

Molecular studies regarding to virulence factors of Streptococcus species isolated from raw milk.

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ABSTRACT

The present study was performed on a total of 124 milk samples from small scale producers, farmers and markets at El-Menofya Governorate. The prevalence of Streptococcus species in the examined samples was (65.3%), where *S. agalctiae*, *S. dysgalactiae*, *S. uberis*, *S. pyogenes* and *S. pneumoniae* were 28.2%, 11.3%, 16.1%, 8.9% and 0.8%; respectively. The antibiogram for Streptococcus spp. revealed that vancomycin and erythromycin were the most proper antibiotics with the highest in vitro efficiency against isolated Streptococcus spp., but were resistant to ceftriaxone and chloramphenicol. Additionally, *S. agalactiae* and *S. dysgalactiae* were sensitive to penicillin, ofloxacin ; respectively. But *S. uberis* was sensitive to amoxicillin and clindamycin. By using PCR, virulence gene *hyaluronidase (hyl)* was detected in 25% of *S. agalactiae*, while monokine induced by IFN-gamma (*mig*) gene was detected in 100% of *S. dysgalactiae*. Also *plasminogen activator (pauA)* gene was detected in 100% of *S. uberis* isolates.

Keywords: Streptococcus spp., Raw milk, PCR.

1. INTRODUCTION

Milk has a complex biochemical composition and its high water activity and nutritional value serves as an excellent medium for growth and multiplication of many kinds of microorganisms when suitable conditions exists (Parekh and Subhash, 2008). Streptococci are a heterogeneous group of bacteria, consisting of as many as 48 species (Facklam, 2002). Streptococcus was isolated frequently from bovine mammary glands (Fortin et al., 2003). *S. agalactiae*, *S. dysgalactiae* and *S. uberis* have been reported as the three most common etiological agents of bacterial intramammary infection (Khan et al., 2003). Streptococci are classified on the basis of colony morphology, hemolysis, biochemical reactions, and

serologic specificity. They are divided into three groups by the type of hemolysis on blood agar: β -hemolytic (clear, complete lysis of red cells), α hemolytic (incomplete, green hemolysis), and γ hemolytic (no hemolysis). Serologic grouping is based on antigenic differences in cell wall carbohydrates (groups A to V), in cell wall pili-associated protein, and in the polysaccharide capsule in group B Streptococci (Maria, 1996). *S. agalactiae* is a major contagious mastitis pathogen and continues to be a major cause of mastitis in dairy cattle and buffaloes (Zadoks and Fitzpatrick, 2009). *S. uberis* has been isolated from many extramammary sites on the cow, including the skin surface, gut, tonsils, and genital tract (Razavi-Rohani and Bramley, 1981). It is also found in high numbers in bedding material, which is a likely source of infection in housed cattle (Bramley, 1982). *S. dysgalactiae* has the unique characteristic of being considered both a contagious and an environmental pathogen. These organisms can spread from cow to cow at milking time and are also commonly found in the cow's environment (Christina and John, 2012). All the bacteria isolated through microbiological procedures should be subjected to antimicrobial susceptibility test by disc diffusion method to identify the most effective drugs for infection treatment in the study area (Hameed et al., 2008). The *hyl* gene is very important for the pathogenesis of *S. agalactiae* (Arpini et al., 2016). *Mig* gene of *S. dysgalactiae* which promote dissemination of the organism into host tissue. (Calvinho et al., 1998). *PauA* gene of *S. uberis* activates plasminogen which has been proposed as an important mechanism to obtain nutrients for optimal growth of the organism (Ward and Leigh, 2004). So the current study aimed to isolate and identify Streptococci and some of their virulence genes using biochemical tests and PCR; respectively.

2. MATERIALS AND METHODS

2.1. Samples:

A total of 124 raw milk samples were collected from different markets and farms. The samples were transferred in an ice box directly within an hour to the laboratory with a minimum delay to be bacteriologically examined.

2.2. Pigment production (Hotis test):

0.5 ml of sterile 0.5% aqueous solution of bromcresol purple was added to 9.5 ml of milk sample in a sterile test tube. The tube was inverted several times to mix the contents before being incubated at 37°C for 24 hours (Atherton and Newlander 1977).

2.3. Bacteriological examination:

The collected milk samples were incubated aerobically at 37°C for 24 hours then divided into two parts: One part was centrifuged at 3000 rpm for 20 minutes. The cream and supernatant fluids were discarded. Methylene blue stain was used routinely to detect the suggestive bacterial causes from the sediment. A Loopful from the second part was streaked on the surface of Crystal violet blood agar (Cruickshank et al., 1975) and Edward's media (oxid). The inoculated plates were incubated at 37°C for 24 - 48 hrs and examined for bacterial growth, suspected Streptococcal colonies were sub cultured, purified and preserved in semisolid media for further identification. The purified colonies were morphologically identified by Gram's stain, Loeffler's methylene blue and biochemical tests (Catalase, Oxidase, haemolysis, Growth at 6.5% NaCl, Arginine decarboxylase, Hippurate hydrolysis, Bile esculin hydrolysis and Fermentation of sugars) (Koneman et al., 1992, Quinn et al., 1994 and MacFaddin, 2000).

2.4. In-Vitro antibiotic sensitivity test:

The *S. agalactiae*, *S. dysgalactiae* and *S. uberis* isolates were subjected to the sensitivity test against eight different antibiotics (Table 2), using the disc and agar diffusion method (Finegold and Martin, 1982).

2.5. Detection of Virulence genes of isolated Streptococcus spp. by PCR:

Primers were used for detection of three virulence genes that may play a role in virulence of Streptococcus spp. These genes were *hyl* gene of *S. agalactiae*, *mig* gene of *S. dysgalactiae* and *pauA* gene of *S. uberis*. It was applied on four isolates of *S. agalactiae*, three isolates of *S. dysgalactiae* and three isolates of *S. uberis* according to QIAamp® DNA mini kit instructions, (Catalogue no.51304), Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit and agarose gel electrophoreses (Sambrook et al., 1989).

3. RESULTS:

Streptococcus spp. were identified by morphological and culture characters as well as identical biochemical tests as following:

3.1. Hotis test:

Positive results indicated by appearance of Canary-yellow granules or flakes (0.5 to 4mm in diameter) attached to the sides and sometimes at the

bottom of the tube due to fermentation of lactose to acid which changes bromocresol purple into yellow in case of *S. agalactiae* isolates.

3.2. Colonial appearance:

On Blood agar media *Streptococcus* spp. showed small colonies that were moist, convex and translucent with less beta hemolysis in case of *S. agalactiae* and well-defined zone of complete or β - hemolytic in *S. pyogenes*. But *S. pneumoniae* showed Smooth, glistening, wet-looking, mucoid colonies, α -Hemolytic, while on Edwards media, *S. agalactiae* and *S. pyogenes* grew as beta hemolytic small transparent bluish grey colonies but *S. dysgalactiae* produced greenish discoloration of the medium but *S. uberis* grew as dark colored colonies surrounded by black or brown zone of colouration due to hydrolysis of aesculin (Table 1).

3.3. Microscopic examination:

3.3.1. Gram's stain:

Streptococcus species showed Gram positive, violet colored cocci, small in size, non sporulated and arranged in pairs or long chain under microscope.

3.3.2. Loeffler's methylene blue:

Streptococci appear as chain of blue color when examined under oil immersion lens of microscope.

3.4. Biochemical reactions:

All *Streptococci* isolates were catalase and oxidase negative. *S. agalactiae* isolates were beta hemolytic, able to hydrolyze Na hippurate and Arginine but unable to hydrolyse esculin. They could not ferment sorbitol, mannitol, arabinose, raffinose but ferment lactose and Ribose. *S. dysgalactiae* isolates were alpha hemolytic, not able to hydrolyze Na hippurate and esculin but able to hydrolyse arginine and could not ferment sorbitol, arabinose, mannitol and raffinose but ferment lactose and ribose. *S. uberis* isolates were alpha/gamma hemolytic, able to hydrolyze hippurate and esculin. All isolates ferment, sorbitol, mannitol and lactose. They were negative for fermentation of raffinose, ribose and arabinose. They can grow on 6.5% NaCl. All *S. pneumoniae* isolates were alpha hemolytic, They were negative for hippurate hydrolysis but positive for arginine and esculin hydrolysis, negative for fermentation of sorbitol, mannitol, but could ferment raffinose, lactose, arabinose and ribose. All *S. pyogenes* isolates were beta hemolytic, could ferment lactose only. They were negative for Na hippurate and esculin hydrolysis but positive for arginine.

3.5. Results of in vitro Antibiotic Sensitivity:

All the tested Streptococcal isolates were sensitive to vancomycin at the percentage of (100%), erythromycin (90%), amoxicillin (80%), clindamycin (60%), ofloxacin (50%), ceftriaxone (50%), penicillin (40%) but resistant to chloramphenicol (100%). On the other hand, *S. agalactiae* isolates were fully sensitive to amoxicillin, followed by penicillin, erythromycin, ofloxacin and clindamycin. *S. dysgalactiae* isolates were fully sensitive to ofloxacin and erythromycin followed by clindamycin but were fully resistant to penicillin followed by amoxicillin. On the other hand; *S. uberis* isolates were fully sensitive to amoxicillin, erythromycin, and clindamycin but resistant to penicillin followed by ofloxacin. According to NCCLS (2016) (Table 2).

3.6. PCR results for virulence genes(*hyl*, *mig* and *pauA*):

One isolate of *S. agalactiae* was amplified at 950bp. Fig.(1). The three isolates of *S. dysgalactiae* were amplified at 188 bp. Fig.(2). The three isolates of *S. uberis* were amplified at 439 bp. Fig.(3). (Table 3).

4. DISCUSSION

Streptococci form a large group of organisms, which are associated with bovine intramammary infections (Wyder et al., 2011). Streptococci are Gram positive cocci in the family Streptococcaceae. They often occur in pairs or chains, especially in fluids. Many members of the genus *Streptococcus* are pathogenic for humans and animals. Some species are proven or suspected to be zoonotic. *S. pyogenes* is, by far, the most common cause of Streptococcal pharyngitis. It also causes relatively mild skin infections such as pyoderma and impetigo, as well as otitis media, sinusitis, abscesses, cellulitis, osteomyelitis, arthritis, endocarditis and rarely, serious infections such as pneumonia, meningitis, septicemia, necrotizing fasciitis or Streptococcal toxic shock syndrome. *S. pyogenes* can also be carried asymptotically. *S. pneumoniae* is an important human pathogen that causes pneumonia, meningitis, otitis media, sinusitis and other diseases (The center for food security and public health, 2005). In most countries, the major mastitis pathogens are *S. agalactiae*, *Staph. aureus* (contagious pathogens), *S. dysgalactiae*, *S. uberis* and *E. coli* (environmental pathogens). The word “major” reflects their considerable impact on cow health, milk quality and productivity (Neave et al., 1969). The action of lipases originating from the mastitis pathogens will contribute significantly to milk fat lipolysis and thus to raw milk deterioration (Janak et al., 2015).

In the present study, eighty one *Streptococcus* species isolates were recovered from 124 milk samples with a percentage of 65.3%. Nearly similar results were recorded by Amosun et al.,(2010), Hala (2011), El-Jakee et al. (2013) and Marguerite et al. (2016).On the other hand; the obtained result was disagreed with David et al. (2013), Fentaye et al. (2014), Gamil et al. (2014), Shaimaa (2014) and Yara (2015).

The prevalence of *Streptococcus* species in bovine milk were 28.22% for *S. agalactiae*, 11.29% for *S. dysgalactiae*, 16.13% for *S. uberis*, 8.87% for *S. pyogenes*, 0.81% for *S. pneumoniae* .(Table 1).This agreed with Fatma (2013), Ararsa et al. (2014),Gharari et al. (2014),Redeat et al. (2014) and Yanliang et al. (2016) and while disagreed with Elizabeth (2010), Leelahapongsathon et al. (2014) and Gürler et al. (2015).

In the present study, the results of in-vitro antibiotic sensitivity tests for the isolated Streptococcal species (Table 2) were agreed with Salah (2008), Kiro (2011), Tanvir et al. (2013), Singh and Roy (2015), Subha et al. (2016) and Yuexia et al. (2016).On the other hand, the recorded results were disagreed with Markus et al. (2014), Vasquez (2014), Preethirani et al. (2015) and Safaa (2016).

In the current study, *hyl* gene of *S. agalactiae* was detected in 1 out of 4 studied *S. agalactiae* isolates. These results go parallel with Clarisse (2011) and disagree with Ayman et al. (2015). *Mig* gene of *S. dysgalactiae* was detected in all isolates of *S. dysgalactiae*. *PauA* gene was detected also in all *S. uberis* isolates and these results go parallel with Melina et al. (2015). Hyaluronidase gene (*hyl*) of *S. agalactiae* facilitates invasion of host cells, Monokine induced by IFN-gamma gene (*mig*) of *S.dysgalactiae* plays a role in virulence of the bacteria by binding to the plasma protein α 2-M or IgG and thus preventing phagocytosis by bovine PMNs (Song et al., 2001).Also plasminogen activator gene (*pauA*) of *S. uberis* has been proposed as an important mechanism for this organism to obtain nutrients for optimal bacterial growth (Oliver et al., 1998).