Detection of Enterotoxin Types Produce by Coagulase Positive Staphylococcus species Isolated from Mastitis in Dairy Cows in Sulaimaniyah Region

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Abstract

This study included 250 dairy cows (985 quarters) selected randomly from six selected dairy herds and also from individual cows from areas around Sulaimaniyah region including Bazyan, Tanjarwo, Arbit, Peramagrwn, Kanakawa and Tagaran during March to August, 2007. It was found that the prevalence of bovine mastitis was 80 % (200) at cow level. Out of 200 cows (785 quarters), it was found that the incidence of clinical mastitis was 26 % cows (65), 25.4% quarters (250), while the incidence of sub-clinical mastitis was 54 % cows (135), 54.3% quarters (535). According to manitol fermentation, coagulase, oxidase, catalase, urease, and DNase tests of 200 cows (785 quarters), it was found that 246 isolates (31.3%) were coagulase positive staphylococci (CoPS). An Api-Staph system was used in assuring a diagnosis for these bacteria, which showed complete matching with traditional biochemical tests. Production of enterotoxin A, B, C and D was detected by reverse passive latex agglutination test (RPLA). Among 246 CoPS isolates, 113 (45.9%) were found to be positive for production of one or more of enterotoxin types A, B and C. The production of enterotoxin types A, B, C, D, E, G, H, I and J were also tested by PCR technology. Among 246 CoPS isolates, 125 (50.8%) were found to be positive for production of one or more enterotoxin types A, B, C and E.

Key words: Detection of Enterotoxin Types, Coagulase, Positive Staphylococcus, Sulaimaniyah Region

Introduction

Bovine mastitis is a major disease that affects the dairy industry, and Staphylococcus aureus is one of the most frequently isolated pathogens from both subclinical and chronic infections (Zhao and Lacasse, 2008). Some Staph. aureus isolates from bovine milk carry different staphylococcal enterotoxins (StE) or toxic shock syndrome toxin-1 (Boerema et al., 2006). These toxins are responsible for food poisoning outbreaks and toxigenic syndrome in humans respectively (Bahatia and Zahoor, 2007); they may also contribute to the persistence of Staph. aureus in bovine mammary glands and increased udder pathogenicity (Hogy et al., 2008).

The pathogenicity of Staphylococcus species is associated with a number of virulence factors. The virulence factors include surface proteins that promote colonization of host tissues, invasions that promote bacterial spread in tissues (leucocidin, hyaluronidase), surface factors that inhibit phagocytic engulfment (capsule), biochemical properties that enhance their survival in phagocytes (catalase production), and membrane-damaging toxins that lyse eukaryotic cell membranes (haemolysins, leukotoxin), (Haven et al., 2007; Fournier et al., 2008).

Several reports (Stephan et al.,2001; Dasilva et al.,2005) have noted that the production of StE in Staph. aureus isolated from bovine mastitis may be determined by environmental and management factors in each geographical area. This genetic variability in StE production contributed to the emergence of distinct epidemiological profiles that were dependent on predominant strains within a herd. It indicates the necessity to identify such strains or subtypes before applying specific measures of mastitis control (Stephan et al.,2001; Dasilva et al.,2005; Cremones et al., 2005). Many molecular epidemiological
studies have already been conducted on enterotoxigenic Staph. aureus isolated from bovine milk, food, and humans (Cremones et al., 2005; Boerema et al., 2006).

The aim of this study is to investigate the prevalence and the cause of mastitis in dairy cows in the Sulaimaniyah region. To determine the prevalence of both coagulase positive Staphylococci species-induced clinical and sub-clinical mastitis in dairy cows in the Sulaimaniyah region. To estimate the level of enzymes and toxins among Staphylococcus species isolates from bovine mastitis.

Materials and Methods

Milk Sampling

Two hundred and fifty lactating cows (985 quarters) of which 15 animals had lost a quarter each was examined from dairy herds and individual cows in different smallholder farms in six regions during March to August 2007. Milk samples were collected using aseptic technique from individual quarters of the cows that were suspected to have subclinical mastitis as detected by high Somatic cell counts (SCC greater than 200,000 cells/ml), and clinical observation, the milk was immediately transported cooled (4°C) to the Microbiology Laboratory, Veterinary Medicine/ University of Sulaimaniyah for samples cultured according to standard protocols suggested by the National Mastitis Council (Harmon et al., 1990).

Isolation and Identification of S. aureus

Staphylococcus aureus was isolated from milk samples in dairy herds according to the protocols of the National Mastitis Council (Harmon et al.,1990). An aliquot of 10 µl from each sample was spread over blood agar plates (Bacto-Agar, Difco, Detroit, MI) containing 5% washed sheep erythrocytes and incubated at 37°C for 24 h. Colonies suspected of being staphylococci were sub-cultured on blood agar plates, and isolation and identification of Staph. aureus was performed by the method of Roberson et al. (Roberson et al., 1992) as follows: Gram staining, coagulase test, Baird-Parker medium culture test, DNase test, mannitol fermentation test, and identification in a commercial biochemical identification system (API-Staph®, bioMerieux, Nürtingen, Germany). Isolates of Staph. aureus were kept refrigerated (−20°C) for this study.

Enterotoxin test. The production of Ste was examined using a staphylococcal enterotoxin A, B, C, D detection by reverse passive latex agglutination) according to the instructions of the kit manufacturer (Oxoid Ltd., Basingstoke, UK).

Enterotoxin Gene Typing

DNA Preparation

Qiagen DNeasy Blood and Tissue kit was performed according to Hein et al., (2001) for isolation the genomic DNA from Staphylococcus species

Multiplex-PCR for the Detection of Genes Ste,

Selection of Primers

These primers (Table 1), were provided by Fermentas (U.S.A.), in lyophilized forms. To prepare working solution they were dissolved in sterile distilled water to a final concentration of 5 pmol/µl.

Protocol of PCR technique

HotStarTaq Master mix was prepared according to its manufacture (Qiagen HotStarTaq Master Mix Kit), by mixing 25 µl of the HotStarTaq Master Mix, 1 µl of diluted primer 1 and 1 µl of diluted primer, 2 µl of template DNA and RNase-Free water was added to a final volume of 50 µl of the reaction mixture. The mixture dispended in PCR tubes. All these steps were done on ice. All prepared reaction tubes were placed in the thermal cycler to carry out amplification. The amplification program used is shown in Table 2. After amplification, reactions were analyzed on 1% agarose gels by gel electrophoresis.

Agarose gel electrophoresis

PCR reaction mix (10 pl) and 3 pl of sample buffer (glycerol 30%, BPB 0.1%) were loaded and separated in an agarose 1% w/v gel; 5 pl of a 1-kb ladder (BRL) was used as a mol. Wt standard with every gel. Fragments were visualized after ethidium bromide staining under UV light.
Table 1. Oligonucleotide sequences used for the detection of staphylococcal enterotoxins.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide sequences (5'–3')</th>
<th>Gen Bank accession no.</th>
<th>Tm</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>sea</td>
<td>TGGCTTAACGTTGACAAACGAATGCA</td>
<td>M118970.1</td>
<td>66.0</td>
<td>343 bp</td>
</tr>
<tr>
<td>seb</td>
<td>GTTTGGTGTTACCACCCCAGCA</td>
<td></td>
<td>63.0</td>
<td></td>
</tr>
<tr>
<td>sec</td>
<td>ACATGATGCCTGCACCAAGGAGA</td>
<td>1M11118.1</td>
<td>64.0</td>
<td>406 bp</td>
</tr>
<tr>
<td>sdc</td>
<td>AGTTTTGACGCAAATGATGCAATG</td>
<td></td>
<td>66.0</td>
<td></td>
</tr>
<tr>
<td>sec</td>
<td>AGCAAACCAGCCCAATTGCACA</td>
<td>1X05815.1</td>
<td>65.0</td>
<td>614 bp</td>
</tr>
<tr>
<td>sdc</td>
<td>TCGCTTGTGACAGCCATCA</td>
<td></td>
<td>63.0</td>
<td></td>
</tr>
<tr>
<td>sed</td>
<td>TGAGCAAGTTGGATAGATTGCAGGCGC</td>
<td>8M28521</td>
<td>65.0</td>
<td></td>
</tr>
<tr>
<td>see</td>
<td>TGCAAATTCGCTTGTGCA</td>
<td></td>
<td>65.0</td>
<td></td>
</tr>
<tr>
<td>sed</td>
<td>TCAATGTCGCTGAGGACACCAAA</td>
<td>6M21319</td>
<td>65.0</td>
<td>831 bp</td>
</tr>
<tr>
<td>seg</td>
<td>AGCCCTTTGCACCTTTACCGCC</td>
<td></td>
<td>66.0</td>
<td></td>
</tr>
<tr>
<td>SEG1</td>
<td>TCGCTTGAGGTCGGACATCGAAGG</td>
<td>1AF064773</td>
<td>66.0</td>
<td></td>
</tr>
<tr>
<td>SEG2</td>
<td>TTTAGTGACGCTTGTGTCGGT</td>
<td>67.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seh</td>
<td>CAA ATCATATGTCAAGGCAAGG</td>
<td>U11702</td>
<td>62.0</td>
<td>202 bp</td>
</tr>
<tr>
<td>seh</td>
<td>CAT CAT CCC AAA CAT TAG CAC C</td>
<td></td>
<td>60.0</td>
<td></td>
</tr>
<tr>
<td>sei</td>
<td>GACATCTGGAACAGGAAAGCTTAGAAGT</td>
<td>3AF064774</td>
<td>67.0</td>
<td>480 bp</td>
</tr>
<tr>
<td>sei</td>
<td>TGTCCTGATTAAGGGCGCCCTCC</td>
<td></td>
<td>66.0</td>
<td></td>
</tr>
<tr>
<td>sej</td>
<td>GCCGGAATCATTCAACCTTGCTCA</td>
<td>1AF053140</td>
<td>68.0</td>
<td></td>
</tr>
<tr>
<td>sej</td>
<td>TCCGGCTTTCCTATGGCACC</td>
<td></td>
<td>65.0</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Optimized polymerase chain Reaction (PCR) cycling program.

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Additional Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial activation step</td>
<td>15 min</td>
<td>95°C</td>
<td>HotStarTaq DNA Polymerase is activated by this heating step.</td>
</tr>
<tr>
<td>3-step cycling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>1 min</td>
<td>94°C</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>1 min</td>
<td>50–68°C</td>
<td>Approximately 5°C below Tm of primers.</td>
</tr>
<tr>
<td>Extension</td>
<td>1 min</td>
<td>72°C</td>
<td>For PCR products longer than 1 kb, use an extension time of approximately 1 min per kb DNA.</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>10 min</td>
<td>72°C</td>
<td></td>
</tr>
</tbody>
</table>

Results

Prevalence of Mastitis in Sulaimaniyah Province

A total of 250 cows (985 quarters) were examined from six selected dairy herds and also from individual cows from areas around Sulaimaniyah province including Bazyan, Tanjarwo, Arbit, Peramagwim, Kanakawa and Tagaran, during March to August, 2007. The incidence rate of clinical mastitis was 26 % (65) as measured on a cow basis, and 25.4 % (250) as measured on a quarters basis, which grouped according to the clinical state of the mammary gland in various groups, see Tables 3 and 4, and Figure 1.

The diagnosis of sub-clinical mastitis was based on a quarter foremilk sample for somatic cell counts (SCCs), together with bacteriological observations. In this study both parameters were included.

Table 3. Clinical mastitis in tested cows (n=65) from region grouped according to the clinical state of the mammary gland in the various groups in different months.

<table>
<thead>
<tr>
<th>Clinical state of the mammary gland of cows</th>
<th>March no. %</th>
<th>April no. %</th>
<th>May no. %</th>
<th>June no. %</th>
<th>July no. %</th>
<th>August no. %</th>
<th>Prevalence no. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute</td>
<td>7 (2.8)</td>
<td>4 (1.6)</td>
<td>3 (1.2)</td>
<td>5 (2.0)</td>
<td>6 (2.4)</td>
<td>5 (2.0)</td>
<td>30 (12.0)</td>
</tr>
<tr>
<td>Chronic</td>
<td>3 (1.2)</td>
<td>4 (1.6)</td>
<td>5 (2.0)</td>
<td>4 (1.4)</td>
<td>3 (0.9)</td>
<td>3 (1.2)</td>
<td>22 (8.8)</td>
</tr>
<tr>
<td>Sub-acute</td>
<td>4 (1.6)</td>
<td>2 (0.8)</td>
<td>3 (1.2)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (0.4)</td>
<td>10 (4.0)</td>
</tr>
<tr>
<td>Gangrenous</td>
<td>1 (0.4)</td>
<td>0 (0.0)</td>
<td>1 (0.4)</td>
<td>1 (0.4)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>3 (1.2)</td>
</tr>
<tr>
<td>Total</td>
<td>15 (6.0)</td>
<td>10 (4.0)</td>
<td>12 (4.8)</td>
<td>10 (4.0)</td>
<td>9 (3.6)</td>
<td>9 (3.6)</td>
<td>65 (26)</td>
</tr>
</tbody>
</table>

Table 4. The prevalence of clinical and sub-clinical mastitis in cows according to Sulaimaniyah province.
Identification of Staphylococcus species

According to the biochemical tests used in this study, it was found that all colonies revealed Gram positive, cocci, catalase positive, oxidase negative were considered 246 isolates coagulase positive. According to Api-Staph Ident System, 246 coagulase positive isolates (31.3%) were found, all of which were Staphylococcus aureus.

Enterotoxin Assay

Bacterial Isolates and Culturing Technique used in Enterotoxin Assay

All Staphylococcus species isolates obtained in this study were investigated for their ability of enterotoxin production by using culture technique on BHIA with phenol red at pH 5.4 as indicator. Among 246 isolates of Staph. aureus, 125 isolates had the ability to produce enterotoxin.

Enterotoxin Types Detection

Staphylococcal enterotoxins (SE) were extracted from 125 isolates of Staph. aureus which had the ability to produce enterotoxin, and then the enterotoxin types were detected by using reverse passive latex agglutination (RPLA) kit. Out of 125 Staph. aureus enterotoxin producer, it found 66 isolates enterotoxin type A producer (52.8 %); 27 isolates enterotoxin type B producer (21.6 %) and 20 isolates enterotoxin type C producer (16 %). None of the isolates tested produce enterotoxin type D. Enterotoxins of these isolates A13-R2, A20-L2, A4-L1, T5-L2, T10-R1, Tg8-L2, Tg9-L2, B15-R2, B16-R2, B21-L1, P1-L2, P3-L1 and K9-L2 were produced in a similar concentration (320ng/ml). In spite of these similarities, the scores of agglutination in different dilutions of the cell-free extracts of these isolates enterotoxin type A and type B were more obvious than enterotoxin type C, which indicated that enterotoxin type C was produced in smaller amount than enterotoxin type A and type B.

Polymerase Chain Reaction Test

The technique of polymerase chain reaction was applied to determine the presence of gene coded for staphylococcal enterotoxin (se) gene.
Staphylococcal Enterotoxin (se) Gene

Among 246 Staphylococcus aureus isolates, 125 isolates (45.9%) were found to be involved in production of one or more se genes, out of 125 Staph. aureus enterotoxin producer, it was found that 66 isolates (52.8 %) produce enterotoxin type A, the PCR product is 340 bp (Figure 2); 27 isolates (21.6%) produce enterotoxin type B, the PCR product is 403 bp (Figure 3); 20 isolates (16 %) produce enterotoxin type C, the PCR product is 613 bp (Figure 4) and 12 isolates (9.6 %) produce enterotoxin type E, the PCR product is 166 bp (Figure 5). None of the isolates tested produce enterotoxin type D.

Figure 2. Agarose gel electrophorsis showing the PCR amplified products of the staphylococcal enterotoxin type A, sea gene 340 base pair.
Lanes M: DNA molecular weight marker (100 bp -3000 bp)
Lanes 1, 2, 3 and 5: positive for the sea gene.
Lane 4: negative with the enterotoxin a gene.

Figure 3. Agarose gel electrophorsis showing the PCR amplified products of the staphylococcal enterotoxin type B, seb gene 403 base pair.
Lanes M: DNA molecular weight marker (100 bp -3000 bp)
Lanes 2, 3 and 4: positive for the seb gene.
Lanes 1, 5, 6 and 7: negative with the enterotoxin b gene.

Figure 4. Agarose gel electrophorsis showing the PCR amplified products of the staphylococcal enterotoxin type C, sec gene 613 base pair.
Lanes M: DNA molecular weight marker (100 bp -3000 bp)
Lanes 4, 5, 6, 9 and 10: positive for the sec gene.
**Discussion**

**Prevalence of Mastitis in the Sulaimaniyah Province**

The prevalence of clinical mastitis in udder quarters in six selected dairy herds and individual cows, as shown in Tables 3, 4 and Figure 1 was found to be higher than those reported in Jordan (15.7%), Egypt (12.08%) (Azmi and Al-Dabbas, 2008), and in Sweden, (Sommerhauser et al., 2003). However, the incidence rates observed in studies on herds from different geographical locations should be compared with caution, since the differences in dairy herds are associated with factors such as climate, breed, level of production, and management.

The prevalence of sub-clinical mastitis recorded in this study was higher compared with that reported in other countries; see Table 3 and Figure 1. The prevalence of sub-clinical mastitis in cows reported for countries such as in Jordan, Israel, Greece, England, Wales, USA (Vermont), Kenya, Ethiopia and Spain has ranged from 12% - 37% (Azmi and Al-Dabbas, 2008; leitener et al., 2001; McDougall et al., 2002 and Dego and Tareke, 2003). The prevalence of sub-clinical mastitis differs among countries. This might be due to the differences in animal breed, management conditions and methodological approaches used.

In this study, the milk samples from animals with bacterial infection of the mammary gland showed significantly higher geometric SCC than did the corresponding milk from healthy animals. The somatic cell counts in this study agree with (Suriyasathaporn et al., 2000). The variation in SCC between these studies might be due to different factors such as type of breed, lactation number, lactation period, volume of milk produced, animal age, methodology and or pathogens producing infections (Beakau et al., 2002).

**Enterotoxin Assay**

The determination of staphylococcal enterotoxin type has a long history of successful use in epidemiological studies in both clinical and environmental microbiology studies. As our knowledge of the molecular genetic structure of these organisms’ increases, it becomes increasingly more difficult to test for all of the known phenotypes, with genotype analysis often providing the only way the diversity of different subspecies types can be identified. Oligonucleotide primers for specific detection of enterotoxin genes sea, seb, sec, sed, see, seh, sei and sej have previously been reported (Omoe et al., 2002). These were used in individual PCR assays, thus requiring several PCRs for each sample to screen for the presence of all of the enterotoxin genes. The results presented showed that it is possible, using a single PCR assay, to rapidly screen staphylococcal isolates for the presence of different enterotoxin genes which are routinely detected by serological tests (Bahatia and Zahoor, 2007 and Suzuki et al., 2002).

**Reverse Passive Latex Agglutination (RPLA) Test**

By using a RPLA kit we found that the Staph. aureus isolated from bovine mastitis in Sulaimaniyah district had the ability to produce either enterotoxin A, SEB, SEC or SEA+ SEC. None produced SED. This result was consistent with previous reports from Japan, Poland and Slovakie (Bahatia and Zahoor, 2007 and Vojtov et al., 2002).
On the other hand, the results were in contrast to other studies from Spain, Kenya, Switzerland, Brazil, South Korea, the USA and Slovakia, where most enterotoxigenic Staph. aureus isolates usually carried the toxin gene sec, sea or sed (Smith et al., 2001 and Soriano et al., 2002). However, one major application of the SET-RPLA assay is the toxin typing of strains for epidemiological purposes when it is not usually essential to know whether or not a gene is expressed. Alternatively, the SET-RPLA test is used for the detection of preformed toxin in processed foods when the toxin-producing cells have been killed.

**Polymerase Chain Reaction Test**

**Enterotoxin Detection**

In this study, the prevalence rate of enterotoxin-producing Staph. aureus from bovine mastitis was 50.8% (125). The predominance of SEA contradicts with reports from countries such as Switzerland, Brazil, and Japan, where SEC-producing Staph. aureus were frequently isolated (Dasilva et al., 2005 and Katsuda et al., 2005). Various reports have indicated that SEA is mainly from humans, whereas SEC is from animals (Bergdoll et al., 1989 and Order et al., 1992). In contrast, a review by Genigeorgis (1989) concluded that there is no predominant type of SE, apart from the isolates from food involved in staphylococcal gastroenteritis, where SEA is the main type of SE recovered (Geningeorgis, 1989). SE-producing strains have been widely isolated from mastitis. Recently, the occurrence of new types of SE, such as staphyloccocal enterotoxin type E have been reported (Zschock et al., 2005), see Figure 5. None of the newly described enterotoxins (SEG, SEH, SEI, and SEJ) have been investigated in this study.

**Conclusion**

The prevalence of clinical and sub-clinical mastitis in dairy cattle is high, and could affect milk production and milk quality in smallholder farms in the Sulaimaniyah region.

This study showed that Staphylococcus aureus was the predominant organism to causing bovine IMIs in the Sulaimaniyah region.

The results indicated that S. aureus isolated from bovine mastitis has the ability to produce enterotoxin. Additionally, it was demonstrated that S. aureus causing mastitis in Sulaimanian dairy herds harbored the sea, seb, sec and see gene and produced the SEA, SEB and SEC toxin, suggesting that it may play a role in bovine mastitis pathogenesis.

**References**


