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# Pathological changes in the mammary gland of rabbits following intra-mammary inoculation of *Mycoplasma agalactiae*

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## Abstract

Contagious agalactia is a disease predominantly of milking sheep and goats caused mainly by *Mycoplasma agalactiae*, clinically manifested as mastitis, arthritis, keratoconjunctivitis and pneumonia. Pathological changes in the mammary gland of rabbits following intra-mammary inoculation of *M. agalactiae* were studied. Clinical mastitis developed within 24 hpi. It was initially acute but became chronic by the end of the experiment at 9 dpi. The disease was characterized by atrophy of the infected mammary glands, resulting in marked agalactia. Histopathology revealed that the mastitis was acute and purulent initially, followed by infiltration of mononuclear cells with fibroplasia in the interacinar tissue, and later by massive fibrosis by 9 dpi.

**Key words: Contagious agalactia, Mycoplasma agalactiae, mastitis, rabbit, mammary gland**

## Introduction

Contagious agalactia (CA) is a disease of sheep and goats caused by mycoplasmas, which is clinically manifested as mastitis, arthritis, keratoconjunctivitis and pneumonia. *Mycoplasma agalactiae* is the main causal organism of CA (Nicholas, 1996). Contagious agalactia is a disease predominantly of milking sheep and goats herd usually in the spring season soon after lactation. The young ones get infected directly at sucking while the adults are contaminated via the milker's hands, milking machines or by bedding material which often provides a rich source of mycoplasmas. The CA infection frequently occurs as an enzootic disease. In lactating female animals, it is usually manifested as mastitis, whereas in males, young animals and non-lactating females, the disease is manifested as arthritis, keratoconjunctivitis and respiratory problems. Contagious agalactia contributes to significant losses in small ruminants. The disease has been estimated to cause annual losses around US$ 30 million in European countries especially the Mediterranean region, primarily as a result of decreased milk production, mortality of young ones, pneumonia and abortions (Nicholas, 2002). Considering the importance of the disease, the suitability of rabbit as a model to study mastitis induced by *M. agalactiae* was evaluated in the present study.

# Materials and methods

## Inoculation

One *Mycoplasma agalactiae* isolate from sheep (S2) and one *Mycoplasma agalactiae* isolate from goat (G4), isolated from mastitic cases were used for experimental studies in rabbits. *M. agalactiae* was cultivated in PPLO broth for 3- 4 days and organisms were pelleted at 12000 rpm for 10 mins in a refrigerated centrifuge. A loopful of pellet was suspended in PBS and the suspension was adjusted to a final concentration of $10^7$, $10^8$ and $10^9$ cfu/ml using McFarland standards.

## Experimental animals

Six female rabbits in 2nd or 3rd lactation were procured from a reputed breeder and the animals were housed in cages under standard husbandry conditions during the experiment. They were provided standard laboratory animal feed and water *ad libitum*. The approval of the Institutional Animal Ethics Committee was obtained prior to start of the experiment. Out of six rabbits, five rabbits were inoculated with *Mycoplasma agalactiae* and one served as control.

## Experimental design

Before infecting, the lactating animals were exhaustively milked by squeezing the mammary gland from the base of the teat. The two *Mycoplasma agalactiae* isolates were inoculated to five rabbits with 0.5 ml bacterial suspension (in PBS) at the base of the teat by using 30 G needle. The sixth rabbit was kept as control. The bacterial count v/s different mammary glands used for induction of mastitis in rabbits were as follows:

| Mammary gland | Left side | Right side |
|---|---|---|
| I | Un-inoculated | Un-inoculated |
| II | 0.5 ml PBS only | 0.5 ml PBS only |
| III | 0.5 ml suspension of S2 ($1.5x10^7$ cfu/ml) | 0.5 ml suspension of G4 ($1.5x10^7$ cfu/ml) |
| IV | 0.5 ml suspension of S2 ($1.5x10^8$ cfu/ml) | 0.5 ml suspension of G4 ($1.5x10^8$ cfu/ml) |
| V | 0.5 ml suspension of S2 ($1.5x10^9$ cfu/ml) | 0.5 ml suspension of G4 ($1.5x10^9$ cfu/ml) |

After inoculation, lesions in the mammary glands, if any, were recorded at 48 hrs intervals up to 9 days. The rabbits were carefully examined for the development of clinical signs of mastitis. One animal was sacrificed by humane method at the end of 24 hrs (Day 1) followed by sacrifice of one animal every 48 hrs up to Day 9. After thorough postmortem examination, the mammary glands were collected for histopathology.

# Results

In the present study, mastitis was induced in five rabbits by intra-mammary inoculation of two isolates of *Mycoplasma agalactiae*, one from sheep S2 and one from goat G4. Both the isolates were successful in inducing clinical mastitis in rabbits. The lesions encountered in the mammary glands infected with G4 isolate were more severe compared to the mammary glands infected with S2 isolate of *Mycoplasma agalactiae* even at 24 hours post-infection (hpi). The lesions in the glands infected with the different doses ($1.5x10^9$, $1.5x10^8$ or $1.5x10^7$ cfu/ml) of the two isolates did not differ much, but a dose dependent increase in the severity of lesions were recorded.

The signs of mastitis commenced as early as 24 hpi. Inflammation was observed in all the mammary glands inoculated with $1.5x10^9$, $1.5x10^8$ or $1.5x10^7$ cfu/ml of S2 or G4 isolates. The infected mammary glands of rabbits euthanized up to 5 days post-infection (dpi) were swollen, oedematous and hyperaemic (Fig. 1), and had a slightly yellowish viscous secretion which contained occasional white flecks. The rabbits evinced pain on palpation of mammary glands. The severity of these lesions gradually declined from 5 dpi. By 7 dpi, the infected glands were slightly atrophied than the un-infected control glands and contained more serous secretion. The infected gland of the rabbit killed at 9 dpi was shrunken and firm with a scant, yellow, viscous secretion. There was marked agalactia in the affected glands from 3 dpi onwards. In this study, both the isolates produced gross changes of similar intensity. The gross lesions produced by the different infective doses were similar. The control glands of all rabbits were normal throughout the study. The control glands of all rabbits injected with PBS were apparently normal on histopathological examination throughout the course of the experiment. The secretory acini were distended with eosinophilic secretory material. The intralobular and interlobular ducts were lined with cuboidal epithelium (Fig. 2). The interlobular septae were thin and the glands showed no evidence of inflammation.

Microscopically, mastitis was seen in the infected glands infused with S2 or G4 isolates of *Mycoplasma agalactiae*. The microscopical changes were similar and varied in magnitude and severity with the different isolates and at different doses of *Mycoplasma agalactiae*. The mammary glands of rabbits euthanized on Day 1 pi showed acute, diffuse purulent mastitis characterized by marked infiltration of heterophils in the lumina of acini and milk ducts. There was moderate vacuolar degeneration of secretory and ductal epithelia with exfoliation of the secretory and the ductal epithelium into the lumen. Hyperaemia, perilobular and interlobular oedema were also evident.

By 3 dpi, the intraluminal exudate was denser than at 24 hpi, which included a few macrophages and lymphocytes. A striking feature at 3 dpi was vacuolar degeneration and necrosis of the secretory epithelium. At 3 and 5 dpi the inflammatory reaction was severe. At this stage, some alveoli were filled exclusively with heterophils (Fig. 3), but most had mixed cellular components in the exudate consisting of macrophages,

lymphocytes, heterophils and occasional plasma cells. The epithelial lining of the acini were hyperplastic. There was multifocal lymphoid infiltration of the interstitium.

By 5 dpi, the acute mastitis subsided and progressed to a chronic mastitis which was characterized by fibrosis in the interlobular septa with infiltration of lymphocytes and macrophages in the interacinar interstitial tissue resulting in thickening of the septa. This resulted in decrease in the size of acini. Severe galactophoritis characterized by periductal fibrosis and stratified squamous metaplasia of the ductal epithelium was also observed.

On 7$^{th}$ day post infection, there was severe infiltration of lymphocytes and macrophages in the interstitial tissue with complete replacement of most of the acini and marked fibrosis resulting in thickening of interlobular septa (Fig. 4). Formation of calcified corpora amylacea was observed in the acini at this stage. The milk ducts showed chronic galactophoritis. Widespread alveolar involution, a diffuse lymphoid cell interstitial infiltration and prominent interstitial fibrosis were more conspicuous.

On 9$^{th}$ day post infection, the infected gland revealed progressive chronic mastitis than the earlier stages. The mammary gland revealed almost entire glandular involution. The interstitium was heavily infiltrated with lymphocytes and macrophages along with fibrosis (Fig. 5). Extensive interlobular and interacinar fibrosis resulted in pseudolobulation and atrophy of secretory lobules. At places, there were cystic dilatations of acini due to fibrosis around them. Lymphoid aggregates were present adjacent to some lactiferous ducts. Most of the glandular parenchyma was replaced by fibrous tissue with islands of small lobules (Fig. 6).

## Discussion

There was marked agalactia in the affected glands from 3 dpi onwards. This might be attributed to the fact that *Mycoplasma* has special affinity for secretory epithelial surface and that it gets firmly attached to the secretory epithelium of acini causing permanent damage by various mechanisms such as increase in local concentration of potent proteolytic enzymes, nucleases and other toxic metabolites like hydrogen peroxide ($H_20_2$) released by the mycoplasmas. Mycoplasmas may also cause cell damage by activating host mediators of inflammation. This damage to the secretory epithelium leads to marked decrease in the production of milk and subsequent agalactia with further damage to the epithelium (Razin *et al.,* 1998).

Due to paucity of literature regarding mastitis induction in rabbits with *M. agalactiae,* the results of the present study have been compared with the experimental mastitis in sheep or goat with mycoplasmas causing disease in small ruminants. This is justified by the findings of Anderson *et al.,* (1976) who compared the response to intramammary inoculation of 13 strains of mycoplasma in the mouse mammary gland and found that there was no histopathological evidence of any major difference in the mechanism of disease production with different mycoplasmal species. The lack of differences between the mycoplasma species was attributed to the short duration of infection in mice and because of the limited number of ways the mammary gland can respond to infection. The clinicolesional course of infection was characteristic of natural and experimental mycoplasmal mastitis (Rana *et al.,* 1992; Hasso *et al*., 1993; Reilly *et al.,* 1993; Bergonier *et al.,* 1997; Sanchis *et al.*, 2000; Castro-Alonso *et al.*, 2009). The present experiment also confirms the findings of previous experiments, in that the control glands remained uninfected, despite the theoretical possibility of transfer of infection by sucklings or by haematogenous spread (Reilly *et al.*, 1993, Kaur *et al.*, 1998). In contrast to this, Castro-Alonso *et al.,* (2009) have reported spread of infection to the contralateral uninoculated half of the udder.

In the present study, clinical mastitis developed within 24 hpi. It was initially acute but became chronic by the end of the experiment at 9 dpi. The findings of initial neutrophil response in the present study are in accordance with the findings of other workers in experimental mycoplasmal mastitis (Reilly *et al.,* 1993, Kaur *et al.*, 1998, Garg *et al.,* 2004, Castro-Alonso *et al.,* 2009) and indicate an unspecific innate immune response. Marked lymphocytic and macrophage infiltration seen after the subsiding of acute mastitis in the present study, have also been reported in experimental mycoplasmal mastitis (Kaur *et al.*, 1998, Garg *et al.,* 2004). These findings suggest that strong cell-mediated immune responses are directed against the invading Mycoplasma. Extensive fibrosis and the subsequent replacement of most of the secretory parenchyma seen from 5 dpi onwards, indicates the repair of the degenerated and atrophied tissue. This indicates that *M. agalactiae* induces permanent damage to the rabbit mammary gland resulting in agalactia.

Although specific protective defence mechanisms such as the production of anti-mycoplasma antibodies and stimulation of cell-mediated immunity are essential for resistance and protection against mycoplasmal infections, they also contribute to the development and exacerbation of mycoplasmal induced lesions and autoimmune responses (Howard and Taylor, 1985; Razin *et al.*, 1998; Rottem, 2003). In light of the previous reports and based on the results of the present investigation, it can be hypothesized that the experimental inoculation of *Mycoplasma agalactiae* in rabbits induces an acute, innate immune response that was unable to prevent initial colonization and spread of infection. This initial cellular immune response was followed by a specific humoral response that was able to reduce the severity of infection but unable to eliminate it completely, resulting in a chronic persistent infection.

Kaur *et al.,* (1998) stated that establishment of mycoplasma organisms and presence of histopathological lesions in mammary glands was the parameters for describing mastitogenic potential of organisms. The re-isolations of injected *Mycoplasma agalactiae* organisms from milk of the infected glands along with the occurrence of histopathological changes were suggestive of mastitis during the entire period of the experiment. Thus, it can be concluded that the mastitogenic capacity of the two isolates of *M. agalactiae* used in the present study has been well established.

Many workers have studied experimental induction of mastitis in various species using different mycoplasma organisms (Anderson *et al.,* 1976, Ball *et al.,* 1987, Taoudi *et al.,* 1987, Misri *et al.*, 1988, Rana *et al.,* 1992, Reilly *et al.*, 1993, Darzi et al., 1998, Kaur *et al.*, 1998, Garg *et al.,* 2004, Castro-Alonso *et al.,* 2009, Castro-Alonso *et al.,* 2010, De la fe *et al.*, 2010). These workers have used either ovine or caprine mammary glands for experimental induction of mastitis, except Kaur *et al.* (1998) and Garg *et al.* (2004) who have used rabbits as experimental model. Some workers have induced mastitis experimentally in goats with *Mycoplasma agalactiae* (Castro-Alonso *et al.,* 2009, Castro-Alonso *et al.,* 2010, De la fe *et al.*, 2010). This is the first study of experimental induction of mastitis in rabbits using *M. agalactiae.*

Further, based on the findings of the present study it is opined that the rabbit can serve as a model to study mastitis due to *M. agalactiae,* as there was no difference in the development of mastitis compared to other animals, especially sheep and goats.

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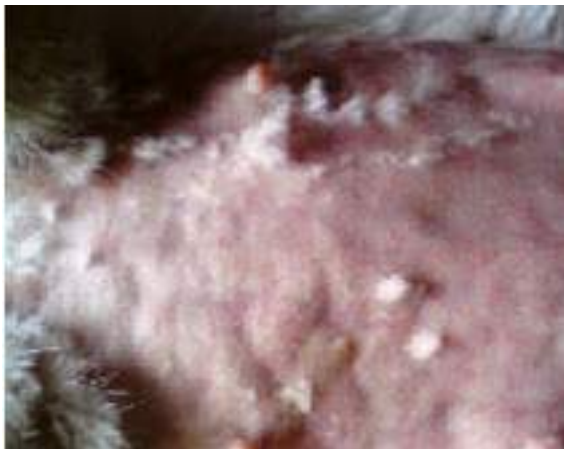

Fig. 1 : Rabbit mammary glands infected with *M. agalactiae* showing swelling and oedema at 24 hpi.

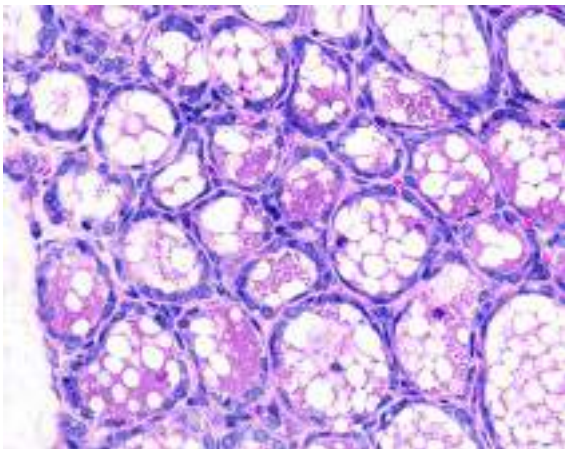

Fig. 2 : Section of uninfected mammary gland showing normal secretory acini with cuboidal epithelium and secretions in the acini. H&E x 200

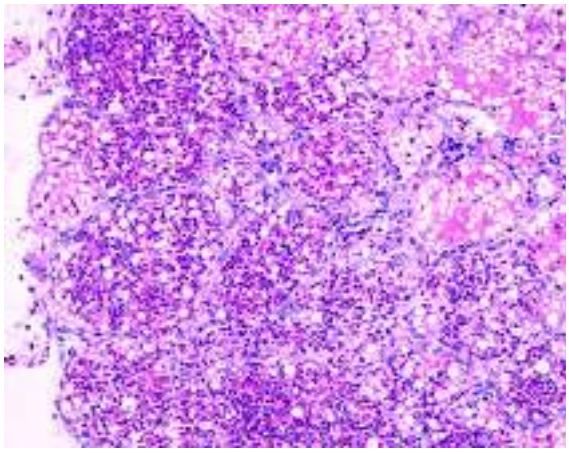

Fig. 3 : Mammary gland infected with *M. agalactiae* at 24 hpi showing diffuse, purulent mastitis with marked infiltration of heterophils in the lumina of acini and milk ducts. H&E x 40

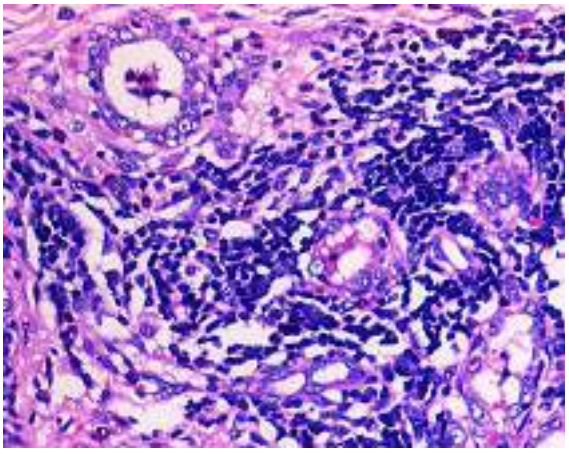

Fig. 4 : Mammary gland infected with *M. agalactiae* at 7 dpi showing marked thickening of interalveolar and interlobular septa, severe infiltration of lymphocytes, destruction and cystic dilatation of alveoli along with hyperplasia of alveolar epithelium. H&E x 200

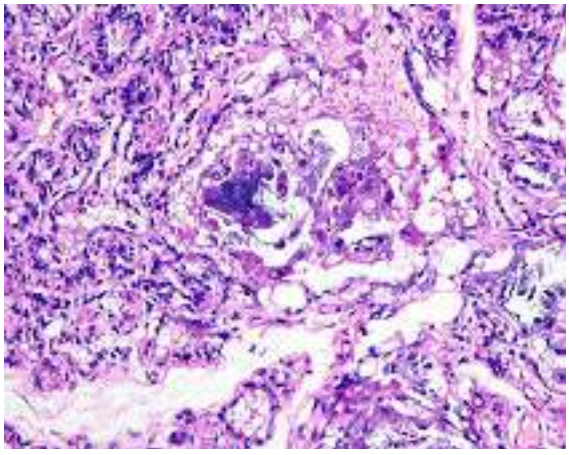

Fig. 5 : Mammary gland infected with *M. agalactiae* at 9 dpi showing necrosis with loss of architecture, fibrosis, desquamation of alveolar epithelium into the acinar lumen and inflammatory cells. H&E x 20

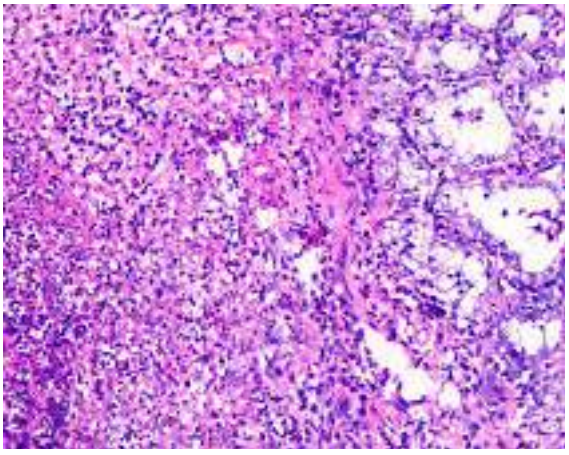

Fig. 6 : Infected mammary gland showing replacement of the glandular parenchyma by fibrous tissue with islands of alveoli without secretions. H&E x 20