Experimental pathogenicity evaluation of *Mycoplasma canadense* from bovine mastitis *in vitro* and *in vivo* laboratory models

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*Mycoplasma canadense*, a clinical isolate from milk of a mastitic buffalo, was experimentally tested for its pathogenic potential in hamster tracheal ring and rabbit fallopian tube explant organ cultures (*in vitro*) and rat and rabbit mammary gland (*in vivo*) models. The activity percentage reduction in *M. canadense* infected hamster tracheal rings was 99.1% in comparison to 16.4% in control rings. *Mycoplasma canadense*, also induced complete ciliostasis at 11-day post-infection in rabbit fallopian tube explants. Histopathological lesions in these infected organ cultures were loss of cilia, desquamation or denudation of epithelium, infiltration of inflammatory cells and proliferation of macrophages as well as oedema in lamina propria. At the end of the experiments, *M. canadense* organisms were reisolated in pure colonies from the infected but not the control organ cultures. In the rat and rabbit mammary glands, *M. canadense* organisms persisted up to 6-day and 7-day postinfection, respectively and caused histopathological changes suggestive of subacute to chronic mastitis during the experimental period. The results indicate that the tested *M. canadense* clinical isolate was virulent.

**Keywords:** Bovine mastitis, Experimental pathogenicity, *Mycoplasma canadense*

*Mycoplasma canadense* was originally reported in 1973 from a case of bovine mastitis, from joint and umbilicus of a week old calf, vaginal mucopurulent discharge of a cow and semen of a bull*. Later, it caused many outbreaks of bovine mastitis* and was commonly isolated from bovine semen/prepuce and cervix* and aborted foetuses*. In India, *M. canadense* was first reported in 1998 from cases of mastitis in cows and buffaloes* and 61% mastitic cows and 63.3% mastitic buffaloes positive by *M. canadense*-capture ELISA and 61% mastitic cows and 63.3% mastitic buffaloes positive by *M. canadense* serum-antibodies by indirect-ELISA*. However, the role of *M. canadense* in various infections of bovine is less clear because of obvious difficulty in conducting experiments in cattle as *M. canadense* do not persist or cause lesion in genital tract*. However, mastitogenic capability of *M. canadense* has been reported previously in cow and sheep udder models*. In the present study, *M. canadense* has been experimentally tested for its pathogenic potential in organ cultures (hamster tracheal, rabbit fallopian tube) and rat and rabbit mammary gland models, which have previously been used successfully to determine the virulence of various mycoplasmas*.

**Materials and Methods**

*Mycoplasma strains and their maintenance—* A field strain of *Mycoplasma canadense* #BM-70A isolated in 1998 from a case of mastitis in a buffalo and the type strain of *M. canadense* (NCTC-10152) were used. The organisms were freshly propagated from freeze dried culture and maintained by serial passages in PPLO-broth without thallium acetate. Their identity were reconfirmed by disc growth inhibition using polyclonal rabbit-anti-*M. canadense* serum raised against the type strain NCTC-10152. In *in vitro* organ cultures and their infection—Hamster tracheal ring (HTR) and rabbit fallopian tube (RFT) organ cultures were prepared as described previously. These were maintained in Eagle’s BME-Basal Medium supplemented with Hank’s salts, L-glutamine without sodium bicarbonate (Himedia, Mumbai). The ciliary activity of epithelial cells at inner surface of HTR and at the fringes of RFT explants was observed using an inverted microscope (×300). Six to eight HTR and RFT explants showing vigorous ciliary activity were
selected and infected with *M. canadense* (0.5 ml having $2 \times 10^6$ cfu) to give finally $10^6$ cfu/ml. Equal volume of sterile PPLO-broth was added to control HTR and RFT explants (3-4 numbers). Infected and control tracheal organ cultures were examined on alternate days for their ciliary activity score. Fallopian tube explants were observed daily for presence of ciliary activity till the day on which ciliary activity ceased. The recovery of *M. canadense* organisms was done on alternate days from both the organ cultures. At the termination of experiments, the number of *M. canadense* organisms per ml was determined separately in the medium and the tissues. Both, the infected and control HRT and RFT explants were subjected to histopathological examination.

In vivo mammary gland infection—Experimental design and method of infection of mammary glands of rat (*Rattus norvegicus*) and rabbit (*Oryctolagus cuniculus*) at 7- and 4-7 day lactation, respectively were as per earlier description\(^6\). The third, fourth and fifth left mammary glands (L-3, L-4, L-5) of five rats and second, third and fourth left mammary glands (L-2, L-3, L-4) of five rabbits were injected with ca. $10^6$ cfu of freshly prepared *M. canadense* culture into each teat. The corresponding right side glands of rat and rabbit served as un inoculated and sterile PPLO-broth inoculated controls. Infected rats and rabbits were clinically observed daily, especially for gross appearance of mammary glands. One rat each was autopsied at 1, 2, 3, 4, 5 and 6 day post-infection whereas one rabbit each was sacrificed at 1, 3, 5, 7 and 8-day PI. Each gland was cut into two halves; one fixed in formal saline for histopathology and the other half used for mycoplasmological examination.

### Results

**Growth and effects of *M. canadense* in vitro organ cultures—** *M. canadense* (# BM-70A) survived in HTR organ culture and ciliary activity of uninfected tracheal rings could be maintained for 12 days. *M. canadense* was reisolated from the infected but not from the control tracheal rings at the end of the experiment. The effect of *M. canadense* on ciliary activity of HTR is given in the Fig. 1. The ciliary activity average score was reduced from 255 at 0-day to 15 at 12-day PI in comparison to uninoculated control rings in which average score of 6 rings decreased from 255 at 0-day to 213 at 12-day PI. Thus, the ciliary activity percentage reduction in *M. canadense* infected tracheal rings was 99.1% in comparison to 16.4% in control rings. The *M. canadense* organisms could be recovered regularly on alternate days from the medium of infected HTR cultures and also from the infected tracheal explants at the end of the experiment. The significant histopathological changes in infected but not in control rings were focal loss of cilia and denudation of lining epithelium and infiltration of lymphocytes, focal oedema and mild proliferation of macrophages in lamina propria.

Vigorous ciliary activity in uninfected control RFT explants was noted up to 13 days; *M. canadense* induced complete ciliostasis at 11-day PI and was recovered in higher concentration ($10^{11}$ cfu/ml tissue homogenate; $10^{17}$ cfu/ml medium) at the end of the experiment. Histopathological lesions in infected RFT explants were intensive loss of cilia, mild to moderate desquamation of the lining epithelium with mild infiltration of mononuclear cells with a few neutrophils and oedema in lamina propria.

![Fig. 1 — Cilia stopping effect (CSE) of *M. canadense* (BM-70A) in hamster tracheal rings](image-url)
Growth and effect of *M. canadense* in vivo mammary gland models—All the infected rats and rabbits appeared healthy and no clinical sign (pyrexia, anorexia, restlessness) were noted throughout the experimental period. In rat mammary model, *M. canadense* organisms could be reisolated in pure colonies up to 4-day PI from glands infected with strain BM-70A organisms and up to 6-day PI from glands infected with strain NCTC-10152 organisms. However, *M. canadense* strain # BM-70A organism injected into rabbit mammary glands could be recovered up to 7-day PI. Gross teat lesions of some but not all the infected mammary glands of rats and rabbits up to 6-day PI included inflamed teats showing mild redness surrounding the glandular tissues on reflected skin and/or reddened swelling.

Histopathological changes in rat and rabbit mammary glands, demonstrative of mastitogenic potential of *M. canadense*, which were observed at different days after infection are given in the Table 1. In rat, subacute type reaction involving interacinar tissues with mild infiltration of lymphocytes and macrophages along with mild desquamation of acinar cell lining were noted in early stage. Later, the acini were atrophied with moderate infiltration of lymphocytes and macrophages along with moderate fibroblastic proliferation in the interstitium. Histopathological changes in rabbit mammary gland observed at 1, 3, 5, 7 and 8-day post-infection with *M. canadense* organisms were of subacute to chronic type reaction involving interacinar tissues with mild infiltration of lymphocytes and macrophages along with mild desquamation of acinar cell lining, fibroblastic cell proliferation and hyperplasia of acinar duct in early stage. Later, the acini were atrophied with moderate infiltration of lymphocytes and macrophages along with moderate to marked proliferation of fibroblast in the interstitium.

**Discussion**

*Mycoplasma canadense* occurs in the genital tract of male and female bovines. However, its less widespread occurrence is attributed to its limited persistence at this site. Bovine mammary gland is also a receptive site for colonization by *M. canadense* causing mastitis. Obiously, many outbreaks of bovine mastitis due to *M. canadense* have been reported in past. It has been concluded that variations in virulence possibly exist in different strains of *M. canadense*. There are problems in carrying out experiments with *M. canadense* in cattle, a high proportion of which may have been previously exposed to it. Nonetheless, experimental production of bovine and ovine mastitis with *M. canadense* has been reported earlier. However, experimental pathogenicity of *M. canadense* has not been previously

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+ to +++ indicates the intensity of described histopathological lesion.

N= neutrophil, L= lymphocyte, M= macrophage, Des= desquamation, Dist= distortion, ND= not done
determined in more convenient and less cumbersome laboratory systems viz. organ cultures, laboratory animal models.

In the present investigation, HTR and RFT explant organ cultures as well as rat and rabbit mammary gland models were used to evaluate the pathogenic potential of *M. canadense* strain # BM 70A isolated from milk of a mastitic buffalo. The results obtained with HTR organ culture showed that the reduction in ciliary activity percentage in *M. canadense* organisms infected rings was 99.1% at 12-day PI in comparison to 16.4% in uninfected control rings, which is indicative of pathogenicity. Likewise, complete ciliostasis in RFT explants infected with *M. canadense* at 11-day PI in comparison to vigorous ciliary activity of uninfected explants up to 13 days was also suggestive of pathogenic nature of tested *M. canadense* isolate. This observation was substantiated by recovery of *M. canadense* organisms from infected but not from uninfected HTR and RFT explants along with significant histopathological lesions viz. loss of cilia, denudation of lining epithelium and infiltration of inflammatory cells (lymphocytes, neutrophils and mononuclear cells) and proliferation of macrophages in lamina propria. Hamster tracheal ring and RFT explant organ cultures have been used previously to evaluate pathogenic potential of mollicutes (*Mycoplasma, Acholeplasma*) of bovine udder and reproductive tract origin and have described almost similar cilia stopping and histopathological effects. Our observations also suggest that both HTR and RFT organ cultures seems equally sensitive to pathogenic determinants of *M. canadense*.

The observed histopathological lesions (Table 1) along with recovery of *M. canadense* organisms from the experimentally inoculated teats of rats and rabbits suggested that *M. canadense* clinical isolate # BM-70A was mastitogenic to former and moderately mastitogenic to the later. The *M. canadense* organisms persisted in mammary tissues of rat and rabbit for 6-7 days in contrast to 11 to 49 days recorded earlier in cow and ewe mammary glands. The failure of *M. canadense* to persist for longer than 6-7 days may be a reflection of strain or more possibly due to host variation. The histopathologic response of rat mammary gland to *M. canadense* was similar to that already described earlier for other bovine mastitis mycoplasmas and bovine reproductive mycoplasmas. In the present study, *M. canadense* produced subacute to chronic type histopathological reaction in rabbit mammary glands. Rabbit mammary gland model was first used to determine mastitogenic potential of a mycoplasma viz, *M. capricolum ss capripneumoniae*, and the recorded histopathological lesions were similar to that produced by *M. canadense* during this study. However, neutrophilic infiltration in acinar lumen and interacinar tissues, considered characteristic of *M. bovis* in cow and rat and *M. capricolum ss capripneumoniae* in rabbit mammary glands, was found absent in *M. canadense* infected rabbit mammary tissues. The recorded basic inflammatory response in rabbit mammary glands infected with *M. canadense* was lymphocytic and macrophagic.

The observations of experimental infection of described in vitro and in vivo model systems with a *M. canadense* clinical strain from buffalo-mastitis suggested it to be pathogenic in former and mastitogenic in later, indicating its vital role in bovine mycoplasmal mastitis. The financial support of ICAR, New Delhi under a national fellow project to the senior author is gratefully acknowledged.

**References**