Molecular Characterization of Tetracycline-Resistant Genes in Staphylococcus aureus Isolated from Dairy Cows and She-camels Suffering from Subclinical Mastitis

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ABSTRACT

The administration and the mass use of antimicrobial therapy without the appropriate dose or exposure time, enhance the emergence of antimicrobial resistance leading to increasing the difficulty to control the infectious bacterial agents in dairy farms and hence impose negative economic impact on dairy industry. Tetracycline resistance represented with several genes in the bacterial chromosome, but this study was concerned with two genes, tet (k) and tet (38). The tet (38) gene had high prevalence than the tet (k) gene and one of the interesting features found in this work that the tet (k) was found only in most of MRSA isolates (mecA gene harboring) while the tet (38) gene was found in all S. aureus isolates (MRSA and MSSA). High level of similarities were found between these genes isolated from different stains and their corresponding reference sequences retrieved from the GeneBank by applying the multiple sequence alignment and the phylogenetic analysis. Further analysis would be done to determine other genes of resistance to explore the whole picture of antibiotic resistance but on the genetic level.

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1. INTRODUCTION

S. aureus is a key and common reason of bovine subclinical mastitis (Wilson et al., 1997). Recent control of S. aureus mastitis includes appropriate milking, sanitization practices, get rid of chronically diseased animals, discrimination of infected animals, and antimicrobial therapy. Carrying out and implementation of well-established preventive measures reduces the transmission of S. aureus as a main cause of contagious mastitis.

Antimicrobial remedy is a main method to overcome S. aureus mastitis and its application via intramammary infusion to combat the infection and the prognosis to treat the penicillin-resistant S. aureus isolates lower the cure rate of those caused by penicillin-sensitive S. aureus with either beta-lactam or non-beta-lactam antibiotics (Barkema et al., 2006).

Due to the increasing rate of Antimicrobial resistance among S. aureus isolates in dairy farms of Egypt as well as other parts of the world, different studies had been carried out in different countries (Shi et al., 2010; De Oliveira et al., 2000; Abera et al., 2010, Sameh et al., 2015, Al-Saaid et al., 2015).

The tetracyclines are bacteriostatic for the staphylococci despite a mechanism of action similar to that of the aminoglycosides and they are considered a class of structurally related compounds that characterized by four interlocking six-carbon rings which inhibit protein synthesis by binding to the 30 S subunit of the ribosome and block the entry of aminoacyl-tRNAs into the acceptor site (Klein, 1995; Schnappinger and Hillen, 1996). The purpose of this study was to determine the level of antimicrobial resistance, especially tetracycline resistance among S. aureus isolates that were detected from subclinical infected cows and she-camels.

2. MATERIALS AND METHODS

A total of two hundred forty milk samples were collected from dairy cattle (140) in Qena Governorate, as well as She-Camels (100) in Red Sea Governorate (Al-Shalateen area), Egypt. All these animals were apparently healthy without the presence of clear clinical manifestations that indicate local mastitic condition or systemic reaction; these samples were processed according to Pamela, (2005).
2.1 California Mastitis test
According to the methods described by Schalm and Noorlander (1957) and Schalm et al. (1971), the California mastitis test (CMT) was done to detect the presence of mastitic cases in dairy cow as well as she-camels.

2.2 isolation and culturing of S. aureus
The preparation of the Bairred-Parker media according to Vanderzant and Splittstoesser, (1992). 3 typical colonies were harvested and picked up by a sterile metal bacteriological loop and then immersed in the glycerol stock in Eppendorf tube and kept immediately at -70 to -80 °C for further studies (Jones et al., 1991).

2.2 Biochemical tests
The coagulase test was performed by two different methods; the slide coagulase test and tube coagulase test (Wichelhaus et al., 1999), and thermostable nuclease test "deoxyribonuclease activity" (Lachia et al., 1971).

2.3 Antibiotic susceptibility test
Antimicrobial susceptibility was tested against different kinds of antibiotics by the single diffusion method according to (Deresse et al., 2012) for S. aureus. They were used to determine the susceptibility of the isolated S. aureus strains (Oxoid Limited, Basingstoke, and Hampshire, UK). Therefore, the antimicrobial susceptibility testing was applied according to the guidelines stipulated by National Committee for Clinical Laboratory Standards "NCCLS" (2001).

2.4 Whole genome sequencing
The S. aureus resistant strains (MRSA) were DNA extracted and the genomic DNA used to obtain the whole genome sequence of S. aureus by shotgun sequencing (Sanger institute, UK) which divided into several contigs and by using several bioinformatics tools, the bacterial chromosome sequence can be obtained, aligned and evaluated.

2.5 Statistical and sequence analysis
To be able to manipulate the whole genome sequence of S. aureus, it is needed to use Artemis which is a free genome browser and annotation tool that allows visualization of sequence features, next generation data and the results of analyses within the context of the sequence, and also its six-frame translation (Rutherford et al., 2000).

Sequence alignments, translations, and comparisons were carried out using BIOEDIT (Version 7.0.9.0, Hall, T.A, 1999). The BLAST algorithm was used to search the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) databases for homologous sequences.

Neighbor-joining trees (Saitou and Nei, 1987), were constructed on the basis of genetic distances, estimated by Kimura’s (1980) two-parameter method, using MEGA 5 (Kumar et al., 2001; http://www.megasoftware.net). The reliability of the trees was estimated by bootstrap confidence values (Felsenstein, 1985) and 500 bootstrap replications were used.

The tet (k) and tet (38) genes sequences used to construct the neighbor-joining tree (Figure 3) were (by NCBI GenBank accession numbers); the tet (k) were (J01764 and U38428) and tet (38) genes sequences were (FR821779 & AY825285).

3. RESULTS

3.1. The California mastitis test (CMT)
The California mastitis test (CMT) confirmed the presence of 24 (17.14%) subclinical mastitis out of 140 dairy cows and 10 (10%) out of 100 she-camels (Table 1). The coagulase test was carried out to isolate S. aureus from other types of Staphylococci (Table 2). Antibiotic sensitivity test estimated the frequency of different levels of resistance to various antibiotics among S. aureus isolates (Table 3).

By using the shogun sequencing, the whole genome sequencing of some local isolates of S. aureus could be annotated and visualized. RAST (Rapid Annotation using Subsystem Technology) is a fully-automated service for annotating complete or nearly complete bacterial and archaeal genomes. It provides high quality genome annotations for these genomes across the whole phylogenetic tree (http://rast.nmpdr.org/) and Artemis which is a free genome browser is used to manipulate the whole genome sequence of S. aureus (Rutherford et al., 2000). This study is concerned with investigation of the tetracycline resistance, two genes had been found which is responsible in conferring this kind of resistance tet (k) and tet(38). The tet (k) gene had been found in 5 local isolates (A1, A3, A5, A6 & A7), and the tet(38) gene had been found in 12 local isolates (A1, A2, A3, A4, A5, A6, A7, A10, A11, A12, A13 &A15). The position of the tet(k) and tet(38) genes within the bacterial genome and its blast similarity with the reference genes had been shown in table 4.
3.2. Tet (K) and tet (38) multiple sequence alignment
The Tet (k) gene found in 5 local isolates were aligned with 2 reference genes (U38428 & J01764), and the amino acid were predicted by using Bioedit software, there were complete similarities between the 7 sequences which indicating high degree of monomorphism except 1 positions; p 266 (Amino acid W), this different amino acid was found in one of the reference genes used for comparison (GeneBank accession number, J01764) while the other reference (GeneBank accession number, U38428) was in complete similarity with the local isolates used in this study (figure 1).

Tet (38) gene found in 12 local isolates were aligned with 2 reference genes ( FR821779 & AY825285), and the amino acids were predicted by using Bioedit software, there were complete similarities between the 14 isolates which indicating high degree of monomorphism except p 63 (amino acid A) in the local isolate A15, p100 (amino acid A) in the local isolates A10, A11, A12, A13 and the reference gene (FR821779), p110 (amino acid V) in the local isolate A10, p283 (amino acid I) in the reference gene (FR821779), and the last variant position p441(amino acid I) in the local isolates A10, A11, A12, A13 and the 2 reference genes (FR821779 and AY821779) (figure 2).

3.3. Phylogenetic analysis of Tet (K) and tet (38)
The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei., 1987). The optimal tree with the sum of branch length = 2.55195861 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 985 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

The phylogenetic tree showed that both genes of tetracycline resistance were gathered with their similar reference genes sequences and formed two main groups, the first one tet(38) group (a1, a2, a3, a4, a5, a6, a7, a10, a11, a12, a13, a15, AY825285 & FR821779) and the other one tet (k) group (a1, a3, a5, a6, a7, J01764 & U38428) which indicating high level of identity between the local isolates genes and their corresponding reference sequences in the GeneBank (figure 3).

Table 1. Incidence of subclinical mastitis in examined animals according to California mastitis test.

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of Animals Examined</th>
<th>CMT +VE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>140</td>
<td>24 (17.14%)</td>
</tr>
<tr>
<td>Camels</td>
<td>100</td>
<td>10 (10%)</td>
</tr>
</tbody>
</table>

Table 2. Incidence of S. aureus isolated from dairy cows and she-camels

<table>
<thead>
<tr>
<th>Species</th>
<th>S. aureus</th>
<th>Other Staphylococci</th>
<th>Other pathogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle (140)</td>
<td>22 (15.7%)</td>
<td>77 (55%)</td>
<td>41 (29.2%)</td>
</tr>
<tr>
<td>Camels (100)</td>
<td>7 (7%)</td>
<td>38 (38%)</td>
<td>55 (55%)</td>
</tr>
</tbody>
</table>

Table 3. Antibiotic resistance profile of S. aureus isolated from dairy cows and she-camels.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td>17 (58.6%)</td>
<td>10 (34.5%)</td>
<td>2 (6.9%)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>18 (62.06%)</td>
<td>7 (24.1%)</td>
<td>4 (13.8%)</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>19 (65.5%)</td>
<td>8 (27.6%)</td>
<td>2 (6.9%)</td>
</tr>
<tr>
<td>Amoxiclav</td>
<td>18 (62.06%)</td>
<td>9 (31.03%)</td>
<td>2 (6.9%)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>18 (62.06%)</td>
<td>7 (24.1%)</td>
<td>4 (13.8%)</td>
</tr>
</tbody>
</table>
Table 4. Tetracycline resistance genes (tet k & tet 38) and their positions in the S. aureus genome

<table>
<thead>
<tr>
<th>strain</th>
<th>mecA gene</th>
<th>Resistance gene</th>
<th>% Identity</th>
<th>Query/HSP length</th>
<th>Position in contig</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>+</td>
<td>tet(K)</td>
<td>100</td>
<td>1380 / 1380</td>
<td>276824..2764203</td>
<td>U38428</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tet(38)</td>
<td>99.85</td>
<td>1353 / 1353</td>
<td>679533..680885</td>
<td>AY825285</td>
</tr>
<tr>
<td>A2</td>
<td>+</td>
<td>tet(38)</td>
<td>99.85</td>
<td>1353 / 1353</td>
<td>849985..851337</td>
<td>AY825285</td>
</tr>
<tr>
<td>A3</td>
<td>+</td>
<td>tet(38)</td>
<td>99.85</td>
<td>1353 / 1353</td>
<td>2402116..2403468</td>
<td>AY825285</td>
</tr>
<tr>
<td>A4</td>
<td>+</td>
<td>tet(K)</td>
<td>100</td>
<td>1380 / 1380</td>
<td>2824205..2825584</td>
<td>AY825285</td>
</tr>
<tr>
<td>A5</td>
<td>+</td>
<td>tet(38)</td>
<td>99.85</td>
<td>1353 / 1353</td>
<td>2011056..2012408</td>
<td>AY825285</td>
</tr>
<tr>
<td>A6</td>
<td>+</td>
<td>tet(K)</td>
<td>99.89</td>
<td>888 / 888</td>
<td>2779341..2780227</td>
<td>J01764</td>
</tr>
<tr>
<td>A7</td>
<td>+</td>
<td>tet(38)</td>
<td>99.85</td>
<td>1353 / 1353</td>
<td>2089322..2090674</td>
<td>AY825285</td>
</tr>
<tr>
<td>A10</td>
<td>-</td>
<td>tet(38)</td>
<td>98.97</td>
<td>1353 / 1353</td>
<td>2765067..2766446</td>
<td>U38428</td>
</tr>
<tr>
<td>A11</td>
<td>-</td>
<td>tet(38)</td>
<td>98.97</td>
<td>1353 / 1353</td>
<td>2461719..2463071</td>
<td>FR821779</td>
</tr>
<tr>
<td>A12</td>
<td>-</td>
<td>tet(38)</td>
<td>99.63</td>
<td>1353 / 1353</td>
<td>680449..681801</td>
<td>FR821779</td>
</tr>
<tr>
<td>A13</td>
<td>-</td>
<td>tet(38)</td>
<td>99.63</td>
<td>1353 / 1353</td>
<td>236737..238089</td>
<td>FR821779</td>
</tr>
<tr>
<td>A15</td>
<td>-</td>
<td>tet(38)</td>
<td>99.56</td>
<td>1353 / 1353</td>
<td>1399661..1401013</td>
<td>AY825285</td>
</tr>
</tbody>
</table>

A11, A12, A13 and A15 isolates from she-camels

4. DISCUSSION

S. aureus is widely spread in dairy farms producing highly significant negative impact on milk production which will be reflected in the economy of dairy industry. The antimicrobial drugs were used routinely either as a prophylaxis or treatment of infectious agents. The presence of different levels of antimicrobial drugs and the bacterial agents in the dairy farm environment would initiate and enhance the development of certain kind of resistance especially in the presence of mass use of these medications. S. aureus acquired resistance against the beta lactam antibiotics through harboring the mecA gene leading to the emergence of MRSA strains, this study is dedicated to investigate the presence of another types of resistance against the tetracycline resistance, the phenotypic methods would give some indications on the presence of tetracycline resistance by the use of antibiotic sensitivity tests (table 3), but these results would not figure out completely the actual status of resistance, hence, it comes the importance of genotyping method which gives a more clear picture at the genetic levels. In this study, the use of a modern technique as shotgun sequencing, it would give a good chance to look for the genes responsible on tetracycline resistance, two genes found, the tet(k) and the tet(38) and the different sequences were extracted from the bacterial chromosome after the annotation process had been done via the use of the RAST (Rapid Annotation using Subsystem Technology) (http://rast.nmpdr.org/).

The tet (38) gene was found in all S. aureus isolates while the tet(k) gene was found only in 5 isolates which indicate the presence of high prevalence of tet (38) more than tet (k) (table 4).

One of the interesting findings was the presence of tet (k) gene in accompany with the presence of mecA gene ( MRSA, 5 isolates out of 7), on the other hand, the tet (38) gene was found in all isolates (12 isolates) both MRSA or MSSA strains (table 4), these results contradict the results obtained by Schmitz (2001), Jones et al., (2006) and El-Mahdy et al. (2010), which indicated that the tet (k) gene is highly prevalent in MSSA and the tet (M) gene is highly prevalent in MRSA, the tet (M) gene was absent in all MRSA isolates in this study.
Figure 1. Showed multiple sequence alignment of the predicted amino acids sequences of tet (k) gene and their corresponding reference sequences
Figure 2. Multiple sequence alignment of the predicted amino acids sequences of tet (38) gene and their corresponding reference sequences.
The presence of tet (k) and the tet (38) genes only in this study indicates high level of relatedness between the two genes conferring tetracycline resistance rather than other genes (Trzcinski et al., 2000).

The multiple sequence alignment was done by using different bioinformatics softwares, the results obtained showed high level of similarities between the local isolates sequences of both tet(k) and tet(38) and the reference sequences which retrieved from the GeneBank databases (NCBI GenBank, http://www.ncbi.nlm.nih.gov/) except minor variations (figure 1 & 2).

The phylogenetic analysis would give more confirmed picture on the inter-relationships between different isolates on the nucleotide level, the sequences had aligned in two groups, one for the tet(k) and their corresponding reference sequences and the same result obtained for the tet (38) (figure 3).

On conclusion, this study was concerned with shedding the light on the tetracycline resistance in *S. aureus*, two genes had been found the tet(k) and tet(38). The tet (38) gene was found in higher prevalence than the tet (k) and high level of similarities between different isolates in the same gene were tested by the multiple sequence alignment and the phylogenetic analysis, tet (k) tetracycline-resistant strains often showed co-resistance with mecA gene (MRSA strains) while the tet (38) gene found in both strains MRSA and MSSA. Further studies are needed to investigate the presence of other genes responsible for tetracycline resistance and this can be done by getting large number of samples and sequenced by the whole genome sequence to get complete picture on these genes.

4. Acknowledgment
This work was funded through the South Valley University higher studies sector and the cultural & missions sector, ministry of Higher Education, Egypt. All thanks to Professor Mark Holmes, University of Cambridge, UK, for his support.

5. Reference:


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