

**STUDIES ON BOVINE MYCOPLASMAL MASTITIS AND
EXPERIMENTAL PATHOGENICITY OF
MYCOPLASMA BOVIS**

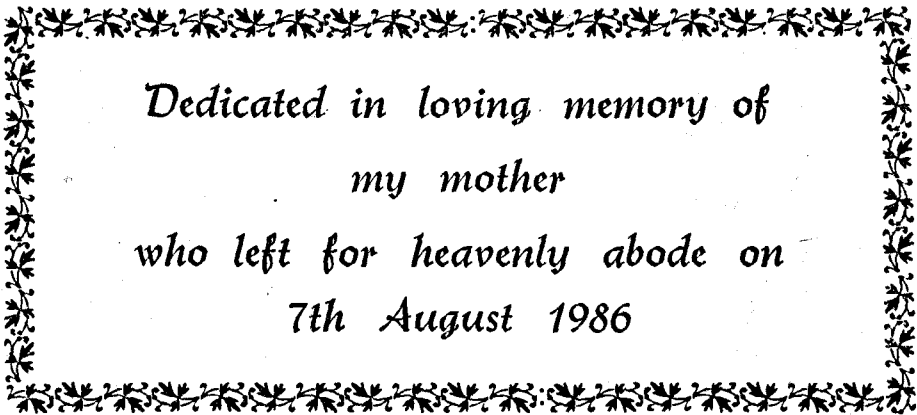
**By
ASHWANI KUMAR**

**DISSERTATION SUBMITTED TO THE HARYANA AGRICULTURAL UNIVERSITY
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
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**DOCTOR OF PHILOSOPHY
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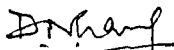
*Dedicated in loving memory of
my mother
who left for heavenly abode on
7th August 1986*

CERTIFICATE I

This is to certify that this dissertation entitled, "Studies on bovine mycoplasmal mastitis and experimental pathogenicity of Mycoplasma bovis" submitted for the degree of Ph.D. in the subject of Veterinary Public Health and Epidemiology of the Haryana Agricultural University is a bonafide research work carried out by Sh. Ashwani Kumar under our supervision and that no part of this dissertation has been submitted for any other degree.

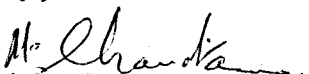
The assistance and help received during the course of investigation have been fully acknowledged.

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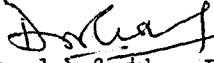
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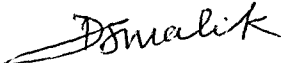
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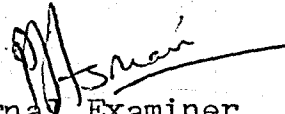
This is to certify that this dissertation entitled "Studies on bovine mycoplasmal mastitis and experimental pathogenicity of Mycoplasma bovis" submitted by Sh. Ashwani Kumar to the Haryana Agricultural University in partial fulfilment of the requirements for the degree of Ph.D. in the subject of Veterinary Public Health and Epidemiology has been approved by the Student's Advisory Committee after an oral examination on the same in collaboration with an External Examiner.


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C O N T E N T S

<u>CHAPTER</u>	<u>PAGES</u>
INTRODUCTION	1
REVIEW OF LITERATURE	
Isolation of <u>Mollicutes</u> organisms from milk of bovine origin	6
Seroprevalence of <u>Mollicutes</u> associated bovine mastitis and associated immune response	14
Experimental pathogenicity of <u>Mollicutes</u> organisms associated with bovine mastitis	19
MATERIALS AND METHODS	
Collection of milk and serum samples	31
Isolation of <u>Mycoplasma</u> , <u>Acholeplasma</u> and <u>Ureaplasma</u>	34
Characterization of <u>Mycoplasma</u> , <u>Acholeplasma</u> and <u>Ureaplasma</u>	35
Isolation and identification of other associated bacteria	41
Seroprevalence of bovine mycoplasmal mastitis	42
Experimental pathogenicity of <u>Mollicutes</u> organisms isolated from bovine milk	52
RESULTS	59
DISCUSSION	125
SUMMARY	144
REFERENCES	i-xxxii
APPENDIX	i-viii

INTRODUCTION

I INTRODUCTION

Bovine mastitis is an economically important disease to the dairy industry because of reduced milk yield, higher culling rate and increased treatment cost. It is now appearing more frequently in geographic areas within and between countries. It is a complex disease of multifactorial etiology, and bacterial agents are well known to play a major role. Sometimes the problem of mastitis has been observed to linger in a herd even after prolonged antibiotic therapy of affected animals. This aspect eventually led to the discovery of association of Mycoplasma with bovine mastitis.

Mycoplasma bovis was the first mycoplasma recognised as a cause of bovine mastitis (Davidson and Stuart, 1960; Stuart et al., 1963). However, the most common cause of mycoplasmal bovine mastitis has been M. bovis and most of the observations and researches on mycoplasmal mastitis relate to this species. The first recognised outbreak of mastitis due to M. bovis occurred in Connecticut in 1961 (Hale et al., 1962; Rovozzo et al., 1963) followed by another outbreak involving 16 herds in New York (Carmichael et al., 1964; Fincher, 1964) in which

etiological agent was found serologically similar to M. bovis. Since that time, M. bovis mastitis or the isolation of M. bovis from bovine milk has been reported from Israel (Bar-Moshe, 1964); Spain (Barrado and Perez, 1967); Australia (Cottew, 1970); Italy (Biancardi, 1970, 1971); Bulgaria (Buchvarova and Veselinova, 1973); France (Gourlay et al., 1974); Hungary (Romvary et al., 1975); Canada (Ruhnke et al., 1976); Yugoslavia (Durisic, 1976); England (Davies and Boughton, 1976); Netherlands (Vander-Molen and Grootenhuis, 1979) and Denmark (Friis, 1984). Jasper (1981) indicated that mycoplasmal mastitis should be suspected whenever there is (a) an increase in severe purulent mastitis cases that resist treatment and show little other evidence of illness, (b) mastitis cases that typically involve more than one quarter, (c) marked loss of production in affected quarters, most of which show severe mastitis but may simply cease lactating, and (d) abnormal secretions with tannish or brownish discolouration, sandy or flaky sediments in watery or serous fluid.

Bovine mastitis due to mycoplasmas other than M. bovis also deserve attention. Other species recorded in bovine mastitis include: M. bovigenitalium (Cho et al., 1976); M. canadense (Ruhnke and Onoviron, 1975);

M.californicum (Dellinger et al., 1977; Jasper, 1977);
M.alkalescens (Brookbanks et al., 1969; Jasper et al., 1979);
M.bovirhinis (Jasper, 1979); M.arginini (Rinaldi et al., 1971);
Mycoplasma sp.gp.7 (Connole et al., 1967) and A.laidlawii
(Pan and Ogata, 1969a,b; Gourlay and Howard, 1979).

Diagnosis of mycoplasmal mastitis has been greatly facilitated by employing laboratory tests, which are mainly based on immunological response of the host. The important tests include: gel double diffusion test (Jain et al., 1969a,b); growth inhibition test (Bennett and Jasper 1978a,c); metabolic inhibition test (Erno et al., 1973); complement fixation test (Kehoe et al., 1967); latex agglutination test (Jain et al., 1969a); indirect haemagglutination test (Cho et al., 1976) and radial growth precipitation test (Howard et al., 1976). However, indirect haemagglutination test has been found to be more sensitive, reliable and specific in comparison to other serological tests for the diagnosis of mycoplasmal mastitis viz. M.bovis.

Pathogenesis of bovine mycoplasmal mastitis has been poorly studied because of non-availability of suitable laboratory models. However, studies have been conducted in cows using M.bovis of bovine-udder origin (Pfutzner et al. 1983b) and in goats using M.bovigenitalium of buffalo bull

semen and vaginal mucus origin (Pal et al., 1983b). Mice-udder model has been exploited to study the pathogenicity of ureaplasmas of human genital-tract origin, bovine origin (Howard et al., 1975), and standard/field isolates of M.bovis (Thorns and Boughton, 1980).

Research work pertaining to bovine mycoplasmal mastitis in India is mainly restricted to the isolation of A.oculi and A.axanthum (Katoch et al., 1983); M.bovigenitalium, M.arginini, Mycoplasma sp. gp. 7, M.alkalescens and A.laidlawii (Pal et al., 1984). Indirect haemagglutinating and complement fixing antibody titres against M.bovis, M.bovigenitalium and A.laidlawii were reported by Pal et al. (1983a) as suitable indicator of genital mycoplasmal infection in buffaloes, whereas counter-current immunoelectrophoresis test was found less sensitive.

In view of the absence of researches on various aspects of bovine mycoplasmal mastitis in India, the present investigation was undertaken with the following objectives:

1. To isolate and characterize Mycoplasma (and Acholeplasma, Ureaplasma) from mastitic and apparently normal udder of cows and buffaloes.

2. To determine the seroprevalence of Mycoplasma (M.bovis) associated bovine mastitis employing sensitive serological tests.
3. To determine the pathogenicity of chief etiological agent of bovine mycoplasmal mastitis viz. M.bovis in a suitable experimental model.

REVIEW OF LITERATURE

II REVIEW OF LITERATURE

A. Isolation of Mollicutes Organisms from Mastitic Milk of Bovine Origin

Mastitis due to Mycoplasma was first reported from England by Davidson and Stuart (1960). During the past 25 years, the role of mycoplasma in bovine mastitis has gained immense importance. Different Mycoplasma species have been reported from milk of mastitic-cows and buffaloes in different parts of the world. Pfutzner et al. (1979a, 1981) named different mycoplasmas as pathogens for causing mastitis. They are Mycoplasma bovis, M. bovirhinis, M. arginini, M. californicum, M. alkalescens, M. dispar, Ureaplasma sp. Acholeplasma laidlawii and A. axanthum.

Mycoplasma bovis

Among all the Mollicutes organisms reported from bovine mastitis, M. bovis is the most frequently isolated species. The first recognized outbreak of mastitis due to this species occurred in Connecticut in 1961 (Hale et al., 1962; Rovozzo et al., 1963). This species earlier named as M. agalactiae var. bovis or M. bovimastitidis was renamed as M. bovis in 1976 (Aska and Erno, 1976; Anon, 1976).

Subsequently, Carmichael et al. (1964), and Fincher (1964) described bovine mycoplasmal mastitis in 16 herds in New York and showed that the causative organism was serologically similar to M. bovis. In 1964 outbreaks of mycoplasmal mastitis occurred in Israel (Bar-Moshe, 1964) and California (Jasper et al., 1966), which were apparently due to M. bovis. M. bovis induced mastitis and the isolation of M. bovis from milk has also been reported from Spain (Marcos-Barrado and Sanz-Perez, 1967); Italy (Rinaldi et al., 1969, 1971, 1972; Biancardi, 1970, 1971; Biancardi et al., 1973; Galli, 1973; Novazzi, 1975); Australia (Cottew, 1970); Yugoslavia (Durisic et al., 1973, Durisic, 1976); Bulgaria (Buchvarova and Veselinova, 1973); France (Gourlay et al., 1974); Hungary (Romvary et al., 1975); Britain (Davies and Boughton, 1976; Bicknell et al., 1978; Boughton and Wilson, 1978); Canada (Ruhnke et al., 1976); Japan (Shimizu and Nagatomo, 1977); The Netherlands (Anon, 1977; Vander-Molen and Grootenhuis, 1979); and Switzerland (Schaeren et al. 1983).

Amongst all the countries of the world, maximum reports of M. bovis mastitis originated from different states of America viz. California. Jasper (1980), and Jasper et al. (1979) reported involvement of 104 herds with clinical mycoplasmal mastitis outbreaks in which M. bovis was

involved in 59 outbreaks. Further 59 per cent of pathogenic isolates from 4 per cent milk tank samples in California were M.bovis (Jasper, 1980; Jasper et al., 1979).

Pfutzner and Schimmel (1979) identified 16 M.bovis out of 32 Mycoplasma/Acholeplasma isolates from mastitic-milk of cows. Pfutzner et al.(1979b) reported mastitis in 3 herds due to M.bovis, A.laidlawii and A.axanthum whereas Buchvarova (1979) isolated M.bovis, A.laidlawii, A.axanthum, A.modicum and M.alkalescens from milk samples of healthy, subclinical and clinical cases of cow-mastitis. Similarly, two strains of M.bovis were also isolated from milk of mastitic-cows (Zalewska, 1979).

Weigt et al.(1981) and Heitman et al. (1981) reported the isolation of M.bovis from cows suffering with mastitis in Westphalia and German Federal Republic. Heitman et al.(1981) reported the isolation of M.bovis not only from mastitic-milk but also from lungs, spleen, tonsils and lymph nodes of affected animals. Hartman et al.(1981), Steiner (1982) and Wienhues (1982) have also described the isolation of M.bovis from milk of mastitic-cows, whereas Wienhues (1982) have reported the isolation of streptococci, E.coli and yeast alongwith M.bovis. Baumgartner (1983) recommended the direct culturing of milk samples on medium I-agar which

reduced laboratory work load. The author tested 50,000 milk samples by this method and could isolate 455 M.bovis, A.laidlawii and A.axanthum. Isolation of M.bovis from mastitic-cows is being continuously reported in the literature (Chen et al., 1983; Wienhues et al., 1984; Friis, 1984; Li et al., 1984; Kunkel, 1985).

Mycoplasma bovigenitalium

M.bovigenitalium was the first Mycoplasma to be recognized as a cause of bovine mastitis (Davidson and Stuart, 1960; Stuart et al., 1963). Later, M.bovigenitalium antibodies were reported from cases of mastitis in Canada (Cho et al., 1976). Counter (1978) described mastitis affecting 75 of 200 cows due to mixed infection with M.bovigenitalium and A.laidlawii. Jasper (1977) and Jasper et al. (1979) have described M.bovigenitalium as the cause of mastitis in 7 herds in California. Wehnert et al. (1983) described outbreaks of severe drug resistant mastitis due to M.bovigenitalium in 2 herds of 1930 and 1000 cows. In one of the herds, 18 M.bovigenitalium were isolated alongwith 9 A.laidlawii, whereas only M.bovigenitalium was recognised in the other herds. Jurmanova et al. (1983) reported that 112 milk samples from mastitic-cows yielded either or both M.bovigenitalium and M.californicum. Pal et al. (1984)

isolated M. bovigentialium from mastitic-milk of buffaloes in India, which is the only report of buffalo-mastitis.

Mycoplasma canadense

In 1973 a strain of Mycoplasma (No. 466), later found to be a new species named as M. canadense (Langford et al., 1976), was isolated in Canada from a case of mastitis and also from other sources viz. joints and umbilicus of a calf, vaginal mucopurulent discharge from a cow and semen of a bull (Ruhnke and Onoviran, 1975). Subsequently, another apparently new strain was isolated from mastitic-milk, the vagina and respiratory tract in 1974, which was later found to be identical to M. canadense (Dellinger et al., 1977; Jasper, 1977 and Jasper et al., 1979). M. canadense was later reported from the outbreaks of mastitis in California (Jasper et al., 1979) and England (Gourlay et al., 1978; Jackson et al., 1981).

Mycoplasma californicum

A new strain of Mycoplasma was recovered from an acute outbreak of mastitis in California designated as ST-6 (Jasper, 1977; Dellinger et al., 1977) and later named as a new species M. californicum (Jasper et al., 1981). It has also been isolated from milk of mastitic-cows in Hawaii (Jasper et al., 1979).

M.californicum was considered to be the second most common pathogen from cases of mycoplasmal mastitis in California (Jasper et al., 1974). Mackie and Logan (1982) isolated M.californicum from 5 cows with chronic incurable mastitis in a herd. In Czechoslovakia, Jurmanova et al. (1983) isolated M.californicum or/and M.bovigenitalium from mastitic-cows.

Mycoplasma alkalescens

M.alkalescens as the cause of mastitis was first reported in New Zealand in 1969 (Brookbanks et al., 1969). Dellinger et al. (1977) isolated M.alkalescens from mastitis in two herds and from bulk milk samples. Outbreaks of mastitis due to M.alkalescens has also now been reported from California (Jasper et al., 1979). Jasper (1979) and Buchvarova (1979) isolated M.alkalescens from mastitic-milk of cows, whereas Pal et al. (1984) reported its isolation from mastitic-milk of buffaloes.

Mycoplasma bovirhinis

Langer and McEntee (1966) isolated a strain 56 R from mammary secretions of all the four quarters of a mastitic-cow. This strain caused acute, purulent mastitis upon intramammary inoculation (Langer and Carmichael, 1964) and was later classified as M.bovirhinis (Leach, 1967). M.bovirhinis was isolated from mastitic-milk (Brownlie et al., 1976) and

also from some composite milk samples of a herd (Jasper, 1979).

Mycoplasma arginini

M.arginini has been isolated from a few cases of mastitis in a herd outbreak which was primarily due to M.bovis. Ruffo et al. (1971) used M.arginini to reproduce experimental mastitis, which was anatomically and clinically similar to that caused by M.bovis. Jasper (1977, 1979) and Jasper et al. (1979) recorded the occurrence of M.arginini in association with mastitis in two bovine herds whereas Pal et al. (1984) isolated M.arginini from milk of the buffaloes having clinical and subclinical mastitis in India.

Mycoplasma sp.gp.7

This Mycoplasma species caused severe mastitis in a herd of Australia in 1967 (Connoles et al., 1967). It produced experimental mastitis in cows in one quarter but did not spread to all the quarters (Jasper, 1981). Alexander et al. (1985) isolated Mycoplasma sp.gp.7 from mastitic-milk of cows and also from arthritis in cows of the same herd.

Mycoplasma dispar

Hodges (1983) isolated M.dispar from yellowish milk secretions from 25 of 35 cows in New Zealand, which was the

first and the only report of field cases of bovine mastitis due to M. dispar. This species of Mycoplasma caused clinical mastitis in cows following experimental inoculation (Brownlie et al., 1976).

Ureaplasmas

Ureaplasmas have been frequently isolated from urogenital tract, respiratory tract and eyes of cattle and possess variable pathogenicity for the bovine-udder. The isolation of Ureaplasma from three out of 12 milk samples from cows having mastitis was reported (Garg and Kapoor, 1985). The isolation of Ureaplasma in these cases was not associated with any other bacterial and fungal pathogen. Mastitis in cow-udder could be experimentally produced with the ureaplasmas isolated from ewes and rams suffering with vaginitis and orchitis (Mackie and Ball, 1984; Rae, 1984).

Acholeplasmas

Isolation of A. laidlawii from sporadic cases of mastitis and from mastitis outbreaks have been reported (Pan and Ogata, 1969a,b; Gourlay and Howard, 1979). Wehnert et al. (1977) described symptoms in both lactating and dry cows infected with A. laidlawii or A. axanthum. References to mixed infections of M. bovigenitalium and A. laidlawii (Counter, 1978; Wehnert, 1983), and M. bovis and A. laidlawii (Steiner,

1982) are available in the literature. Besides, heavy contamination of both clinical and bulk tank milk with A.laidlawii has also been reported (Jasper et al., 1979). Other reports of isolation of different species of Acholeplasma from milk of diseased and healthy cows/buffaloes are: A.axanthum (Pfutzner and Schimmel, 1979; Pfutzner et al., 1979b; Buchvarova, 1979; Baumgartner, 1983; Katoch et al., 1983); A.oculi (Katoch et al., 1983); A.modicum (Buchvarova, 1979); and A.laidlawii (Pfutzner and Schimmel, 1979; Pfutzner et al., 1979b); Jasper, 1979; Buchvarova, 1979; Zalewska, 1979; Baumgartner, 1983; Pal et al., 1984).

B. Seroprevalence of Mollicutes Associated Bovine Mastitis and Associated Immune Response

Seroprevalence

Mycoplasma infections in animals and men result in marked increase in humoral antibodies, which are detected by various serological tests. Boughton (1979) has critically reviewed the various serological tests applicable for the diagnosis of infections with mycoplasmas. M.bovis has been shown to stimulate complement fixing, CF (Kehoe et al., 1967; Howard et al., 1976, 1977); growth inhibiting, GI (Jain et al., 1969a,b; Al-Aubaidi and Fabricant, 1971); film inhibiting, FI (Ruhnke et al., 1976; Cho et al., 1976; Howard et al.,

1976; Thorns and Boughton, 1978; Pfutzner et al., 1983); agglutinating (Jain et al., 1969a,b); latex agglutinating, LA (Jain et al., 1969a,b; Ruhnke et al., 1976); metabolic inhibiting (Al-Aubaidi and Fabricant, 1971, Erno et al., 1973); growth precipitin (Krosgaard-Jensen, 1972; Erno and Jurmanova, 1973; Howard et al., 1976, 1977; Bennett and Jasper, 1978c); gel-double diffusing (Jain et al., 1969a,b; Erno and Jurmanova, 1973); tetrazolium reduction inhibiting, TRI (Carroll et al., 1977; Bennett and Jasper 1978c); single radial hemolysin, SRH (Howard et al., 1977); indirect haemagglutinating, IHA (Howard et al., 1976, 1977; Cho et al., 1976; Jasper, 1977; Bennett and Jasper, 1978a,c; Pfutzner et al., 1983a) and Enzyme-linked-immuno-sorbent-assay, ELISA (Boothby et al., 1981) antibodies. IHA test, CF test and ELISA have been employed to determine the seroprevalence of mycoplasmal mastitis.

Howard et al. (1976, 1977) found CF titres 1:80 in naturally and experimentally infected calves and considered CF test as the most sensitive but complicated test. Kehoe et al. (1967) reported that in experimentally infected cows with M. bovis the CF titres may rise upto 1:500 and can appear as early as 19-day postinfection.

Jain et al. (1969a) and Bennett and Jasper (1978a) reported the strong reactions in GI test, which is usually

negative even when antibodies are detectable by other tests like IHA test. FI test was found useful to detect antibodies against M.bovis (Ruhnke et al., 1976; Thorns and Boughton, 1978; Pfutzner et al., 1983a). However, Cho et al.(1976) and Howard et al.(1976) described this test as less reliable and satisfactory.

IHA test as applicable to M.bovis was studied by Cho et al. (1976) and Howard et al.(1976), who detected mycoplasmal antibodies in sera from cattle which had symptoms of mastitis due to natural infection with either M.bovis or M.bovigenitalium. Cho et al.(1976) considered IHA titres $\geq 1:160$ as positive, $= 1:80$ as suspicious, and $\leq 1:40$ as negative or nonspecific. Benett and Jasper (1978a,c) found very high IHA titres, upto 20,480, in infected cows. Jurmanova et al.(1983) tested 49 serum samples from naturally infected mastitic-cows by IHA test and 84 per cent and 61 per cent sera were found positive for M.bovigenitalium and M.californicum, respectively. IHA titres against these two species of Mycoplasma ranged from 1:16 to 1:2048. Su et al.(1984) detected haemagglutinating antibodies to M.bovirhinis in 29, to A.laidlawii in 21, to Mycoplasma sp.gp. 7 in 18, and to M.dispar in 12 out of 106 cattle. The CF test with same antigens and sera gave similar results while gel-diffusion test gave only a few positive results.

Serological studies using blood and milk samples from a herd of cows with prolonged intractable acute mastitis and arthritis revealed that antibodies against M.bovis were detectable by IHA, CF test and ELISA (Heitmann et al., 1983). In this study CF test showed low titres which disappeared earlier. In an outbreak of M.bovis mastitis in Switzerland, Schaeren et al.(1983) used micro-ELISA on serum samples from 30 cows, which were found all positive for M.bovis. Antibodies to M.californicum, M.bovigenitalium, M.bovirhinis, M.bovis and M.arginini were found in milk and serum samples from 143 dairy cows in two localities of Czechoslovakia by ELISA and IHA (Jurmanova et al., 1985).

Immune response

Roberts (1968) studied the immunological response of cows to M.bovigenitalium, strain-1836 following inoculation of 4 cows via the udder, and measured the response by CF and plate agglutination tests. The positive agglutination test with 1:4 to 1:32 as highest titres was recorded at 5-day and 14-day postinfection. The corresponding CF titres varied from 1:40 to 1:640 at 2-week postinfection.

Infections of the bovine mammary-glands due to M.bovis usually elicit substantial systemic antibody response (Jain et al., 1969a; Jasper, 1977). Bennett and Jasper(1979)

in their study of local and systemic antibody response against intramammary inoculation of small numbers (70 CFU) of M.bovis observed that IgM was the predominant immunoglobulin response for IHA titres upto 50 days after inoculation, as was found previously for M.bovigentialium and M.bovis induced mastitis (Roberts, 1968; Bennett and Jasper, 1978a). After 57 days, IgG was found to be predominant. It has been observed by Jain et al. (1967, 1969a) that serum agglutinating titres (latex and plate test) developed in cows following M.bovis antigen inoculation intravenously or intramammary. IHA titres of 1:80 and GI antibodies to M.bovis were recorded in cows with a history of udder infection in Canada (Cho et al., 1976).

Immunological aspects of M.bovis infection in rabbits were studied using three field isolates of M.bovis from mastitic-bovines (Carroll et al., 1976). GI antibodies were absent while precipitins were present. IHA antibodies appeared in all the rabbits at 1-week postinfection, followed by 1.0×10^5 to 3.3×10^5 titres in 4-6 weeks postinfection but decreased to low levels inspite of intravenous antigenic stimulation.

Carroll et al. (1977) studied the immune response of cow-calves against M.bovis antigen utilizing IHA, GI and TRI tests. IHA test proved to be the best as it detected IHA

titres as high as 1:1280 and 1:20480 after 4-week and 15-18 weeks postinfection, respectively.

C. Experimental Pathogenicity of Mollicutes Organisms Associated with Bovine Mastitis

The pathogenicity of Mollicutes isolated from bovine-udder has been determined in both the natural and experimental systems. Cow-udder (Karbe, 1967; Bennett and Jasper, 1978b; Vander-Molen and Grootenhuis, 1979; Vandeplassche et al., 1979; Seffner and Pfutzner, 1980; Stanarius et al., 1981; Mackie and Logan, 1982; Seffner et al., 1983; Mackie and Ball, 1984) and mice-udder (Howard et al., 1975; Thorns and Boughton, 1980) as in-vivo experimental models, and organ culture viz. tracheal organ culture and fallopian tube organ culture as in-vitro experimental models have frequently been employed to determine the pathogenicity of Mollicutes organisms. Goat-udder (Pal et al., 1983b) has also been used as an experimental model to determine the pathogenicity of two strains of M.bovigentialium isolated from genital tract of buffaloes.

Organ culture

Mollicutes organisms are extracellular pathogens and have the epithelial cells as their target. Organ

cultures permit the maintenance of organised mucosal surface in-vitro and allow to observe the direct injury done by Mollicutes. A large number of mycoplasmas have been studied in several different organ culture systems by several investigators (Collier et al., 1969, 1971; Collier and Clyde, 1971; Butler, 1969; Butler and Ellaway, 1970; Cherry and Taylor-Robinson, 1970b; Taylor-Robinson and Cherry, 1972; Stanbridge, 1971). Cherry and Taylor-Robinson (1973) used chicken tracheal culture; tracheal, nasal and synovial organ cultures of human embryo origin; human oviduct; mouse tracheal; bovine embryo tracheal; bovine embryo lung and calf tracheal culture to study the effect of Mycoplasma/Acholeplasma of avian, bovine, murine, ovine, caprine, porcine, and human origin. Chicken embryo tracheal organ culture was found superior for the study of pathogenicity of Mollicutes organisms.

Tracheal organ culture: Butler (1969) and Butler and Ellaway (1970) have examined the effect of several mycoplasmas viz. M.hominis, M.orale, M.salivarium, M.pneumoniae, Ureaplasma urealyticum, M.mycoides subsp. capri, M.mycoides var. mycoides, M.agalactiae, M.gallisepticum and M.synoviae on human fetus tracheal organ culture. Cessation of ciliary activity and epithelial cell damage was produced by

M.mycoides subsp. capri and M.pneumoniae only. Collier and Clyde (1971) reported that ciliostasis and cytopathological changes were produced by M.pneumoniae only on human fetal tracheal organ culture.

Collier et al. (1969) studied the effect of M.pneumoniae, M.fermentans, M.hominis, M.orale, M.salivarium on hamster tracheal organ culture and found that M.pneumoniae produced ciliostasis and epithelial cell damage. Other mycoplasmas did not produce ciliostasis and cytopathology.

Reed (1971) examined the interaction of M.hyorhinis and swine influenza virus in bovine tracheal organ culture and found no evidence of interaction. Pijoan et al. (1971) assessed the pathogenicity of M.hyopneumoniae, M.hyorhinis, A.granularum, M.hyosynoviae in porcine tracheal organ culture. In this study, M.hyorhinis produced the marked effect on ciliary activity and caused necrosis of the epithelium. A.granularum and M.hyosynoviae produced a decrease in ciliary activity but no epithelial necrosis.

The spectrum of growth and cilia stopping effect (CSE) of approximately 60 mycoplasmas have now been studied in the chicken tracheal organ culture (Cherry and Taylor-Robinson 1970a,b,c, 1971, 1973; Taylor-Robinson and Cherry, 1972; Butler and Ellaway, 1970). Cherry and Taylor-Robinson (1970b) studied the growth and pathogenicity of M.mycoides

var. capri in chicken embryo tracheal organ cultures whereas Cherry and Taylor-Robinson (1971) used chicken tracheal organ culture to study the effect of M.gallisepticum. The CSE and associated tissue damage was observed which was either due to accumulation of toxic products in the medium or the organisms themselves.

Cherry and Taylor-Robinson (1973) reported that M.mycoides subsp. capri grew and produced ciliostasis in bovine-embryo tracheal and mouse tracheal organ cultures. Thomas and Howard (1974) studied the effects of M.dispar, M.bovirhinis, A.laidlawii and Ureaplasma on bovine tracheal organ cultures and found that only M.dispar produced ciliostasis and epithelial destruction. Ciliostasis and epithelial cell cytopathology was produced by M.pneumoniae membranes on hamster tracheal organ culture. Chandler and Barile (1980) found that extract of glass grown M.pneumoniae inhibited 72 to 100 per cent of the ciliary activity of hamster tracheal organ culture (ciliostasis). Rosengarten and Kirchhoff (1981) reported CSE of 28 M.hyorhinis strains in tracheal organ cultures of gnotobiotic pigs and hamsters due to production of hydrogen peroxide and presence of organisms.

Araake (1982) compared the ciliostatic effect of mycoplasma of human and animal origin in mouse and chicken tracheal organ cultures. The mycoplasmas used in this study

were categorised in three subgroups on the basis of intensity of CSE viz. M.pulmonis, M.mycoides (PG1), M.gallisepticum (S6) showing a strong CSE; M.arthritis, M.capri, M.maleagris, M.neurolyticum showing mild effect; and M.pneumoniae, M.salivarium, M. orale and A.laidlawii showed weak CSE.

Moorthy and Spradbrow (1985) inoculated chicken embryo tracheal culture with equine strains of M.arginini, M.equigenitalium, M.subdolum, A.laidlawii and A.oculi. All strains were established and multiplied in the explant cultures but only M.subdolum and A.oculi produced a cytopathic effect in ciliated epithelial cells causing sloughing of cells and cilia after 6 days.

Fallopian tube organ culture: Taylor-Robinson and Carney (1974) studied the effect of genital strains of M.hominis, M. orale (type 5), various genital and oral T-Mycoplasma of human origin, M.hyorhinis of porcine origin and T-Mycoplasma of simian and bovine origin in human fallopian tube organ cultures. In no instance damage was observed as evident either by loss of ciliary activity or by histopathological changes. Stalheim and Gallagher (1975) made microscopic observations in sections of normal bovine uterine tube (oviduct/and fimbriae) maintained

in-vitro and infected them with M.bovis (Donetta), M.pneumoniae (FH, 1428) and M.bovirhinis, but found no effect. Stalheim et al.(1976) tested the effect of ureaplasmas isolated from the human genital tract and from the respiratory and genital tract of cattle in organ culture of bovine oviduct. All strains of Ureaplasma multiplied, stopped ciliary activity and caused histological lesions. Employing light scanning and transmission electron microscopy, CSE was correlated with collapse and sloughing of cilia with bulging and vacuolation of secretory and ciliated cells and finally with necrosis, desquamation and disorganisation of the epithelium.

Mice-udder

Chandler (1970) developed a simple technique for the intramammary inoculation of mice using isolates of Staphylococcus aureus, Streptococcus agalactiae, Corynebacterium pyogenes, Pseudomonas aeruginosa and Escherichia coli from bovine mastitis. Mice-model has also been used on two occasions to study the pathogenicity of Mycoplasma and Ureaplasma. Howard et al. (1975) produced mastitis in mice employing ureaplasmas of human genital tract origin and of bovine origin. The strains multiplied and neutrophils were observed in sections of infected glands, some of which were macroscopically inflamed

but none of the glands had any sign of disease. Mice mammary-glands appeared more susceptible to ureaplasma than the udder of cows and goats. Therefore, mouse as a suitable small animal model has been suggested for the study of Ureaplasma pathogenicity.

Thorns and Boughton (1980) studied the effect of serial passages through the liquid medium on the virulence of M.bovis for the mouse mammary-gland. The strains of M.bovis which were passaged in liquid medium more than 60 times were markedly less virulent and produced only minor histopathological changes than the strains with fewer passages, which produced a systemic response in some mice and severe gross and histopathological changes in mammary-glands of all mice.

Cow udder

M.bovis: Boughton (1979) and Jasper (1981) have given numerous descriptions of the lesions found in cow-mastitis caused by M.bovis. Seffner and Pfutzner (1980) examined 60 udders from cows, which were infected experimentally with M.bovis and killed between 2 and 21 days postinfection. At about 7-day postinfection, the affected quarters appeared engorged, slightly enlarged and hard; their cut surfaces were nodular and moderately grey-yellowish. By 2 to 3 weeks postinfection, they were smaller than

unaffected quarter and the cut surfaces were light-brown to brown-yellow. Formation of multiple abscesses ranging from 5 to 10 cm in diameter were noticed (Jasper, 1981; Seffner and Pfutzner, 1980; Bennett and Jasper, 1978a). Gland cisterns, milk duct and teats were filled with thick purulent exudate at the height of the inflammatory reaction (Jain et al. 1969b; Bennett and Jasper, 1978a). Supramammary lymphnodes became oedematous with prominent lymphoid follicles and areas of haemorrhage have been observed (Bennett and Jasper, 1978a; Jasper, 1981). Illing (1979) and Horvath et al. (1980) found swollen and inflamed udders of cows infected with M. bovis.

Kehoe et al. (1967) found variability in microscopic changes of lobules and observed congestion and necrosis in the udder tissue. Bennett and Jasper (1978b) described marked hyperplasia of alveolar epithelium, distended epithelial cells with vacuoles, intense mononuclear cells infiltration in the interstitium alongwith hyperplasia of ductile epithelium and fibrosis in alveoli and interstitium in udder tissue of cows infected with M. bovis.

Karbe (1967), Karbe and Mosher (1968) and Karbe et al. (1967) in their studies on experimental mastitis with M. bovis (Donetta) emphasised that the basic inflammatory cells invading alveoli were eosinophils rather than the

neutrophils. Buchvarova and Veselinova (1973) also considered the eosinophils to predominate early in inflammatory response. Bennett and Jasper (1978c) supported that the eosinophils infiltrate the interstitial tissues but neutrophils were present in the alveoli.

Vander-Molen and Grootenhuis (1979) observed different lesions in udders of lactating cows infected with M. bovis. The inflammation was characterized by exudation of mostly eosinophils in the alveoli during acute stage which was followed by eosinophilic and mononuclear cells infiltration in the interstitial tissue in later stage. However, progressive fibroplasia around ductuli and alveoli with hypertrophy of alveolar epithelium was characteristic of chronic stage. Vandeplasseche et al. (1979) observed not only eosinophils but also neutrophils and macrophages in the early stage of experimental mycoplasmal mastitis. Infiltration of lymphocytes, plasmocytes, histiocytes with alveolar atrophy were observed in chronic mastitis.

Seffner and Pfutzner (1980) observed pronounced degeneration of alveolar epithelium and exudation of neutrophils 4-9 days postinfection. Similar changes, though less pronounced were found 2 to 3 weeks postinfection with extensive alveolar atrophy, interalveolar infiltration of plasma cells, lymphocytes and histiocytes. Stanarius et al.

(1981) described all leucocytes present in the lumen as neutrophils based on electron microscopy of udder tissues. Besides, cortical hyperplasia of the supramammary lymphnodes with active lymphoid follicles and marked increase in plasma cells in medullary areas of mammary-glands have also been described (Carmichael et al., 1964).

M.bovigenitalium: Roberts (1968) reproduced mastitis in four cows by inoculating M.bovigenitalium (strain 1836) which was earlier isolated from mastitis outbreak (Stuart et al., 1963). The infection did not spread to non inoculated quarters. Erno (1967) and Jurmanova et al. (1978) produced mastitis in cows by inoculating M.bovigenitalium isolates from urogenital tract of cows and bulls. Pal et al. (1983b) produced mastitis in goats 48 hr. postintramammary inoculation of two strains of M.bovigenitalium. The histological lesions in cow-udder infected with M.bovigenitalium was characterized by exudation of neutrophilic granulocytes, degeneration of alveolar epithelium and interalveolar accumulation of plasma cells, lymphocytes and macrophages (Seffner et al., 1983). Mackie and Ball (1984)

investigated the pathogenicity of three strains of M.bovigentialium, one each from bovine mastitis and vaginal mucus of aborted cow and the type strain-PG11. Only the M.bovigentialium from mastitis was found pathogenic to cow-udder. Wehnert (1983) and Pfutzner et al.(1983b) reported that M.Bovigenitalium produced mild clinical mastitis.

M.californicum: Mackie and Logan (1982) produced chronic mastitis in three cows by intramammary inoculation of M.californicum of mastitic-udder origin.

Acholeplasma: The pathogenic status of Acholeplasma, especially A.laidlawii is controversial. Gourlay et al. (1979a) observed only transient inflammatory responses in mammary-glands of lactating cows inoculated with A.laidlawii and A.axanthum. Wehnert (1983) and Pfutzner et al.(1983b) reported that field isolates of A.laidlawii could not produce clinical mastitis but resulted only short term mild disorders. Seffner et al.(1983) observed that A.laidlawii caused moderate subclinical mastitis when inoculated in the mammary-glands of cows. The histological lesions were very mild and degenerative changes in alveolar epithelium were hardly discernable. Hauke (1979) reported that 12 of 16 isolates of A.laidlawii were found pathogenic

to cow-udder and symptoms of acute udder inflammation were recorded. Severe mastitis comparable to that caused by M.bovis in cows has also been recorded (Pfutzner et al., 1979c). with field isolates of A.laidlawii and A.axanthum. The histological changes in the mammary-glands of cows inoculated with A.laidlawii includes proliferation of lymphoid cells in interstitium and alveoli, sporadic occurrence of histiocytes and eosinophils and glandular fibrosis in a few cases (Buchvarova, 1979).

MATERIALS AND METHODS

III MATERIALS AND METHODS

A. Collection of Milk and Serum Samples

Six hundred and nine milk samples from 275 cows and 201 buffaloes were collected from the dairy herds of the department of Animal Breeding, department of Livestock Production and Management, Veterinary Clinics, Haryana Agricultural University, Hisar; Government Livestock Farm (Sector I, II, III), Hisar; Central Institute for Research on Buffaloes, Hisar; Veterinary hospitals at village Bhuna, Jamalpur (district Hisar); village Sisani (district Rohtak); National Dairy Research Institute, Karnal; Dairy herds of College of Veterinary Sciences and Animal Husbandry, Mathura (Uttar Pradesh). The milk from affected quarter(s) was collected separately in sterilized tubes from mastitic animals, while milk from healthy quarter(s) was collected separately and pooled. The milk of all the four quarters from healthy animals was collected and pooled. The collected samples were transported to the laboratory on ice and immediately screened for subclinical mastitis employing Modified White-Side test and Chloride test before subjecting to the isolation procedures for Mycoplasma, Acholeplasma, Ureaplasma and other associated bacteria. Number of milk samples examined alongwith their other details is given in Table 1.

Table 1 Sources and Number of Milk Samples Examined

Source	Number examined			
	Cows		Buffaloes	
	Mastitis	Normal	Mastitis	Normal
HAU, Animal Farm	46(56)*	107(107)	8(10)	52(52)
Government Livestock Farm, Hisar(I,II,III)	36(55)	12(12)	-	-
Veterinary Clinics (HAU, Bhuna, Sisani, Jamalpur)	19(36)	-	43(81)	-
National Dairy Research Institute, Karnal	26(60)	3(3)	9(16)	3(3)
Veterinary College Animal Farm, Mathura	3(4)	23(23)	5(5)	34(34)
Central Institute for Research on Buffaloes, Hisar	-	-	34(39)	13(13)
Total	130(211)	145(145)	99(151)	102(102)

* Figures in parentheses are number of milk samples examined

Blood samples from 321 cows and 97 buffaloes whose milk samples were screened for Mycoplasma, Acholeplasma, Ureaplasma and other associated bacteria, were collected and serum was separated and merthiolated (1:10,000). These were stored at -20C till their serological examination.

Detection of subclinical mastitis

Modified White-Side Test: The test was performed as described earlier (Murphy and Hanson, 1941). One drop of 1N NaOH was added to 5 drops of milk under test on a clean glass slide and mixed thoroughly for about 20 seconds. The formation of weak to strong flocculation and gel formation was considered positive while their absence was considered negative.

Chloride Test: It was performed as per the method of Tompkins et al. (1946). Five ml of silver nitrate (1.3415 gm per litre of distilled water) was taken in a test tube. Two drops of 10 per cent potassium chromate were added. A red colour developed at once. One ml of milk under test was added to the mixture. The appearance of a yellow colour within one min or less was considered positive for subclinical mastitis indicating chloride content of 0.14 per cent or above, whereas the unchanged red colour was considered negative (chloride content less than 0.14%).

B. Isolation of Mycoplasma, Acholeplasma and Ureaplasma

Standard liquid medium (SLM) for Mycoplasma/Acholeplasma (Appendix-I), and U-9B urease colour test medium (Appendix-I) for Ureaplasma were used for primary isolation.

The milk samples were diluted in SLM upto 10^{-4} and incubated at 37C for 4 days. If the growth was suspected in the medium, it was confirmed by transfer of 0.2 ml onto solid agar medium, SAM (Appendix-I). Four to five broth to broth blind passages at 4-day intervals were given if no visible growth was appreciated. The finally passaged broth was plated onto SAM to observe the colonized organisms. Purification of isolates was done by the transfer of an agar-block having single colony to SLM followed by 4-day incubation and reinoculation onto SAM. This procedure was repeated thrice to obtain pure colony-cloned culture. The stock culture for further identification and characterization was prepared in 20 ml SLM which was distributed in 2 ml aliquots and stored (-20C to -40C). Cultures were also lyophilized for long term preservation.

Milk samples were diluted in U-9B medium upto 10^{-5} for the isolation of ureaplasmas and incubation at 37C. The growth of ureaplasmas was indicated by the change in colour of the medium from yellow to pink-red without any turbidity.

The colour change usually commenced from the bottom of the tube to the upward direction. Ureaplasma growth was generally obtained within 18 to 24 hr. incubation at 37C. However, negative tubes were incubated and inspected daily upto 7 days before considering them negative for Ureaplasma. The number of viable ureaplasmas present in a specimen was recorded as colour changing unit (CCU) of activity per ml. The highest dilution of a specimen that produced colour change contained 1 CCU.

C. Characterization of Mycoplasma, Acholeplasma and Ureaplasma

Isolates of Mycoplasma and Acholeplasma were characterized as per the recommendations of Sub-committee on the taxonomy of Mycoplasmatales (Anon, 1979). The isolates were classified as belonging to class Mollicutes on the basis of characteristic 'fried egg' colonial appearance employing Diene's staining (Appendix-I) and absence of reversion to bacterial forms. The classification to the respective orders, Mycoplasmatales and Acholeplasmatales, and into families Mycoplasmataceae and Acholeplasmataceae was done on the basis of sensitivity to digitonin (Clyde, 1964). Filter paper discs (6 mm) were soaked with 0.02 ml of a 1.5 per cent(w/v) ethanolic solution of digitonin ($C_{56}H_{92}O_{29} = 1229.3$) and dried overnight at 37C. The SAM plates were inoculated with 0.1 ml

of test culture having Ca. 10^5 org./ml. The digitonin discs were pressed gently in the middle of the inoculated area and the plates were incubated at 37C in a candle jar for 4 days (Chandiramani et al., 1966). A zone of inhibition of 1 mm or more was considered to be a criterion of sensitive-ness of the organism to digitonin (Freundt et al., 1973).

The isolates were characterized as to their (a) production of acid in glucose, galactose and xylose, (b) deimination of L-arginine, (c) hydrolysis of urea and arbutin, (d) hydrolysis of esculin, (e) reduction of 2',3',5' triphenyl tetrazolium chloride, (f) film and spots formation, (g) test for phosphatase activity, (h) antibiotic sensitivity, and (i) serological identification.

Fermentation of carbohydrates

One per cent carbohydrate viz. glucose/galactose/xylose in phenol red broth (Appendix-II) was used to examine the fermentation ability of the isolates. The medium was inoculated with 0.2 ml of 48 hr. old broth culture of the isolate, and incubated for 21 days. Acid production was manifested by the change of colour from pink to yellow. Uninoculated medium was always kept as control.

Deimination of L-arginine

Each tube of medium (Appendix-II) for deimination of L-arginine was inoculated with 0.2 ml of 48 hr. growth of test culture and incubated alongwith uninoculated tube for three weeks. A distinct alkaline colour change (yellow to pink) when compared with the control tube indicated positive reaction.

Hydrolysis of urea and arbutin

Test media for urea and arbutin hydrolysis (Appendix-II) were inoculated with 0.2 ml of 48 hr. old test culture. Urea hydrolysis was recorded positive when a distinct alkaline colour change (yellow to pink) was evident within three weeks. Hydrolysis of arbutin was manifested by yellowish colouration of the test medium (Appendix II) within a period of 21 days.

Hydrolysis of esculin

The ability of the isolates to hydrolyse esculin was tested on agar medium (Appendix- II). The plates were inoculated with 48 hr. old test culture and incubated at 37C. The results were recorded at 3-, 7- and 14-day of incubation. The appearance of black colour in the test medium was indicative of a positive reaction whereas uninoculated plate incubated simultaneously served as control.

Reduction of 2',3',5' triphenyl tetrazolium chloride

Duplicate tubes of liquid medium having triphenyl tetrazolium chloride (Appendix-II) was inoculated with 0.2 ml of 48 hr. old test culture and incubated, one tube each aerobically and anaerobically at 37C for 2 weeks. The appearance of pink or red colour was recorded as positive test which usually commenced from the bottom of the tube.

Film and spots formation

The medium used was SAM with 20 per cent inactivated normal horse serum. A plate was inoculated with 0.1 ml of 48 hr. old broth culture, incubated for two weeks at 37C in candle jar, and examined after 3 to 4 days for the formation of film over the inoculated area. The plates were also examined under stereoscope (X 80) for dark coloured spots in the upper layer of the medium.

Test for phosphatase activity

Three plates of the medium having phenolphthalein phosphate (Appendix-II) were inoculated for each test culture and incubated at 37C alongwith uninoculated plates. One inoculated and one uninoculated plates were examined at 3,7- and 14-day incubation by flooding the plate with 5N NaOH. Appearance of pink or red colour within approximately 30 seconds was considered positive for phosphatase

activity.

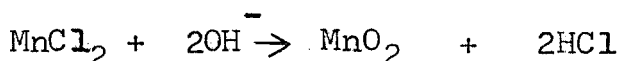
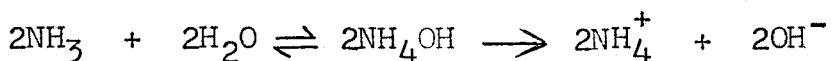
Antibiotic sensitivity

The in-vitro single disc diffusion technique (Bauer et al., 1966) was employed using SAM and various antibiotic discs viz. spiramycin (30 mcg), vibramycin (30 mcg), ledermycin (30 mcg), tetracycline (30 mcg), erythromycin (15 mcg), oxytetracycline (30 mcg), lincomycin (2 mcg), neomycin (30 mcg), kanamycin (30 mcg), streptomycin (10 mcg), ampicillin (10 mcg) and tylosin (15 mcg). The results were interpreted according to the diameter of zone of inhibition as per the manufacturers (Span Diagnostics, Surat, India) recommendations after 5-day incubation at 37°C in candle jar except tylosin in which case the results were recorded as sensitive or resistant irrespective of diameter of zone of inhibition.

Identification of Ureaplasma

Identification of ureaplasmas was based on the direct urease test (Shepard and Howard, 1970) on primary agar cultures employing the indicator reagent (1% aqueous urea + 0.8% mangnous chloride). Ureaplasma colonies under stereoscope (X 80) were visible as dark, golden brown coloured colonies which were due to the deposition of manganese on the surface of colonies (and sometimes in a circular zone on the agar surrounding colonies) as per the

following reactions:



Further characterization of Ureaplasma isolates could not be done as these could not be propagated in U-9B colour test medium for longer periods.

Serological identification of Mycoplasma and Acholeplasma

The serological identification of Mycoplasma and Acholeplasma isolates was done using growth inhibition test (Clyde, 1964). The test culture (0.1 ml having Ca. 10^5 CFU/ml) was spread on SAM plates, dried for 30 min at room temperature and filter paper discs impregnated with 0.02 ml undiluted hyperimmune rabbit anti-mycoplasma serum were pressed gently. The plates were incubated in a candle jar for 4 days at 37C after which the zone of inhibition was measured in mm from the rim of the disc to the beginning of the growth. A clear zone of inhibition of 1 mm or more indicated the homologous inhibition. The isolates were serogrouped utilizing standard rabbit hyperimmune sera against M.bovis (Donetta), M.bovigenitalium (PG-11), M.bovirhinis (PG-43), Mycoplasma sp.gp.7 (PG-50),

M. bovoculi (M-165/69), M. mycoides var. mycoides (SC type, PG-1; LC type, Y-goat), M. mycoides var. capri (PG-3), M. fermentans (PG-18), M. dispar (462/2), M. alkalescens (PG-51), M. arginini (G-230), A. laidlawii (PG-8), A. oculi (19-L), A. granularum (BTS-39), A. axanthum (S-743), A. modicum (PG-49), and A. hippikon (C-1). These sera were kindly supplied by Dr. E.A. Freundt, Director, FAO/WHO Collaborating Centre for Animal Mycoplasmas, Aarhus, Denmark. The isolated and identified strains in our laboratory were submitted for confirmation to (i) Dr. R.H. Leach, PHLS, Mycoplasma Reference Laboratory, Norwich, England, (ii) Dr. T. Shimizu, Laboratory of Veterinary Hygiene, Miyazaki University, Japan, and (iii) Dr. H. Kirchhoff, Institut für Microbiologie und Tierseuchen, der Tierärztlichen Hochschule, Hannover, West Germany.

D. Isolation and Identification of Other Associated Bacteria

Milk samples were streaked on sheep blood agar, nutrient agar, Carter's medium and McConkey's Lactose Agar (Cruickshank, 1975) to isolate other bacteria associated with mycoplasmal bovine mastitis. The plates were incubated at 37C for 48 hr. and cultures were purified and preserved in soft-agar stabs at 4C. Bacterial isolates which were associated with bovine mycoplasmal mastitis were characterized upto the generic level (Cowan, 1977). The in-vitro single disc diffusion technique (Bauer et al., 1966) using various

antibiotics discs viz. ampicillin (10 mcg), chloramphenicol (30 mcg), cephaloridine (30 mcg), cloxacillin (1 mcg), colistin (10 mcg), erythromycin (15 mcg), furadantin (300 mcg), gentamycin (10 mcg), kanamycin (30 mcg), ledermycin (30 mcg), lincomycin (2 mcg), neomycin (30 mcg), oxytetracycline (30 mcg), penicillin (10 IU), tetracycline (30 mcg), streptomycin (10 mcg), sulphadiazine (300 mcg), tylosin (15 mcg), vibramycin (30 mcg), and spiramycin (30 mcg)^{was employed.} The results were interpreted according to diameter of zone of inhibition as per the manufacturer's (Span Diagnostic, Surat, India) recommendations except tylosin, in which case the results were recorded as sensitive or resistant irrespective of zone of inhibition.

E. Seroprevalence of Bovine Mycoplasmal Mastitis

M. bovis and M. bovis genitalium are considered as the most important species which cause bovine mastitis. Therefore, the serum samples collected from mastitic and healthy cows and buffaloes were screened through the application of three serological techniques viz. Enzyme-Linked-Immuno-Sorbent Assay (ELISA), Indirect Haemagglutination (IHA) and Agar-Gel-Immuno-Precipitation Test (AGIPT) to determine the seroprevalence of M. bovis and M. bovis genitalium antibodies. A brief procedure for raising hyperimmune sera against M. bovis and M. bovis genitalium for standardization of ELISA, IHA and AGIPT with the test procedures is given below:

Production of hyperimmune serum in rabbits

The freeze dried cultures of the M.bovis (NCTC-10131) and M.bovigenitalium (NCTC-10122) were reconstituted in 0.5 ml of SLM having 20 per cent normal rabbit serum (4 days at 37C). Initially cultures were inoculated in 10 ml and 100 ml SLM and further inoculated to obtain growth in 450 ml SLM (37C, 48 hr). The resultant growth was centrifuged (10,000xg, 30 min) at 4C using refrigerated centrifuge (Sorvall, RC-58). Pellete of the organisms was washed thrice with PBS (pH 7.2), and final pellete was suspended in 10ml PBS, which was stored at 4C. It served as stock-antigen for immunization of rabbits.

Healthy Mycoplasma-free rabbits (NewZealand-White), 2 for M.bovis and 2 for M.bovigenitalium, were immunized as per schedule given in Table 2 and bled one week after the last injection to harvest hyperimmune serum.

Production of hyperimmune serum in cow-calves

The antigen to immunize cow-calves was prepared as described for rabbit immunization except that normal calf serum was used in SLM instead of rabbit serum. The growth in 1800 ml medium was pelleted and resuspended in 36 ml PBS (pH 7.2). This was further diluted with PBS to 1:5 (4×10^{11} CFU/ml) and used for immunization of cow-calf. Healthy

Table 2 Immunization schedule for raising hyperimmune serum against M. bovis/M. bovis genitalium in rabbit and cow-calf

Week (s)	Inoculum*	Administration
<u>Schedule for Rabbit</u>		
1/7	1.8 ml + 1.8 ml CFA**	Subcutaneously 0.4 ml in each rear foot pad, 0.8 ml distributed at 4 sites on the back, and 2.0 ml intramuscularly in the thigh region
3	1.25 ml	1.0 ml intramuscularly and 0.25 ml intravenously
4	0.25 ml	0.25 ml intravenously
5	0.25 ml	0.25 ml intravenously
8	0.25 ml	0.25 ml intravenously
<u>Schedule for cow-calf</u>		
1/7	5 ml antigen + 5 ml CFA	10 ml subcutaneously at 4 places in prescapular region
4/7	5 ml	5 ml subcutaneously at prescapular region
2	5 ml	5 ml subcutaneously at prescapular region
4	3 ml	3 ml intravenously
7	5 ml	5 ml subcutaneously at prescapular region

* Stock antigen diluted to Brown's opacity tube no. 10 with PBS and sonicated for 10 min at 150-200 watts using Brounsonic-1510

** CFA = Difco's Freundt Complete adjuvant

mycoplasma-free bovine calves, 4-6 weeks of age, in groups of 2 per Mycoplasma species were immunized as per the schedule given in Table 2 and bled one week and 15 days after the last injection to harvest hyperimmune serum.

Pre-immunization sera of rabbits and cow-calves served as known negative sera for conducting various serological tests.

Agar-gel-immuno-precipitation-test (AGIPT)

The Ouchterlony's technique (1953) was employed using 75X75 mm slides containing 8 ml of one per cent agarose in PBS (pH 7.3) with sodium azide (0.1%) to prevent bacterial growth. The melted agar was added to the slides with a pipette and solidified at 4C for 60 min. Wells, 7 mm in diameter and 3 mm apart were cut in the agar in a hexagonal pattern.

The antigen used in AGIPT was sonicated-whole-cell antigen (180 to 200 watts for 15 min using Braunsonic-1510) which was adjusted to Brown's opacity tube no. 10. Approximately 70 μ l antigen in central well and equal amount of test-sera in outer wells were added. The slides were incubated in humidified chambers at 37C for 12 hr. and results were recorded. Later, the test was observed at 4C for 7 days before recording the final results. Every

serum collected from mastitic and healthy bovine was screened for M.bovis and M.bovigenitalium antibodies.

Cross reactions, if any, of M.bovis and M.bovigenitalium antigens were determined with hyperimmune sera of 15 other Mollicutes organisms: M.mycoides var. mycoides, SC type (PG-1), LC type (Y-goat); M.mycoides var. capri (PG-3); M.capricolum (California-kid); Mycoplasma sp.gp.7 (PG-50); M.bovis (Donetta); M.bovigenitalium (PG-11); M.dispar (462/2); M.alkalescens (PG-51); M.agalactiae (PG-2); M.bovirhinis (PG-43); M.bovoculi (M-165/69); M.canadense (C-275); M.verecundum (107); A.laidlawii (PG-8) and A.granularum (BTS-39).

Indirect haemagglutination test (IHA) (Cho et al., 1976)

Antigen preparation: M.bovis (NCTC-10131) and M.bovigenitalium (NCTC-10122) were used for antigen preparation. Organisms were grown in SLM for 4 days at 37°C. The growth was centrifuged at 10,000xg for 30 min at 4°C using refrigerated centrifuge (Sörvall, RC-58). The pellete was washed thrice with PBS (7.2) and finally suspended in PBS to give a final protein concentration of 3.75 mgm/ml for M.bovis, and 5.5 mg/ml for M.bovigenitalium measured by Lowry's method (Lowry et al., 1951). Sodium azide (0.01%) was added to the antigen and stored at 4°C.

Preparation of glutaraldehyde-fixed sheep erythrocytes: Sheep erythrocytes collected in Alsever's solution (glucose 2.05 gm, sodium chloride 0.42 gm, trisodium citrate 0.80 gm, citric acid 0.055 gm, distilled water 100 ml) were washed 4 times with phosphate buffered glucose, PBG (76 ml or 0.15M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 24 ml of 0.15M KH_2PO_4 , 100 ml of 5.4% glucose, pH 7.2). An equal volume of 0.2 per cent glutaraldehyde dissolved in PBG was mixed with 20 percent erythrocytes in PBS and incubated at 37C in water bath for 15 min. The glutaraldehyde-fixed cells were washed 5 times with 0.85 per cent saline having 0.1 per cent sodium azide and stored at 4C until use.

Sensitization of glutaraldehyde-fixed sheep erythrocytes with M.bovis and M.bovigenitalium: Stored glutaraldehyde-fixed sheep erythrocytes were washed twice with 0.01M PBS (pH 7.0) and 20 per cent suspension of erythrocytes was made in PBS. Sensitization procedure included mixing 2 ml of M.bovis or M.bovigenitalium antigen, 5 ml of 20 per cent glutaraldehyde-fixed sheep erythrocytes and 0.7 ml sodium azide (1%). The mixture was shaken thoroughly and incubated at 37C for 16 to 18 hr. with intermittent agitation. Thereafter, the erythrocytes were washed thrice with PBS at 250xg for 10 min. The sensitized erythrocytes were resuspended in 50 per cent glycerine in PBS to give 20 per cent concentration and stored at 4C until use.

IHA test procedure: Bovine serum under test and normal rabbit serum (both inactivated at 56C for 30 min) were preabsorbed with fresh sheep erythrocytes. Equal volumes of serum and erythrocytes were incubated at 37C for 30 min in water bath. Absorbed serum was separated by centrifugation at 250xg for 10 min. Serial two-fold dilutions of bovine sera (starting 1:10) under test were prepared using PBS having 1 per cent normal rabbit serum as diluent in 96-well microtitre U-shaped wells plastic trays (Laxbro, India). The mycoplasma sensitised glutaraldehyde-fixed sheep erythrocytes were washed thrice in PBS and resuspended at 2 per cent concentration in PBS. This antigen in 0.025 ml was added to each well having 0.025 ml serum dilution. The contents of the wells were mixed by gentle tapping and incubated at 37C for 2 hr. Antigen, known positive and negative serum controls were included in each test-batch. The maximum dilution showing characteristic 50 per cent erythrocytic agglutination was recorded as end-titre. The results were interpreted as per Cho et al. (1976), considering a titre of $\geq 1:160$ as positive, $= 1:80$ as suspicious and $\leq 1:40$ as negative. Heterologous cross reactions, if any were recorded for the hyperimmune serum against M. bovis genitalium (PG-11), M. bovis (Donetta), M. bovoculi (M-165/69), M. canadense (C-275), M. capricolum (California-kid), M. mycoides (SC type, PG-1; LC type, Y-goat), M. bovirhinis (PG-43), M. mycoides var. capri (PG-3), M. dispar (462/2), Mycoplasma sp.gp.7 (PG-50),

M. agalactiae (PG-2), A. laidlawii (PG-8), A. axanthum (S-743), A. granularum (DTS-39) and A. oculi (19-L).

Enzyme-linked-immuno-sorbent-assay (ELISA) (Boothby et al., 1981)

Antigen: Strains used for antigen preparation were M. bovis (NCTC-10131) and M. bovis genitalium (NCTC-10122). The sonicated whole-cell antigen as prepared for calf immunization was diluted 1:25 with carbonate-bicarbonate buffer (pH 9.2). It was centrifuged at 10,000xg for 30 min, the supernatant fluid was collected and the protein was adjusted so that each well received 1.75 µg/well and 3.31 µg/well for M. bovis and M. bovis genitalium, respectively.

Enzyme-antiglobulin conjugate: Horseradish-peroxidase (type VI, Sigma) labelled rabbit anti-cow-Ig obtained from M/s Dakopatts, Denmark (product no. P-159) was used.

Substrate: A 0.2 mM solution (w/v) of 40 mM 2,2'-azino-di-(3-ethyl-benzy-thiazoline-sulfonic acid) i.e. ABTS in 2 mM hydrogen peroxide solution with 0.5 M citrate buffer was prepared fresh (Appendix-III).

Optimization of antigen and conjugate: Disposable microtitre trays (Linbro) type IS-FB-96 (Flow Lab., UK) were used as test vehicle for soluble Mycoplasma antigen.

The optimum antigen concentration was determined by checker board titration with known positive and negative hyperimmune sera against M.bovis and M.bovigenitalium. Antigen (3.5 mg/ml for M.bovis, 6.6 mg/ml for M.bovigenitalium) was diluted 1:25, 1:50, 1:100 and 1:200 in carbonate-bicarbonate buffer (Appendix-III) before application to the plate. The conjugate was diluted 1:100, 1:200, 1:400 and 1:800 in 0.1M PBS (Appendix-III) and known positive and known negative sera were diluted from 1:40 to 1:5120. The ELISA was performed using different dilutions of antigens, conjugate and serum. Optimum dilution of the coating antigen in this study was 1:100, which was the highest dilution when reacted with highest dilution of serum resulted in maximum colour change in the substrate solution and maximum contrast between positive and negative serum. The optimum dilution for the anti-cow gamma globulin conjugate used in this study was 1:400, which was the highest dilution of the conjugate which produced maximum colour change in wells having highest dilution of the serum with maximum contrast between wells having positive and negative serum.

ELISA procedure: Distilled-water rinsed plate-wells were coated for 3 hr. at 37°C with 0.05 ml of dilution of antigen (1:100) which contained protein 1.75 µg/well for M.bovis antigen and 3.31 µg/well for M.bovigenitalium antigen. The antigen coated plates were stored at 4°C in

plastic covers without loss of the activity until use, which was about 3 months in this study. The plate-wells were rinsed with 0.15 M NaCl with 0.5 per cent Tween-20 (pH 7.0) using wash bottle. Serum diluted in 0.1M PBS (pH 7.6) containing 0.5 per cent Tween-20 (Appendix-III) was added to each well (0.05 ml) and kept at room temperature (25 to 37C) for 15 min with intermittent shaking. Serum was removed and wells were washed 4-times with washing solution. Optimum dilution of conjugate (1:400) in same buffer as for the serum was added to each well (0.05 ml) and again kept at room temperature for 15 min. After again washing the plates, 0.1 ml of enzyme substrate was added to each well. The plates were kept for 15 min at room temperature, 0.1 ml of stopping reagent (Appendix-III) was added, and the absorbance was measured at 450 nm in a Titertek-Multiscan (Flow laboratories, UK).

With each test-batch, known mycoplasmal antibodies were included for comparison. The results were recorded considering the absorbance of known negative serum. The range of maximum absorbance of reference negative serum varied from 0.043 to 0.195 for M.bovis antigen and 0.043 to 0.143 for M.bovigenitalium antigen on the basis of atleast 65 readings of the same negative serum in our study and taken as cut-off absorbance value for calculating the end-titre of the test serum.

F. Experimental Pathogenicity of Mollicutes Organisms Isolated from Bovine Milk

The pathogenicity of significant Mollicutes organisms isolated from bovine mastitis was studied employing two experimental systems viz. hamster tracheal-ring organ culture and rat mammary-gland system.

Hamster tracheal-ring organ culture

Adult golden hamsters (Mesoericetus auratus), weighing 80 to 100 gm, were obtained from the Disease Free Small Animal House, Haryana Agricultural University, Hisar. Hamsters were deeply anaesthetised with ether. Tracheas were dissected out under a strict aseptic conditions and were cut into circular rings of about 3-4 mm, which were kept immersed for 15 to 20 min in prewarmed Hank's balanced salt solution, pH 7.2 (HBSS) containing 2000 IU penicillin/ml, nystatin 50 µg/ml. The exudate and cell debris from tracheal rings were removed by 2 to 3 washings in HBSS. Following Hoorn's (1966) technique, tracheal rings were planted on scratched 35 mm disposable plastic petridishes (Steriware, India) (Fig.1), each containing Eagle's BME-basal medium (pH 7.2) supplemented with 10 per cent (v/v) heat inactivated bovine fetal-calf serum (Flow laboratory, UK), 1 per cent 2 mM glutamine, 1000 IU/ml penicillin and 50 µg/ml nystatin. These cultures were incubated at 37°C in candle jar. The

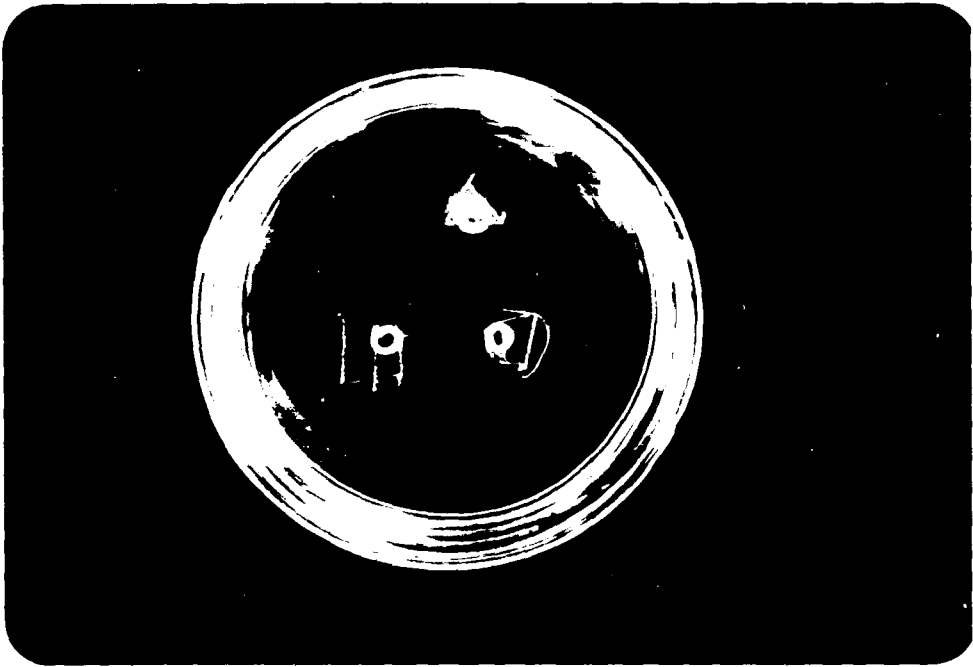


Fig.1 Hamster trac cell ring on a culture system (A 1.5)

culture medium was changed after 24 hr. and incubated further for 48 hr.. Cultures were observed daily for ciliary activity. The normal ciliary activity could be maintained for atleast 12 days under these conditions.

Tracheal rings exhibiting vigorous ciliary activity were selected for inoculation with Mycoplasma/Acholeplasma culture under test. Each culture dish was inoculated with 0.5 ml of Mycoplasma/Acholeplasma culture having 1×10^6 to 2.7×10^6 CFU to give a final concentration of Ca. 10^6 organisms/ml of BME nutrient medium. Five rings were inoculated per culture, and 2 to 3 rings were kept as uninoculated controls.

Inoculated and control rings were observed at every 48 hr. upto 12 days for ciliary activity and reisolation of inoculated organisms was attempted simultaneously from organ culture medium. At 12-day postinfection, tracheal rings were subjected to histopathology and reisolation attempted from infected and control rings. The presence of endotoxin in the nutrient medium was estimated by Limulus amoebocyte lysate test (Technical bulletin no. 210, Sigma). The ciliary activity was observed at X200 with an inverted microscope (CK-Model, Olympus, Japan) and the ciliary activity score given was the product of ciliary vigour (from 0 for no detectable activity which is due to complete ciliostatis, and 3 for normal activity), times the per cent of tracheal ring retaining the ciliary activity. Scores ranged

from 0, no ciliary activity on any portion of the ring to a maximum of 300 for normal vigorous activity over the entire ring (i.e. 3 times 100%). The culture under test which caused reduction of ciliary activity to a score of 20 or less was considered pathogenic (Chandler and Barile, 1980). Eight Mycoplasma/Acholeplasma isolates, (7 from mastitic and 1 from normal bovine milk) and a type strain of M.bovis (NCTC-10131) were tested for their pathogenicity in hamster tracheal ring organ culture (Table 3).

Rat mammary-gland model

Norway albino rats (Rattus norvigeus, strain Wistar) at 7 to 10 days lactation, bred at Disease Free Small Animal House, Haryana Agricultural University, Hisar were used. The technique of Chandler (1970) was employed to inoculate rats by the intramammary route using nontoxic pyrogen free 30Gx $\frac{1}{2}$ " needles (Thomas, USA). The third, fourth and fifth pairs of mammary-gland from the front on each side (Fig.2) were used and the volume of the inoculum was 0.1 ml having Ca. 10^6 org./teat. The right glands (R-3,R-4) served as uninoculated controls whereas right R-5 served as inoculated control, which was given sterile SLM. Left glands (L-3, L-4, L-5) were injected with Mycoplasma/Acholeplasma culture under test.

Table 3 Mycoplasma/Acholeplasma isolates tested for their pathogenicity in experimental systems

Sr.No.	<u>Mollicutes</u> species	Isolate no.	Source
1	<u>M.bovis</u>	NCTC-10131	Cow milk
2	<u>M.mycoides</u> var. <u>capri</u>	CM-111	Mastitic-cow milk
3	<u>Mycoplasma</u> F-38	CM-193	Mastitic-cow milk
4	<u>M.bovigenitalium</u> + <u>M.bovirhinis</u> + <u>Mycoplasma</u> F-38	CM-195	Mastitic-cow milk
5	<u>M.bovirhinis</u> + <u>M.bovigenitalium</u>	CM-196	Normal-cow milk
6	<u>M.bovis</u> + <u>M.bovigenitalium</u> + <u>Mycoplasma</u> F-38 + <u>A.laidlawii</u>	CM-197	Mastitic-cow milk
7	<u>A.axanthum</u>	CM-212	Mastitic-cow milk
8	<u>A.laidlawii</u>	CM-230	Mastitic-cow milk
9	<u>Acholeplasma</u> sp.*	CM-448	Mastitic-buffalo milk

* The isolate could not be serogrouped employing antisera against A.laidlawii (PG-8), A.granularum (BTS-39), A.oculi (19-L), A.axanthum (S-743), A.modicum (PG-49) and A.hippikon (C-1)

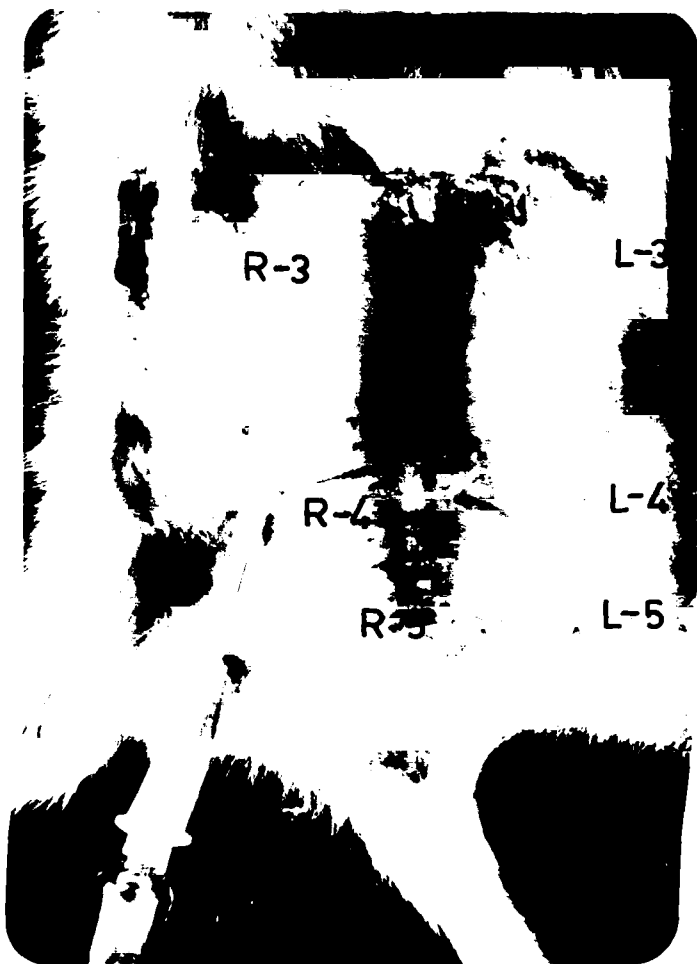


Fig.2 Rat mammary-gland model. L-3, L-4, L-5 left mammary-glands; R-3, R-4, R-5 right mammary-glands (x1.5)

Five rats per culture were injected in order to observe progress of infection in terms of gross and microscopic lesions. One rat each was observed and sacrificed at 24, 48, 72, 96 and 144 hr. postinfection. Gross lesions of infected glands at necropsy were recorded and glands were cut into two; one half fixed in formal-saline for histopathology, the other half was ground with sterile sand in 1.8 ml SLM having 50 µg/ml ammonium reineckate in order to recover the inoculated Mycoplasma/Acholeplasma. Ammonium reineckate was used in the medium to overcome inhibitory activity of murine tissue homogenates (Mardh and Taylor-Robinson, 1973). The control glands were processed similarly for comparison. All the Mycoplasma/Acholeplasma isolates (Table 3) were tested for their pathogenicity in rat-udder.

RESULTS

III RESULTS

A. Isolation of Mycoplasma, Acholeplasma and Ureaplasma

Two hundred and eleven milk samples from 130 mastitic-cows and 151 milk samples from 99 mastitic-buffaloes were screened to isolate Mollicutes organisms and other associated bacteria (Table 1). Besides, 145 milk samples from healthy cows and 102 milk samples from healthy buffaloes were also tested.

1. Isolation of Mollicutes organisms from mastitic-cows and-buffaloes

Fifteen isolates comprising of 20 strains belonging to class Mollicutes from milk of 130 mastitic-cows (Table 4) and 5 isolates from 99 mastitic-buffaloes (Table 5) were isolated. Twenty strains from mastitic-milk of cows comprised of 8 mycoplasmas (1 M.bovis, 2 M.bovigenitalium, 3 Mycoplasma, F-38, 1 M.bovirhinis, 1 M.mycoides var. capri), 7 acholeplasmas (4 A.laidlawii, 2 A.axanthum, 1 Acholeplasma sp. unidentified), and 5 isolates of Ureaplasma. The isolates no. CM-195 and CM-197 were considered to be mixed isolates on the basis of results of indirect epiimmuno-fluorescence test on the colonies of isolates (done in the laboratory of Dr.H.Kirchhoff, Hannover, Germany) as the former consisted of M.bovigenitalium, M.bovirhinis and

Table 4 Isolation of Mycoplasma, Acholeplasma and Ureaplasma from milk of mastitic-cows

Sample/Isolate number/	Source	Mollicutes organism(s)	Other associated bacteria
CM-193	GLF III, Hisar	<u>Mycoplasma</u> F-38	-
CM-195*	" " "	<u>M.bovigenitalium</u> , <u>M.bovirhinis</u> , <u>Mycoplasma</u> F-38	<u>S.epidermidis</u>
CM-197*	" " "	<u>M.bovis</u> , <u>M.bovigenitalium</u> , <u>Mycoplasma</u> F-38, <u>A.laidlawii</u>	<u>S.epidermidis</u>
CM-198	" " "	<u>A.axanthum</u>	-
CM-192	" " "	<u>Acholeplasma</u> sp.	-
CM-202	HAU, Animal Farm	<u>A.laidlawii</u>	<u>S.epidermidis</u>
CM-203	" " "	<u>A.laidlawii</u>	<u>Micrococcus</u> sp.
CM-212	" " "	<u>A.axanthum</u>	-
CM-230	" " "	<u>A.laidlawii</u>	<u>Micrococcus</u> sp.
CM-111	NDRI, Karnal	<u>M.mycoides</u> var. <u>capri</u>	-
CM-290	" " "	<u>Ureaplasma</u> (5×10^2)**	-
CM-105	" " "	<u>Ureaplasma</u> (5×10^2)	-
CM-110	" " "	<u>Ureaplasma</u> (5×10^3)	<u>S.epidermidis</u>
CM-111	" " "	<u>Ureaplasma</u> (5×10^2)	-
CM-113	" " "	<u>Ureaplasma</u> (5×10^2)	-

* These isolates were considered mixed isolates on the basis of indirect epiimmunofluorescence test in the laboratory of Dr. H.Kirchhoff, Hannover, Germany

** Colour changing units (CCU)/ml, of original sample, GLF = Government Livestock Farm, HAU = Haryana Agricultural University, NDRI = National Dairy Research Institute

Table 5 Isolation of Mycoplasma, Acholeplasma and Ureaplasma from buffalo-milk

Sample/Isolate number	Source	Mollicutes organism(4)	Other associated bacteria
<u>Mastitic-buffaloes</u>			
CM-115	National Dairy Research Institute, Karnal	<u>Ureaplasma</u> (5×10^3)*	-
CM-117	National Dairy Research Institute, Karnal	<u>Ureaplasma</u> (5×10^3)	<u>Micrococcus</u> sp.
CM-42	Veterinary Hospital, Bhuna	<u>Mycoplasma</u> **	<u>S. epidermidis</u>
CM-43	Veterinary Hospital, Bhuna	<u>Mycoplasma</u> **	-
CM-448	Haryana Agricultural University Animal Farm, Hisar	<u>Acholeplasma</u> sp.	-
<u>Normal-buffalo</u>			
CM-462	Haryana Agricultural University Animal Farm, Hisar	<u>Acholeplasma</u> sp.	<u>Micrococcus</u> sp.

* Colour changing units (CCU)/ml. of original sample

** Cultures could not be colonized

Mycoplasma F-38 whereas the latter consisted of M. bovis, M. bovis genitalium, Mycoplasma F-38 and A. laidlawii. The biochemical characters and serogrouping of 11 isolates of Mycoplasma and Acholeplasma from milk of mastitic-cows and a buffalo are given in Table 6, whereas their antibiotic sensitivity characters are given in Table 7.

Out of 5 isolates of class Mollicutes (2 Mycoplasma, 1 Acholeplasma, 2 Ureaplasma) from mastitic-milk of buffaloes, 2 mycoplasma could not be colonized and 1 Acholeplasma sp. remained unidentified (isolate no. CM-448) by growth inhibition test utilizing antisera of A. laidlawii, A. axanthum, A. granularum, A. oculi, A. modicum and A. hippikon (Table 5). The biochemical and serogrouping of isolate no. CM-448 from mastitic-buffalo milk is given in Table 6 and its antibiotic sensitivity is given in Table 7. All the 11 isolates from 10 mastitic-cows and 1 mastitic-buffalo were sensitive to spiramycin, vibramycin, ledermycin, lincomycin, tetracycline, oxytetracycline and tylosin, but were resistant to ampicillin. Only 1 isolate (no. CM-111) was resistant to neomycin, kanamycin and erythromycin and 7 isolates (no. CM-193, CM-195, CM-198, CM-203, CM-212, CM-111, CM-192) were resistant to streptomycin.

Evidences of Ureaplasma infection were noted in 5 and 2 milk samples from 130 mastitic-cows and 99 mastitic-buffaloes, respectively. Number of ureaplasmas measured in

Table 7 Antibiotic sensitivity of 11 isolates of Mycoplasma and Acholeplasma from milk of mastitic-cows and -buffaloes

Antibiotic discs/concentration	Antibiotic sensitivity of isolate number from										Buffal
	Cows										CM-44
	CM-193	CM-195	CM-197	CM-198	CM-202	CM-203	CM-212	CM-230	CM-111	CM-192	
Spiramycin (30 mcg)	S	S	S	S	S	S	S	S	S	S	S
Vibramycin or doxycycline (30 mcg)	S	S	S	S	S	S	S	S	S	S	S
Ledermycin or demeclocycline (30 mcg)	S	S	S	S	S	S	S	S	S	S	S
Tetracycline (30 mcg)	S	S	S	S	I	S	S	S	S	S	S
Erythromycin (15 mcg)	S	S	S	S	S	S	S	S	R	S	S
Oxytetracycline (30 mcg)	S	S	S	S	S	I	S	S	S	S	S
Lincomycin(2 mcg)	S	S	S	S	S	S	S	S	S	S	S
Neomycin (30 mcg)	S	S	S	S	S	S	S	S	S	S	S
Kanamycin (30 mcg)	S	S	S	S	S	S	S	S	R	S	S
Streptomycin(10 mcg)R		R	S	R	I	R	R	S	R	R	S
Ampicillin(10 mcg) R		R	R	R	R	R	R	R	R	R	R
Tylosin(15 mcg)	S	S	S	S	S	S	S	S	S	S	S

S = Sensitive, R = Resistant, I = Intermediate depending upon the diameter of zone of inhibition ranges prescribed by manufacturer of discs (Span Diagnostic, Surat) except tylosin for which only R or S was recorded

terms of colour changing units (CCU/ml) in original milk samples ranged between 5×10^2 to 5×10^3 . All the strains of ureaplasma were characterised on the basis of their urease production on primary cultures in U9-B urease colour test medium and primary agar culture plates employing indicator reagent (1.0% aqueous urea + 0.8% MnCl_2). Ureaplasma strains could not be propagated for longer period in either liquid or solid medium making it impossible to characterize further.

The percentage of organisms of class Mollicutes from mastitic-cows was 15.38, which was higher than the 5.05 per cent isolation of Mollicutes organisms from mastitic-buffaloes (Table 8, Fig.3). The isolation of Mycoplasma (6.15%), Acholeplasma (5.38%) and Ureaplasma (3.84%) was also higher in mastitic-milk of cows than their isolation from mastitic-buffalo milk (Mycoplasma 2.02%, Acholeplasma 1.0% and Ureaplasma 2.02%) (Table 8).

2. Isolation of Mollicutes organisms from milk of healthy cows and buffaloes

A total of 609 milk samples from 275 cows and 201 buffaloes were examined. This included 145 and 102 normal milk samples from healthy cows and buffaloes, respectively (Table 1). Ten isolates comprising of 11 strains belonging to class Mollicutes from 145 healthy cows were isolated (Table 9) whereas only 1 isolate was from 102 healthy buffaloes (Table 5).

Table 8 Prevalence of Mollicutes organisms associated bovine mastitis

Animals with numbers in parentheses	<u>Mycoplasma</u>	<u>Acholeplasma</u>	<u>Ureaplasma</u>	Prevalence of <u>Mollicutes</u> organisms
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Prevalence of Mollicutes organisms in mastitic-milk

Cows (130)	8(6.15)*	7(5.38)	5(3.84)	20(15.38)
Buffaloes (99)	2(2.02)	1(1.01)	2(2.02)	5(5.05)

Prevalence of Mollicutes organisms in normal-milk

Cows(145)	3(2.06)	4(2.75)	4(2.75)	11(7.58)
Buffaloes(102)	0(0)	1(0.98)	0(0)	1(0.98)

* Figures in parentheses indicate percentage

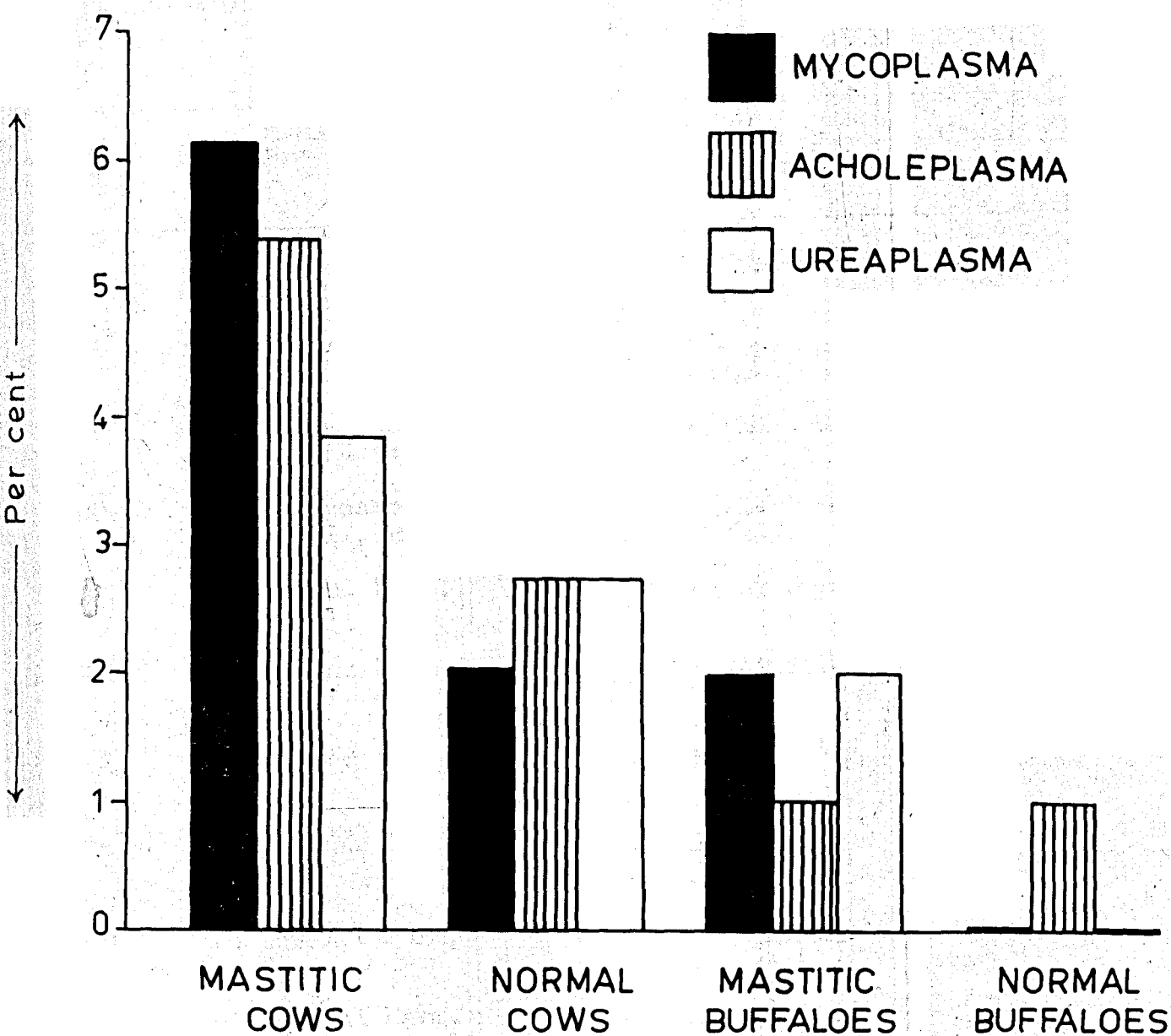


Fig.3 Prevalence of various Mollicutes amongst mastitic and normal milk of cows and buffaloes

Table 9 Isolation of Mycoplasma, Acholeplasma and Ureaplasma from milk of normal cows

Sample number	Source	Mollicutes organism(s)	Other associated bacteria
CM-194	Govt. Livestock Farm Sector III, Hisar	<u>M. bovirhinis</u>	-
CM-196*	Govt. Livestock Farm Sector III, Hisar	<u>M. bovirhinis</u> <u>M. bovigenitalium</u>	-
CM-199	Govt. Livestock Farm Sector III, Hisar	<u>A. axanthum</u>	-
CM-200	Govt. Livestock Farm, Sector III, Hisar	<u>A. axanthum</u>	<u>S. epidermidis</u>
CM-209	HAU Animal Farm, Hisar	<u>A. laidlawii</u>	<u>Enterobacter</u> sp.
CM-210	HAU Animal Farm, Hisar	<u>A. laidlawii</u>	<u>Micrococcus</u> sp.
CM-129	HAU Animal Farm, Hisar	<u>Ureaplasma</u> (5×10^1)**	-
CM-136	HAU Animal Farm, Hisar	<u>Ureaplasma</u> (5×10^1)	<u>Citrobacter</u> sp.
CM-139	HAU Animal Farm, Hisar	<u>Ureaplasma</u> (5×10^2)	-
CM-213	HAU Animal Farm, Hisar	<u>Ureaplasma</u> (5×10^1)	-

* The isolate was considered mixed isolate on the basis of indirect epiimmunofluorescence test in the laboratory of Dr.H.Kirchhoff, Hannover, Germany

** Colour changing units (CCU)/ml in original sample

Eleven strains belonging to class Mollicutes from healthy cows represented 2 M.bovirhinitis, 1 M.bovigenitalium, 2 A.laidlawii, 2 A.axanthum and 4 Ureaplasma sp. The isolate no. CM-196 was considered to be a mixed isolate on the basis of results of indirect epiimmunofluorescence test on the colonies of isolate (done in the laboratory of Dr.H.Kirchhoff, Hannover, Germany) as it consisted of M.bovirhinitis and M.bovigenitalium. The biochemical characters and serogrouping of Mycoplasma and Acholeplasma isolated from milk of healthy cows and buffaloes are given in Table 10 whereas their antibiotic sensitivity characters are given in Table 11. All the 6 isolates from cow-milk and one isolate from normal buffalo-milk were sensitive to spiramycin, vibramycin, lincomycin, neomycin, kanamycin and tylosin but were resistant to ampicillin. One strain of A.laidlawii (no. CM-209) from normal cow-milk was intermediary sensitive to ledermycin, tetracycline and oxytetracycline whereas only strain resistant to erythromycin was A.laidlawii (isolate no. CM-210). All the isolates of Mycoplasma and Acholeplasma from normal cow-milk were resistant to streptomycin, whereas the only isolate of Acholeplasma sp. from normal buffalo-milk was sensitive.

The only isolate (no. CM-462) from normal buffalo-milk belonged to genus Acholeplasma (Fig.4) which could not be

Table 10 Biochemical characters and serogrouping of Mycoplasma and Acholeplasma isolated from milk of healthy cows and buffaloes

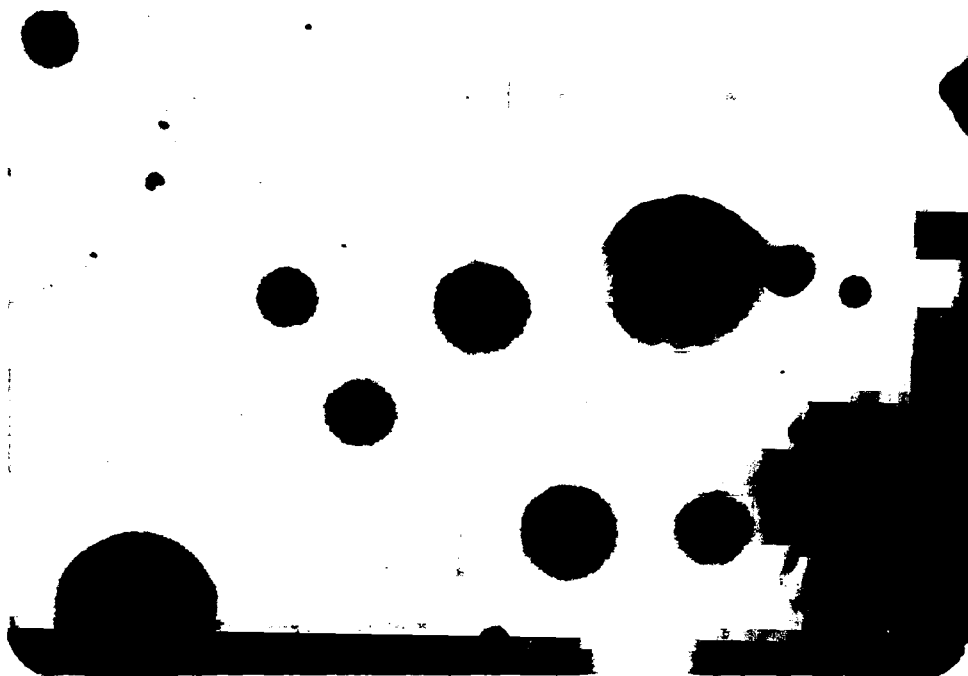
Test	Isolate number from						
	Cows						
	CM-194	CM-196	CM-199	CM-200	CM-209	CM-210	Buffalo CM-462
Sensitivity to digitonin:	S	S	R	R	R	R	R
Fermentation of:							
Glucose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+
Deimination of L-arginine:	-	-	-	-	-	-	-
Hydrolysis of:							
Urea	-	-	-	-	-	-	-
Arbutin	+	+	+	+	-	-	-
Esculin	-	-	-	-	+	+	+
Reduction of tetrazolium chloride	-	-	-	-	-	-	+
Film and spots formation	-	-	-	-	-	-	-
Test for phosphatase activity	+	+	+	+	-	+	-
Serotyping by disc growth inhibition test/epimmunofluorescence	M.bovirh.	M.bovirh.	M.bovirh. + M.bg.	A.axan.	A.laid.	A.laid.	Achol.

S = Sensitive, R = Resistant, M.bovirh. = M.bovirhinis, M.bg. = M.bovigentialium, A.axan. = A.axanthum, A.laid. = A.laidlawii, Achol. = Acholeplasma unidentified

Table 11 Antibiotic sensitivity of Mycoplasma and Acholeplasma isolated from milk of healthy cows and buffaloes

Antibiotic discs/ concentration	Antibiotic sensitivity of isolate number from						
	Cows						Buffalo
	CM-194	CM-196	CM-199	CM-200	CM-209	CM-210	CM-462
Spiramycin (30 mcg)	S	S	S	S	S	S	S
Vibramycin or doxycycline (30mcg)	S	S	S	S	S	S	S
Ledermycin or demeclocycline (30mcg)	S	S	S	S	I	S	S
Tetracycline (30 mcg)	S	S	S	S	I	S	S
Erythromycin (15 mcg)	S	S	S	S	S	R	S
Oxytetracycline (30 mcg)	S	S	S	S	I	S	S
Lincomycin (2 mcg)	S	S	S	S	S	S	S
Neomycin (30 mcg)	S	S	S	S	S	S	S
Kanamycin (30 mcg)	S	S	S	S	S	S	S
Streptomycin (10 mcg)	I	R	R	R	R	R	S
Ampicillin (10 mcg)	R	R	R	R	R	R	R
Tylosin (15 mcg)	S	S	S	S	S	S	S

S = Sensitive, R = Resistant, I = Intermediate, depending upon the diameter of zone of inhibition ranges prescribed by manufacturer of discs (Span Diagnostic, Surat) except tylosin for which only R or S was recorded



g.4 Characteristic "fried egg" colonies of an unidentified isolate number CH-462 (Diene's strain, 180)

speciated employing growth inhibition test with hyperimmune serum against A.laidlawii, A.axanthum, A.granularum, A.oculi, A.modicum and A.hippikon.

Ureaplasmas were not isolated from normal buffalo-milk (Table 5) but found associated with 4 of the 102 normal cow-milk samples (Table 9). The concentration of Ureaplasma organisms in healthy cow-milk ranged between 5×10^1 to 5×10^2 CCU/ml which was lower than their number in mastitic cow-milk (5×10^2 to 5×10^3) (Table 4,9).

The per cent isolation of the Mollicutes organisms from milk of healthy cows was 7.58 per cent and from healthy buffalo-milk was 0.98 per cent (Table 8, Fig.3), which was lower than the per cent isolation of Mollicutes from mastitic-cows (15.38%) and mastitic-buffaloes (5.05 %) (Table 8, Fig.3).

B. Isolation of other Bacteria Associated with Mollicutes-Mastitis

All the milk samples were screened for other bacteria associated with Mollicutes bovine-mastitis. Some of the Mollicutes organisms were isolated alone from mastitic and normal milk of cows and buffaloes, whereas other Mollicutes organisms were isolated along with other bacteria (Table 4,5,9). Micrococcus sp. and Staphylococcus epidermidis were associated with 11 Mollicutes organisms from milk of 130 mastitic-cows. However, no bacteria were found associated with remaining 9 organisms of class Mollicutes (Table 12).

Table 12 Association of other bacteria with Mycoplasma/Acholeplasma/Ureaplasma in mastitic and normal milk of cows and buffaloes

Status of animals with no. in parentheses	<u>Mycoplasma</u>	<u>Mycoplasma*</u> with other bacteria	<u>Acholeplasma</u>	<u>Acholeplasma</u> with other bacteria	<u>Ureaplasma</u>	<u>Ureaplasma</u> with other bacteria
Mastitic-cows (130)	2(1.5)**	6(4.6)	3(2.3)	4(3.07)	4(3.07)	1(0.7)
Normal-cows (145)	0(0)	3(2.06)	1(0.68)	3(2.06)	3(2.06)	1(0.68)
Mastitic-buffaloes (99)	1(1.01)	1(1.01)	1(1.01)	0(0)	1(1.01)	1(1.01)
Normal-buffaloes (102)	0(0)	0(0)	0(0)	1(0.9)	0(0)	0(0)
Overall positivity of <u>Mollicutes</u> organisms						

Status of animals with number in parentheses	<u>Mollicutes</u> only	<u>Mollicutes</u> with other bacteria
Mastitic-cows (130)	9(6.9)	11(8.4)
Normal-cows (145)	4(2.7)	7(4.8)
Mastitic-buffaloes (99)	3(3.03)	2(2.02)
Normal-buffaloes (102)	0(0)	1(0.9)

* Other bacteria found associated with S. epidermidis, Micrococcus sp., Citrobacter sp., and Enterobacter sp.

** Figures in parentheses indicate percentage

Two of the 5 isolates of class Mollicutes from 99 mastitic-buffaloes were associated with Micrococcus sp. and S.epidermidis, whereas no bacteria was associated with remaining 3 Mollicutes organisms from mastitic buffalo-milk (Table 12).

Isolates of Mollicutes organisms from milk of healthy cows and buffaloes were also associated with other bacteria viz. S.epidermidis, Enterobacter sp., Citrobacter sp. and Micrococcus sp.(Table 9). The only isolate of Acholenplasma sp. from normal milk of a healthy buffalo was associated with Micrococcus sp. (Table 5).

The biochemical character and identification of Gram-positive bacteria, and Gram-negative bacteria found associated with Mollicutes mastitis is given in Table 13 and 14, respectively. The antibiotic sensitivity of bacterial isolates is given in Table 15.

C. Seroprevalence of Bovine Mycoplasmal Mastitis

Appearance of precipitin, IHA and ELISA antibodies in experimentally antigenized calves

Appearance of precipitin, ELISA and IHA antibodies in experimentally antigenized cow-calves with M.bovis and M.bovigenitalium is given in Table 16. The results indicated that ELISA antibodies gradually increased from nil (preimmune titre) to 1:10,240 in 9 weeks time in M.bovis antigenized calf. However, anti-M.bovigenitalium ELISA antibodies in equal time

Table 13 Biochemical characters of Gram-positive bacteria associated with Molluscus organisms from milk of cows and buffaloes

Isolate no.	Biochemical character				Identification		
	Production of Coagulase	Catalase	Fermentation of glucose				
			Oxidase	Aerobi- cally			
				Anaerobi- cally	Fermentation of mannitol		
CM-110	-	+	-	+	-	-	<u>S. epidermidis</u>
CM-117	-	+	+	-	-	-	<u>Micrococcus sp.</u>
CM-195	-	+	-	W ⁺	-	-	<u>S. epidermidis</u>
CM-197	-	+	-	+	-	-	<u>S. epidermidis</u>
CM-200	-	+	-	+	W ⁺	-	<u>S. epidermidis</u>
CM-202	-	+	-	+	W ⁺	-	<u>S. epidermidis</u>
CM-203	-	+	+	-	-	-	<u>Micrococcus sp.</u>
CM-210	-	+	+	-	-	-	<u>Micrococcus sp.</u>
CM-230	-	+	+	-	-	-	<u>Micrococcus sp.</u>
CM-462	-	+	+	-	-	-	<u>Micrococcus sp.</u>
CM-42	-	+	-	+	+	-	<u>S. epidermidis</u>

+W = Weak positive

Table 14 Biochemical characters of Gram-negative bacteria associated with Mollicutes organisms from milk of cows and buffaloes

Test	Isolate number	
	CM-209	CM-136
Production of Indole	-	+
Methyl-red	-	-
Voges-Proskauer	-	+
Citrate utilization	+	+
Fermentation of:		
Glucose	+	+
Lactose	+	+
Sucrose	+	-
Salicin	+	+
Maltose	+	+
Mannitol	+	+
Reduction of nitrate	+	-
Hydrolysis of urea	-	-
H ₂ S production (TSI)	+	+
Genus identification	<u>Enterobacter</u> sp.	<u>Citrobacter</u> sp.

Table 15 Antibiotic sensitivity of bacteria associated with the Mollicutes organisms from milk of cows and buffaloes

Antibiotic discs/ concentration	Antibiotic sensitivity of isolates no. from												
	Cows						Buffaloes						
	Mastitis						Normal				Mastitis		Normal
	CM 110	CM 195	CM 197	CM 202	CM 203	CM 230	CM 136	CM 200	CM 209	CM 210	CM 42	CM 117	CM 462
erythromycin(30mcg)S	R	S	S	S	S	S	R	R	R	S	S	S	R
erythromycin(30mcg)R	R	I	I	S	I	I	R	R	R	S	R	R	R
erythromycin(30mcg)R	S	S	S	S	I	I	R	R	R	S	R	I	R
tetracycline (30mcg)S	I	R	S	S	S	S	R	S	I	S	S	I	R
chloramphenicol (15 mcg)S	S	S	S	S	S	S	R	S	R	S	S	S	R
tetracycline (30 mcg)R	S	I	S	S	S	S	R	R	I	S	R	I	I
erythromycin(2 mcg)R	R	R	R	S	R	R	R	R	R	R	R	R	R
erythromycin (30 mcg) I	S	S	S	S	S	S	I	S	R	S	I	S	S
erythromycin(30 mcg)I	S	S	S	S	S	S	I	S	R	S	I	S	S
erythromycin (10 mcg)S	I	R	S	S	S	S	I	I	S	S	S	R	S
penicillin (10mcg)S	S	R	S	S	S	S	S	S	R	S	S	S	S
tylosin (15 mcg)R	R	R	S	S	R	R	R	R	R	S	R	R	R
penicillin(10 IU)R	R	I	R	R	R	R	R	R	R	R	R	R	R
chloramphenicol (30mcg)S	S	I	S	S	S	S	I	S	I	S	S	I	S
chloramphenicol (30mcg)S	S	I	S	S	S	S	I	S	I	S	S	I	S
oxacillin(1 mcg)S	R	R	S	S	S	S	R	S	R	S	S	R	R
erythromycin(10 mcg)S	S	S	S	S	S	S	S	S	I	S	S	S	S
cephadizine (300 mcg)R	R	R	R	R	S	R	R	R	R	S	R	R	R
erythromycin(10 mcg)R	R	R	R	R	R	R	R	R	R	I	R	R	I
erythromycin (300 mcg)R	R	R	S	S	R	R	I	R	I	R	R	R	S

S = Sensitive, R = Resistant, I = Intermediate, depending upon the diameter of zone of inhibition ranges prescribed by manufacturer of discs (Span Diagnostic, Surat) except tylosin for which only R or S was recorded

Table 16 Appearance of precipitin, IHA, ELISA antibodies in experimentally antigenised cow-calves with M.bovis (NCTC-10131) and M.bovigentialium (NCTC-10122)

Week(s) postimmuni- zation(1st injection)	Anti- <u>M.bovis</u> antibodies in cow-calf no.B-184 detected with			Anti- <u>M.bovigentialium</u> antibodies in cow-calf no. B-179 detected with		
	AGIPT* (1 line)	IHA titre	ELISA titre	AGIPT*	IHA titre	ELISA titre
0/1	-	1:10	-	-	-	-
1/1	-	1:10	-	-	-	-
3/1	-	1:10	-	-	-	-
1	-	1:10	-	-	-	-
2	-	1:20	1:20	-	1:10	1:20
3	+	1:20	1:640	-	1:80	1:640
4	+	1:160	1:1280	-	1:160	1:160
5	+	1:40	1:1280	-	1:160	1:320
6	+	1:160	1:2560	-	1:160	1:640
7	+	1:160	1:1280	-	1:160	1:640
8	+	1:320	1:5120	-	1:160	1:640
9	+	1:1280	1:10,240	-	1:320	1:1280
10	NR	NR	NR	-	-	1:320

* Results recorded at 12 hr. incubation at 37C

NR= ELISA, IHA and AGIPT reactions could not be recorded as calf died suddenly due to inclement weather

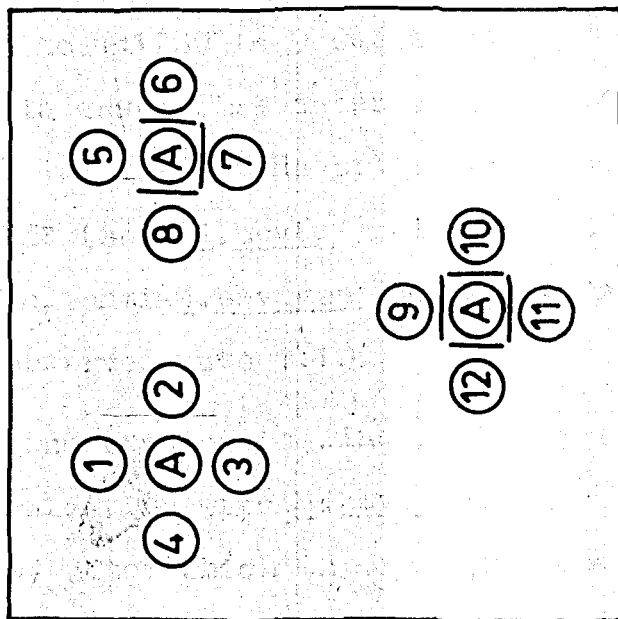


Fig.5 Agar-gel-immuno-precipitation test (AGIPT): Central wells(A) contained M.bovis antigen whereas wells at periphery contained homologous immune calf serum at 0-day (1), 1-day(2), 3-day (3), 6-day (4), 2-week(5), 3-week(6), 4-week(7), 5-week(8), 6-week(9), 7-week(10), 8-week(11), 9-week(12)

interval mounted from nil (preimmune titre) to 1:1280 which later declined to 1:320 at 10-week. It was noted that maximum homologous M.bovis and M.bovigenitalium ELISA titres were 1:10,240 and 1:1280, respectively. However, ELISA antibodies cross reacted with heterologous antigens (anti-M.bovis antibodies with M.bovigenitalium antigen, anti-M.bovigenitalium antibodies with M.bovis antigen) upto 1:80.

IHA titres against M.bovis increased from 1:10 (preimmune titre) to 1:1280 in 9 weeks time whereas anti-M.bovigenitalium titre in equal time interval increased from nil (preimmune titre) to 1:320. IHA cross reactions with heterologous antigens (anti-M.bovis antibodies with M.bovigenitalium antigen, anti-M.bovigenitalium antibodies with M.bovis antigen) were observed upto 1:10.

One precipitin line was observed at 3-week post antigenization with M.bovis which was noted upto 9-week (Fig.5) after which the results were not recorded. No precipitin line was observed with M.bovigenitalium antigen and homologous hyperimmune calf serum.

Anti-M.bovis and anti-M.bovigenitalium rabbit hyperimmune sera

Rabbit hyperimmune sera against M.bovis and M.bovigenitalium were harvested at 9-week postimmunization (1st injection) which gave one sharp precipitin line and 1:1280 IHA titre with

homologous antigen.

Standardization of AGIPT, IHA and ELISA

AGIPT: AGIPT using sonicated M.bovis (NCTC-10131) antigen and its rabbit hyperimmune serum gave one sharp line within 18 hr. at 37C. It cross reacted with M.agalactiae (2 lines) but none with 12 other mycoplasmas (M.mycoides var. mycoides SC type, PG-1; M.mycoides var. mycoides LC type, Y-goat; M.mycoides var. capri; M.capricolum; Mycoplasma sp.gp.7; M.bovigenitalium; M.dispar, M.alkalesens; M.bovirhinis; M.bovoculi; M.canadense; M.verecundum) and 2 acholeplasmas (A.laidlawii, A.granularum).

AGIPT using sonicated M.bovigenitalium (NCTC-10122) antigen and its rabbit hyperimmune serum gave 2 lines within 18 hr. at 37C. No line was observed with M.bovigenitalium antigen and hyperimmune sera of 13 other mycoplasmas and 2 acholeplasmas mentioned in the preceding para.

IHA: M.bovis (NCTC-10131) sensitized glutaraldehyde-fixed sheep erythrocytes and rabbit hyperimmune serum yielded a titre of 1:1280 at 8-week postantigenization whereas hyperimmune calf-serum gave this titre at 9-week postimmunization (Fig.6). M.bovis sensitized glutaraldehyde-fixed sheep erythrocytes cross reacted upto 1:10 with rabbit hyperimmune serum of M.bovigenitalium, M.agalactiae, M.dispar, and A.laidlawii. Hyperimmune serum of M.canadense cross reacted

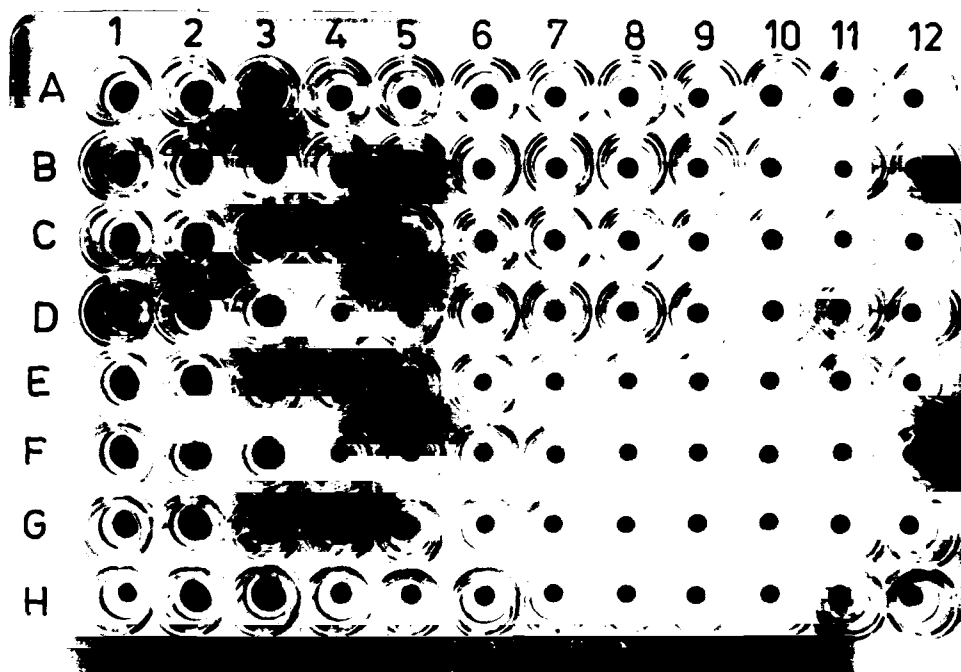


Fig.6 Indirect haemagglutination test with *M. bovis* sensitized sheep erythrocytes as antigen and sera from homologous immune calf serum, 8-week post immunization (1), homologous immune calf serum, 9-week postimmunization (2), homologous immune rabbit serum, 9-week postimmunization (3) and unknown negative cow sera (4 to 12) except 1:10 titre serum (6)

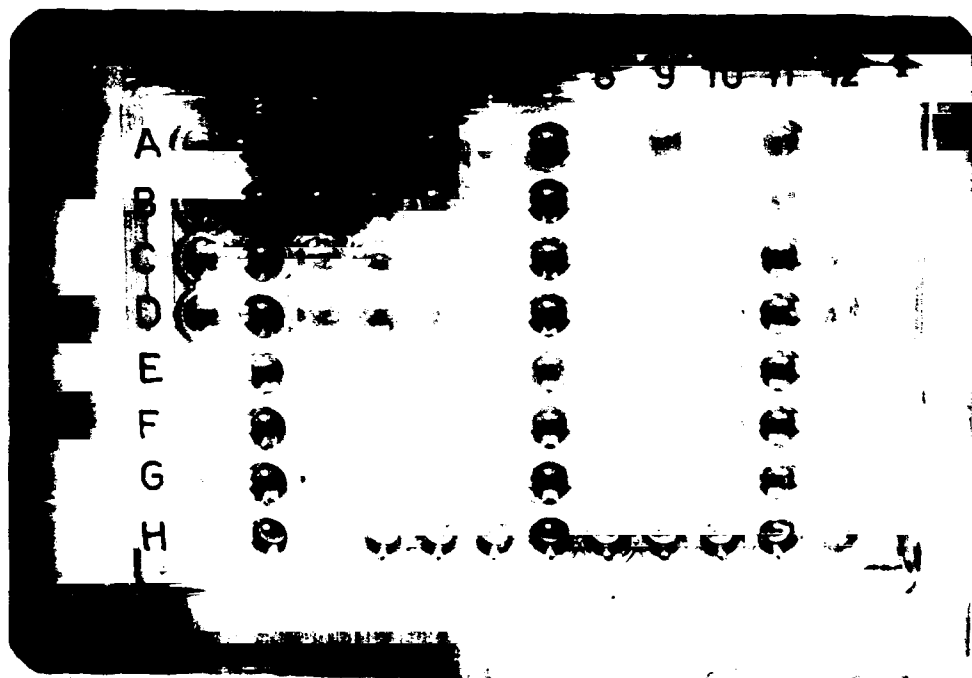


Fig.7 Enzyme-linked-immuno-sorbent-assay (ELISA) with *M. bovis* antigen and sera from preimmune calf serum (1), homologous immune calf serum, 9-week postimmunization, (2,3) unknown cow sera (4 to 12)

upto 1:20 whereas hyperimmune sera of M.bovoculi, M.capricolum, M.mycoides var. mycoides SC type and LC type; M.mycoides var. capri, M.bovirhinis, Mycoplasma sp.gp.7; A.axanthum, A.granularum, and A.oculi did not show cross reactions.

IHA using M.bovigenitalium sensitized sheep-erythrocytes and rabbit hyperimmune serum had a maximum of 1:1280 titre at 6-week postimmunization. It cross reacted with hyperimmune sera of M.bovis and M.canadense to a titre of 1:10 but not with hyperimmune sera of 9 mycoplasmas and 4 acholeplasmas mentioned in preceding para.

Enzyme-Linked-Immune-Sorbent-Assay(ELISA): The maximum homologous M.bovis and M.bovigenitalium ELISA titres in the serum of antigenized cow-calves was 1:10,240 and 1:1280, respectively (Fig.7). Both the hyperimmune calf sera cross reacted with heterologous antigen (anti-M.bovis antibodies with M.bovigenitalium antigen, anti-M.bovigenitalium antibodies with M.bovis antigen) upto 1:80 titre.

ELISA, IHA and AGIPT antibodies of M.bovis and M.bovigenitalium positive and negative reference sera from cow-calf employed as controls in serotesting are given in Table 17.

Table 17 ELISA, IHA and AGIPT antibodies of positive and negative reference cow-calf sera used as controls in serotesting

Sera	ELISA titre	IHA titre	AGIPT (1 line)
Calf <u>M. bovis</u> -positive sera	1:1280	1:160	+
Calf <u>M. bovis</u> -negative sera	-	1:10	-
Calf <u>M. bovigenitalium</u> -positive sera	1:1280	1:160	-
Calf <u>M. bovigenitalium</u> - negative sera	-	-	-

Seroprevalence of anti-M. bovis and anti-M. bovigenitalium antibodies in sera collected from mastitic/healthy cows and buffaloes

Seroprevalence in cow sera Sera from 321 cows (116 mastitic, 205 healthy) were examined for M. bovis and M. bovigenitalium antibodies by ELISA, AGIPT and IHA tests.

ELISA Antibodies: Seroprevalence of M.bovis and M.bovigentialium ELISA antibodies amongst 116 mastitic-cows (33 subclinical, 24 acute, 59 chronic) is given in Table 18. Seventy two (21 subclinical, 18 acute, 33 chronic) of 116 (62.06%) cases of cow mastitis had M.bovis ELISA antibodies whereas 51 (21 subclinical, 10 acute, 20 chronic) of 116 (43.9%) cases had M.bovigentialium ELISA antibodies. However, specific M.bovis and M.bovigentialium ELISA antibodies were present in 25 (7 subclinical, 2 acute, 16 chronic) and 11 (8 subclinical, 3 chronic) cases of cow mastitis, respectively. Dual ELISA antibody titres against M.bovis and M.bovigentialium were recorded in 1 subclinical (1:320 for both antigens) and one chronic case (1:1280 for both antigens) of mastitis in cows.

Observations on healthy cows revealed that specific M.bovis ELISA antibodies were present in 28 of 205 (13.6%) and M.bovigentialium in 17 of 205 (8.2%) cows (Table 19).

The results of ELISA have suggested a higher seroprevalence of specific M.bovis (21.5%) and M.bovigentialium (9.4%) ELISA antibodies in mastitic-cows than those in healthy cows (13.6% M.bovis and 8.2% M.bovigentialium) (Table 20). Per cent specific M.bovis ELISA antibodies was also found higher (21.5% mastitic-cows, 13.6% healthy cows) than specific

Table 18 Seroprevalence of M. bovis and M. bovis genitalium ELISA antibodies amongst 175 cases of bovine mastitis

ELISA titre	Number of positive cases of cow mastitis for						Number of positive cases of buffalo mastitis for					
	<u>M. bovis</u>			<u>M. bovis genitalium</u>			<u>M. bovis</u>			<u>M. bovis genitalium</u>		
	Sub-clinical	Acute	Chronic	Sub-clinical	Acute	Chronic	Sub-clinical	Acute	Chronic	Sub-clinical	Acute	Chronic
1:10	2	6	5	2	2	3	1	-	1	-	1	1
1:20	2	3	4	5	1	7	4	3	-	4	1	-
1:40	6	3	4	5	3	4	-	-	-	-	-	2
1:80	4	4	4	1	4	3	-	-	-	-	-	2
1:160	5	-	6	6	-	1	2	-	-	-	2	2
1:320	2	2	3	1	-	1	1	-	1	-	1	-
1:640	-	-	3	-	-	-	-	-	-	-	-	-
1:1280	-	-	4	1	-	1	-	-	1	-	-	-
Nil	12	6	26	12	14	39	22	9	14	26	7	10
Total	33	24	59	33	24	59	30	12	17	30	12	17

Table 19 Seroprevalence of M. bovis and M. bovisgenitalium
ELISA antibodies amongst healthy cows and
buffaloes

ELISA titre	ELISA antibodies			
	Number of cows positive for		Number of buffaloes positive for	
	<u>M. bovis</u>	<u>M. bovisgenitalium</u>	<u>M. bovis</u>	<u>M. bovisgenitalium</u>
1:10	14	19	2	2
1:20	33	33	3	1
1:40	14	12	1	-
1:80	8	10	1	-
1:160	16	13	3	1
1:320	7	2	2	-
1:640	1	-	2	-
1:1280	4	2	1	-
Nil	108	114	23	34
Total	205	205	38	38

Table 20 Seroprevalence of M. bovis and M. bovis genitalium antibodies in cows and buffaloes

Status of animals with number in parentheses	Number of sera positive for <u>M. bovis</u> antibodies detected by				Number of sera positive for <u>M. bovis genitalium</u> antibodies detected by			
	AGIPT	ELISA	IHA	IHA and ELISA	AGIPT	ELISA	IHA	IHA and ELISA
Mastitic-cows (116)	-	25(21.5)*	10(8.6)	1(0.8)	-	11(9.4)	5(4.3)	1(0.8)
Normal-cows (205)	-	28(13.6)	15(7.3)	6(2.9)	-	17(8.2)	13(6.3)	5(2.4)
Mastitic-buffaloes (59)	-	5(8.4)	1(1.6)	1(1.6)	-	5(8.4)	2(3.3)	2(3.3)
Normal-buffaloes (38)	-	8(21)	-	-	-	1(2.6)	2(5.2)	-

* Figures in parentheses indicate percentage

M.bovigenitalium ELISA antibodies (9.4% mastitic-cows, 8.2% healthy cows) (Table 20, Fig. 8).

IHA antibodies: Seroprevalence of M.bovis and M.bovigenitalium haemagglutinins amongst 116 cases of cow-mastitis and 205 healthy cows is given in Table 21 and 22. The sera were classified considering IHA titre of 1:160 and above as serodiagnostic, 1:80 as suspicious and 1:40 or below as insignificant.

Ten of 116 (8.6%) cases of cow mastitis had serodiagnostic/suspect M.bovis haemagglutinins, whereas only 5 of 116 (4.3%) had M.bovigenitalium serodiagnostic/suspect haemagglutinins (Table 20). One chronic case of cow-mastitis had both M.bovis and M.bovigenitalium haemagglutinins at the level of 1:80.

Fifteen of 205 (7.3%) and 13 of 205 (6.3%) healthy cows had M.bovis and M.bovigenitalium haemagglutinins (serodiagnostic/suspect), respectively (Table 20).

It is evident from Table 20 that per cent positivity of M.bovis specific haemagglutinins was higher in mastitic (8.6%) and healthy cows (7.3%), than the per cent positivity of M.bovigenitalium specific haemagglutinins (4.3% mastitic cases, 6.3% healthy cows). The per cent M.bovis

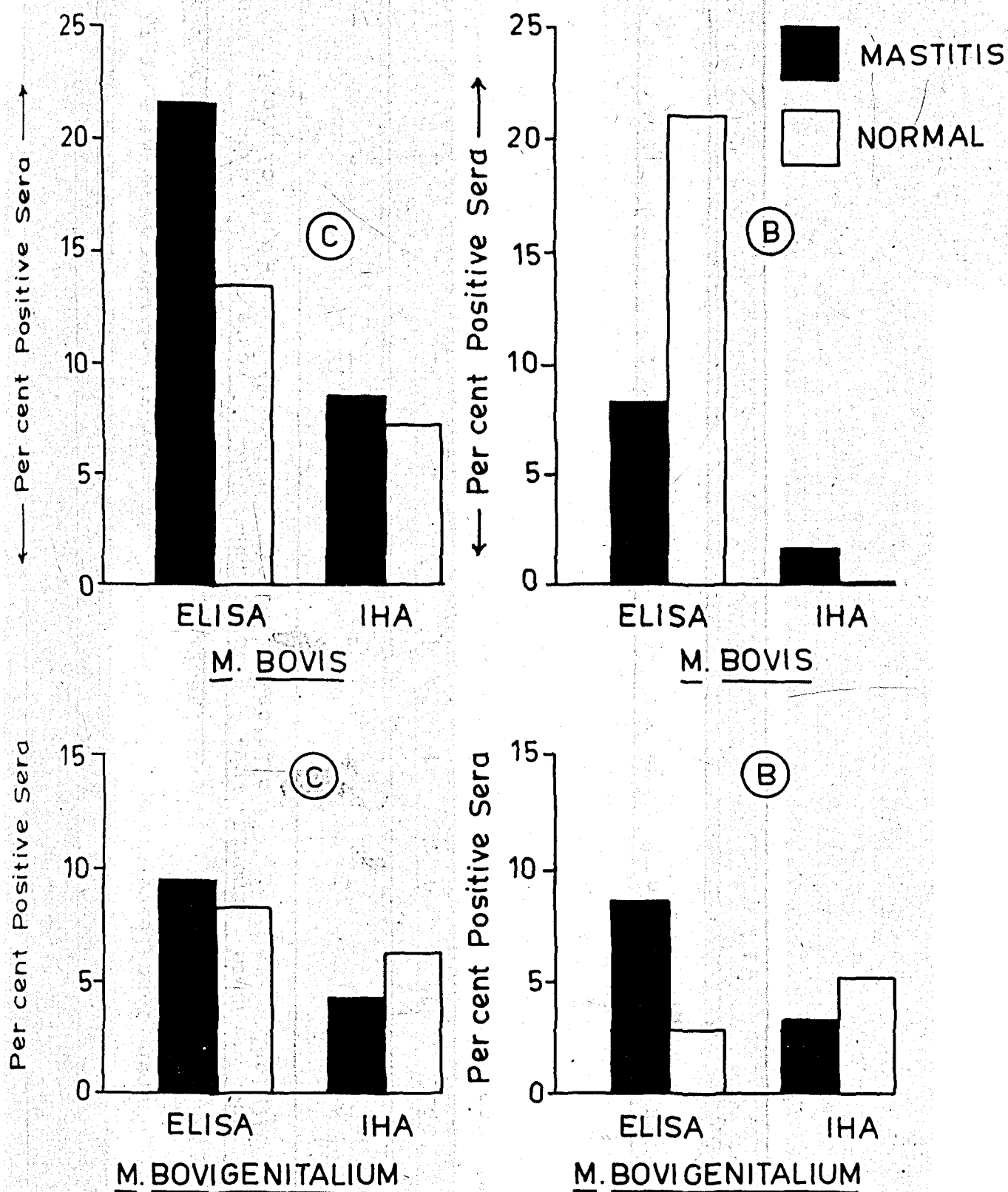


Fig.8 Seroprevalence of *M. bovis* and *M. bovis genitalium* antibodies in cows (C) and buffaloes (B)

Table 21 Seroprevalence of *M. bovis* and *M. bovis genitalium* IHA antibodies amongst cases of bovine mastitis

IHA titre	Number of positive cases of cow mastitis for			Number of positive cases of buffalo mastitis for											
	<u>M. bovis</u>			<u>M. bovisgenitalium</u>			<u>M. bovis</u>						<u>M. bovisgenitalium</u>		
	Sub-clinical	Acute	Chronic	Sub-clinical	Acute	Chronic	Sub-clinical	Acute	Chronic	Sub-clinical	Acute	Chronic	Sub-clinical	Acute	Chronic
1:160 or more	2	-	-	-	-	-	1	-	-	-	-	-	-	-	1
1:80	3	1	4	1	1	3	-	-	-	-	-	-	-	-	1
1:40 or low	22	20	40	24	19	50	9	7	13	15	5	7	15	7	7
Nil	6	3	15	8	4	6	20	5	4	15	7	8	15	7	8
Total	33	24	59	33	24	59	30	12	17	30	12	17	30	12	17

Table 22 Seroprevalence of M.bovis and M.bovigenitalium
IHA antibodies amongst healthy cows and buffaloes

IHA titre	Number of cows positive for		Number of buffaloes positive for	
	<u>M.bovis</u>	<u>M.bovigenitalium</u>	<u>M.bovis</u>	<u>M.bovigenitalium</u>
1:160 or more	3	7	-	-
1:80	12	6	-	2
1:40 or low	151	148	8	27
Nil	39	44	30	9
Total	205	205	38	38

haemagglutinins was higher in mastitic-cows (8.6%) than the healthy cows (7.3%), but the per cent M.bovigenitalium specific haemagglutinins were found higher in healthy cows (6.3%) than the mastitic-cows (4.3%) (Fig.8).

Precipitins: None of the 321 cows (116 mastitic, 205 healthy) sera was AGIPT positive for M.bovis and M.bovigenitalium precipitins.

Seroprevalence in Buffalo-sera Ninety seven sera collected from 59 mastitic and 38 healthy buffaloes were examined for M.bovis and M.bovigenitalium antibodies by ELISA, AGIPT and IHA test.

ELISA antibodies: Five (3 subclinical, 2 chronic) of 59(8.4%) cases of buffalo mastitis had M.bovis specific ELISA antibodies whereas ELISA M.bovigenitalium specific antibodies were present in 5(3 acute, 2 chronic) of 59 (8.4%) cases (Table 18,20). All the chronic cases of buffalo mastitis had dual ELISA antibodies specific to both M.bovis and M.bovigenitalium. Corresponding observations in 38 healthy buffaloes revealed that 8 of 38 (21.0%) and 1 of 38 (2.6%) had M.bovis and M.bovigenitalium specific ELISA antibodies, respectively (Table 19,20). It was observed that the prevalence of M.bovis ELISA antibodies was higher than the M.bovigenitalium ELISA antibodies in healthy buffaloes (Table 20, Fig. 8).

IHA antibodies: IHA detected M.bovis and M.bovigenitalium serodiagnostic/suspect haemagglutinins in 1 of 59(1.6%) and 2 of 59(3.3%) cases of buffalo-mastitis (Table 21) respectively. Corresponding observations in healthy buffaloes indicated the presence of M.bovigenitalium serodiagnostic/suspect haemagglutinins in 2 of 38(5.2%) healthy buffaloes while none of the 38 buffaloes had diagnostic/suspect M.bovis haemagglutinins (Table 22).

AGIPT: None of the 97 buffalo-sera (59 mastitic, 38 healthy) was AGIPT positive for M.bovis and M.bovigenitalium precipitins.

D. Experimental Pathogenicity of Mollicutes Organisms Isolated From Bovine Milk

Hamster tracheal ring organ culture

All the isolates of Mollicutes survived and the ciliary activity of tracheal rings could be maintained in Eagle's BME-nutrient medium throughout the period of observations i.e. 12 days postinfection (PI) (Fig.9). The growth of inoculated culture and ciliary activity of tracheal rings remained unaffected by the inhibitors viz. penicillin, nystatin used in the nutrient medium. Each culture dish was inoculated with 0.5 ml of culture under test having 1×10^6 to 2.7×10^6 CFU to give a final concentration of Ca. 10^6 org./ml of BME-nutrient medium.



Fig.9 Uninfected hamster tracheal lining showing cilia (X100)

Ciliary activity scores at different days PI with different isolates of Mollicutes including the type strain of M.bovis (NCTC-10131), are given in Table 23. It is evident that ciliary activity score, indicative of ciliostatic effect exerted by M.bovis (NCTC-10131) organisms, gradually decreased from 300 on 0-day to 12 on 12-day PI. Considering a score of 20 (>90% reduction) as significant, the strain M.bovis (NCTC-10131) was found pathogenic. Eight isolates of Mollicutes from bovine-milk were tested for their pathogenicity in hamster tracheal ring organ culture (Table 23). Four isolates viz. Mycoplasma F-38 (no. CM-193); M.bovigenitalium + Mycoplasma F-38 + M.bovirhinis (no. CM-195); M.bovigenitalium + M.bovirhinis (no. CM-196); and M.mycoides var. capri (no. CM-111) were found pathogenic. The ciliary activity in these cases was reduced from a score of 300 to 18.7 (12-day PI), to 0 (6-day PI), to 4 (8-day PI) and to 16.0 (12-day PI) by isolate numbers CM-193, CM-195, CM-196 and CM-111, respectively (Fig.10,11). The remaining four isolates viz. A.axanthum (no. CM-212), A.laidlawii (no. CM-230), unidentified Acholeplasma sp. (no. CM-448) and M.bovis + M.bovigenitalium + Mycoplasma F-38 + A.laidlawii (no. CM-197) proved nonpathogenic (Fig.10, 11).

Corresponding histopathological lesions in the hamster tracheal rings at 12-day PI are given in Table 24, whereas normal histological appearance of hamster tracheal rings is

Table 23 Effect of Mycoplasma/Acholeplasma isolates of bovine milk origin on hamster tracheal ring ciliary activity

Molluscites organism(s)	Ciliary activity score (mean of at least 5 rings) at days post infection						Pathogenicity
	0	2	4	6	8	10	12
<u>M. bovis</u> (NCTC-10131) 300(0)*	300(0)	224(22)	164(34)	92(41)	48(62)	12(88)	+
<u>Mycoplasma</u> F-38 (CM-193)	300(0)	200(0)	133(23.8)	90(40)	40(60)	32.5(6.7)	18.7(81.3)
<u>M. bovis genitalium</u> + <u>Mycoplasma</u> F-38 + <u>M. bovirhinis</u> (CM-195)	300(0)	125(7.5)	81(19)	0(90)	-	-	+
<u>M. bovis genitalium</u> + <u>M. bovirhinis</u> (CM-196)	300(10)	212(4)	57(43)	22(78)	4(96)	-	+
<u>M. bovis</u> + <u>M. bovis genitalium</u> + <u>Mycoplasma</u> F-38 + <u>A. laidlawii</u> (CM-197)	300(0)	262.5(3)	158.5(20)	124(26)	66.4(43)	53.5(47)	24.2(76)
<u>M. mycoides</u> var. <u>capri</u> (CM-111)	300(0)	140(0)	134(14)	91.0(25)	91.0(22)	28.0(72)	16.0(84)
<u>A. axanthum</u> (CM-212)	300(0)	250(0)	216(0)	200(0)	200(17)	190(10)	116(24)
<u>A. laidlawii</u> (CM-230)	300(0)	300(0)	275(0)	250(0)	220(2.5)	155(12.5)	155(12.5)
Unidentified <u>Acholeplasma</u> (CM-448)	300(0)	250(0)	200(0)	150(0)	109(7.5)	105(11.7)	84(15.9)

Note: During the entire 12-day period of observations, inoculated culture could be recovered from medium every 48 hr.

Figures in parentheses is per cent inhibition of ciliary activity, which is related to score indicating the extent of pathogenicity of Molluscites organisms

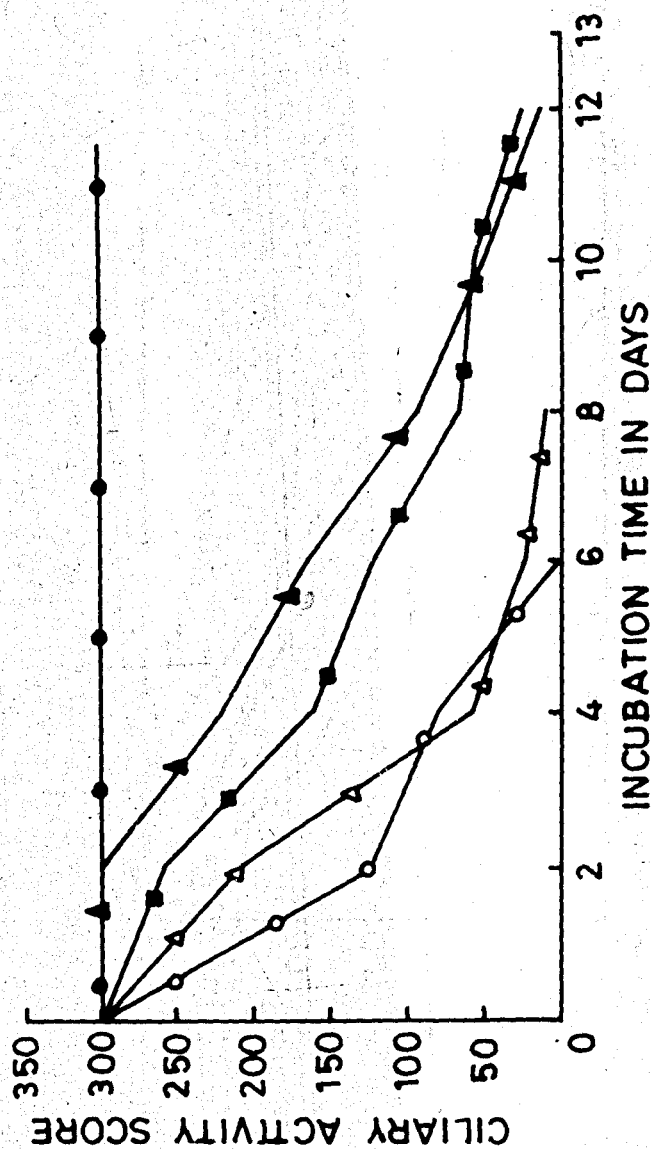


Fig.10 CILIA STOPPING EFFECT (CSE) OF MYCOPLASMA/ACHOLEPLASMA
IN HAMSTER TRACHEAL ORGAN CULTURE

▲ M. BOVIS, ● UNINOCULATED CONTROL,

△ M. BOVIGENITALIUM + MYCOPLASMA F-38 + M. BOVIRHINIS

○ M. BOVIGENITALIUM + M. BOVIRHINIS

■ M. BOVIS + M. BOVIGENITALIUM + MYCOPLASMA F-38 + A.LAIDLAWII

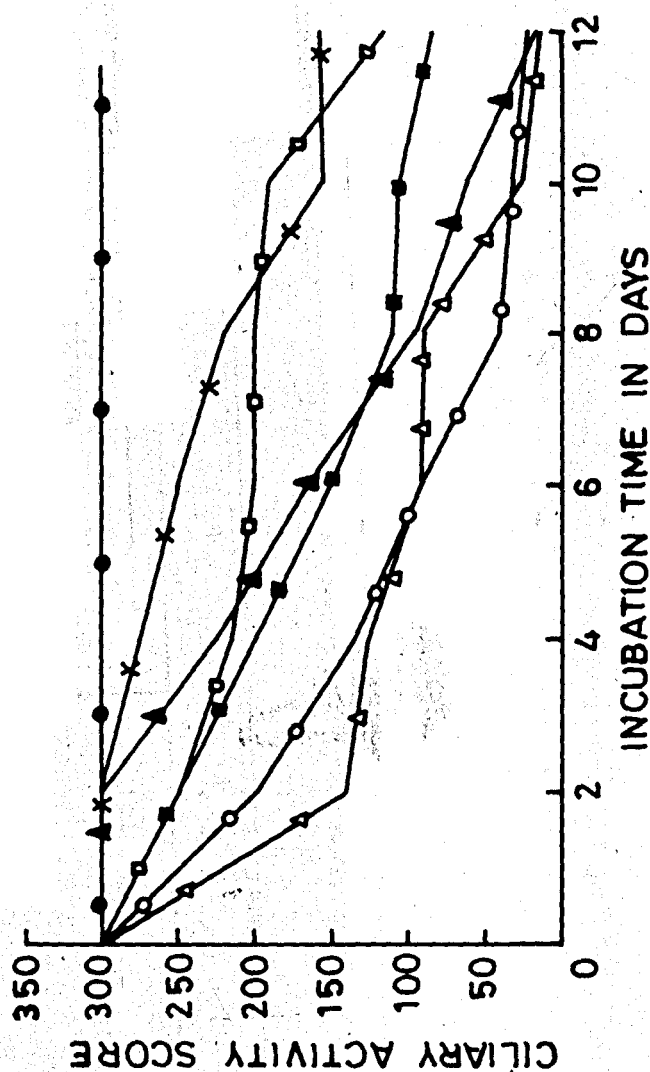


Fig. 11 CILIA STOPPING EFFECT (CSE) OF MYCOPLASMA/ACHOLEPLASMA IN HAMSTER TRACHEAL ORGAN CULTURE

▲ M. BOVIS, ● UNINOCULATED CONTROL, ○ MYCOPLASMA F-38
 ▲ M. MYCOIDES VAR CAPRI, □ A. AXANTHUM, ■ UNIDENTIFIED
 ISOLATE (CM 448), x A. LAIDLAWII

Table 24 Mycoplasmological and histopathological observations in hamster tracheal rings infected with various isolates of Mollicutes from bovine-milk

<u>Mollicutes</u> <u>organism(s)</u>	<u>Reisolation of the infected</u> <u>organisms from</u>	Nutrient medium	Tracheal ring	<u>Histopathological lesions</u>			
				<u>Inflammatory</u> <u>cell* response</u> <u>in lamina</u> <u>propria/sub-</u> <u>epithelium</u>	<u>Loss**</u> <u>of</u> <u>cilia</u>	<u>Hyperplasia</u> <u>of epithelial</u> <u>lining cells</u>	<u>Desquamation</u> <u>of epithelial</u> <u>lining cells</u>
<u>M. bovis</u> (NCTC-10131)	+		+	+	+++	-	+
<u>Mycoplasma</u> F-38 (CM-193)	+		+	+	++	+	-
<u>M. bovis genitalium</u> + <u>Mycoplasma</u> F-38 + <u>M. bovirhinis</u> (CM-195)	+		+	-	++	+	+
<u>M. bovis genitalium</u> + <u>M. bovirhinis</u> (CM-196)	+		+	-	+++	+	+
<u>M. bovis</u> + <u>M. bovis genitalium</u> + <u>Mycoplasma</u> F-38 + <u>A. laidlawii</u> (CM-197)	+		+	-	++	+	-
<u>M. mycoides</u> var. <u>capri</u> (CM-111)	+		+	-	++	-	-
<u>A. axanthum</u> (CM-212)	+		+	-	+	-	+
<u>A. laidlawii</u> (CM-230)	+		+	-	+	-	+
Unidentified <u>Acholeplasma</u> (CM-448)	+		+	-	-	-	-

* 1+ to 3+ indicates degree of cell infiltration, N = Neutrophils; L = Lymphocytes
** 1+ to 3+ indicates the degree of denudation of cilia from epithelial cells

given in Fig. 12 & 14. The salient histopathological lesions noticed in Mollicutes organisms infected tracheal rings were neutrophilic and lymphocytic infiltration in lamina propria and subepithelium, loss of epithelial cilia (Fig. 13, 15), and hyperplasia with or without desquamation of epithelial cells. The degree of histopathological lesions in infected tracheal rings varied from isolate to isolate (Table 24).

There were very mild to no pathological changes in the tracheal rings infected with A. axanthum (isolate no. CM-212), A. laidlawii (isolate no. CM-230) and unidentified Acholeplasma (isolate no. CM-448), whereas the remaining 5 isolates viz. Mycoplasma F-38 (no. CM-193), M. bovigenitalium + Mycoplasma F-38 + M. bovirhinis (no. CM-195), M. bovigenitalium + M. bovirhinis (no. CM-196), M. bovis, + M. bovigenitalium + Mycoplasma F-38 + A. laidlawii (no. CM-197) and M. mycoides var. capri (no. CM-111) and M. bovis type strain NCTC-10131 produced moderate to severe histopathological lesions in hamster tracheal rings described vide-supra.

Endotoxin was detected by Limulus lysate test in nutrient medium of hamster tracheal ring organ culture infected with 7 of the 9 Mollicutes organisms at 12-day PI viz. M. bovis (NCTC-10131), Mycoplasma F-38 (isolate no. CM-193), M. bovigenitalium + Mycoplasma F-38 + M. bovirhinis (isolate no. CM-195), M. bovigenitalium + M. bovirhinis (isolate no. CM-196), M. bovis + M. bovigenitalium + Mycoplasma F-38 + A. laidlawii (isolate no. CM-197), M. mycoides var. capri (isolate no.



- .12 Hamster tracheal ring organ culture maintained in Earle's MEM - basal medium for 48 hr: uninfected ciliated epithelium (H&E, X200)



- .13 Hamster tracheal ring organ culture infected with isolate no. CU-196 (*H. bovis* + *B. bovis*) at 12-day postinfection. The epithelium showed loss of cilia and lymphocytic infiltration in lamina propria (H&E, X200)

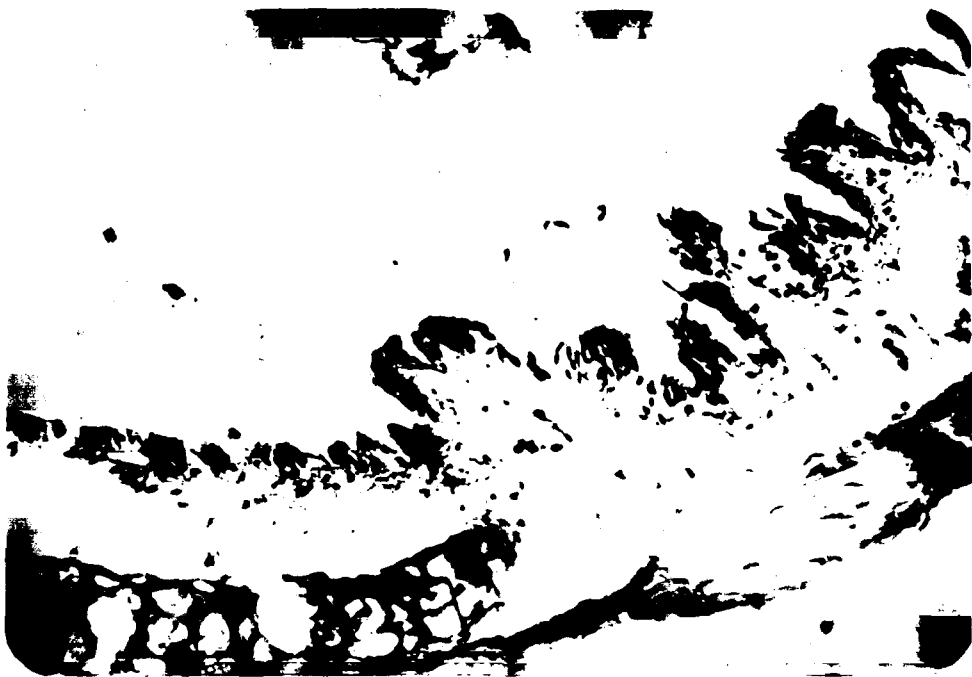


Fig.14 Hamster tracheal ring organ culture: uninfected ciliated epithelium maintained in Earle's BME nutrient medium for 12 days (H&E, X200)

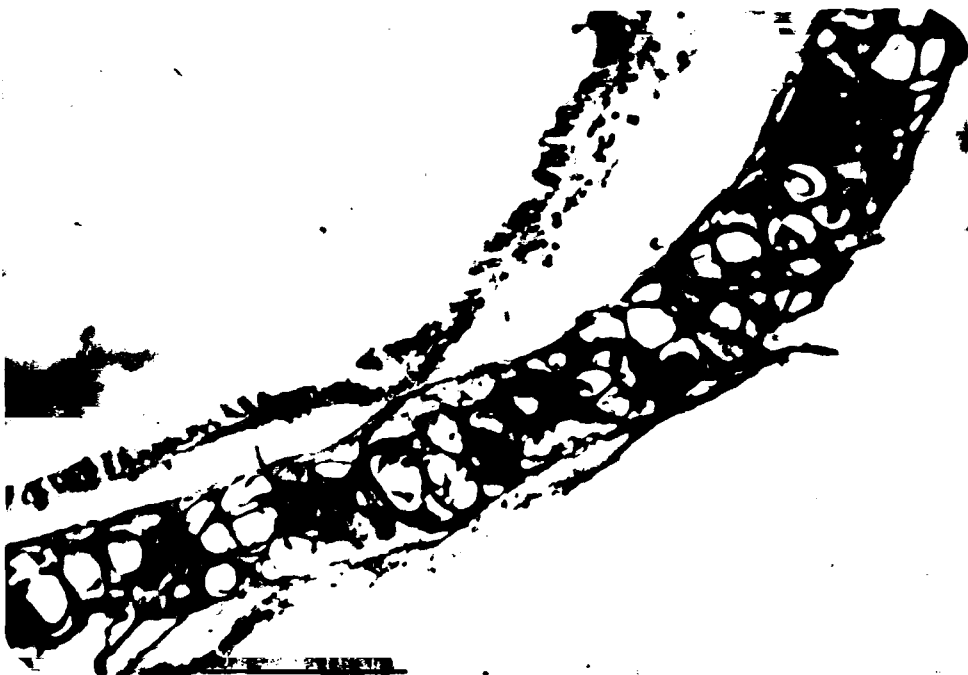


Fig.15 Hamster tracheal ring organ culture infected with isolate no. CV-197 (M.bovis + M.bovisgenitulum + Mycobacterium F-38 + A.layii). The epithelium showed focal loss of cilia and mild lymphocytic infiltration in subepithelium(H&E, X200)

CM-111), and unidentified Acholeplasma (isolate no. CM-448). Endotoxin was not detected with A. axanthum (isolate no. CM-212) and A. laidlawii (isolate no. CM-230).

Rat-mammary gland

All the Mycoplasma/Acholeplasma isolates from bovine milk (Table 3) were tested for their pathogenicity in rat mammary-gland. The recovery of the infected organisms alongwith gross pathological lesions in rat teats infected experimentally with various isolates of Mycoplasma/Acholeplasma of bovine-udder origin is given in Table 25. It is evident that the isolates no. CM-197, M. bovis + M. bovigenitalium + Mycoplasma F-38 + A. laidlawii; CM-111, M. mycoides var. capri could be recovered from the infected teats throughout the 6-day period of experiment. However, 4 isolates (no. CM-230, A. laidlawii; no. CM-448, unidentified Acholeplasma; no. CM-196, M. bovigenitalium + M. bovirhinis; no. CM-195, M. bovigenitalium + Mycoplasma F-38 + M. bovirhinis) were recovered upto 4-day PI, whereas 3 isolates (no. CM-193, Mycoplasma F-38; no. CM-212, A. axanthum; M. bovis, NCTC-10131) were recovered upto 3-day PI. All the isolates were recovered in pure form and no other bacteria ^{were} was found associated during the entire period of observations.

Some of the rat teats that had received isolates of Mollicutes viz. M. bovis, NCTC-10131; M. bovigenitalium +

Table 25

Recovery of infected organisms along with gross pathological lesions in rat teats infected experimentally with various isolates of Mycoplasma/Acholeplasma of bovine-udder origin

Test organism(s)	Recovery of organisms at postinfection (at day)					
	1	2	3	4	6	
<u>M. bovis</u> (NCTC-10131)	+(GI)	+(GI)	+(GI)	-(GI)	-	
<u>Mycoplasma</u> F-38 (CM-193)	+	+	+	-	-	
<u>M. bovis genitalium</u> +	+(GI)	+(GI)	+(GI)	+(GI)	-	
<u>Mycoplasma</u> F-38 +						
<u>M. bovirhinis</u> (CM-195)						
<u>M. bovis genitalium</u> +	+	+	+	+	-	
<u>M. bovirhinis</u> (CM-196)						
<u>M. bovis</u> + <u>M. bovis genitalium</u>	+	+	+	+	+	
+ <u>Mycoplasma</u> F-38 +						
<u>A. laidlawii</u> (CM-197)						
<u>M. mycoides</u> var. <u>capri</u> (CM-111)	+(GI)	+(GI)	+	+	+	
<u>A. axanthum</u> (CM-212)	+	+	+	-	-	
<u>A. laidlawii</u> (CM-230)	+	+	+	+	-	
Unidentified <u>Acholeplasma</u> (CM-448)	+	+	+	+	-	

GI = Gross teat inflammatory changes included mild to marked areas of congestion surrounding the glandular tissue on reflected skin of udder

Mycoplasma F-38 + M. bovirhinis, no. CM-195; M. mycoides var. capri, no. CM-111, showed mild to marked areas of congestion (upto a maximum period of 4-day PI) indicative of inflammation (Fig.16). Subsequently, the inflammation subsided and by 6-day PI infected teats became normal in gross appearance but slightly reduced in size. No clinical signs of illness were observed in infected rats.

The isolate-wise details of the histopathological changes in udder-tissues were as under in comparison with the normal histological appearance of uninoculated rat mammary-gland (Fig.17,18) and inoculated with sterile standard liquid medium (Fig.19).

M. bovis (NCTC-10131)

1-day postinfection: There was mild infiltration of neutrophils and desquamated cells in the lumen of some of acini and mild mononuclear cells infiltration with neutrophils in the interstitium. Interlobular blood vessels were congested. Focal infiltration of mononuclear cells with few neutrophils were also observed in interlobular septae particularly at periphery of the gland (Fig. 20).

2-day postinfection: The lumen of acini and ducts contained large number of intact and degenerating neutrophils mixed with some desquamated epithelial cells. Mild oedema in interstitium was noticed. Other changes in interstitium were similar to those observed at 1-day PI (Fig.21).



Fig.16 M.bovis infected rat mammary-gland (L-4) showing gross pathological lesions (red inflamed area) in comparison to normal right mammary-gland (R-4) injected with sterile standard liquid medium.

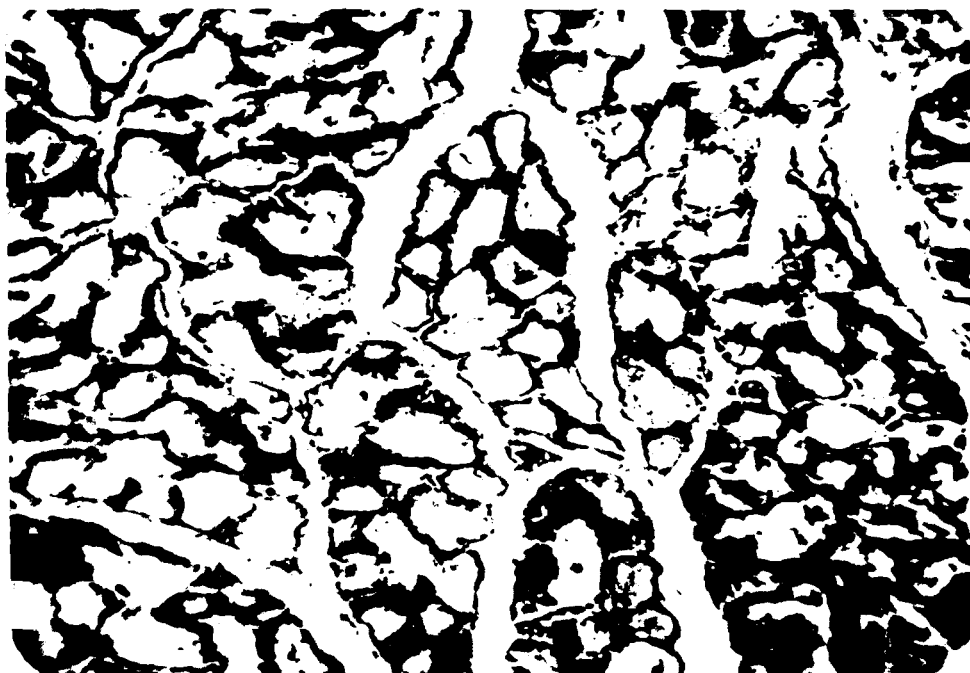


Fig.17 Uninoculated normal rat mammary-gland showing lobules of normal acini which are separated by interlobular septae (H&E, X100)

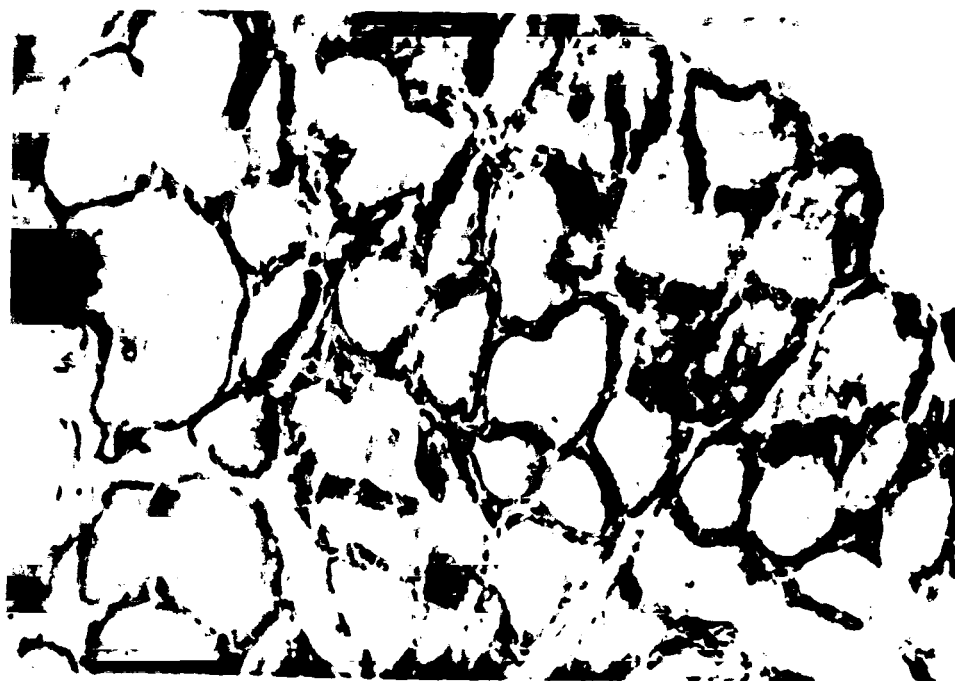


Fig.18 Uninoculated normal rat mammary-gland, showing the normal acini having milk secretion and epithelial lining (H&E, X200)

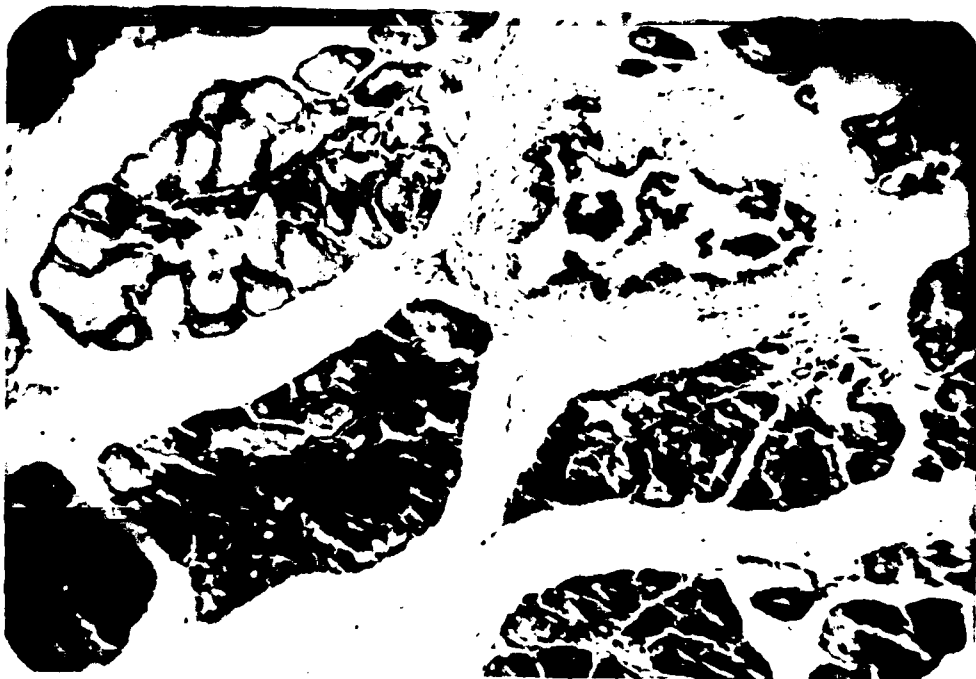


Fig.19

Normal rat mammary-gland, 1-day postinoculation of sterile standard liquid medium showing normal architecture of acini containing milk deposits (H&E, X100)



Fig.20

Rat mammary-gland, 1-day postinfection with M. bovis (NCTC-10131) showing mild neutrophilic infiltration in acini and interstitium and congested interlobular blood vessels (H&E, X100)

3-day postinfection: Mild infiltration of the neutrophils with desquamated cells were seen in a few acini. Focal infiltration of lymphocytes, macrophages/reticular cells were seen in interstitium (Fig.22).

4-day postinfection: Lumen of acini and intralobular duct were infiltrated with lymphocytes, macrophages and with a few neutrophils and desquamated cells. There was marked infiltration of lymphocytes and macrophages in the interstitium with fibroblastic proliferation. Lumen of acini became distorted and reduced due to proliferation of lining cells and thickened interstitium (Fig.23).

6-day postinfection: Only mild focal infiltration of lymphocytes and macrophages distinguished the mycoplasma inoculated glands from control.

Mycoplasma F-38 (isolate no. CM-193)

Heavy infiltration of neutrophils were seen in the lumen of acini and intralobular and interlobular ducts at 1-day PI. Interstitium was oedematous and at places contained few macrophages, lymphocytes and neutrophils. Interlobular blood vessels were congested. At 2-day PI, the changes were comparable to those observed at 1-day PI. Besides, the infiltration of neutrophils, a few macrophages and detached acinar cells were also seen in the lumen of acini.

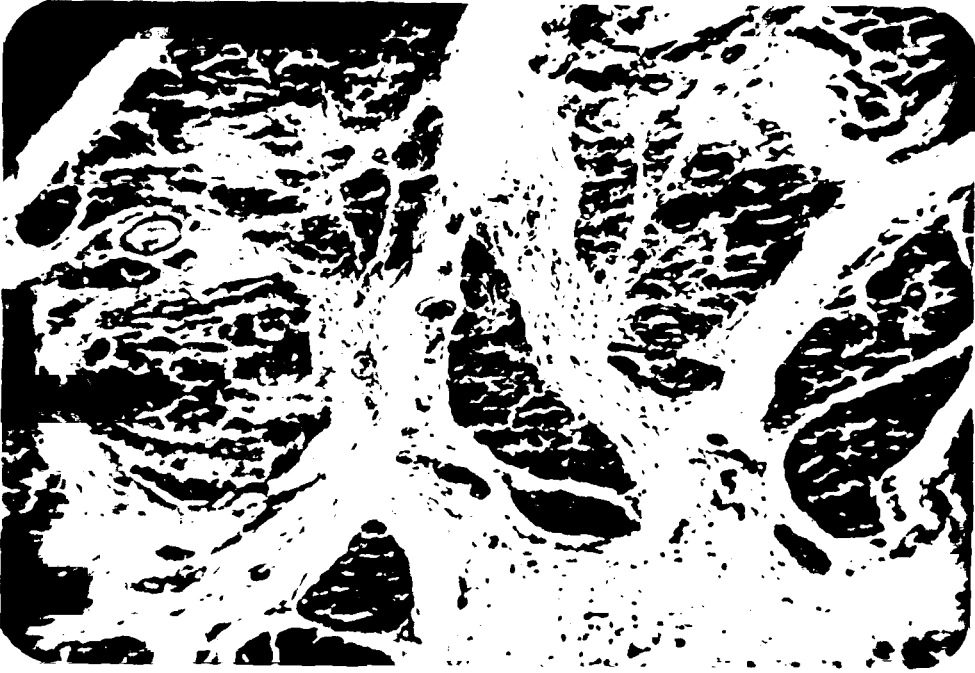


Fig.21 Rat mammary-gland, 2-day postinfection with M.bovis (NCTC-10131) containing intact and degenerating neutrophils in acini and interlobular septæ (H&E, X100)



Fig.22 Rat mammary-gland, 3-day postinfection with M.bovis (NCTC-10131) showing infiltration of mononuclear cells in acini and interstitium (H&E, X200)



Fig.23 Rat mammary-gland, 4-day postinfection with M.bovis (NCTC-10131) showing the distorted acini thickened interstitium with fibroblastic proliferation (H&E, X200)

Congestion of interlobular blood vessels was comparatively less. On 3-day PI with isolate no. CM-193, the number of macrophages were somewhat increased in the acinar lumen and in the interstitium. On 4-day PI, the lumen of acini as well as of intralobular and interlobular ducts were filled with disintegrated neutrophils, desquamated lining cells and macrophages. There was hyperplasia of lining cells of acini and interlobular duct. By the 6-day PI the number of macrophages in the lumen of acini had further increased and neutrophils were few in numbers. The interstitium was thickened due to fibroblastic proliferation and infiltrated with macrophages and lymphocytes. The thickening of the interstitium alongwith hyperplasia of lining cells resulted in the reduced lumen of acini and interlobular duct(Fig.24).

M.bovigenitalium + Mycoplasma F-38 + M.bovirhinis (isolate no. CM-195)

Mild inflammatory changes were present in the mammary-gland at 1-and 2-day PI. There was mild neutrophilic infiltration in the lumen of acini. Interlobular blood vessels were congested. At 3-day PI, infiltrating neutrophils and detached epithelial cells were seen in acini and interlobular duct. The interstitium was infiltrated with neutrophils, macrophages and lymphocytes. The histopathological changes at 4-day PI were similar to that seen at 3-day PI except that fibroblastic proliferation had

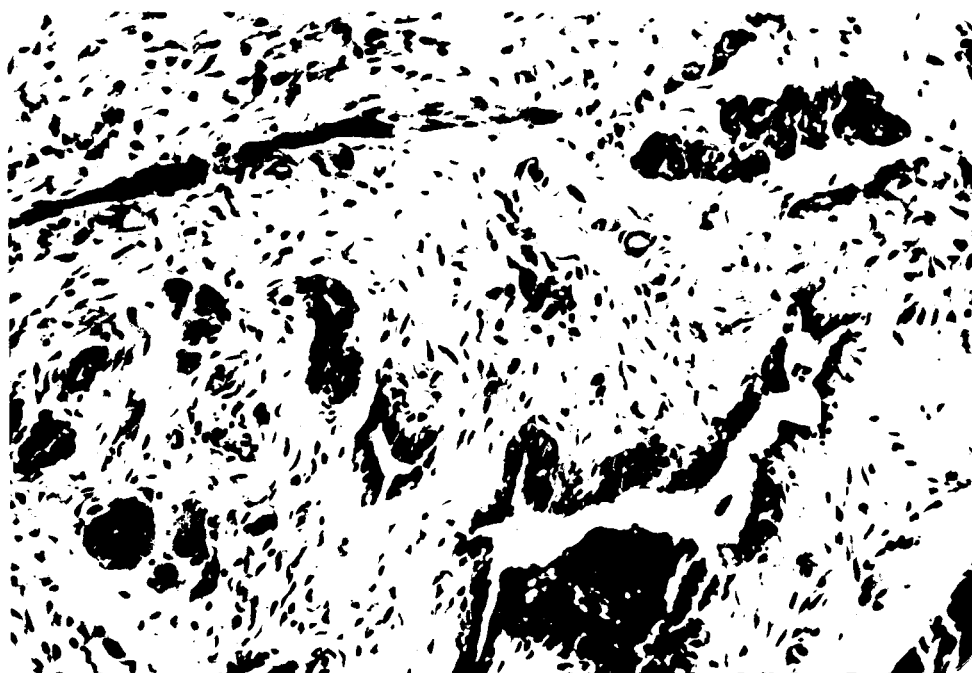


Fig.24 Rat mammary-gland at 6-day postinfection with isolate no. CM-193 (Mycoplasma F-58) showing thickened interstitium due to fibroblastic proliferation along with infiltration of macrophages and lymphocytes. Lumen of acini are narrowed with hyperplastic lining cells of acini and interlobular duct (H&E, X200)

resulted into thickened interstitium at 4-day PI, which became infiltrated with macrophages and lymphocytes at 6-day PI and caused atrophy of the acini.

M.bovigenitalium + M.bovirhinis (isolate no. CM-196)

At 1-day PI, a few neutrophils were present in the acini and interstitium. There was oedema of interstitium and the interlobular blood vessels were congested. By 2-day PI, the neutrophilic infiltration increased in acini, intralobular and interlobular ducts. A few macrophages were also seen in intralobular and interlobular ducts. The interstitium was also infiltrated with macrophages, lymphocytes and neutrophils. At places hyperplasia of lining cells of intralobular and interlobular ducts was seen. Interlobular blood vessels were highly congested (Fig.25). At 3-day PI, the atrophy of acini was seen (Fig.26) alongwith infiltration of mononuclear cells and fibroblastic proliferation in interstitium. Partial hyperplasia of lining cells alongwith disintegrated neutrophils and macrophages were present in acini, intralobular and interlobular ducts. At 4-day PI, the lumen of acini had further reduced in size due to hyperplastic changes and thickened interstitium. The interlobular connective tissue septae were occluded with adipose tissue. On 6-day PI the desquamated epithelial cells were also seen in lumen



Fig.25 Rat mammary-gland at 2-day postinfection with isolate no. CM-196 (M.bovigenitalium + M.bovirhinis) showing marked neutrophilic infiltration in interlobular duct and acini (H&E,X200)

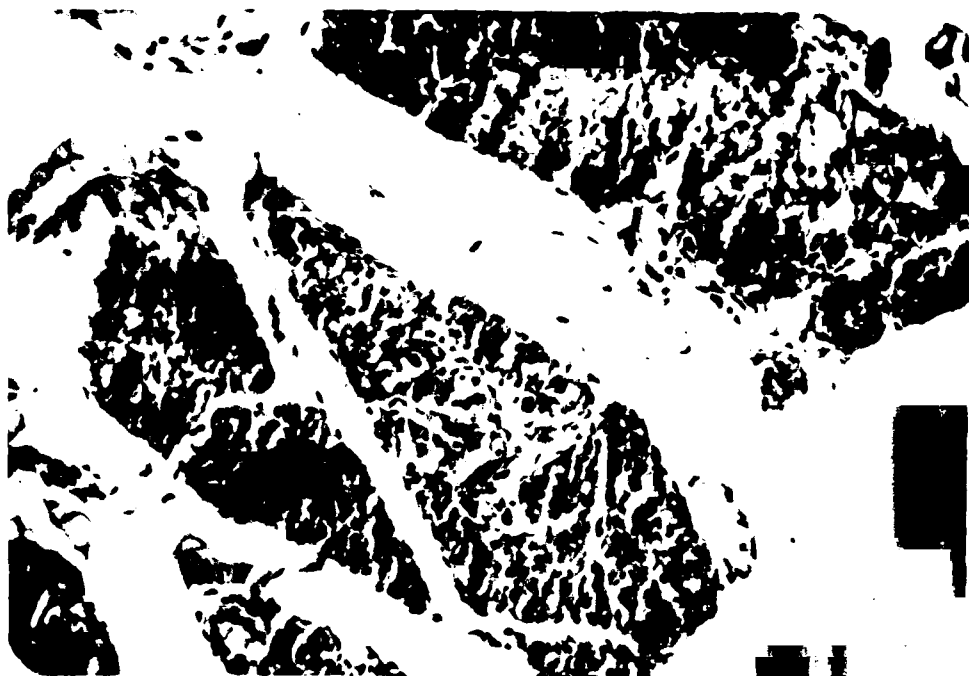


Fig.26 Rat mammary-gland at 3-day postinfection with isolate no. CM-196 (M.bovigenitalium + M.bovirhinis) showing acinar atrophy (H&E,X100)

of acini, intralobular and interlobular ducts alongwith hyperplasia of their lining cells. Fibroblastic proliferation of interstitium was seen infiltrated with macrophages and lymphocytes.

M.bovis + M.bovigenitalium + Mycoplasma F-38 + A.laidlawii
(isolate no. CM-197)

At 1-day PI, mild infiltration of neutrophils and detached cells in the lumen of acini and interlobular duct were seen. The lining cells of acini and intralobular ducts were swollen. There was mild oedema of the interstitial tissues and the interlobular blood vessels were congested (Fig. 27). The histopathological changes at 2-day PI were comparable to those observed at 1-day PI except the increased infiltration of neutrophils, lymphocytes and macrophages in the interstitium. At 3-day PI, moderate infiltration of neutrophils was seen in the lumen of acini and interlobular duct alongwith vacuolation and degenerative changes in the lining cells. At places hyperplasia of lining cells of acini and interlobular duct resulted in the narrow lumen. In the interstitium, there was infiltration of neutrophils, lymphocytes and macrophages. At 4-day PI, the lumen of a few acini and interlobular ducts were filled with intact and degenerated neutrophils alongwith desquamated epithelial cells. The fibroblastic interstitium was infiltrated with



Fig.27

Rat mammary-gland at 1-day postinfection with isolate no. CM-197 (M.bovis + M.bovis genitalium + Mycoplasma F-38 + A.laidlawii) showing oedema of the interstitial tissues with mild neutrophilic infiltration in acini (H&E, X200)

macrophages, lymphocytes and neutrophils causing the distorted and reduced lumen of acini (Fig.28). At 6-day PI, hyperplasia was seen in lining cells of acini and in intralobular and interlobular ducts. Their lumen were occluded with desquamated epithelial cells, macrophages and neutrophils (Fig. 29).

M.mycoides var. capri (isolate no. CM-111)

There was mild (1-day PI) to moderate (2-day PI) infiltration of neutrophils in lumen of a few acini. Heavy infiltration of neutrophils in the lumen of intralobular and interlobular ducts was seen at 2-day PI. Intralobular blood vessels were congested. There was moderate, focal infiltration of macrophages, lymphocytes and few neutrophils in the interstitium at 2-day PI (Fig. 30). At 3-day PI there was abscess formation characterized by degenerated and intact neutrophils surrounded by a few lymphocytes and macrophages in the fibroblastic tissues. At 4- and 6- day PI, the lumen of acini and interlobular duct were seen occluded with neutrophils, macrophages and hyperplastic lining cells. The thickened interstitium due to infiltration of macrophages, lymphocytes and fibroblasts caused the narrow lumen of acini and intralobular duct.

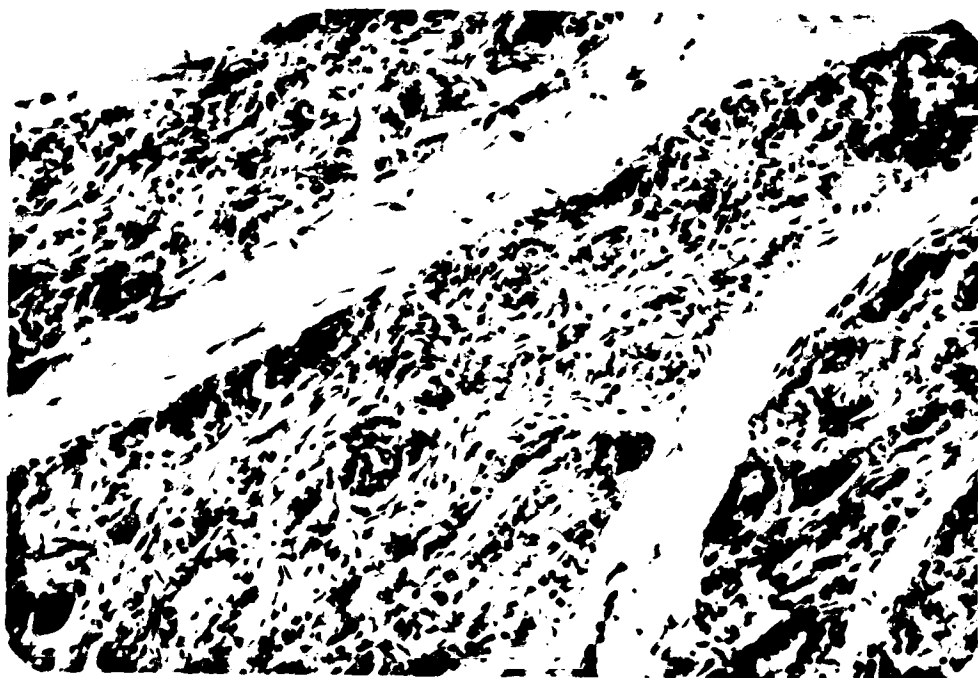


Fig.28 Rat mammary-gland at 4-day postinfection with isolate no. CM-197 (M.bovis + M.bovis cellulium + Myco-plasma F-38 + A.laidlawii) showing the distorted and reduced lumen acini due to fibroblastic proliferation of interstitium. Infiltration of inflammatory cells in acini and in interstitium is also visible (H&E, X200)

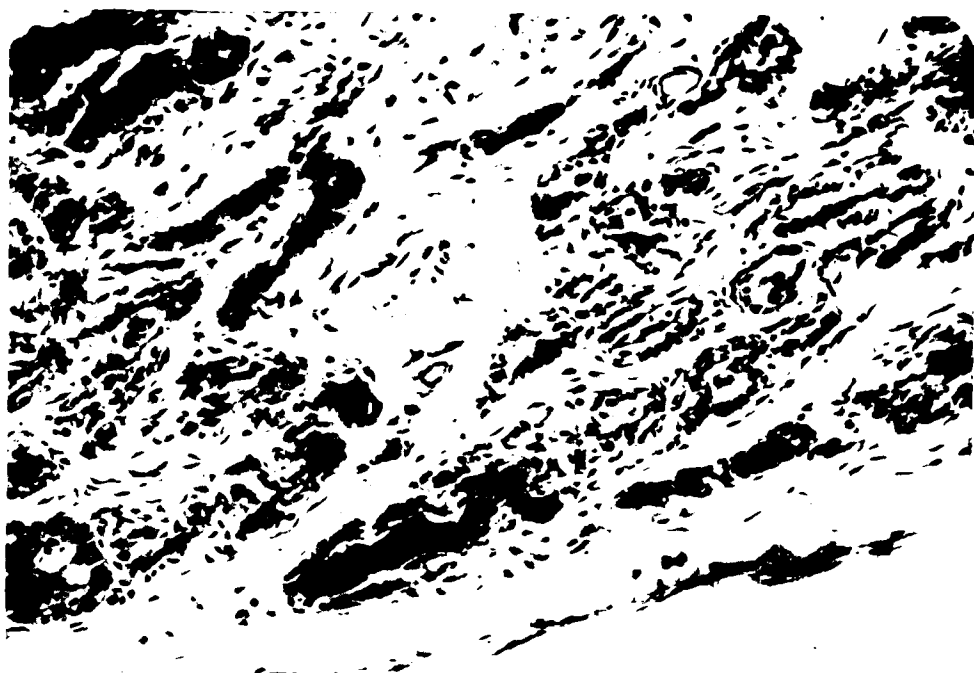


Fig.29 Rat mammary-gland at 6-day postinfection with isolate no. CM-197 (M.bovis + M.bovis cellulium + Myco-plasma F-38 + A.laidlawii) showing hyperplastic cell lining of acini and ducts with thickened interstitium (H&E, X200)



Fig. 30 Rat mammary-gland at 2-day postinfection with isolate number CM-111 (M. mycoides var. capri) showing marked neutrophilic infiltration in acini and interlobular duct with congested interlobular blood vessels (H&E, X200)

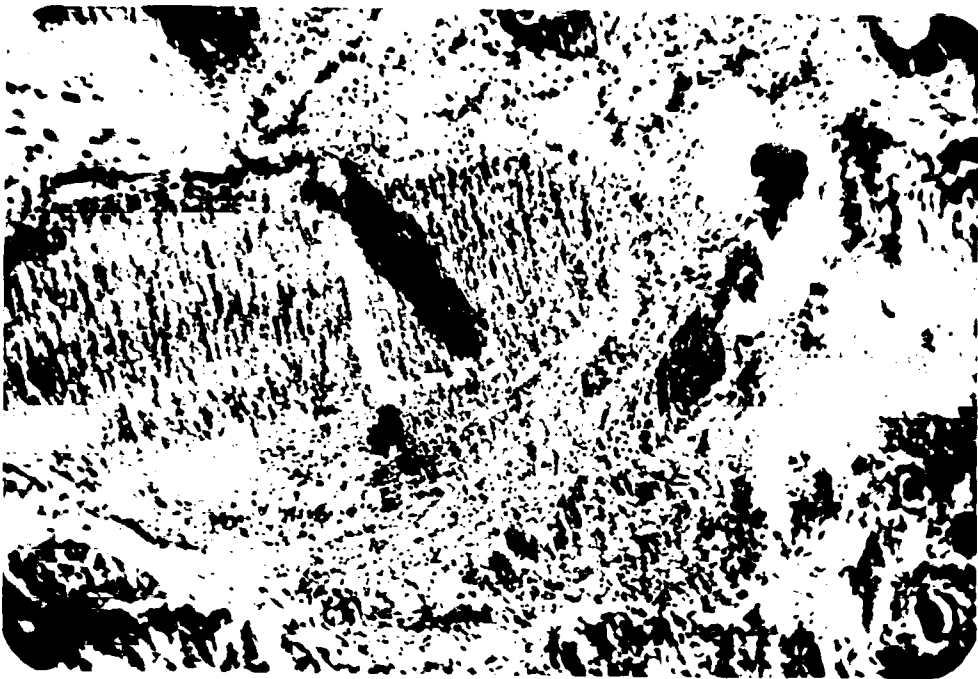


Fig. 31 Rat mammary-gland at 2-day postinfection with isolate number CM-448 (unidentified Leptospira sp.) showing abscess in mammary-gland (H&E, X200)

Unidentified Acholeplasma (isolate no. CM-448)

At 1-day PI, the histopathological changes included: mild infiltration of neutrophils alongwith desquamated cells in the lumen of acini, and interlobular and intralobular ducts, mild neutrophilic infiltration in interstitium and abscess formation characterized by neutrophils in the centre and small number of macrophages and fibroblasts at periphery. Similar changes were noted at 2-day PI. However, the infiltration of neutrophils, macrophages and lymphocytes was moderate in acini, intralobular and interlobular ducts and in interstitium. The abscesses were surrounded by comparatively more number of macrophages, lymphocytes and fibroblasts (Fig. 31). At 3-, 4-, and 6-day PI, the histopathological changes were similar to those noted at 2-day PI but abscess was not seen. Besides, focal hyperplasia of lining cells of acini, intralobular and interlobular ducts alongwith thickened interstitium resulted in the narrow lumen of acini and interlobular duct.

A. axanthum (isolate no. CM-212)

On 1-day PI, mild infiltration of neutrophils were seen in lumen of acini and intralobular ducts. At places hyperplasia of lining cells of interlobular duct was seen and interstitium was slightly thickened due to proliferation of reticular cells. On 2-day PI, the significant

histopathological changes included: hyperplasia of lining cells of acini and duct resulting in the narrow lumen, mild infiltration of neutrophils and macrophages in the lumen of acini and in interstitium. Histopathological changes on 3-day PI were comparable to those observed at 2-day PI except that a few eosinophils infiltrated in the lumen of acini. The changes on 4- and 6-day PI were similar to those observed at 2- and 3-day PI.

A.laidlawii (isolate no. CM-230)

With A.laidlawii (CM-230), no significant histopathological changes were observed in the rat-udder tissue except vacuolation and hyperplasia of epithelial cells lining the acini at some places and mild infiltration of macrophages and lymphocytes in interstitium.

DISCUSSION

IV DISCUSSION

Mycoplasmas have emerged as important pathogens of bovine mastitis during the last two decades. Though several reports pertaining to the outbreaks of mycoplasmal bovine mastitis are available from various parts of the world (Hale et al., 1962; Cottew, 1970; Durisic et al., 1973; Gourlay et al., 1974; Davies and Boughton, 1976; Ruhnke et al., 1976), only 3 reports (Katoch et al., 1983; Rahman et al., 1984; Pal et al., 1984) of mycoplasmal bovine mastitis exist in India. This study was designed to assess the involvement of mycoplasmas, acholeplasmas and ureaplasmas in bovine udder-infection. A total of 362 milk samples from 130 mastitic-cows and 99 mastitic-buffaloes were examined. This included 211 samples from 130 mastitic-cows and 151 milk samples from 99 mastitic-buffaloes. In addition, 247 milk samples from healthy bovines (145 cows, 102 buffaloes) were also examined for comparison. The isolates belonging to class Mollicutes were characterized and their pathogenicity was evaluated in two experimental systems viz. hamster tracheal ring and organ culture, rat mammary-gland. Seroprevalence of M.bovis and M.bovigenitalium (two important etiological agents of bovine mycoplasmal mastitis) antibodies was determined employing Enzyme-Linked-Immuno-Sorbent Assay (ELISA), Indirect Haemagglutination (IHA) and Agar-Gel-Immuno-Precipitation Test (AGIPT) to have an overview of the prevalence of bovine mycoplasmal mastitis.

Isolation of Mycoplasma, Acholeplasma, Ureaplasma and other Bacteria

Fifteen isolates comprising of 20 strains belonging to class Mollicutes from 130 mastitic-cows (15.38%) and 5 isolates of Mollicutes from 99 mastitic-buffaloes (5.05%) were isolated. Rahman et al. (1984) could not isolate any Mollicutes from 62 cows and 52 buffaloes affected with clinical mastitis, whereas Katoch et al. (1983) reported only two acholeplasmas from 73 mastitic-cows (2.7%) and none from 56 mastitic-buffaloes. However, Pal et al. (1984) reported 41 isolates belonging to class Mollicutes (26 Mycoplasma, 15 Acholeplasma) from 156 mastitic-buffaloes (26.2%). The higher percentage of mycoplasmal mastitis in buffaloes as reported by Pal et al. (1984) in comparison to 5.05% in this study might be due to the regional variation.

Mollicutes organisms isolated in this study from bovine (cows and buffaloes) mastitis comprised of M. bovis, M. bovis genitalium, Mycoplasma F-38, M. bovirhinis, M. mycoides var. capri, A. laidlawii, A. axanthum, Acholeplasma sp. and Ureaplasma. Out of these Mollicutes organisms, M. bovis, M. bovis genitalium, M. bovirhinis, A. laidlawii, A. axanthum and Ureaplasma have already been reported from cases of cow-mastitis (Pfutzner et al., 1979a, 1981; Garg and Kapoor, 1985).

Mycoplasma F-38 and M.mycoides var. capri associated with bovine mastitis have not been reported earlier, and the isolation of these two species from cow mastitic-milk appears the first isolations. Mycoplasma F-38 was found associated with contagious caprine pleuropneumonia (CCPP) in Kenya (MacOwan and Minette, 1976) which was later characterized to have cross reactions with M.mycoides subsp. mycoides, SC and LC varieties, M.primatum, M.equigenitalium and Mycoplasma sp.gp.7 in growth precipitation, complement fixation, growth inhibition, immunofluorescence, ELISA and immunoblotting (MacOwan and Minette, 1976; Erno and Salih, 1980; Erno, 1983; Erno et al., 1983; Kibe et al. 1985). M.mycoides var. capri had been isolated from outbreaks of highly fatal oedema and cellulitis in goats (Razin and Freundt, 1984). As such the isolation of Mycoplasma F-38 and M.mycoides var. capri, which are usually goat pathogens, from milk of cows suffering with mastitis is not surprising in view of the suggestion that capacity of a given mycoplasma to colonize is not confined to one class of animals (Gois et al., 1973). The report of isolation of M.bovigenitalium from lungs of aborted equine fetus (Langford, 1974), M.bovis from sputum of a woman with lobar pneumonia (Madoff et al., 1979) also supports the suggestion made by Gois et al. (1973).

Employing growth inhibition and indirect epiimmunofluorescence tests, one isolate of Acholeplasma from a mastitic-cow could not be identified with the help of antisera of

common Acholeplasmas viz. A.laidlawii, A.granularum, A.axanthum, A.modicum, A.oculi, A.equi and A.hippikion. This Acholeplasma isolate could not be tested against the antiserum of A.morum, A.parvum and A.florum. Thus, either it may be an undescribed new Acholeplasma species or it may be one of the untested species.

Ureaplasmas have not yet been shown as significant naturally occurring udder-pathogen. Ureaplasmas are known to cause urethritis in man, pneumonia in cattle and urogenital diseases in several animal species including cattle (Taylor-Robinson, 1979). Although certain strains of Ureaplasma can cause experimental mastitis in cows (Mackie and Ball, 1984; Rae, 1984), but reports of natural infections of cows due to ureaplasmas are a few (Morar, 1969; Gourlay et al., 1979b; Garg and Kapoor, 1985). Thus, nothing conclusive can be stated as to the significance of Ureaplasma as bovine-udder pathogen. In the present study, 5 of the 130 mastitic-cows (3.8%), 4 of the 145 normal cows (2.75%) and 2 of the 99 mastitic-buffaloes (2.02%) were found positive for ureaplasmas. These isolates of ureaplasmas were not associated with any Mycoplasma or other known significant bacterial udder-pathogen. Since the Ureaplasma isolates of this study could not be propagated for longer periods, these could not be speciated. Hence, the pathogenicity of the isolates could also not be tested in experimental models and the ability of isolated ureaplasmas to cause bovine mastitis remained a matter of speculation.

Milk samples from cows without mastitis were also examined for obtaining a comparative profile of Mollicutes organisms in healthy and mastitic-bovines. The results indicated that M.bovirhinis, M.bovigenitalium, A.laidlawii, A.axanthum and Ureaplasma were from the milk of healthy bovines, whereas the isolated Mollicutes from mastitic bovines comprised of M.bovis, Mycoplasma F-38, M.mycoides var. capri, M.bovigenitalium, M.bovirhinis, Mycoplasma sp., Acholeplasma sp., A.laidlawii, A.axanthum and Ureaplasma.

The present isolation of Mollicutes organisms from healthy cows (7.58%) was significantly lower than their isolation from milk of mastitic-cows (15.38%). It became evident that M.bovis, Mycoplasma F-38, M.mycoides var. capri were isolated from cases of cow-mastitis whereas M.bovirhinis, M.bovigenitalium, A.laidlawii, A.axanthum and Ureaplasma were isolated from milk of both mastitic and healthy cows. Although ureaplasmas were isolated from milk of both mastitic- and healthy cows but the concentration of ureaplasmas in terms of CCU/ml of original milk sample was higher in mastitic-milk (5×10^2 to 5×10^3 CCU/ml) than the milk of normal cows (5×10^1 to 5×10^2 CCU/ml). M.bovis is the primary etiological agent in mycoplasmal mastitis and in this study it has only been isolated from mastitic-milk and not from milk of normal cows. M.bovirhinis and M.bovigenitalium though have also been isolated from normal cow milk, their pathogenicity for other

tissues is well documented, e.g. M.bovirhinis and M.bovigenitalium have been considered pathogens of bovine respiratory and genital tract, respectively (Gourlay and Howard, 1979). A.axanthum and A.laidlawii as reported from healthy and mastitic-cows in this study have also been reported earlier from milk of mastitic-buffaloes in this region (Katoch et al., 1983).

Five isolates of class Mollicutes (2 Mycoplasma, 1 Acholeplasma, 2 Ureaplasma) were isolated from 99 mastitic-buffaloes in the present study. These isolates could not be sufficiently characterized and as such, does not require further discussion. However, M.bovigenitalium, Mycoplasma sp.gp.7, M.arginini, M.alkalescens and A.laidlawii from the buffalo mastitic-milk has been reported previously (Pal et al., 1984). Isolation of ureaplasmas from milk of mastitic-buffaloes has not been reported earlier. Occurrence of acholeplasmas including A.laidlawii in bovine milk is not unusual as these occur widely in a variety of host and tissues. In comparison to the isolation of Mollicutes organisms from mastitic buffalo-milk (5.05%), the isolation from healthy buffaloes was significantly lower (0.98%).

To obtain a comparative overall view of bovine mastitis, it was noted that the per cent isolation of Mollicutes was significantly higher in mastitic-milk of cows (15.38%) and buffaloes (5.05%) than the milk of normal cows (7.58%) and buffaloes (0.98%). It was also evident that the per cent isolation of Mollicutes from cow milk was higher (15.38%) in

mastitic-milk, 7.58% normal milk) than that of buffalo-milk (5.05% mastitic-milk, 0.98% normal milk), irrespective of their health status.

In an attempt to delineate the association of other bacteria in milk, if any, with mycoplasmal bovine mastitis, it was noted that S.epidermidis and Micrococcus sp. were associated with 5 (4 cow mastitis, 1 buffalo mastitis) and 3 (2 cow mastitis, 1 buffalo mastitis) cases of bovine mastitis, respectively. Neither S.epidermidis nor Micrococcus sp. are known for their udder pathogenicity and hence their role in causation of mycoplasmal mastitis does not need any discussion. However, earlier S.aureus was found associated with M.bovigenitalium, A.laidlawii, Mycoplasma sp.gp.7; Streptococcus with M.bovigenitalium, Mycoplasma sp.gp.7, M.arginini, A.laidlawii; E.coli with A.laidlawii; and Corynebacterium with unidentified Mycoplasma sp. (Pal et al., 1984).

Antibiotic susceptibility testing of Mycoplasma and Acholeplasma isolates from normal and mastitic-cow and buffalo milk revealed that all the isolates of Mycoplasma, Acholeplasma were sensitive to spiramycin (30 mcg), vibramycin (30 mcg), lincomycin, (2 mcg) and tylosin (15 mcg) but resistant to ampicillin (10 mcg). Only 4 (2 Acholeplasma spp., 1 A.laidlawii, 1 mixed isolate no. CM-197 of M.bovigenitalium + M.bovis +

Mycoplasma F-38 + A.laidlawii) out of 18(22.2%) isolates of Mollicutes were sensitive to streptomycin (10 mcg).

All the isolates of Mycoplasma and Acholeplasma were sensitive to ledermycin (30 mcg), tetracycline (30 mcg), neomycin (30 mcg) oxytetracycline (30 mcg) and erythromycin (15 mcg) except isolate nos. CM-202, CM-203, CM-209, CM-210 and CM-111 which had intermediate sensitivities. Available literature suggests that tylosin and spiramycin are the drugs of choice in containment of mycoplasmal infections in domestic animals and birds (Ozasay et al., 1964; Singh and Singh, 1967; Rao and Kapoor, 1967; Mohan et al., 1975; Sharma, 1977; Shawki Ali et al., 1981), which can also be used in limiting bovine mycoplasmal mastitis (Biancardi et al., 1973).

Seroprevalence of Bovine Mycoplasmal Mastitis

The seroprevalence of M.bovis and M.bovigenitalium antibodies in cows and buffaloes in this study was determined using AGIPT, IHA and ELISA. Three hundred and twenty one sera (116 mastitic, 205 healthy) from cows, and 97 sera (59 mastitic, 38 healthy) from buffaloes were examined. With AGIPT, antibodies against M.bovis and M.bovigenitalium could not be detected in any of the tested sera. IHA test detected 8.6% per cent and 4.3 per cent of sera from mastitic-cow, positive for M.bovis and M.bovigenitalium haemagglutinins, respectively. However, only 1.6 per cent

and 3.3 per cent mastitic-buffalo sera were positive for M.bovis and M.bovigenitalium haemagglutinins, respectively. The per cent positivity of M.bovis and M.bovigenitalium haemagglutinins in sera of healthy cows and buffaloes was lower as compared to mastitic-cows and buffaloes. Employing ELISA, 21.5 per cent and 9.4 per cent sera of 116 mastitic-cows were found positive for M.bovis and M.bovigenitalium ELISA specific antibodies, respectively, and 8.4 per cent mastitic-buffalo sera each were positive for M.bovis and M.bovigenitalium specific ELISA antibodies. Although several serological tests have been used for the diagnosis of mycoplasmal infections, complement fixation, growth and film inhibition and indirect haemagglutination tests have been recommended and used (Boughton, 1979). Recently, ELISA has been used to detect antibodies to M.bovis, M.bovigenitalium, M.bovirhinis, M.californicum and M.arginini in sera of cows (Jurmanova et al., 1985).

AGIPT was included in the present study as a possible diagnostic test because of its simplicity but it did not yield results when applied to field serum samples. It appears that AGIPT detects the antibodies quite late (Jain et al., 1969a) and hence it may not be a test of choice for serosurvey though it remains a good test for laboratory studies. Su et al. (1984) have also reported only a few cow-sera positive for Mycoplasma antibodies using AGIPT in comparison to IHA.

An alternative to AGIPT viz. immunoelectrophoresis (Kenny, 1983) may be tried to screen field serum samples which might yield better results.

IHA test was found specific for detecting antibodies against M.bovis and M.bovigenitalium in sera of mastitic and healthy cows and buffaloes in this study. The IHA test had earlier been used to detect antibodies in cattle against M.bovis (Cho et al., 1976; Jurmanova et al., 1985), M.bovigenitalium (Cho et al., 1976; Jurmanova et al., 1985), M.mycoides subsp. mycoides (Chima and Onoviran, 1982), M.bovirhinis (Su et al., 1984; Jurmanova et al., 1985), Mycoplasma sp.gp.7 (Su et al., 1984; Jurmanova et al., 1985), M.dispar (Su et al., 1984), M.californicum (Jurmanova et al., 1985), M.arginini (Jurmanova et al., 1985) and A.laidlawii (Su et al., 1984). While employing IHA in our study it was found that sensitization of glutaraldehyde-fixed sheep erythrocytes was more successful with freshly prepared M.bovis and M.bovigenitalium antigen than the refrigerator-stored antigen. The fixed erythrocyte was used successfully upto 2-months of storage in this study but can be used upto 7 months as suggested earlier (Cho et al., 1976).

The seroprevalence of M.bovis specific ELISA antibodies was higher in both mastitic (21.5%) and healthy (13.6%) cows than M.bovigenitalium specific ELISA antibodies (9.4% mastitic-cows, 8.2% healthy cows). Similarly, M.bovis specific

haemagglutinins in mastitic (8.6%) and healthy cows (7.3%) was found higher than the M. bovis genitalium specific haemagglutinins. However, M. bovis genitalium specific ELISA antibodies (8.4%) and M. bovis genitalium specific haemagglutinins (3.3%) were equal/higher than M. bovis specific ELISA antibodies (8.4%) and M. bovis specific haemagglutinins (1.6%) in mastitic-buffaloes. However, M. bovis specific ELISA antibodies (21%) were more than the M. bovis genitalium ELISA specific antibodies (2.6%) in healthy buffaloes.

Under optimum conditions, ELISA yielded reproducible results and was less time consuming. It proved more sensitive than IHA as it detected more number of positive sera which were found negative with IHA and AGIPT. Therefore, ELISA can be recommended as a sensitive serodiagnostic test for the seroepidemiology of bovine mycoplasmal mastitis.

Experimental Pathogenicity of Mollicutes Organisms Isolated from Bovine Milk

The pathogenicity of mycoplasmas, acholeplasmas and ureaplasmas has been studied in the cow-udder (Illing, 1979, Seffner and Pfutzner, 1980; Horvath et al., 1980; Mackie and Logan, 1982), goat-udder (Pal et al., 1983), chicken tracheal organ culture (Cherry and Taylor-Robinson, 1973), bovine

fallopian-tube organ culture (Taylor-Robinson and Carney, 1974), hamster tracheal organ culture (Chandler and Barile, 1980) and mice mammary-gland (Howard et al., 1975; Thorns and Boughton, 1980). The specific pathogen free (SPF) mice/rats and organ culture (tracheal, fallopian) have greatly facilitated the studies pertaining to the pathogenicity of Mollicutes organisms. Howard et al. (1975) preferred to use mice-udder over cow-udder to study the pathogenicity of ureaplasmas of human and bovine origin because the infection established easily in mice-udder and the results are obtained in shorter period.

In the present study, hamster tracheal ring organ culture and rat-udder models were employed to study the pathogenicity of Mollicutes organisms isolated from bovine milk. Cilia stopping effect (CSE) indicative of their pathogenic nature was noted with Mycoplasma F-38 (isolate no. CM-193), M. mycoides var. capri (isolate no. CM-111), M. bovis genitalium + Mycoplasma F-38 + M. bovirhinis (isolate no. CM-195), M. bovis genitalium + M. bovirhinis (isolate no. CM-196). The 4 isolates (A. laidlawii, CM-230; A. axanthum, CM-212, unidentified Acholeplasma CM-448, M. bovis + M. bovis genitalium + Mycoplasma F-38 + A. laidlawii, CM-197) did not exhibit significant CSE in hamster tracheal ring organ culture. Mastitis could be produced experimentally in rat mammary-gland by the isolates of Mollicutes viz. Mycoplasma F-38

(no. CM-193), M. mycoides var. capri (no. CM-111), unidentified Acholeplasma (no. CM-448), M. bovis genitalium + Mycoplasma F-38 + M. bovirhinis (no. CM-195), M. bovis genitalium + M. bovirhinis (no. CM-196), M. bovis + M. bovis genitalium + Mycoplasma F-38 + A. laidlawii (no. CM-197), along with histopathological lesions. However, A. laidlawii (isolate no. CM-230) did not produce mastitis in rat and A. axanthum (isolate no. CM-212) resulted in mild histopathological inflammatory response in rat-udder tissues. These two Acholeplasma species were also found nonpathogenic to hamster tracheal ring organ culture. Four isolates (Mycoplasma F-38, CM-193; M. mycoides var. capri, CM-111; M. bovis genitalium + M. bovirhinis, CM-196; M. bovis genitalium + Mycoplasma F-38 + M. bovirhinis CM-195) and the type strain of M. bovis, NCTC-10131 were found pathogenic in both the hamster tracheal ring organ culture and rat-udder model.

Several workers have successfully used organ cultures to study the pathogenicity of Mollicutes organisms. Araake (1982) had examined the effects of various mycoplasmas in mouse and chicken tracheal organ cultures, whereas Stalheim et al. (1976) studied the effect of ureaplasmas in bovine fallopian tube organ culture. In the present study, hamster tracheal ring organ culture was employed successfully and normal ciliary activity of tracheal rings could be maintained for a period of 12 days and the inoculated Mollicutes organisms survived till the end period of experiment which was 12-day. The

M.bovis (NCTC-10131) decreased the ciliary activity score in 12-day period from 300 to 12. Mycoplasma F-38 (CM-193), M.mycoides var. capri (CM-111), M.bovigenitalium + M.bovirhinis (CM-196), M.bovigenitalium + Mycoplasma F-38 + M.bovirhinis (CM-195), isolated from cow-milk resulted in the significant reduction of ciliary activity score from 300 to a range of 0 to 18.7. Cherry and Taylor-Robinson (1973) observed the mild CSE due to M.bovis and significant CSE due to M.mycoides var. capri in chicken tracheal organ cultures. A.axanthum and the unidentified Acholeplasma sp. was found nonpathogenic to hamster tracheal ring organ culture in this study as the reduction in ciliary activity score was not significant. Insignificant reduction in ciliary activity by A.laidlawii in mouse and chicken tracheal organ culture has been reported earlier (Aarkee, 1982; Cherry and Taylor-Robinson, 1973). The organ culture studies using various acholeplasmas suggest their questionable pathogenicity to the hosts, which already exist in the literature.

The presence of endotoxin in the nutrient medium of hamster tracheal ring organ culture infected with various isolates of Mollicutes in this study was detected at the end of the experiment i.e. 12-day postinfection by Limulus lysate test. Endotoxin was detected in organ culture medium infected with type strain M.bovis, NCTC-10131, Mycoplasma F-38, CM-193, M.mycoides var. capri, CM-111; M.bovigenitalium + Mycoplasma

F-38 + M.bovirhinis, CM-195, M.bovigenitalium + M.bovirhinis, CM-196, M.bovis + M.bovigenitalium + Mycoplasma F-38 + A.laidlawii, CM-197 and an unidentified Acholeplasma, CM-448, but could not be detected in cultures infected with A.laidlawii (CM-230) and A.axanthum (CM-212). Thus, these two acholeplasmas were considered nonpathogenic in hamster tracheal ring organ culture because neither the endotoxin was detected nor the histopathological and cilia stopping effect was noted. On the other hand, M.bovis (NCTC-10131), Mycoplasma F-38 (CM-193), M.mycoides var. capri (CM-111), M.bovigenitalium + Mycoplasma F-38 + M.bovirhinis (CM-195) and M.bovigenitalium + M.bovirhinis (CM-196) were considered pathogenic to hamster tracheal rings because these liberated endotoxin in the nutrient medium and produced significant histopathological and cilia stopping effects. However, the isolate no. CM-197 (M.bovis + M.bovigenitalium + Mycoplasma F-38 + A.laidlawii) and unidentified Acholeplasma (CM-448) liberated the endotoxin but the significant histopathological and cilia stopping effects were not encountered. Therefore, further investigations are needed to establish the relation of mycoplasma endotoxin and cilia stopping effects, if any. However, Chandler and Barile (1980) suggested that proteolytic substance(s) extracted from M.pneumoniae possess the property of ciliostasis. A thermolabile exotoxin has also been shown to be liberated by M.neurolyticum which caused rolling

disease in rats and mice (Thomas et al., 1966).

Mollicutes organisms from mammals are extra cellular pathogens that have the epithelial cells of mucosal surface as their target. In organ culture the injury produced due to interaction of mycoplasmas and target epithelial cells can be observed. In the present study histopathological lesions observed in the hamster tracheal rings infected with mycoplasmas and acholeplasmas from bovine-milk were loss of cilia and degeneration and desquamation of tracheal epithelium and cellular infiltration (neutrophils, macrophages) in lamina propria and subepithelium. Dykstra et al. (1985) have also reported similar histopathological lesions in the chicken tracheal rings infected with M. gallisepticum.

Rat mammary-gland was also adopted successfully for evaluation of pathogenicity of Mollicutes isolates from bovine-udder in the present study. Mice-udder model was first used for experimental production of mastitis by the bacteria (Chandler, 1970). Subsequently, Howard et al. (1975) produced mastitis in mice by inoculation of ureaplasmas in the mammary-gland. The inoculated rat-teats were observed upto 6-day postinfection in our study because lactating tissues are known to decrease to less than 1 per cent after 8 days of removal of offspring (Anderson, unpubl. data). Mice model is preferred over cow or goat-udder because inoculated

organisms are washed out with the milk-stream in the later and the infection may be difficult to establish. In the present study the inoculated organisms were recovered from the infected rat-teats upto 4-day postinfection only and not afterwards. Mycoplasmacidal effect of lecithin which is present in murine tissues has been reported in the literature (Kaklamanis et al., 1969). In spite of the use of 50 µg/ml of ammonium reineckate (which counteract the effect of lecithin) in SLM to reisolate the Mycoplasma organisms from rat tissues, it might be possible that the concentration of ammonium reineckate may not have been optimum for some rat-udder tissues. Humoral mycoplasmacidal factor, similar to those found in whey of bovine-milk (Brownlie et al., 1974; Howard et al., 1975) might also be in operation in certain rats.

Histopathologically the basic inflammatory response in the mycoplasma infected rat-udder tissues was neutrophilic which was evident even at 1-day postinfection. The neutrophilic response in experimental ureaplasma mastitis in mice has earlier been noted at 48 hr. postinfection (Howard et al., 1975). However, abscess formation was also seen in the rat-udder tissues inoculated with an unidentified Acholeplasma (CM-448) in this study. Similar abscesses which may be microscopic or range from 5 to 10 cm in diameter have also been observed in cow-udder experimentally inoculated with

M. bovis (Bennett and Jasper, 1978a; Jasper, 1981). Neutrophilic infiltration, in acinar lumen and interlobular ducts, hyperplasia of epithelium of acini and reduction in size of acini were some of the histopathological changes observed in rat-udder tissues infected with Mycoplasma/Acholeplasma in the present study. The similar changes have been described earlier for Ureaplasma infection in mice-udder (Howard et al., 1975). In addition, some other histopathological changes in rat udder-tissues infected with various isolates of Mollicutes in this study were infiltration of macrophages and lymphocytes in acini and interstitium, fibroblastic proliferation in the interstitium, oedema of interstitial tissues and desquamation of epithelium which have been recorded earlier in cow-udder experimentally infected with M. bovis (Jasper, 1982).

In hamster tracheal ring organ culture, M. bovis type strain NCTC-10131 and 4 Mycoplasma isolates (Mycoplasma F-38, CM-193; M. bovigenitalium + Mycoplasma F-38 + M. bovirhinis, CM-195; M. bovigenitalium + M. bovirhinis, CM-196; M. mycoides var. capri, CM-111) from bovine mastitic-milk were found pathogenic, whereas A. laidlawii, CM-230; A. axanthum, CM-212; unidentified Acholeplasma, CM-448; M. bovis + M. bovigenitalium + Mycoplasma F-38 + A. laidlawii, CM-197 were nonpathogenic. However, mastitis in rat-udder could be produced not only by the isolates which were pathogenic to hamster tracheal ring organ culture but also by other two isolates (no. CM-448, Acholeplasma sp.; no. CM-197, M. bovis + M. bovigenitalium + Mycoplasma F-38 +

A.laidlawii) which were nonpathogenic to hamster tracheal rings. Thus, the rat-udder model appears to be a better choice for evaluating mastitogenic potentiality of Mollicutes organisms isolated from bovine-udder. The rat-udder model is also convenient, quicker, easy to handle and less expensive.

SUMMARY

V SUMMARY

The present investigation was undertaken to study the role of Mollicutes (Mycoplasma, Acholeplasma, Ureaplasma) in bovine mastitis and to study the experimental pathogenicity of important isolates including M.bovis (NCTC-10131) associated with bovine mastitis. A total of 609 milk samples from 275 cows (211 from 130 mastitic-cows, 145 from 145 healthy cows) and 201 buffaloes (151 from 99 mastitic-buffaloes and 102 from 102 healthy buffaloes) were collected and processed for the isolation of Mycoplasma, Acholeplasma, Ureaplasma and other associated bacteria. Serum samples from 321 cows (205 mastitic, 116 healthy) and 97 buffaloes (59 mastitic, 38 healthy), whose milk samples were also examined for Mollicutes, were tested for prevalence of antibodies against M.bovis and M.bovigenitalium which are commonly associated with bovine mycoplasmal mastitis.

Fifteen isolates comprising of 20 strains belonging to class Mollicutes from milk of 130 mastitic-cows and 10 isolates comprising of 11 strains from 145 healthy cows were isolated. The isolates from mastitic cow-milk comprised of M.bovis, M.bovigenitalium, Mycoplasma F-38, M.bovirhinis, M.mycoides var. capri, A.laidlawii, A.axanthum, Acholeplasma (unidentified) and Ureaplasma whereas the isolates from healthy cows

consisted of M.bovirhinis, M.bovigenitalium, A.axanthum, A.laidlawii and Ureaplasma. Five isolates of Mollicutes (2 Mycoplasma, 1 Acholeplasma, 2 Ureaplasma) from milk of 99 mastitic-buffaloes and one isolate (Acholeplasma sp.) from milk of 102 healthy buffaloes were isolated. All the isolates were characterized as per the recommendations of Subcommittee on the Taxonomy of Mycoplasmatales. The isolates were also tested for their in-vitro sensitivity to various antibiotics, and it was found that all the isolates of Mycoplasma and Acholeplasma from bovine milk were sensitive to spiramycin, vibramycin, lincomycin and tylosin but resistant to ampicillin. The milk samples were simultaneously tested for the presence of other bacteria associated with mycoplasmal mastitis. It was found that some isolates of Mollicutes from bovine mastitis were associated with S.epidermidis and Micrococcus spp.

Seroprevalence of M.bovis and M.bovigenitalium antibodies was determined employing 3 serotests viz. Enzyme-Linked-Immuno-Sorbent-Assay (ELISA), Indirect Haemagglutination (IHA) and Agar-Gel-Immuno-Precipitation Test (AGIPT). Sera from 321 cows (116 mastitic, 205 healthy) and 97 buffaloes (59 mastitic, 38 healthy) were examined. The results of ELISA have suggested a higher prevalence of specific M.bovis (21.5%) and specific M.bovigenitalium (9.4%) antibodies in mastitic-cows than the healthy cows (13.6% M.bovis, 8.2%

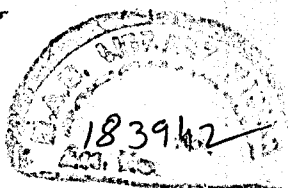
M.bovigentialium). Ten of 116 (8.6%) cases of cow-mastitis had specific M.bovis haemagglutinins whereas only 5 of 116 (4.2%) had M.bovigentialium specific haemagglutinins. In comparison, 15 of 205 (7.3%) and 13 of 205 (6.3%) healthy cows had M.bovis and M.bovigentialium specific haemagglutinins, respectively. None of the sera from mastitic and healthy cows was AGIPT positive. It became evident that per cent positivity of M.bovis specific ELISA antibodies and specific haemagglutinins was higher than the per cent positivity of M.bovigentialium specific ELISA antibodies and specific haemagglutinins in mastitic and healthy cows. The per cent positivity of M.bovis and M.bovigentialium specific ELISA antibodies was higher in mastitic than the healthy cows. Results on 97 sera from 59 mastitic and 38 healthy buffaloes indicated that the prevalence of M.bovis specific ELISA antibodies was higher/equal than the M.bovigentialium specific antibodies in buffaloes. IHA detected serodiagnostic/suspect titre against M.bovis and M.bovigentialium in both mastitic and healthy cows and buffaloes whereas AGIPT did not detect M.bovis and M.bovigentialium antibodies. ELISA proved to be a more sensitive serotest than IHA and AGIPT.

The pathogenicity of significant Mollicutes organisms isolated from milk of mastitic and healthy cows and buffaloes was studied in two experimental systems viz.

hamster tracheal ring organ culture and rat mammary-gland. Eight isolates of Mollicutes and the type strain of M.bovis (NCTC-10131) were tested for their pathogenicity in both the test systems. The effect of Mollicutes organisms under test on ciliary activity of tracheal ring was studied and the organisms were considered pathogenic if they reduced the ciliary activity to a score of 20 or less. The type strain M.bovis (NCTC-10131) and 4 isolates viz. Mycoplasma F-38 (isolate no. CM-193), Mycoplasma bovigenitalium + Mycoplasma F-38 + M.bovirhinis (isolate no. CM-195), M.bovigenitalium + M.bovirhinis (isolate no. 196), and M.mycoides var. capri (isolate no. CM-111) were found pathogenic whereas the other four isolates viz. A.axanthum (isolate no. CM-212), A.laidlawii (isolate no. CM-230), unidentified Acholeplasma (isolate no. CM-448) and M.bovis + M.bovigenitalium + Mycoplasma F-38 + A.laidlawii (isolate no. CM-197) proved nonpathogenic. Rat mammary-gland was successfully adopted as a suitable small animal model for the study of pathogenicity of mycoplasmas and acholeplasmas. The mastitis could be produced in the rat-mammary gland by the type strain M.bovis (NCTC-10131), Mycoplasma F-38 (CM-193), M.mycoides var. capri (CM-111), M.bovigenitalium + Mycoplasma F-38 + M.bovirhinis (CM-195), M.bovigenitalium + M.bovirhinis (CM-196), M.bovis + M.bovigenitalium + Mycoplasma F-38 + A.laidlawii (CM-197) and an unidentified Acholeplasma (CM-448). A.laidlawii (CM-230) did not produce mastitis in rat

whereas A. axanthum (CM-212) caused mild inflammatory reactions in the rat mammary tissue.

Two important etiological agents of bovine mycoplasmal mastitis viz. M. bovis and M. bovigenitalium were demonstrated culturally and serologically during the present investigation. However, more efforts are required for complete characterization of ureaplasmas from cases of mastitis in bovines in order to assess their role in bovine mastitis. ELISA was found to be a more sensitive serotest than IHA and AGIPT to determine the seroprevalence of M. bovis and M. bovigenitalium antibodies in bovines. Rat mammary-gland was found to be more suitable experimental model to study the pathogenicity of mycoplasmas and acholeplasmas because mastitis in rat-udder system could be produced not only by those isolates which were pathogenic to hamster tracheal ring organ cultures but also by other isolates which were not pathogenic in organ culture system.



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APPENDIX

Media for Isolation and Characterization of Mycoplasma, Acholeplasma and Ureaplasma

A. Standard liquid medium (SLM)

PPLO-broth (Difco)	80 ml	} sterilized by autoclaving at 15 lbs pressure and 121°C for 15 min
Yeast extract (Difco)	1%	
Sterile, inactivated, normal horse serum	20%	
Sterile Deoxyribonucleic-acid (BDH)	0.02% (1 ml of 0.2%)	
Thallium acetate	1:2000 (0.2 ml of 10%)	
Benzyl Penicillin	1000 IU/ml	
pH 7.8		

B. Solid agar medium (SAM):

Solid agar medium had the same composition as SLM with 1% agarose.

C. U-9B Urease colour test medium (Shepard and Lunceford, 1970)

Basal broth:

Tryptic digest broth	0.75 gm
Sodium chloride	0.50 gm
Potassium acid phosphate(KH_2PO_4)	0.02 gm
Distilled water	100 ml.

Adjusted the mixture to a pH 5.5 with 1N-HCl and sterilized in the autoclave at 121°C for 15 min.

(ii)

Complete U-9B medium (pH 6.0)

Sterile basal broth, pH 5.5	90	ml
Sterile unheated normal horse serum	10	ml
Sterile 10 per cent urea stock solution	0.5	ml
Sterile 2% L-cysteine hydrochloride	0.5	ml
Sterile 0.2% deoxyribonucleic acid(DNA)	1	ml
Sterile 1% Phenol red	0.1	ml
Penicillin G potassium 100,000 IU/ml	1.0	ml

Stored U-9B medium in freezer (-20C) or prepared fresh each week.

D. Solid U-9B colour test medium

Solid U-9B colour test medium had the same composition as U-9B colour test medium with 1% agarose

E. Diene's Stain(Diene, 1939; Madoff, 1959)

Methylene blue	2.5 gm
Azure II	1.25 gm
Maltose	10 gm
Sodium carbonate	0.25 gm
Benzoic acid	0.2 gm
Distilled water	100 ml

APPENDIX II

Media for biochemical characterization

Basal medium

PPL0 broth (Difco)	1	litre
Yeast extract (Difco)	10	gm

Adjusted the pH to 7.8 with 1N-NaOH

Autoclaved at 15 lbs. pressure for 15 min.

Phenol red broth

Sterile basal medium	80	ml
Sterile normal horse serum	10	ml

Sterile deoxyribonucleic acid(0.2%)	1	ml
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Phenol red (1%)	0.5	ml
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Each carbohydrate (glucose, galactose, xylose) was prepared as 10 per cent stock solution, seitz filtered and stored in screwcapped bottles at 4C. When required, 10 ml sterile 10% carbohydrate was added into 90 ml of complete phenol red broth. This medium was finally distributed in 3.6 ml quantity in sterilized test tubes and incubated at 37C for 24 hr. before use.

(iv)

Medium for deimination of L-arginine

Sterile basal medium	80	ml
Sterile normal horse serum	10	ml
Sterile deoxyribonucleic acid (0.2%)	1	ml
L-arginine (10%)	10	ml
Phenol red (1%)	0.5	ml

Adjusted the pH 7.0, sterilized by seitz filtration and distributed in 3.6 ml quantity in sterilized test tubes.

Test medium for Urea hydrolysis

Sterile basal medium	80	ml
Sterile normal horse serum	10	ml
Sterile deoxyribonucleic acid(0.2%)	1	ml
Seitz filtered urea (10%)	10	ml
Phenol red (1%)	0.5	ml

Adjusted the pH to 6.5 with 1N-HCl

Test medium for arbutin hydrolysis

Sterile basal medium	80	ml
Sterile normal horse serum	10	ml
Sterile deoxyribonucleic acid(0.2%)	1	ml
Sterile Arbutin (10%)	10	ml
Phenol red (1%)	1	ml

Adjusted the pH to 7.8 with 1N-NaOH and distributed the medium in 3.6 ml quantity in sterilized test tubes.

(v)

Medium for hydrolysis of esculin

Solid agar medium (SAM)	100	ml
Sterile Esculin (10% w/v)	1.2	ml
Sterile Ferric citrate (5% w/v)	1.2	ml

Adjusted the pH 7.8. Distributed the medium in petri-dishes and incubated at 37C for 24 hr. before use.

Medium for reduction of 2',3',5' triphenyl tetrazolium chloride

Sterile basal broth	80	ml
Sterile normal horse serum	10	ml
Sterile deoxyribonucleic acid(0.2%)	1	ml
Sterile 2',3',5' triphenyltetrazolium chloride (1%)	10	ml

Adjusted pH 7.8. Distributed the medium in 3.6 ml quantity in sterile test tubes.

Medium for phosphatase activity

Solid agar medium	99	ml
Sodium phenolphthalein phosphate (1%)	1	ml

pH 7.8

The medium was dispensed in petridishes and incubated at 37C for 24 hr. before use.

Reagents for Enzyme-Linked-Immuno-Sorbent-Assay (ELISA)

1. Substrate (ABTS): The substrate was prepared as per the recommendations of USDA, APHIS, Diagnostic Reagent laboratory, Ames, USA and the procedure is given briefly as below:

Solution A₁ (0.05 M Citrate buffer)

Citric acid (Mol.wt. 192.12)	9.6 gm.
Distilled water	1 litre
pH 4.0 with 1N NaOH	

Solution A₂ (40 mM 2,2'-azino-di-(3-ethyl-benzy, thiazoline sulfonic acid i.e. ABTS stock solution, Mol. wt. 548.7)

ABTS	274.4 mg
Distilled water	12.5 ml

Stored at 4C and protected from light in amber coloured bottle.

Solution A₃ (0.5 M hydrogen peroxide stock solution)

H ₂ O ₂ (30% of 8M solution)	0.5 ml
Distilled water	7.5 ml

Stored at 4C (stable for 1 month only)

8M H₂O₂

2.47 ml of H₂O₂ (30% w/v) was added to
7.53 ml distilled water

(vii)

Working solution of ABTS

Solution A₁, A₂ and A₃ were mixed in the following ratio to be used in ELISA:

0.025 ml of A₂ (final molarity 0.2 mM) +

0.020 ml of A₃ (final molarity 2 mM) +

5.0 ml of A₁ (final molarity 0.5M)

2. Stopping Reagent (0.1 M hydrofluoric acid)

2 ml hydrofluoric acid in 1 litre of distilled water, adjusted pH 3.3 with NaOH

(approximately 15 ml N NaOH was required in 500 ml of 0.1 M hydrofluoric acid)

3. Antigen diluent (Carbonate-bicarbonate buffer)

a) 0.2mM solution of anhydrous solution of sodium carbonate (21.2 gm in 1 litre of glass distilled water)

b) 0.2mM solution of sodium bicarbonate (16.8 gm in 1000 ml glass distilled water)

Mixed 16.0 ml of solution(a) + 34.0 ml of solution(b) and diluted upto 200 ml (pH 9.6)

4. Serum/conjugate diluent (Phosphate buffer saline, pH 7.6 with 0.5% Tween-20)

a- 0.2 M solution of monobasic sodium phosphate (31.2 gm NaH₂PO₄ · 2H₂O in 1000 ml glass distilled water)

(viii)

b- 0.2 M solution of dibasic sodium phosphate
(28.39 gm of Na_2HPO_4 or 71.7 gm $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ or
35.6 gm of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$)

13.0 ml of solution (a) + 87.0 ml of solution (b) and
diluted to 200 ml, pH 7.6. Added 8.5 gm of sodium
chloride to 1000 ml of phosphate buffer, pH 7.6

