Laboratory Differentiation between Streptococcus Species Isolated from Different Sources

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Key words
- Streptococcus
- Galactia
- streptococcus pyogenes
- Milk,

ABSTRACT:
This study was planned to throw light on the most important Streptococcus bacteria that isolated from the milk and pus and the most important laboratory tests that used in differentiation between the streptococcal bacterial species.
This study was carried-out on a total number of 100 random samples of milk which were collected from different areas at Behera Governorate. Also, 100 pus samples from closed abscesses from different parts of cattle body where collected and examined bacteriologically for streptococcus introduction.
Our results concluded that, the streptococcus agalactiae and streptococcus pyogenes are the most important bacterial isolates that causes severe losses to milk industry, also the streptococcus pyogenes of zoonotic importance as it transmitted to human. The catalase test, oxidatse test and hemolytic test are the main tests used for diagnosis and differentiation between streptococcus agalactiae and streptococcus pyogenes. The bacitracin sensitivity test is main test for differentiation between streptococcus agalactiae and streptococcus pyogenes The results also indicated that the level of streptococcus agalactiae in milk higher than that of streptococcus pyogenes and reached to 7 % and 3 % for streptococcus agalactiae and pyogenes, while, in pus its levels reached to 0 and 40 % for streptococcus agalactiae and pyogenes in pus. Also the results cleared that the PCR method for detection of mastitis considered as the best method.

1. INTRODUCTION
Raw milk may contain a variety of disease causing pathogens as demonstrated by numerous scientific studies. These studies, along with numerous foodborne outbreaks, clearly demonstrate the risk associated with drinking raw milk. Pasteurization effectively kills raw milk pathogens without any significant impact on milk nutritional quality. (Lin et al., 1991 and Werner et al. (2012)).

The Streptococcus is the main cause of pharangitis and tonsillitis in human especially in children and is the main bacteria transmitted from the milk to human and also can transmitted from the human to animals. (Siddique et al., 1988 and Schröder et al. (2005). Also, (Shome et al., 2012) reported that, streptococci are one among the major mastitis pathogens which have a considerable impact on cow health, milk quality, and productivity.

Identification of bacterial pathogens especially Streptococcus spp. in milk from cows with mastitis is the definitive diagnosis of mastitis infections. It also provides information important for prevention and control of the disease. (Phuektes et al. 2001).

The infection with Streptococcus pyogenes, a beta-hemolytic bacterium that belongs to Lancefield serogroup A, also known as the group A streptococci (GAS), causes a wide variety of diseases in humans. (Khan, 2012)
The main reservoir of streptococcus pyogenes include, man, rarely cattle. S. pyogenes is almost exclusively associated with man, and contact with infected individuals or asymptomatic carriers is the most common source of infection. (McDougall, 2005).

So, this study was planned to throw the light on the most important Streptococcus bacteria that isolated from the milk and pus and the most important laboratory tests that used in differentiation between the streptococcal bacterial species.

2. MATERIALS AND METHODS

a-Milk samples:- A total number of 100 random samples of milk were collected from different areas at Behera Governorate. The udder of each cow was palpated before sampling for detecting any abnormalities such as swelling ,asymmetry or any other physical changes. Each udder was washed and carefully dried with clean towel.
Table (1): Distribution of collected milk pus samples.

<table>
<thead>
<tr>
<th>Milk samples</th>
<th>Total number of samples</th>
<th>Apparent normal</th>
<th>Mastitis milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pus swab</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Milk samples and pus swab samples were transported in ice box (BSL, 1976) to Lab. of Dept. of microbiology, Fac. Of Vet. Med. Alex. University for bacteriological examination as soon as possible.

The teats were then swabbed with 70% alcohol. The first jets of milk were rejected, then 15-20 ml of milk were drawn from each quarter into a sterile screw-capped MacCarteny bottles, the milk samples were labeled and kept in ice containers and transported to the laboratory for examination.

b-Abscesses: 100 samples from closed abscesses have been obtained with sterile syringe which transferred directly and rotated in enrich selective media. The sample were collected from the skin lesions and pus discharge from the genital tracts using wooden spatual and take the swab from pus found in the lesion. Also the pus samples collected from closed pus using sterile syringe (Bessen, 2009). The number of collected samples were clear in the following Table.

c-Bacteriological examination

1. Isolation of bacteria from milk samples:
A total number of 200 milk samples were incubated overnight at 37 °C then loopfull from the sample were cultured on blood agar. All plates were incubated at 37 c for 18-24 hs and examined for bacterial growth. While, from pus were touched with a sterile bacteriological loop was introduced, then the inoculum was streaked on the surface of blood agar. The inoculated plates were incubated for 48 hrs at 37 C. (Sherbina, 1973).

The St. agalactia growth showed a small translucent colonies of beta haemolytic (Clear zone).

Smears were picked-up for gram stain, well-developed isolated colonies (creamy, white, raised) were transferred by sterile loop for purity and identification.

Then, loopful of each pure culture was inoculated into two tubes of semi-solid media of TSA, one of which was used as stock culture and other tube used for detection of motility and further biochemical tests (Lennette et al., 1980).

2. Identification and biochemical characterization of the isolates:
Identification of the bacterial isolates was carried-out by determining their morphological, cultural and biochemical characters.

a. Colonies characters and bacterial morphology:
The pure isolates were subjected for identification morphologically:-

1. Morphological examination:
Dry heat fixed smears were prepared from pure recovered isolates and stained with Gram stain. The stained smears were examined microscopically for the morphological characters of streptococci (shape and arrangement).

2. Cultural characters:
The pure isolates were inoculated onto TSA and incubated for 24hrs. at 25C and the growing colonies were subjected for morphological (transparency, elevation and entire).

3. Biochemical activities:
All biochemical activities were carried out using the methods of Cruckshank (1982).

Catalase test (Koneman et al., 1988).
A drope of H2O2 3% was put on clean slide. Loopfull of bacterial growth was emulsified with the H2O2 drops.
Positive test: rapid production of air bubbles was appeared.

Oxidase test (Koneman et al., 1988).
Few drops of reagents (1% solution of tetra methyl-P-phenylene-diamine dihydro chloride) placed on the colony to be tested on filter paper
Positive reaction: deep blue color within seconds.
Urease test (Koneman et al., 1988).
Streaking with the tested organism was made on the surface of urea slant then incubated at 37 C and examined after 24 hrs overnight incubation.
Positive result: indicated by development of pink colour.

Indole test (Koneman et al., 1988).
The tested organism was inculated onto peptone water and incubated at 37C for 18-24hours then 5 drops of kovac's reagent were done on the inner wall of the tubes. Development of stable red colour at interface of the reagent and the peptone water within seconds means positive result.

Hippurate test. (Fidanoski and Fidanoski, 2007).

Bacitracin sensitivity test. (Fidanoski and Fidanoski, 2007).

Oxidation fermentation test. (Fidanoski and Fidanoski, 2007).

4-Haemolysis test :
All isolates were streaked on surface of 5% sheep blood agar and incubated at 25 C for 48 hr.

5- Molecular characterization using (PCR-test)
1-Bacterial isolates :
Selected a total of biochemically and serologically well identified streptococcus isolates for molecular characterization.

2-Extraction of whole cell protein by gell electrophoresis :
The whole cell protein of the selected streptococcus isolates were separated using the method described by Eliott et al. (1990).

3-Electrophoresis of whole cell protein antigens:
The plates were used according to the manufactures instructions. The separating solution was poured into the gap between the glass plates and sufficient space was left for stacking gel (the length of the teeth of the comb.). After polymerization (30 minutes), the gel was washed by deionized water to remove any unpolymerized acrylamide. The gel was mounted in the electrophoresis apparatus and Tris glycine electrophoresis buffer was added. The samples was heated for 5 minutes at 100°C with equal volume of loading buffer (denaturation proteins). Up to 25 µl of each sample was loaded to each well. The prestained standard protein marker must be loaded in the same gel (about 10 µl). Constant current of 32.5 mA per gel at 10°C was applied and the gel was run until the bromophenol blue reached the bottom. The gel was removed, left in the staining solution for about 2 hrs then was distained (Bomed Instruments, Inc.) (Sadwska et al., 2003).

5-Statistical analysis:
The statistical analysis was made using Chi²-test for examining the significance of the incidence of yeasts and molds among the examined samples according to (SAS, 2004).

3. RESULTS AND DISCUSSION
Streptococcal agalactiae and streptococcal pyogenes causes a great economic losses to milk industry as through discarding of the milk that contaminated with streptococcal bacteria as well as this bacteria reduced the amount of milk production., as the milk is very essential to human as we can synthesis from it cheese, butter, ice-cream and a lot of other products. (Cohen et al., 2005 and Bessen., 2009).

A-Results of bacteriological examination of milk and pus samples:-
The results of bacteriological examination of milk and pus samples cleared that, by Gram's staining (to ensure Gram's positivity, and shapping under micro-sco pic examination.) from 100 samples 40 (samples showed gram-positive cocci arranged in chains isolated from milk and 45 samples from pus. Pigmentation production 5% and 10 % of the milk samples isolates and pus samples out of 100 samples of each of Cultured samples on blood agar, pin point small colonies were 40 and 45 isolates with an incidence of streptococci. This results agreed with those of (Phuektes, et al., 2001) where they, reported that identification of bacterial pathogens especially Streptococcus spp. in milk from cows with mastitis or from pus is the definitive diagnosis of mastitis infections. It also provides information important for prevention and control of the disease.
B. Physical characters of the collected milk and pus samples:

The results observed in Table (2) cleared that, the incidences of streptococci in milk reached to 40 % from the total number of examined samples, followed by staphylococci 15 %, E. coli (10 %) while, the incidences of other bacteria reached to 35 %. While, the incidences of streptococci isolates in pus samples reached to 45 % from the total number of examined samples, followed by staphylococcus (40 %), E. coli (5 %) and the incidences of other bacteria reached to 10 %.

C. Bacteria isolated from milk and pus samples:

Table (3) cleared that the number of milk samples that showed normal colour reached to 70 % from the total number of examined samples and the abnormal colour that showed yellowish or greenish colour observed in 30 % from the examined samples. And the normal consistency of the milk observed in 60 % from the examined samples and the thick consistency observed in 40 % from the total number of examined samples.

While, in the pus samples 55 % from the examined samples showed yellowish colour and 45 % showed greenish colour. While, the pus consistency showed slightly liquid in 70 % of the examined samples and the thick pus observed in 30 % from the examined samples. This results agreed with those of Selem et al. (2002) where they reported that, the main bacterial isolates from milk and pus are streptococcus species, staphylococcus sp., E. coli.

D. Morphology of the bacterial isolates:

The microscopical examination of colony morphology results that observed in Table (4) showed that, Gram positive cocci arranged in chains observed in milk reached to 10 % from the total examined samples but its incidences in the pus reached to 45 %, production of the colony that produce Golden-yellow pigments reached to 5 % while, in pus reached to 10 % from the examined samples. This results agreed with those of Bonnett et al. (1991) the gram positive cocci isolated from pus reached to 40 – 50 % from the total bacterial isolates, while, in milk it reached to 10 – 15 % from all bacterial isolates.

E. Biochemical activities of the isolated bacteria:

Table (5) cleared that, the significant differences of the sensitivity of the isolated streptococci from milk and pus among different biochemical tests.

The bacteria that showed +ve results for catalase test were 3 % and 10 % for milk and pus, respectively. While, oxidase test showed positive results in 80 % and 77.78 % for the bacteria showed positive results for streptococci and urease test showed positive results in 70 % and 66.67 % for the isolated bacteria from milk and pus, respectively.

<table>
<thead>
<tr>
<th>Samples</th>
<th>colour of milk</th>
<th>No. of samples</th>
<th>Consistency of milk</th>
<th>No of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>Normal</td>
<td>70</td>
<td>Normal</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Abnormal colour</td>
<td>30</td>
<td>Thick</td>
<td>40</td>
</tr>
<tr>
<td>Pus</td>
<td>yellowish</td>
<td>55</td>
<td>Slightly liquid</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Greenish</td>
<td>45</td>
<td>thick</td>
<td>30</td>
</tr>
</tbody>
</table>

\[ \text{Chi}^2 = 10.56^{**} \quad \text{Chi}^2 = 12.33^{**} \]

** = Significant at (P< 0.01).

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Milk</th>
<th>Pus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
</tr>
<tr>
<td>Streptococci</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>E. coli</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Other bacteria</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

\[ \text{Chi}^2 = 13.37^{**} \]

** = Significant at (P < 0.01).
This results supported by the results of (Fidanoski and Fidanoski, 2007) where they reported that, the catalase, oxidase and urease test are the main test used for diagnosis and detection of streptococci and the catalase test is the main test that differentiated the staphylococcus species from streptococcus species as all streptococcus species are catlase –ve while, staphylococcus species are catalase +ve.

Table (6) cleared that, the significant differences (P < 0.01) of the results of the oxidation fermentation and aerobic fermentation of mannnitol among bacteria isolated from milk and pus. The oxidation fermentation test showed a positive results in 70 % and 77.78 for the samples gave +ve results for streptococci. Whilethe results of the aerobic fermentation of mannnitol showed +ve results for 70 % and 77.78 % of the isolated streptococci.

Table (7) cleared that, the significant differences (P < 0.01) of the sensitivity of the isolated streptococcal bacteria for coagulase test and haemolysis test. The coagulase test gave positive results in 70 % and 66.67 % of the streptococcal samples. While, the haemolysis test gave +ve results in all samples that gave +ve results for streptococci +ve samples. Our results agreed with those of (Khan, 2012) where he reported that, the infection with Streptococcus pyogenes, and streptococcus agalactiae associated with a beta-hemolytic bacterium that belongs to Lancefield serogroup A, also known as the group A streptococci (GAS), causes a wide variety of diseases in humans and animals..

Table (4): The colony morphology of the % obtained suspected isolates recovered from culturered milk samples on nutrient agar.

<table>
<thead>
<tr>
<th>Total N. of Samples</th>
<th>Gram-positive cocci</th>
<th>Golden-yellow pigmented colony arranged in chains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>%</td>
</tr>
<tr>
<td>Milk</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Pus</td>
<td>45</td>
<td>45</td>
</tr>
</tbody>
</table>

Chi² = 4.29”** Significant at (P< 0.01).

Table (5): Biochemical activities of the obtained suspected Streptococcus agalactiae isolates from examined milk samples:

<table>
<thead>
<tr>
<th>Total No. of samples</th>
<th>Number of positive streptococci samples</th>
<th>Catalase</th>
<th>Oxidase</th>
<th>Urease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Milk</td>
<td>10</td>
<td>3</td>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td>Pus</td>
<td>45</td>
<td>10</td>
<td>22.23</td>
<td>35</td>
</tr>
</tbody>
</table>

Chi² = 6.28”** Significant at (P< 0.01).

Table (6): Biochemical activities of the suspected Streptococcus isolates:

<table>
<thead>
<tr>
<th>Samples</th>
<th>Samples</th>
<th>Oxidation-Fermentation</th>
<th>Aerobic fermentation of mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Milk</td>
<td>10</td>
<td>7</td>
<td>70</td>
</tr>
<tr>
<td>Pus</td>
<td>45</td>
<td>35</td>
<td>77.78</td>
</tr>
</tbody>
</table>

Chi² = 6.28”** Significant at (P< 0.01).

Table (7): Results of estimating pathogenic determinants of the isolated S.aureus.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total No. of isolates</th>
<th>Coagulase test</th>
<th>Haemolysis test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Milk</td>
<td>10</td>
<td>7</td>
<td>70</td>
</tr>
<tr>
<td>Pus</td>
<td>45</td>
<td>30</td>
<td>66.67</td>
</tr>
</tbody>
</table>

Chi² = 3.44”** Significant at (P< 0.01).
Table (8): Differentiation of the samples of Streptococcus agalactiae and streptococcus pyogenes.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total No. of isolates</th>
<th>Beta-Haemolysis</th>
<th>Bacteracine sensitivity</th>
<th>Hippurate test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Milk</td>
<td>10</td>
<td>100</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>Pus</td>
<td>45</td>
<td>100</td>
<td>40</td>
<td>88.89</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 7.88^{**} \] ** Significant at (P< 0.01).

Table (9): Incidence of Streptococcus agalactiae and streptococcus pyogenes in milk and pus samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Bacteria</th>
<th>Total No. of isolates</th>
<th>Incidences</th>
<th>Percentage from all examined samples (100)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Milk</td>
<td></td>
<td></td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Pus</td>
<td></td>
<td></td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>40</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 5.29^{**} \] ** Significant at (P< 0.01).

Table (8) cleared that, the streptococcal bacteria that isolated from examined samples differ in its sensitivity to different tests. The results cleared that, all streptococcal samples gave +ve beta haemolysis, while, the bacteracine sensitivity test showed +ve results for 3 % and 88.89 % for the samples of milk and pus while the hippurate test gave +ve results in 70 % and 88.89 % from all of the examined milk and pus samples. Our results agreed with those of Cruickshank et al. (1975); Fidanoski and Fidanoski, (2007) where they reported that, streptococcus agalactiae and streptococcal pyogenes have a beta haemolysis.

Table (9) cleared that, the incidences of streptococcus agalactiae in in milk samples showed +ve results for streptococci about 70 % and streptococcal pyogenes 30 % while, in pus samples there is no incidences for streptococcal agalactiae, while, the higher incidences observed 88.89 % from all of the examined samples.

F- Incidences of streptococcal agalactiae and streptococcal pyogenes in examined samples:

The results observed in Table (9) cleared that, the isolated streptococci can be grouped into two main categories. In milk the higher streptococcal isolates observed in streptococcus agalactiae 70 % and streptococcus pyogenes 30 % while, in pus samples the higher incidences observed in streptococcus pyogenes 88.89 % and from all examined samples (100 ) reached to 7 % and 3 % for streptococcus agalactiae and streptococcus pyogenes isolated from milk and 0 % and 40 % for streptococcus agalactiae and streptococcus pyogenes, respectively.

This results agreed with those of (McDonald and McDonald, 1976) where they, observed that, the incidence of streptococcus agalactiae in milk reached to 1.5 %. Also, (Hashim et al., 1990) reported that, the main streptococcal spp. Isolated from milk and causes a great economic importance were streptococcus agalactiae and streptococcus pyogenes.

G. Polymerase chain reaction:

The results of polymerase chain reaction for detection of streptococcus bacteria gave +ve results by ordinary and biochemical tests, the results obtained indicated that the virulent strains identified gave +ve results with PCR (Fig. 1).
These results agreed with those of (Pollard et al. 1990; Pollard et al., 1991 and Sadowska et al., 2003) where they reported that, Polymerase chain reaction was used to detect the relationships between the bacteria isolated from milk and pus swab.

Our results concluded that, the streptococcus agalactiae and streptococcus pyogenes are the most important bacterial isolates that causes severe losses to milk industry, also the streptococcus pyogenes of zoonotic importance as it transmitted to human. The catalase test, oxidatse test and hemolytic test are the main tests used for diagnosis and differentiation between streptococcus agalactiae and streptococcus pyogenes. The bacitracin sensitivity test is main test for differentiation between streptococcus agalactiae and streptococcus pyogenes The results also indicated that the level of streptococcus agalactiae in milk higher than that of streptococcus pyogenes and reached to 7 % and 3 % for streptococcus agalactiae and pyogenes, while, in pus its levels reached to 0 and 40 % for streptococcus agalactiae and pyogenes in pus. Also the results cleared that the PCR method for detection of mastitis considered as the best method.

4. REFERENCES


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