymph samples were obtained from paralysed or dead adult tick females by piercing one of their legs with a sterile pair of scissors. The outflowing haemolymph was collected onto a sterile cover glass and examined with a light microscope for yeasts.

Cultivation of yeast cells. The yeast cells from the haemolymph were cultivated on Sabouraud dextrose agar dishes (SDA) with addition of antibiotic (200 µg/ml ampicillin 1.0; Biotika) at 25°C for 14 days.

Identification of yeast cells. The pure strain was obtained by a routine slicing method. This strain on the SDA was submitted to the Yeast Identification Service (CBS-Delft, Netherlands) for further identification.

Artificial infection by yeast cells. Yeast cells were transferred by a sterile loop into 1 ml of sterile phosphate buffered saline (PBS) and stirred well. The suspension of yeast cells was adjusted to a final concentration 2.8×10^9/ml using a Bürker counting chamber. A group of 125 adult female ticks was injected through the dorsolateral cuticle with 15 µl

Address for correspondence: P. Kopáček, Institute of Parasitology, Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic. Phone: ++ 420 38 777 2207; Fax: ++420 38 5300388; E-mail: kopajz@paru.cas.cz

of yeast suspension (4.2×10^7 yeast cells). The injection was performed using 1-ml disposable syringes mounted on a microapplicator. The control group of 125 adult females was inoculated with sterile PBS. The ticks were placed into sterile Petri dishes and kept at 25°C. Mortality was recorded daily for 33 days.

Infection *per os*. One ml of the yeast suspension with concentrations: 1.5×10^7/ml; 3.7×10^9/ml; 1.0×10^{10}/ml was added to 100 ml tick blood meal. For each feeding experiment 25 adult females were used. The control group of 125 adult females was fed with non-infected blood meal.

Preparation of fluorescent particles. Fresh *C. haemulonii* cells were labelled with fluorescein isothiocyanate (FITC) according to the method described for the baker's yeast by Wiesner et al. (1998). Briefly, *C. haemulonii* were inoculated into 200 ml autoclaved culture medium (1% yeast extract, 2% D (+) glucose monohydrate and 0.05% peptone) and incubated at 37°C overnight. Cells were harvested from 1-ml aliquots by centrifugation (200× g, 10 min) and washed three times with 1 ml of sterile PBS. The last washing was performed in distilled water. The yeast cells were then resuspended in 1 ml of sterile 0.2M sodium carbonate-bicarbonate buffer, pH 9.4 to which 1 mg of FITC was added. The cell suspension was incubated on a shaker for 30 min (room temperature, darkness). The unbound FITC was thoroughly washed out with PBS (four washes) and once with distilled water. The pellet from the final wash was resuspended in 1 ml of Grace's insect medium (GIM, Serva). FITC-labelled yeast cells were counted in a Bürker counting chamber. The density of yeast cells was adjusted to 1×10^8 cells/ml with GIM and stored in aliquots at –20°C.

Phagocytosis assay. The phagocytic activity of haemocytes was determined using the *in vitro* microscopic fluorescence assay described by Rohloff et al. (1994). The assay was performed using 4-well chamber slides™ (Nunc). Aliquots (200 µl) of freshly collected haemolymph with predetermined concentration of haemocytes were pipetted into each testing well and haemocytes were allowed to settle and attach for 30 min at room temperature. Then, 100µl aliquots of fluorescent yeast suspension were added dropwise to each well. Concentration of yeast cells in the suspension was pre-adjusted to assure the optimum ratio of haemocytes : yeast cells to be roughly 1 : 10. The chamber slides were incubated at 31°C in the dark for 2 hours. Then, 200 µl of culture medium from each well was replaced by the same volume of trypan blue solution (4 mg/ml dissolved in GIM, filtered). After 20 min, quenching of the yeast cells not ingested by haemocytes was completed. The procedure was stopped by rinsing the cell cultures three times with GIM. After the last washing step, the haemocyte nuclei were stained with 150 µl DAPI-solution (10^{-4} M 4',6'-diamidino-2-phenylindole in 6.4 mM Na_2HPO_4, 3.6 mM KH_2PO_4, pH 7.2) for 20 min. Then supernatant was removed and the monolayers were fixed with 4% formaldehyde in GIM. Phagocytic activity was determined as a percentage of phagocytically active haemocytes (containing at least one FITC-labelled yeast cell) out of total 600 haemocytes counted using a BX 60 Olympus fluorescence microscope.

Photography. All images were photographed using an Olympus PM 20 camera.

RESULTS

A relatively high rate of mortality among laboratory colony of *O. moubata* was caused by extreme paralysis that led to death. Microscopic examination of haemolymph from dead ticks revealed the presence of ellipsoidal budding yeast-like microorganisms.

The samples of these microorganisms were submitted to the specialised laboratory of Yeast Identification Service in Delft, Netherlands. After both, routine and extended determination procedures the pathogen was identified as *Candida haemulonii* (van Uden et Kolipinski, 1962) Meyer et Yarrovi, 1978, a species of the imperfect yeasts. This strain has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures in Delft as No. G98-115.

Ticks, artificially infected by intrahaemocoelic inoculation of 15 µl yeast suspension (4.2×10^7 cells), displayed typical symptoms as described above. The growth of *C. haemulonii* within haemolymph of dead and paralysed ticks was apparently not suppressed. An occasional formation of elongated chains of yeast cells was recorded (Fig. 1). The artificial infection led to a total 37% mortality; 27% of the infected ticks died in 21 days and another 10% died in 22–33 days after inoculation (Fig. 2). The mortality within control group was 1.6%, and it was attributed to damage caused to ticks during inoculation.

The mortality of ticks infected *per os* by contaminated blood meal was positively related to yeast cell concentration (Fig. 3). The maximum mortality recorded, achieved by 1.0×10^{10} cells/ml, was 13%. The mortality of 10% was obtained already after 14 days, while in the case of the lowest concentration (1.5×10^7 cells/ml) the mortality gradually reached 10% after 28 days. In the control experiment with non-infected blood meal, no mortality was recorded. The yeast cells of *C. haemulonii* were isolated directly from the haemolymph of dead and paralysed ticks. Re-isolated yeast cells were apparently identical with primary yeast cells, without loss of reproductive ability. *Per os* infection experiments with contaminated blood meal indicated that feeding was most likely the natural route of infection within our laboratory colony.

The majority of ticks infected in both experiments mentioned above survived and displayed no evident symptoms of the infection. The presence of yeast cells in the haemolymph of surviving ticks was not detected. Thus it seems that the resistant ticks possessed an efficient mechanism that made it possible to clear out the yeast cells from their haemolymph.

The *in vitro* phagocytosis assay performed with FITC-labelled yeast cells showed that about 4% of tick haemocytes were phagocytically active against the pathogenic yeast cells (Fig. 4). Thus phagocytosis seems to be a potent defence reaction against spreading and multiplying of the yeast *C. haemulonii* within the tick haemocoel.

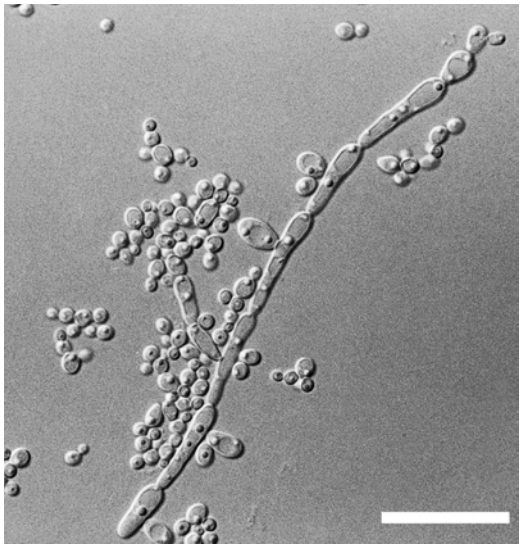

Fig. 1. *Candida haemulonii* (pseudomycelium), Nomarski differential interference contrast. Scale bar = 10 μm.

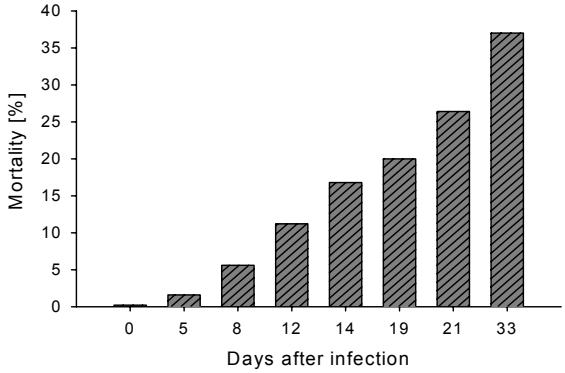

Fig. 2. Cumulative mortality of the adult females of *Ornithodoros moubata* caused by *Candida haemulonii* on various days after experimental intrahaemocoelic infection (2.8×10^9 cells/ml).

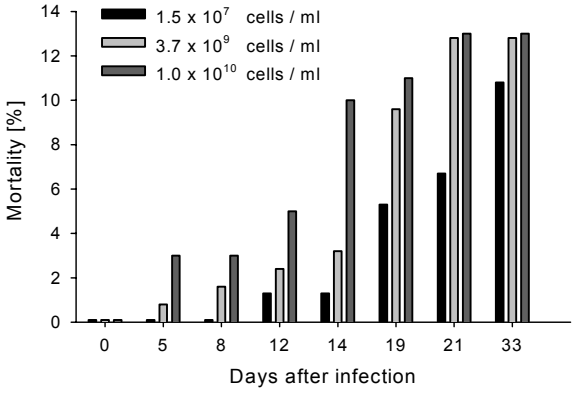

Fig. 3. Cumulative mortality of the adult females of *Ornithodoros moubata* caused by different doses of *Candida haemulonii* on various days after infection *per os.*

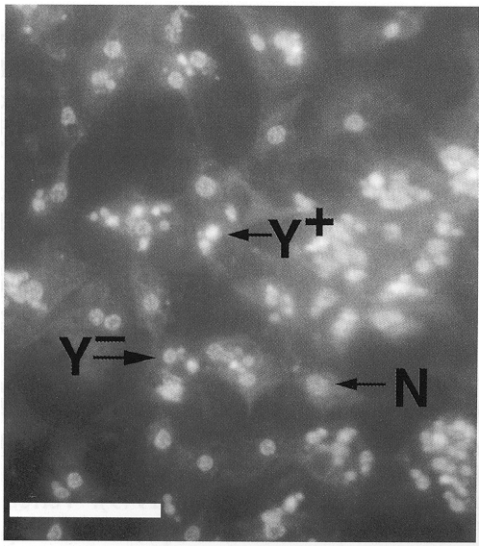

Fig. 4. Micrograph of *Ornithodoros moubata* haemocytes after phagocytosis of FITC-labelled yeast cells. Fluorescence. Ingested yeast cells retained their fluorescence after quenching with trypan blue. Non-ingested cells became dark. N – nucleus of haemocyte, stained by DAPI-solution; Y^+ – ingested yeast cell; Y^- – non-ingested yeast cell. Scale bar = 20 μm.

DISCUSSION

Studies on naturally occurring pathogens of ticks have been done in an attempt to establish their importance in regulating natural tick populations (Mwangi et al. 1995). Except for the entomopathogenic fungi, little attention has been paid to the other microorganisms pathogenic for ticks.

Sidorov and Scherbakov (1973) reported viral infection in laboratory colonies of *Ornithodoros moubata*, *O. pavlovskyi*, *O. lahorensis* and *Argas persicus*. Virus-like particles caused ulcers in different body areas, particularly deformities of the mouthparts. A few species of bacteria have been reported to be pathogenic for ticks. Brown et al. (1970) noted an endemic disease among laboratory populations of the hard tick *Dermacentor andersoni*. The causative agent was isolated from these dead ticks and identified as the bacterium *Proteus mirabilis*. Four Gram-negative bacteria (*Proteus* sp., *Klebsiella pneumoniae*, *Pseudomonas* sp., *P. mirabilis*) and one Gram-positive bacterium (*Staphylococcus* sp.) were isolated from the laboratory colonies of the hard tick *Boophilus decoloratus* (Hendry and Rechav 1981).

Most organs of laboratory-bred *O. moubata* contain rickettsia-like microorganisms. Weyer (1953) succeeded in isolating *Coxiella burnetii* from digested host blood and coxal fluid of parenterally inoculated *O. moubata.* Multiplication of *Rickettsia australis* was observed in the haemolymph of the same tick species (Řeháček 1965). Reinhardt et al. (1972) recorded the presence of long rickettsia-like microorganisms in all examined organs of both sexes of *O. moubata* with the exception of spermioduct and the testicles.

The entomopathogenic fungi play a major role in reducing natural populations of ticks. Natural infection of hard ticks by entomopathogenic fungi was recorded by Lipa (1971), Samšiňáková (1957), Samšiňáková et al. (1974) and Estrada-Peña et al. (1990). Many ixodid ticks were found to be infected with several species of fungi, belonging mostly to the group of Fungi Imperfecti (*Aspergillus, Beauveria, Fusarium, Paecilomyces, Verticillium*). Krylova (1972) isolated from the argasid tick *Argas persicus* the fungi of the genera *Penicillium* and *Aspergillus*.

The species of the genus *Candida* are not often reported as entomopathogens. *Candida zeylanoides* has been identified as a pathogen in Douglas-fir tussock moths, *Orgyia pseudotsugata* (Martignoni et al. 1969) and in *Blatta orientalis* (Rozoni 1949). Verrett et al. (1987) described a new *Candida* species to be a parasite of the American cockroach *Periplaneta americana*. El Said (1992) investigated yeast-like microorganisms associated with the sperm cells of the ixodid ticks *Hyalomma marginatum*, *H. dromedarii* and *Amblyomma hebraeum*. These microorganisms represented extracellular symbionts, produced by male and introduced in the genital system of the female through copulation. They occurred in the form of compact spherules and morulae that did not alter their shape and did not attach to the sperm cells. In contrast to this observation, Feldman-Muhsam and Havivi (1962) described microorganisms, that attached to a specific zone of the sperm cells in the spermatophores of the soft tick *Ornithodoros tholozani.*

Candida haemulonii was originally isolated from the gut of the bluestriped grunt fish *Haemulon sciurus* and to date has not been reported to be part of natural microbial flora of the soft tick *O. moubata.* Our experiments demonstrated the possible transmission of the pathogen via contaminated bovine blood meal. In order for the pathogen to kill susceptible ticks, it is apparently necessary for the yeast particles to overcome the intestinal barrier and penetrate into haemolymph. From the results presented in this paper it is obvious that isolated haemocytes are phagocytically active against yeast cells *in vitro*. The phagocytic activity of non-stimulated tick haemocytes against *C. haemulonii* was relatively high (4%). For comparison, less than 1% of nonstimulated insect haemocytes (*Galleria mellonella*) were phagocytically active against cells of the yeast *Saccharomyces cerevisiae* (Wiesner et al. 1998). It remains an unresolved issue if the intestinal barrier and/or immune response within the haemocoel are compromised in the ticks susceptible to candidiasis. Further research is in progress about the mechanisms involved in tick defence against the pathogenic yeast *C. haemulonii*.

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