

CLINICAL STUDIES ON MASTITIC BUFFALOES
NATURALLY INFECTED WITH MYCOPLASMA
BOVIGENITALIUM IN ASSIUT
GOVERNORATE - EGYPT

(With 3 Tables & 9 Fig.)

By

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**دراسات اكلينيكية علي التهاب الضرع في الجاموس
المصاب طبيعياً بالميكوبلازما بوفيجينيتاليم
بمحافظة اسيوط**

أحمد زيتون ، صبرى عيسى

خلال المده من يناير ١٩٩١ حتى فبراير ١٩٩٣ تم فحص عدد ٩٩٦ جاموسه حلوب اكلينيكيًا لبيان حالات التهاب الضرع . هذا وقد وجد أن ٥١ حاله كانت تعاني من أعراض التهاب الضرع فى ربع واحد أو ربعين . من بين هذه الحيوانات المصابه بالتهاب الضرع كانت هناك ٢٢ حاله لم تستجب للعلاج بالمضادات الحيويه . من هذه الحالات التى لم تستجب للعلاج كانت هناك إثني عشر جاموسه تعاني من ورم غير مؤلم فى الأرباع المصابه باستثناء حالة واحده كانت تعاني من ورم مصاحب بأعراض الألم والتحجر فى ربع واحد . وكان يشاهد هذا الورم فى الجزء العلوى من الربع المصاب أكثر من الحلمه فى سبع حالات وفى الحالات الباقية (٥) كان الورم يشمل كل الربع بما فيه الحلمه . وبالرؤيه الخلفيه للحيوان المريض كان الربع المصاب يأخذ الشكل الهرمى المقلوب . هذا ولم يلاحظ أى تغيير اكلينيكى فى الغدد الليمفاويه لضروع هذه الحيوانات المصابه وكذلك لم يلاحظ أى أعراض مرضيه عامه . الأرباع المصابه من هذه الحيوانات (١٢) كانت تدر لبنًا ضاربًا إلى البياض - مع وجود بعض الاستثناءات نتيجة لوجود الميكروبات الصيديه - عديم الرائحه مصحوبًا بتغيرات مرثيه بالعين المجرده . من هذه الأثنى عشر جاموسه تم عزل ميكروب الميكوبلازما بوفيجينيتاليم من تسع حالات . وكان افراز هذا الميكروب فى اللبن غير مستديم . الحالات الثلاثه المتبقية كانت تعاني من أعراض مشابهه بالحالات الايجابيه للميكوبلازما ولكن لم تتمكن من عزل الميكوبلازما أو أى ميكروب ممرض آخر منها .

من لبن الجاموس المصاب بالتهاب الضرع والذى تم عزل الميكوبلازما بوفيجينيتاليم منه (٩)

حالات) تم عزل ميكروب الـيستيريا مونوسيتوجين مختلط مع الميكوبلازما من الحالة التى أظهرت
ورماً مصحوباً بأعراض الألم والتحجر فى الربع المصاب هذا وقد تم وصف الأعراض المرضيه للحالات
الايجابيه للميكوبلازما باسهاب وكذلك ذكرت الميكروبات الأخرى المصاحبه للميكوبلازما فى
جدول .

وبعمل اختبار الحساسيه بالمضادات الحيويه على عترات الميكوبلازما المعزوله فى المعمل
وجد أن الكلورامفينيكول والثيامفينيكول كانوا ذو تأثير فعال . على النقيض وجد أن مجموعات
شبيهات البنسلين والسيفالوسبرين كانت عديمة التأثير . ووجد أيضاً أن ٧٨ ر ٧٧ % من عترات
الميكوبلازما المعزوله لم تتأثر بالأوكسيتتراسيكلين . هذا وقد أجريت محاولة علاج لثلاث حالات
مصابه بالميكوبلازما بدواء الثيامفينيكول ولكنها باءت بالفشل وعزلت الميكوبلازما من هذه
الحالات بعد مضى أسبوعين من العلاج .

على الجانب الآخر . كان هناك ثلاث حالات أخرى من بين ٢٢ جاموسه مصابه بالتهاب الضرع
ولم تستجب للعلاج تعانى من أعراض التهاب ضرع صديدى حيث عزل منها ميكروب الكورينى
الصديدى مختلط مع ميكروبات أخرى . الحالات المتبقية من هذا الجاموس (٢٢) كانت تعانى من
أعراض التهاب ضرع تقيحى دون الحاد وعزل منها الميكروب العنقودى .

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SUMMARY

Over two years investigations (Jan., 1991-Feb., 1993), of 996 dairy buffaloes were examined clinically for mastitis, 51 cases showed signs of mastitis in one or two quarters. Of fifty one clinical mastitic buffaloes, twenty two cases were resistant to antibiotics therapy. Twelve of drug resistant mastitic buffaloes had non painful swellings quarters with exception of one case showed signs of firmness and pain in one swelled quarter. These painless swellings were observed in the upper parts of the affected quarters rather than the teats in seven cases. Concerning the rest cases (5), the whole quarters including the teats were swollen. By posterior inspection, the affected quarter appeared like the "inverted pyramid" in shape. Neither supramammary lymph nodes involvement nor systemic illness were noticed in these cases. The affected quarters yielded whitish (some exceptions were reported due to the involvement of pyogenic microorganisms) odorless milk secretion with macroscopical changes. Of these twelve buffaloes, *Mycoplasma bovis* (*M. bovis*) was isolated from nine cases. Shedding of *M. bovis* was intermittent. The remainder cases (3 of 12) showed clinical signs fairly similar to *M. bovis* mastitic buffaloes but no mycoplasma or other significant pathogens could be isolated. Of *M. bovis* mastitic buffaloes, *Listeria monocytogenes* (*L. monocytogenes*) mixed with mycoplasma was isolated from a case which showed signs of firmness and pain in one swelled quarter. Detailed description on history and clinical signs of mycoplasma mastitic cases was described, and on other microorganisms associated with *M. bovis* was tabulated. In vitro, both chloramphenicol and thiamphenicol had strong inhibitory effect on all isolated mycoplasma strains. Contrariwise, penicillins analogous and cephalosporins groups were ineffective. 77.78% of tested mycoplasma strains were oxytetracycline resistant. Therapeutic trial with thiamphenicol was

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carried out on three mycoplasma mastitic buffaloes without successful response, and mycoplasma organism was re-isolated from milk of these cases after two weeks of treatment.

On the other side, three cases of twenty two drug resistant mastitic buffaloes were suffered from signs of suppurative mastitis where *Actinomyces* (*Corynebacterium*) *pyogens* (*A. pyogens*) coupled with other organisms were isolated. The other cases of these drug resistant mastitic buffaloes showed signs of subacute purulent mastitis where *Staphylococcus aureus* was isolated.

INTRODUCTION

Both *M. bovis* and *M. bovigentialium* were incriminated as pathogenic agents responsible for incurable mastitis of dairy cows with high culling rates (WEHNERT et al., 1983; STALHEIM, 1990 and JACKSON and BOUGHTON, 1991).

First report on isolation of mycoplasma (*M. bovigentialium* either alone or coupled with bacteria) from milk of buffaloes suffered from clinical mastitis was by PAL et al (1984) in india.

In Egypt, the first report on mycoplasma mastitis in cattle was recorded by EL-EBEEDY et al (1985) at El-Kaluobia Governorate with highest culling rate due to ineffective therapy and dramatic decline in milk yield. Thereafter, reports on the incidence of mycoplasma (*M. bovis*, *M. bovigentialium* and *M. arginini*) mastitis in dairy Friesian cows had been increased in Egypt with variable ratios (EISSA, 1986; AHMED, 1987; EL-SHABINI et al., 1989; ZAITOUN, 1990 and ZAITOUN, 1991). The increased reports on mycoplasma mastitis may refer to spread of the disease at different localities of Egypt.

Mastitis of Egyptian buffaloes due to bacterial and mycotic infection was documented (AHMED, 1981 and EISSA, 1986). Contrariwise, reports on buffaloes' mycoplasma mastitis are still scanty in the available literature. However, both *M. bovis* and *M. arginini* (mixed infection) were isolated from milk of a dairy buffalo with chronic mastitis yielding abnormal milk secretion (ZAITOUN, 1990). Consequently, the goal of the present work was to study the clinical signs of mycoplasma mastitic buffaloes, and to clear up the bacterial and mycotic pathogens which may be involved with mycoplasma. Antibigram of the isolated mycoplasma strains and therapeutic trial for some mycoplasma positive cases with selected drug were also achieved.

MATERIAL and METHODS

Animals:

Over two years investigations (JAN., 1991 - FEB., 1993), 300 dairy buffaloes of El-Hawatka herd, 689 private cases belonged to some neighboring villages of Assiut Governorate (El-Malgaa, El-Assara, Bani-Mour, Kom Abu-chial and Abnoub), and 7 buffaloes of veterinary clinic (Faculty of Veterinary Medicine-Assiut University) were examined clinically for mastitis. Mastitic cases were treated by broad spectrum antibiotic (oxytetracycline combined with oleandomycin) intramammary infusion for 5-7 days. Some cases apparently responded to the given treatment while others were resistant. The oxytetracycline-resistant mastitic cases were retreated by cloxacillin intramammary infusion for three days without clinical improvement. Detailed clinical examinations of these drug resistant mastitic buffaloes were described.

Samples collection:

Milk sample from each affected quarter of the drug resistant mastitic buffaloes was collected aseptically and subjected to mollicutes (mycoplasma and acholeplasma) analysis. Another trials (two further occasions) for isolation of mollicutes from the same cases were adopted. Bacterial and mycotic analysis for the collected milk samples were also conducted. Eighteen milk samples of clinically normal dairy buffaloes in the same areas of the diseased cases were also collected and subjected to mollicutes analysis.

Culture techniques:

Five drops of each collected sample were added to 2.5 ml of modified Hayflik's broth medium (STALHEIM, 1990) in screw capped bijou bottle (5ml) and incubated at 37°C for 48 h. for mycoplasmal enhancement. Half milliliter of the inoculated broth was spread over a 5 cm plate of modified Hayflik's agar and incubated in 5% carbon dioxide atmosphere and examined at 2, 3, 5, 7 and 10 days for characteristic growth of mycoplasma colonies (Fig. 5). The possibility of "L" form of bacterium was excluded according to the methods described by STALHEIM (1990). The isolated mollicutes were identified biochemically on the basis of sterol dependence, dextrose and arginine utilization, and film and spots tests (STALHEIM, 1990). Disc

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growth inhibition test (CLYDE, 1964) with specific immune sera* was used for serological identification of isolated mycoplasma strains.

On the other hand, for screening the other pathogens which may be involved with mastitis, each sample was cultured on 5% ovine blood agar and Sabouraud's glucose agar plates for bacterial and mycotic growth, respectively. The isolated strains were identified on the lines of CARTER and COLE JR (1990) criteria. Mice inoculation and Anton tests (CARTER and COLE JR, 1990) were used for isolated listeria strains to determine the degree of virulence.

Antibiogram and therapeutic trial:

Antibiogram of the isolated mycoplasma strains was achieved by growth inhibition test with different members of antibiotic discs (Table 3). Therapeutic trial with thiophenicol** (1 gm per affected quarter, intramammary infusion, and 15 mg per kg body weight, intramuscular injection, for five successive days) was carried out on three mycoplasma positive cases (Table 2). The treated cases were re-sampled after two weeks of treatment to determine the degree of the responsiveness.

RESULTS

Fifty one of the examined buffaloes (996) were clinically mastitic in one or two quarters (Table 1). The cardinal signs of acute inflammation including painful and hotness swellings of the affected quarters without supramammary lymph nodes involvement nor marked systemic reactions were the predominant observed signs. Of fifty one clinical mastitic buffaloes, twenty nine were apparently improved after oxytetracycline-oleandomycin intramammary infusion therapy while the remainders (22) were resistant (Table 1). The oxytetracycline resistant mastitic cases were treated again by another type of antibiotic (cloxacillin) without successful

*: Specific immune sera were prepared locally in rabbit with references strains of *m. bovis* and *M. boviqenitalium* as protocol described by Stalheim (1990). References strains of *M. bovis* and *M. boviqenitalium* were obtained kindly from Prof. Dr. M.B. Brown, Department of Infectious Diseases, College of Veterinary Medicine, University of Florida, USA.

** : Thiophenicol= Vial contain 750 mg thiamphenicol produced by the Nile Co. for pharmaceuticals Cairo-A.R.E. under License from CLIN-MIDY-Paris.

response. Two weeks later, the cardinal signs of inflammation of these cases were subsided. However, the quarters swelling persisted in 17 cases.

Affected quarters (16) of some drug resistant mastitic buffaloes (12 of 22) had non painful swellings with exception of one case showed signs of firmness with pain in one swelled quarter. These painless swellings were observed in the upper parts of the affected quarters rather than the teats in seven cases out of these twelve buffaloes. In the rest cases (5), the whole affected quarters (including the teats) were swollen and milk was dripping from the external teats orifices. The surrounding muscles of these teats were relaxed. By inspection from behind the infected animal, size of the affected quarter was nearly more than twice as large as the opposite one and appeared like the "inverted pyramid" in shape (Fig. 1). Milk of these affected quarters was streamed like normal but it was pale whitish in color and odorless. These collected milk samples was rapidly separated into two layers on standing (Fig. 2). The supernatant was whitish and watery in six cases and yellowish white with slightly thickness in the remainders while the sediment (in all cases) contained either fine granules of clotted milk like sand (Fig. 3) or large particulate clots (Fig. 4). Of these twelve buffaloes, *M. bovigentialium* (either alone or coupled with other organisms) was isolated from nine cases (Table 2). The remainders (3 of 12) showed signs fairly similar to mycoplasma mastitic cases but no mycoplasma or other significant pathogens could be isolated.

Of *M. bovigentialium* mastitic buffaloes, *L. monocytogenes* was isolated in association with mycoplasma from a case which showed signs of firmness and pain in one swelled quarter. Results of mice inoculation* and Anton** tests revealed that the isolated listeria strain was virulent. All normal milk samples were mollicutes free.

*Inoculated mice were died within two days post intraperitoneal inoculation with 24 h. listerial tryptose culture broth.

** Twenty four hour listerial tryptose culture broth, when instilled onto the conjunctiva of rabbit's eye, showed sever purulent kerato-conjunctivitis within 3oh. The eye-lids were thickened with profuse thick exudate leading to adherence of the eye-lids at the end of the course (Fig. 6, 7, 8 & 9). These lesions persisted up to one week.

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Two weeks later, mycoplasma mastitic buffaloes were re-sampled again with positive results in five cases only. However, the swelled quarters and macroscopical changes of the milk samples were still observed in all mycoplasma mastitic cases. After 20 days, the affected quarters of these five cases fairly regained its normal appearance however the secreted milk was cloudless fluid without separation on standing and was mycoplasma free.

The three buffaloes which showed signs fairly similar to *M. bovigentalium* mastitic cases without isolation of mycoplasma from their milk were also subjected to another trial for detection of mycoplasma with negative results. In this trial, the affected quarters of these three cases became small when compared with the non affected ones yielding little amount of watery fluid like blood-serum without floccular deposits on standing. Signs of fibrosis thereafter were developed.

Antibiogram of the isolated mycoplasma strains revealed that both chloramphenicol and thiamphenicol had strong inhibitory effect on all tested strains while penicillins analogous (amoxycillin, ampicillin and cloxacillin) and cephalosporins (cefxitine, cefotaxime and cephoperazone) groups were ineffective. 77.78% of tested mycoplasma strains were oxytetracycline resistant (Table 3). The therapeutic trial with thiophenicol on three mycoplasma mastitic buffaloes was ineffective and *M. bovigentalium* was re-isolated from milk of these cases after two weeks of treatment.

On the other side, three cases of the drug resistant mastitic buffaloes (22) had hardness quarters with sings of pain yielding very thick light coffee colored pus secretions with putrid odor, where *A. pyogens* coupled with micrococci and Gram negative bacteria were isolated. Sings of subacute purulent mastitis were observed in the remaining resistant cases where *Staphylococcus aureus* was isolated.

DISCUSSION

Good hygienic measures and milk sanitation, and early diagnosis of mastitis during subclinical stage were considered the fundamental bases in control of bovin mastitis (AHMED, 1981; EISSA, 1986 and ZAITOUN, 1990). This may interpret the high incidence of clinical mastitis of private buffaloes in comparison with El-Hawatka herd and veterinary clinic farm of Assiut University.

No records had been encountered in the available literature concerning the clinical manifestation of *M. bovigentalium* mastitic buffaloes namely the non painful

swellings of the affected quarters with the appearance of these swellings in the upper parts of the quarters rather than the teats in most cases. In addition, the secreted odorless milk with characteristic macroscopical changes and the absence of the involvement of neither supramammary lymph nodes nor systemic reactions could not also be traced in the available literature. However, *M. bovigentialium* developed mastitis in cows with similar clinical findings (EISSA, 1986, and JACKSON BOUGHTON, 1991). It is worth to mention that the observed signs of firmness and pain in one case of *M. bovigentialium* mastitic buffaloes was probably due to the involvement of *L. monocytogenes* in association with mycoplasma.

The characteristic macroscopical changes of the obtained milk of *M. bovigentialium* mastitic buffaloes, the secreted milk was streamed like normal which rapidly formed either floccular deposits of clotted milk or large particulate clots and whitish cloudless supernatant on standing, were also recorded in mastitic cows infected with *M. bovis* and/or *M. bovigentialium* (EL-EBEEDY *et al.*, 1985 and Ahmed, 1987). In addition, these characteristic findings of the obtained mastitic milk may be due to the presence of great increase in casine nitrogen which resulted in the appearance of insoluble casine (BOUGHTON, 1979). It is worth to notice that the yellowish coloration of the supernatant of the some collected milk samples of *M. bovigentialium* mastitic buffaloes may be related to the involvement of pyogenic bacteria (staphylococci) with mycoplasma.

As far as the second trial for isolation of mycoplasma was conducted, five of nine *M. bovigentialium* mastitic buffaloes yielded mycoplasma in their milk and the remainder cases mycoplasma could not be isolated. However the swelled quarters and the characteristic macroscopical changes of the collected milk samples were still observed in all mycoplasma mastitic buffaloes. The secreted milk of these five positive cases thereafter was mycoplasma free at the third trial of isolation. This may indicate that shedding of *M. bovigentialium* through milk was intermittent. The intermittent shedding of *M. bovigentialium* via milk was probably not as prolong as *M. bovis* (Jackson and Boughton 1991).

The presence of mixed infection (*M. bovigentialium* and *Staphylococcus aureus*) in some *M. bovigentialium* mastitic buffaloes was also recorded in buffaloes (PAL *et al.*, 1984) and in cows (EISSA, 1986 and JACKSON and BOUGHTON, 1991). Furthermore, the coupling between mycoplasma and bacteria in

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mastitis might reflect a synergistic situation (PAL et al., 1984).

The obtained results of antibiogram of the isolated *M. bovigentialium* strains may be explained on the basis that mycoplasma is a wall free prokaryotic cell (STALHEIM, 1990) and consequently both penicillins analogous and cephalosporins interfere with cell wall formation of the microorganism while chloramphenicol and thiamphenicol are protein synthesis interference (BYWATER, 1991). Although the pharmacological action of oxytetracycline is similar to chloramphenicol (BYWATER, 1991) yet 77.78% of the tested mycoplasma strains were oxytetracycline resistant. In spite of the isolated *M. bovigentialium* was sensitive, in vitro, to thiamphenicol, therapeutic trial (in vivo) with this drug was ineffective and mycoplasma organism was re-isolated after two weeks of treatment. Consequently, mastitic buffaloes with painless swelling quarters yielding abnormal milk secretion and did not respond to antibiotic therapy, mycoplasma infection should be suspected. In addition the mycoplasma positive cases showed clinical signs must be culled (WEHNERT et al., 1983, EL-EBEEDY et al., 1985 and EISSA, 1986).

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Table 1 : Incidence of mycoplasma mastitis :

Localities	Number of examined buffaloes	Number of clinical mastitic cases	Number of mastitic quarters	Number of drug resistant mastitic cases	Number of drug resistant mastitic cases yielded mycoplasma
EL-Hawatka herd	300	6 (2.00%)	7 (0.58%)	1	0
Private cases	689	45 (6.53%)	52 (1.88%)	21	9
Veterinary clinic cases	7	0	0	0	0
Total	996	51 (5.21%)	59 (1.48%)	22	9

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Table 2 : Cultural results of milk samples from the drug resistant mastitic buffaloes (22)*

Number of cases	Microbial isolates
4** (18.18%)	<i>Mycoplasma bovis</i>
1 (4.54%)	<i>Mycoplasma bovis</i> <i>Acholeplasma</i> (unclassified)
2+ (9.09%)	<i>Mycoplasma bovis</i> <i>Acholeplasma</i> (unclassified) <i>Staphylococcus aureus</i>
1+ (4.54%)	<i>Mycoplasma bovis</i> Coagulase negative staphylococci <i>Staphylococcus aureus</i>
1 (4.54%)	<i>Mycoplasma bovis</i> <i>Listeria monocytogenes</i>
3 (13.64%)	<i>Actinomyces pyogenes</i> Micrococcus species Gram negative bacteria
7 (31.82%) *	<i>Staphylococcus aureus</i>

*: Three cases of the resistant mastitic buffaloes showed sing fairly similar to *M. bovigelitalium* positive cases but no mycoplasma or other significant pathogens could be isolated after two trial of isolatin with two weeks interval.

** : Three of these cases were subjected to therapeutic trial with thiophenicol.

+ : The supernatant of the collected milk from these cases was yellowish white in color.

Table 3 : Antibiogram of isolated Mycoplasma bovis strains

Drugs		Number of tested mycoplasma strains								
		1	2	3	4	5	6	7	8	9
Amoxycillin	(25 µg)	-	-	-	-	-	-	-	-	-
Ampicillin	(25 µg)	-	-	-	-	-	-	-	-	-
Cefxitine	(30 µg)	-	-	-	-	-	-	-	-	-
Cefotaxime	(30 µg)	-	-	-	-	-	-	-	-	-
Cefoperazone	(30 µg)	-	-	-	-	-	-	-	-	-
Cloxacillin	(25 µg)	-	-	-	-	-	-	-	-	-
Chloramphenicol	(30 µg)	++	++	++	++	++	++	++	++	++
Oxytetracycline	(30 µg)	-	-	-	-	++	-	-	+	-
Thiamphenicol	(30 µg)	++	++	++	++	++	++	++	++	++

- Inhibition zone (0 - 2 mm).
 + Inhibition zone (6 - 8 mm).
 ++ Inhibition zone (19 - 21 mm).



Fig.1: Buffalos' swelled quarter due M. bovis infection. Note: a) Size and shape ("inverted Pyramid") of the affected quarter. b) Displacement of the middle line of the udder towards the right side, c) The difference in level of the two hind teats.

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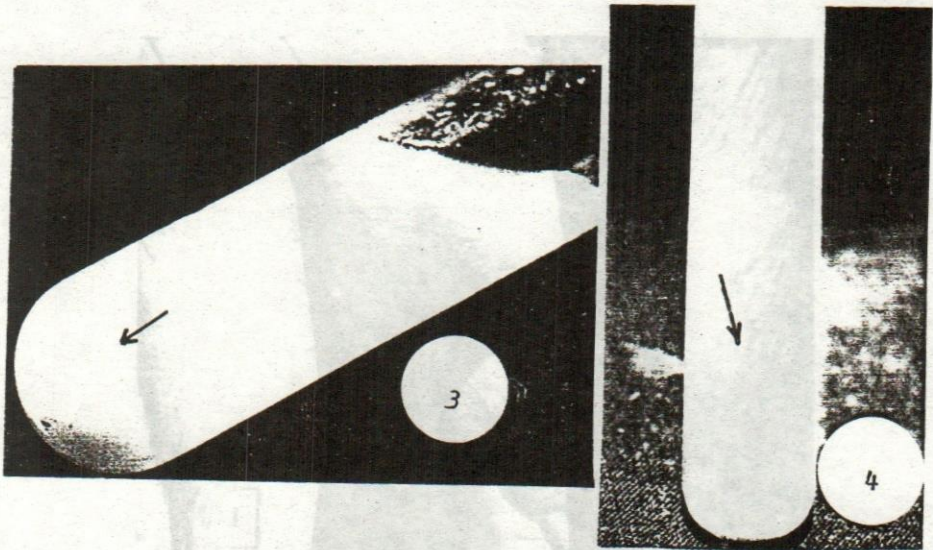
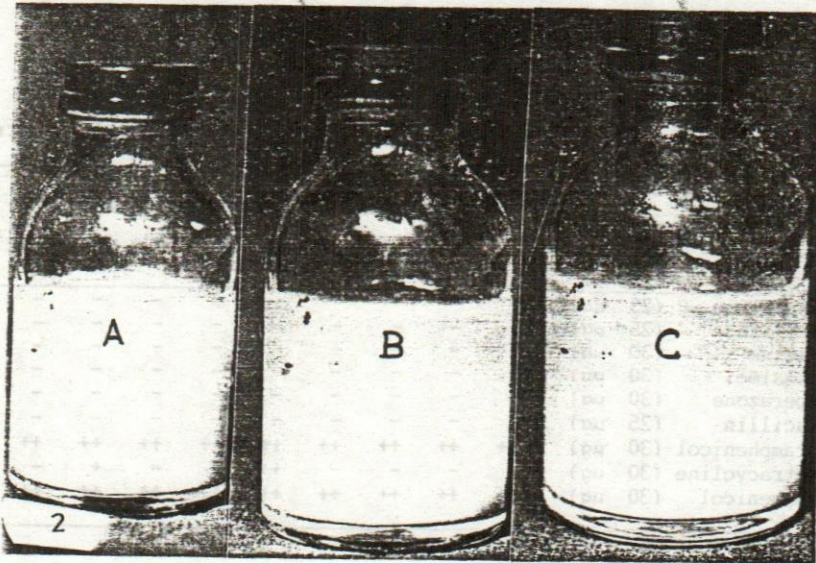


Fig. 2, 3 & 4: Secreted milk from mycoplasma mastitic buffaloes was streamed fairly normal and thereafter was separated, on standing, into two layers. The supernatant was cloudless whitish in color while the sediment was either fine granules of clotted milk like sand (Fig. 3) or large particulate clots (Fig. 4).

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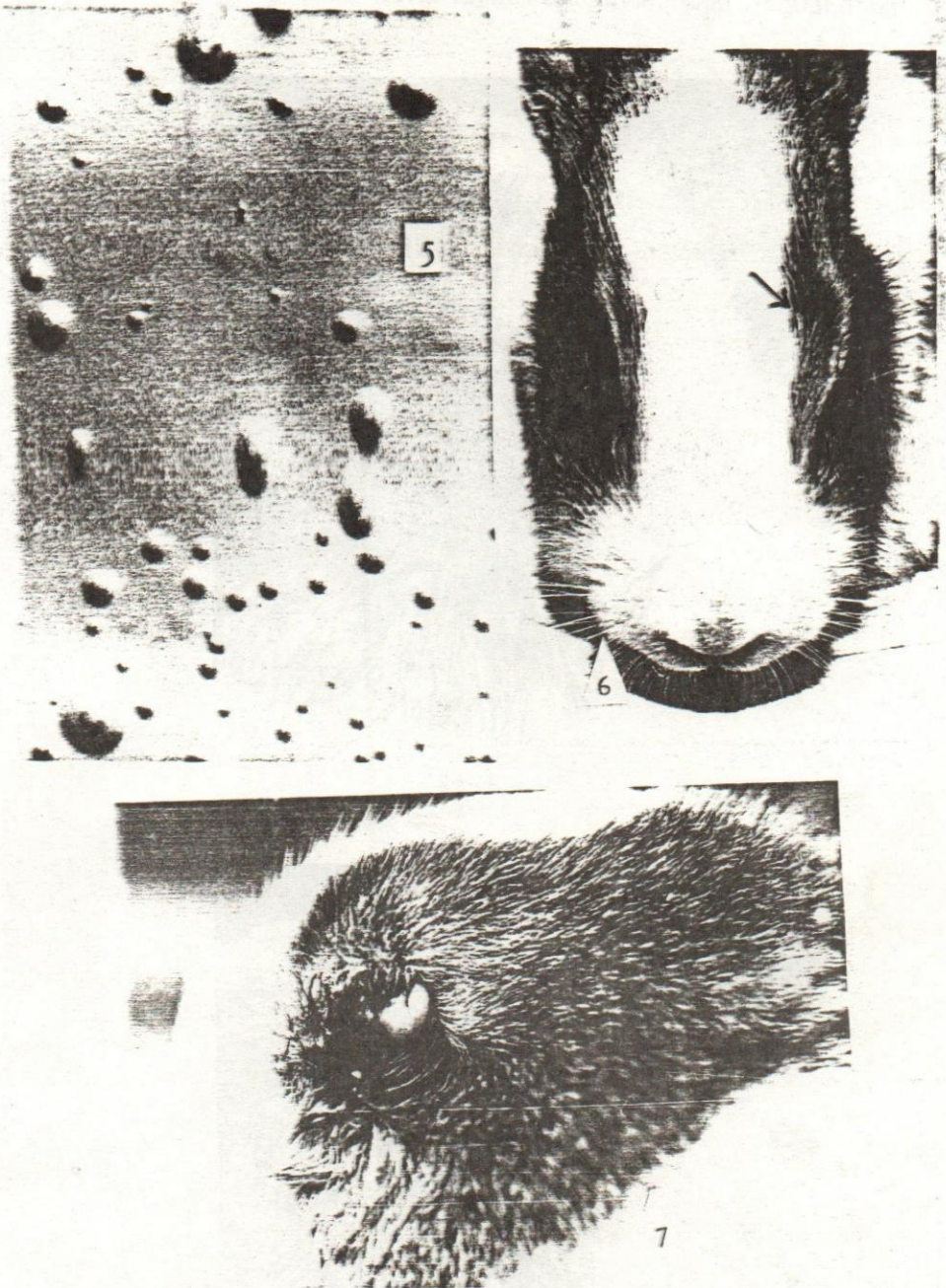


Fig. 5: Characteristic shape of mycoplasma colonies under dissecting microscope (x 40). Note the fried egg appearance (nipped colonies).

Fig. 6&7: The right rabbit's eye was used as control (Fig. 6). Severe purulent kerato-conjunctivitis in the left rabbit's eye with profuse thick exudate (Fig. 7) due to *Listeria monocytogenes* tryptose culture broth (24 h.) instillation.

BUFFALOES' MCOPLASMA BOVIGENITALIUM MASTITIS-ASSIUT



Fig. 8: The eye-lids of the instilled rabbit were markedly thickened and congested.

Fig. 9: Complete adherence of the rabbit's eye-lids was developed within 30 h.