STUDY ON CANINE MYCOPLASMAS

THESIS

Submitted to

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Vishwavidyalaya Evam Go Anusandhan Sansthan,
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By

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CERTIFICATE

We hereby certified that **Dr. Vikendra Singh** a candidate of **M.V.Sc. (final)** examination of **2006** in ***"Epidemiology & Veterinary Preventive Medicine"*** has been working under our supervision during this session and that the accompanying thesis entitled, **"Study on canine Mycoplasmas"**, which he is submitting is his genuine work.

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Guide

Acknowledgement

Any formal statement of acknowledgement can hardly express my gratitude to all those who have helped me in one or the other during the period of my study here.

First and foremost I would like to thank **God**, without Him none of the accomplishment of the past years would have been possible. I have been truly blessed by God's presence and guidance in my life.

I also feel privilege to have this opportunity to express profound gratitude to my guide **Dr. S. K. Yadav**, Associate Professor, Department of Epidemiology & Veterinary Preventive Medicine, U. P. Pt. D. D. U. Pashu Chikitsa Vigyan Vishwavidyalaya Evem Go-Anusandhan Sansathan, Mathura (U.P.).

I extend my esteemed and profound sense of gratitude and honors to my co-guide **Dr. B. C. Pal**, Professor and Head, Department of Epidemiology & Veterinary Preventive medicine, for his constructive counsel, erudite suggestions, above all for painstaking efforts in planning and execution of this study, and in preparation of this thesis. I wish, I should live up to his expectations. Acknowledging him, I could only say that I was fortunate enough to have a visionary like him to guide me on my first journey through the corridors of science.

My grateful record and heartiest thanks are offered to **Dr. Raja Ram**, Professor and Head, and **Dr. M. M. Farooqi** Associate Professor, Department of Anatomy for providing material for Histopathological examination of canine mycoplasmas.

I sincerely thanks to **Dr. Atual Sexana** Associate Professor, Department of Gynaecology **& Dr. Servejeet Yadav** Associate Professor, Department of Physiology for their technical support

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(Vikendra Singh)

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Introduction

INTRODUCTION

In India dog population has been recorded to be 21.17 million in 1992 with a growth rate of 3.93% from 1987-92 (Anonymous, 1997). It is now evident that the dog has become a fixture in modern man's life because, the role of dogs in providing protection, transport, recreation, companionship, security, religion, crime investigation and defense services. Dog as a pet is becoming more and more important in today's world.

The first documented occurrence of mycoplasmas in dogs was in 1934 (Shoetensack, 1934). Within the last 70 years, 15 known mycoplasma species and two species of mycoplasma that have not yet been fully described and named have been reported from dogs as ***Acholeplasma laidlawii, Mycoplasma arginine, M. canis, M. cynos, M. edwardii, M. feliminutum, M. felis, M. gateae, M. haemocanis, M. maculosum, M. molare, M. opalescens, M. spumans and ureaplasma canigenitalium***.

In dogs, mycoplasmas are thought to be part of the normal microflora in upper respiratory tract (Rosendal, 1982), but there are conflicting reports above the presence of mycoplasmas in lower respiratory tract of healthy dogs. The role of individual Mycoplasma sp. in respiratory infections in dogs is not well understood, but they are thought to colonize in lungs during pneumonia (Rosendal, 1982). Mycoplasmas have been questionably associated with respiratory tract infection by Appel and Binn 1987 and Binn ***et al.,*** 1968.

Many reports are available on the recovery of isolation, that causes different diseases in canine but no single report is available on the association of Mycoplasmas with clinical condition viz pneumonia, arthritis and delay conception in bitch. However, the purpose of this study was to recovery mycoplasmas from different clinical condition of the male and female dogs.

Mycoplasmas are wall-less bacteria that are found in a variety of avian, insect, mammalian, plant and reptilian hosts. Several species are pathogenic and a variety of infections, such as anemia, arthritis, infertility and respiratory disease, have been attributed to infection by mycoplasmas.

In the past 20 years, work on canine mycoplasmas has been extremely limited, with only a dozen publications on mycoplasmas in dogs.

The role of mycoplasma spp. as a primary cause of respiratory disease in dog and cat has been debated for several years. The genus was first described as a pleuropneumonia like organism in 1932 when it was cultured from the nasal secretions, lung, and liver of dogs with distemper. Since that time, Mycoplasma spp. have been thought to play a role, mostly secondarily, in disease processes. A number of studies have shown several mycoplasma spp. to be inhabitants of the mucosal surfaces of the respiratory and genital tracts, as well as the eyes , elementary canal, mammary, and joints of animals; some of these studies also provide evidence that mycoplasma spp. may be a causal factor of inflammation in these areas.

Many of the mycoplasma spp. were isolated from the respiratory tract. It is now accepted that mycoplasma spp. are normal flora of the upper airway (i.e., pharynx, larynx, oral cavity, nasal cavity). Mycoplasma spp. are not thought to colonize the lower airways (i. e., trachea and lungs) of normal dogs or cats. However, mycoplasma spp. have been isolated alone and in combination with other bacteria from the lungs of dogs and cats with pulmonary disease.

Review of Literature

Review of Literature

Mycoplasmas are the smallest self-replicating organisms closely related to sgram negative organisms with a low G + C content (Manniloff ***et al.,*** 1992) and distinguished phenotypically from other bacteria by their minute, highly pleomorphic size and absence of cell wall (Razin 1968).

The mollicutes are wide spread in nature as parasite of human, mammals, reptiles, fish, arthropods and plants (Razin, 1992). Several species of mycoplasmas have been shown to be pathogenic to canine, bovine, caprine, ovine, porcine and avian species (Tully and Razin,, 1983). Recent molecular developments have tremendously improved the diagnosis of mycoplasma infection especially of those with in "Mycoides clusters" by the adaptation of novel diagnostic tools like, polymerase chain reaction, DNA probes and monoclonal antibodies.

Isolation of canine mycoplasmas

Edward & Fitzgerald (1951a) studied a pleuropneumonia- like organism associated with infertility and epididymitis in a dog led to the examination of throat and vaginal swabs from all the animals in a breeding cannel. Pleuro-pneumonia like organism were isolated from the majority of samples. Strains differed in colonial appearances, and a classification into types of based on these difference was confirmed by the serological demonstration that each type of antigenically distinct. Nearly all the strains belonged to one of three types, provisionally called as ά, β and γ. Each type regarded as a distinct species of the pleuro-pneumonia group, and its biological properties were studied.

Edward & Fitzgerald (1951b) representated strains of the pleuropneumonia group, originally isolated from animals, grew well on a basal medium containing infusion broth, peptone, yeast extract and agar when this was further enriched with horse serum. The serum could be replaced by an ethereal extract of egg- yolk and fractionation of this suggested that cholesterol might be the active substence promoting growth. Growth was obtained when cholesterol (0.01 mg/ml.) was added

to the basal medium, together with starch or bovine albumin. Addition of the acetone- insoluble fraction of egg- yolk with cholesterol gave better results, growth of six of the eight strains tested being equal to that on serum agar: the lecithin and kephalin fractions after purification were less effective. No growth was obtained when starch, bovine albumin or the acetone-insolubie lipid was added to the medium without cholesterol. Cholesterol and stigmasterol were as effective as cholesterol in promoting growth. Oleic acid in high concentration(.0.05 mg/ml) inhibited growth. Both lipid and protien fraction of serum appeared to be concerned in its ability to promote growth.

Skalka & Krejcer (1968) Mycoplasma organism were isolated in liquid medium from the external genital organs of 25 of 47 dogs chosen at random. Nasal swabs from 17 of these dogs were also positive in 7 cases. Serotyping by growth inhibition tests of 36 strains isolated, classified 10 strains as ***M. canis***, 10 as ***M. spumans*** and 14 as ***M. maculosum***. Two strains were unclassified. Biochemical tests were reliable only for the isolation of ***M. spumans*** strains.

Brennan and Simkins (1970) culture of throat swabs of 467 Beagles in a closed breeding colony revealed a high incidence of Mycoplasma species (100%), coliforms (82%) , ***Pasteurella multocida*** (79.5 %) and lancefield group-G B-hemolytic streptococci 41.8 %. The flora was relatively stable of 12 month period, although younger dogs (1-4 years old) had a consistently lower incidence of beta-heamolytic streptococci then older dogs. The incidence of other organisms regarded as part of throat flora was similar to that reported in man.

Kato ***et al.*** (1972) isolated strains of ***Mycoplasma canis (48), M. spumans (12)*** and ***M. maculosum (7)***, from 107 dogs. They further reported that there was no difference in prevalence of the species in between healthy and sick dogs.

Mortensen & Stadsvold (1972) isolated a species of mycoplasma from three dogs having lung lesions which were died due to severe respiratory distress and symptom of canine distemper and in further studies, they observed 1:28 antibody titer from clinical cases.

Kirchhoff (1973) Mycoplasmas were isolated from 105 of 112 healthy and sick dogs received for distruction. They were present in the tonsils of 98 dogs, oral cavity of 55, nose of 16; also in the urogenital systemof 36% of females and 41% males. Occasional isolates were recovered from lung, small intestine and joints. There was no apparent relationship between the isolation of mycoplasma and the presence of pathological changes.

Rosendal (1973) cultivated mycoplasmas from conjunctive, respiratory and genital tract of male and female dogs. He also cultivated mycoplasma from lungs with pneumonic lesions. The author identified mycoplasma colonies in pharynx of 85% dogs examined by immunofluroscence technique. He reported frequent presence of mycoplasma in upper respiratory tract. He further isolated 11 glucose fermenting mycoplasma strains from respiratory and genital tract of dogs which differ from other established canine species and glucose fermenting species of other animals. He classified above strains as ***Mycoplasma cynos***. Rosendal & Laber (1973) reported 6 strain of ***A. laidlawii*** in dogs out of 38 strains isolated from vagina of dogs.

Koshimizu & Ogata (1974) identified, characterized and differentiated mycoplasma. From nasal, tracheal and lung pieces viz 100 (30.8%) of 324specimens collected from 81 apparently healthy or diseased dogs. These isolates were from the nasal cavity (17/23: 73.9%) oral cavity (7/7: 100%) trachea (33/46: 71%) lungs (9/46: 19.5%) liver (1/40: 2.5%) kidneys (2/38: 5.2%) spleen (2/40: 5%) lymph nodes (4/35: 11.4%) vagina (18/30: 60%)and urethra (7/10: 70%).

Rosendal (1974a) isolated first time a strain of ***Mycoplasma molar*** from the pharynx of dog which differ in biological and serological characters than the established glucose fermenting mycoplasmal species of canines.

Eberle ***et al.*** (1977) isolated mycoplasma from pericardium (Acholeplasma laidlawii) and heart valves (***M. canis***) of dogs and were investigated for infectivity in rats, mice and gerbils (Meriones unguiculatus). He also studied ***M. spumans, M. maculosum, M. edwardii*** and ***M. molar*** in mice. Interaperitoneally the mycoplasmas were given without and with incomplete Freund's adjuvants. The injected mycoplasmas could not be reisolated from rats and gerbils and they have

not produced any disease. From mice ***A. laidlawii, M. canis, M. edwardii*** were recovered from lung, liver and spleen only; ***A. laidlawii*** was recovered from heart muscles too.

Kirchner ***et al.*** (1990) isolated three mycoplasma spp. From one crossbred, two male and three females and four beagles of five laboratory dogs. Three animals showed respiratory disease as dyspnoea chronic coughing and moist rales, but other 2 dogs were observed during thoracic surgery to have microscopic lesion suggestive of pnemonia. Two dogs also had infection of ***Bordetella bronchiseptica, Pasteurella multocida*** and ***β- haemolytic Streptococcus sp.***

Binder ***et al.*** (1986) detected unknown canine species of mycoplasma in bitch with reproductive disorder. One of the bitches showed abortion and the other infertility with vaginitis and vaginal discharge. The mycoplasmas were isolated from the dog and from one of the bitches 6 times in 18 months despite repeated intramuscular and intra-vaginal treatment with various antibiotics. The role of the mycoplasmas in above condition could not certained.

Randolph ***et al.*** (1993) studied the prevalence of mycoplasmal and ureaplasmal infection from pharyngeal swab specimens from dogs with (n =38) or without (n=26) pulmonary disease. These mycoplasmal infections rates were found from tracheobronchial lavage specimens from dogs ≥1 year old with (21%) or without (25%) pulmonary disease. They reported prevalence of mycoplasmal recovery from tracheobronchial ravage was significantly associated with pulmonary disease among dogs <1 year old, and with dogs that had concurrent Bordetella sps. and Streptococcus sps. isolation. hey isolated Ureaplasma from a tracheobronchial lavage specimen of one dog with pulmonary disease and from none of the dogs without pulmonary disease. Most dogs with (84%) and all dogs without pulmonary *disease had mycoplasmas isolated from the pharynx.*

*Zoldag **et al.** (1993) isolated mycoplasmas from the genitalia of healthy dogs* and those with reproductive disorders. Mycoplasmas were isolated from 106 of 145 bitches and 28 of 44 male dogs. Of 108 bitches and 29 males with clinical signs of reproductive disorders (cervicitis, vaginitis, abortion, mortality of puppies, infertility, balanoposthitis) mycoplasmas were recovered from 87 bitches and 24 males.

Breeding indices of infected bitches or those kept in infected kennels proved the negative effect of mycoplasma infection. Similarly breeding indices of a male dog also confirmed the above finding; the average litter size was 4.8 before infection (data from 16 mating) and decreased to 0.7 after infection (data from 14 mating). The most frequently species were ***M. canis***, and ***M. bovigenitalium***.

Jameson ***et al.*** (1995) studied 93 dogs with bacterial pneumonia from which transtracheal aspiration samples were obtained for culturing of mycoplasma spp. and aerobic bacteria, were reviewed. On the basis of culture results, there were 65 Mycoplasma positive dogs including 7 dogs for which only Mycoplasma spp. Were isolated, and 28 Mycoplasma negative dogs. Most dogs were more then 5 years old, and difference in breed or six distributions among the three groups of dogs were not detected. Hematological and serum biochemical analysis results did not differ significantly between Mycoplasma- positive and Mycoplasma- negative dogs. 53of 93 (57%) dogs had a concurrent medical problem that may have predisposed them to developing bacterial pneumonia as a squeal to aspiration of immunosuppression. Mycoplasma- positive dogs were significantly more likely to have more then one species of bacteria isolated from their transtracheal aspiration samples. Clinical outcome was favorable when antimicrobial were selected on the basis of antimicrobial susceptibility results for the other bacterial isolates and not on results of the antimicrobial activity against Mycoplasma spp. Were primary pathogens or only opportunistic.

Walker ***et al.*** (1995) studied recovery of mycoplasma species from abscesses in a cat following bite wounds a dog. Two species of mycoplasmas, previously unreported from necrotic lesion associated with a bite wound from a dog. For bacteriological examination, ***M. canis*** and ***M. spumans*** was isolated and identified and the chemotherapy was successfully changed to ciprofloxacin

L, Abee-Lund ***et al.*** (2003) identified ***Mycoplasma canis*** in urogenital disease of dogs. They isolated ***M. canis*** from 9 dogs with clinical signs of urogenital disease. Seven of the dogs had a urinary tract infection, one had chronic purulent epedimymitis and one had chronic prostatitis, overt haematuria was frequently observed among the dog with cystitis. They isolated ***M. canis*** in pure culture from

seven of the dogs and in mixed culture from the other of two. In three cases the mycoplasma. was cultivated only from urinary sediment and it was typically obtained in smaller number then would be considered indicative of the urinary tract infection.

Chalker ***et al.*** (2004) reported mycoplasma infection in respiratory system that occurs worldwide predominantly in kenneled dogs and several and micro-organisms have been associated with outbreaks of canine mycoplasma infectious respiratory disease (CIRD). However, few studies have comprehensively examined the species of mycoplasma present in healthy dogs and those with CIRD. As part of an extensive study investigating the micro-organisms involved in CIRD were determined.

Morphological, biochemical and serological characterization

Armstrong & Yu (1970) Strains that had been divided in to four groups (A, B, C, D) on the basis of biological and serological methods. They were studied further by means of polyacrylamide gel electrophoresis (PGE) and double diffusion in agar gel. ***M.canis M. maculosum*** and ***M. spumans*** had distinctive PGE patterns. A group Bstrain, indistinguishable for ***M. canis*** in PGE and growth inhibition, reacted serologically with a wider range of field isolates than ***M. canis***. The group A strain were similar to ***M. edwardii***. Group C and group D strains were distinct from other canine mycoplasmas. PGE patterns of two strains differed at different intervals during incubation.

Armstrong ***et al.*** (1972) studied Canine pneumonia associated with mycoplasma infection. In most of the cases cultures made from infected tissue yielded one or more mycoplasma. These were classified on the basis of belonging to groups A, B, C, C' or D. Serological examination of three groups of dogs indicated result of canine mycoplasma, which caused pneumonia.

Eberle (1973) isolated mycoplasmas by Immunofluorcent differentiation from dogs. The indirect fluorescent antibody test using serum against eight canine strains of mycoplasmas (including ***M. spumans, M. canis, M. maculosum, M. edwardii***) and ***Acholeplasma laidlawiiwas***. He compared 220 strains isolated from 19 organs in 103 dogs. 73 did not react with any of the antisera. Of the 91 that were

typed, 72 were mycoplasma canis, 13 were similar to canine strain 'MH 6013' and the remainder reacted with other reference strains.

Kirchhoff (1973) studied Biochemical properties of 238 mycoplasma isolates indicated that 146 were ***M. canis*** or ***M. edwardii***, 4 were ***M. – mous***, 3 ***M. maculosum*** and 8 ***M. lardawii;*** 77 strains could not be typed.

Koshimizu & Ogata (1974) cloned a total of 158 strains cloned from these positive cultures could be differentiated into 6 types on the basis of their colonial morphology and various biological and serological characteristics; of these, 72 strains were identified as ***M. canis,*** 28 as ***M. edwardii***, 23 as ***M. spumans*** and 21 as ***M. maculosum***. A strain not requiring sterol and isolated from the vagina of a bitch was identified as ***Acholeplasma laidlawii***. Eleven strains isolated from the urethra of male dogs and from the vagina of the female were clearly distinguished from any other previously described canine mycoplasma

Rosendal (1974b) described biochemical characterization and serological classification of canine mycoplasmas. They isolated 118 mycoplasma strains from the conjunctiva and the respiratory and genital tract of 78 dogs. Three biochemical tests (glucose fermentation, catabolism of arginine and phosphates activity) divided the strains into six groups that did not show any antigenic overlapping. Serologically, 35 isolates were identified as ***M. canis***, 21 ***M. edwardii***, 10 ***M. cynose***, 10 ***M. gaeteae***, 30 ***M. spumans***, 2 ***M. bovigenitalium***, and 5 ***M. feliminutum***. The three remaining strains which were glucose positive and arginine and phosphatase negative were plasedin a separate serogroup, group A, as they were serologically different from the established canine species.

Rosendal (1975) further worked on cultural and biochemical studies. He typed reference strain of canine mycoplasmas. The type and strains of all mycoplasma species or " serogroups " presently known to occur in dogs were compared serologically. Test of growth inhibition and metabolism inhibition as well as indirect immunofluoresence tests did not disclose any cross reaction. Examination by double immunodiffusion revealed at least one antigen specific for each strain tested. In addition, the common antigens were found with in the glucose fermenting group and with in the arginine metabolizing group of mycoplasma

strains, respectively. No antigens were shared by these groups. The antigen positive strains MH5408, serogroup D, differed serologically from all other arginine positive Mycoplasma species. Serogroup D is therefore a new species for which the name Mycoplasma opalescence is proposed. The type strain is MH 5408 (ATCC27921and 10149) Finally, serological data are presented which relate strain HRC 689 to ***M.mycoides***.

Adegboye ***et al.*** (1979) studied two type of mycoplasma, one producing smooth colonies and the other rough colonies, which were isolated from the vaginal exudates of a dog with pyometra (VB 48, abst. 4112). Typing by the paper disc growth inhibition test and immunoflurescence showed that both types were ***Mycoplasma canis***; a smooth colony form was isolated from one and a rough form from two of six other pyometra cases examined

Eberle & Kirchhoff (1978) investigated 121 isolates, identified as ***M. canis***, 5 as ***M. edwardii,*** 3 as ***M. spumans***, 1 as ***M. maculosum*** and 3 as ***A. laidlawii***. 20 isolates were serologically identical with a serogroup MH 6010, 3 with the serogroup MH 4942. 64 isolates could not be identified. The percentage of isolate which could be determined by MIT was 71%, by IFT was 44.6% and by GIT 42%. An increase number of identification was possible by a combination of method, 56% could be identified using IFT, GIT and MIT.

Chalker ***et al.*** (2004) identified mycoplasma culture from tonsillar, tracheal and bronchial lavage samples, and to the species level by PCR and sequencing. ***Mycoplasma cynos*** was demonstrated on the ciliated tracheal epithelium by in situ hybridization and was the only mollicute found to be associated with CIRD, but only in the lower respiratory traci .Isolation of ***M. cynos*** was correlated with an increased severity of CIRD. Younger age and a longer time in the kennel

Experimental pathogenicity

Campbell & Okuda (1975) reported isolation of mycoplasma from conjunctiva and production of corneal immune response in guinea pigs. ***M. canis*** was the most frequent mycoplasmal isolate obtained from the conjunctival surface of the dogs. From the lower cul-de-sac of 101 dogs nine mycoplasma isolates recovered in which 5 were ***M. canis***. Experimentally induced immune keratitis was produced by

sensitizing guinea pigs to three antigen preparation of ***M. canis*** and then administering intracranial challenge inoculation. The guinea pigs were examined by the leucocytes inhibition (LMI) technique and by histopathological study of their corneas. The corneal reaction of the guinea pigs was not identical to a clinical entity of the dogs.

Eberle ***et al.,*** (1977) studied the pathogenicity of a laidlawii strain A42 isolated from the pericardial sac of the dog and was investigated by infection of 14 young dogs, belonging to three titers. The mycoplasmas were administered by oral, intervenous routes. The strain produced clinical illness when it was administered intraperitoneally in emulsion with freund's adjuvant or given to the dogs after treatment with ACTH. An inflammatory process on the heart valves was observed in one dog only, which received seven intravenous injection.

Rosendal & Vinther (1977) studied the experimental pneumonia caused by mycoplasma, detection and confirmed by electron microscopy of infected tissue. Thin section of lung tissue from 3 dogs killed 2, 3 and 4 weeks after pneumonia had been induced by endobronchial inoculation of ***Mycoplasmas cynos*** (strain D19) were examined by electron microscopy. Mycoplasma was observed extracellulary in lumen of bronchus and in alveoli in the earlier stage of the infection. The infection also resulted in degenerative changes in the bronchial epithelial cells, including in particular destruction and loss of cilia.

Rosendal (1978) studies pathohgenicity of mycoplasmas associated with distemper pneumonia of canine mycoplasmas. He isolated mycoplasma from the lungs of dog with distemper were ***M. cynos, M. bovigenitalium, M. canis, M. spumans***, and ***M. gateae***. These species of mycoplasma were inoculated in one week old pups by endobronchially routes .***M. cynos*** induced a focal pneumonia that was characterized histologically by sever inflammation of bronchi and adjacent respiratory tract tissue. The inflammatory response was initially neutrophilic and later was recovered from the lungs of inoculated dogs until three weeks after inoculation. A mid inflammatory response was observed in lung tissue of the dog inoculated with ***M. bovigenitalium***, whereas ***M. canis, M. spumans*** and ***M. gateae*** did not induce any visible lesion.

Rosendal (1972) Comparison was made between direct and indirect immunofluorescence using unfixed and hot-water-fixed colonies. Mycoplasma strains used were, 6 of canine and 1 of human (***M. pneumoniae***) origin. Indirect immunofluorescence of unfixed colonies was a useful serological method of identification. It was more sensitive than and equally specific as, direct immunofluorescence while being free from the background fluorescence associated with the latter method. Direct and indirect immunofluorescence of hot-water-fixed colonies was too unspecific for the identification of canine mycoplasma species ***M. pneumoniae*** appears to differ in this respect, as its antigenic specificity is apparently better retained during hot water fixation.

Materials & Methods

MATERIALS & METHODS

MATERIALS:-

In present study a total of 141 dogs from different locations and different breeds were screened by collection of nasal, genital and ocular swabs from healthy and diseased animals. The details is given in table 1 and table 2.

Table 1: Samples collected from different locations

	Location	No. of samples	Domestic dogs	Streets dogs	Details of specimen		
					nasal	genital	ocular
1	Kothari hospital	34	31	3	10	17	7
2	PFA	38	-	38	11	22	5
3	BSF	37	37	-	8	26	3
4	From field	32	21	11	12	17	3
Total of samples		141	89	52	41	82	18

Table 2: Collection of specimens from different breeds

S. No.	Source of material	No. of samples	Different breeds of domestic dogs						Street dogs
			German shepherd	Great dane	Pomer-anian	Dober mann	Bull dog	Dalmatian	
1	Nasal	41	7	-	4	8	1	-	21
2	Genital	82	18	3	8	16	2	1	34
3	Oculars	18	5	-	2	4	-	-	7
Total samples		141	30	3	14	28	3	1	62

A. Media employed:

The liquid and solid medium used for isolation and identification of mycoplasmas was prepared as advocated by L, Abee-Lund *et al.* (2003)

(Appendix). The solid medium contained almost all the same ingredients as liquid except that it contain PPLO agar 2.5% (Difco).

B. Collection of material from :

I- Nasal swab:-

Both nostrils of animals were thoroughly cleaned with rectified spirit. A sterilized swab was inserted in, to the one nostril and rub the mucas membrane of it in order to collect mucus in the nasal cavity. This swab was collected in 1.8 ml of mycoplasma broth medium. In the same manner another swab was taken from other nasal cavity.

II- Vaginal or perpetual swabs:-

Cleaned the vagina or prepuce with rectified spirit. A sterilized swab was rub the mucas membrane of vagina and prepuce it in order to collect mucus. This swab was collected in 1.8 ml of mycoplasma broth medium. In the same manner another swab was taken from other nasal cavity.

III- Ocular swab:-

Eyes were thoroughly cleaned with rectified spirit or sterilized water and a swab was touched gently into the conjunctiva of an eye. This swab was collected in 1.8 ml of mycoplasma broth medium. In the same manner swabbing was done from another eye.

IV- Experimental animals:-

For observation the nasal &genital swabs were collected the pieces of lung and spleen from sacrificed dogs were collected for isolation and in 10% formalin for histopathological examination.

1. Processing of sample

i- Liquid medium

Processing of specimen containing material was processed for detection of mycoplasma as per the method advocated by Taylors- Robinson ***et al.*** (1963). The sample was taken in mycoplasma broth and 2-3 dilution were made in mycoplasma broth, then the tubes were incubated at 37°C for 3-4 days and examined daily for

granularity. A clear broth with granularity having no turbidity and sediment was indicative of mycoplasma growth.

ii- Solid medium

From each dilution, a loopful culture as inoculated on mycoplasma solid medium. There after, the plates were put in desecrator with some moisture and CO2. The whole assembly was kept in incubator at 37 °C for 5-7 days.

2. Purification (cloning) of primary culture

a. Cloning in liquid medium

A plate with discrete colonies was selected. Individual colony was removed by cutting out a small block of agar containing single colony and transferred to 2.5 ml of modified mycoplasma broth in a tube and incubated for 48 hours. The culture was filtered with 0.22 um syringe filter. The first 2-3 drop discarded and then subsequent filtrate was collected in sterilized tube. From this filtrate 1:10, 1:100, and 1:1000 dilution in mycoplasma broth were made and 0.05 ml of each dilution was then spread in separate plate of solid medium. The plates were then incubated at 37° C in desiccators with a moist cotton swab and CO_2.

D. Examination of colonies on solid medium

I. Colonial appearance

Colonies on the incubated plates were examined with 4X magnification microscope for presence of "fried egg appearance".

II. Absence of reversion

The "fried egg appearance" colonies were rubbed on neutrient agar (antibiotic free) medium then there was no growth confirming mycoplasma colonies.

III. Staining

a- Giemsa's staining

The 24-48 hours liquid culture was centrifuged at 12,000 rpm for 30 minutes and smears were prepared from the sediment of each batch of mycoplasma cultures. The smear was also prepared by rubbing the colony surface from the agar

block containing colonies on a clean grease free slide. The smears were fixed in methyl alcohol for three minutes. A mixture of 1:10 Giemsa's stain (Appendix) was prepared with buffer saline pH 7.1-7.2 in a couplin jar and the slide was kept vertically in the jar for 30 minutes and washed with buffer saline (Appendix). Opposite side of smear was thoroughly cleaned then rinsed in air, it was examined under oil immersion

b- Hot water fixed colonies:

Agar block containing colonies of about 1.0 cm square size cut and put on glass slide colonies facing towards the slide. The slide was immersed in a beaker of hot distilled water at 80° C at an angle of 45°. The slide was removed with forceps and gently rinsed to drain the distilled water.

c- Hot water fixed colonies (Dienes staining)

The dienes staining procedure was adopted as described by Dienes (1939). *Stain the colonies with 1:10 dilution of dienes stain (Appendix) for 15 minutes and rinsed with distilled water, Light blue periphery of mycoplasma colonies with deep* blue central nipple was observed under low power microscope.

d. Hot water fixed colonies (Acridine orange staining)

Acridine orange staining of hotwater fixed colonies were done by the technique as advocated Rosendal, S. (1975). Composition acridine orange is given in Appendix.

F. Characterization of isolates

a. Sensitivity to digitonin

The technique of Freundt ***et al*** (1973) was used to classify the isolates of family mycoplasmataceae and acholeplasmataceae. The test was performed as the disc growth inhibition test.

For this test digitonin discs were prepared in advance by impregnating 6 mm paper disks with 0.025 ml of a 1.5% (w/v) ethanolic solution of digitonin (appendix). They can be used fresh or allow to dry and the stored at 4° C (heated for 30 minutes at 56° C in water bath for clear solution) and dried overnight at 37° C. The mycoplasma plates were incubated with 0.01 ml of culture using the running drop

technique. The discs were pressed gently into the middle of the inoculated area and plates were incubated at 37°C in desiccators. Mycoplasmas were sensitive to digitonin and acholeplasmas were resistant. A clear zone of inhibition after 3-5 days indicates sensitivity.

2. Biochemical characterization

To determine the fermenting activity of different canine mycoplasma isolates, the technique was used as advocated by Rosendal (1972).

I. Catabolism of glucose

Glucose medium (appendix) was inoculated with 0.05 ml culture incubated at 37° C aerobically. Observations were recorded every second day for two weeks. A color change from pink to yellow (acid) is indicated a positive reaction.

II. Catabolism of Mannose

Mannose medium was inoculated with 0.05 ml culture incubated at 37° C aerobically. Observation were recorded every second day for to weeks. A color change from pink to yellow is indicated a positive reaction.

III. Hydrolysis of arginine

Arginine incorporate medium (appendix) with ph 4.7 was inoculated with 0.05 ml culture incubated at 37° C aerobically. Changes were recorded continue up to 14 days. A color change from yellow to dark red (alkaline) is indicated a positive reaction.

IV. Phosphatase production test

Phosphatase agar (Appendix) plates were prepared. Inoculated and uninoculated plates were incubated in triplicates. After incubation for 3, 5 and 7 days, plates were flooded with 2ml of 5N NaOH. Appearance of a red color in 30 seconds indicated a positive reaction. The uninoculated plates developed red color after some minutes.

V. Reduction of triphenyl-tetrazolium chloride

a. aerobic

b. anaerobic

The test was performed as described by Freundt ***et al.*** (1973) using the same procedure as in testing of catabolism of carbohydrates. Mycoplasma broth was incorporated with 2, 3, 5 triphenyl tetrazolium chloride (Appendix) aerobically. The development of pink color with formation of a red precipitate indicated a positive reaction.

VI. Film and Spot formation

The medium (Appendix) used in this test was as employed by Freundt ***et al.*** (1973). After inoculation the plates were examined for formation of film and spot after 3, 7 and 14 days of incubation. A crinkled appearance of the film with small black dots in the upper layer of the medium seen under a dissecting microscope indicates a positive reaction.

VII. Liquefaction of coagulated serum

Two slant of serum digestion medium (Appendix) were inoculated with pasture pipette and then incubated at 37°C anaerobically. Readings were recorded at frequent intervals during 14 days of incubation. Liquefaction was revealed by the development of shallow depressed area with a moist base when growth was confluent and of small pits when growth was scattered. In the case of heavy liquefaction, fluid accumulates in the angle between the base of the slope and the wall of the tube.

3. Mycoplmasmal growth on blood agar plate –

Two drop of mycoplasma culture on blood agar plate and incubate 0n 37 °C in incubator after two to three days examine this plate on 4x microscope. This was advocated by Chalker ***et al.*** (2004)

4. Serological Characterization

a. Growth inhibition test

The method for this test was employed as described by Chalker, V. J. (2005). The growth inhibition test was performed on a solid medium using 6 mm filter paper disks impregnated with 0.025 ml antiserum. After loading the disks were subjected to vacuum drying overnight in desiccators and stored at –20° C in screw capped

vials. The 3 days incubated liquid culture allowed to run down the plate (running drop technique). The surface of the plate should be dried and then applied the antiserum disks. The plates were incubated at 37 °C anaerobically. The standard antisera were borrowed FAO/WHO Aarhus, Denmark. A clear zone with no colony beyond 0.5 mm from disc was considered positive inhibition with inhibition of growth.

c. Preparation of antigen for countercurrent immunoelectrophoresis

The preparation of antigen was done as per the technique of Freundt **et al** (1979).

EXPERIMENTAL DESIGN-

I- Preparation of inoculums

A single colony of Mycoplasma was picked up from mycoplasma agar plate and inoculated in mycoplasma broth. Broth was incubated for 72-96 hours at 37^0 C. After growth of mycoplasma culture, containing 10^5-10^6 CFU/ml diluted and again incubated for 2-3 days. For the check the growth of organism, one drop of prepared inoculum taken on mycoplasma solid medium. This confirmed mycoplasma culture were used for inoculation of experimental animals in different groups which was described as vide infra.

II- Animals:

Six weeks old four street healthy pups were obtained from Veterinary College, Mathura campus and examined for any disease condition against Mycoplasma infections. These pups were used for experimental trial as under-

1- For Pathogenecity test
2- For examination of contagious status
3- For clinicopathomorphological changes
   (a) Clinical signs
   (b) Pathomorphological examination
      - Gross observation
      - Histopathological Changes

All four pups divided in four groups

Group I- Pup was inoculated with 2 ml of inoculum via oral route

Group II- Pup was inoculated with 1 ml of inoculum via nasal route

Group III- Pup was inoculated with 0.4 ml of inoculum via intramuscular route

Group IV- Pup was treated as negative control

III- Processing of morbid material for Histopathological examination

For the histopathological studies the lung tissue of different level from each lobe of lung for experimental pups collected immediately after sacrifice of the pups. Small pieces of lung tissue were fixed in 10% suffered natural formalin to facilitate histological study (Luna, 1968). The fixed tissue were processed for routine paraffin embedding technique using standard alcohol- cedar wood oil schedule and were sectioned at 5-6µ thickness. The paraffin sections were stained Haematoxylin and Eosin method Luna, 1968. The stained sections were observed under light microscope.

Results

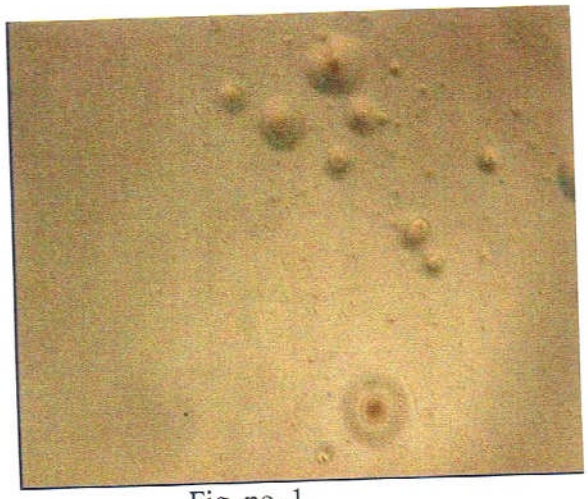

Fig. no. 1

Egg fried appearance of canine mycoplasmas from vaginal mucous

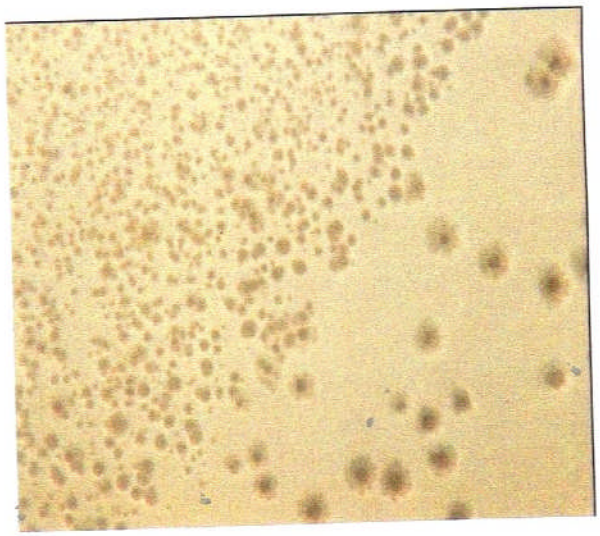

Fig. no. 2

Colony of canine mycoplasma from nasal cavity

RESULTS

Mycoplasma Isolation

The various types of material collected from male and females dogs were first cultured in the liquid mycoplasma broth medium. There was visible granularity without turbidity in the medium on second and third day of inoculation, which increased during the subsequent days up to seven days. The subculture was than made on solid mycoplasma agar. Mycoplasma or Acholeplasma colonies were confirmed by the microscopic examination on the solid medium as fried egg appearance.

A total of 7 (4.96%) mycoplasma isolates were recovered from 41 nasal swabs (domestic dogs (20) and street dogs (21)), 82 genital (domestic dogs (48) and street dogs (34)) and 18 oculars domestic dogs (11) and street dogs (7) with clinical history of respiratory distress, healthy, arthritis suspected, infertile, pneumonia, parvovirus suspected, fever and digestive problem, abortion and paralysis suspected. Mycoplasma organisms were isolated from 1 nasal swabs out of 41 (2.45%) nasal swabs collected from healthy and diseased dogs. Six mycoplasma isolates could be recovered from 82 (7.32%) genital samples from healthy and diseased dogs. No mycoplasma were isolated from 18 ocular samples.

Morphological characteristics

The morphology of the organism was observed after Giemsa staining. The organisms were of varying shape highly pleomorphic revealing spherical, ovoid, pear shape and short filamentous.

Colony characteristics

The typical fried egg appearance colonies (fig 1&2) of mycoplasma or acholeplasmas were observed on solid media. However, no growth was observed on routine media without antibiotics viz., blood agar and nutrient agar indicating the purity of isolates. The size of colony was depended on its density of colonies on the solid medium in the plate. Colonies were stained with Dien's stain and with acridine

orange gave blue and orange color respectively (Fig. 3). It was special observation that the growth of canine mycoplasma was presented on blood agar medium.

Digitonin sensitivity

The isolates with fried egg appearance were tested for differenction of mycoplasmas and acholeplasmas. Only 7 isolates could find sensitive to digitonin No digitonin resistance isolates could recovered. (Fig. 5).

Biochemical Characteristics for Speciation

The isolates were characterized by using different biochemical tests viz., 1. Catabolism of glucose, 2 Catabolism of arginine, 3. Phosphatase production, 4. Film and spot formation 5. Tetrazolium reduction (arobic and anrobic), 8. Catabolism of mannose, and 7. hemadsorption (rabbit & calf RBC). (Table No. 3.) They were grouped in three categories. These were divided into three categories, Ist category Glucose positive arginine negative, IInd category glucose positive arginine positive, and IIrd category glucose negative and arginine positive. In the present study a total of 141 samples yielded 7 mycoplasmas from different geographical location. Of total samples examined seven isolates were recovered with the characteristics of the mycoplasma. The isolates were speciated by using biochemical and serological tests, On the basis of biochemical tests.

One isolate CG_1 which, glucose positive and arginine negative and recovered from genital tract of German Shepherd bitch with clinical history of infertility. Thus identified as ***M. canis.*** This isolate catabolized mannose could not produced phosphated and did not reduced tetrazolium and found negative for rabbit and calf RBC. Thus it was identified as M. canis.

In category II, 4 isolates were found glucose positive and arginine positive (CG_2, CG_3, CG_4 & CD_1), among these one CG_2 was from the nasal cavity of dog with clinical history of pneumonia and CG_4 from vagina of healthy bitch while $CD_1$and CG_3 were from prepuce of male dogs. CD_1 was having clinical history of lameness and CG_3 was having fever with cuffing. The two isolates of categiry II (CG_2 & CG_4) were negative for phosphatase and film & spot formation table no. 5. They were

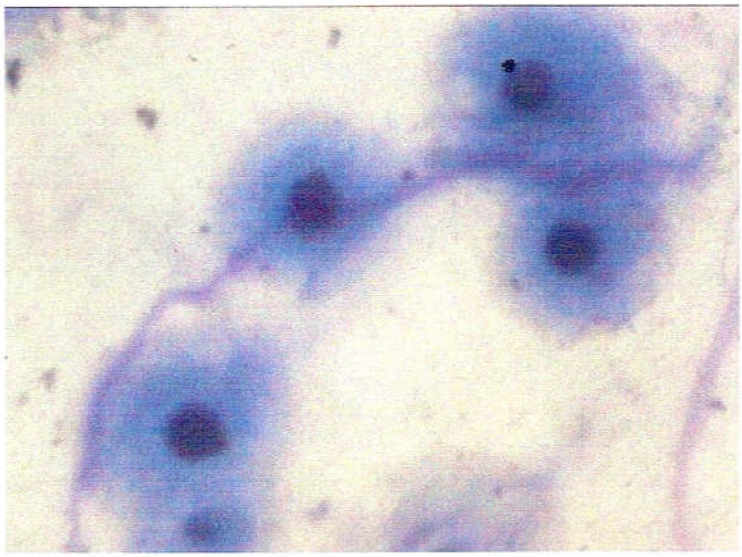

Fig. no. 3
Dense stains colonies of canine mycoplasmas

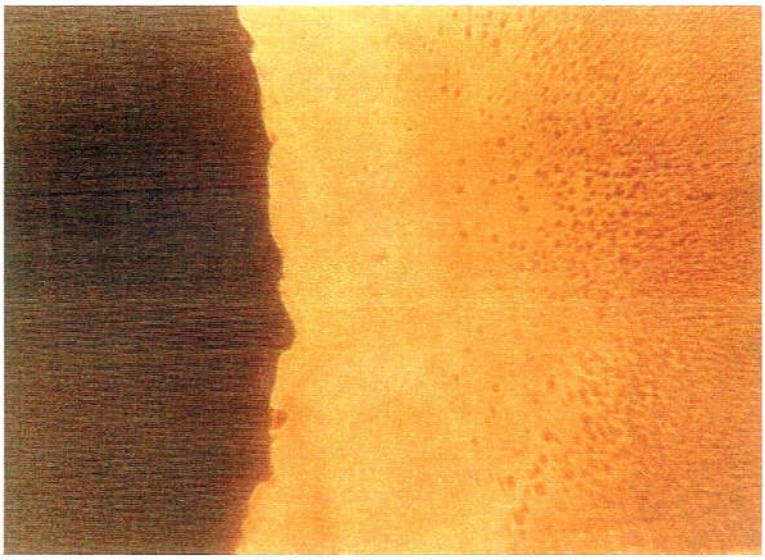

Fig. no. 4
Growth inhibition test

biochemically identified as M. gateae. But two other isolates (CG_3 & CD_1) were not identified and remained unknown.

In category III, 2 isolates (CD_2 and CG_5) were negative for glucose and positive for arginine among these CD_2 and CG_5 were from vagina from bitchs with clinical history of abortion and other was having clinical history for Parvov. These isolates were put for put for further identification with antisera.

Table 3: Biochemical parameters of seven isolates

	Location	Glucose	Mannose	Arginine	Phosphatase	Teteazolium		Film & spot	hemadsorption	
						Arobic	Anerobic		Rabbit RBC	Calf RBC
CG_1	Vagina	+	+	-	-	-	-	-	-	-
CG_2	Nasal	+	+	+	-	-	-	-	-	-
CG_3	Prepuce	+	+	+	-	-	+	-	-	-
CG_4	Vagina	+	+	+	-	+	+	-	-	-
CD_1	Prepuce	+	+	+	-	+	+	-	-	-
CD_2	Vagina	-	-	+	-	-	-	-	-	-
CG_5	Vagina	-	-	+	-	-	-	-	-	-

CG- Canine German Shepherd, CD- Canine Doberman

Growth inhibition test

In growth inhibition test, the isolate CG_1 tested with standard antiserum against *M. canis* revealed a good zone of inhibition, thus identified as *M. canis*. The isolates CG_1 and CG_4 put for growth inhibition against M. canis, M. gateae and M. arginini but they revealed a zone of growth inhibition with *M. gateae* antiserum and were identified as ***M. gateae.***

But the remaining 2 isolates (CG_3 and CD_1) of this group could not revealed a zone of growth inhibition with *M. gateae* and also with other antisera like ***M. canis*** *and M. arginini*. Two CG_3 and CD_1 remained unidentified. These isolates were from pneumonia and nasal secretion respectively.

Isolates CD_2 and CG_5 were further tested with antiserum of *M. alkalescens* and *M. arginini*. It could give a good zone of growth inhibition with *M. arginini* and thus identified as ***M. arginini***. Both isolates were from healthy female bitch with clinical history of abortion. (Fig. 4).

Table 4: Growth Inhibition test with Standard antiserum impregnated disc

S. No.	No. of Isolates	Antiserum impregnated disc with				Results
		M. canis	*M. gateae*	*M. arginini*	*M. alkeline*	
1	CG_1	+++	-	-	-	*M. canis*
2	CG_2	-	+++	-	-	*M. gateae*
3	CG_3	- -	-	-	-	*Unidentified*
4	CG_4	-	+++	-	-	*M. gateae*
5	CD_1	-	-	-	-	*Unidentified*
6	CD_2	-	-	+++	+	*M. arginini*
7	CG_5	-	-	+++	+	*M. arginini*

Strong zone of growth inhibition +++ , Moderate zone of growth inhibition +, No growth inhibition –.

Antibiotic sensitivity

Antibiotic sensitivity of the all field strains of mycoplasma was carried out by using 6 antibiotics as Erythromycin, spiramycin, sparfloxacin, tylosin, enrofloxacin and oxytetracycline. All 7 isolates (100%) sensitive to tylosin, 57% were found strong sensitive, while 43% were week sensitive to Enrofloxacin. Antibiotic spiramycin could revealed moderate sensitivity (43%), while 57% with moderate sensitivity with Spiromycin. Antibiotic Erathomycin, sparfloxacin and oxytetracyclin could not inhbit the growth of any canine mycoplasma.

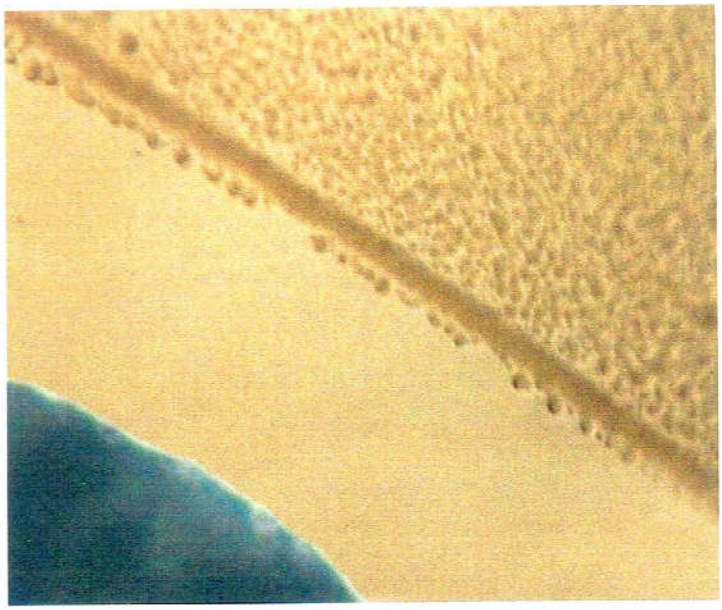

Fig No. 5
Digitonin test of mycoplasma

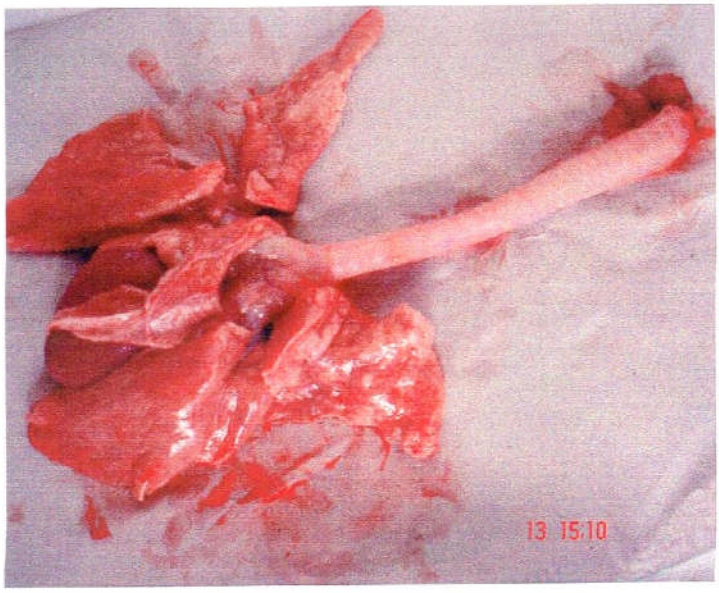

Fig. no. 6
Pneumonia in experimental pup with congestion

Table 5: Antibiogram of canine Mycoplasmas

S. No.	No. of Isolates	Erythromycin	spiramycin	sparfloxacin	tylosin	enrofloxacin	oxytetracycline
1	CG_1	-	++	-	+++	+++	-
2	CG_2	-	++	-	+++	+++	-
3	CG_3	-	+	-	+++	++	-
4	CG_4	- -	+++	-	+++	++	-
5	CD_1	-	++	-	+++	++	-
6	CD_2	-	+++	-	+++	+++	-
7	CG_5	-	+++	-	+++	+++	-

Very sensitive +++, sensitive ++, Less sensitive +, and resistant –

Experimental design

Pathogenecity

After inoculation via different routs (oral, nasal and intramuscular routs) to see the pathogenecity. The animal which were given inoculum by oral routs increase in temperature after 17 days of 107 °F and decrees intake food. The animal which were given through inoculum nasal rout there was high in temperature after 13 days of 107 °F and decrese intake food. The animal which was given inoculum with intramuscular rout revealeda high temperature after 8 days of 107 °F with anorexia.

Contagious status

The fourth dog which was not given any inoculums showed high temperature after 21 days (107-108 °F). The mycoplasma was recovered.

Clinical signs

Disease was observed in four dogs with defined clinical manifestations viz., fever, nasal secretion, coughing, dullness and anorexia.

Pathomorphological examination

a-Gross observation-

Immediately after the sacrifice, the lungs were examined. The borders of apical lobs of both lungs were highly congested. Hemorrhagic patches were also

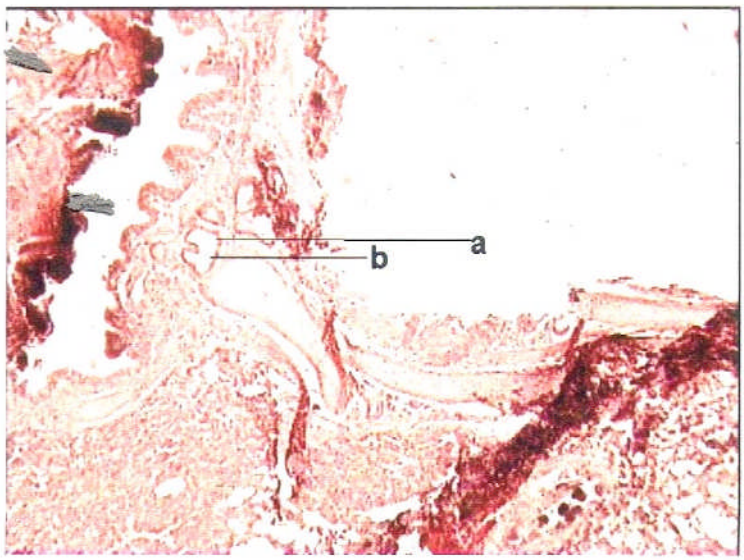

Fig. no. 7

a- Elongation of glandular cells,
b- dilation of bronchial glands

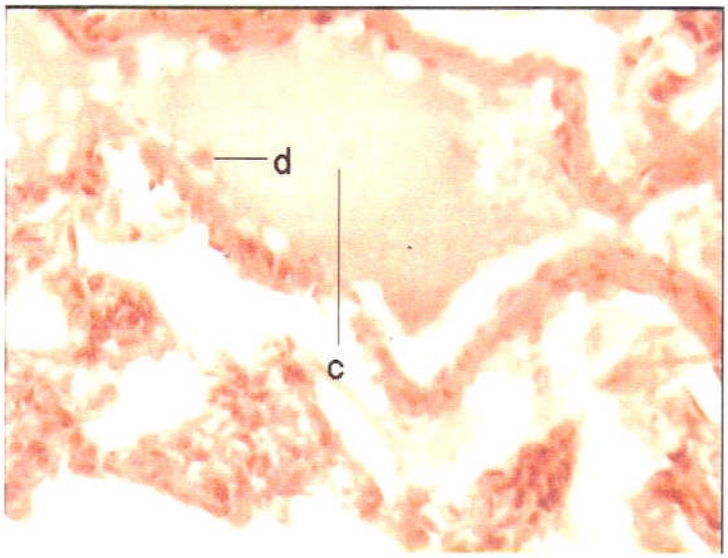

Fig. no. 8

c- Exudates filled in blood vessels,
d- Fibrin in distorted blood Vessels which containing mononuclear cells

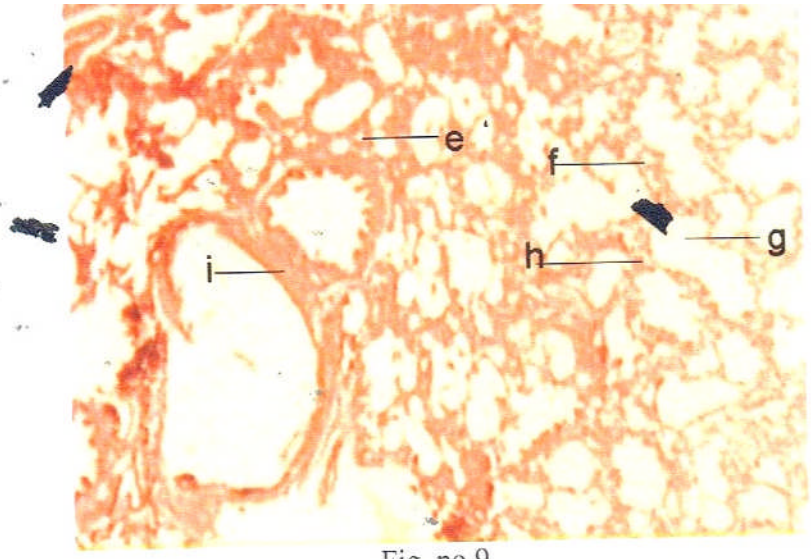

Fig. no.9

e- Cellular aggregation around tertiary bronchiole, f & g- inter alveolar septae and alveolar ducts become thickened (fibro cellular),

h- Infiltrated by lymphocyte, monocyte and neutrophils, i- Tertiary bronchiole, respiratory bronchiole, alveolar ducts and blood vessels were surrounded by cellular and fibrous

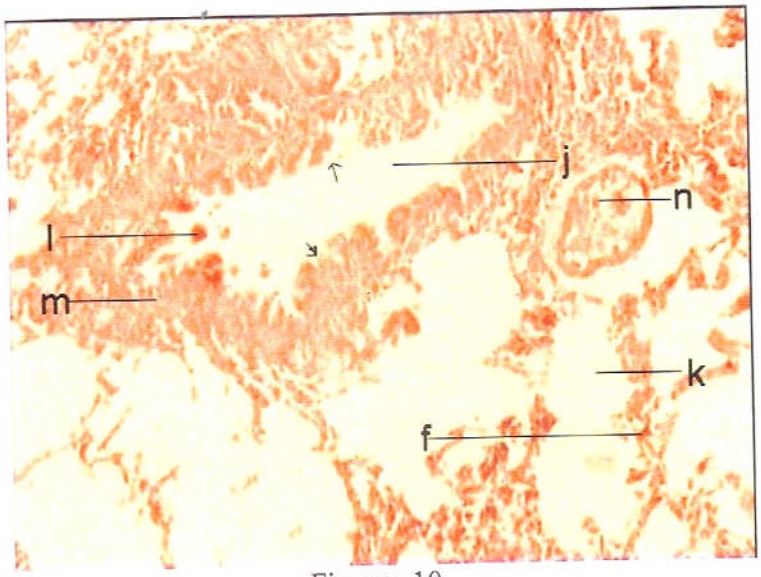

Fig. no. 10

J & k- epithelium of respiratory bronchiole and alveolar duct was denuded

l- Mononuclear cells were observed in the lumen of respiratory bronchiole

m-The fibrosis of lamina propria of respiratory bronchiole was also recorded, n- Fibrin in distirted blood vessels which containing mononuclear cells

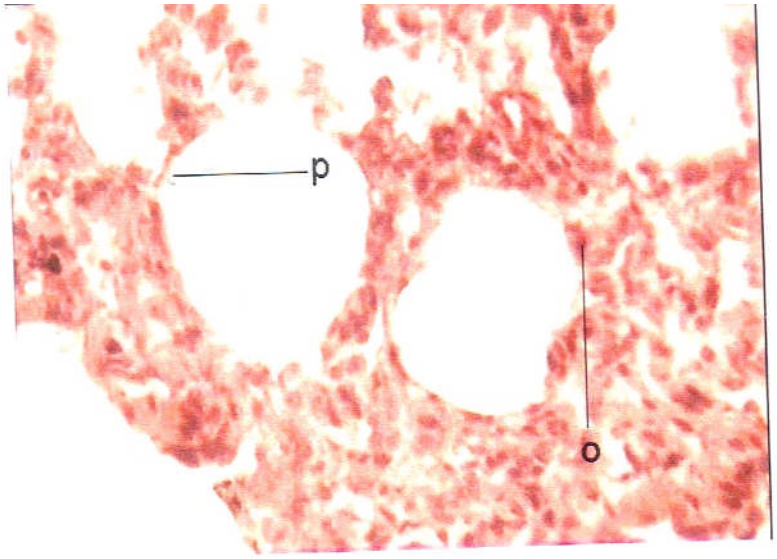

Fig. no. 11

o- The epithelium of pulmonary alveoli became thickened

p- Epithelium was covered by mucoid layer

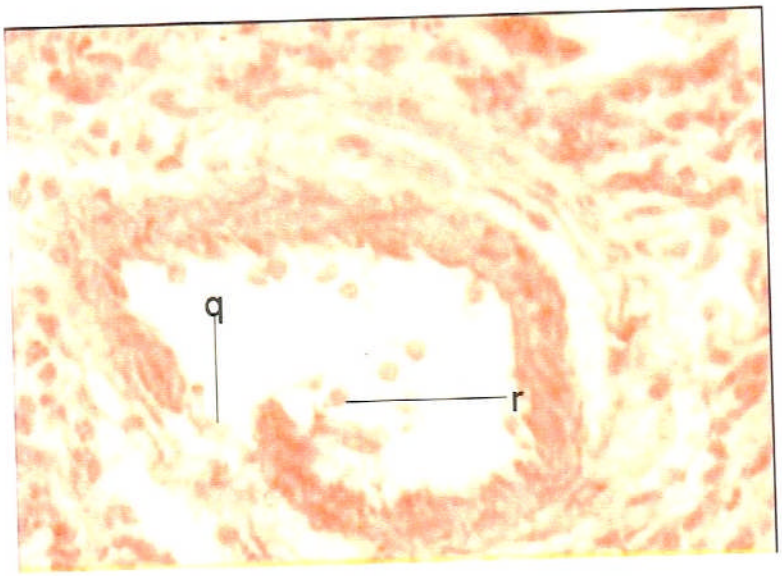

Fig. no. 12

Q & r- epithelium of respiratory bronchiole and alveolar duct was denuded and wherever present it was inflamed and heavily infiltrated with chronic inflammatory cells

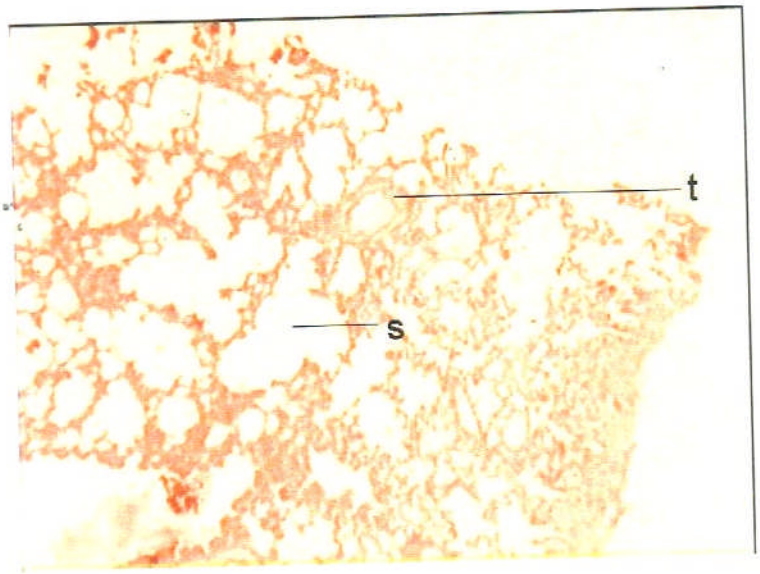

Fig. no. 13

s- Alveolar duct, sacs and alveoli were highly enlarged throughout the lung (Emphysema)

t- Epithelium of the secondary and tertiary bronchiole was inflamed the suprenuclear zone was enlonged and contained highly

noticed all over the lungs. Lungs showed mild to moderate degree of consolidation. (Fig. 6)

b-Histopathology

Histopathological Changes were not marked in secondary bronchus. However, Elongation of their glandular cells indicated enhanced secretary activity. The dilatations of bronchial glands were suggestive of apocrine type of secretion. (Fig. 7).

Change started in lower respiratory tract they are as fallows: the cellular aggregation around the tertiary bronchiole was observed.The Inter alveolar septae and alveolar ducts became thickened (fibro cellular). Mostly they were infiltrated by Lymphocytes, monocytes and neutrophils. Tertiary bronchiole, respiratory bronchiole, alveolar ducts were surrounded by large amount of cellular fibrous aggregation (Fig. 9).

Fibrous aggregation mostly contained collagenous fibrous. Blood vessels were also encircled by fibrous aggregation. At most of the place the epithelium of respiratory bronchiole, alveolar ducts was denuded and wherever presents it was inflamed, fibrous and heavily infiltrated with chronic inflammatory cells (Fig. 12). Intra epithelial mononuclear cells were observed in the lumen of respiratory bronchiole. The fibrosis of lamina propria of respiratory bronchiole was also recorded. Exudates filled blood vessels were noticed. The fibrin in distorted blood vessels was also observed which also contained mononuclear cells (Fig. 8 & 10).

The epithelium of pulmonary alveoli which is normally thin and difficultly observed became thickened. The distorted blood vessels contained fibro prudent material containing mononuclear cells, deformed erythrocytes and fine fibers of fibrin suggestive of intravascular coagulation. Dilation of blood vessels, interalveolar septum, alveolar ducts contained mononuclear cells. Intra alveolar septa become thickened while Alveolar duct, sacs and alveoli were highly enlarged throughout the

lung (Emphysema) (Fig. 13). Inter alveolar septa become thickened with abundance of mononuclear cells. Fibrin was also noticed in the lumen of blood vessels. The lumen of the blood vessels contained eosinophilic mass having few mononuclear cells. in the lumen of these vessels plasma cells within the wall were also noticed. Epithelium of the secondary and tertiary bronchiole was inflammed the suprenuclear zone was enlongated and contained highly eosiophilic material. At places epithelium was covered by mucoid layer. (Fig. 11).

Discussion

Discussion

No research work is reported on the recovery of canine mycoplasmas in the ocular, genital and respiratory tract of dogs in India. Indian conditions the lack of literature on this topic is due to inherent difficulties in isolation and propagation of mycoplasmas which are highly fastidious in nature. Moreover, their was no published work until on their isolation and characterization.

Number of recipes of media reported by different workers (Rosendal, 1975, Randolph ***et al.,*** 1993, Chalker ***et al***.2004) were tried but those given in the material and methods gave good results in present research work. The colonial appearance of certain mycoplasma species especially those that were several days old can be visualized by the naked eye for observing fried egg appearance. However microscopic examination is still required to accurately confirm typical mycoplasma fried-egg colonial morphology.

During present work it was observed that colonial morphology was of little use in the distinction of canine mycoplasma species. The majority of canine mycoplasmas identical to fried-egg like colonial morphology were seen within individual species of canine mycoplasma and assumptive identification should not be based upon colonial morphology alone .Mixed infection were extremely common and can not be determined by colonial morphology due to reversion which was a characteristic of L- forms bacteria. Therefore, it is good practice to identify several colonies from the same sample with increasing the use of labor for identification.

Traditionally, the identification of canine mycoplasma was confirmed by serological methods that were dependant upon specific antisera to each individual species (Rosendal, 1975). He used different tests viz. growth inhibition, immunofluorescence for differentiation canine mycoplasma species successfully (Rosendal, 1975). But in present study only growth inhibition test was used due to time bound programme of the post graduate study. The cross reactions were observed as observed by Rosendal, 1975 in growth inhibition test.

Due to non availability of some species standard antisera, it was possible to characterize some canine mycoplasmas by their biochemical profiles using glucose fermentation, arginine hydrolysis and phosphatase production, film and spot formation and tetrazolium chloride which reduces the range of antisera needed for identification (Rosendal, 1974a). No doubt, growth of the organism increases the identification time and cost on using biochemical tests.

In present work two mycoplasma isolates could not be identified on bio chemical and growth inhibition and remained as unidentified such mycoplasmas may be or may not be associated with a important role in clinical disease as reported by Binder ***et al.,*** 1986; Kirchner ***et al.,*** 1990; Randolph ***et al.,*** 1993; and Jameson ***et al.,*** 1995. Thus, present findings were similar to the workers as reported earlier.

Many workers (Bowe ***et al.,*** 1982; Rosendal, 1973b; Rosendal and Laber, 1973; Jang ***et al.,*** 1984; Barton ***et al.,*** 1985; Chalker ***et al.***, 2004) reported difficulties in identifying canine mycoplasma species and the majority of studies have investigated the presence or absence of mycoplasmas in a variety of clinical samples .Similarly, In the present study, a total of 141 samples (82 genital swabs, 41 nasal swabs and 18 ocular swabs) were collected from different locations. Out of these samples, Mycoplasma sps. could be isolated from 7 samples with 4.96%. Of six isolates were from the genital tract of male and female dogs with 7.32%.The percentage 2.44 % and 4.88%were harbored by male and female genital tract of dogs. It was observed that female genital tract was more prone for mycoplasma infections.

During present study ***M. canis*** has been isolated from the German Shepard bitch with clinical history of infertility. It was reported by Rosendal, 1982 that ***M. canis*** produced chronic urethritis, and endometritis. ***M. canis*** is also linked to genital tract infections and infertility (Ayling ***et al.,*** 2004; L'Abee-Lund ***et al.,*** 2003). Doige ***et al.*** (1981) have also isolated several species of mycoplasma from the vagina/prepuce from infertility in male dogs. Similarly in present work ***M.arginin and M.gateae*** were also reported from the genital tract. However conclusive evidence is lacking due to presence of unusual mycoplasma species (***M. gateae***).

Rosendal, 1982; Randolph et al 1993 reported that all dogs have mycoplasmas in the upper respiratory tract where in they are thought to form part of the normal bacterial flora. They observed 20-25% of healthy dogs harbour mycoplasma in the trachea and lungs (Randolph ***et al.,*** 1993). The lungs of dogs are colonized by mycoplasma during pneumonia (Rosendal, 1982) and mycoplasma have been isolated from 78% of younger dogs (<1 yr) with pulmonary disease (Randolph ***et al.,*** 1993). Randolph ***et al.***(1993) also found that young dogs with concurrent infection with bordetella or streptococci in the lower airway were more likely to be infected with mycoplasma, and that the presence of mycoplasma was associated with septic inflammation.

Speculation as to the role of mycoplasmas as primary or secondary pathogens in canine pulmonary disease continues despite several studies describing the isolation of mycoplasmas in pure culture from respiratory specimens (Randolph ***et al*** 1993; Candler and Lappin, 2002).

During present work of 41 nasal swab a single mycoplasma isolate (2.44%) was recovered and was identified as ***M. gateae.*** It was from the male dog with clinical history of pneumonia. Though the isolates were recovered but some time due to electric failure most of the isolates lost during maintenance. The presence of ***M. gateae*** in respiratory tract is in confirmation the findings reported earlier.

All the canine mycoplasma isolates were sensitive to digitonin this confirms that these were Mycoplasmas not Acholeplasmas. In present study, the prevalence of canine mycoplasmosis is 4.96%. In similar studies Kirchhoff (1973), Koshimizu & Ogata (1974), Eberle & Kirchhoff (1978), Randolph *et al.* (1993), Jameson ***et al.*** (1995) showed similar results. Out of seven isolates, one was ***M. canis*** (CG1), two were ***M. arginini*** (CG2 & CG4), two were ***M. gateae*** (CD_2 & CG_5), and two (CG_3 & CD_1) was unidentified.

No mycoplasma recovered from ocular samples collected from male and females dogs indicating as they are difficult to culture, making interpretation of the positive result difficult.

Experimental infection with ***M. canis***, ***M. gateae*** and ***M. spumans*** failed to reproduce respiratory disease in dogs (Rosendal, 1978) and the role played by other mycoplasma species in pulmonary disease remaining to be established.

The purpose of the study reported here was to determine the recovery of mycoplasmas dogs with or without pulmonary disease. In addition, the prevalence of mycoplasmal in isolation from nasal cavity of dogs with or without pulmonary disease was reevaluated.

Antibiotic sensitivity test was performed on all the isolates by using Tylosin, Erythromycin, spiromycin, ciprofloxacin, oxytetracyclin and enrofloxacin. All the isolates were sensitive to tylosin, spiromycin and enrofloxacin while they were resistant to erythromycin, ciprofloxacin, and oxytetracycline.

The pathogencity of ***M. canis*** (CG_1) was tested by inoculation in three experimental pups via different route viz., oral, nasal and intramuscular. The respiratory symptoms like coughing, nasal secretions, high temperature were observed in first dog which was given infection by I/M route (within 8 days) followed by dog with nasal route (13 days) and dog with oral route (17 day). The possible reason for early appearance of symptoms in the dog which was given infection through I/M route may be that on I/M inoculation the infection reached the blood earlier in comparison to other route like nasal and oral as reported by Campbell & Okuda (1975), Eberle ***et al.***, (1977), Rosendal & Vinther (1977), Rosendal (1978).

Histopathological were seen with massive infiltration of lymphocytes and macrophages around the bronchioles, alveoli and blood vessels. There was development of lymphoid hyperplacia in the peribronchial and perivascular areas. Brochial epithelium showed hyperplasia with lymphocytic and macrophages infilteration. Alveoli showed coupious serocellular exudates consisting primarily of desquamated epithelial cells, mononuclear cell infilteration. Alveolar duct, sacs and alveoli were highly enlarged throughout the lung (Emphysema). Inter alveolar septa become thickened with abundance of mononuclear cells. Fibrin was also noticed in the lumen of blood vessels. The lumen of the blood vessels contained eosinophilic mass having few mononuclear cells. in the lumen of these vessels plasma cells within the wall was by Rosendal (1978).

There is a need for critical assessment of pathogenicity and pathogenesis of reported canine mycoplasma species in this study. This study on the use of pups for pathogenecity with M. canis need similar studies to be extended to other isolates.

The study on the presence of canine mycoplasmas not yet known and those reported for the first time in this study from genital and respiratory tract of dogs have not only added to the existing knowledge but the experimental work under taken employing a new technique have opened new dimensions for further investigation of canine mycoplasma infection in the prevailing Indian conditions.

Summary

SUMMARY

During the present study, a total of 141 samples were collected from domestic dogs (89) and street dogs (52). The samples include nasal, ocular and vaginal swabs.

A total of 7 (4.96%) mycoplasma isolates were recovered from 41 nasal swabs (domestic dogs (20) and street dogs (21)), 82 genital (domestic dogs (48) and street dogs (34)) and 18 oculars domestic dogs (11) and street dogs (7) with clinical history of respiratory distress, healthy, arthritis suspected, infertile, pneumonia, parvovirus suspected, fever and digestive problem, abortion and paralysis suspected. Mycoplasma organisms were isolated from 1 nasal swabs, out of 41 (2.45%) nasal swabs collected from healthy and diseased dogs. Six mycoplasma isolates could be recovered from 82 (7.32%) genital samples from healthy and diseased dogs. No mycoplasma were isolated from 18 ocular samples.

All mycoplasma isolates were characterized by using morphological, cultural and biochemical tests. The digitonin sensitivity test was performed for differentiation of mycoplasmas and acholeplasmas. To minimize the use of standard antiserum, biochemical tests were used viz., glucose and mannose catabolism, arginine hydrolysis, phosphatase production, formation of film and spot, tetrazolium chloride and hemadsorption.

In this study 7 mycoplasmas were identified as ***Mycoplasma canis*** (1), ***Mycoplasma gateae*** (2), ***Mycoplasma arginine*** (2) and two samples remained unidentified for serological testing whole cell antigen (WC) was prepared. Growth inhibition test was performed by using homologous antiserum.

These isolates of canine mycoplasmas were tested against six antibiotics, erythromycin, spiramycin, sparfloxacin, tylosin, oxytetracycline and enrofloxacin. Tylosin, enrofloxacin and spiramycin were found to be sensitive for all these ***Mycoplasma*** isolates above antibiotics.

In the present study, isolate (***M. canis***) was inoculated in three experimental dogs via different route viz., oral, nasal and intramuscular. The respiratory symptoms like coughing, nasal secretions, high temperature were observed.

One pup was sacrificed for histopathological examinations. On gross examination, lungs showed congestion and mild to moderate consolidation.

Histopathological changes include massive infilteration of lymphocytes and macrophages around the bronchioles, alveoli and blood vessels.

There was development of lymphoid hyperplacia in the peribronchial and perivascular areas. Brochial epithelium showed hyperplasia with lymphocytic and infilteration macrophages. Alveoli showed coupious serocellular exudates consisting primarily of desquamated epithelial cells, mononuclear cell infilteration. Alveolar duct, sacs and alveoli were highly enlarged throughout the lung (Emphysema). Inter alveolar septa become thickened with abundance of mononuclear cells. Fibrin was also noticed in the lumen of blood vessels. The lumen of the blood vessels contained eosinophilic mass having few mononuclear cells. in the lumen of these vessels plasma cells within the wall was also noticed.

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APPENDIX

1. Mycoplasma broth:

PPLO broth broth (Difco)	70.0 ml
Sterilized by autoclaving	
Horse serum (unheated)	20.0 ml
Yeast extract (25%), (Taylor-Robinson *et al.*, 1963)	10.0 ml
Thallium acetate (1%) (w/v) solution)	1.0 ml
Deoxyribonucleic acid sodium salt (from calf Thymus	1.2 ml
Sigma 0.2 %(w/v solution)	
Penicillin (1000 IU/ml)	1.0 ml
pH 7.8	

2. Mycoplasma Agar:

PPLO agar (Difco)	70.0 ml
Sterilized by autoclaving	
Horse serum (unheated)	20.0 ml
Yeast extract (25%), (Taylor-Robinson *et al.*, 1963)	10.0 ml
Thallium acetate (1%) (w/v) solution)	1.0 ml
Deoxyribonucleic acid (from calf Thymus,	1.2 ml
Sigma 0.2 %(w/v solution)	
Phenol red (0.06 % (w/v) solution)	5.0 ml
Penicillin (1000 IU/ml)	1.0ml
pH 7.8	

3. Medium for biochemical tests

(a). Glucose fermentation

PPLO broth (Difco)	70.0 ml
Sterilized by autoclaving	
Horse serum (unheated)	20.0 ml
Yeast extract (25%), (Taylor-Robinson *et al.*, 1963)	10.0 ml

Glucose (50% (w/v) solution)	1.0 ml
Phenol red (1% (w/v) solution)	0.4 ml
Thallium acetate (10%) (w/v) solution)	1.0 ml
Penicillin (1000 IU/ml)	1.0 ml
pH7.8	

(b). Hydrolysis of arginine

PPLO broth (Difco)	70.0 ml
Sterilized by autoclaving	
Horse serum (Inactivated)	20.0 ml
Yeast extract (25%), (Taylor-Robinson *et al.*, 1963)	10.0 ml
L-arginine Hcl 10% solution	5.0 ml
Phenol red (1% (w/v) solution)	0.4 ml
Thallium acetate (10%) (w/v) solution)	1.0 ml
Penicillin (1000 IU/ml)	1.0 ml
PH 6.8	

(c). Phosphatase test:

PPLO broth (Difco)	70.0 ml
Sterilize by autoclaving	
Horse serum (inactivated)	20.0 ml
Yeast extract (25%) (Taylor-Robinson *et al.*, 1963)	10.0 ml
Sodium phenolphthalein diphosphate, (1% w/v solution)	1.0 ml
Thallium acetate (1% (w/v) solution)	0.8 ml
Penicillin (1000 IU/ml)	0.2 ml
pH 7.8	

(d). Triphenyl tetrazolium chloride:

Rabbit meat infusion	100.0 ml
Rabbit serum	10.0 ml
Yeast extract (Taylor and Robinson *et al.*)	10.0 ml
Deoxyribonucleic acid (Sigma 0.2 %(w/v) solution)	1.2 ml

2,3,5, triphenyl-tetrazolium chloride (merck)	5.0 ml
(1 %(w/v) solution)	
Thallium acetate (10 %(w/v) solution)	1.0 ml
Penicillin (1000 IU per ml)	1.0 ml
pH 7.8	

(e). Film and spot formation test (Medium By):

PPLO agar (Difco)	90.0 ml
Sterilize by autoclaving	
Horse serum	20.0 ml
Egg Yolk emulsion	13.6 ml
Yeast extract (25%) (Taylor-Robinson et al., 1963)	10.0 ml
Deoxyribonucleic acid (Sigma 0.2 %(w/v) solution)	1.2 ml
Thallium acetate (10% (w/v) solution)	1.0 ml
Penicillin (1000 IU/ml)	1.0 ml
pH 7.8	

Diene's stain:

Methylene blue	2.5g
Azore II	2.25g
Maltose	10g
Sodium carbonate	0.25g
Benzoic acid	0.20g
Distilled water up to	100 ml
Used 1:5 dilution of distilled water for staining	

Acridine Orange Stain:

Sol. A. Citrate Phosphate Buffer pH 3.8

Sol. B. Stock solution (0.1%)

Acridine Orange R	100 mg

Distilled water	100 ml

Sol. A. & Sol. B. prepares fresh and store at 4°C

1 ml of Stock acridine orange solution diluted in 9 ml citrate phosphate buffer and used as a working solution.

Giemsa Stain:

Stock sol.

Gurr's Giemsa Stain Powder	3.8 gm
Glycerine	250 ml
Methanol	250 ml

Working sol.

1ml of Giemsa stain solution mixed with 9 ml of buffer saline.