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

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A B S T R A C T

The present study was performed on a total of 124 milk samples from small scale producers, farmers and markets at El-Menofyia 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 efficiency against isolated Streptococcus spp., but they were resistant to cefatriaxone and chlormphenicol. Additionally, *S. agalactiae* and *S. dysgalactiae* were sensitive to penicillin, ofloxacin ;respctively. However, *S. uberis* was sensitive to amoxicillin and clindamycin. By using PCR, virulence gene *hyalurinidase (hyl*) was detected in 25% of *S. agalactiae*, while a surface expressed M-like protein (*mig)* gene was detected in 100% of *S. dysgalactiae*. Also *plasminogen activator (pauA*) gene was detected in 100% of *S. uberis* isolates.

Keywords: Streptococcus, raw milk, virulence gene, PCR.

(http://www.bvmj.bu.edu.eg) (BVMJ-32(1): 145-152, 2017)

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. dysgalatiae* 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 (Oliver et al., 1998; 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 37ºC 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 (oxoid). 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, hemolysis, Growth at 6.5% NaCl, Arginine decarboxylase, Hippurate hydrolysis, Bile esculin hydrolysis and Fermentation of sugars) (Koneman, 1992; MacFaddin, 2000; Quinn et al., 1994).

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:

3.1. Morphological and culture character of Streptococcus species:

Streptococcus species are Gram positive cocci, small in size, non sporulated and arranged in pairs or long chain. In addition, 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 coloration due to hydrolysis of aesculin (Table 1). Microscopically, Streptococci appear as chain of blue color when examined under oil immersion lens of microscope using methylene blue stain. On the other hand, all Streptococcal 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. Also, Hotis positive results in case of *S. agalactiae* isolates was 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.

3.2. 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%), cefatriaxone (50%), penicillin (40%) but resistant to chlormphenicol (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).

3.3. 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).

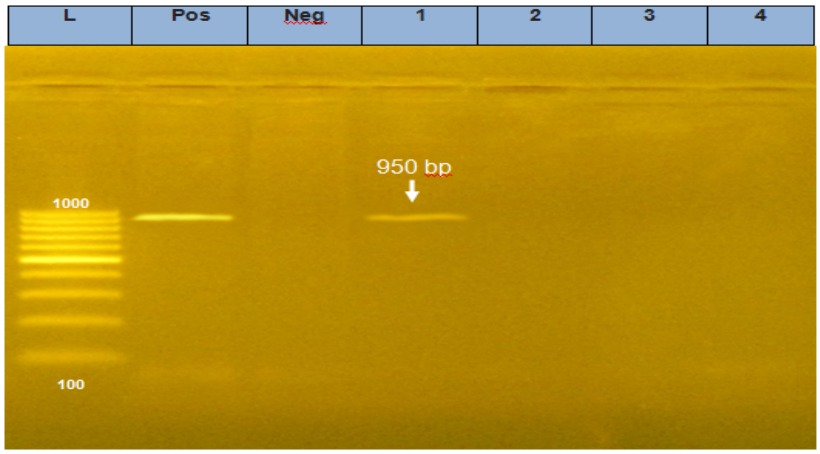

Figure (1): Gel electrophoresis for *hyl* gene of *S. agalactiae:* L: molecular weight marker (100-1000 pb). Pos: positive control (at 950pb). Neg: Negative control. Lane1: *S. agalactiae* at 950 pb . Lane 2, 3, 4: Negative.

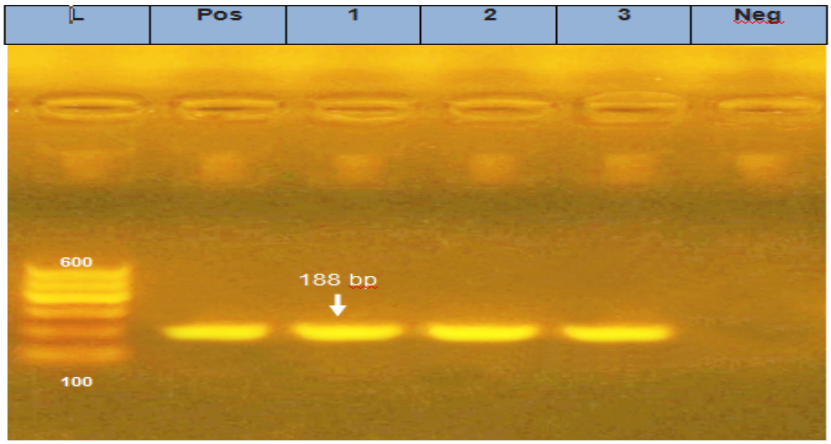

Figure (2): Gel electrophoresis for *mig* gene of *S. dysgalactiae:* L: molecular weight marker (100-600 pb). Pos: positive control (at 188pb). Neg: Negative control. Lane1, 2, 3: *S. dysgalactiae* at 188 pb.

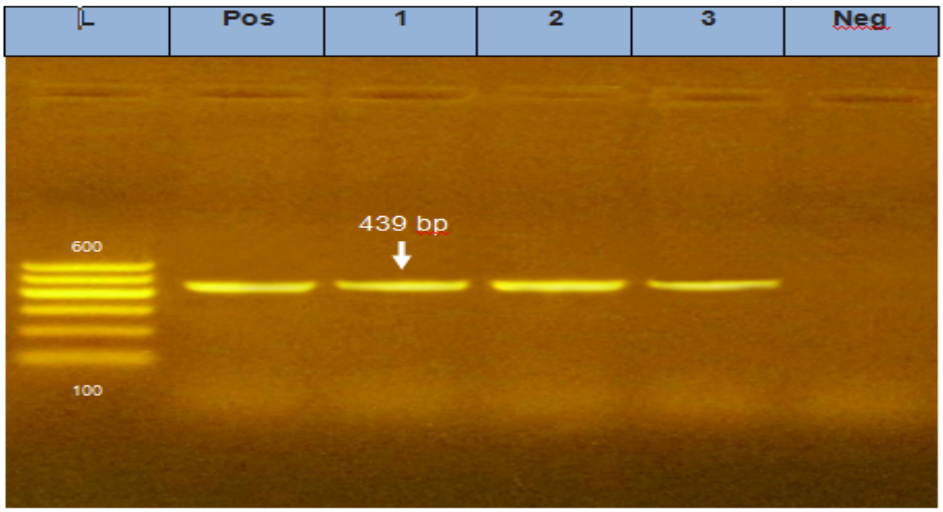

Figure (3): Gel electrophoresis for *pauA* gene of *S. uberis:* L: molecular weight marker (100-600 pb). Pos: positive control (at 439pb). Neg: Negative control. Lane1, 2, 3:*S. uberis* at 439pb.

Table (1): Prevalence of Streptococcus species among collected milk samples using Crystal violet blood agar and Edward's media. (n=124).

Isolates	No.of positive			
media used	Crystal violet blood agar		Edward's media	
	No	%	No	%
S. agalactiae	37	29.9	35	28.2
S. dysgalactiae	14	11.3	14	11.3
S. uberis	21	17	20	16.1
S. pyogens	13	10.5	11	8.9
S. pneumoniae	3	2.4	1	0.8
Total	88	71%	81	65.3%

*Percentage in relation to total number samples (n=124).

Table (2): In vitro, antibiotic sensitivity test of *S. agalactiae*, *S.dysgalactiae* and *S.uberis* isolated from the examined milk

Type of Antibiotic	Conc. (µg)	Isolates								
		*S.agalactiae**			*S.dysgalactiae*+			*S.uberis***		
		S	I	R	S	I	R	S	I	R
Penicillin G_{10}	10	4	0	1	0	0	3	0	0	2
Cefatriaxone	30	0	0	5	0	0	3	0	0	2
Amoxicillin	25	5	0	0	1	0	2	2	0	0
Ofloxacin	5	2	2	1	3	0	0	0	1	1
Vancomycin	30	5	0	0	3	0	0	2	0	0
Erythromycin	15	4	0	1	3	0	0	2	0	0
Clindamycin	2	2	1	2	2	1	0	2	0	0
Chloramphenicol	30	0	3	2	0	0	3	0	0	2

S: sensitive. I: intermediate sensitive. R: resistant. * n =5. + n =3. ** n =2

Table (3): Detection of Streptococcus species' virulence genes (*hyl*, *mig* and *pauA*).

Virulence gene	*S.agalactiae*			*S.dysgalactiae*			*S.uberis*		
	No.of tested isolates	No.of positive	%	No.of tested isolates	No.of positive	%	No.of tested isolates	No.of positive	%
Hyl	4	1	25	-	-	-	-	-	-
Mig	-	-	-	3	3	100	-	-	-
PauA	-	-	-	-	-	-	3	3	100

2. DISCUSION

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 asymptomatically. *pneumoniae* is an important human pathogen that cause 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); 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); 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 came in agreement with Ararsa et al. (2014); Fatma (2013); Gharari et al. (2014); Redeat et al. (2014) Yanliang et al. (2016) and while disagreed with Elizabeth (2010); Gürler et al. (2015); Leelahapongsathon et al. (2014).

In-vitro antibiotic sensitivity tests for the isolated Streptococcal species revealed that all of the tested Streptococcal isolates were sensitive to vancomycin and erythromycin but resistant to chloramphenicol and cefatriaxone as shown in (Table 2).This come in agreement with Kiro (2011); Salah and Abd-El (2008); Singh and Roy (2015); Subha et al. (2016); Tanvir et al. (2013) Yuexia et al. (2016). On the other hand, the recorded results were disagreed with (Markus et al., 2014); Preethirani et al. (2015); Safaa (2016); Vasquez (2014). *Hyl* gene of *S. agalactiae* was detected in 1 out of 4 studied S. agalctiae 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. dysgalctiae*. *PauA* gene was detected also in all *S. uberis* isolates and these results go parallel with Melina et al. (2015). Hyalurinidase gene (*hyl*) of *S. agalactiae* facililates invasion of host cells, a surface expressed M-like protein (*mig*) gene of *S.dysgalactiae* plays a role in virulence of the bacteria by binding to the plasma protein α2-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).

5. CONCLUSION

Presence of streptococcus in milk represents a human health hazard as it is associated with milk-

borne diseases of man. Milk should be pasteurized or ultra-heat treated. The virulence genes play an important role in increasing the pathogenesis of Streptococcus species so further studies should be done to produce effective vaccines against Streptococcus spp. and these virulence genes to minimize the high spread of Streptococcal infection among dairy cows.

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