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Isolation and identification of bacterial causes of clinical mastitis in cattle in Sulaimania region

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Abstract

A total of 51 cases of bovine clinical mastitis in Sulaimani district were investigated for their bacteriological causative agents; 76 milk samples were cultured on primary and selective media and the isolated bacteria were tested for their susceptibility to antimicrobial agents used in commercial intramammary infusion products. Eighty two bacterial isolates were obtained and further identified using biochemical tests. *Escherichia coli* was the most common bacteria followed by *Staphylococcus aureus, Streptococcus agalactia* and coagulase–negative staphylococci. Two other bacterial species (*Pseudomonas aeruginosa* and *Streptococcucs uberis*) were also isolated but in a lower proportion. Antibacterial susceptibility testing showed that the use of florfenicol, cephalexin and gentamicin may be useful for the treatment of clinical mastitis cases in cows.

Keywords: Bovine; Mastitis; Intramammary infection

عزل وتشخيص المسببات الجرثومية لالتهاب الضرع السريري في الابقار في منطقة السليمانيه

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الخلاصة

أخـذت عينات حليب من ٧٦ ربع مصاب mastitic quarters وجدت في ٥١ بقره تعاني من التهاب الضرع السريري clinical mastitis في منطقـة السـليمانيـه لأجل التحري عن المسببات الجرثوميه لهـذه الحاله. زرعت عينات الحليب على أوساط زرعيه جرثوميه أوليه وأختياريه ومن ثم تم فحص حساسية الجراثيم المعزوله للمضـادات الجـرثـوميـه التي تستخدم في الحقن التجاريه (commercial intramammary infusion products)المستخدمه للعلاج الموضعي لحالات التهاب الضرع. تم الحصول على ٨٢ عزله جـرثـوميـه و قـد أظهرت الفحوصـات الكيمياحيويـه أن أغلب هـذه العزلات تعود لجراثيم الـ *Escherichia coli* تلتـهـا جـراثيم الـ *Staphylococcus aureus* و الـ *Streptococcus agalactia* و الـ Coagulase Negative Staphylococci . كمـا تـم تشخيـص نوعين أخـرين من الجـراثيـم (الـ *Pseudomonas aeruginosa* و الـ *Streptococcucs uberis*) ولكـن بنسـب أقـل. أظهـرت فحـوصـات حسـاسيـة الجـراثيـم المعـزولـه للمضـادات الجـرثـومـيـه ان الأستخدام المتزامن للفلـورفنيـكـول والسيفالكسين والجنتامايسين قـد يكـون نافعا لعلاج التهاب الضرع السريري في منطقـة السـليمانيـه.

Introduction

Bovine mastitis is the most costly disease facing the dairy industry throughout the world. It creates estimated losses of about two billions dollars per year in the United States (1). The majority of losses is due to reduced milk production, production of lower quality milk, cost of drugs and veterinary services, increased culling rate and reduced reproductive efficiency (2).

Mastitis or intramammary infection can generally be classified as clinical and subclinical (3) Clinical mastitis is characterized by an abnormal secretion containing clots or flakes (4, 5). Sudden onset of clinical mastitis (acute clinical mastitis) is accompanied by swelling, hardness and increased temperature and it also may be accompanied by systemic signs such as loss of appetite, fever, dehydration, or depression (6).

No apparent changes in the udder or milk are expected with subclinical mastitis, although microorganisms can be isolated by appropriate culture techniques. Compositional changes and increased somatic cell count in milk usually accompany subclinical mastitis and can be detected by appropriate tests (3, 7).

Many of intramammary infections originate during the dry or nonlactating period and result in clinical or subclinical mastitis during early lactation (4). More than 135 different microorganisms have been isolated from bovine intramammary infections (3). The causative organisms of mastitis are categorized as contagious pathogens including *Staphylococcus aureus*, *Streptococcus agalactia*, and *Mycoplasma bovis* or as environmental pathogens such as environmental streptococci (e.g., *Streptococcus dysagalactia* and *Streptococcus uberis*), and the enterobacteriaceae (8-10). Coagulase–negative staphylococci, which colonize bovine teat skin and teat canals, are classed as skin flora opportunists (3).

Rapid reduction in herd mastitis level requires identification of infected glands and specific pathogens, followed by drug therapy and/or culling of affected cows. Thus, microbiological culturing is the single most reliable tool for developing a specific mastitis control program for a dairy herd (3, 11).

The objectives of this study were to isolate and identify mastitis associated bacteria in Sulaimani district and to determine the susceptibility of the isolated bacteria to antimicrobial agents used in commercial intramammary infusion products.

Materials and Methods

Sample collection

A total of 51 individual clinical cases of bovine mastitis were investigated from July 2003 through August 2006 at the teaching veterinary hospital and at several small private cattle herds in Sulaimani district.

Milk samples were collected from 76 mastitic quarters which were recognized by swelling, hardness, warmth and/or abnormal secretions (abnormal color or consistency and/or presence of clots or flacks). Sampling of milk was performed according to Watts, (3) as below:

- The udder was carefully washed, dried and several streams of milk were forcibly stripped from the mastitic quarter. The teat of the affected quarter was then scrubbed with a cotton pledget soaked in 70% alcohol.
- Two streams of milk were collected into a sterile screw capped vials.
- Collected samples were immediately kept in an insulated container with ice packs and transferred to the laboratory for bacterial culturing.

Bacterial Isolation and identification

Milk samples were brought to room temperature and mixed thoroughly. A 0.1 ml of each sample was surface plated on 5% calf blood agar, MacConkey's agar and nutrient agar.

The inoculated plates were subsequently incubated aerobically at 37°C and examined for growth after 16 to 48 hours of incubation.

Primary cultures were evaluated by visual examination of the morphology of the bacterial colonies and were subcultured on mannitol salt agar, eosin methylene blue agar and nutrient agar slants.

Identification of the purified bacterial cultures was applied using conventional bacteriological and biochemical procedures as described by Carter and Carter *et al*. (12 and 13) as well as commercial identification kits including the enterosystem 18R (Liofilchem s.r.l., Italy) and the Analytic Profile Index -API- (Biomériux, France).

Cultures were considered to be negative when no bacterial growth was observed on the culture plates and they were considered to be positive when only one or two species of bacteria, known to cause mastitis, were isolated from a sample or when contagious pathogens such as *Staphylococcus aureus* or *Streptoococcus agalactia* were recovered, even in a mixture of environmental bacteria. On the other hand, cultures were considered to be contaminated when they show mixed growth of three or more environmental bacteria (11).

Susceptibility testing

The Bauer Kirby procedure (14), on Muller–Hinton agar plates was used to determine the susceptibility of the bacterial isolates that obtained in the present study to antimicrobial agents used in commercial intramammary infusion products such as ampicillin, cephalexin,

erythromycin, florfenicol, gentamicin, penicillin, streptomycin, and tetracycline.

Following 16-18 hours of aerobic incubation, the plates were examined and the diameter of the zone of inhibition was measured by a ruler. The zone diameters were expressed as resistant, intermediate or susceptible according to Chengappa (15).

Results and Discussion

The identification of etiological agents of mastitis along with their susceptibility or resistance to antimicrobial agents used in intramammary infusion products, may help in controlling bovine mastitis, one of the most costly diseases affecting the dairy industry, and in developing therapy protocols for particular dairy farms (11). To our knowledge, this is the first investigation of clinical mastitis cases in cattles in Sulaimani district.

Of the seventy six milk samples examined in the present study, six samples revealed negative cultures and eleven samples revealed mixed cultures of three or more environmental bacteria and accordingly considered contaminated. The remaining 59 samples revealed positive cultures of which, 23 samples showed dual bacterial isolation, i. e., a total of 82 different bacterial isolates were recovered and the biochemical tests revealed these isolates belong to 7 species. The isolation frequency of the bacterial strains and their responses to the most important biochemical tests are summarized in table 1 and 2 respectively.

The high prevalence of *Escherichia coli*–induced clinical mastitis encountered in the present study is in agreement with the findings of other authors (16-18) who considered the *Escherichia coli* organisms as major etiological agents of clinical mastitis. They are opportunistic environmental or enteric pathogens and it may be possible for the infected quarter to serve as reservoir for recurrent episodes of *Escherichia coli*–induced clinical mastitis (19,20). It is worth mentioning that cases of *Escherichia coli*–induced clinical mastitis are often of very short duration and milk samples may reveal negative cultures in approximately 20% of such cases (11,16), accordingly, the six culture–negative milk samples encountered in the present study may be attributed either to *Escherichia coli* or to other intramammary pathogens could not be detected in the present study such as *Mycoplasma* species.

Staphylococcus aureus and *Streptococcus agalactia*–induced clinical mastitis cases were also encountered in a high prevalence in this study. This finding is similar to those of other authors (4, 21,22). However, it disagrees with findings of some other authors (6,23) who mentioned that these two bacteria are currently classified as causes of subclinical rather than clinical mastitis. This disagreement can be attributed to the poor mastitis control measures applied in Sulaimani district wherein, cattle breeding is of the semi–intensive type and management of animals is the whole family duty especially females who have little or even no idea about the principle concepts of animal management compared to the effective control measures followed in dairy herds of the developed countries. Nevertheless *Staphylococcus aureus* and *Streptococcus agalactia* are considered significant organisms associated with clinical and subclinical bovine mastitis worldwide (9, 10, 13 and 24) due to persistent cow –to– cow spread, possibly via milking machines and perhaps by the hands of milkers (25). Their main reservoirs are infected quarters (3, 26). In addition, *Staphylococcus aureus* can also be isolated from the skin of the udder and teats and from many other sites in dairy cows as well as feed and caretakers (27, 28).

The other bacterial species frequently isolated in the present study are coagulase–negative staphylococci including *Staphylococcus epidermidis and Staphylococcus xylosus*. The importance of such bacterial species as a cause of clinical mastitis has come under increased scrutiny in dairy herds used effective recommended mastitis control measures, they were previously considered as mastitis minor pathogens associated with a mild inflammatory reaction but they are now known to cause clinical mastitis (29, 30). They colonize bovine teat skin and teat canals, thus they are classed as skin flora opportunists (3).

Other bacteria including *Pseudomonas aeroginosa* and *Streptococcus uberis* were also isolated from mastitic quarters milk but in a lower proportion compared with those mentioned above (table 1). These findings are generally in agreement with those reported by other authors (4, 21, 22).

The natural habitat of *Pseudomonas aeruginosa* organisms is water, soil, and decaying vegetation; they also may be found on the skin and mucous membranes, and in feces, thus they are classified as environmental mammary gland pathogens (13).

Streptococcus uberis is classified as an environmental mammary gland pathogen; it colonizes various body sites including teat canal and has been isolated from bedding material. (3).

The susceptibility of the bacterial species isolated in the present study to the antimicrobial agents used in commercial intramammary infusion products is shown in table 3. These results are generally in agreement with the findings of other authors (3, 11, 13, 31). They showed that except *Pseudomonas aeruginosa*, all bacterial isolates obtained in the present study were susceptible or at least intermediately susceptible to florfenicol and cephalexin. In addition, the *Escherichia coli* isolates were also susceptible or intermediately susceptible to ampicillin, gentamicin and streptomycin; the *Staphylococcus* isolates were intermediately susceptible to erythromycin, gentamicin, and

streptomycin; and the streptococcal isolates showed high susceptibility to penicillin G, ampicillin, and erythromycin. Regarding the *Pseudomonas aeruginosa* isolates, they were resistant to most antibacterial agents; however they showed moderate susceptibility to gentamicin. Thus, the simultaneous use of florfenicol, cephalexin and gentamicin may be useful for the treatment of bovine clinical mastitis cases in Sulaimani district.

Table 1: Bacterial isolates from milk samples obtained from the mastitic quarters.

Bacteria	Number of isolates	%*
Escherichia coli	31	37.8
Staphylococcus aureus	19	23.2
Streptococcucs agalactia	15	18.3
Staphylococcus epidermidis	9	10.98
Staphylococcus xylosus	3	3.66
Pseudomonas aeruginosa	3	3.7
Streptococcucs uberis	2	2.4
Total	82	

* The percentage is with respect to the total number of isolates (82).

Table 2: The biochemical properties of the bacterial isolates obtained from milk samples of mastitic quarters.

Bacteria	Indole production	Methyl red	Voges - proscauer	Citrate utilization	Hydrogen sulfide (TSI)	Oxidase	Coagulase	Catalase	Hemolysis	Nitrate reduction	Arginine dihydrolase	Ornithin decarboxylase	Lysine decarboxylase	Lactose	Maltose	Mannitol	Xylose	Gelatin hydrolysis	Esculin hydrolysis
Escherichia coli	+	+	–	–	–	*			α ,β		–	V	V	+	+	+		–	V
Staphylococcus aureus						–	+	+	β	+	–	–		+	+	+			
Streptococcucs agalactia						–	–	–	α ,β					+	+	–			–
Staphylococcus epidermidis						–	–	+	–	+	–	–		+	+	–			
Staphylococcus xylosus						–	–	+	–	+	–	–		+	+	+	+	+	+
Pseudomonas aeruginosa	–				–	+			β	+	+	–	–	–	–			+	
Streptococcucs uberis						–	–	–	α					+	+	+			+

* : not tested; V: Variable

Table 3: Antimicrobial susceptibility testing of the bacterial isolates obtained in the present study to the antimicrobial agents used in commercial intramammary infusion product.

Antimicrobial agent	Disk potency	Inhibition zone diameter *					
		Resistant		Intermediate		Susceptible	
		Diameter " mm "	No. of isolates	Diameter " mm "	No. of isolates	Diameter " mm "	No. of isolates
Ampicillin							
Escherichia coli	10 µg	≤ 11	2	12 – 13	7	≥ 14	22
Staphylococcus aureus	10 µg	≤ 28	19	__ **	__	≥ 29	__
Streptococcus agalactia	10 µg	≤ 21	__	__ **	__	≥ 30	15
Cephalexin							
Escherichia coli	30 µg	≤ 14	5	15 – 17	14	≥ 18	12
Staphylococcus aureus	30 µg	≤ 14	__	15 – 17	5	≥ 18	14
Streptococcus agalactia	30 µg	≤ 14	__	15 – 17	2	≥ 18	13
Erythromycin							
Escherichia coli	15 µg	≤ 13	28	14 – 17	3	≥ 18	__
Staphylococcus aureus	15 µg	≤ 13	4	14 – 17	9	≥ 18	6
Streptococcus agalactia	15 µg	≤ 13	__	14 – 17	5	≥ 18	10
Florfenicol							
Escherichia coli	30 µg	≤ 12	__	13 – 17	10	≥ 18	21
Staphylococcus aureus	30 µg	≤ 12	2	13 – 17	5	≥ 18	12
Streptococcus agalactia	30 µg	≤ 12	3	13 – 17	5	≥ 18	7
Gentamicin							
Escherichia coli	10 µg	≤ 12	4	13 – 14	9	≥ 15	18
Staphylococcus aureus	10 µg	≤ 12	3	13 – 14	8	≥ 15	8
Streptococcus agalactia	10 µg	≤ 12	15	13 – 14	__	≥ 15	__
Penicillin G ***							
Escherichia coli	10 U	≤ 11	28	12 – 21	3	≥ 22	__
Staphylococcus aureus	10 U	≤ 20	16	21 – 28	3	≥ 29	__
Streptococcus agalactia	10 U	≤ 11	__	12 – 21	__	≥ 22	15
Streptomycin							
Escherichia coli	10 µg	≤ 11	7	12 – 14	13	≥ 15	11
Staphylococcus aureus	10 µg	≤ 11	5	12 – 14	8	≥ 15	6
Streptococcus agalactia	10 µg	≤ 11	15	12 – 14	__	≥ 15	__
Tetracycline							
Escherichia coli	30 µg	≤ 14	26	15 – 18	5	≥ 19	__
Staphylococcus aureus	30 µg	≤ 14	17	15 – 18	2	≥ 19	__
Streptococcus agalactia	30 µg	≤ 14	3	15 – 18	7	≥ 19	5

* The interpretive standards of the inhibition zone diameter in this table are mentioned according to Chengappa, 1990 (15).
** Not available or not recommended.
*** Benzyl-penicillin.

Table 3. (continued)

Antimicrobial agent	Disk potency	Inhibition zone diameter *					
		Resistant		Intermediate		Susceptible	
		Diameter " mm "	No. of isolates	Diameter " mm "	No. of isolates	Diameter " mm "	No. of isolates
Ampicillin							
Coagulase – negative staphylococci	10 µg	≤ 28	9	__ **	__	≥ 29	3
Pseudomonas aeruginosa	10 µg	≤ 11	3	12 – 13	__	≥ 14	__
Streptococcus uberis	10 µg	≤ 21	__	__ **	__	≥ 30	2
Cephalexin							
Coagulase – negative staphylococci	30 µg	≤ 14	__	15 – 17	4	≥ 18	8
Pseudomonas aeruginosa	30 µg	≤ 14	3	15 – 17	__	≥ 18	__
Streptococcus uberis	30 µg	≤ 14	__	15 – 17	__	≥ 18	2
Erythromycin							
Coagulase – negative staphylococci	15 µg	≤ 13	4	14 – 17	3	≥ 18	5
Pseudomonas aeruginosa	15 µg	≤ 13	3	14 – 17	__	≥ 18	__
Streptococcus uberis	15 µg	≤ 13	__	14 – 17	__	≥ 18	2
Florfenicol							
Coagulasen – negative staphylococci	30 µg	≤ 12	__	13 – 17	2	≥ 18	10
Pseudomonas aeruginosa	30 µg	≤ 12	3	13 – 17	__	≥ 18	__
Streptococcus uberis	30 µg	≤ 12	__	13 – 17	__	≥ 18	2
Gentamicin							
Coagulase – negative staphylococci	10 µg	≤ 12	1	13 – 14	8	≥ 15	3
Pseudomonas aeruginosa	10 µg	≤ 12	__	13 – 14	3	≥ 15	__
Streptococcus uberis	10 µg	≤ 12	2	13 – 14	__	≥ 15	__
Penicillin G ***							
Coagulase – negative staphylococci	10 U	≤ 20	9	21 – 28	__	≥ 29	3
Pseudomonas aeruginosa	10 U	≤ 11	3	12 – 21	__	≥ 22	__
Streptococcus uberis	10 U	≤ 11	__	12 – 21	__	≥ 22	2
Streptomycin							
Coagulase – negative staphylococci	10 µg	≤ 11	3	12 – 14	5	≥ 15	4
Pseudomonas aeruginosa	10 µg	≤ 11	1	12 – 14	2	≥ 15	__
Streptococcus uberis	10 µg	≤ 11	2	12 – 14	__	≥ 15	__
Tetracycline							
Coagulase – negative staphylococci	30 µg	≤ 14	8	15 – 18	1	≥ 19	3
Pseudomonas aeruginosa	30 µg	≤ 14	3	15 – 18	__	≥ 19	__
Streptococcus uberis	30 µg	≤ 14	__	15 – 18	1	≥ 19	1

* The interpretive standards of the inhibition zone diameter in this table are mentioned according to Chengappa, 1990 (15).
** Not available or not recommended.
*** Benzyl-penicillin.

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