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MOLECULAR DETECTION OF MYCOPLASMA INFECTION IN MILK OF CLINICALLY MASTITIC BUFFALOES

(With 3 Tables and 5 Figures)

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البيولوجيا الجزيئية في اكتشاف عدوى الميكوبلازما في ألبان الجاموس المصاب بالتهاب الضرع الظاهري

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الهدف من إجراء البحث معرفة مدى إنتشار التهاب الضرع المسبب بالميكوبلازما في الجاموس الحلوبة. خلال فترة البحث تم الفحص الظاهري لعدد 1250 جاموسة بقرى مختلفة بمحافظة أسيوط وسوهاج بصعيد مصر – ووجد أن 89 حالة مصابة بأعراض إلتهاب الضرع الظاهري وأخذ عينات من إفرازات ضروعها للكشف عن وجود عدوى الميكوبلازما بأستخدام إختبار البلمرة التسلسلي التفاعلي (PCR). وقد تم تقسيم الحيوانات المريضة إكلينيكيا - طبقا لشدة الأعراض المرضية على الضرع المصاب - الى ثلاث مجموعات: فوق الحاد (تسع حيوانات) و حاد (36 حيوان) وتحت الحاد/مزمن (44 حيوان). واتضح أن 20.22 % من الحيوانات المختبرة كانت ايجابية لعدوى الميكوبلازما وتم تحديد ثلاثة أنواع مختلفة من الميكوبلازما: 1- *Mycoplasma bovigenitalium* 2- *Mycoplasma bovirhinus* 3- *Mycoplasma arginini* بنسب عزل تكرارية قدرها 58.62 % و31.03 % و10.35 % على التوالي. وأظهر إختبار PCR ان جميع العينات المختبرة كانت سالبة لوجود عدوى ميكوبلازما الأبقار (*Mycoplasma bovis*) وكانت عدوى ميكوبلازما الأبقارالتناسلية (*Mycoplasma bovigenitalium*) هي الميكوبلازما السائدة في حالات الإصابة التحت الحادة و/أو المزمنة (29.54 %) أكثر من حالات الإصابة الحادة (13.88 %). أن جميع الحيوانات المصابة بالتهاب الضرع فوق حادة كانت سالبة لعدوى الميكوبلاوما. هذا وقد نوقشت الأهمية الأكلينيكية لكل من *(Mycoplasma bovirhinus), Mycoplasma (arginini)* كأحد الميكروبات المسببة لإلتهاب الضرع في الجاموس الحلوبة. أجملت نتائج هذا العمل أن عدوى ميكوبلازما الأبقار التناسلية تبوءت مرتبة عليا كأحد مسببات التهاب الضرع في الجاموس الحلوبة مقارنتاً بدراسات سابقة مشيرا الى إنتشار العدوى. وأن أختبار PCR كأداة كشف كان سهل الأداء وسلس ولكنه مازال نفيس بالمعامل كأجراء روتيني للكشف عن مرض التهاب الضرع خاصة في المزارع الصغيرة أو حالات الأهالي.

SUMMARY

Prevalence of Mycoplasma infection in private cases of buffaloes with clinical mastitis was fundamentally goaled. During five years investigation, 1250 primiparous and multiparous dairy buffaloes located in different villages of Assiut and Sohag Governorates, South Egypt were clinically inspected. Eighty-nine cases showed signs of clinical mastitis and their mammary secretions were tested for the presence of Mycoplasma infection using Polymerase Chain Reaction (PCR). The clinically mastitic buffaloes were categorized into peracute (nine cases), acute (n = 36) and subacute/chronic (n = 44) forms. Mycoplasma infection was detected in 20.22 % of the tested buffaloes. All molecularly Mycoplasma positive—buffaloes were multiparous and characterized by recurrent attacks of mastitis. *Mycoplasma bovigenitalium*, *Mycoplasma bovirhinus* and *Mycoplasma arginini* were detected with frequent detection, 58.62 %, 31.03 % and 10.35 %, respectively. All PCR—tested samples were negative to *Mycoplasma bovis*. *Mycoplasma bovigenitalium* was a predominant Mycoplasmal mastitis pathogen detected in buffaloes with subacute/chronic (29.54 %) mastitis rather than acute (13.88 %) form. Mycoplasma infection could not be detected in buffaloes with peracute mastitis. Clinical significance of the detected *Mycoplasma bovirhinus* and *Mycoplasma arginini* as mastitis pathogen was discussed. It can conclude that *Mycoplasma bovigenitalium* become occupying a considerable grade as a contagious mastitis pathogen in dairy buffaloes in comparison with previous studies referring to spread of infection. PCR as a detecting tool is a manageable contrivance and is easier than conventional culturing procedure, but it is still precious in our laboratories as a routine test for diagnosis of mastitis particularly in smallholder farms and/or private cases.

Key words: *Buffaloes, South Egypt, Clinical Mastitis, M. bovigenitalium, M. bovirhinus M. arginini, PCR*

INTRODUCTION

Clinical mastitis incriminated as a critical problem of the dairy animals causing dramatic economic losses during the lactation season. These losses are not only due to losses of quality and quantity of milk production but also due to the spread of infection to the neighboring animals. Moreover, costs of veterinary service, therapeutic trials and control amplify the seriousness of clinical mastitis (Palanivel *et al.*, 2008; Zaitoun, 2008 and Petrovski *et al.,* 2009). Microorganisms causing mastitis are numerous, but mastitis pathogens can be categorized

as environmental (*Escherichia coli*) or contagious (*Staphylococcus aureus* and Mycoplasma species) depending on their primary reservoir (environment versus infected mammary gland) (Edmonson and Bramely, 2004). Mastitis caused by contagious pathogens appears to be more prevalence than the environmental mastitis (Riekerink *et al.,* 2006; Ali *et al.,* 2008 and Zaitoun, 2008 and Petrovski *et al.,* 2009).

Contagious mastitis caused by Mycoplasma infection in cows and buffaloes is a worldwide disease (Nicholas, 2002; Roy *et al.,* 2008 and Nicholas *et al.,* 2009). In Egypt, Mycoplasma isolated from milk of cows by El-Ebeedy *et al.* (1985), Eissa (1986) and Ahmed (1987) and from buffaloes' milk by Zaitoun (1990). The authors corroborated that Mycoplasma mastitis coming Egypt through importation of infected Friesian cows. Twenty years later, several works on Mycoplasma mastitis in dairy cows and buffaloes on farms and in individual (private) cases at different localities of Delta-region and of South Egypt's Governorates were subsequently published (Zaitoun *et al.,* 1991; El-Shabiny, and Abo-El-Makarem, 1994; Zaitoun and Eissa, 1994; Zaitoun, 2000; Ibrahim *et al.,* 2007 and Abdelhameed and Sharaf, 2009). Adbelhameed and Sharaf, (2009) carried out a wide survey on Mycoplasma infection on various smallholder farms of large dairy ruminants in mid-area of Delta region of Egypt. They concluded that Mycoplasma infection is develop into a significant risk on Friesian farms and occupied a considerable level as mastitis pathogen in Delta region of Egypt. These may noticeably refers to the spread of Mycoplasma infection throughout the Egyptian farms and it may become inherent or endemic in some areas. Various types of Mycoplasma were isolated from large dairy ruminants with intramammary infections in Egypt. However, *Mycoplasma bovis* and *Mycoplasma bovigenitalium* are still the most important causes of Mycoplasma mastitis in Egypt (El-Ebeedy *et al.* (1985); Zaitoun *et al.,* 1991; El-Shabiny, and Abo-El-Makarem, 1994; Ibrahim *et al.,* 2007 and Abdelhameed and Sharaf, 2009).

Buffaloes consider the first source of animals' resources for milk yield rather than meat production in Egypt. They produce approximately 65 % (or may be more) of milk production (Mostageer, 1989 and Metry, 1996). More than 90% of the Egyptian buffaloes' population is apparently still under the villagers' hands as private cases rather than that those kept on farms (Mostageer, 1989). However, the most of published literature on mastitis in Upper Egypt are focused on dairy Friesian and buffaloes farms (El-Gamal, 1989; El-Shabiny, and Abo-El-Makarem, 1994 and Seddek *et al.,* 1999). During the past 15 years, field data on

prevalence of Mycoplasma mastitis of dairy buffaloes in south Egypt could not be traced on the available literature and appears to be scarce. Consequently, the aim of the present work was to elucidate the prevalence of Mycoplasma mastitis of dairy buffaloes with clinical mastitis in different villages of Assiut and Sohag Governorates, South Egypt, using PCR technique.

MATERIALS and METHODS

Animal:

During the period, June 2002 to September 2007, a total number of 1250 dairy buffaloes (private cases) located in different villages of Assiut and Sohag Governorates, Upper Egypt, were clinically inspected and their mammary glands were carefully examined for detection of gross udders and milk abnormalities. Eighty-nine cases of the inspected buffaloes showed signs of clinical mastitis (udder and/or milk abnormalities). Based on the number of parturitions (lactation seasons), the diseased cases were grouped into primiparous (n = 14) and multiparous (n = 75) and they were manually milked twice daily. The majority of the owners have no awareness concerning pre— and/or post—milking sanitations or dried-period therapy. History of previous mastitis or reduction of milk yield due to known and/or unknown cause, prior treatment was serially enrolled. Clinically, buffaloes with mastitis were categorized into three forms: peracute (n = 9), acute (n = 36), subacute/chronic (n = 44) with and/or without systemic reaction based on the outlines illustrated by Jackson and Cockcroft (2002).

Samples collection and technical procedure:

A composite sample (approx. 25 ml of mammary secretion) from the infected quarter(s) of each diseased buffalo was collected in a sterile screw capped bottle. The collected samples were reserved frozen until examination. The time interval between the samples collection and the Mycoplasma examination was a month. The frozen sample was thawed and thoroughly vortexed and half milliliter of the content was pipetted, immersed into 1.5 ml of modified Hayflick's broth medium containing inhibitors to suppress bacterial population and to enhance the growth of Mycoplasma cells and incubated at 37 ^{o}C (Zaitoun, 1990). Two days later, a milliliter of the incubated broth was transferred to 10 ml of modified Hayflick's broth medium and incubated at 37 °C for three consecutive days. These broths were tested for the presence of Mycoplasmas.

PCR procedure for identification of Mycoplasma:

Specific-species primers of *Mycoplasma bovis*, *Mycoplasma bovigenitalium*, *Mycoplasma bovirhinus* and *Mycoplasma arginini* were used for molecular examination of the collected samples.

The PCR procedure for detection of *Mycoplasma bovis* was carried out according to the protocol illustrated by Lunini *et al.* (2006) and Radaelli *et al.* (2008). Briefly, the incubated broth sample was harvested for 30 min at 14000 xg. The pellet was washed and resuspended in 300 µl of distilled water treated with 0.1% of diethylpyrocarbonate and the DNA was extracted from 100 µl using the DNeasy Tissue Kit (QIAamp®DNA Mini, Qiagen®) based on outlines of the manufacturer.

Specific primers of *Mycoplasma bovis* were designed for PCR (MYCBV-Fw – 5'-TAT CGG TGA CCC TTT TGC AC-3' ; MYCBV-Rw – 5'-TTC CAC TTC CTG ACT CAC CA-3') that would amplify a fragment of 348 bp of the oppD (oligopeptide permease D) gene of *Mycoplasma bovis*. A final volume of 20 µl was used containing 0.2 µM of each primer, 200 µM of dNTPs, 2 units of Taq polymerase (Roche Diagnostics), 1 x 1.5 mM of $MgCl_2$ and 5 µl of the extracted DNA. The following amplification procedure was used: 95 °C for 5′ , 36 cycles at 95 °C for 1′ , 59 °C for 30″ and 72 °C for 30″ and 72 for 7′ . Ten microliters of the amplified product were analyzed by electrophoresis in a 1.8 % (wt / vol) agarose gel (Agarose, MP, Multi purpose agarose, Boehringer Manheim) and thereafter stained with ethidium bromide (0.5 µg/ml) stain and photographed.

On the other hand, specific primers of *Mycoplasma bovigenitalium* (Kobayashi *et al.*, 1998), *Mycoplasma bovirhinus* (Kobayashi *et al.*, 1998) and *Mycoplasma arginini* (Timenetsky *et al.*, 2006) were also incorporated in molecular detection of the examined samples according to the protocol illustrated by the publishers. The sequences of the selected primers are tabulated in Table 1. Unfortunately, reference strains of Mycoplasmas could not be obtained. Consequently PCR products were sequenced with an automatic capillary system (Applied-Biosystem®) to confirm the specificity of the reaction. The PCR procedure was carried out in Dept. of Animal Med., Faculty of Vet. Med., Assiut University.

RESULTS

PCR Technique and Prevalence of Mycoplasma infection:

PCR technique revealed that 18 of 89 tested samples were positive to Mycoplasma infection and three different species of Mycoplasma were detected. All peracute cases of mastitis were molecularly negative to Mycoplasma infection, in contrast to the remained cases (acute and chronic mastitis) (Table 2). On the other hand, all Mycoplasma positive—buffaloes were multiparous and characterized by recurrent attacks of mastitis.

The prevalence (% affected) of Mycoplasma infection of the examined diseased buffaloes with different forms of clinical mastitis is illustrated on Table 2, which revealed that 20.22 % of the PCR-tested samples were infected by Mycoplasma. Thirteen buffaloes (72.22 %) of the Mycoplasma-infected-cases (n = 18) were associated with subacute/chronic mastitis, and the remainders (27.78 %) were associated with acute mastitis (Fig. 1).

Detection of different species of Mycoplasma infection of the examined diseased buffaloes with Mycoplasma mastitis (n = 18) was tabulated on Table 3. The frequent distribution of the molecularly detected Mycoplasma species was figured in Fig. 2

Mycoplasma bovigenitalium was the predominant detected Mycoplasmas (Fig. 3). Both *Mycoplasma bovirhinus* (Fig. 4) and *Mycoplasma arginini* (Fig. 5) were also molecularly detected in the investigated samples. On the other hand, all PCR—tested samples were negative to *Mycoplasma bovis*.

Table 1: Nucleotide sequences of the PCR primers used for detection of *Mycoplasma bovigenitalium* (Mbg) *Mycoplasma bovirhinus* (Mbr) and *Mycoplasma arginini* (Marg).

Species	Sequence	Size of PCR product (bp)
Mbg	5'-CGT AGA TGC CGC ATG GCA TTT ACG G-3' 5'-CAT TCA ATA TAG TGG CAT TTC CTA C-3'	**312**
Mbr	5'-GCT GAT AGA GAG GTC TAT CG-3 5'-ATT ACT CGG GCA GTC TCC-3	**316**
Marg	5' GCATGGAATCGCATGATTCCT 3' 5' GGTGTTCTTCCTTATATCTACGC 3'	**545**

Table 2: Prevalence of Mycoplasma infection of the examined dairy buffaloes with different forms of clinical mastitis.

Number of diseased buffaloes	Clinical condition of the infected udder	Number of the diseased cases	Mycoplasma infection		
			Positive cases	% of infection	Total
89	Peracute	9 (10.11 %)	0	0	18 (20.22 %)
	Acute	36 (40.45 %)	5	13.88	
	Subacute/chronic	44 (49.44 %)	13	29.54	

The numbers between parentheses are the percentages of infection to all mastitis cases (n = 89)

Table 3: Detection of Mycoplasma species in the diseased buffaloes with Mycoplasma mastitis (n = 18).

Clinical condition of the diseased udder	Number of diseased buffaloes	Type of Mycoplasma, detected by PCR technique
Acute mastitis	3	*Mycoplasma bovigenitalium*
Acute mastitis	2	*Mycoplasma bovigenitalium* *Mycoplasma bovirhinus*
Subaute/chronic mastitis	1	*Mycoplasma arginini*
Subaute/chronic mastitis	4	*Mycoplasma bovigenitalium*
Subaute/chronic mastitis	5	*Mycoplasma bovigenitalium* *Mycoplasma bovirhinus*
Subaute/chronic mastitis	2	*Mycoplasma bovigenitalium* *Mycoplasma bovirhinus* *Mycoplasma arginini*
Subaute/chronic mastitis	1	*Mycoplasma bovigenitalium*

Fig. 1: Mycoplasma infection (% affected) in the various forms of clinical mastitis of buffaloes

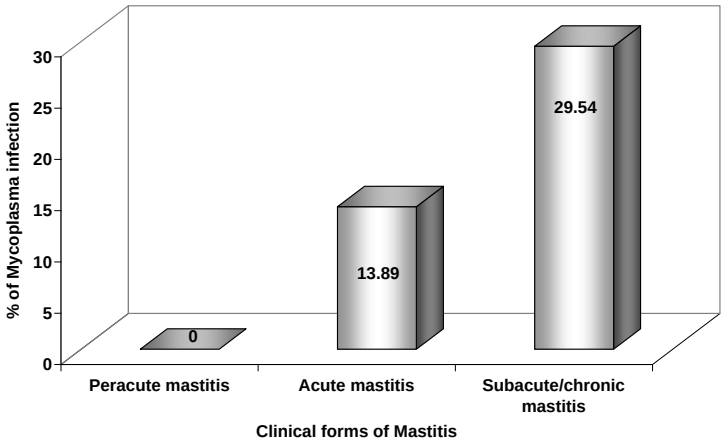

Fig. 2: Frequent distribution of the molecularly detected mycoplasma species of the examined diseased buffaloes with mycoplasma mastitis (n = 18)

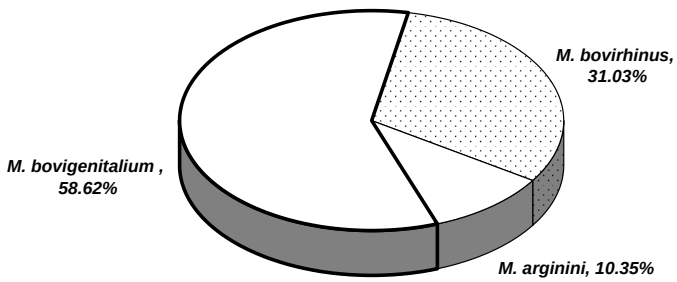

Fig. 3: Ethidium bromide-stained, 1.8 % agarose gel showing the reaction product of PCR amplification. The white arrow refers to the size of PCR product of *Mycoplasma bovigenitalium*, 312 bp

Lane 1: Molecular weight marker, (Sigma, 100 bp ladder)
Lane 2 -5: Positive samples to *Mycoplasma bovigenitalium*
Lane 6: Negative sample
Lane 7 – 12: Positive samples to *Mycoplasma bovigenitalium*

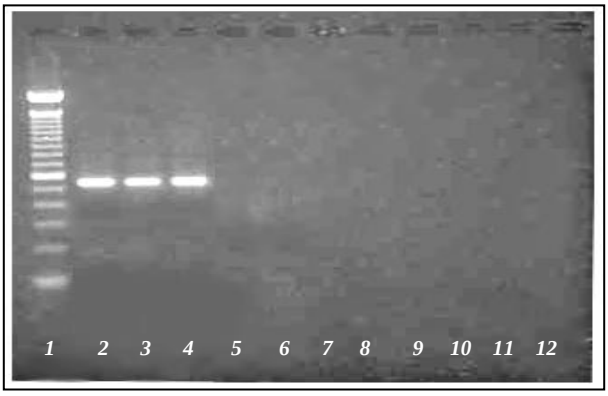

Fig. 4: Ethidium bromide-stained, 1.8 % agarose gel showing the reaction product of PCR amplification. The white arrow refers to the size of PCR product of *Mycoplasma arginini*, 545 bp

Lane 1: Molecular weight marker, (Sigma, 100 bp ladder)
Lane 2 - 4: Positive samples to *Mycoplasma arginini*
Lane 5 – 12: Negative samples

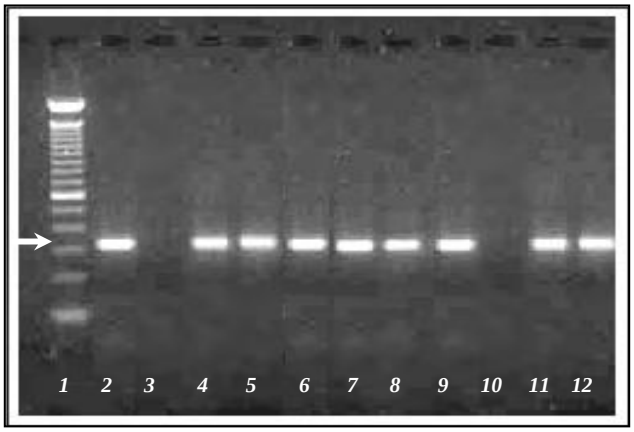

Fig. 5: Ethidium bromide-stained, 1.8 % agarose gel showing the reaction product of PCR amplification. The white arrow refers to the size of PCR product of *Mycoplasma bovirhinus*, 316 bp
Lane 1: Molecular weight marker, (Sigma, 100 bp ladder)
Lane 2: Positive samples to *Mycoplasma bovirhinus*
Lanes 3 & 10: Negative samples
Lane 4 – 9: Positive samples to *Mycoplasma bovirhinus*
Lanes 11 &12: Positive samples to *Mycoplasma bovirhinus*

DISCUSSION

The current work revealed that 20.22 % of the examined diseased buffaloes with clinical mastitis were molecularly positive to Mycoplasma infection concluding that Mycoplasma occupied a considerable magnitude as an etiologic agent of contagious mastitis pathogen of private dairy buffaloes. Previously Eissa (1986), Zaitoun (1990) and Zaitoun *et al.* (1991) concluded that the prevalence of Mycoplasma mastitis of dairy buffaloes ranged from 0 to 2.04 % referring to the limitation of Mycoplasma infection at that time. Four years later, Zaitoun and Eissa (1994) carried out a wide-scale survey on Mycoplasma mastitis of dairy buffaloes at different localities of Assiut Governorate using conventional culturing procedure. They found that 17.65 % of the examined animals with clinical mastitis were culturally infected by Mycoplasma. In the present study the higher rate of Mycoplasma infection (20.22 %) than that previously reported by Zaitoun and Eissa (1994) may primarily ascribe to the high sensitivity rate of the applied molecular assay in comparison with conventional culturing procedure. On the other side, highest incidence of clinical

mastitis caused by Mycoplasma infection in dairy buffaloes was reported by El-Shabiniy and Abo-El-Makarrem (1994). They indicated that the infection rate with Mycoplasma was 25 % of the examined buffaloes with clinical mastitis at Beni-Suef Governorate, south Egypt.

The high level of mastitis caused by contagious mastitis pathogens including Mycoplasma infection in the dairy animals attributes to several causes illustrated by Edmonson and Bramely, (2004) and Palanivel *et al.* (2008). However, unhygienic sanitary measures during milking and lake and/or defects of pre— and post—milking precautions may play an astonishing role. Moreover, omission or slackness in culling of the dairy buffaloes infected by Mycoplasma mastitis in particular *Mycoplasma bovis* or *Mycoplasma bovigenitalium*; consider the utmost point in increasing the prevalence of Mycoplasma mastitis in Egyptian field (Zaitoun, 1990 and Zaitoun,1991).

The results of the current work reveal that *Mycoplasma bovigenitalium* (58.62 %), *Mycoplasma bovirhinus* (31.03 %) and *Mycoplasma arginini* (10.35 %) are molecularly detected. The former Mycoplasma is the predominant Mycoplasmal pathogen involved in clinical mastitis of dairy buffaloes in Egypt and is agreement with data from previous studies carried out in north and south Egypt (Ahmed, 1987; Zaitoun, 1990; Zaitoun *et al.,* 1991; Zaitoun and Eissa, 1994 and El-Shabiny and Abo-El-Makarrem, 1994). *Mycoplasma bovigenitalium* is a worldwide infection and is frequently incriminated as a major pathogenic agent capable of inducing a rang of clinical diseases including mastitis and serious reproductive disorders in cattle and buffaloes in numerous countries including Egypt (Nicholas, 2002). In addition to the incrimination of *Mycoplasma bovigenitalium* as mastitis pathogens of dairies, this microorganism could induce a severe inflammatory reaction in undeveloped mammary gland of an immature calf (Roy *et al.,* 2008).

On the other side, the present study indicates that *Mycoplasma bovirhinus* is occupied the second grade following *Mycoplasma bovigenitalium* of the detected Mycoplasmas from the examined diseased buffaloes with clinical mastitis and this may imply the importance of that germ as a mastitis pathogen in buffaloes. Previous result published by El-Shabiny and Abo-El-Makarrem (1994) referred to *Mycoplasma bovirhinus* as an unconventional mastitis pathogen responsible for severe clinical mastitis in dairy buffaloes at Beni-Seuf Governorate (south Egypt). However, Gourlay and Howared (1979) and Hirose *et al.* (2001) concluded that *Mycoplasma bovirhinus* was a

secondary respiratory infection rather than mastitis pathogen. Moreover, Ayling *et al.* (2004) indicated that *Mycoplasma bovirhinus* was commonly found in ruminants but was thought to be more opportunistic than pathogenic. On the other hand, the current work reveals that *Mycoplasma arginini* was less frequently detected of milk speciemens of the clinically diseased buffaloes with mastitis and this coincided with previous reports of Eissan (1986), Zaitoun (1990), Zaitoun *et al.* (1991) and Eissa, and Zaitoun (1994). In fact, role of *Mycoplasma arginini* in diseases of Egyptian dairy buffaloes is still unclear and need investigations. In cattle, Hirose *et al.* (2003) and Gagea *et al.* (2006) indicated that *Mycoplasma arginini* was isolated from the nostrils and pneumonic lungs of calves and from the lower respiratory tract of cattle with signs of respiratory disease.

Clinically, it is appreciate to indicate that all molecularly positive cases to Mycoplasma infection showed signs of subacute and/or chronic mastitis (49.45 %) rather than acute (40.45 %) form, and all tested animals with peracute mastitis were molecularly Mycoplasma free. This may refers that the positive cases are chronically infected with Mycoplasma and the infection may become inherent. Moreover, based on history, buffaloes with subacute/chronic mastitis did not respond well to the antimastitic therapy. Therefore, Mycoplasma testing in paralleling with conventional bacteriological examinations should carry out to buffaloes with incurable mastitis. Moreover, causes of incurable mastitis of private dairy buffaloes should be focused.

The current work concludes that Mycoplasma, in particularly *Mycoplasma bovigenitalium*, occupied a considerable level as an important etiologic agent responsible for contagious mastitis in buffaloes located in villages of south Egypt. PCR is a rapid detecting assay for diagnosis of Mycoplasma mastitis in contrast to the conventional culturing procedure, which tedious and time consuming. PCR procedure can carried out directly on milk samples for detection of Mycoplasma (Hirose *et al.,* 2001) and this may makes it as a practical for clinical testing. However, from practical point of view, PCR is still an expensive detecting procedure in our laboratories as a routine test for diagnosis of mastitis particularly in smallholder farms and/or private cases.

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