

Prevalence and Bacterial Etiology of Subclinical Mastitis in Dairy Cows in Al Sulaimaniyah District

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Abstract:

Comprehensive data regarding subclinical mastitis in dairy cows are essential for implementation of an appropriate control programs for this economically significant disease. Such data are unavailable in Al Sulaimaniyah district in Iraqi Kurdistan Region, therefore, this study was designed to determine the prevalence, bacterial causative agents and antimicrobial susceptibility of subclinical mastitis in dairy cows in this district. A total of 288 milk samples obtained from 72 dairy cows in 5 different regions in Al Sulaimaniyah district were investigated for subclinical mastitis using California mastitis test (CMT). Milk samples with positive results to CMT were subjected to bacteriological analysis.

Out of the 72 cows tested, 28 (38.89%) showed positive results for subclinical mastitis, of which, 15 were Holstein-Friesian cows, 4 were native cows and 9 were crossbred cows. Regarding age susceptibility, 7 infected cows were 2-4 years old and the other 21 were 5-7 years old. A total of 62 bacterial isolates were recovered and the biochemical tests revealed these isolates belonging to 8 species. *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus agalactiae* were the most common bacteria followed by *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus* and *Staphylococcus chromogenes* respectively. Antibacterial susceptibility testing showed that the simultaneous use of florfenicol, cephalexin, erythromycin and ampicillin may be useful for the treatment of subclinical mastitis cases in cattle in Al Sulaimaniyah district.

Keywords: subclinical mastitis; Intramammary infection, dairy cows.

الخلاصة:

تعد بيانات التهاب الضرع تحت السريري في حقول ابقار الحليب ضرورية جدا لاعداد برامج مناسبة للسيطرة على هذا المرض المهم جدا من الناحية الاقتصادية، ولافتقار منطقة السليمانية الى مثل هذه البيانات صممت هذه الدراسة التي تهدف الى تحديد نسبة التهاب الضرع تحت السريري في ابقار الحليب ومسبباته البكتيرية وحساسيتها للمضادات الجرثومية في محافظة السليمانية في اقليم كردستان العراق، حيث تم التحري عن التهاب الضرع تحت السريري في 288 عينة حليب أخذت من 72 بقرة في 5 مناطق مختلفة من

المحافظة باستخدام اختبار كاليفورنيا لالتهاب الضرع، وبعد ذلك أخضعت عينات الحليب التي اظهرت نتائج موجبة لهذا الاختبار للتحليل الجرثومي.

أظهرت 28 من مجموع الـ 72 بقرة التي شملتها هذه الدراسة نتائج موجبة لالتهاب الضرع تحت السريري، كان منها 15 من فصيلة ابقار الهولشتاين-فريزيان و 4 محلية و 9 مضربة. وفيما يخص العمر، كانت 7 من الابقار المصابة بعمر 2-4 سنوات فيما كانت الـ 21 الاخرى بعمر 5-7 سنوات. تم عزل 62 عزلة بكتيرية، وقد اظهرت الاختبارات الكيمياحيوية انها تعود الى 8 انواع، حيث كانت الـ *Staphylococcus aureus* والـ *Escherichia coli* والـ *Streptococcus agalactiae* هي الغالبة ومن ثم تلتها الـ *Streptococcus uberis* والـ *Streptococcus dysgalactiae* والـ *Staphylococcus epidermidis* والـ *Staphylococcus saprophyticus* والـ *Staphylococcus chromogenes* على التوالي. أظهرت اختبارات حساسية البكتريا المعزولة للمضادات الجرثومية أن الاستخدام المتزامن للفلورفينيكول والسيفالكسين والاريثرومايسين والامبسلين قد يكون نافعا في لعلاج حالات التهاب الضرع تحت السريري في ابقار الحليب في منطقة السليمانية.

Introduction:

Mastitis is a serious problem causing enormous economic losses in dairy industry throughout the world (1-5). Many of the intramammary infections (IMI) originate during the dry or non lactating period and result in clinical or subclinical mastitis during early lactation (6). Subclinically infected udder quarters can develop clinical mastitis and the rate of new infections can be high (7). Cows with subclinical mastitis maintain a reservoir of infection within the dairy herd and increase the potential exposure of uninfected cows to contagious pathogens (8). The identification of the microorganisms responsible for subclinical mastitis in cattle is significant, in order to establish specific and efficient management of dairy flocks to avoid the development of clinical mastitis (9-11).

The causative organisms of mastitis are categorized as contagious

pathogens including *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Mycoplasma bovis* or as environmental pathogens such as environmental streptococci (e.g., *Streptococcus dysgalactiae* and *Streptococcus uberis*), and the enterobacteriaceae (12-14). The objectives of this study was to determine the prevalence and bacterial etiology of subclinical mastitis in Al Sulaimaniyah district and to investigate the susceptibility of the isolated bacteria to antimicrobial agents used in commercial intramammary infusion products.

Materials and Methods:

1. Study areas and milk sampling

A total of 288 milk samples were obtained from 72 apparently healthy cattle in some villages "Tagaran, Damrkan, Mahmudiya and Zrgwez" in Chwarta, Arbat, Bazian and Qaradagh

regions of Al Sulaimaniyah district and in a cattle breeding farm in Bakrajo in Al Sulaimaniyah center during three months extended from the June to August of the year 2008. Out of these 72 cattle, 26 were Holstein-Friesian, 15 were native and 31 were crossbred. The ages of the sampled cattle ranged from 2-4 years (23 animals) to 5-7 years (49 animals).

Sampling of milk was performed according to (15). Briefly, Teats were washed, dried and sterilized with cotton soaked in 70% ethyl alcohol. The first 3-4 streams of milk were discarded. 15 ml. of milk were collected from each quarter into sterile vials. The collected milk samples were immediately kept in an insulated container with ice packs and were transferred to the laboratory for CMT and bacterial culturing.

2. California Mastitis Test

The CMT was applied to all milk samples involved according to (16) as follows: Three ml of milk were taken from each of the 4 milk samples that were collected from each cow involved in this study and poured into the 4 shallow cups of the plastic paddle used in this test. Following that, an equal volume of the CMT reagent (Bovivet CMT Test Liquid, USA) was added to each cup and mixed thoroughly by a gentle circular motion of the paddle.

The results of the CMT were reflected by the degree of precipitation or gel formation and they were scored

as follows: "Negative" when the consistency of the mixture is homogenous, liquid and not associated with visible changes; "trace" when the reaction was associated with slight precipitate that tended to disappear with continued movement of the paddle; "1+" when a distinct precipitate was formed but with no tendency toward gel formation; "2+" when the mixture was thickened immediately with a suggestion of gel formation; "3+" when a distinct gel was formed, tended to adhere to the bottom of the paddle and during swirling a distinct central peak was formed.

3. Bacteriological cultures

According to (17), quarters with a positive CMT ($\geq 1+$) reaction were considered as subclinically inflamed and accordingly milk samples of these quarters were subjected to bacteriological analysis as follows: After thorough mixing, 10 μ l aliquots were taken from each milk sample and were streaked on 5% sheep blood agar, MacConkey agar and nutrient agar. Following that, these media were incubated under aerobic conditions at 37°C and examined for bacterial growth after 16 to 48 hours of incubation.

Primary cultures were analysed by colony morphology, hemolysis and Gram stain 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 (18-19), as well as commercial identification kits including the enterosystem 18R (Liofilchem s.r.l., Italy), API Staph-Ident System (Montalieu- Vercieu, France) and API 20 Strep (API system, La Balme les Grottes, 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 *Streptococcus agalactiae* 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 (20).

4. Antimicrobial susceptibility testing

The Bauer Kirby procedure (21), 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 including ampicillin, cephalixin, erythromycin,

florfenicol, gentamicin, penicillin G, streptomycin, and tetracycline. Following 24 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 the National Committee for Clinical Laboratory Standards (22).

5. Statistical analysis

Prevalence values of subclinical mastitis were analyzed by the Chi square test (23). *P* values less than 0.05 were considered significant.

Results

1. Prevalence of subclinical mastitis

Out of the total 288 milk samples of the 72 cows tested in this study, 61 samples (21.18%) of 28 cows (38.89%) showed positive results for subclinical mastitis by the CMT. These results showed that the prevalence of subclinical mastitis at the cow and udder levels was significantly higher in Friesian cows than in the native and crossbred cows (Table 1).

In addition, results of the CMT showed that the prevalence of subclinical mastitis at the cow and udder levels was significantly higher in cows aged 5-7 years than in those aged 2-4 years (Table 2).

Table 1: Prevalence of subclinical mastitis* in relation to breeds.

| Breeds of cattle | Number of tested cattle | Number of infected cattle | Prevalence rates of infected cattle | Number of tested quarters | Number of infected quarters | Prevalence rates of infected quarters |
|------------------|-------------------------|---------------------------|-------------------------------------|---------------------------|-----------------------------|---------------------------------------|
| Friesian | 26 | 15 | 57.69% ^a | 104 | 41 | 39.42% ^a |
| Native | 15 | 4 | 26.67% ^b | 60 | 7 | 11.67% ^b |
| Crossbred | 31 | 9 | 29.03% ^b | 124 | 13 | 10.48% ^b |
| Total | 72 | 28 | 38.89% | 288 | 61 | 21.18% |

* Detection of subclinical mastitis was carried out by CMT.

Within a column, prevalence rate values with different small letter superscripts (^a and ^b) vary from each other (< 0.05).

Table 2: Prevalence rates of subclinical mastitis* in relation to age.

| Age | Number of cattle tested | Number of infected cattle | Prevalence rates of infected cattle | Number of quarters tested | Number of infected quarters | Prevalence rates of infected quarters |
|-----------|-------------------------|---------------------------|-------------------------------------|---------------------------|-----------------------------|---------------------------------------|
| 2-4 years | 23 | 7 | 30.43 % ^a | 92 | 16 | 17.39% ^a |
| 5-7 years | 49 | 21 | 42.86 % ^b | 196 | 45 | 22.96% ^a |
| Total | 72 | 28 | 38.89% | 288 | 61 | 21.18% |

* Detection of subclinical mastitis was carried out by CMT.

Within a column, prevalence rate values with different small letter superscripts (^a and ^b) vary from each other (< 0.05).

2. Bacterial isolation and identification

A positive bacterial isolation was obtained from 47 samples out of the total 61 milk samples that showed positive results by the CMT. Out of these 47 samples, 32 cultures revealed a single bacterial isolate (pure

cultures) and 15 cultures revealed a mixture of 2 bacterial isolates (dual bacterial isolates), i.e., a total of 62 different bacterial isolates were recovered and the biochemical tests revealed these isolates belonging to 8 species (Table 3).

Table 3: Bacterial isolates recovered from milk samples of the infected quarters.

| Bacteria | Number of isolates | % * |
|-------------------------------------|--------------------|-------|
| <i>Staphylococcus aureus</i> | 21 | 33.87 |
| <i>Escherichia coli</i> | 15 | 24.19 |
| <i>Streptococcus agalactiae</i> | 11 | 17.74 |
| <i>Streptococcus uberis</i> | 7 | 11.29 |
| <i>Streptococcus dysgalactiae</i> | 3 | 4.84 |
| <i>Staphylococcus epidermidis</i> | 2 | 3.23 |
| <i>Staphylococcus saprophyticus</i> | 2 | 3.23 |
| <i>Staphylococcus chromogenes</i> | 1 | 1.61 |
| Total | 62 | 100% |

* The percentage is with respect to the total number of isolates (62).

4. Antimicrobial susceptibility test

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 4. They showed that 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 susceptible or intermediately susceptible to erythromycin, gentamicin, and streptomycin; and the streptococcal isolates showed high susceptibility to penicillin G, ampicillin, and erythromycin.

Table 4: Antimicrobial susceptibility testing of the bacterial isolates obtained in the present study to the antimicrobial agents used in commercial intramammary infusion products.

| Bacteria | No. of isolates | Antimicrobial agents | | | | | | | | | | | | | | | | | | | | | | | |
|-------------------------------------|-----------------|----------------------|---|----|------------|---|----|--------------|---|---|-------------|---|----|------------|----|----|--------------|---|----|--------------|---|---|--------------|---|---|
| | | Ampicillin | | | Cephalexin | | | Erythromycin | | | Florfenicol | | | Gentamicin | | | Penicillin G | | | Streptomycin | | | Tetracycline | | |
| | | R | I | S | R | I | S | R | I | S | R | I | S | R | I | S | R | I | S | R | I | S | R | I | S |
| <i>Staphylococcus aureus</i> | 21 | 21 | - | - | - | 4 | 17 | 4 | 8 | 9 | 1 | 1 | 19 | 3 | 10 | 8 | 20 | 1 | - | 9 | 9 | 3 | 20 | 1 | - |
| <i>Escherichia coli</i> | 15 | - | 3 | 12 | 1 | 9 | 5 | 13 | 1 | 1 | - | 3 | 12 | 1 | 3 | 11 | 12 | 3 | - | 2 | 6 | 7 | 14 | 1 | - |
| <i>Streptococcus agalactiae</i> | 11 | - | - | 11 | - | 1 | 10 | - | 3 | 8 | - | 3 | 8 | 11 | - | - | - | - | 11 | 11 | - | - | - | 7 | 4 |
| <i>Streptococcus uberis</i> | 7 | - | - | 7 | - | 2 | 5 | - | 1 | 6 | - | - | 7 | 6 | 1 | - | - | - | 7 | 6 | 1 | - | - | 5 | 2 |
| <i>Streptococcus dysgalactiae</i> | 3 | - | - | 3 | - | - | 3 | - | 1 | 2 | - | 1 | 2 | 3 | - | - | - | - | 3 | 3 | - | - | - | - | 3 |
| <i>Staphylococcus epidermidis</i> | 2 | 2 | - | - | - | - | 2 | - | 1 | 1 | - | - | 2 | - | 1 | 1 | 2 | - | - | 1 | 1 | - | 2 | - | - |
| <i>Staphylococcus saprophyticus</i> | 2 | 2 | - | - | - | 1 | 1 | - | 1 | 1 | - | - | 2 | - | 1 | 1 | 2 | - | - | 1 | 1 | - | 2 | - | - |
| <i>Staphylococcus simulans</i> | 1 | 1 | - | - | - | - | 1 | - | 1 | - | - | - | 1 | - | 1 | - | 1 | - | - | - | 1 | - | 1 | - | - |

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- The Bauer Kirby procedure (21), 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.
 - The bacteria were considered to be R, resistant; I, intermediately susceptible or S, susceptible depending on the diameters of the inhibition zones which were measured by a ruler and compared with the interpretive standards of the inhibition zone diameter according to Chengappa, 1990 (22).

Discussion:

The result of CMT performed in the present study showed that the prevalence of subclinical mastitis among the sampled cattle was 38.89%. This high prevalence can be ascribed to the type of animal raising, since most of the cattle involved in this study were raised in rural areas in which the small dairy unit owners have no concept of subclinical mastitis, do not practice teat dipping or dry cow treatment and usually do not keep adequate herd records (24), however, this prevalence is relatively low in comparison with prevalence values reported by other authors (25-27). This prevalence variation can be attributed to the fact that in rural areas (to which the sampled cattle of the present study belong), animal breeding is of the semi-intensive type wherein the cattle are bred in large areas resulting in a lower opportunity for cow-to-cow transmission of IMI compared to the intensive type of cattle breeding in the dairy farms (from which the sampled cattle of prevalence studies mentioned above belong), wherein large numbers of cattle are bred in restricted areas resulting in a higher opportunity for cow-to-cow transmission of IMI since the animals in such breeding conditions are restricted to smaller areas that may be wet and dirty, permitting the exposure of teats to such a dirty environment (28-30).

The result of CMT performed in the present study also showed that the prevalence of subclinical mastitis at the cow and udder levels was significantly higher in Friesian cows than in the native and crossbred cows. This finding is generally in agreement with those reported by other authors (27, 31-33) and is can attributed to the fact that the native, relatively low milk yield cows and crossbred cows are genetically resistant to IMI and are more adapted to the local environment and climate than the Friesian cows (24, 34).

In addition, the CMT showed that the prevalence of subclinical mastitis in the current study at the cow and udder levels was significantly higher in cows aged 5-7 years than in those aged 2-4 years. This age-related variation in the prevalence of subclinical mastitis is attributed to the fact that older cattle with multiple parturitions and lactations are more exposed to IMI than younger cows and is in agreement with findings reported by other authors (33, 35-36).

Microbial culturing is considered the most suitable, accurate and reliable method to confirm the causative organisms; and many investigations had assured that bacterial culturing is the gold standard method for identifying IMI and for developing a specific mastitis control program for a dairy herd (15, 20, 37-39). Forty seven milk samples showed

positive results for bacterial culturing out of the total 61 samples that showed positive results for the CMT. Out of these 47 milk samples, a total of 62 different bacterial isolates were recovered and the biochemical tests revealed these isolates belonging to 8 species, the most prevalent of which is *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus agalactiae*. The high prevalence of these bacterial species in the present study is in agreement with the findings of other authors (11, 24, 25, 26, 33, 40-41) who considered these organisms as major etiological agents of clinical and sub clinical mastitis worldwide due to teat-to-teat and cow-to-cow spread, possibly via milking machines and perhaps by the milker's hands under the lack of hygiene (42).

The other bacterial species isolated in the present study are *Streptococcus uberis*, *Streptococcus dysgalactiae* and three species of coagulase negative staphylococci. The importance of such bacterial species as a cause of bovine mastitis has come under increased scrutiny in dairy cattle, they were previously considered as mastitis minor pathogens associated with a mild inflammatory reaction but they are now known to cause bovine mastitis (43-45). They colonize bovine teat skin and teat canals, thus they are classified as skin flora opportunists (15).

The results of susceptibility testing of the bacterial species isolated in the present study to the antimicrobial agents used in commercial intramammary infusion products are generally in agreement with the findings of other authors (15, 19, 20, 40, 46). All bacterial isolates were susceptible or at least intermediately susceptible to florfenicol and cephalixin. In addition, the *Escherichia coli* isolates were also susceptible or intermediately susceptible to ampicillin, gentamicin and streptomycin; the *Staphylococcus* isolates were susceptible or intermediately susceptible to erythromycin, gentamicin, and streptomycin; and the streptococcal isolates showed high susceptibility to penicillin G, ampicillin, and erythromycin. Thus, the simultaneous use of florfenicol, cephalixin, erythromycin and ampicillin may be useful for the treatment of subclinical mastitis cases in cattle in Al Sulaimaniyah district.

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