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SOME STUDIES ON MASTITIS OF DAIRY BUFFALOES WITH A PARTICULAR EMPHASIS TO MYCOPLASMA INFECTION

(With 8 Tables and 6 Figures)

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بعض الدراسات على التهاب الضرع في الجاموس الحلوب مع تشديد خاص لعدوى الميكوبلازما

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خلال فترة البحث – من أكتوبر ١٩٩٧ إلى أغسطس ٢٠٠٠ – تم فحص عدد ١٠٨٣ جاموس حلوب–بقرى مختلفة في الشمال الشرقي والغربي لمحافظة أسيوط بصعيد مصر– لمرض التهاب الضرع. ووجد أن ٥,٨٢ % و ١٦,٧١ % من الجاموس كانت إيجابية لالتهاب الضرع الظاهري والغير ظاهري– على التوالي. تم عزل ميكوبلازما "بوفيس" من ١٧ (١,٥٧%) من الجاموس المختبر (١٠٨٣) منهم ١١ حالة أظهرت التهاب ضرع ظاهري و٦ حالات التهاب ضرع خفي. الجاموس السليم (السالب لمرض التهاب الضرع الظاهري أو الخفي) كان خاليا من الميكوبلازما. من الجائز استخدام اختبار التحليل الرأسي الكهربائي كاختبار سريع ودقيق في تصنيف الميكونلازما المعزولة. تم رصد معدل انتشار الأنواع المختلفة لمرض التهاب الضرع في مواسم الإد رار المختلفة. كانت أهم الأعراض المرضية للحيوانات الإجابية للعدوى الميكوبلازما هي ورم غير مقترن بألم أو حرارة وذو طبيعة متصلبة في الربع (أو الأرباع) المصاب الذي يدر لبنا عديم الرائحة مع تغيرات مرئية (التي تحدث عقب الادرار خلال بضع دقائق) مع عدم وجود أعراض مرضية عامة على جسم الحيوان المصاب. هذا وقد تم وصف الأعراض المرضية لأنواع التهاب الضرع المسببة بالبكتيريا. جميع الحالات المصابة بالميكوبلازما وجدت في الشمال الشرقي لمحافظة أسيوط. حالات التهاب الضرع المسبب بالميكوبلازما كانت عالية الانتشار خلال شهور الشتاء. كانت جميع العترات (البكتريا والميكوبلازما) المختبرة معمليا شديدة الحساسية لدواء الانروفلكساسين. كانت المحاولات العلاجية بالانروفلكساسين مع مضادات الالتهاب ناجحة في علاج حالات التهاب الضرع باستثناء الحالات الإكلينيكية المسببة بالميكروب العنقودي الذهبي. الحالات المريضة بالميكوبلازما بوفيس أظهرت تحسنا ظاهريا بعد العلاج بالانروفلكساسين دون القضاء إلى ذات الميكروب الذي ظل يدر في ألبانها بصورة متقطعة.

لذلك الحالات الإيجابية لعدوى ميكوبلازما بوفيس يجب استبعدها في أسرع وقت لتجنب الانتشار الأفقي واحتمال الانتشار الرأسي لهذه العدوى.

SUMMARY

From Oct. 97 to Aug. 2000, 5.82 % and 16.71 % of the examined dairy buffaloes (n = 1083) in different villages of Eastern-north and Western-north of Assiut Governorate-Egypt, were positive to clinical and subclinical mastitis, respectively. *Mycoplasma bovis (MB)* was isolated from 17 (1.57%) cases out of the tested buffaloes (n =1083); 11 cases showed severe clinical mastitis and 6 had subclinical mastitis. The normal pooled-samples were mycoplasma free. SDS-PAGE* can be used as a rapid test for identification of the isolated mycoplasma. Prevalence of mastitis in buffaloes at various lactation seasons was described. Painless and firmness gross swelling of the affected quarters yielding milk with characteristic changes, which appeared within few minutes after collection, and without systemic illnesses were the predominant findings of *MB* positive cases. Clinical signs of the bacterial mastitis were described. All *MB* positive cases were found in the villages of the Eastern-north of Assiut, and most of these cases were more prevalent during the cool-months. All tested isolates were enrofloxacin-sensitive. Therapeutic trials with enrofloxacin and anti-inflammatory drugs clinically improved the mastitic cases with exception of the clinical *Staphylococcus aureus* mastitis. The udder and milk abnormalities of *MB* positive cases were apparently reduced after enrofloxacin-therapy but *MB* were intermittently shed in their milk. The *MB* positive cases should culled as early as possible to avoid the horizontal and probably the vertical transmissions of infection.

Key words: *Buffaloes' mastitis, clinical, etiology, epizootiology & therapy.*

INTRODUCTION

Mastitis is still implicated as a serious problem of dairy animals causing a considerable level of economic losses particularly in the developing countries where the hygienic measures and the milking sanitation are often insufficient. Several bacteria belonged to class Schizomycetes particularly streptococci and staphylococci were frequently encountered as mastitis pathogens responsible for curable and/

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*: **SDS-PAGE:** Sodium dodecyl sulphate polyacrylamide gel electrophoresis

or incurable mastitis of dairy animals (Carter and Cole Jr., 1990). On the other side, mycoplasma (Gram negative microorganism, class Mollicutes) was also incriminated as one of the major non-traditional mastitis pathogens causing drug—resistant mastitis with extremely decline of the milk yield of the affected cases, and increased the culling rate of the infected herds (Bisping and Amtsberg, 1988 and Carter and Cole Jr., 1990; Gunning and Shepherd, 1996 and Mettifogo et al., 1996).

Since the original reports on mastitis due to mycoplasma infections (*Mycoplasma bovigenitallium*, in England, Davidson and Stuart, 1960 and *Mycoplasma bovis*, in Connecticut, USA, Hale et al., 1962) of dairy herds, mastitis caused by these agents became an important disease of dairy cows and buffaloes in much of the world (Pal et al., 1984; Gunning and Shepherd, 1996; and Mettifogo et al., 1996).

In Egypt, severe outbreaks of incurable mastitis of dairy Friesian herds with colossal economic losses due to *Mycoplasma bovis* and *Mycoplasma bovigenitalium* infections were recorded for the first time by El-Ebeedy et al. (1985) and Eissa (1986), respectively. Thereafter, during the last 14 years, several reports on mastitis caused by mycoplasma (*Mycoplasma bovis, Mycoplasma bovigenitallium, Mycoplasma arginini and Mycoplasma bovirhinus*) infection in dairy Friesian cows located in different localities of Egypt were published by many authors (Ahmed 1987; Zaitoun, 1990; Akl, 1993; and El-Shabiny, 1994). This may reflect an increase in spread of the mycoplasma infection throughout the Egyptian land.

In regard to buffaloes, Metry (1996) epitomized that dairy buffaloes gave more than 65 % of the total milk production in Egypt. However, reports on mastitis of Egyptian dairy buffaloes due to mycoplasma infection are apparently still brief in the available literature. Eissa (1986) carried out frequent attempts for isolation of mycoplasma from mastitic buffaloes with negative results. On the same year, Sabry and Ahmed (1986) reviewed a full document on the different types of mycoplasmas (*Mycoplasma bovis, Mycoplasma bovigenitalium, Mycoplasma arginini* and *Mycoplasma bovirhinus*) that culturally isolated from the diseased and apparently normal buffaloes in Egypt. They concluded that respiratory dysfunction, and infertility and reproductive disorders in both female and male buffaloes were the major associated diseases with mycoplasma infection. However, their review did not refer to the role of mycoplasma infection as mastitis pathogens in

the Egyptian dairy buffaloes. Such review might reveal that mycoplasma mastitis was still restricted only in the Egyptian Friesian herds on that time. Four years later, Zaitoun (1990) isolated *Mycoplasma bovis* coupled with *Mycoplasma arginini* from milk of a dairy buffalo (1 out of 49 mastitic cases, 2.04%). He also reported that no mycoplasma could be isolated from milk of the normal and the subclinically mastitis buffaloes (n=104). Thereafter, by using culturing procedure synchronized with fluorescent antibody technique, Akl (1993) found that 12.10 % and 8.7% of the clinically and subclinically mastitis buffaloes, respectively, in north-Egypt were mycoplasma (*Mycoplasma bovis* and *Mycoplasma bovigenitalium*) positive. In addition to *Mycoplasma bovis, Mycoplasma bovigenitalium* and probably *Mycoplasma arginini* as causative agents of mastitis in the Egyptian dairy buffaloes, El-Shabiny (1994) added that 33.33% of the examined mycoplasma positive cases of a dairy buffalo herd (Giza Governorate, south-Egypt) showed clinical mastitis yielded *Mycoplasma bovirhinus*. Furthermore, on the same year (1994), El-Shabiny and Abou-El-Makarem (1994) investigated 40 randomly selected diseased buffaloes with clinical mastitis in a dairy buffalo's herd located in Beni-Suef Governorate (south-Egypt). They found that 10 (25 %) cases yielded mycoplasmas in their milk. These mycoplasmas were identified as *Mycoplasma bovigenitalium* (50 %), *Mycoplasma bovis* (30%) and *Mycoplasma bovirhinus* (20 %). Such results may refer to the high prevalence of buffaloes' mycoplasma mastitis in south-Egypt and it may also reveal that *Mycoplasma bovirhinus* can be considered as additional mycoplasma beside *Mycoplasma bovis* and *Mycoplasma bovigenitalium* are responsible for mastitis in the Egyptian dairy buffaloes. However, Sabry and Ahmed (1986), Bisping and Amtsberg (1988) Carter and Cole Jr. (1990) encountered *Mycoplasma bovirhinus* as one of the respiratory mycoplasmas. The available reports on buffaloes' mycoplasma mastitis in Egypt focused principally on detection of mycoplasma infection by cultural or serological methods rather than the clinical and epizootiological descriptions of the disease in the infected buffaloes' herds only. However, the individual dairy buffaloes under the farmers' hands appear to be more denseness than that of herds in Egypt. Moreover, these reports also neglected the role of other mastitis pathogens that probably involved with mycoplasma infection. Consequently, the fundamental goals of the following work were directed to describe the subclinical and clinical mastitis of the individual dairy buffaloes with a particular emphasis to mycoplasma

infection, and to reveal some field and laboratory notes on mycoplasma mastitis in Assiut Governorate (south-Egypt). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as a rapid test for identification of the isolated mycoplasma derived from mastitis of dairy buffaloes in comparison with reference strains was achieved. Antibiotic sensitivity test of the isolated mastitis pathogens was preformed. Therapeutic trials of some diseased cases with follow up (prognosis) were also carried out.

MATERIAL and METHODS

ANIMAL:

During the period of investigation (Oct. 97 – Aug. 2000), a total number of 1083 dairy buffaloes located in different villages of Eastern-north and Western-north of Assiut Governorate (Table 5) were clinically and subclinically examined for the presence of mastitis. Clinical examinations depend mainly on visual inspection and palpation of the mammary glands for detection of clinical udder and milk abnormalities. Moreover, the general health condition and the systemic reactions of the examined animals were also monitored. For subclinical examinations, the non-clinically mastitic buffaloes were milked manually and their quarters' milk were subjected to the indirect tests (California mastitis test, CMT and/or modified Whiteside test, MWT) for detection of the subclinical mastitis cases. Owner's complaint and history of the clinical curable and incurable mastitis cases were recorded and discussed.

COLLECTION OF THE SAMPLES:

Milk samples:

The apparently normal examined buffaloes were tested for the presence of subclinical mastitis using CMT and/or MWT as the methods described by Schalm and Noorlander (1957) and Murphy and Hanson (1941) respectively. The subclinically mastitis—positive cases were sampled as a composite sample for each infected case included one or more quarter(s) and subjected to bacteriological culturing with minimum of delay. Thereafter, these collected milk samples were stored in - 20°C till the time of mycoplasmal analysis. On the other hand, a composite milk sample of each clinically mastitis case was collected and subjected primarily to bacterial and mycotic culturing, and thereafter stored in the deep freezer temperature till mycoplasmal analysis. The remained buffaloes (clinically and subclinically mastitis negative cases) were also

sampled as pooled samples (each pooled sample for 4 – 5 cases) and tested for the presence of mycoplasma.

LABORATORY PROCEDURES:

Bacteriological examinations:

Brain heart infusion agar (BHIA, Gibco) containing 5 % sheep blood were prepared and used for bacterial isolation. Biochemical identification of the purified suspected colonies were carried out according to the methods described by Carter and Cole Jr. (1990).

Mycological examinations:

BHIA and Sabouraud's dextrose agar (Oxoid) plates supplemented with antibiotics (penicillin and streptomycin) were used for isolation of mycotic agents. These plates were incubated at 37°C and at 25°C for 2-3 week with daily observation, respectively. The isolates were morphologically and biochemically identified according to the methods described by Carter and Cole Jr. (1990). The mycological analysis focused principally on the pathogenic agents and discounted the saprophytes.

Mycoplasma examinations:

The time interval between samples collection and mycoplasma analysis was 1 - 2 weeks. The stored milk samples were thawed, and 0.2 ml. of each thorough mixed sample was inoculated into Hayflick liquid medium (Bisping and Amtsberg, 1988) and incubated, and thereafter cultured onto Hayflick agar plates and incubated in 5 % Co_2 atmosphere. The characteristic smooth grains fried egg-like colonies were picked and purified, and stored at -80°C. in Dept. of Mycoplasma, Animal Health research Institute, Dokki, Giza-Egypt until the time of identification. Bacterial irreversibility, genus differentiation, and biochemical identification of the purified strains were achieved according to the criteria of Bisping and Amtsberg (1988). The biochemically identified mycoplasma strains were thereafter subjected to serological identification by using growth inhibition test according to the methods reported by Clyde (1964). Reference antisera against *Mycoplasma bovis* and *Mycoplasma bovigenitallium* were kindly supplied by Dr. S. J. Geary, Dept. of pathobiology, University of Connecticut, U.S.A.

Milk smears of the mycoplasma-tested samples were prepared and stained by Giemsa's and Gram's stains, and examined under oil-immersion lens of light microscope (X 1250).

Cell protein electrophoresis procedure:

Cell protein electrophoresis procedure was used as a rapid test for characterization of mycoplasmas derived from mastitis in buffaloes and compared with the reference strains.

1- Reference strains:

Mycoplasma bovis (Donetta strain) and *Mycoplasma bovigenitallium* (PG 11 strain) reference strains were obtained from Dr. S. J. Geary, Dept. of pathobiology, University of Connecticut, U.S.A. and subjected to SDS-PAGE.

2- Field strains:

Randomly some strains of the isolated mycoplasma, which previously serologically identified by growth inhibition test, were subjected to SDS-PAGE.

3- SDS-PAGE technique:

Preparation of SDS-PAGE antigen was prepared (Thirkell et al., 1990) and SDS-PAGE technique were carried out according to the methods described by Laemmli, (1970).

Antibiotic sensitivity test:

Some strains of bacterial and mycoplasmal isolates were tested to the different members of antibiotics (BioMerieux, France) that commonly used in the veterinary field by disc diffusion methods. Muleller-Hinton medium (BioMerieux, France) supplemented with and/or without normal sterilized horse serum (El-Agouza Institute, Giza-Egypt) used to bacterial isolates, and Hayflick medium to the tested mycoplasma strains. Results of the antibiotic sensitivity test were interpreted according to the guidelines of National Committee for Clinical Laboratory Standards (1984) and to Carter and Cole Jr. (1990).

Therapeutic trials and follow up:

Lines of the therapeutic trials of some mastitic buffaloes due to mycoplasma infection or other mastitis pathogens were carried out with the choice drug according to the results of the antibiotic sensitivity test (Table 8). The therapeutic course persisted for 5 successive days. The treated cases were subjected thereafter to clinical examinations synchronized with re-isolation of the mastitis pathogens for a forecast of the probable course and termination of a disease. Two occasions with 10 – 15 day's intervals for re-isolation of the mastitis pathogens were carried out.

Localities and seasonal influences:

Localities and seasonal influences on the prevalence of mycoplasma mastitis of the examined buffaloes were studied.

Statistical analysis:

Statistical analyses were carried out according to Milton and Tsokos (1983).

RESULTS

Prevalence (% affected) of clinical and subclinical mastitis:

Sixty-three (5.82 %) and 181 (17.46 %) cases out of the examined buffaloes (n = 1083) showed clinical and subclinical mastitis, respectively, (Table 1). Prevalence of the different types of the clinical and subclinical mastitis (according to the isolated microbial pathogens, Table 2) at various lactation seasons of the tested buffaloes was illustrated in Figures 1 and 4.

Mycoplasma mastitis:

Seventeen cases (1.57 %) out of the tested buffaloes (n =1083) yielded *Mycoplasma bovis* in their milk; 11 cases showed severe clinical mastitis and 6 had subclinical mastitis (Table 3). The tested normal samples were mycoplasma free (Table 3). Of these mycoplasma positive buffaloes, 35.29 % yielded *Mycoplasma bovis* in a pure culture, and 64.71 % yielded *Mycoplasma bovis* coupled with coagulase negative staphylococci (Table 4). Neither pathogenic fungi nor yeast could be isolated from the mycologically examined mycoplasma positive samples.

Fig. D shows the electrophoretic pattern (SDS-PAGE) of the tested mycoplasma strains (n = 10) and the two reference strains (Donetta strain), on 10 % polyacrylamide gel with coomassie stain. The majority of proteins were ranged between 37000 – 75000 molecular weight. A high degree of similarity between the most strains was established with minor differences confined to the lower portion of the gel (molecular weight region of 19 – 27 KDa).

Clinical findings:

The whole affected quarter including the teat of the clinically mycoplasma positive case was markedly swollen with complete disappearance of the annular fold. On posterior inspection, size of the swelled quarter was more than triple as large as the opposite unaffected one and its shape was similar to the inverted conical flask with short pointed neck, where the teat was "invaginated" in the swelled quarter (Fig. A). By palpation, there were no hotness or pain of the infected

quarter and it consistency was similar to a plastic bag filled with extra amount of the pressed spongy material. Teat orifice of the ill quarter was relaxed with dripped milk. The mammary lymph nodes of the infected cases appeared to be normal. The streamed milk of the positive *Mycoplasma bovis* mastitis buffaloes was apparently normal immediately after collection, but it lacks the characteristic odor of the buffalo's milk. Within few minutes post collection, the sampled milk was gradually separated into two layers on standing position (Fig. B_1 and B_2). The supernatant was watery and colorless in 7 cases, and was thickly and yellowish in the remaining cases (n = 10). The sediment of all infected cases contained white flakes that deposited onto the bottom or adhered on the wall of the collected vials. Neither offensive odor nor abnormal contents like blood were macroscopically noticed in the positive *Mycoplasma bovis* samples. The *Mycoplasma bovis* positive cases were not systematically ill and they continued to eat and drink well. The prepared milk smears failed to demonstrate Mollicutes microorganisms under 1250-magnification power but it showed extra amount of mononuclear and polymorphnuclear cells. Gram's positive staphylococcus clusters were demonstrated in 9 out of 11 milk smears. History taken with the owners elicited that the clinically infected buffaloes with *Mycoplasma bovis* did not respond to various types of anti-mastitis drugs with drastic decline in milk yield, while the subclinically infected cases showed decrease of the milk yield about 2.5 – 4 liter per day. They also added that the milk of the subclinically infected buffaloes failed in manufacturing of "green (kahries)" cheese (useless milk) referring to the economic losses.

On the other side, subacute purulent mastitis with variable degree of pain and without marked systemic reactions were the prominent clinical findings of mastitis yielding *Staphylococcus aureus*, either alone or coupled with streptococci. The secreted milk of these cases had a pussy odor and it was yellowish in color.

Remarkable inflammatory pain and hotness of the affected quarters were the characteristic findings of clinical *E. coli* mastitis. These quarters were moderately swollen; purplish pink in color, and yielded odorless watery milk contained reddish flakes. Anorexia, pyrexia ($40 \pm 0.2^{\circ}C$, average), tachycardia (83 ± 6 / min.) and the respiratory rate ranged from 28 to 36 per minute were the predominant systemic illness in the diseased cases with *E. coli* mastitis. Slightly ejection of diarrhea soiled the hind udder' quarters were noticed in two cases.

Localities influence on the prevalence of mycoplasma mastitis:

Table 5 revealed that the different localities in the Western-north and Eastern-north villages of Assiut were not a governing factor on the prevalence of clinical and subclinical mastitis of the examined buffaloes. However, all mycoplasma mastitic cases were found in the Eastern-north villages (Table 5).

Seasonal influence on the prevalence of mycoplasma mastitis:

Table 6 summarized the seasonal influence on the prevalence (% affected) of mastitis of dairy buffaloes in Assiut Governorate. It was found that the percentage of infection with bacterial mastitis was apparently higher in the cool months (24.67 %) than the summer months (19.74 %). Such variation was statistically insignificant ($P>0.05$). Mycoplasma mastitis was highly significantly ($P<0.01$) prevalent during the cool months (Table 6).

Antibiotic sensitivity test:

Table 7 revealed that all tested isolates were highly sensitive to enrofloxacin (one of the quinolon groups) with exception of one strain (N°. 3) of *Mycoplasma bovis* was resistant. Sixty percentage and 75 % of the tested Mycoplasma *bovis* and *Staphylococcus aureus* strains were oxytetracycline-resistant, respectively. The tested *E. coli* isolates were generally resistant to the applied antibiotics with exception of enrofloxacin and chloramphenicol.

Therapeutic trails and prognosis:

Regarding groups A and B, two weeks post the start of the therapeutic trial (Table 8); the mycoplasma positive cases showed mildly reduction in the local swelling of the affected quarter(s). However, the milk yield was still watery with grossly changes, and mycoplasma was isolated from one case, whereas staphylococci were disappeared. Two week later, the affected quarter(s) of the mycoplasma positive cases showed great reduction of the swelling and the gross changes of the secreted milk were largely reduced but it was still watery. Mycoplasma (on this trial of re-isolation) was isolated from 3 cases, including the previous positive case, of the apparently recovered cases (unfavorable prognosis). Staphylococci were still disappeared.

Concerning group C (clinical *staphylococcus aureus* mastitis, Table 8), one week post treatment, enrofloxacin did not reduce the clinical abnormalities of the infected quarters and their milk was became more thickness yellowish pussy discharge with repulsive odor. The skin

of the teat of one case was chapped. *Staphylococcus aureus* but not streptococci could be isolated from their milk (bad prognosis). Conversely, enrofloxacin was supreme in curing the subclinical mastitis caused by *Staphylococcus aureus* mixed with streptococci (group D, Table 8) and their milk gave negative results to MWT and to the bacteriological examinations (excellent prognosis).

Enrofloxacin with supportive treatments and frequent evacuation of the udder were successful to reduce the remarkable udder and milk abnormalities of *E. coli*-mastitic buffaloes (group E, Table 8). The causative agent(s) could not be re-isolated. Moreover, the systemic illnesses of these cases were declined. However, the reduced amount of milk yield was the main owner's complaint. Three weeks post treatment, the affected quarters were apparently normal during palpation and their milk was restored to the normal appearance. The general health condition of the diseased cases returned to the normal. Prognosis is favorable with some reservations where the amount of the milk yield per day was reduced.

DISCUSSION

The obtained results revealed that the most cases of clinical and subclinical mastitis of the examined dairy buffaloes occurred during the beginning months of the first lactation season (Table 1 and Fig. 1) suggesting the increased susceptibility of the primiparous buffalo to the intramammary infection. This may attributed to the decreased number and killing capacities of the phagocytes (cellular barriers) that occurred shortly after calving of the primiparous lactating animals (Kehrli et al., 1989a, Kehrli et al., 1989b and Weller and Davies, 1998). Rapid diapedesis of sufficient amount of phagocytes from blood into milk considered the major defense mechanism of the mammary gland against the invading pathogens (Rebhun et al., 1995). On the other side, the highly significantly decreased ($P<0.01$) prevalence of mastitis (Fig. 2 and 3) of the aged dairy buffaloes (multiparous) may ascribed to the increased numbers of the phagocytes in their milk (Weller and Davies, 1998). They concluded that the somatic cell counts (phagocytes) were markedly increased with advancing age and stage of lactation of the dairy animals.

Mastitis due to *Staphylococcus aureus* either alone or coupled with streptococci (Table 2 and Fig. 4) was the predominant type of mastitis of the examined buffaloes at the various lactation seasons. Poorer management precautions during pre- and post-milking were

usually the contributing factor of the examined buffaloes in the different villages, and the most owners has no knowledge concerning post milking teat dips with a germicidal solution. Blowey and Edmondson (1998) reported that teat-dipping germicidal solutions killed the majority of organisms that colonized the teat canal before invading the udder like staphylococci and the most strains of streptococci, and they also prevented the further colonization. Consequently, the higher prevalence of staphylococcus and streptococcus mastitis may interpreted (Fig. 5). *E. coli* and the other coliform bacteria are soil-borne infections and they did not colonize the teat canal (Blowey and Edmondson, 1998). Therefore the possibilities of spread of infection from animal to another become low, and this may interpret the less frequently occurrence of *E.coli*-mastitis (Fig. 5).

The microbiological analysis of the examined samples (Table 2) declared that 10.25 % of the mastitic buffaloes were culturally negative. This may attributed to several probable reasons including (a) too few mastitis pathogens to be detected; (b) haphazard application of anti-mastitic drugs without detection of the causal agents and (c) antibiotic residues may temporarily inhibit the mastitis pathogens. Furthermore, it was also probable that the mastitic pathogens inhibited or killed after the sample was collected but before culturing (Zorah et al., 1993).

From the epizootiological point of view, mycoplasmal analysis (Table 3) referred to the lower prevalence (1.57 %) of *Mycoplasma bovis* mastitis in the individual dairy buffaloes under field circumstances. This may ascribed to the decreased possibilities of spread of infection from animal to another. Contrariwise, the results obtained by El-Shabiny (1994) and El-Shabiny and Abou-El-Makarem (1994) concluded that mycoplasma mastitis was a drastic problem for the investigated buffaloes' dairy herds in Giza and Beni-Suef Governorates, respectively. They found that 30 % and 25 % of the culturally examined samples were positive to three pathogenic types of mycoplasmas including *Mycoplasma bovis*, respectively. Although the prevalence of *Mycoplasma bovis* mastitis of the individual buffaloes is low, special precautions should be taken to avoid the horizontal and vertical transmissions of that infection. Gonzalez et al. (1992) reported that the field practitioners were responsible for spreading the infection to the other areas; mycoplasma was cultured from a metal syringe and from treatment material being dispensed. Experimentally, Pfutzner and Schimmel (1985) concluded that *Mycoplasma bovis* was vertically

transmitted from the infected udder of the pregnant cow to the fetus and to the respiratory system of the newborn calf in which it remain infective up to sexual maturity. Pfutzner et al. (1981) reported that transmission of mycoplasma mastitis could be occurred by contact between animals at markets, both of dams and their offspring, and by means of bull semen. The latter mode may be refers to the hematogenous spread of mycoplasma infection from the deposition site to the udder. Bennett and Jasper (1977) experimentally incriminated the hematogenous spread of *Mycoplasma bovis* infection from the inoculated quarter to the non-inoculated quarters of the previously non-infected cows. In Egyptian villages, the most villagers are still prefers the natural mating than artificial insemination for pregnancy. Sabry and Ahmed (1986) reported that 40 % of the examined buffaloes-bulls that bred for natural service were harbor different types mycoplasmas including *Mycoplasma bovis* in their prepuces and semen. However, intra-mammary infections of bovine udders through entrance of mastitis pathogens via teat orifice are still the major mode of infection (Rebhun et al., 1995).

The bacteriological analysis of mycoplasma positive cases (Table 4) indicated that 64.71 % of these cases yielded *Mycoplasma bovis* in association with non-pathogenic or possibly mild pathogenic staphylococci (Bisping and Amtsberg, 1988 and Carter. and Cole Jr, 1990). Such association seems to be a synergistic situation between mycoplasma and bacteria. Probably, the presence of bacteria may induce a favorable microenvironment for growth or overgrowth of mycoplasma infection in the udder tissue. Wienhus and Kirchhoff (1984) found that the smallest detectable number of *Mycoplasma bovis* in milk was 5 colony-forming units (CFU) in the absence of bacteria, or 50 CFU (10 times more) in the presence of bacterial contaminants.

Results of SDS-PAGE (Fig. D) shows the similarity between the protein patterns of the field mycoplasma strains and *Mycoplasma bovis* reference strains with minor difference. Such difference may attribute to the ecological and climatic variations. The agreement percentage between the results of SDS-PAGE and growth inhibition test with specific antiserum was 100 %. This may conclude that SDS-PAGE can be use as a satisfactory and rapid test (within hours) for classification of unknown mycoplasma isolates.

From the clinical point of view, painless grossly diffuse swellings of the whole quarters with characteristic changes of their milk without systemic illnesses were the prominent signs of *Mycoplasma bovis*

mastitis. Similar clinical findings could not be traced in the available literature of buffaloes' *Mycoplasma bovis* mastitis. However, similar findings in cows were fairly reported by Eissa (1986), Bisping and Amtsberg (1988), and Mettifogo et al. (1996) with some clinical differences. Number of the affected quarters appeared to be the major clinical difference between the infected cows and buffaloes. In the latter, one or two quarters were found to be ill whereas in the infected cows the four quarters were commonly involved. Gross changes in milk of the clinical *Mycoplasma bovis* positive cases were remarkable suggesting changes in the physical characteristics and chemical compositions of milk. These grossly alterations were apparently not explored in *Mycoplasma bovis* positive-buffaloes. However, Ruffo and Resmini (1971) investigated the chemical alterations of milk of *Mycoplasma bovis* and *Mycoplasma arginini* mastitis in cows. They found that both organisms induced reduction in fat, lactose, α-lactalbumin and β-lactoglobulin contents, and increased in the casein nitrogen (insoluble compound), sodium, chloride and blood serum albumin. Consequently the lake of odor and the gross changes of milk of the mycoplasma positive cases may explained. The yellowish coloration of the supernatant layers of some milk samples ascribed to the involvement of staphylococci with mycoplasma.

On the other side, purulent mastitis without systemic reactions were the characteristic findings of mastitic buffaloes due to *Staphylococcus aureus* either alone or coupled with streptococci. Conversely, remarkable inflammatory pain of the affected quarter(s) with systemic reactions including pyrexia was the predominant signs of *E. coli* mastitis. Such variations may attributed to the endotoxemic condition induced by *E.coli* (Oz et al., 1985). Consequently, it is suggest that, in the absence of the laboratory facilities, signs of severe local reactions of the affected quarter(s) associated with systemic illnesses including pyrexia may be consider a valuable guide to differentiate between mastitis caused by Gram's negative bacteria and that caused by Gram's positive one. White et al. (1987) suggested that rectal temperature could be used, as a predictor to revealed the cause of mastitis was Gram's positive or Gram's negative bacteria.

Concerning environment, the obtained results illustrated in Table 5 indicated that there was no significant variations ($P>0.05$) between the prevalence of mastitis in buffaloes that located in the Western-north villages than those located in the Eastern-north villages. However, all

Mycoplasma bovis mastitis cases were found in the Eastern-north villages. Such distribution could not be accounted. However, according to the nature of land, the Eastern-north villages are ecologically classified into cultivated areas parallel to the River Nile and its branches, whereas the western-north villages are desert or semi-desert areas and appears to be more dryness. Pfutzner (1984) and Rosenbusch (1994) concluded that the non-dryness and moisture environment increased the survival period and the resistance of *Mycoplasma bovis*. Consequently, the dryness areas may consider a hostile environment for spread of mycoplasma infection.

Regarding the seasonal influence, Table 6 and Fig.6 revealed that the prevalence of *Mycoplasma bovis* mastitis of the examined animals was highly significantly increased ($P<0.01$) during the cool months (non-rainfall-months) in Assiut Governorate. Experimentally, Pfutzner (1984) concluded that the moisture-lowest temperature (below 20°C) media activated and increased the survival period of *Mycoplasma bovis*. Naturally, Gonzalez et al. (1992) found that the highest frequency of *Mycoplasma bovis* mastitis occurred during winter season, decreased in mid spring and thereafter sharply declined during the summer months. This may reveal that the cool environment is act as a favorable media for growth and spread of *Mycoplasma bovis* infection.

Results of the antibiotic sensitivity test and the therapeutic trials of some diseased cases with mycoplasma revealed that enrofloxacin was successful in reduction of the clinical abnormalities of the infected cases. Similarly, Pulgiese et al. (1994) concluded that enrofloxacin supramammary-injection was superior in comparison with intra-muscular route to eliminate the majority of mastitis pathogens including mycoplasma infection. The results of re-isolation trials revealed that *Mycoplasma bovis* was intermittently shed from the treated cases. Intermittent shedding of *Mycoplasma bovis* infection considered one of the major problems of the infected dairy animals and herds (Gonzalez et al., 1992 and Gunning and Shepherd, 1996). Failure elimination and the intermittent shedding of *Mycoplasma bovis* from the infected cases may ascribed to the intra– and intercellular parasitism of this microbe (Stanarius et al., 1981 and Rosenbush, 1994). Stanarius et al. (1981) found that some of *Mycoplasma bovis* microorganisms were within the phagocytes and they possessed a great coat on the outer part of the unit membrane. This coat may be act as a protective cover.

On the other side, the results of therapeutic trials of the diseased buffaloes with bacterial mastitis suggested that enrofloxacin associated with anti-inflammatory drugs appears to be the choice treatment. However, clinical *Staphylococcus aureus* mastitic cases were incurable. *Staphylococcus aureus* (pus-producing bacteria) mastitis was poorer treatment due to the degenerative changes of the mammary tissues and the pussy material was act as a protective cover against the antibiotics (Carter and Cole Jr., 1990 and Rebhun et al., 1995). Conversely, enrofloxacin was superior in treating of the subclinical *Staphylococcus aureus* mastitis.

In conclusion prevalence *Mycoplasma bovis* mastitis of the individual buffaloes under field conditions in Assiut Governorate appeared to be non-problematic. However, mycoplasma mastitis positive cases should culled as early as possible to avoid the horizontal and probably vertical transmissions of that disease. Buffaloes shows painless and non-hotness gross swellings of their quarters (which yield abnormal milk) without systemic illnesses and they did not respond to treatment, mycoplasma mastitis should be suspected. Staphylococcus and streptococcus mastitis were frequently still the major mastitis. *E. coli* mastitis were less frequently occurred.

It is worthily to report that milk of the subclincally mastitis buffaloes appeared to be like normal, and some villagers prefer drink the milk as raw milk without sterilization. From the public health point of view, Bisping and Amtsberg (1988) reported that some staphylococci causing mastitis may produced entrotoxin that causes food poisoning for human consumption. The zoonotic importance of *Mycoplasma bovis* appears to be still obscure. However, Carter and Cole Jr. (1990) declared that mycoplasmas were mostly host-specific but *Mycoplasma bovis* was isolated from human patients with respiratory disease. They also declared that because of the pathogenicity of mycoplasmas of animal origin for laboratory personnel was unknown, all reasonable precautions should be taken to minimize self-exposure. Such declarations may give warning from the probabilities of transmission of this microbe from animal to man.

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Table 1: Prevalence (% affected) of the clinical and subclinical mastitis of the examined dairy buffaloes during different lactation seasons.

Lactation seasons	Number of the examined animals	Clinically mastitic buffaloes (CMBs) and quarters(Qs)			Subclinically mastitic buffaloes (SCMBs) and quarters(Qs)		
		Number of CMBs	Number of affected Qs#	% of infection to all mastitic cases	Number of SCMBs	Number of affected Qs#	% of infection to all mastitic cases
1st	240	31(12.92%)@**	34 (3.54 %)	49.20 %+	87 (36.25 %)**	94 (9.79 %)	48.07 %+
2nd	237	17(7.17 %)	21 (2.21 %)	26.98 %	41 (17.30 %)	46 (4.85 %)	22.65 %
3rd	245	9 (3.46 %)	10 (1.02 %)	14.29 %	33 (13.47 %)	38 (3.88 %)	18.23 %
4th	215	5 (2.32 %)	5 (0.58 %)	7.94 %	11 (5.12 %)	16 (1.86 %)	6.08 %
5th and >5th	146	1 (0.68 %)**	1 (0.17 %)	1.56 %	9 (6.16 %)	10 (1.71 %)	4.97 %
Total	1083	63 (5.82 %)	71 (1.64 %)		181 (16.71 %)	204 (4.71 %)	

@: Most cases of the clinical and subclinical mastitis were between the first and third months of the first lactation (primiparous buffaloe).

**: Highly significant variation ($P < 0.01$).

+: Clinical and subclinical mastitis of the examined buffaloes are more prevalent (highly significant increase, $P < 0.01$) during the first lactation seasons than other seasons.

#: Rear quarters appeared to be more prominent affected quarters than the others are.

Table 2: Microbiological analysis of the collected milk samples from the clinical (n = 63) and subclinical (n = 181) mastitis of the examined dairy buffaloes.

Isolated Organism	Clinical mastitis		Subclinical mastitis		% to all mastitic cases (n= 244)
	Number of cases.	% to all clinically mastitic cases.	Number of cases.	% to all subclinically mastitic cases.	
Staphylococci#	21	33.33 %	62	34.42 %	34.02 %
Streptococci@	9	14.28 %	51	28.18 %	24.58 %
Staphylococci & Streptococci	12	19.05 %	42	23.20 %	22.13 %
Mycoplasma*	11	17.46 %	6	3.31 %	6.97 %
Coliform**	4	6.35 %	1	0.55 %	2.05 %
N.C.$	6	9.52 %	19	10.50 %	10.25 %

#: 73 % out of the isolated staphylococcus strains were coagulase positive *stayphlococcus aureus.*

@ ; *Streptococcus agalactia was* the predominant (89.5 %) isolated streptococci.

*: *Mycoplasma bovis* was isolated either alone with other microbial agents.

**: *E. coli* coupled with/without other unidentified gram-negative bacteria.

S: Negative culture, mastitis pathogen(s) could not be isolated.

Table 3: Prevalence of *Mycoplasma bovis (MB)* mastitis of the examined dairy buffaloes.

Number of the examined buffaloes	Clinical mastitis			Subclinical mastitis*			Normal cases+	
	Number of cases	Number of the positive cases yielded *MB***	% of infection with *MB* to all clinical mastitic cases	Number of cases	Number of the cases yielded *MB***	% of infection with *MB* to all subclinical mastitic cases	Number of cases	Number of positive cases yielded *MB*
1083	63 (5.82%)$	11 (1.02 %)$	17.46 %	181 (16.71%)$	6 (0.55 %)$	3.31 %	839	-

*: Scores of the indirect test (CMT) ranged from +2 to +3.

+: Pooled-sampling system was carried out (each pooled sample for 4 or 5 cases).

**: *Mycoplasma bovis* was culturally isolated either alone or coupled with other microbial agents.

S: This percentage between the parenthesis is the percentage to the examined buffaloes (n = 1083).

-: Mycoplasma could not be isolated.

Table 4: Frequent distribution of the isolated microorganisms with *Mycoplasma bovis* mastitis of dairy buffaloes (n = 17).

Microorganisms	Number of cases	% to all positive
Mycoplasma bovis	6	35.29 %
Mycoplasma bovis & Coagulase negative staphylococci.	11	64.71 %

Table 5: Ecological distribution of *Mycoplasma bovis* mastitis of buffaloes in different villages of Assiut Governorate-Upper Egypt.

Locality	Number the examined animals	Clinical mastitis			Subclinical mastitis		
		Number Of the mastitic cases	Chi-square value (χ^2)	Number of Cases yielded *Mycoplasma bovis*	Number of the mastitic cases	Chi-square value (χ^2)	Number of Cases yielded *Mycoplasma bovis*
Eastern North[@] Villages	686	45 (6.56 %)	1.88[+]	11 (1.60 %)	125 (18.22%)	3.06[#]	6 (0.87 %)
Western north[$] Villages	397	18 (4.53 %)		--	56 (14.10%)		--

@ : Eastern-north villages, Wa'lidia, Kom-Abu-chial, Bani-mour, Abnoub, Abnoub-El-Ham'mam.
$: Western-north villages, Gahdam, Bani-Ghaleb, Bani-Adi, and Awlad-Eliew.
+: Non-significant variation (P > 0.05).
#: Non-significant variation (P > 0.05).
--: *Mycoplasma bovis* could not be isolated.

Table 6: Seasonal influence on the prevalence of *Mycoplasma bovis* mastitis of dairy buffaloes in Assiut Governorate.

Months	Climatic-temperature+ (Mean ± SD)	Number of the examined buffaloes++	Number of mastitic cases	*Mycoplasma bovis* positive cases
Cool-months@	19.58 ± 3.36 °C	612	151[a] (24.67 %)	15[c] (2.45 %)
Hot-months#	35.42 ± 4.89 °C	471	93[b] (19.74 %)*	2[d] (0.42 %)**

+: Mean climatic temperature obtained from Meteorological station, Faculty of Agriculture, Assiut University.
++: Mastitic cases included the clinical and subclinical forms (n = 244).
@: Cool-months; the 2nd half of October ~ the end of Marsh.
#: Hot-months; April ~ the 1st half of October.
χ^2 (a • b) = 3.70 , non-significant difference (p > 0.05).
χ^2 (c • d) = 7.29 , highly significant difference (p < 0.01)**.

Table 7: Antibiotic sensitivity test of some isolated strains.

Antibiotic disc	*Mycoplasma bovis* Tested strains					Staph. aureus Tested isolates				CAN-Staph. Tested isolates			Streptococci. Tested isolates			E. coli Tested isolates	
	1	2	3	4	5	1	2	3	4	1	2	3	1	2	3	1	2
Pen.	no	no	No	no	no	r	r	r	r	s	r	s	s	ss	ss	r	r
Amoxy.	r	r	r	r	r	s	ss	s	ss	s	ss	ss	ss	sss	sss	s	r
Oxy.	s	r	r	r	s	r	r	ss	r	ss	sss	sss	sss	sss	sss	s	r
Chlo.	ss	ss	s	s	ss	sss	sss	s	sss	sss	sss	sss	sss	sss	sss	ss	ss
Enro.	ss	sss	r	ss	sss	sss	sss	sss	sss	sss	sss	sss	sss	sss	sss	sss	sss
Ery.	s	ss	s	r	r	ss	s	r	s	s	ss	ss	ss	s	ss	r	r
Strepto.	s	s	r	r	s	no	no	no	no	no	no	no	no	no	no	s	r

CAN-staph: coagulase negative staphylococci .
Pen.: Penicilline G sod. (30 IU). Amoxy.: Amoxycilline (10µg). no: not tested.
Chlo.:Chloramphenicol (30µg). Enro.: Enrofloxacin (10µg) Oxy.: Oxytetracycline (30µg).
Strepto.: Streptomycin (10µg) Ery.: Erythromycin (15µg).
r: Resistant strain.
s: Intermediate sensitivity, the inhibition zone ranged from 12 to 18 mm.
ss: Sensitive, the inhibition zone ranged from 19 to less than 23 mm.
sss: Highly sensitive, the inhibition zone was more than 23 mm.

Table 8: Therapeutic trials of some mastitic cases of the examined buffaloes.

Group/ Status	Number of cases	Isolated organisms	Therapeutic lines
(A) Clinical mastitis	3	*Mycoplasma bovis*	1-Enrofloxacin: 10 mg / Kg B.W., IM /day, and 75 mg / IQ / twice daily. 2- Flunixin meglumine: 1.5 mg / Kg B.W., IM / day, and 100 mg / IQ / twice daily. 3- Chymotrypsine (Alphachemotrypsin, Lemquin Co.) : 10 mg., IM / head/ daily.
(B) Clinical mastitis	2	*Mycoplasma bovis* Staphylococci	1-Enrofloxacin: 10 mg / Kg B.W. IM /day, and 75 mg / IQ / twice daily. 2- Flunixin meglumine: 1.5 mg / Kg B.W., IM / day, and 100 mg / IQ / twice daily. 3- Chymotrypsine: 10 mg. IM / head/ daily.
(C)Clinical mastitis	3	*Staphylococcus aureus* Streptococci	1-Enrofloxacin: 10 mg / Kg B.W. IM /day, and 75 mg / IQ / twice daily. 2- Flunixin meglumine: 1.5 mg / Kg B.W., IM / day, and 100 mg / IQ / twice daily. 3- Chymotrypsine: 10 mg., IM / head/ daily.
(D) Subclinical mastitis	2	*Staphylococcus aureus* Streptococci	1-Enrofloxacin: 10 mg / Kg B.W. IM /day and 75 mg / IQ / twice daily. 2- Flunixin meglumine: 1.5 mg / Kg B.W., IM / day, and 100 mg / IQ / twice daily.
(E) Clinical mastitis	2	*E. coli* Gram negative bacteria	1- Fluid therapy: NaCl 0.9% + Dext. 5%, 20ml / Kg B.W., IV / daily. 2-Enrofloxacin: 15 mg / Kg B.W. IM /day, and 75 mg / IQ / twice daily. 3- Flunixin meglumine: 1.5 mg / Kg B.W., IM / day, and 100 mg / IQ / twice daily. 4- Chymotrypsine: 15 mg., IM / head/ daily. 5- New-Diaclean (oral, anti-diarrheal drug): Two sachets / head/ daily

IM : Intra-muscular. IQ: Infected quarter. Enrofloxacin: (Enro-Flox, 10% w/v), VETWIC, produced by EL-Naser for Pharmaceutical chemicals Co.
Flunixin meglumine:: (Finadyne) Non-steroid anti-inflammatory drug, produced by Schering Plough Animal Health. IV: Intravenous.
New-Diaclean: products of Pharma Swede-Egypt (under license of AVICO-Jordan.)

Fig. 1 : Prevalence of clinical (CM) and subclinical (SCM) mastitis of dairy buffaloes during the different lactation seasons.

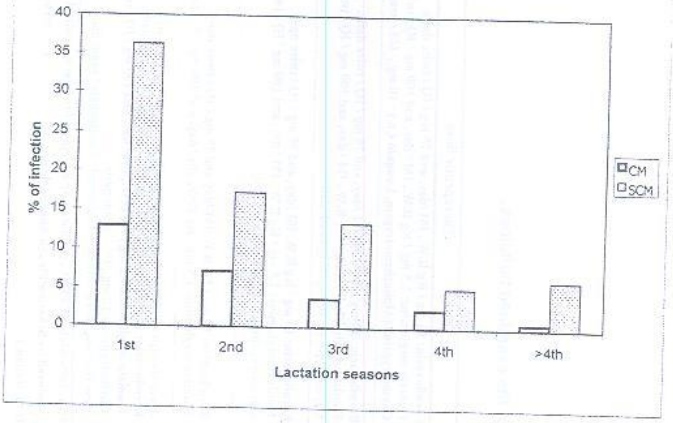


Fig. 2 : Regression line of the prevalence of clinical mastitis of the examined buffaloes on the different lactation seasons (age).

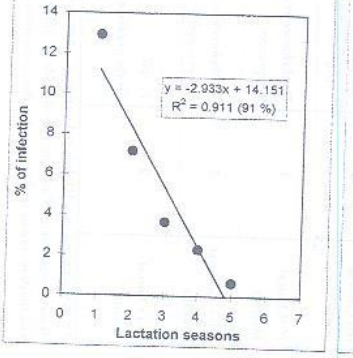
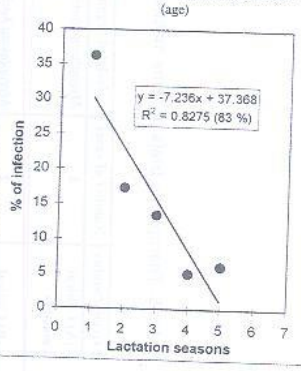


Fig. 3: Regression line of the prevalence of subclinical mastitis of the examined buffaloes on the different lactation seasons (age)



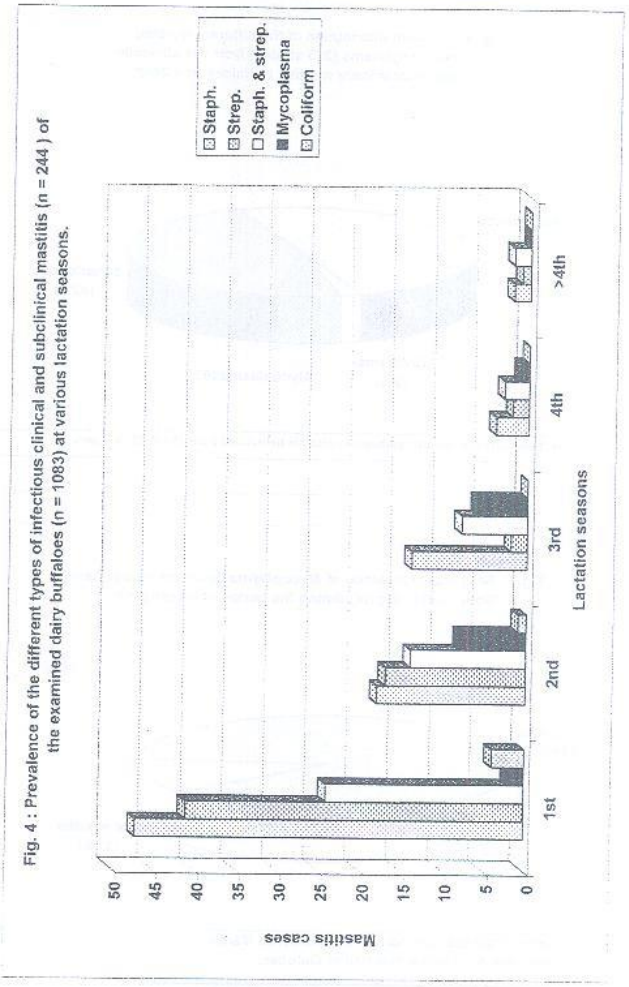
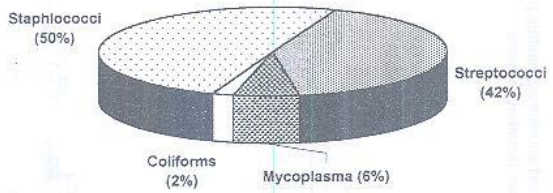


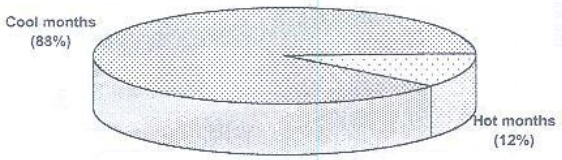
Fig. 4 : Prevalence of the different types of infectious clinical and subclinical mastitis (n = 244) of the examined dairy buffaloes (n = 1083) at various lactation seasons.

Fig. 5 :Frequent distribution of the different isolated microorganisms (273 strains) from the clinically and subclinically mastitic buffaloes (n = 244)*.



*: Out of these buffaloes, mastitis pathogens could not be isolated from 25 (10.25 %) cases.

Fig. 6: Seasonal prevalence of *Mycoplasma bovis* mastitis in Assiut Governorate (Egypt) during the period of investigation.



Cool-months; from the second half of October to Marsh.
Hot-moths; from April to the frist half of October.

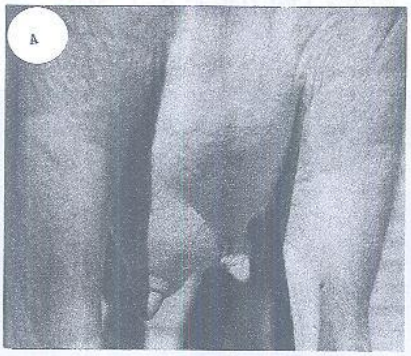


Fig.A: Gross enlargement of the hind-left quarter of a dairy buffalo yielded mycoplasma. Note: 1) the size and the shape of the affected quarter (inverted conical flask with pointed end, invaginated teat). 2) disappearance of the annular fold. 3) the middle line of the udder is displaced to the right side. 4) the difference in level of the two hind teats.

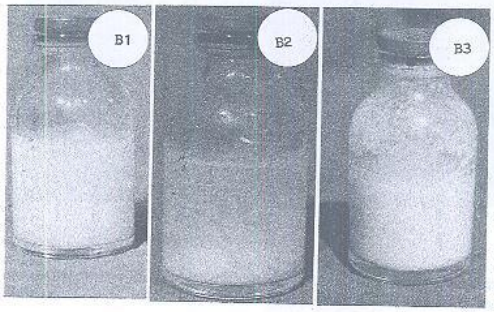
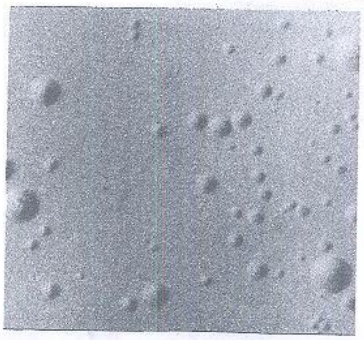


Fig.B: A milk sample from a mycoplasma positive case. It appears as normal but it lacks the characteristic milky-white color of buffalo's milk (wateriness). On standing position (Fig.B2), the collected milk separated into two layers. The supernatant is cloudy watery liquid while the sediment is a collection of white flakes that deposit into the bottom. By vigorous shaking (Fig. B3), these flakes adhered onto the wall referring to the sticky nature.



Characteristic shape of mycoplasma colonies under stereo-dissecting microscope (X = 30). Note: the fried-egg like appearance (nipple-colonies).

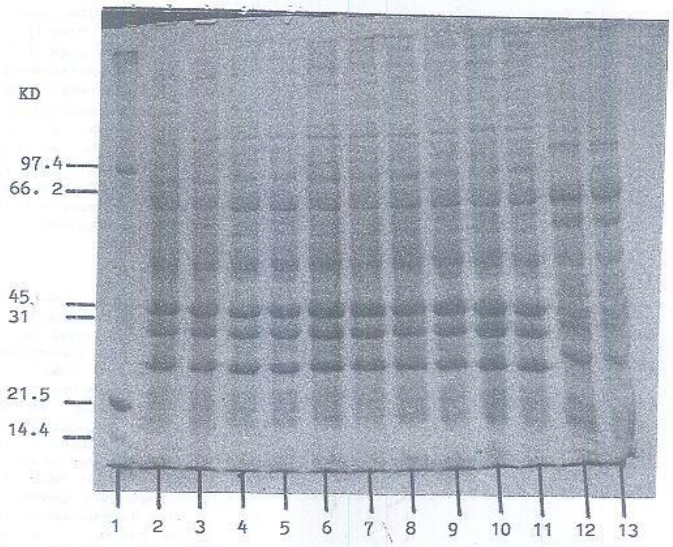


Fig. D: Electrophoretic patterns of ***Mycoplasma bovis*** isolated from buffaloes with mastitis.
1: Low molecular wieght standard (Bio-Rad).
2 - 11: ***Mycoplasma bovis*** (field strains).
12-13: Reference strains of ***Mycoplasma bovis*** (donetta)