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Pathogenicity of *Mycoplasma bovis* isolates from some states in Nigeria inoculated intramammarily into lactating New Zealand white rabbits

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Abstract

The focus of this study was to test the pathogenicity of *Mycoplasma bovis* isolated from cases of clinical mastitis in cattle in some states of Nigeria. Pathogenicity study of *Mycoplasma bovis* was carried out using five New Zealand White lactating rabbits, obtained from a local breeder in Ibadan, Nigeria. The left mammary glands of each rabbit were inoculated intracisternally with 1ml of 2.4 x 10^5 cfu/ml of *Mycoplasma bovis* obtained from cases of clinical mastitis in Nigeria. The right mammary glands served as control and received 1 ml of sterile Tryptose Soy broth each. The clinical signs, post-mortem and histological findings were recorded. Clinically, the rabbits were weak and anorexic with mortalities. Grossly, lesions were observed in the spleens, lungs and the ovaries, while the mammary glands were atrophied. This clearly showed the septicaemic nature of the *Mycoplasma bovis* isolated from the clinical mastitis cases hence proper hygienic practices should be implemented during milking for public health reasons.

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Introduction

Mycoplasma bovis infection causes huge financial losses and health effects in animal production especially in the cattle industry (Nicholas & Ayling, 2003) and increases the utilization of antibiotics. Different sources of *Mycoplasma bovis* infection such as artificial insemination (AI) (Wrathall *et al*., 2007), contaminated equipment and environment (Piccinini *et al*., 2015), embryo transfer (Bielanski *et al*., 2000) and contact with other animal species (Spergser *et al*., 2013) have been described. Animal contact has been identified as the principal means of this infection and the key factors responsible for infection are connected to animal movement, animal purchase and animal fairs (Aebi *et al*., 2015). A diversity of clinical signs such as bronchopneumonia, mastitis and arthritis (Byrne *et al*., 2001), otitis media (Maeda *et al*., 2003), genital disorders (Hermeyer *et al*., 2012). meningitis (Ayling *et al*., 2005), or keratoconjunctivitis (Alberti *et al*., 2006) are usually observed in this infection. The evasion of host immune response and mechanism of tissue damage by this organism are not clearly comprehended (Caswell & Archambault, 2007). Even though, the chronicity of pneumonic lesions and the persistence of *M. bovis* indicate an

inadequacy in eradicating the pathogen (Gagea *et al*., 2006), potential virulence factors of *M. bovis* are related to variable surface membrane proteins (Maunsell *et al*., 2011). In clinical and subclinical mycoplasma mastitis, the milk of affected animals usually contains the organism at concentrations of $\geq 10^8$ and $\leq 10^6$ cfu/ml respectively (Byrne *et al*., 2005). Calves become infected with *M. bovis* through ingestion of infected milk from their mother (Maunsell *et al*., 2012). Elimination of this organism is problematic due to its contagious nature, role of subclinical carrier animals and unresponsiveness to antibiotic therapy (Maunsell *et al*., 2011). Studies on clinical isolates of mycoplasma associated with mastitis had been described (Maunsell *et al*., 2012) with little information on the pathogenicity of those isolates in laboratory animal models. This study was carried out in rabbits to test the pathogenicity of *Mycoplasmas bovis* isolated from cases of clinical mastitis in Nigeria.

Materials and Methods

Milk samples were collected from the mammary gland of 200 mastitic cow located in Ibadan (Southwest), Ilorin (Northcentral) and Kaduna (Northwestern Nigeria). The breeds of cows sampled in this investigation were Kuri, Red Bororo, White Fulani, Friesian cross with White Fulani and Sokoto Gudali. These animals were managed under-semi intensive system. Milk samples were obtained aseptically from the affected udders and the initial stream of fore-milk was discarded. About 5ml of milk from each mastitic cow was collected respectively into a sterile bottle. Immediately after collection, two sterile swabs were used to collect the mastitic milk from each sample bottle. One swab was inoculated into mycoplasma broth and the second into Tryptose Soya broth (TSB). Samples were transported on ice packs to the Department of Veterinary Microbiology and Parasitology, University of Ibadan, Ibadan for bacteriological analysis.

The broth cultures were incubated in air at 37°C. A liquid to solid culture technique was used (Freundt, 1983). After 24h inoculation of TSB culture and 72h for mycoplasma broth culture, subcultures were made onto blood agar, MacConkey agar from (TSB) and Mycoplasma medium N from mycoplasma broth culture (Ernϕ & Stipkovits, 1973) with a slight modification; glucose was omitted because glucose favours the growth of contaminating organisms that may be present in the samples. The blood agar and MacConkey agar were incubated aerobically at 37°C for 24h. The colonies were examined and identified bacteriologically according to standard methods (Barrow & Feltham, 1993). The mycoplasma agar plates were incubated in 5-10% CO_2 at 37^0C in an anaerobic candle jar.

The plates were examined using dissecting microscope, up to the 10th day, for isolation of mycoplasma. The suspected colonies were tested for sensitivity to digitonin and sensitive isolates were purified by cloning (Ernϕ & Stipkovits, 1973). The mycoplasma isolated were further analysed biochemically and serologically according to standard methods (Poveda & Nicholas, 1998). The *mycoplasma* isolates were identified by growth inhibition test (GIT) using *an*tisera produced in rabbits against *M. bovis.* (Poveda & Nicholas, 1998)

A modification of the method used by Chandler (1970) was employed to determine the colony forming unit of each microorganism species isolated, including *Mycoplasma bovis*. Colonies were inoculated in mycoplasma broth. After 48 hours of incubation in mycoplasma broth, the test isolates were centrifuged at 2000g for 30 minutes. The supernatants were discarded and 0.1 ml of the well-mixed deposit was added to 0.9 ml of mycoplasma broth to make a 1 in 10 dilution. From this, other 10-fold dilutions were made to give (10^2, 10^3, up to 10^{14}). These dilutions were subsequently cultured on Mycoplasma agar to determine the number of respective colonies present in 1.0ml of the original sample.

From the results of the preparation of inocula, colony-forming units were observed only at the first 5 dilutions (1 in 10 to 10^5). Of these, 1ml of 10^3 showing 2.4x 10^5 colony-forming unit per ml was used. The mammary glands on the left-hand side of the animals in cages 1-5 were inoculated with the *Mycoplasma bovis* organism under investigation. The right-hand side nipples were used as control. The same volumes of sterile broth were inoculated into the right-side nipples of the mammary glands of the same animals.

Detailed systemic post-mortem examination was conducted on all the infected organs. The gross pathologic lesions were recorded. Tissue samples from lungs and enlarged spleen were collected in 10% neutral buffered formalin for histopathological examination.

Tissues obtained from post-mortem were inoculated onto Mycoplasma broth. These were subsequently sub cultured onto Mycoplasma agar, and incubated at 37^0C anaerobically for possible recovery of the organisms.

Results and Discussion

All the screened rabbits' milk sampled before inoculation were negative for *Mycoplasma* organism. The agar surface was sterile.

From the five lactacting female rabbits inoculated with overnight broth culture of *Mycoplasma bovis,* three rabbits died within 48 hours after inoculation with the signs of weakness, anorexia, and prostration. All the inoculated nipples were atrophied. The remaining two rabbits died within 96 hours of inoculation.

The mammary glands were atrophied, the spleens were enlarged and congested. The lungs were also congested while the uterine horns and the ovaries were haemorrhagic.

The lungs of rabbits infected with *Mycoplasma bovis* showed interstitial pneumonia characterized by distension of interalveolar septa due to oedema, fibrin and cellular infiltration. The spleen showed marked lymphoid depletion. The mammary gland; teats and cistern were all atrophied while there were haemorrhages in the uterine horn and the ovaries.

Mycoplasma bovis isolate was recovered from the tissues of the inoculated rabbits. The finding in this study agree with Koch's postulate that the organism can be recovered in pure culture from experimentally infected animals (Koch, 1882).

This investigation has been able to elucidate the pathogenicity and virulent roles of *Mycoplasma bovis* in clinical mastitis. These pathogens produced serious effects such as death of the rabbits within 24 hours to 96 hours of inoculating the pathogens into the rabbits. This is in agreement with an earlier study by Nicholas & Ayling (2003) who observed various effects caused by *Mycoplasma bovis* on animal health. Although the mechanisms leading to tissue damage and how *M. bovis* induces disease in their host are incompletely understood (Caswell & Archambault, 2007), previous studies have shown that experimental infection of animals with *M. bovis* has both stimulating and suppressing effects on the host immune response (Wiggins *et al*., 2011). Grossly, the spleens of the infected rabbits were enlarged; the lungs were congested and consolidated while the spleen in all cases showed marked lymphatic depletion. These findings can be explained by initial similar experiments which stated that there is hematogenous spread of *M. bovis* from lungs to other organs such as spleen, uterine horns and ovaries (Caswell & Archambault, 2007). This statement is further confirmed histopathologically by the atrophy of the mammary glands and the haemorrhages observed in the ovaries. These findings indicated that the pathogens were virulent and should the milk contaminated by these pathogens be consumed by calves or kids they may cause lesions and death in the consumer if the milk is not properly pasteurized.

Thus, it is suggested that lactating cows should be kept in hygienic environment. Good functioning milking machines should be used for milking. The milking parlour should be clean and should be disinfected to prevent pathogens from contaminating the teat and udder. Milkers should wash their hands, care should be taken that they do not introduce pathogens into the teats and udder during hand milking.

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Conflicts of Interest

The authors declare no conflict of interest.

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