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Role of *Amblyomma lepidum* in the transmission of *Mycobacterium farcinogenes*, the causal agent of Bovine farcy

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ABSTRACT

The present study was conducted to investigate the role of *Amblyomma lepidum* in the transmission of *Mycobacterium farcinogenes*, the causal agent of bovine farcy in Sudan. A total of 22 samples comprising of prescapular, submaxillary, mediastinal, parotid, retropharengeal and pulmonary lymph nodes, and subcutaneous nodules were collected for this study. *M. farcinogenes* was successfully isolated from the specimens by following traditional bacteriological techniques, and the bacteria were identified using standard biochemical assays, lipid analysis using Thin Layer Chromatography, and duplex Polymerase Chain Reaction. Larvae and nymphs of *A. lepidum* acquired *M. farcinogenes* from experimentally infected rabbits, and subsequently transmitted the infection to fresh rabbits within a maximum period of ten weeks. In contrast, adult *A. lepidum* failed to contract the organism from the infected rabbits; so, trans-ovarian transmission could be ruled out. In conclusion, this study confirmed the trans-stadial transmission of *M. farcinogenes* through larvae and nymphs of *A. lepidum*.

Keywords

Amblyomma lepidum, *Mycobacterium farcinogenes*, Rabbit, trans-stadial transmission, Sudan

ARTICLE HISTORY

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INTRODUCTION

Bovine farcy (BF) is a chronic granulomatus inflammation of the skin lymphatic and draining lymph nodes of cattle. The disease has been reported in 19 different countries of Asia, Africa, Latin America and Caribbean with tropical and subtropical climates, but the disease is dominant mostly in the sub-Saharan African countries (Hamid, 2012). The causative agent of BF was first isolated, and was described in 1988 (Nocard, 1988). During the last 50 years, there were reports of BF infections from Chad, Mauritania, Senegal, Ghana, Nigeria and Somalia (Diguimbaye-Djaibé et al., 2006; Alawa et al., 2011; Hamid, 2012). In Sudan, BF was confirmed in Kordofan and Darfur States where both *A. lepidum* and *A. variegatum* were reported (Mohammed et al., 2005). Hamid et al. (1991) conducted a survey among 14,192 cattle belonging to 17 different herds of Baggara cattle in one region of South Kordofan, and reported an average 21.2% occurrence of BF. In addition, out of 200 caseated lymph nodes of slaughtered cattle collected during routine meat inspection at Omdurman, Sudan, 9% showed *Mycobacterium farcinogenes*, whereas 5.5% revealed *M. bovis* (Osman, 2007). Epidemiological data have not reported BF in wild and other domestic animals (Hamid, 2012).

The role of ticks in the transmission of BF is controversial. Transmission of BF by ticks in field condition has not been completely established. However, it has long been said in the Sudan that the ticks might be involved in the transmission process. Some authors associate BF with tick infestation (notably the ixodid tick, *A. variegatum*) (Hamid, 2014).

In addition, AL-Janabi et al. (1975) could transmit *N. farcinica*, which was believed at that time to be the causal agent of bovine farcy, from experimentally infected rabbit to a control one via *A. variegatum*. This study was aimed to investigate the epidemiological role of the tick *A. lepidum* in the transmission of *M. farcinogenes* in cattle.

MATERIALS AND METHODS

Collection of specimens: Twenty two specimens comprising of prescapular, submaxillary, mediastinal, parotid, retropharengeal and pulmonary lymph nodes as well as subcutaneous nodules were collected from Northern and Southern Kordofan States and from the Sabaloga abattoir, West Umdorman, Khartoum State during the period from January to February, 2007. The specimens were sent to laboratory in sterile plastic bags in ice box for bacteriological analysis.

Histopathological findings: The collected lymph nodes (approximately 1 gram each) were trimmed, grounded and treated with 2% Sodium hydroxide (NaOH). Then, the suspension was strained, and incubated at room temperature for 30 min. The mixture was centrifuged at 3000 rpm for 15 min. The sediment was resuspended and washed twice in sterile distilled water, and centrifuged again as above. The sediment was then used for preparation of smears for the detection of acid fast bacilli by Zeil Neelsen (ZN) stain. From the suspended sediment, 20 mL was inoculated on Lowenstein Jensen (LJ) medium, and incubated at 37°C for 5-7 days.

Mycolic acid profile of the isolates: The isolates designated as M10, M13 and M16 in addition to the re-isolated strain from rabbits, and the reference strain M39 (positive control) were subjected to analyze with thin layer chromatography (TLC) to delineate their mycolic acid profiles, as per the method of Minnikin et al. (1975). In brief, 5 mg of dried biomass of the strains M13, M10 and M16 in addition to a re-isolated strain and reference strain (M39) were placed in separate caped corning tubes. One mL of methanol, toluene and concentrated H_2SO_4 mixture (30:15:1; v/v) was added and heated overnight at 75°C. The preparation was cooled and shacked after addition of 1 mL petroleum ether. The upper layer, that contained the fatty acid esters, was discarded while the lower was evaporated to become dry at 37°C.

Infection of rabbits: Two rabbits (R1 and R2) were injected intravenously with *M. farcinogenes* (M13) at a concentration of $1.5x10^8$ CUF/mL. Strict aseptic measure was taken before and after the inoculation. The rabbits were injected with betamethasone dosed at 0.5 mL/animal for four days subcutaneously as an immunosuppressant. Once the infection of the rabbits with *M. farcinogenes* was observed and confirmed in their blood, larvae of *A. lepidum* were applied to the left and right ears of both R1 and R2 according to the method described by Bailey (1960). Each ear was infested by about 150 larvae, from which about 30 engorged larvae were taken after dropping, that were washed in 70% alcohol, crushed, and decontaminated according to Al-Janabi et al. (1975), and finally examined for the presence of *M. farcinogenes* by smear and culturing on LJ medium. The same process of detection of the causative agent was applied to the molted nymphs.

Trans-stadial transmission:

Larval-Nymph transmission: Emerged nymphs were used to transmit the infection to fresh rabbits. Fifty nymphs were applied to the left ear of each fresh rabbit (Ra, Rb and Rc). Betamethazone was injected as previously described. The right ear was left plain for blood sampling. Nymphs completed engorgement within 7-9 days. Within this period, the rabbits were bled daily from the right ear vein until the first appearance of the organism (day 14). The blood samples were subjected to a series of smearing and culturing procedures to detect the presence of *M. farcinogenes*.

Nymph-Adult transmission: A total of 150 non-infected nymphs were applied to the left and right ears of each infected rabbits (R3 and R4). Betamethazone treatment was applied as previously described. The nymphs completed engorgement within 7-9 days and molted to adults in 16-30 days. To confirm that the adults were infected with *M. farcinogenes,* a random sample of 10 ticks were washed in 70% alcohol for 30 min, ground up, and decontaminated according to the method described by Al-Janabi et al. (1975). The sediment was used for direct smearing and cultivation on LJ medium, and incubated at 37°C for 5-7 days. After hardening, 4 males were applied to the left ear of each rabbit (Rd, Re, Rf and Rg). This was followed by the application of 4 females 4 days later, and betamethazone was injected as before. The right ear was left free for blood sampling. The blood samples were smeared and cultured on treptose phosphate broth (TPB) and LJ medium.

Trans-ovarian transmission: Total 12 females and 12 males of non-infected *A. lepidum* were applied to the left and right ears of *M. farcinogenes* infected rabbits (R5, R6 and R7). Engorgement was completed within 16-30 days. When dropped, the *A. lepidum* was incubated at 27°C and 90-95% relative humidity for oviposition. Egg clusters were crushed, decontaminated with 2% NaOH solution, centrifuged at 3000 rpm for 15 min, and washed with distilled water. The sediment was used for direct smears and culture on TPB and LJ medium to confirm the presence of *M. farcinogenes*. This process was repeated weekly for three weeks.

Duplex polymerase chain reaction (PCR) was used for the confirmatory identification of *Mycobacterium*, using the primers and methods described by Hamid (2012).

RESULTS AND DISCUSSION

Isolation and identification of *Mycobacterium farcinogenes*: Four strains of *M. farcinogenes* were isolated and designated as M10, M13, M16, and the reisolated strain of M13. They were found to be identical to the reference strain (M39) when subjected to a series of biochemical tests such as oxidase, catalase, urease, nitrate reduction tests and fermentation of glucose, fructose and sucrose. Oxidase was negative while all other biochemical tests were positive for all isolates. According to Hamid (2014), many actinomycetes showed similar biochemical results so further confirmation methods were carried out.

Mycolic acid analysis: The TLC analysis revealed the presence of dark spots for the investigated strains. These dark spots represented the presence of α, α′ and epoxymycolate methyle esters that were specific for *M. farcinogense*. Mycolate esters from the field strains were found to be identical to that of the reference strain of *M. farcinogenes* (**Figure 1**).

Duplex PCR: The amplicons obtained from the PCR analysis showed 235-bp band for the *M. tuberculosis* reference strain (positive control) and 136-bp for the tested isolates which are represented here by the *M. farcinogenes* isolates as well as the reference strain used (**Figure 2**).

Trans-stadial transmission:

Nymph stage transmission: Nymphs emerging from experimentally infected larvae contracted the *M. farcinogenes* infection and transmitted the infection to the fresh rabbits (Ra, Rb, and Rc) from day 14th up to 25th day. These results were confirmed by the growth of the organism on LJ medium (**Table 1**).

Adult stage transmission: Clean nymphs contracted the infection from the infected rabbits. When molted within 26-34 days, 10 of the emerged adults were ground and cultured on TPB medium, where growth of *M. farcinogenes* was obtained. The remaining adult ticks were allowed to feed, where they transmitted the infection to the rabbits (Rd, Re and Rf) from the day 19 onwards. The rabbit Rg died before being subjected to blood sampling (**Table 2**).

Trans–ovarian transmission: The eggs produced by the infected ticks were found to be negative for *M. farcinogenes* after culturing on TPB and LJ media slants for up to three weeks.

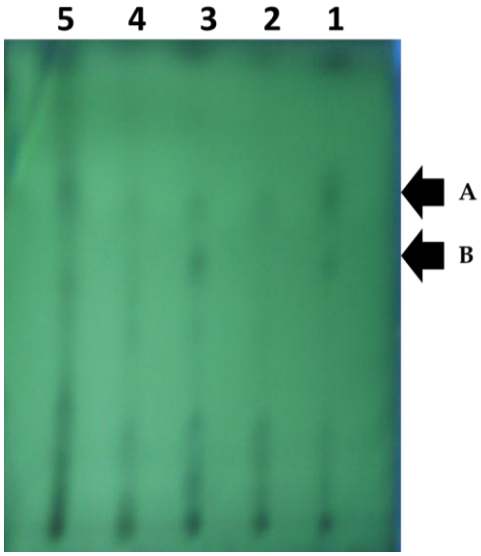

Figure 1. Thin layer chromatography (TLC) of mycolic acid of *M. farcinogenes* showing A = (α, α′) and B = (epoxymycolate methyl ester). *Lane 1* = strain M39 (reference strain), *lane 2* (control negative), *lane 3* = *M. farcinogenes* (strain M13), *lane 4* = strain M16 and *lane 5* = M10.

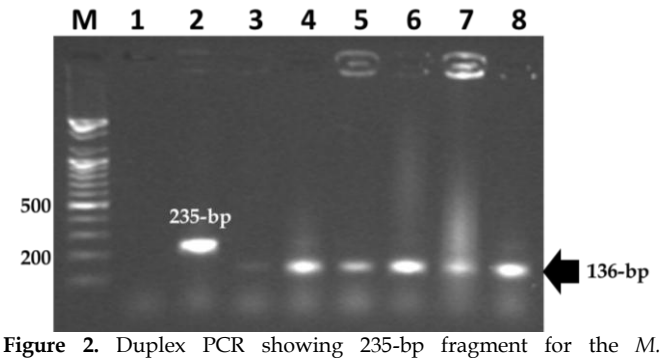

Figure 2. Duplex PCR showing 235-bp fragment for the *M. tuberculosis* reference strain (Positive control) and 136-bp for the NTM (*M. farcinogenes* M10, M13, M16 and reference strain M39). *Lane M* = Molecular marker, *Lane 1* = negative control, *lane 2* = *M. tuberculosis*, *Lane 3* = *M. paratuberculosis*, *lane 4* = reference strain M39, *lane 5* = M16, *lane 6* = M.13, *lane 7* = M10 and *lane 8* = re-isolate M13.

Table 1: Transmission of *M. farcinogenes* by nymphs of *A. lepidum* ticks

Experimental Rabbits	No. of infected nymph	Period of engorgement (days)	Period of moulting (days)	Date of blood sampling	Infection
R (a)	50	8 - 11	13 - 20	14 day	+
R (b)	50	8 - 11	14 - 19	14 day	+
R (c)	50	7 - 11	15 - 21	14 day	+

* = Rabbit received dexamethasone dosed at 0.5 mL/animal.
R = Rabbit (a, b and c)

Table 2: Infection of rabbits with *M. farcinogenes* by the adult ticks of *A. lepidum*

Experimental rabbits	No. of ticks (male+female)	Period of engorgement	Date of blood sampling	Infection
R (d)	4 + 4	16-22	Day 19	+ ve
R (e)	4 + 4	18-20	Day 19	+ ve
R (f)	4 + 4	17-30	Day 19	+ ve
R (g)	4 + 4	-	-	Died

* = Rabbit received dexamethasone dosed at 0.5 mL/animal.
R = Rabbit (d, e. f and g).

Pathological findings in rabbits:

Macroscopic appearance: Autopsy of two infected rabbits with *M. farcinogenes* after 90 days revealed the presence of profuse, creamy-white pus in the lymph nodes. Necrosis was observed in the kidneys, spleen, liver, and lungs.

Macroscopic appearance of infected lymph nodes showed enlargement, caseation and tuberculous nodules. Histopathological examination of the lymph nodes and subcutaneous nodules of cattle infected with bovine farcy, revealed a severe granomulomatous reaction. A central zone of caseation and necrosis surrounded by an inflammation zone was clearly marked. The central zone comprised different types of inflammatory cells mainly neutrophils, lymphocytes, macrophages and giant cells that were surrounded by a thick capsule showing proliferation of fibroblast.

Histopathological findings of rabbit tissue sections showed inflammatory changes. In the lungs; emphysema, thickening of alveoli septa with hemorrhages and inflammatory cells especially lymphocytes and plasma cells were seen. Degenerative changes of tubular cells and shrinking of the glomeruli were observed in the kidneys. Prominent necrosis, degeneration and dilatation of liver sinusoids were encountered in the liver sections.

The present study was investigated the role of *A. lepidum* in the transmission of *M. farcinogenes* using rabbits as experimental animals. Rabbits were selected as a model in these experiments; since they were recommended as suitable hosts for tick feeding. The idea of this work emerged from suggestions of many investigators. In addition, the experimental demonstration of trans-stadial transmission of the pathogen via *A. variegatum* was confirmed by Al-Janabi et al. (1975). There might be other risk factors that enhance the pathogenicity of the causative agent of BF. However, the risk factors of BF are poorly investigated (Hamid, 2012).

M. farcinogenes was successfully isolated from the collected specimen following the standard bacteriological procedures. The bacteria were identified using standard biochemical assays, lipid analysis through using TLC techniques as well as molecular techniques represented by Duplex PCR to confirm the results. The amplicons obtained from the PCR analysis were 136-bp for the tested isolates which are represented here by the *M. farcinogenes* isolates as well as the reference strain used.

Extraction and analysis of mycolic acid was proved to be of value in confirming the identity of the isolates as members of the genus *Mycobacterium*. TLC analysis of mycolic acid patterns for the reference strain of *M. farcinogenes* and the isolates showed dark spots that were typical to those obtained by Hamid et al. (1993), El Hussein (2002), and Eiman (2003); this indicated the presence of α, α′ and epoxymycolates. The obtained results confirmed the presence of α, α′ and

epoxymycolates in the mycolic acid of *M. farcinogenes*, the causal agent of BF. Many species of the genus *Mycobacteria* namely *M. farcinogenes, M. senegalense, M. fortuitum, M. peregrium, M. smegmatis, M. chitae* and *M. porcinum* produce the mycolic acid patterns of α, α' and epoxymycolates (Luquin et al., 1991). From these species only *M. farcinogenes* and *M. senegalense* revealed branching filaments (Hamid, 2014). Hamid et al. (1993) used the test of glycolipids on TLC to differentiate between these two species, because differentiation between these two species was found to be difficult when using morphological characteristics.

Histopathological examination was performed for the collected bovine specimens as well as for rabbits after experimental infection. Histipathology findings were in agreement with those described by Al-Janabi et al. (1975).

The present study demonstrated the trans-stadial transmission of *M. farcinogenes* through the developmental stages of the *A. lepidum* ticks. The organism was transferred from the larvae to the nymphs and subsequently the infection was experimentally established in the rabbits. The same process was repeated, where the nymphs conveyed the organism to adults that produced the infection in the rabbits. The eggs produced from clean females of *A. lepidum* fed on infected rabbits were confirmed free from the infection when cultured on LJ medium. This finding indicated that *M. farcinogenes* is not transmitted by the trans-ovarian route of transmission. This result was in agreement with Al-Janabi et al. (1975) who reported that *A. variegatum* did not pass the infection through this route.

To the best of our knowledge, this is the first report confirming the role of *A. lepidum* in the transmission of *M. farcinogenes* infection from experimentally infected rabbits in Sudan.

The immunosuppressant drug (Betamethazone) was used in these experiments with a view to reduce immunity and to raise the pathogen level in the blood. The maximum period between acquisition of infection and transmission of *M. farcinogenes* by *A. lepidum* was about ten weeks in this experimental trial but it is presumed that this interval is potentially very much longer.

During the course of the current study, the feeding trails showed that *A. lepidum* females attached only in juxtaposition with the males which had been feeding for at least 5 to 7 days. This behavior elongated the feeding period of adult ticks and may in turn play a role in the slower rate of transmitting the infection by the adults.

CONCLUSION

The nymphal and adult stages of *A. lepidum* ticks can acquire *M. farcinogenes* infection from experimentally infected rabbits, and subsequently transmits the infection trans-stadially to fresh rabbits within a maximum period of ten weeks. Further studies are warranted to investigate the role of other tick species in transmission of Bovine farcy.

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