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Article *in* American Journal of Animal and Veterinary Sciences · May 2014

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American Journal of Animal and Veterinary Sciences 9 (2): 116-121, 2014
ISSN: 1557-4555

doi:10.3844/ajavssp.2014.116.121 Published Online 9 (2) 2014 (http://www.thescipub.com/ajavs.toc)

PREVALENCE OF MASTITIS PATHOGENS AND THEIR RESISTANCE AGAINST ANTIMICROBIAL AGENTS IN AWASSI SHEEP IN AL-BALQA PROVINCE OF JORDAN

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Received 2014-03-14; Revised 2014-04-30; Accepted 2014-05-03

ABSTRACT

The primary objective of this study was to establish data on mastitis in Awassi Sheep in Al-Balqa Province of Jordan. Milk samples were collected from 260 lactating ewes that selected randomly from eight flocks. California Mastitis Test (CMT) gave result with 220 milk samples; 122 samples (55.5%) showed positive CMT. Infection with some bacterial species was associated with positive CMT. About 26% of the ewes revealed clinical signs of mastitis. The highest percentage of bacterial count, which range from 3×10^2 to <3.0 10^3 cfu mL^{-1} was founded in the milk samples. The most predominant bacteria isolated were *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus* spp., *Escherichia coli*, *Corynebacterium* spp. and *Coagulase negative Staphylococci*. Sensitivity tests were applied to different isolated strains., Gentamycim, Ampicillin and Tetracycline were the most effective antimicrobial agents against the bacterial isolates.

Keywords: Awassi Sheep, Mastitis in Ewes, California Mastitis Test, Jordan

1. INTRODUCTION

Mastitis, similar to most livestock disease, is a result of the interaction between the host, pathogen and environment, although stress and physical injuries may cause inflammation of mammary gland, infection by invading bacteria or other microorganisms (fungi, yeast) is the primary cause of mastitis. It is the course of multiple hazardous effects on human health and animal production.

This inflammation of the mammary gland (mastitis) is known to be a complex and costly disease (Radostitis *et al*., 1994) The disease is associated with a decrease in milk production, an increase of veterinary services, treatment, labour costs and culling (Fthenakis, 1994). Mastitis is one of the most series economic and health problems of small ruminates flocks worldwide (Las Heras *et al*., 1999; Corrales *et al*., 2004; Osman *et al*., 2013). Current Knowledge of mastitis in small ruminants has been reviewed by some authors (Bergonier *et al*., 2003; Bergonier and Berthelot, 2003; Lafi *et al*., 1998; Contreras *et al*., 2007). The causative organisms of mastitis are categorized as major or minor pathogens (Harmon, 1994). The most common major pathogens include *Staphylococcus aureus*, *Streptococcus agalaciae*, *Coliforms* and *Enterococci*, while other pathogens such as *Streptococcus spp*., *Pseudomonas aeruginosa*, *Mannheimia hemolytica*, *Corynebacteria*, Coagalase negative *Staphylococci* and Fungi, are considered to be minor pathogens which can produce Intramammary Infection (IMI) in small ruminants, but occurance rates are

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lower (Contreras *et al*., 2007). In Asia bovine major mastitis causing organisms are *Staphylococcus aureus, Streptococci, E. Coli., Corynebacterium* spp *and klebsiella* spp., but recent reports indecating the changing trend from *Staphylococcus aureus* to *Coagulase Negative Staphylococci* (Sharma *et al*., 2012).

In North Greece, clinical mastitis of ovine was recorded in 11.4% of ewes examined. Mycoplasma spp. and *Staphylococcus aureus* were the important pathogens, as they were isolated from 45.9 and 38.5 percent respectively of mammary secretions samples, while other microorganism were isolated at a lower rate (Fthenakis and Jones, 1990). The annual incidence of clinical mastitis in small ruminants is generally lower they 5%, but this incidence can increase sporadically (Contreras *et al*., 2007). The prevalence of subclinical mastitis has been estimated at 5-30% or even higher (Bergonier and Berthelot 2003; Contreras *et al*., 2003).

In Egypt coagulase-negative *Staphylococci* were isolated from the examined subclinical mastitic sheep and goats with percentages of 50 and 55.6% respectively (El-Jakee *et al*., 2013).

In Jordan there are about 2.4 million Awassi sheep. The good adaptability of this breed to semi-dry climate encouraged sheep farmers to raise this breed in Jordan. This breed is raised for meat, milk and wool production. As Jordan lacks reliable information concerning the approporiate treatment of mastitis and due to the unregulated use of veterinary drugs, the objective of this study to isolate and identify the major udder pathogens and to determine the incidence of clinical and subclinical mastitis in ewes, a further objective was to determine the susceptibility of these bacteria to 6 antimicrobial agents that are or have been commonly used in Jordan.

2. MATERIALS AND METHODS

This study was conducted during the year 2012 and 2013. Milk samples were collected from 260 lactating ewes that selected randomly from eight flocks in Al-Balqa province. All udders were subjected to clinical examinations such as swelling and presence of lesions or anatomical malformation. Clinical mastitis was defined by the presence of abnormal udder secretions (clots, flakes, or abnormalities in color or consistency) and detection of mastitis pathogens by bacteriological culture, whereas subclinical mastitis was recognized by apparently normal milk and increase in leukocyte counts as evidenced by California Mastitis Test (CMT) and a positive culture result. CMT was used to give an indication of the number of somatic cells, it based upon a gelling reaction between the nucleic acid of the cells and a detergent reagent. The CMT was chosen in several investigation because it is more perfect, efficient and reliable than other field and chemical tests for diagnosis of subclinical mastitis (Dingwell *et al*., 2003; Sargeant *et al*., 2001; Sharma *et al*., 2011, Osman *et al*., 2013). CMT score 0 was taken as negative, while CMT socres trace, 1+, 2+ and 3+ were considered positive. All milk samples irrespective of CMT result was bacteriologically examined. For determination the total bacterial count, a volume of 0.1 mL of each milk sample was spread on Plate Count Agar (Oxoid); plates were incubated at 37°C 24 h and then developing colonies were counted. Direct streaking was done on duplicate 7% sheep blood agar and Macconkey agar plates; plates were incubated aerobically and anaerobically using Gas Pack System at 37°C and examined after 24 and 48 h. Bacteriological examinations were carried out following standard methods (Quinn *et al*., 1994; Sears *et al*., 1993). Presumptive identification of bacterial isolated was made based on colony morphological features, Gram-stain reaction, hemolytic characteristic and a catalase test.

Staphylococci and *Micrococci* were identified based on their growth characteristics on mannitol salt agar, coagulase production, catalase and oxidase test. Streptococci were evaluated according to CAMP reaction, growth characteristics on Edward's medium, hydrolysis of esculin, sodium hippurate, catalase production and sugar fermentation tests. Gram-negative isolates were subcultured on MacConkey agar and further tested using Triple Sugar Iron (TSI) agar (Oxoid), the IMVIC test (indol, methyl red, Voges-Proskuer and citrate utilizing test), urea, lysine and ornithine decarboxylase and oxidase reactions.

Sensitivity tests were carried out by using Muller-Hinton Agar (oxoid) and susceptibility discs (oxoid) to test the susceptibility of the isolates to some antibiotics, 10 µg Ampicillin, 10 µg Gentamycin, 10 IU Penicillin, 30 µg Tetracycline, 30 µg Neomycin and 25 µg Sulfamethoxazole.

All statistical analyses were performed using SAS/STAT Version 9.2 SAS (Institute Inc., Cary, NC) and Analysis of Variance (ANOVA) was conducted by the PROC GLIMMIX procedure.

3. RESULTS

Two hundred twenty milk samples out of 260 collected from individual ewes were scored by the CMT technique, Ewes with signs of inflamed udders had a

mean lactation of about three months. About one fourth (26%) of the ewes had clinical signs of mastitis.

Table 1 shows the relationship between positive and negative CMT scores and the percentages of ewes milk samples of different bacterial counts. The positive and negative samples distributed in three different bacterial count ranges namely $<3.0\times10^2$, 3.0×10^2 to $<3.0\times10^3$ and $>3.0\times10^3$ cfu mL^{-1}, the highest percentage of CMT positive samples (60.3) was found in the range of 3.0×10^2 to $<3.0\times10^3$ cfu mL^{-1}, while the highest percentage of CMT negative samples (65.5) was found in the total bacterial count of $<3.0\times10^2$ cfu mL^{-1}.

Bacteria identified and percentage of ewe milk samples with different CMT scores were illustrated in **Table 2**. This indicates the relationship between specific organisms, which mostly are the causative agent of mastitis and the respective percentage of samples with negative and positive CMT. The bacteria (*Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus* spp.) showed the highest percentages for positive CMT; the bacteria (*Corynebacterium pyogenes*, *Corynebaterium pseudotuberculosis*, *Pseudomonas aeruginosa* and *Brucella melitensis*) showed only positive CMT; while the bacteria (*Pasteurella multocida* and *Mannheimia haemolytica*) showed only negative CMT.

Table 3 shows the percentage of ewes milk samples that included in two different bacterial counts of various organisms. The total bacterial count range for different bacteria infecting ewes udder was most commonly 3.0×10^2 to 3.0×10^3 rather than $>3.0\times10^3$ cfu mL^{-1}. The most frequent bacterial flora from different ewes were: *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus spp*., *E. Coli*, coagulase-negative Staphylococci, *Corynebactenium* spp. and Pseudomonas aeruginose. Five other aerobic bacteria were isolated. Yeast was isolated from two samples.

Table 4 shows the result of sensitivity tests of organisms isolated bacteria to antibiotics. The *in vitro* susceptibility testing of bacterial isolates showed that the most effective drugs were Gentamycin and Ampicillin. The less effective drug was penicillin.

Table 5 shows analysis of variance for six antibiotics and twelve bacteria.

3.1. Statistical Analysis

The analysis of variance for antibiotics sensitivity shows that there are significant differences between antibiotics treatment at ($p\leq0.1$). Meanwhile, there are no significant differences between isolated bacteria.

Table 1. The relationship between positive and negative CMT scores and the percentages of ewes milk samples of different bacterial counts

		Percentage of samples within the total bacterial count range		
CMT score	No. of samples	$<3.0\times10^2$	3.0×10^2 to $<3.0\times10^3$	$>3.0\times10^3$
Positive	122	25.2	60.3	14.5
Negative	98	65.5	29.2	5.3

Table 2. Bacteria identified and percentage of ewes milk samples with different CMT scores

		Percentage of samples within CMT scores range		
Bacterial isolates	No. of samples	No. of positive	Positive	Negative
Staphylococcus aureus	45	37	82.20	17.80
Streptococcus agalactiae	40	32	80.00	20.00
Streptococcus spp. (non-groupable)	25	22	88.00	12.00
Coagulase-negative *staphylococci*	12	9	75.00	25.00
Escherichia coli	22	2	9.10	90.90
Corynebacterium pyogenes	8	8	100.00	0.00
Streptococcus dysagalactiae	4	3	75.00	25.00
Yeast	2	1	50.00	50.00
Corynebacterium pseadotuberculosis	6	6	100.00	0.00
Pasteurella multocida	4	0	0.00	100.00
Mannheimia haemolytica	5	0	0.00	100.00
Pseudomonas aeruginosa	8	8	100.00	0.00
Brucella melitensis	3	3	100.00	0.00

Table 3. The percentage of ewes milk samples that included in two different bacterial counts of various organisms

		Percentage of samples within bacterial count range*	
Bacterial species	No. of samples	>3.0×10^2 to <3.0×10^3	>3.0×10^3
Staphylococcus aureus	42	80.9	19.1
Streptococcus agalactiae	35	77.1	22.9
Streptococcus spp. (non-groupable)	20	80.0	20.0
Escherichia coli	20	70.0	30.0
Coagulase negative staphylococci	11	72.7	27.3
Crynebacterium pyogens	8	62.5	37.5
Pseudomanas aeruginosa	8	50.0	50.0
Corynebacterium pseudo tuberculosis	5	80.0	20.0
Pasteurella multocida	4	50.0	50.0
Mannheimia hemolytica	5	60.0	40.0
Yeast	2	100.0	0.0
Brucella melitensis	2	100.0	0.0
Klebsiella pneumoniae	2	100.0	0.0
Enterococcus spp.	2	100.0	0.0

Table 4. Sensitivity test for bacterial isolates against different antibiotics

		Percentage of sensitivity to antibiotic					
Bacterial species	No. of Isolates	AM	GM	P	TE	NEO	SUL
Staphylococcus aureus	42	88.3	95.2	23.8	95.2	47.6	95.2
Streptococcus agalactia	35	77.1	80.0	28.6	71.4	77.1	71.4
Streptococcus spp.	20	100.0	100.0	25.0	95.0	75.0	71.4
Escherichia coli	20	30.0	95.0	0.0	25.0	25.0	85.0
Coagulase negative steph.	11	90.9	90.9	27.3	90.9	45.9	100.0
Pseudomonas aeruginosa	8	0.0	100.0	0.0	37.5	75.0	75.0
Corynebacterium pyogenes	8	100.0	62.0	75.0	75.0	37.5	75.0
Corynebacterium pseudotuberculosis	5	100.0	100.0	0.0	75.0	40.0	40.0
Pasteurella multocida	4	100.0	50.0	0.0	50.0	25.0	50.0
Mannheimia hemoltytica	5	100.0	100.0	20.0	80.0	40.0	60.0
Enterococcus spp.	2	100.0	100.0	0.0	100.0	50.0	50.0
Klebsiella pneumonia	2	100.0	100.0	0.0	100.0	25.0	50.0
Mean		81.78^{ab}	89.43^{a}	16.64^{d}	74.58^{ab}	46.93^{c}	68.58^{b}

Means followed by the same letter are not significantly different based on Fisher's Protected LSD at p≤0.05.
*AM = Ampicillin (10 µg), GM = Gentamycin (10 µg), P = Penicillin (10 IU), TE = Tetracycline (30 µg), NEO = Neomycin (30 µg), SUL = Sulfamethoxazole (25 µg)

Table 5. Analysis of variance for six antibiotics and twelve bacteria

Source of variation	DF	SS	MSS	F ratio
Antibiotics	5	43483.55	8696.71	18.07
Bacteria	11	8909.92	809.99	1.68
Error	55	26472.23	481.31	
Total	71	78865.70		

4. DISCUSSION

Several studies in different parts of the world have been conducted for the assessment of the occurance of clinical and subclinical mastitis in different breeds of sheep (Al-Majali and Jawabreh, 2003; Lafi *et al*., 1998; Contreras *et al*., 2007; Gebrewahid *et al*., 2012). The relation among CMT, the presence of inflamed udders and the bacteriological findings indicated that ewe milk is like that of cows and camels (Djabri *et al*., 2002; Hawari and Al-Dabbas, 2008); it also indicated that ewes have phogocytic cells, which constitute one of the essential defences against microbial infection of the mammary glands. An increase of somatic cells in milk is a good indication of inflammation as shown in **Table 2** which indicates that the majority of ewes react to

infecting bacteria by raising the somatic cells in milk. So the CMT is a useful screening test in the detection of mastitis and may serve to segregate mammary glands infected with major pathogens in a subclinical form (Schuppel and Schwope, 1998; Clements *et al*., 2003; Gebrewahid *et al*., 2012). **Table 1 and 2** indicated that bacterial infection was involved in mastitis of ewes. Higher bacterial counts were present in positive CMT than in negative ones as shows in (**Table 1**).

In many cases of infection with a variety of bacteria, the organisms are present at less than 3.0×10^3 mL^{-1} and a minority exceed this level as shows in **Table 3**. This may indicate that there is a limit to bacterial multiplication in ewes udder probably due to complex immune system.

Staphylococcus aureus, *Streptococcus agalactiae* and *E. coli* were the main aetiological agents of mastitis in ewes of the present study (**Table 3**). Similar results had been reported by (Lafi and Hailat, 1998; Fthenakis and Jones, 1990) While in other study the most common organisms isolated from subclinical mastitis cases were coagulase-negative *Staphylococci* and *E. Coli* (Lafi *et al*., 1998). The in vitro susceptibility test of the bacterial isolates indicated that the bacterial flora showed greatest resistance to penicillin, this drug are the most commonly used for domestic animals in Jordan and this may lead to an accumulation of resistant bacteria to this drug. The percentage average of resistance of Gram-positive cocci to penicillin was 70.1% as shown in **Table 4**.

5. CONCLUSION

In conclusion, the results of this study indicated that mastitis was prevalent in Awassi sheep in Jordan and the Gram-positive cocci were the dominant mastitis pathogens. Thus, good attention and management practices is require to control the occurance of the disease. The proper isolation and identification of the causative organism plays a significant rol in control of the disease. Further epidemiological studies should be conducted to determine the prevalence of the disease at regional and national levels taking in consideration using effective antibiotics therapy during lactation and at drying off; this would be essential part of such a program.

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