Detection of *Mycoplasma species* in raw milk of lactating animals in Assiut and Qena city of Egypt

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Abstract

The incidence of *Mycoplasma Species* in raw milk of different animal species was determined. A total of 240 random raw milk samples were collected from cows, buffaloes, sheep and goats in Assiut and Qena cities, Egypt (30 samples each). Fifteen strains of *Mycoplasma* were isolated from raw milk. The strains were biochemically characterized followed by PCR assay for confirmation. On the basis of biochemical characterization the strains were divided into 5 species as follow *M. arginini*, *M. bovirhinis*, *M. bovis*, *M. species group 7*, and *M. dispar*. *M. bovis* could not be detected. The most prevalent isolated species was *M. bovirhinis* which was isolated from 6.67% of cow's milk samples collected from Assiut City. Whereas *M. arginini*, was the most prevalent species in the examined milk samples collected from Qena City. None of the five *M. bovirhinis* strains that were identified biochemically were confirmed by PCR assay through amplification of 16S rRNA region of the gene that was amplified at 316 bp. Efforts should be made to protect animals against *mycoplasma* infection.
Key Words: Raw milk, *Mycoplasma bovirhinis,*PCR assay.

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Introduction

Mycoplasmas belong to the class Mollicutes and are among the smallest free-living microorganisms capable of auto-replication. They are fastidious bacteria. The term "mycoplasma" (Greek, mykeys= fungus and plasma= formed) emerged in the 1950s (Edward *et al.*, 1956). In the 1960s, *mycoplasmas* were designated members of a class named Mollicutes, which derives from Latein words meaning soft (mollis) and skin (cutis) (Waites *et al.*, 2001). Many species are important veterinary pathogens causing respiratory infection, mastitis, conjunctivitis, arthritis, and abortion (Nicholas, 1998).

Mycoplasmal organisms are nearly ubiquitous in both the plant and animal kingdoms as colonizers and pathogens. The first *mycoplasma* to be isolated in a culture was the bovine pleuropneumonia agent now known as *Mycoplasma mycoides subsp. Mycoides*, which was described initially by Nocard and Roux in 1898. The first *mycoplasma* isolated from humans was detected by Dienes and Edsall in 1937 which we know now as *Mycoplasma hominis*. Other human *mycoplasmas*, *Mycoplasma salivarium*, *Mycoplasma fermentans*, in addition to *Mycoplasma pneumoniae* which was first isolated by Eaton *et al.*, (1944).

Mycoplasma pneumoniae remains an important cause of pneumonia, and is also associated for its extra pulmonary manifestation, nausea, vomiting and abdominal pain are the most common symptoms for extra pulmonary manifestion (Kim *et al.*, 2005). Hepatitis, hematuria, skin rash, gastro-enteritis and myepicarditis were reported in some cases (Lee *et al.*, 1986). Although scientists have isolated 17 species of *Mycoplasma* from human, 4 species of the organism are responsible for most clinically significant infection (Waites, 2009).

In animals, *Mycoplasma* is an emerging and extremely contagious mastitis pathogen. Several species have been associated with mastitis, *Mycoplasma bovis*, *M. califoranicum*, *M. canadense*, *M. arginini*, *M. bovigenitalium*, *M. alkalescens*, *M. bovirhinis* and *M. dispar* (Kumar and Garg, 1991). The bacterium can be shed in milk in large numbers before clinical signs appears with relatively few organisms required to infect a quarter so, 25 to 30 % or more of a dairy herd can be become infected during an outbreak of *Mycoplasma* (Bayoumi *et al*., 1988).

Microbiological culture is generally used for detection and identification of *mycoplasmas*. However, the former techniques are time consuming and not sensitive enough in some cases. Therefore, PCR assay was originally developed for confirmation of *mycoplasma species* using the gene for16S rRNA (Kobayashi *et al*., 1998).

Owing to *Mycoplasma* is a unique bacterium that does not always receive the attention it merits, considering the number of illness it causes and the degree of morbidity associated with it, this study was planned to know its prevalence in raw milk of cows, buffaloes, sheep and goats.

Materials and methods

The present study was carried out during the period between April, 2008 and, March 2010 in the department of Food Hygiene, Faculty of Veterinary Medicine Assiut University.

a) Collection of samples: A total of 240 random raw milk samples were collected from cows, buffaloes, sheep and goats in Assiut and Qena cities, Egypt (30 samples each). Each sample was mixed and tested for heat treatment using Storch test (Lampert, 1975).

b) Isolation of *Mycoplasma species*: Enrichment of *Mycoplasma species* was adopted using *Mycoplasma* enrichment broth and then incubated at 37°C for 3 days. Isolation was done using surface spreading technique on *Mycoplasma* agar according to Carter and wise, (1995). The plates were incubated at 5-10 % Co_2 incubator for 72 hours at 35-37°C. Plates are held for 7 days before reported as negative. Suspected colonies should be optimized by seeing"fried egg" shaped colonies.

c) Identification of isolates: Digitonin sensitivity was performed as the disc inhibition test according to Erno and Stipkorits, (1973). The biochemical procedures in *mycoplasma* identification have been standardaaized by Aloutto *et al*., (1970). They involve the hydrolysis of arginine, phosphatase activity, film and spots production and tertazolium reduction.

d) DNA extraction and PCR amplification of 16S rRNA *M. bovirhinis*: Specific primer of *M. bovirhinis* was used in the molecular detection of the isolated organism according to the protocol illustrated by Kobayashi *et al*., (1998) with some modification as follow: bacterial DNA was extracted following grown up in the phosphate buffered saline (PBS), one millilitre of inoculated PBS was centrifuged at 15,000 rpm g for 10 minutes. The NucleoSpin® Tissue Kit (Germany) was used to obtain bacterial DNA, according to the manufacturer recommendations and stored at -20°C until use.

DNA amplification: PCR were carried out in 50 µl reaction volumes containing 5 µl template DNA, 1 mM $MgCl_2$, 1 mM of dNTP, 5 µl of x 10 PCR buffer (Quiagen), 1.25 unit of *Taq* DNA polymerase *(Ampli Taq Gold* Quiagen) and 20 pM of each primer. The sequences of the primers are given in Table -1.

Unfortunately, reference strain of *Mycoplasma* could not be obtained by Molecular Biology Research and Genetic Engineering Center, Assiut University, Egypt.

The PCR cycles consisted of pre-heating at 94°C for 9 min, denaturation at 94°C for 30 sec. min, annealing at 60°C for 1 min and extension at

Table-1. Nucleotide sequence of the PCR primers used for detection of *M. bovirhins*

Species	Designation	Sequence	Size of PCR product (bp)
M. bovirhinis	Mbr F	5´- GCT GAT AGA GAG GTC TAT CG-3´	316
	Mbr R	5´- ATT ACT CGG GCA GTC TCC-3´	

Table -2: Incidence of Mycoplasma species in the examined milk samples

Types of examined samples	Sources of samples			
	Assiut City		Qena City	
	No. of positive samples/30	%	No. of positive samples/30	%
Cow's milk	2	6.67	1	3.33
Buffaloe's milk	1	3.33	2	6.67
Sheep milk	1	3.33	3	10.00
Goat's milk	2	6.67	3	10.00
Total	6	20.00	9	30.00

Table -3: Incidence of different Mycoplasma species recovered from the examined milk samples collected from Assiut City

Isolated strain	Cow's milk		Buffaloe's milk		Sheep milk		Goat's milk	
	No./30	%	No./30	%	No./30	%	No./30	%
M. arginini	-	-	-	-	1	3.33	1	3.33
M.bovirhinis	2	6.67	-	-	-	-	1	3.33
M. bovis	-	-	-	-	-	-	-	-
M. species group 7	-	-	1	-	-	-	-	-

72°C for 1 min. the amplifications were performed for 35 cycles in a model T professional basic 070-701 thermocycler, in the Molecular Biology Research and Genetic Engineering Center, Assiut University, Egypt, with a final extension step at 72°C for 7 min. The PCR products were visualized using a 2.5% agarose gel containing 0.5 µg of ethidium bromide/ml in relation to DNA mass ladder standard (1000-bp DNA ladder Quiagen).

Results and Discussion

Many *Mycoplasma species* are pathogenic to animals, human and plants and they are, therefore, of great concern in human and veterinary medicine (Maniloff *et al*., 1992 and Ross, 1993). *Mycoplasma* are often host different species, and ruminants especially cattle, harbour a number of different species.

Results illustrated in Table-2 revealed that *Mycoplasma species* was isolated from 6.67, 3.33, 3.33 and 6.67% of the examined cow milk, buffalo milk, sheep milk and goat milk samples collected from Assiut City, while, 3.33, 6.67, 10.00 and 10.00% of the same samples that collected from Qena City proved to harbor this bacterium, respectively. Presence of *Mycoplasma* in milk is not surprising in view of the fact that they are widely distributed in nature and contaminate milk. The problem was complicated by the absence of cool system that may enhance the multiplication of pathogenic microorganisms.

The highest incidence (30.00%) of *Mycoplasma species* was recorded from the examined milk samples collected from Qena City. Nearly similar results were recorded by El-Shabiny and Abo-el Makarrem, (1994). Zaitoun and Eissa, (1994) found that 17.65% of the examined buffaloe's milk with clinical mastitis were culturally infected by *Mycoplasma*. Infection with *Mycoplasma* in the dairy animals is attributed to several causes illustrated by Edmonson and Bramely, (2004) and Polanivel *et al*., (2008). However, unhygieneic sanitary measures during milking and /or defects of pre-miliking and post- milking may play an astonishing role.

From the results recorded in Table 3 it is clear that the most prevalent isolated species was *M. bovirhinis* which was isolated from 6.67% of cow's milk samples collected from Assiut City. Concerning *M. bovis*, similar results was obtained by McDonald *et al*., (2009) as *M. bovis* was not detected in any tank milk samples by PCR or culture, where 7 of 66 (10.61%) and 6 of 51 (11.76%) cows were infected with the organism (Gonzalez *et al*., 1992). Data recorded in Table 4 showed that *M. arginini*, was the most prevalent species in the examined milk samples collected from Qena City. *M. arginini*, *M. bovirhinis*, *M. species group 7* and *M. dispar* could be isolated in a variable percentage from the examined cows, buffaloes, sheep and goats milk

Table -4: Incidence of different Mycoplasma species recovered from the examined milk samples collected from Qena City

Isolated strain	Cow's milk		Buffaloe's milk		Sheep milk		Goat's milk	
	No./30	%	No./30	%	No./30	%	No./30	%
M. arginini	-	-	-	-	2	6.67	-	-
M.bovirhinis	-	-	1	3.33	-	-	1	3.33
M. bovis	-	-	-	-	-	-	-	-
M. species goup 7	1	3.33	1	3.33	-	-	1	3.33
M. dispar	-	-	-	-	1	3.33	1	3.33

samples collected from Qena City.

The important *Mycoplasma* causes natural disease in both sheep and goat it occurs primarily in Mediterranean countries but is also reported from many other areas of the world (Jones, 1987). The existence of *Mycoplasma* in goat milk clarify that the intra mammary pathogen in goats are associated with poor hygienic condition in housing and in the milking parlors (Contreras *et al*., 2002). Previous results published by El-Shabiny and Abo-el Makarrem, (1994) referred to *M. bovirhinis* as an unconventional mastitis pathogen responsible for sever clinical mastitis in dairy buffaloes at Beni- Suef, Egypt. However, Hirose *et al*., (2001) concluded that *M. bovirhinis* was a secondary respiratory infection rather than mastitis pathogen.

Using PCR for detection of *M. bovirhinis*, non of the 5 isolated strains, gave a positive results as indicated in Figure-1. Although PCR based methods offer a substantial advantage in that they reduce the average diagnostic time. Positive results from PCR assays enable a full investigation to take place, however negative results should not be considered definitive. This may be due to the fact that *mycoplasmas* are the smallest self-replicating microorganisms known (Poveda *et al*., 2002). The small size and correspondingly lower concentrations of cellular constituents of *mycoplasmas* results in smaller signals that are more difficult to resolve from the background noise than those obtained from mammalian cells or even from larger micro-organisms. Additionally, the genetic variation between strains isolated from local samples and the others all over the world affect the results, so, we suggested making a genetic sequencing for the isolated microorganisms and creating specific primers for the locally isolated strains.

There is no treatment of *mycoplasmal* infection, its control relies on identification of infected animal by culture of composite or quarter milk samples from all milking and dry animals in the herd (Bushnell, 1984). Great care should be used when purchasing the animals. All action should be used upon the understanding of the high contagious nature, slow recovery rates and the in–effectiveness of treatment of *Mycoplasma* infection is likely to increase as a consequence of a perceived bioterrorism threat.

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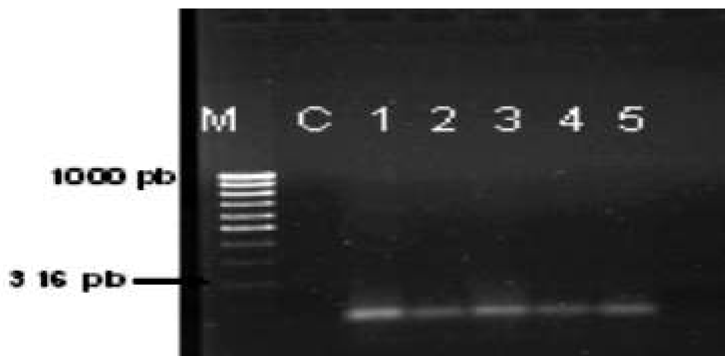

Figure- 1:
PCR products for *M. bovirhinis*

Line M - molecular size marker 1000 bp.
Line C: negative control
Line 1, 2, 3, 4 and 5, negative samples

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Conflict of interest

Authors declare that they have no conflict of interest.

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