Research Article

Bovine Mastitis in Oman is Mainly Associated with Environmental Bacteria that Show High Resistance to Commonly Used Antibiotics

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Abstract

In Oman, mastitis is an important disease that affects the dairy animals, especially cows. In this study, bacteria and fungi from subclinical and clinical mastitis were identified using 16S rDNA and 18S rDNA, respectively, in 76 milk samples from 30 cows. The frequency of subclinical mastitis (75%) was higher than clinical mastitis (25%). Bacterial isolates were detected in 82% of the samples, out of which 12% showed mixed bacterial cultures. The most predominant isolated bacteria were environmental bacteria rather than minor and contagious bacteria from subclinical (53.6%, 42.8% and 3.6%, respectively) and clinical mastitis (62.5%, 25% and 12.5%, respectively).

Antibiotic resistance profiles of the isolated bacteria for six commonly used antibiotics showed an increase in resistance compared to a previous study in 1991. Most isolated bacteria were resistance to AMP, while they were more sensitive for SXT and TE. Eleven percent of the isolated bacteria were resistance to four of the antibiotics tested or more.

About half of the samples (47%) were positive for fungal growth. Most of those samples were positive for bacteria, which suggested that detected fungi may be opportunistic. However, 3% of the investigated samples were negative for bacterial growth, which may indicate pathogenic involvement in mastitis.

In conclusion, the major association of mastitis with environmental bacteria and the detected multi-antibiotics resistance emphasized the need for using appropriate control protocols by allowing to investigate each case and determine whether antibiotic treatment is necessary and which antibiotics to be used.

Keywords: Mastitis; Oman; Antibiotics resistance; Contiguous pathogens; Environmental pathogens; Fungi; Minor pathogens

Abbreviations

PCR Polymerase chain reaction
ITS Internal transcribed spacer
EF Elongation factor
MWT Modified White Side Test
SCC Somatic cell count
CM Clinical mastitis
SCM Subclinical mastitis
AMP Ampicillin
AML Amoxicillin
SXT Trimethoprim-sulfamethoxazole
GE Gentamicin
TE Tetracycline
S Streptomycin
NCBI National Center for Biotechnology Information
BOLD Barcode of Life Data System
AST Antibiotic Susceptibility Testing
CNS Coagulase Negative Staphylococci
CPS Coagulase Positive Staphylococci
GNB Gram Negative Bacteria

Introduction

Mastitis is an important, complex and multifactorial disease. It is the most common and costliest disease affecting dairy farms in the western world (Barkema *et al*., 2009; Gelasakis *et al.,* 2015). It affects the udder of dairy animals causing several changes to milk and udder. These changes can be chemical, physical and pathological in the glandular tissues (Sukumar and James, 2012). It may result from the interaction of host, pathogen(s) and environmental factors (Sharif *et al*., 2009; Rofaida, 2010).

According to the pathogens associated, transmission mode and primary reservoir, mastitis is classified into contagious and environmental mastitis (Makovec and Ruegg, 2003). Contagious pathogens are pathogens that live in the mammary glands of the host and are able to cause subclinical infection. They are transmitted during milking from cow to cow through the hands of the milking person, milking machine or udder cloths (Blowey and Edmondson, 2010). Environmental pathogens are opportunistic pathogens that live in cow environment (Kivaria, 2006). They can enter and attack the udder after milking (Blowey and Edmondson, 2010).

Different species of bacteria are associated with mastitis in different geographical areas due to variation in the management practiced in the different countries (Ahmed *et al*., 2016). Mastitis caused by bacteria represents a major risk for human health, as pathogenic bacteria and their toxins increase the chance of foodborne diseases (Ikiz *et al*., 2013; Bhatt *et al*., 2011; Sharma *et al*., 2011). Many mastitis bacteria are responsible for several diseases in human such as tuberculosis, streptococcal intoxication, colibacillosis, streptococcal sore throat, and brucellosis (Tesfaheywet and Gerema 2017).

Overuse of antibiotics for mastitis treatment or for protection during the dry periods was associated with the development of antibiotic resistant strains of bacteria (Bradley, 2002) and also, the increase of mastitis incidence caused by yeast (Erbaş *et al.*, 2017; Wawron *et al*., 2010). Fungal infections are associated with additional factors including the lack of hygiene, high humidity, high temperature, wet teat and when animal barn is crowded (TalebkhanGaroussi *et al*., 2009; Pachauri *et al*., 2013; Lagneau *et al*., 1996).

From several dairy farms in Oman, *staphylococcus aureus* reported to be the predominant causal pathogens of clinical mastitis in dairy cattle, cow, goats and sheep (Harby *et al.*, 1991). Also, other bacteria were reported by theses authors to be *Streptococcus dysgalactiae, Streptococcus galactiae, Streptococcus uberis, Escherichia coli, Klebsiella spp, Micrococcus, Enterbacter aerogenes*and *Corynebacterium pyogenes*. Notably, sensitivity to 14 commonly used antibiotic were evaluated in the same study.

To the best of our knowledge, there were no studies on mastitis in Oman since 1991, except reports from the central lab of animal health, which identified the pathogens using culture method and the vitecx machine (2016 and 2017). Identified species were *Staphylococcus* spp, *Coliform mastitis, Pseudomonas luteola, Enterobacte raerogenes, Enterobacter*spp, *Streptococcus uberis, Enterococcus faecalis, Proteus mirabilis, Klebsiella pneumonia* spp, *Salmonella enteric, Bacillus* spp, *Staphylococcus mastitis, coliform* and *Escherichia coli*.

Thus, this study aimed to identify the major microorganisms associated with bovine mastitis cases in Oman including bacterial and mycotic pathogens by sequencing and to evaluate the sensitivity of pathogens being isolated to the commonly used antibiotics.

Materials and Methods

Sampling of milk

Five to 15 ml Milk samples were obtained from 30 cows suspected to have clinical or subclinical mastitis during an eight months period (6/2017-1/2018). Samples of milk obtained from eight healthy animals (from the animal research centre Al-Rumais, MoAF) were used as controls. All animals were hand milked by veterinary technicians and veterinarians in veterinary clinics after disinfecting the teats with 70% alcohol and discarding the first streams of milk. Five to 15ml of milk were taken into sterile vials and were labelled as from which quarter they were taken. The samples were transported in a cool box with ice packs to the bacteriology laboratory at the central laboratory of animal health. At the laboratory, the samples were kept at 4°C until used.

Laboratory analysis of milk

Consistency and colour evaluations were carried out according to Quinn *et al*., (1994). The milk samples were checked for colour change and the presence of blood, clots or flakes.

The pH of the milk samples were checked using pH test strips.

Modified White Side Test (MWT)

The test was performed as described by Kahir *et al.*, 2008. Briefly, 100 µL of sodium hydroxide solution 4% was added to 250 µL of cold milk on slide on black background and then stirring the mixture vigorously for 20 seconds. The milk of normal quarter will have no reaction with addition of sodium hydroxide solution and remains uniformly opaque. While the milk of cow suffering from mastitis shows reaction with addition of sodium hydroxide solution.

The reaction was scored as follow:

Negative(N): opaque, milky mixture, no precipitant

(+): Clumping of slight degree is present

(++): Mixture thickness, coagulated materials are present

(+++): Large mass of precipitants

Culturing of bacteria and fungi

For bacteria, the milk samples were cultured in nutrient broth for 1 day at 37°C. Then, the samples were cultured in nutrient agar and were examined for bacterial growth after 24 hours according to (Demme and Abegaz, 2015). The pure cultures were subjected to Gram staining according to manufacture protocol (TCS biosciences, UK). For fungi, the milk samples were cultured in Potato Dextrose Agar and were incubated at 37°C and examined for growth after 2 weeks according to (Pachauri *et al*., 2013; Sukumar and James, 2012).

DNA based identification

Genomic DNA was extracted from all isolated bacteria using High Pure PCR Template Preparation Kit (ROCH). Fungi were isolated using Power Soil DNA kit (MO BIO). The extracted DNA from bacteria and fungi were quantified using NanoDrop spectrophotometer (Thermo Scientific, Germany) at wavelength of 260/280 nm and checked on 1% agarose gel stained with ethidium bromide. Then, the 16S rDNA gene fragment was amplified using two universal primers (27F , 1492R) (Miller *et al*, 2013). The polymerase chain reaction (PCR) mixture consisted of 12.5µL of master mix (Thermo Scientific), 11.3µL of nuclease free water, 0.1µL of each primer, 1-2µL of DNA template. The PCR reaction was performed in 25µL volumes and three stages for 35 cycles. The first stage consisted of five cycle was initiated with 5 min at 94°C, followed by denaturation for 30 seconds at 94°C, and annealing for 30 seconds at 60°C. The extension was carried out for 2minutes at 72°C. The second stage consisted of five cycle started with denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C and extension for 4 minutes at 72°C. The last stage consisted of 25cycles initiated with denaturation for 30 seconds at 94°C, annealing for 30 seconds at 50°C and extension for 4 minutes at 72°C. Additional extension step was carried out for 2 minutes at 72°C.

The internal transcription spacer (ITS) region and elongation factor (EF) gene fragments were amplified using ITS1 and ITS4primers (White *et al*., 1990), and EF4 and fung5primers (Smit *et al*, 1999), respectively. The PCR mixture consisted of 12.5µL of master mix (Thermo Scientific), 11.3µL of nuclease free water, 0.1µL of each primer, 1-2µL of DNA template. The PCR reaction was performed in 25-26µL volumes started with 10 min at 95°C. The 35 cycles initiated with denaturation for 1 min at 95°C, followed by annealing for 5 min at 55°C. The extension was carried out for 2 minutes at 72°C. Additional extension step was carried out for 10 minutes at 72°C.

The PCR products was purified using DyeEx Spin kit (QIAGEN) following the manufacturer's protocol. The sequencing reaction was carried using Big Dye Terminator v3.1 Cycle Sequencing Big dye kit (Applied Biosystems, USA). The sequencing reaction product was purified in 96-Well Plates using sodium acetate purification. The purified DNA was sequenced using the genetic analyzer (3130 XL, Applied Biosystems, USA).

The sequencing results were edited using Bioedit software. The edited results were searched against the sequences available in National Centre for Biotechnology Information (NCBI) database and The Barcode of Life Data System (BOLD) database.

Antibiotic susceptibility testing (AST)

Antibiotic sensitivity tests was determined using disc diffusion method to each isolated strain. Mueller-Hinton agar medium and an antibiotic disc dispenser were used. Individual colonies were

dipped in nutrient broth and then they were spread evenly on petri dishes contain the medium. A total of 6 antibiotic discs were tested against each strain and these were: Gentamicin (GE) (10µg), Amoxicillin/clavulanic acid (AML) (30 µg), Ampicillin/sulbactam (AMP) (10µg), Co-trimoxazole (SXT) (25µg), Streptomycin (S) (10 µg), Tetracycline (TE) (10 µg). The plates were incubated for 24 h and zone of inhibition were measured in mm according to (Bauer *et al.*, 1966; Bhat *et al.*, 2017). The zones of inhibition (mm) were compared to the standards of the antibiotic supplier and the tested strains were recorded as sensitive, intermediate or resistant.

Results and Discussion

Results showed that subclinical mastitis (75%) is more common in the investigated samples than clinical mastitis (Table 1), which is similar to which reported by other studies (Türkyilmaz *et al*., 2010; Abera *et al*., 2012). The increase in subclinical cases since 1991 could be attributed to increased awareness among farmers about milk characteristics from infected animals (i.e. reduction in quality and quantity of milk and complaints from consumers).

Table1: The Frequency of Clinical and Subclinical Mastitis in this study compared to other studies

Study	Clinical	Subclinical
Oman 2018	25%	75%
Türkyilmaz *et al*., 2010	11%	89%
Abera *et al*., 2012	23%	77%

The majority of mastitis cases were associated with environmental and minor pathogens (89% clinical and 96% subclinical) than contagious pathogens (11% clinical and 4% of subclinical) (Table 2). Notably, there is a clear increase in the association of environmental and minor bacteria (50.5% to 89%) and a decrease in contagious pathogens (49.5% to 11%), compared to what was reported earlier by Harby *et al*. (1991). However, the same was reported by others (Kivaria and Noordhuizen 2007 and Carrillo-Casas and Miranda-Morales, 2012).

Table 2: The frequencies of Environmental, minor and contagious bacteria in the clinical and subclinical mastitis

	Clinical		Subclinical	
	1991	2018	1991	2018
Environmental and minor bacteria	50.5%	89%	NA	96%
Contagious	49.5%	11%	NA	4%

Staphylococcus aureus was the only identified contagious bacteria, while coagulase negative *staphylococci* (CNS), which is considered minor pathogens, was the most isolated microbes from clinical (24%) and subclinical (43%) cases (Table 3). In the earlier study, *Staphylococcus aureus* and *Streptococcus agalactiae* were reported (Harby *et al*., 1991) but many recent studies found that both

contagious pathogens decreased, while CNS and *Corynebacterium bovis* are becoming more common (Pitkälä *et al*., 2004). In fact, CNS are emerging as common pathogens associated with mastitis (Zeryehun and Abera 2017; Adwan *et al*. 2015).

Table 3: The number of identified contagious, environmental and minor bacteria

Environmental	Contagious	Minor
Achromobacter insolitus (1) *		
Bacillus velezensis (1)*		
Bacillus australimaris (2)*		
Bacillus licheniformis (2)		
Bacillus cereus (1)		
Bacillus xiamenensis (1)*		
Bacillus methylotrophicus (1)		****Staphylococcus xylosus* (5)
Bacillus aryabhattai (1) *		****Staphylococcus succinus* (1)
Brevibacillu sborstelensis (1)*	*Staphylococcus*	****Staphylococcus chromogenes* (4)
Brevibacillus agri (2)*	*aureus* (4)	****Staphylococcus sciuri* (9)
Chryseobacterium indologenes (1)		****Staphylococcus saprophyticus* (5)
Cosenzaea myxofaciens (1)*		****Staphylococcus epidermidis* (1)
Enterobacter cloacae (1)		*****Staphylococcus agnetis* (1)
Enterococcus faecium (1)		*****Staphylococcus hyicus* (1)
Enterococcus faecalis (1)		*Macrococcus caseolyticus* (1)
Enterococcus lactis (1)		
Escherichia fergusonii (4)		
Klebsiella pneumonia (3)		
Lactococcus lactis (1)		
Pantoea agglomerans (1)		
Proteus mirabilis (5)		
Providencia rettgeri (1)*		
Pseudomonas aeruginosa (3)		
Pseudomonas alcaligenes (1)		
Shigella dysenteria (1)		

The classification of contagious and environmental pathogens according to Coulona, 2002.

*First record of mastitis association

**Coagulase-negative species; ***coagulase-positive and coagulase-variable species (based on Becker *et al*, 2014)

Coagulase positive*:* *Staphylococcus xylosus, Staphylococcus saprophyticus, Staphylococcus aureus*

It is worth highlighting that different species were associated with the environmental cases, nine of which were not previously associated with mastitis**.** However, some of these bacteria were reported in other studies and had been isolated from clinical and subclinical mastitis (Salih, 2013; Olivares-Pérez *et al*.,2015; Banerjee *et al*., 2017; Srednik *et al*., 2017; Munoz *et al*., 2007; or considered as potential pathogens in different diseases (Li *et al*., 2017).

The increase in percentage and diversity of environmental and minor bacteria as a cause of mastitis in Oman could be attributed to the resolution power of sequencing in the identification at the species level compared to the culture-based methods, the unhygienic milking procedures and poor housing practices and/or over use of antibiotics as discussed later. Fortunately, mastitis caused by environmental pathogens can be controlled by increasing the hygiene of environment and pre-dipping (Blowey and Edmondson, 2010) and therefore, cutting cost.

In this study, 18% of the samples were culture negative for bacteria. Although the percentage we are reporting is less than others (49.7%) (Makovec and Ruegg, 2003) these cases are of big concern in management. They could be due to anaerobic bacteria (Du Preez, 1989), algae (Ranjan *et al*.,2006), or mycoplasma infections and/or environmental factors like trauma and drought (Kuehn *et al*., 2013).

Almost half of the examined samples (47%) with mastitis were positive for fungal growth. Different types of fungi were detected in the subclinical (33%) and clinical mastitis milk samples (16 %) (Table 4). Fungi were isolated either mixed with bacteria (45%) or in pure cultures (3%). High frequency of mixed infection is comparable to what Dworecka-Kaszak *et al.* (2012) recorded (57%).

Table 4: The number of identified fungal species associated with clinical and subclinical mastitis

Clinical		**Subclinical**	
Pichia manshurica	2	*Cyberlindnera jadinii*	1
Clavispora lusitaniae	1	*Clavispora lusitaniae*	3
Saccharomycopsis fibuligera	1	*Aspergillus spp (A. tubingensis (2), oryzae(1), flavus (1)*	4
Talaromyces primulinus	1	*Talaromyces pinophilus,*	5
Pichia kudriavzevii	1	*Pichia manshurica*	1
Aspergillus flavus	1	*Saccharomycopsis capsularis*	1
Candida glabrata	1	*Pichia kudriavzevii*	1
		Galactomyces geotrichum	1
		Geotrichum vulgare	2

Candida found to be the most predominant fungi in clinical and subclinical mastitis (66%), followed by *Penicillium spp* (28%), *Aspergillus spp* (16%), and *Galactomyces geotrichum* (12%). This is comparable with what was reported by others (Krukowski and Saba 2003; Kumar *et al.*, 2016; Pachauri *et al*., 2013; Erbaş*et al*., 2017; Wawron *et al., 2010;* Krukowski*et al*. 2001).

The negative bacterial cultures that were positive for fungi (3%) were associated with *Candida glabrata*and *Aspergillus ustus*. Notably, one of the isolated fungi was *Geotrichum candidum*, which is an opportunistic, keratinophilic yeast-like growth. Few reports around the world reported its involvement in mastitis and reported the genues (Costa *et al.*,1993).

Fungi are opportunistic organisms that are considered as normal flora in the udder skin and soil but are able to establish disease when immune system is weak (dos Santos and Marin, 2005).

Weakness of the cow immune system may result from several factors like; changeable weather, mineral-vitamin deficiencies and antioxidant deficiencies (Wawron *et al.*, 2010). Relatively high isolation of fungi from mastitis cases suggested potential unhygienic conditions and poor management practices to be associated. Moreover, fungal association can be attributed to prolong treatment with antibiotics (Pachauri *et al*., 2013). In fact, large doses of antibiotic without bacteriological examination cause vitamin A deficiency that damage the udder's epithelium and teat injuries can facilitate infection by yeast (Krukowski *et al*., 2001).

Seven antibiotics that are commonly prescribed were used to evaluate the antibiotics sensitivity of the isolated bacteria; including Ampicillin/sulbactam, Amoxicillin/clavulanic acid, Gentamicin, Streptomycin, Co-trimoxazole and Tetracycline (Table 5). Notably, *S. aureus* isolates from different regions showed different resistance to different antibiotics. However, they all showed sensitivity for AML.

Table 5: Frequency of antibiotics sensitivity (Number of sensitive isolates /total isolates) for Bacteria isolates

Bacteria isolates	**AMP**	**AML**	**CN**	**SXT**	**S**	**TE**
Escherichia fergusonii	25%		75%	75%	0%	25%
Klebsiella pneumoniae	0%	0%	33%	33%	0%	100%
Proteus mirabilis	20%	20%	100%	80%	40%	80%
Pseudomonas aeruginosa	33%	33%	100%	66%	66%	66%
Staphylococcus sciuri	0%	22%	67%	67%		100%
Staphylococcus chromogenes	0%	50%	50%	25%		50%
Staphylococcus saprophyticus	0	50%	80%	100%		100%
Staphylococcus aureus	25%	100%	75%	50%		50%
Staphylococcus xylosus	40%	50%	100%	100%		100%
Brevibacillusagri	0%		0%	100%	0%	
Bacillus australimaris	50%	100%	0%	100%	100%	50%
Bacillus licheniformis	0%	0%	50%	50%	0%	0%

Sensitivity: Number of isolates sensitive to antibiotic/total isolates

Although, CNS show different antibiotics resistance patterns, generally their response to treatment is higher than treating mastitis caused by *S. aureus* (Taponen and Pyörälä, 2009). CNS exhibited a high degree of resistance to AMP and AML (92%, 67%respectively), while showed high sensitivity to CN (69%), SXT (69%) and TE (84%). Notably, most of the CNS isolates (77%) were resistant to more than one antibiotic. Our results is consistent with what was reported earlier by other (Gentilini *et al*., 2002; Mahami *et al*., 2011; Bansal *et al*., 2015; Beyene *et al*., 2017; Sumathi *et al.*, 2008).

In this study, variety of Gram negative bacteria (GNB) were isolated from clinical and subclinical mastitis. GNB showed resistance to S (60%) and AMP (60%) and AML (52%) and sensitivity to SXT (64%), CN (60%) and TE (62%). Similar findings were observed by Nam *et al.* (2009) but a significant number of GNB isolates (72%) in this study had resistance to more than one

antimicrobial, which is higher (35%) than what was reported earlier (Younis *et al*. 2017).

Compared to the study by Harby *et al.* (1991), it was noted that the patterns of bacterial sensitivity of *Klebsiella* spp and *Staph. aureus* to CN, TE and AMP had changed towards increased resistance to the tested antibiotics. The development of resistance strains to some antibiotic can be due to overuse in the farm as reported by many (Kumar *et al*., 2010; Bhatt *et al.,* 2011; Sharma *et al.,* 2007; Argaw, 2016; Ventola, 2015).

The main challenges faced with the emergence of antibiotic resistance are difficulty of treatment, severity of infection and increase of mortality rates (Abdel-Rady and Sayed, 2009). Moreover, the bacteria and their genes can be transmitted to humans through consumption of non-pasteurized milk, wild animals, contaminated waterways and food chain (Abdel-Rady and Sayed,2009; Manie *et al*.,1999).

Variations in antibiotic sensitivity profiles of some species was detected, and this was reported by others and attributed to differences in the use of antimicrobials (Sadashiv and Kaliwal, 2014; Kalińska *et al.,* 2017). *Staphylococcus agnetis*, which is a coagulase-positive and isolated from clinical cases, showed resistance to all antibiotics used. Different studies reported different resistance to antibiotics (León *et al*., 2015;Taponen *et al.,* 2012).

Conclusion

Although mixed infections suggested to be treated with broad spectrum antibiotics, what we found recommends informed antibiotic selection to intervene with the emergence of resistant bacteria. In addition, to minimize antimicrobial resistance, the use of antibiotics in animal health should be optimized and the nontherapeutic use of antimicrobials as growth promoters in agriculture should be limited.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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