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EVALUATION OF *M. AGALACTIAE* EXPERIMENTAL INFECTION ON THE GENITAL TRACT AND MAMMARY GLAND OF GOATS USING THE HISTOPATHOLOGICAL EXAMINATION AND ULTRASONOGRAPHY

(With 2 Tables, 12 Figures and 2 Images)

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تقييم أثر العدوى التجريبية بالميكوبلازما أجالاكتيا على القناة التناسلية والضرع فى الماعز باستخدام الفحص الهستوباثولوجى والموجات فوق الصوتية

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للوقوف على قدرة الميكوبلازما أجالاكتيا فى احداث الاصابة فى القناة التناسلية والضرع فى الماعز، فقد خطط البحث لإحداث عدوى تجريبية وذلك بحقن ميكروب الميكوبلازما أجالاكتيا فى مهبل عدد ثلاثة من الماعز وضرع اثنين منهم بينما تم استخدام حيوان واحد كضابط للتجربه. تم ملاحظة الأعراض المرضية الناتجة ومتابعة حدوث التغيرات التى نشأت فى القناة التناسلية بواسطة استخدام الموجات فوق الصوتية. بعد الذبح تم فحص التغيرات الباثولوجية فى مختلف أنسجة الماعز المحقونه بالميكروب. كذلك فقد تم زرع عينات من المسحات المهبلية وعينات الأنسجة واللبن لإعادة عزل الميكوبلازما المحقونة خلال فترة التجربة. كما تم تقييم الأستجابه السيرولوجية باستخدام اختبار التلزن الدموى الغير مباشرعلى سيرم الدم للماعز المختبرة. بالإضافة الى ذلك فقد تم استخدام اختبار الأميونوفلورسنت الغير مباشر لأستبيان الميكروب فى أنسجة المهبل والضرع والغدد الليمفاوية فوق الضرع. كذلك تم الفحص الميكروسكوبى لأفلام من عينات اللبن للحيوانات المحقونة من خلال قناة الضرع وذلك بعد صبغها بصبغة الليشمان. وقد لوحظ حدوث إحتقان وارتشاحات فى الغشاء المخاطى الدبطن للحيا مع وجود افرازات مخاطية تحولت الى مخاطية صديدية. كذلك فقد ظهرت درجات متفاوتة من الألتهاب فى الأنصاف المحقونة للضرع. وقد تم اعادة عزل ميكروب الميكوبلازما أجالاكتيا من افرازات المهبل وكذلك من عينات اللبن بعد ٢-٣ أيام من الحقن . كذلك تم عزل الميكروب من أنسجة المهبل و عنق الرحم والرحم والرئة والطحال وكذلك أنسجة الضرع ومن الغدد الليمفاوية فوق الضرع المقابلة للأنصاف المحقونة من الضرع. كما تم استبيان تواجد الميكوبلازما أجالاكتيا فى أنسجة المهبل والضرع والغدد الليمفاوية فوق الضرع باستخدام اختبار الأميونوفلورسنت

الغـير مباشر. وقد أظهرالفحص السونوجرافى زيادة فى سمك جدار المهبل وعنق الرحم مع زيـــادة فـــى قطر كل منهم. كذلك لوحظ وجود افرازات فى المهبل وعنق الرحم وقناة عنق الـــرحم كانت مفتوحة. وأظهر الفحص الميكروسكوبى وجود التهاب فى أنسجة الحيا والمهبل وعـــنق الـــرحم والـــرحم وكذلك قناة فالوب للماعز المحقونة والذى تزداد شدته مع الوقت. وأظهـــر الفحص الميكروسكوبى لأنسجة أنصاف الضرع المحقونة وجود التهاب مزمن تميز بوجـــود عـــدد كبير من خلايا الليمفوسايت وخلايا البلازما وتجمع لخلايا الليمفوسايت يشبه الحويصلات الليمفاوية مع تليف بين فصيصات الضرع وحول الحويصلات المفرزه للبن ، كمـــا تبيـــن وجـــود كثرة عددية للحويصلات داخل الغدد الليمفاوية فوق الضرع والطحال. وبـــالفحص الميكروسكوبى لأفلام اللبن التى صبغت بصبغة الليشمان، تبين وجود عدد كبير من خلايا النيوتروفيل مقارنة بالعينات الضابطة.

SUMMARY

To assess the pathogenicity of *Mycoplasma agalactiae* on the genital tract and mammary gland of goats, experimental intravaginal and intramammary inoculation of the organism into the anterior vagina of 3 non-pregnant does and via the intra mammary route of 2 of them. One animal was kept as control. Clinical signs and ultrasonography findings were recorded. Pathological changes in the genital tract, udder tissue and the internal organs were recorded after sacrification of the inoculated goats. Cultural examination of the vaginal swabs, tissue specimens and milk samples for re-isolation of the inoculated *Mycoplasma* species was carried out. Also, evaluation of the serological response of the infected goats using the indirect haemagglutination (IHA) test at various stages of the experiment was done. Moreover, indirect immunofluorescent antibody technique (IFAT) to detect the causative agent in the fresh tissues of vagina, udder, spleen and supramammary lymph nodes was applied. Milk films from the intramammary inoculated does were microscopically examined. Congestion and development of edema in the vulvar mucosa of the infected does with moderate vaginal mucous discharge that changed to mucopurulent exudate were observed. Varying degrees of mastitis were developed in the inoculated udder halves. *M.agalactiae* was reisolated from the vaginal discharge and milk samples of the inoculated glands, 2-3 days PI. After slaughter, the organism was also re-isolated from the tissues of vagina, cervix, uterus, lungs and spleen as well as from the inoculated udder tissues and their corresponding supramammary lymph nodes. Moreover, the inoculated species was detected in udder parenchyma, supramammary lymph nodes and in the vaginal mucosa of infected animals using IFAT. Ultrasonogram showed thickening in the vaginal and cervical walls with

increase in their diameters. Non-echoic cervical and vaginal secretions with opened cervical canal was also observed. Microscopically, all the inoculated goats developed vulvitis, vaginitis, cervicitis, endometritis as well as salpingitis which increased in severity with increasing period after infection. The tissues of the inoculated udder halves revealed diffuse chronic mastitis characterized by moderate to severe infiltration of the interstitial tissue with lymphocytes and few plasma cells. Lymphoid follicles and extensive interlobular and periacinar fibrosis replaced many of the secretory acini of the inoculated udder. Also, hyperplasia of lymphoid tissues of the supramammary lymph nodes and the spleens was evident. Microscopic examination of milk films of the intramammary inoculated goats showed numerous polymorphnuclear leucocytes compared to the control.

Key words: *Mycoplasma, genital tract, mammary gland, ultrasonography histopathology, goats.*

INTRODUCTION

Mycoplasma-associted reproductive disorders in domestic animals lower the reproductive efficiency of both females and males (Rana *et al.*, 1993). Ovine vulvovaginitis due to *Mycoplasma* and *Acholeplasma* was recorded by Cottew *et al.*, 1974; Cottew, 1979 and Tiwana *et al.*, 1984. Singh *et al.*, 1974& 1975 and Chima *et al.*, 1982 were experimentally produced granular vulvovaginitis (GVV) in female goats by topical application of *M. agalactiae* on scarified vulvar mucosa. They stated that the organism could be re-isolated from all infected goats accompanied by mucopurulent exudate from the vagina which clearly implicates *M.agalactiae* as a cause of GVV in sheep and goat. Cottew *et al.* (1974) and Livingston and Gauer (1983) isolated *Mycoplasma 2-D* from the vaginal swabs of ewes with vulvovaginitis and reproductive problems. They stated that the isolated strain possessed similar biochemical characteristics to *M. agalactiae.* Singh *et al.* (1974) experimentally reproduce granular vulvo-vaginitis in ewes by applying a culture of *M. agalactiae* to scarified mucous membrane of the vulva. They stated that, gross lesions of GVV were observed in 80.33% of the experimentally infected ewes at 11 up to 34 days after inoculation. *M. agalactiae* was reisolated from the infected group starting from 7 days up to 70 days after inoculation.

M. agalactiae, commonly known as the cause of "contagious agalactia of sheep and goats", is one of the most devastating mycoplasmal pathogens DaMassa (1983). Ojo and Ikede (1976) produced clinical mastitis in three goats following inoculation into the mammary gland with *M. agalactiae*. The infection was characterized by pyrexia, acute purulent inflammation of the lactiferous sinus and ducts, necrosis of the duct epithelium and proliferation of chronic inflammatory cells in the parenchyma. Kinde *et al.* (1994) reported sudden onset of clinical mastitis, arthritis, and sudden death in does of a commercial dairy goat herd. They isolated *M. agalactiae* and *M. mycoides subsp. mycoides* LC. Fifteen percent of does were died or culled because of mastitis. Gross necropsy of affected does, showed purulent discharge from udder, enlarged supramammary lymph nodes, firm spleens and swollen livers. Jones (1985) concluded that the goats are the better experimental animal for screening virulence of *Mycoplasma* species.

Ultrasound evaluation of small ruminants offers an unparalleled range of diagnostic information (Kahn, 1992). Haibel (1990) used the real–time ultrasonography for the diagnosis of pyometra, hydrometra and fetal mummification in sheep and goat and he stated that, the advantages of real–time ultrasonography are its sensitivity, speed, safety and accuracy of various diagnosis.

From available literature it is evident that GVV was previously experimentally produced in goats by topical application of *M. agalactiae* culture on the scarified vaginal mucosa by Singh *et al.* (1974&1975) and Rana *et al.*(1993), but there is a paucity of information about a systemic approach to the etio-pathological studies of genital tract inflammation resulted from *M. agalactiae* inoculation. So, to determine the possible association of *Mycoplasma agalactiae* with the genital tract infection and mastitis, experimental intravaginal and intramammary inoculation of the organism with continuous observation of the clinical findings and the developed inflammatory changes in the reproductive tract using ultrasound waves were performed. Also, detection of the sequential histopathological changes of the genital tract, udder and the internal organs of the inoculated goats after scarification was studied. Cultural examination of the vaginal swabs and milk samples for re-isolation of the inoculated *Mycoplasma* species and evaluation of the serological responses of the infected goat during various stages of the experiment were carried out. Also, immunofluorescent antibody

technique (IFAT) was applied to detect the causative *Mycoplasma* species responsible for the lesion, in the fresh frozen tissues.

MATERIALS and METHODS

Experimental animals:

For experimental induction of genital tract infection and mastitis, four healthy, adult non-pregnant female goats aged 2-3 years were obtained and kept under observation for any signs of illness for about 2 weeks preceding the start of the experiment. Vaginal swabs and milk samples were cultured for mycoplasma examination. During observation, neither gross visible vulvar/vaginal lesions were noticed nor any mycoplasma organism could be isolated from vaginal swabs.

Preparation of the inoculum:

The inoculated culture was prepared from *M. agalactiae* (PG 2) reference strain which was kindly obtained from Dr. Renate Rosengarten (head of the institute of bacteriology, mycology and hygiene, university of veterinary medicine), Vienna, Austria. The organism was cultured and propagated in heart infusion (HN) broth which prepared according to Freundt (1983). Thallium acetate was omitted from media used for inoculation as described by DaMassa *et al.* (1986). Serial dilutions of forty-eight hours old culture of *M. agalactiae* were streaked onto agar plates and after 48-72 hours were subjected to colony counts according to Rodwell and Whitecomb (1983). The organisms were pooled and standardized to approximately $10^6 - 10^7$ colony forming units (CFU) /ml.

Experimental design:

A- Intra-vaginal inoculation:

Three goats (No. 1, 2 and 3) were inoculated intravaginally from which two were subjected also to intramammary inoculation. Five ml of 48 hours culture of *M.agalactiae*, containing approximately $10^6 - 10^7$ CFU/ml was inoculated into the vaginal canal of the 3 does using a sterile catheter and the does were retained for 5 minutes by tight holding of the vulvar lips. The inoculum was deposited into the anterior portion of the vagina and the experiment was continued for 28 days post-inoculation (PI). The control animal (one goat) was handled similarly with 5 ml of sterile heart infusion (HN) broth.

B-Intramammary inoculation:

The udder of goats No. 2&3 were thoroughly cleaned with soap and water, dried and the teats were disinfected with 70% ethanol. The left half of each udder in both goats was inoculated via the teat canal by

2 ml of *M. agalactiae* broth culture with 10^6 -10^7 CFU/ml as described by Rana *et al.* (1992). Similarly, the right half of each udder of these two goats was inoculated aseptically via the teat canal with 2ml of sterile HN broth and served as control.

Post-inoculation monitoring and samples collection:

The inoculated does were examined daily for clinical signs, development of gross lesions, vaginal discharges, abnormal changes in the mammary glands and for any changes in the milk character. Daily rectal temperature was recorded and the mammary glands were palpated. Vaginal swabs and milk samples were collected daily during the early stages of infection (the first 5 days) and three times a week following the establishment of infection, for mycoplasma examination and colony counts. Milk films were prepared from a loopful of milk samples (7, 12, 17 and 22 DPI) and stained with Leishman's stain according to Dacie and Lewis (1975). Blood samples were also collected each five days for serum separation to evaluate the serological response of the inoculated goats using the indirect haemagglutination test (IHA) according to Cho *et al.* (1976).

Ultrasound examination:

Ultrasound examination was performed as described by Inskeep (1993) and Abo El-Maaty (2001). All does were examined by transrectal ultrasonography before inoculation. Post intravaginal inoculation ultrasonography was performed each five days per rectum and the findings were recorded. Does were examined by ultrasound scanner 200 (Pie Medical, Nertherlands), equipped with two transducer, the first one is 7.5 MHz real time B- mode linear array endorectal transducers and the second was applied transabdominally (3.5-5 MHz) which modified to be used in goat and sheep to visualize both ovaries and uterus endorectally. The scanner is equipped with electronic calipers that measure distance, length and area. Deoxymethyl cellulose gel was used to lubricate the transducer before insertion into the rectum of the animal, while another amount was injected inside the rectum to avoid air bubbles. Does were scanned in a dorsal recumbence position and the urinary bladder and uterus were used as a guide.

Postmortem examination and sampling:

The three inoculated does (Nos. 1, 2 and 3) were sacrificed, on days 15, 21 and 28 PI, respectively. The control doe was sacrificed 28 days PI. Gross examination of the genital tract, udder and viscera was carried out at the time of sacrification and the macroscopical lesions were recorded. At necropsy, representative tissue samples of each organ

(vagina, uterus, oviducts, lungs, spleen, mammary gland and supramammary lymph nodes) of all sacrificed does were collected aseptically for mycoplasma cultural examination. Also, for histopathological examination, tissue specimens from each organ were collected and fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4-5µm, stained with hematoxylin and eosin (H&E) and examined by light microscopy (Bancroft and Gamble, 2002). For immunofluorescent technique, representative sample of each organ was frozen and sectioned by the cryostat.

Antigen preparation: according to Senterfit (1983).

Preparation of hyperimmune rabbit sera: The hyperimmune sera against *M. agalactiae* were produced in rabbits according to the method described by Senterfit (1983).

Indirect haemagglutination (IHA) test:

For the detection of specific antibodies against the inoculated *Mycoplasma* species, the serum samples of goats were subjected to IHA test as described by Cho *et al.* (1976). The results were interpreted considering 1/160 as a positive titre, 1/80 as a suspicious and 1/40 as a negative.

Indirect immunofluorescent Technique (IFT): The frozen part of each organ was sectioned at 8 µm thickness in the cryostat. Hyperimmune rabbit serum raised against *M. agalactiae* was applied to acetone-fixed tissue sections, then FITC conjugate (anti-rabbit IgG, whole molecule, antibody developed in goat, Sigma) was applied according to Stulle *et al.* (1988). The slides were examined using Carl-Zeiss fluorescent microscope.

RESULTS

Clinical signs:

Two days post intra–vaginal (I.V) and intramammary (I.M) inoculation of *M.agalactiae*, the rectal temperature of the inoculated does (No. 2&3) rose up and ranged between (40.3°C - 41.6°C) and remained high up to 6 DPI (Table 1). On the other hand, the body temperature of the goat which inoculated intra vaginally only (No.1) remained within normal range (39.0 - 39.5°C) during the experiment.

Edema of the vulvar lips and congestion of the vulvar mucosa of the three inoculated goats was recognized at the 4th, 5th and 7th DPI and continued during the period of the study. Moderate mucus discharge was observed from the vulva of does No. 1&3 at the 3rd up to 7th DPI, which changed to mucopurulent vaginal exudate up to slaughter. Mucopurulent

exudate was observed from the vulva of does No. 2 at the 4th up to 15th DPI which progressed to creamy vaginal discharge tinged blood up to slaughter (Table 1). The genitalia and the vital organs of the control animal didn't show any gross lesion with normal vaginal mucosa and scanty clear mucus on the surface of the vagina.

The inoculated udder halves of does No. 2&3 developed varying degrees of mastitis and became hot, swollen and painful during the first 5 days PI, as compared to the control halves (Right). Ten days later (13-15 DPI), the inoculated halves became smaller and by 18 DPI they were firm in consistency and markedly reduced in size (Table 1).

The mastitic milk progressed to yellowish, slightly flocculent then thick during the first 5 DPI. Thereafter it became watery secretion with occasional clots at the 6th - 9th DPI. Then the secretion changed to yellowish supernatant serous fluid and coarse granular sediment up to the 15th DPI, as the milk proteins precipitated as aggregates leaving a why–like supernatant fluid. Then the milk changed to thick, turbid, purulent exudation tinged blood up to 21DPI. Cessation of milk flow of goat No. 3 occurred 23 DPI (Table 1).

Mycoplasma re-isolation:

M.agalactiae was re-isolated from the vaginal discharge of the experimentally inoculated does (No. 1,2&3) from the 2nd DPI till the end of the experiment. The number of the re-isolated colony forming units of the organism ranged from 10^2 up to 10^7 CFU/ml and differed according to the stage of infection and from one animal to the other (Table 2).

Two days post inoculation, *M. agalactiae* was re-isolated with $10^3 - 10^5$ CFU/ml, from the milk samples of the inoculated udder halves of does No. 2&3. Generally, 7 up to 15 DPI the CFU rises up to 10^5 - 10^7CFU/ml, then it was declined to $10^3 - 10^4$ CFU/ml till slaughter (goat No. 2) or cessation of milk flow 23 DPI of doe No.3 (Table. 2). *Mycoplasma* couldn't be isolated from the milk samples of the doe No. 1.

After sacrification, *M.agalactiae* was re-isolated from the tissues of the vagina, cervix and uterus of the three inoculated goats. On the other hand the organism was also re-isolated from the tissues of the inoculated udder halves and their corresponding supramammary lymph nodes, from lungs and spleen of does No. 2&3 with $10^6 - 10^8$ CFU/ml.

Mycoplasma couldn't be isolated from the vaginal swabs or milk samples of goats at the pre-inoculation period. Also, *Mycoplasma* couldn't be isolated neither from milk samples of the right udder halves of does No.2&3 nor from the vaginal and milk samples of the control animal.

Serological responses:

The blood sera of goats No.2&3 showed an increase in the antibody titre ≥1/160 against *M. agalactiae* by mean of the indirect hacmagglutination (IHA) test starting at the 15th DPI (doe No.2) and 20 DPI (doe No.3) and continued up to 20 and 25 DPI, respectively (Table 2.). On the other hand, antibodies against *M. agalactiae* couldn't be detected neither in the serum of goat No. 1 nor in that of the control animal up to sacrification.

Table 1. Evaluation of the post inoculation clinical signs.

	Goat No. 1	Goat No. 2	Goat No.3	Control goat
Rectal temperature	(39.0 -39.5°C) Normal	Rose up to (40.5 - 41.2°C) 48h -4DPI	Rose up to (40.3 - 41.6°C) 24h- 6 DPI	(39.0 -39.3°C) Normal
The vagina & vaginal discharge	.Edema&congestion of the vulvar lips (7 DPI) .Moderate mucus discharge(3-5DPI) .Mucopurulent exudates 6 DPI up to slaughter (15DPI)	.Congestion of the vulvar mucosa (4DPI) .Mucopurulent exudate (4 - 15 DPI) .Creamy discharge tinged blood up to slaughter (21DPI)	.Congestion of the vulvar mucosa (5DPI) .Moderate mucus discharge (5 -7 DPI) .Mucopurulent exudate 8 DPI up to slaughter (28DPI)	.Normal vaginal mucosa .Scanty clear mucus
Inoculated left gland	Normal	.Hot, swollen and painful at 2-15DPI .Reduced in size and became firm till slaughter	.Hot, swollen and painful at 4 - 14DPI .Reduced in size and became firm till slaughter	Normal
Character of the milk	Normal	.Yellowish, turbid & flocculent secretion during the first 5 DPI .Watery secretion with occasional clots up to 9 DPI .Milk separated into supernatant serous fluid and a coarse granular sediment .Thick, turbid purulent exudation up to slaughter	.Yellowish, turbid & flocculent secretion during the first 5 DPI .Watery secretion with occasional clots up to 9 DPI .Milk separated into supernatant serous fluid and a coarse granular sediment . Thick, turbid purulent exudation tinged blood up to 16DPI .Cessation of milk flow 23DPI	Normal

Table 2: Reisolation of *M.agalactiae* from the vaginal swabs and milk samples of the inoculated does and from their tissues after sacrification correlated to their serological response measured by indirect haemagglutination (IHA) test.

Goat No.	Reisolation of *M.agalactiae* from			Serological test (IHA)
	Vaginal swabs	Milk	Variuos Tissue at sacrification	
Goat No.1	+ve at the 2nd 5th DPI (10^3-10^4CFU/ml) +ve at 6th- 12th DPI (10^5–10^6 CFU/ml) +ve at 13th DPI till slaughter (10^2-10^3CFU/ml)	-ve	Vagina, cervix &uterus	Serum antibody titer ≤1/40 at 10&15 DPI
Goat No. 2	+ve at 2- 4DPI (10^3 -10^4 CFU/ml) +ve at 6-15 DPI(10^5–10^7 CFU/ml) +ve at 16 DPI till slaughter (10^3-10^4CFU/ml)	+ve at 2 -6 DPI (10^3 - 10^4CFU/ml) +ve at 7-12 DPI (10^5 – 10^6 CFU/ml) +ve (10^3-10^4 CFU/ml) till slaughter	Vagina, cervix, uterus, mammary gland, supramammary LN and spleen	Serum antibody titre ≥1/160 at 15 &20 DPI
Goat No. 3	+ve at 2 -8 DPI (10^3 CFU/ml) +ve at 9-15DPI (10^4 -10^6 CFU/ml) +ve at 16 DPI till slaughter (10^2 - 10^3CFU/ml)	+ve at 2-9 DPI (10^5 CFU/ml) +ve at 10-15 DPI (10^5-10^7CFU/ml) +ve (10^3-10^4CFU/ml) till cessation of milk	Vagina, cervix, uterus, mammary gland, supramammaryLN, lungs and spleen	Serum antibody titre ≥1/160 at 20 & 25 DPI
Control goat	-ve	-ve	-ve	Serum antibody titer ≤1/40

Ultrasound examination:

Endorectal ultrasonography of does No. 1&2 and 3 indicated thick walled vagina, presence of vaginal secretion, and increase of the cervical diameter. Ultrasonogram of goat No.1, 10 DPI showed that, the

vagina appeared with thick wall and its diameter reached to (2.5cm) (Image 1B), compared to that of the control goat (1 cm). The cervical canal was opened with the presence of cervical secretion and the cervical mucosa appeared hyperechoic (Image 1A) which indicated cervicitis. Also, thickening and increase of the cervical diameter (2.4 cm) compared to the normal (1-1.5 cm) in cycling does. Ultrasonogram of goat No. 3 (20 DPI) revealed that the vagina was still containing secretions (Image 2B). The cervical canal was opened with hyperechoic cervical mucosa and anechoic cervical secretions (Image 2A).

Post-mortem macroscopical examination:

Gross examinations of the genital tract, udder and viscera were done at the time of sacrification. At necropsy, the mucosa of the vulva and posterior part of the vagina of the three inoculated does was severely congested and edematous. Moreover, the vaginal mucosa of the doe (No. 2) which sacrificed after 21 days PI showed variable mucopurulent exudates. On the other hand, no abnormality was detected in the vagina of the control doe.

The spleens of the two goats which inoculated by both routes (No. 2&3) were swollen and firm.

The inoculated left halves of the udder of goats (No. 2&3) were firm in consistency and contained thick yellowish secretions. The cut surfaces of the inoculated halves revealed brownish tissue with hemorrhagic spots at places as compared with the faint pink tissue of the cut surface of the right uninoculated control halves. Purulent exudate escaped from the cut surfaces of the left halves in both does. The left supramammary lymph nodes were enlarged with red grayish cut surface. On the other hand, the supramammary lymph nodes corresponding to the right halves and those of the intravaginally inoculated goat (No.1) and of the control animal didn't show any gross abnormalities.

Histopathological findings of the genital tract:

Microscopically, all the infected does showed lymphocytic vulvitis, vaginitis, cervicitis and endometritis and that increased in severity with increased time after infection. The microscopic changes in goats (No. 1,2&3) inoculated with *M.agalactiae* were consisted of a lymphocytic infiltration in the epithelium (exocytosis) and into the subepithelium (lamina propria) of the vulvar and vaginal mucosa, lymphocytic perivascular cuffing of the blood capillaries and stromal edema (Fig. 1). The lymphocytic infiltration in the epithelial and subepithelial layers of vulva and vagina along with prominent perivascular cuffings was markedly increased in goats No.2 & 3, which

sacrificed at 21 and 28 DPI, respectively (Fig. 2). Additionally, the presence of lymphoid aggregates in the form of lymphoid follicles in the lamina propria of vagina (Fig. 3) were also seen in the later goats. Sloughing and denudation in the surface epithelium of the cervical mucosa (Fig. 4) as well as lymphocytic infiltration of the tunica muscularis of the cervix were evident in goats No.2&3. Moreover, uteri of goats (No.1, 2&3) showed mild to moderate lymphocytic infiltration around and in between the endometrial glands (Fig.5). Mild lymphocytic infiltration of the epithelial and subepithelial layers with patches of vacuolar degeneration and necrosis was also observed in fallopian tubes of goat No.3 (Fig. 6). The ovaries of the all sacrificed does hadn't any significant microscopic lesion.

Histopathological findings of the inoculated udder:

Microscopically, the tissues of the left inoculated udder halves of the goat sacrificed at 21 DPI (No. 2) and that sacrificed at 28 DPI (No.3) revealed diffuse chronic mastitis characterized by moderate (goat No. 2) (Fig. 7) to severe infiltration of the interstitial tissue with lymphocytes and few plasma cells that at places appeared as aggregates in the form of lymphoid follicles replacing many of the secretory acini (goat No. 3) **(Fig. 8).** The acini contained very little or no milk secretion. Lymphoid tissues of supramammary lymph nodes and the spleens of goats No. 2&3 showed hyperplasia (Fig. 9). The inoculated gland, revealed extensive interlobular and interacinar fibrosis at 28 days PI. There was acute inflammation of the supramammary lymph node characterized by neutrophilic infiltration and edema.

Generally, the inoculated animals via the simultaneous intravaginal and intramammary routes (does No. 2&3) had the most extensive clinical manifestations, macroscopic, and microscopic lesions in their genital tracts, mammary glands, and supramammary lymph nodes.

Immunofluorescent antibody examination: Examination of udder sections stained with FAT revealed positive fluorescence against *M.agalactiae* in many acinar cells of the udder parenchyma (Fig. 10) and in some cells within the lactiferous ducts. The supramammary lymph nodes corresponding to the inoculated gland showed positive fluorescence against *M. agalactiae* (Fig. 11). Examination of the vaginal tissues revealed positive fluorescence in the mucosal and submucosal surfaces.

Microscopical examination of milk films:

Microscopical examination of milk films of does No. 2&3 showed the presence of numerous polymorphnuclear cells and few lymphocytes at 7 DPI (Fig. 12). Fifteen DPI, the milk smear showed only few lymphocytes.

DISCUSSION

Several *Mycoplasma* species may infect sheep and goats, some seem to be of little significance, but few produce diseases that were well recognized and economically important (Cottew, 1979 and Singh *et al.*, 1975). *M.agalactiae* is primarily a cause of mastitis leading to agalactia and arthritis (Cottew, 1979; Kinde *et al.*, 1994 and Bergonier *et al.*, 1997). *M. agalactiae* as a cause of granular vulvovaginitis (GVV) and infertility was recorded by Singh *et al.* (1974& 1975) and Chima *et al.* (1982&1995). Singh *et al.* (1974) showed the association of *M. agalactiae* with a severe form of granular vulvo-vaginitis (GVV) of goats. The pathologic features of *M.agalactiae* induced GVV and the genesis of the lesions were reported by Singh *et al.* (1975).

In the present study, the gross and microscopic lesions of the vagina were almost similar to those of the spontaneous and experimental infection resulted from *M.agalactiae* Singh *et al.* (1974&1975), *M.ovine/caprine* serogroup 11 (2-D) Rana *et al.* (1993), and *Acholeplasma laidlawii,* Gupta *et al.* (1990) but differ in severity. Reasons for these variations may be due to the differences in host response, or to the strain of mycoplasma used and its level of passage in artificial media.

Absence of the gross GVV and reduced the severity of the microscopic findings in the vaginal tissues in the present study, may be attributed to the application of the *M.agalactiae* culture into the vaginal canal without scrapping of the mucous membrane of the vagina. On the other hand, Singh *et al.* (1974&1975) and Rana *et al.* (1993) could produce the gross lesions of granular vulvovaginitis (GVV) only when vulvar epithelium was scrapped prior to the inoculation of the organism.

In the present study, the histopathological changes in the genital tract of the inoculated goats were recorded in cervix, uterus in addition to mild lymphocytic infiltration of the epithelial and subepithelial layers with patches of vacuolar degeneration and necrosis in the lining epithelium of fallopian tubes. On the contrary, the reported lesions by Singh *et al.* (1974&1975), were confined only to the vulva and vagina.

However, the microscopic findings of the vulva, vagina of the goat No. 1, that was inoculated by the intravaginal route only as well as its negative antibody titre against the inoculated species by the IHA test was in agreement with the findings of Singh *et al.* (1975) and Rana *et al.* (1993) who explained that the infiltration of a large number of lymphocytes and plasma cells in the epithelial, subepithelial, muscular and serosal layers of the genital tract along with the negative results of serology indicated that strong cell-mediated response more than humoral response was directed against the inoculated *Mycoplasma* species into the vulvovaginal canal of goats.

On the contrary, the reduced severity of lymphocytic infiltration in the genitalia as well as in the internal organs in association with the marked serological response in the sera of the inoculated goats (No. 2&3) (titre ≥1/160) by IHA test that simultaneously inoculated by intravaginal and intramammary routes may be indicative for the strong humoral response than cell-mediated response, which previously reported by Ojo and Ikeda, (1976). Taoudi *et al.* (1987) stated that intramammary inoculation of ewes with *M. capricolum* developed acute mastitis and a significant increase of antibodies against *M. capricolum*. Additionally, similar observation was also recorded after intramammary inoculation of sheep with *M. Mycoides subsp. mycoides* (Banga and Gupta, 1989), *M. ovine caprine* serogroup 11 (2-D) of goat (Rana *et al.*, 1992), and *M. mycoides subsp. capri* (Misiri *et al.*, 1988).

The results of *Mycoplasma* re-isolation from the inoculated goats after scarification showed that *M. agalactiae* was re-isolated from the genital tract (vagina, cervix, uterus), from the tissues of the internal organs (lungs &spleen), from the mammary tissues and supramammary lymph nodes of goats No.2&3, while it could only be re-isolated from the genital tract of goat No. 1. On the other hand, estimation of serological response of the inoculated goats by IHA test revealed that, the sera of goats No.2&3 had high antibody titre (≥ 1/160) against *M. agalactiae*, while no antibodies could be detected in the serum of goat No.1 (Table 2). The present results may be indicative for the systemic dissemination of the organism in goats No. 2&3 but the infection was confined to the genital tract of goat No. 1. These findings coincides with those of Singh *et al.* (1974) and Rana *et al.* (1993) who explained that the re-isolation of *Mycoplasma* species from the vaginal swabs of the intravaginally inoculated goats along with the negative results of serology indicates that the local infection remained confined to the genital tract and didn't cause systemic reaction. On the other hand, Jain

et al. (1969) stated that in experimental mycoplasmal mastitis, a systemic spread was demonstrated as the inoculated *Mycoplasma* species was re-isolated from lymph nodes, uterus, lungs, liver, kidney and spleen as well as frequent, but intermittent isolations were achieved from nasal and vaginal swabs. Also, Pal *et al.* (1983) re-isolated *M. bovigenitalium* from the udder tissues, supramammary lymph nodes as well as from the visceral organs of two goats which previously inoculated via the teat canal of one half of their udder with 2 ml broth culture (10^6 CFU/ml) of the same species. They concluded that the inoculated udder became painful, swollen 5 days postinfection. Also, variable degrees of degenerative changes and focal desquamation of epithelial cells lining the acini were reported.

The results of the present study showed that, the inoculated *M. agalactiae* was continuously re-isolated from the vaginal discharge of the 3 intravaginally inoculated goats (No. 1,2&3) as well as from milk samples and from the tissues of left halves of the udder and their corresponding lymph nodes of does (No. 2&3)(Table 2). In this concern the results of Hartman *et al.* (1964) explained that, the persistence of the organism in the reproductive tract for relatively long periods of time indicates the ability of the organism to multiply, tolerate the environment and causing infection. Moreover, Singh *et al.* (1974) concluded that, the produced lesions and the re-isolation of the organism from the inoculated animals clearly implicates *M. agalactiae*. DaMassa (1983) recovered *M.agalactiae* from mastitic milk of goat and stated that, the high concentration of the organism ($6.3x\ 10^7$ CFU/ml) indicates its pathogenic potential.

Contagious agalactia of small ruminants is a syndrome which affects mainly the mammary gland, joints and lungs and caused by *M. agalactiae* in sheep and *M. agalactiae, M. mycoides subsp. mycoides LC, M. capricolum subsp. capricolum* and *M. putrefaciens* in goats (Bergonier *et al.*, 1997). Sanchis *et al.* (2000) reported the onset of severe mastitis and excretion of *Mycoplasma* in the milk of six groups of lactating ewes which inoculated by the intramammary route with 10^8 CFU/ml of viable *M. agalactiae* organisms.

Concerning the mammary gland in the present study, microscopical examination of the tissues of the left inoculated udder halves of goat No. 2 (sacrificed 21 DPI) and goat No.3 (sacrificed 28 DPI) revealed diffuse chronic mastitis characterized by moderate to severe infiltration of the interstitial tissue with lymphocytes and few plasma cells that at places appeared as aggregates in the form of

lymphoid follicles replacing many of the secretory acini. Similar microscopic findings were described by Cottew, 1979 and Barton and Cottew, 1980 in contagious agalactia caused by *M. agalactiae*. Rana *et al.* (1992) explained that these findings indicated that mycoplasma induced permanent damage to the caprine mammary gland resulting in agalactia.

The clinical response, macroscopic, and microscopic changes observed in the udder of the intramammary inoculated goats in the present experiment were similar, but less in severity to those findings reported in the experimentally induced mastitis in goats by *M. agalactiae var bovis* (Ojo and Ikede, 1976), *M. bovigenitalium* (Pal *et al.*, 1983), *M. mycoides subsp. capri* (Misri *et al.*, 1988), *M. mycoides subsp. mycoides* LC in sheep (Banga and Gupta, 1989) and by *M. ovine/caprine serogroup 11(2-D)* (Rana *et al.*, 1992). Darzi *et al.* (1998) inoculated the right mammary gland of 12 lactating goats intracisternally with 1ml (10^6 CFU/ml) of *M. capricolum subsp. capripneumoniae (Mcc)*. Acute clinical mastitis was developed but at the end of the experiment 24 days PI it became chronic with increased somatic cells count. *Mycoplasma* was re-isolated from mammary secretion up to 16 DPI. Histopathologically, the mastitis was acute and purulent followed by lymphocytic infiltration and fibroplasia in the interacinar tissue and then by massive fibrosis.

In the present study, the results showed that, a febrile clinical mastitis was developed in goats No.2&3 and *M. agalactiae* was re-isolated from the udder tissues as well as from their milk with high numbers of colony forming units (10^6– 10^8 CFU/ml). Also, their blood sera showed a high antibody titre (≥1/160) by IHA test against the inoculated species. These results were similar to those of Ball *et al.* (1987) and Ball (1990). In addition, Bocklisch *et al.* (1991) stated that, the mycoplasma concentration in the udder tissue is probably responsible for the intensity of the lesions and shedding of the organism. They explained that, when high numbers of the infective agent were isolated from the udder tissue, more severe clinical symptoms and a more intensive shedding were recorded. Razin (1978) explained that, *Mycoplasma* has special affinity for secretory epithelial surface, so it get firmly attached to the secretory epithelium of acini and causes permanent damage to it by mechanisms such as increase in the local concentration of potent proteolytic enzymes and nucleases and other toxic metabolites as hydrogen peroxide (H_2O_2). Rana *et al.* (1992) stated

that, mycoplasmas may also cause cell damage by activating host mediators of inflammation.

Microscopical examination of milk films of does No. 2&3, showed the presence of numerous polymorphnuclear cells at 7 DPI. Fifteen DPI, the milk smear showed only few lymphocytes. Similar findings were reported by Alder *et al.* (1980) in the milk of goats experimentally infected with *M.putrifaciens* via the intramammary route within 7 days post-infection.

In the present study, by application of indirect immunofluorescent technique (IFT), *M. agalactiae* could be detected in the epithelium of many acinar cells of the mammary tissue of both goats No. 2&3 as well as in the supramammary lymph nodes corresponding to the inoculated udder halves. L'Ecuyer and Boulanger (1970) stated that IFT might offer a rapid and relatively simple technique for the detection of *Mycoplasma* organism in tissues even when the specific antigen was in small number. Bancroft and Gamble (2002) stated that FAT is the best in diagnosis if fresh frozen tissues are available.

Results of the ultrasound examination of the genital tract of inoculated goats showed the presence of vaginal secretion. Vagina appeared with thick wall and its diameter reached to (2.5cm), compared to that of the control goat (1 cm) (normally the vaginal lumen couldn't be scanned due to the pressure of the transducer from the rectum). Thickening and increase of the cervical diameter (2.4 cm), compared to the normal cervical diameter which not exceeding 1-1.5 cm in cycling does, indicated cervicitis.

In the present study, ultrasonography was chosen to facilitate continuous observation of the developing lesions in the genital tract, rather than slaughter every short period of time. Also, by ultrasonography, lesions were readily distinguishable from the normal cyclic changes. Ultrasound examination gave positive predictive value of ultrasound for diagnosis of genital tract affections (Haibel, 1990).

The observations reported in the present experimental study, indicated that *M. agalactiae* is pathogenic to the genital tract of does and produced severe vulvovaginitis, lymphocytic cervicitis, endometritis and mild salpingitis and confirmed the pathogenicity of *M. agalactiae* for the lactating mammary glands of goats.

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Fig. 1: Vaginal tissue of goat No.1 inoculated with *M. agalactiae*, sacrificed 15 days postinoculation showing moderate lymphocytic infiltration of the mucosa (exocytosis) and lamina propria (H&E, X10).

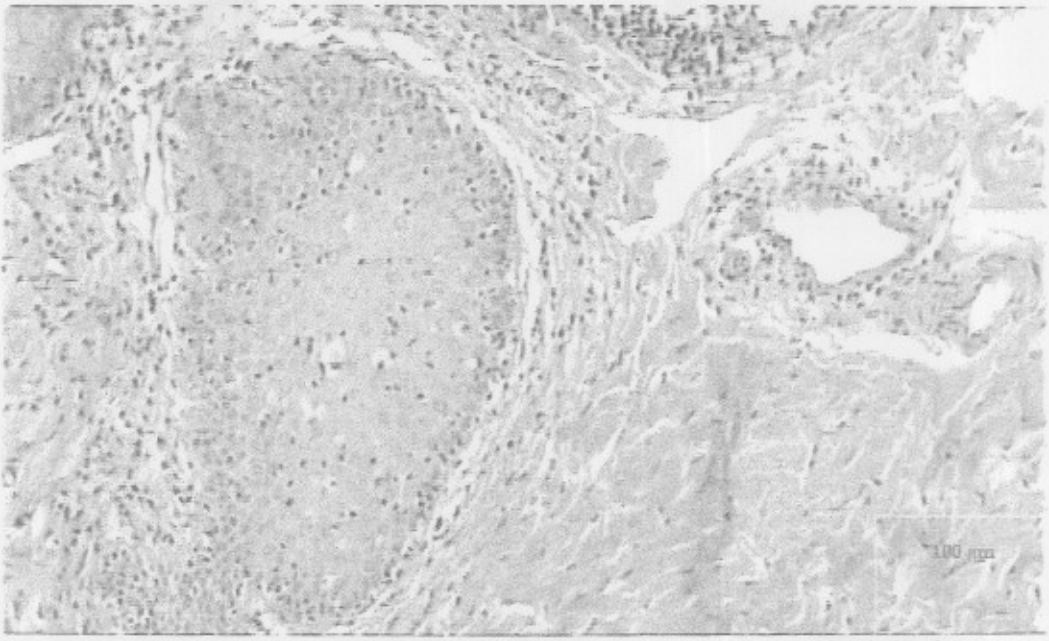

Fig. 2: Vaginal tissue of goat No.3 inoculated with *M. agalactiae*, sacrificed 28 days post inoculation showing moderate lymphocytic infiltration of the mucosa (exocytosis) and lamina propria along with mild lymphocytic cuffing of the blood vessels. Also, moderate edema was evident in the lamina propria (H&E, X10).

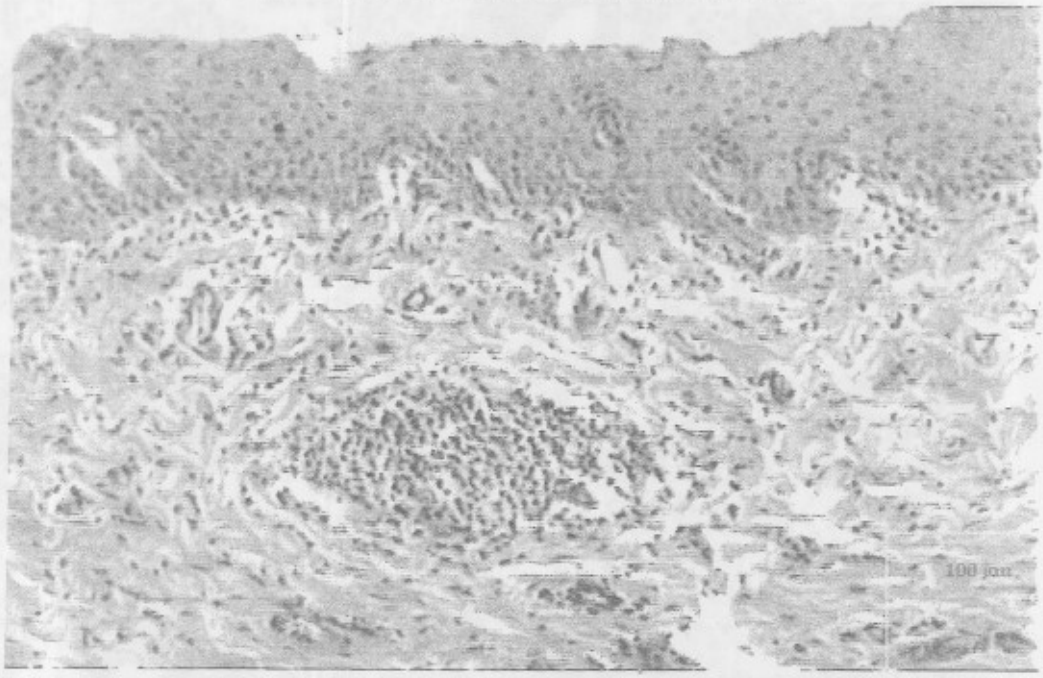

Fig. 3: Vaginal tissue of goat No.3 inoculated with *M. agalactiae*, sacrificed 28 days postinoculation showing subepithelial aggregates of lymphocytes in the form of a lymphoid follicle (H&E, X10).

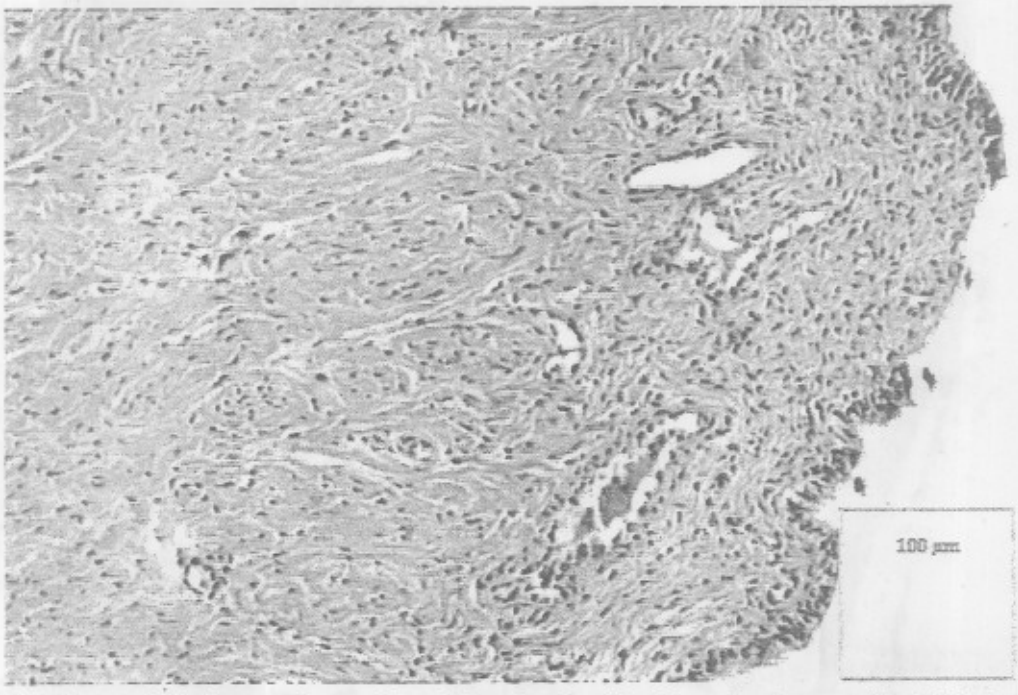

Fig. 4: Cervical tissue of goat No. 2 inoculated with *M. agalactiae*, sacrificed 21 days postinoculation, showing sloughed and denuded epithelium of the cervical mucosa in multiple patches along with moderate lymphocytic infiltration in the lamina propria and in the tunica muscularis (H&E, X10).

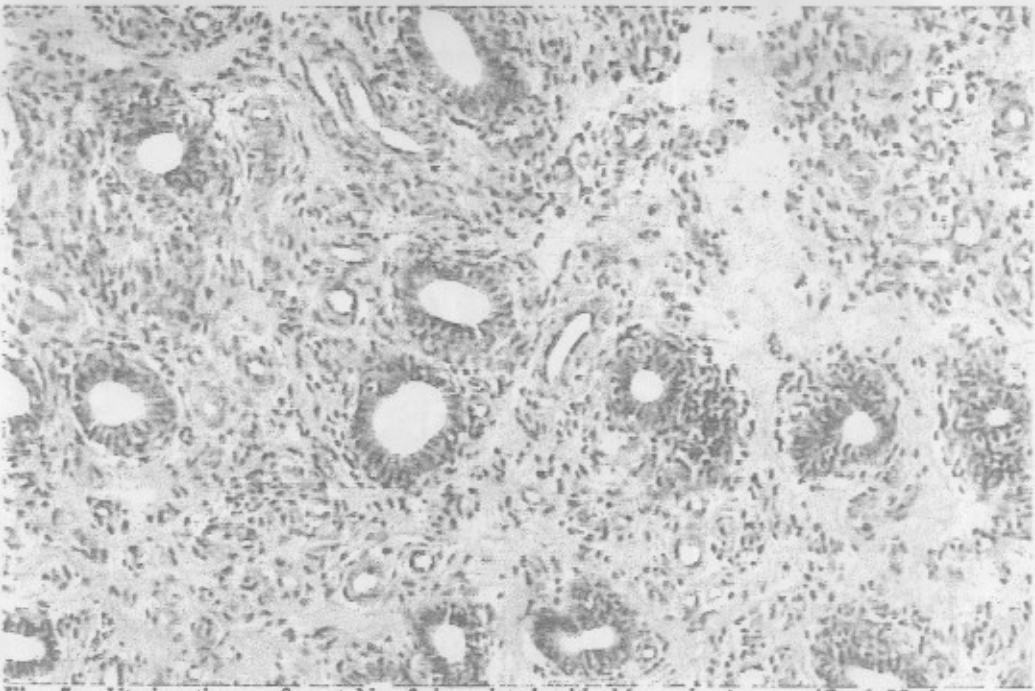

Fig. 5: Uterine tissue of goat No. 3 inoculated with *M. agalactiae*, sacrificed 28 days post inoculation, showing marked lymphocytic and plasma cells infiltrations around the endometrial glands along with mild edema in the endometrial stroma. (H&E, X10).

Fig. 6: Section of fallopian tube of goat No. 3 inoculated with *M. agalactiae*, sacrificed 28 days post inoculation, showing mild lymphocytic infiltration of epithelial and sub epithelial layers along with patches of vacuolar degeneration and necrosis in the lining epithelium of the tube. (H&E, X10).

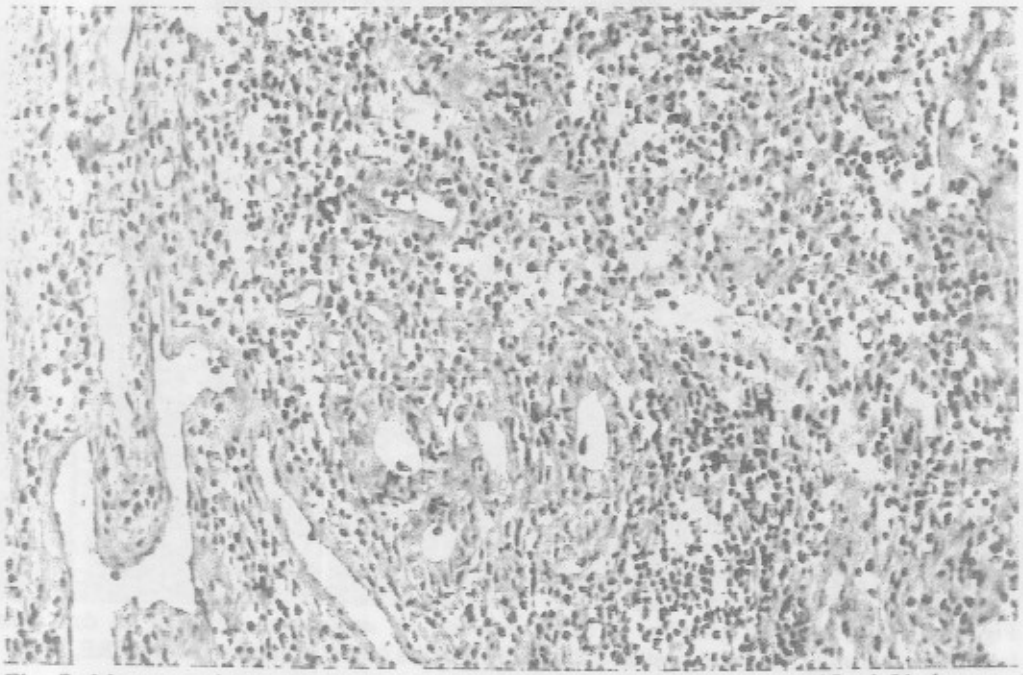

Fig. 7: Mammary tissue of goat No. 2 inoculated with *M. agalactiae*, sacrificed 21 days post inoculation, showing moderate diffuse mononuclear cells infiltration (mostly lymphocytes and few plasma cells) in the interacinar spaces (H&E, X10).

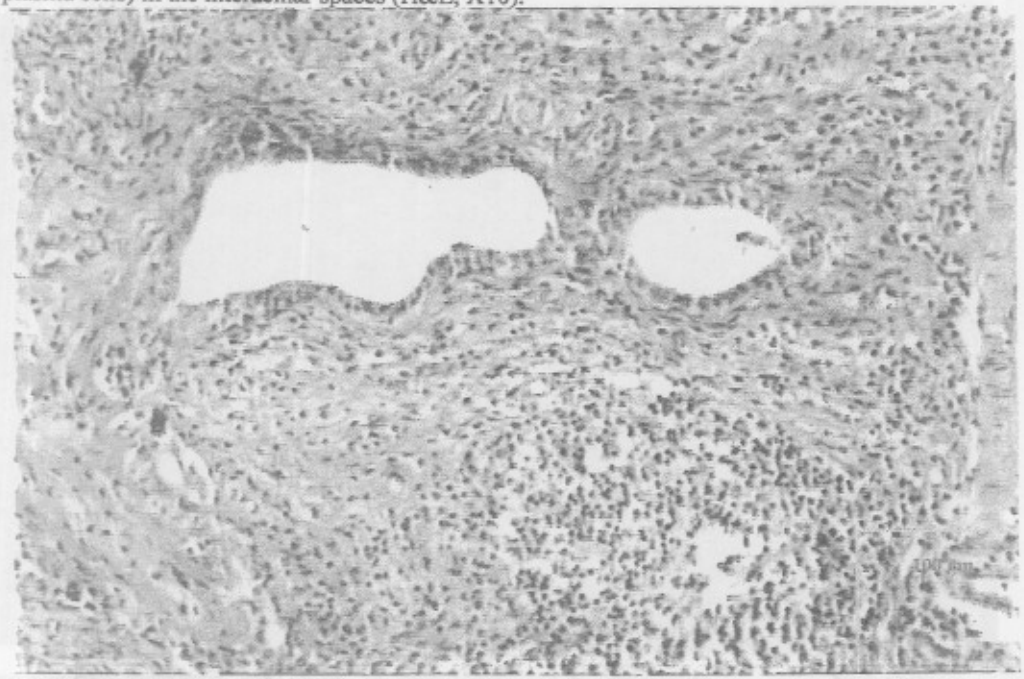

Fig. 8: Mammary tissue of goat No. 3 inoculated with *M. agalactiae*, sacrificed 28 days post inoculation, showing severe diffuse mononuclear cells infiltration (mostly lymphocytes and few plasma cells) and lymphoid follicle formation with fibroblastic proliferation in the interstitial tissue that replacing most of the secretory parenchyma of the gland. (H&E, X10).

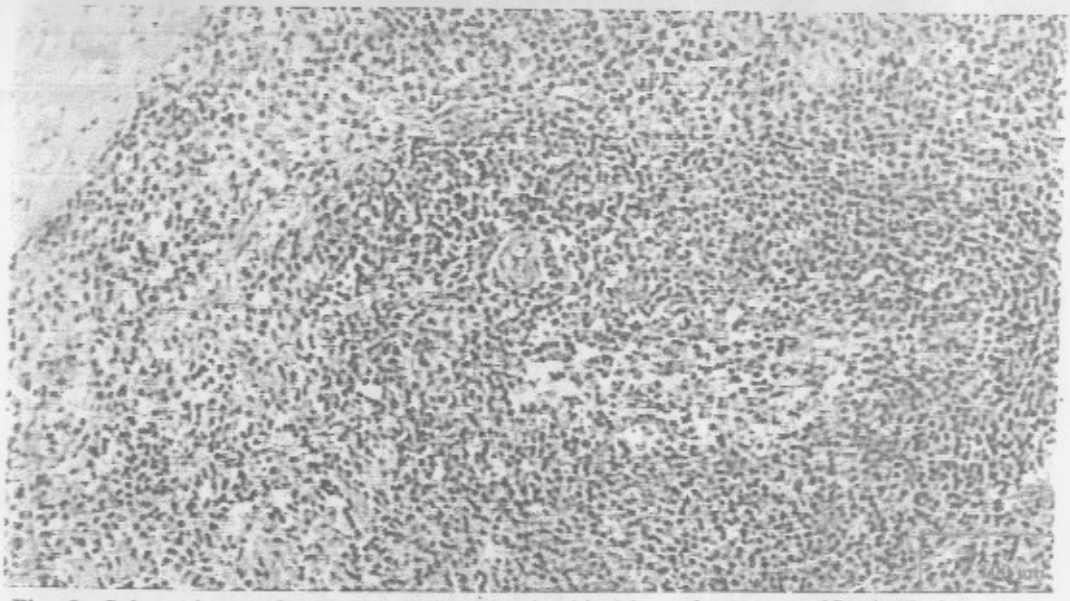

Fig. 9: Spleen tissue of goat No. 3 inoculated with *M. agalactiae*, sacrificed 28 days post inoculation, showing marked activation of lymphoid follicles (wide germinal center). (H&E, X10).

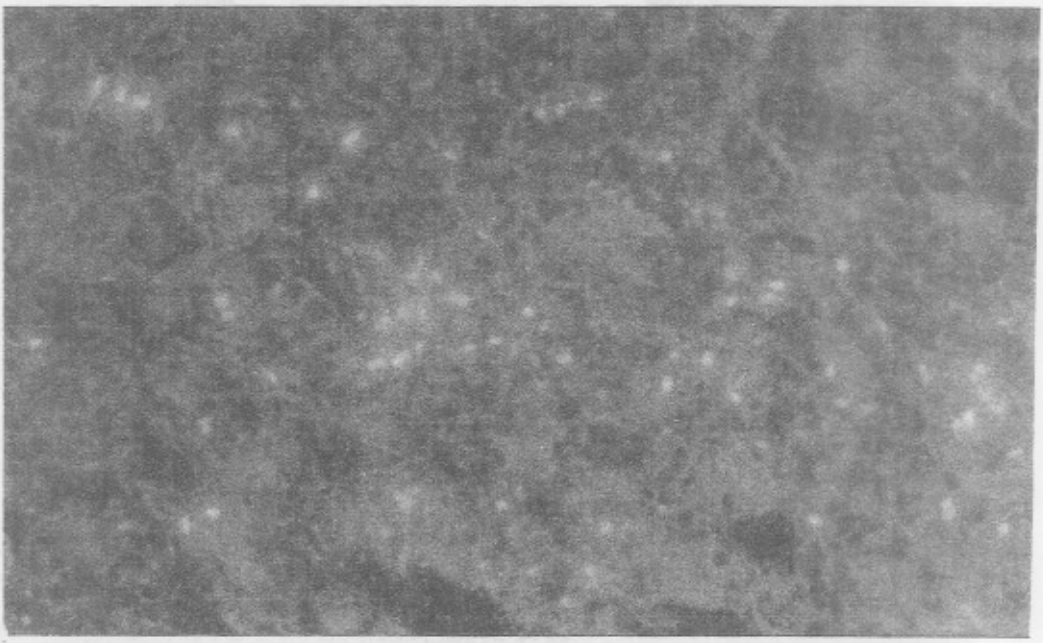

Fig. 10: Mammary tissue of goat No. 3 inoculated with *M. agalactiae*, sacrificed 28 days post inoculation, showing positive fluorescence against *M. agalactae* in the epithelium of many acinar cells (X200).

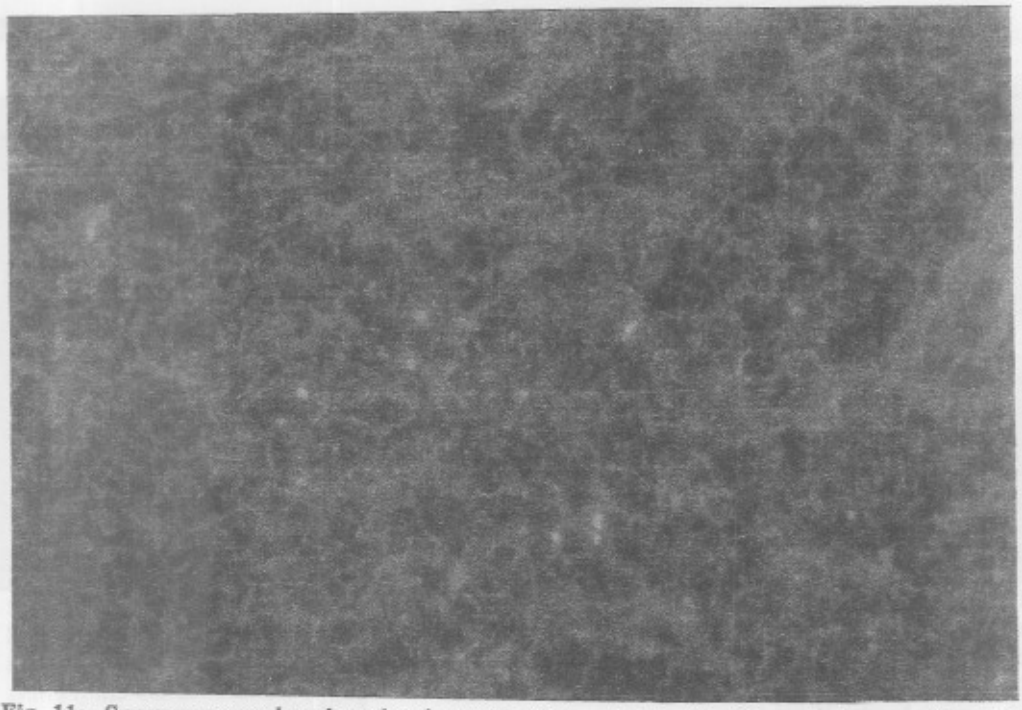

Fig. 11: Supramammary lymph node of goat No. 2 inoculated with *M. agalactiae*, sacrificed 21 days post inoculation, showing positive fluorescence against *M. agalactae*. (X200).

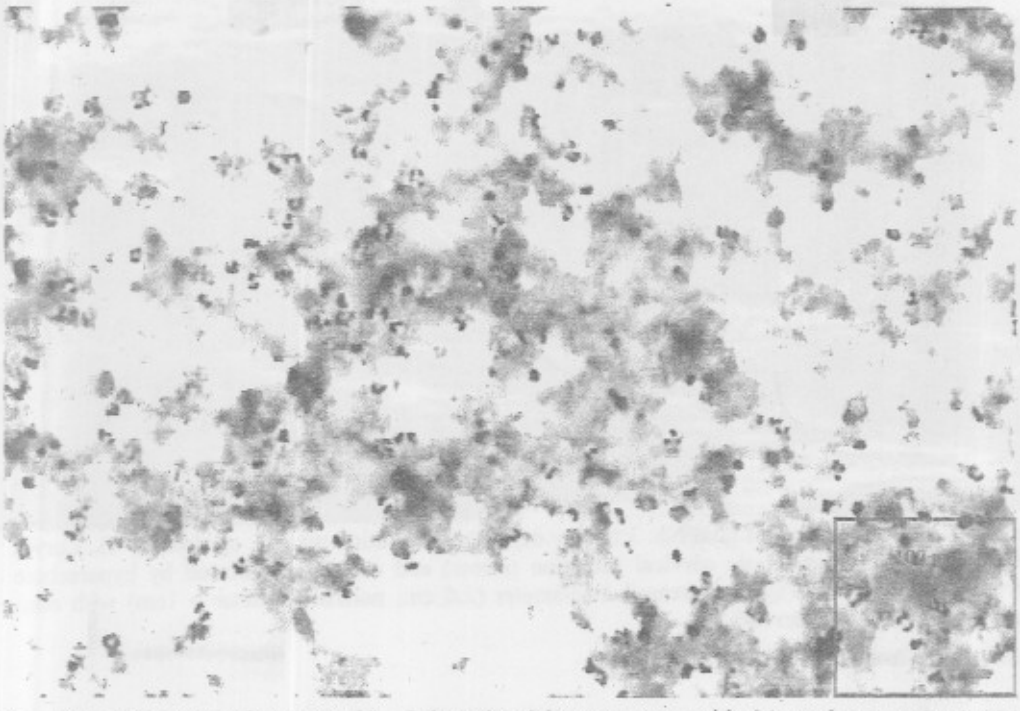

Fig. 12: Milk smear from goat No. 2 inoculated intramammry with *M. agalactia* stained with Leishman stain showing numerous polymorphnuclear leucocytes and few lymphocytes. (X10).

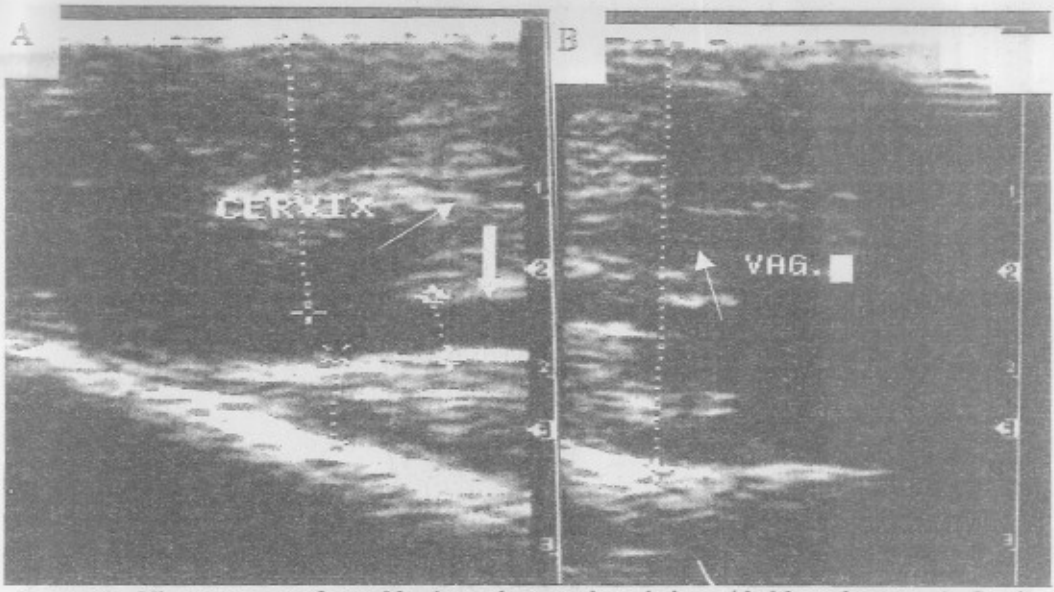

Image 1. Ultrasonogram of goat No. 1 ten days postinoculation with *M. agalactiae* **A.** Cervix showing non-echoic cervical secretion with opened cervical canal (thin arrow) and increased diameter to 2.4 cm (normal diameter = 1cm) and non-echoic vaginal secretion under cervical os (thick arrow) **B.** Vagina appeared with thick wall and with increased diameter to 2.5 cm (normal diameter = 1cm).

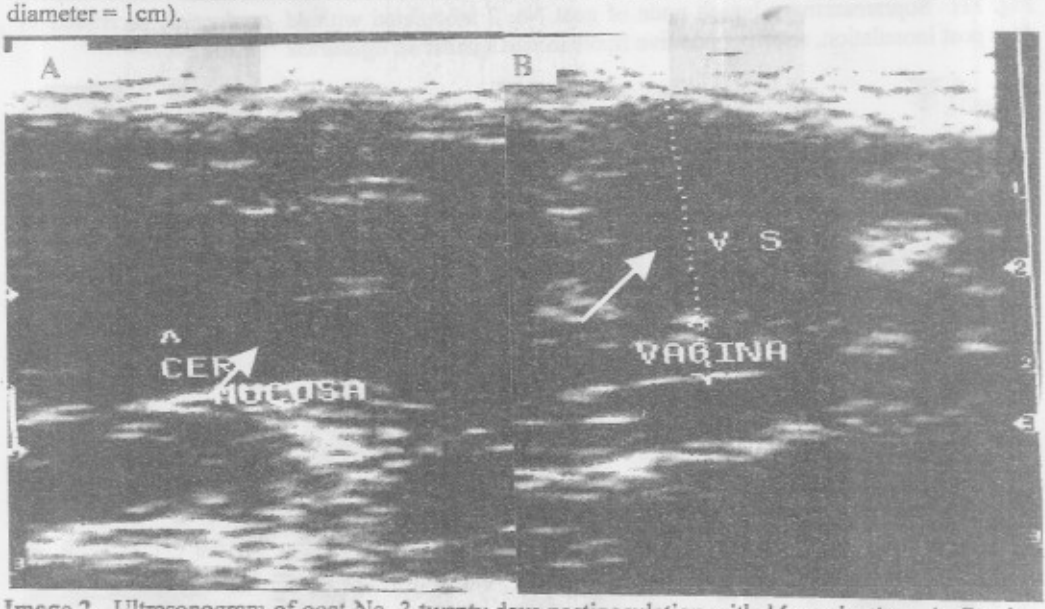

Image 2. Ultrasonogram of goat No. 3 twenty days postinoculation with *M. agalactiae*. **A.** Cervix was opened with non-echoic cervical secretion (arrow) and cervicitis indicated by hyperechoic cervical mucosa. **B.** Vagina increased in diameter (1.6 cm; normal diameter = 1cm) with non-echoic secretion (VS) (arrow).