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Antibiotic Resistance Patterns of *Streptococcus agalactiae* Isolated from Mastitic Cows and Ewes in Egypt

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Abstract: Streptococcal mastitis causes great economic losses in dairy industries allover the world. For humans, *S. agalactiae* is responsible for persistent disease in adults and neonates. The present study investigated the prevalence of streptococci in mastitic cows and ewes as well as, the antibiotic resistance patterns of *Streptococcus agalactiae* isolates. A total of 570 milk samples collected from cow (n=300) and ewes (n=270) were examined. Streptococcus species were isolated from cows (55 %) and ewes (50.4%). *S. agalactiae*, *S. dysgalactiae S. uberis, S. pyogenes* and *S. pneumoniae* were isolated from the examined cow samples with incidence of 19.3, 17, 15.3, 2.7 and 0.7% respectively. Also, *S. agalactiae*, *S. dysgalactiae S. uberis, S. pyogenes* and *S. pneumoniae* were isolated from the examined ewes with incidence of 20.4, 15.9, 10.4, 2.6 and 1.1% respectively. The objective of this work was to characterize *S. agalactiae* isolated from mastitic cows and ewes by RAPD and antibiotic sensitivity test to detect the diversity of the isolates. *S. agalactiae* isolated from dairy cows and ewes was sensitive to ampicillin, penicillin and cefotaxime. Resistant to vancomycin, chloramphenicol, tetracycline and clindamycin was recorded among the examined S. *agalactiae* isolated from cows and ewes. The data indicated the existence of different RAPD patterns among the multidrug resistant *S. agalactiae* isolates.

Key words: Mastitis · Ewes · Cows · Streptococci · Antibiotics · Rapd-Pcr

INTRODUCTION

Mastitis remains one of the most economically important problems of the dairy farms. Slama *et al.* [1] recorded that streptococcal bacteria are one of the most important pathogens causing different type of bovine mastitis. Estimated costs ranged from 149 euro to 570 euro per mastitic case and were highest for contagious pathogens such as *S.aureus* and coagulase-negative staphylococci and lowest for *S. dysgalactiae* and *S. uberis* [2]. Streptococci are a heterogeneous group of bacteria, consisting of as many as 48 species, including important human pathogens such as *S. pneumoniae*, *S. pyogenes* and *S. agalactiae* [3]. *S. agalactiae* is an important cause of serious neonatal infections characterized by sepsis and meningitis. Group B streptococcus is a leading cause of morbidity and mortality in infants in Europe, the Americas and Australia [4]. Most other streptococci are members of the normal human flora [5]. The present study investigated the prevalence of streptococci in mastitic cows and ewes as well as, the antibiotic resistance patterns of *Streptococcus agalactiae* isolates.

MATERIALS AND METHODS

Collection of Milk Samples: A total of 570 milk samples collected from cow (n=300) and ewes (n=270) were investigated. Samples were collected from private and governmental dairy farms in Cairo and Giza. The udder of each animal was examined before sampling for detection of clinical signs of mastitis such as inflammation, asymmetry, hotness, swelling or any physical changes. Each udder was washed and carefully dried with clean towel then the teats were swabbed with 70% alcohol. Before sampling, the first jets of milk were rejected and

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each quarter milk sample was collected into sterile screw capped McCartney bottle [6]. The milk samples were sent immediately to the laboratory for bacteriological examination in an ice container. The milk samples collected from apparently healthy animals were examined by California mastitis test [7] to detect subclinical mastitis from samples collected from apparently normal quarters.

Bacteriological Examination of Milk Samples [8]: The collected milk samples were incubated aerobically at 37°C for 24 hours and centrifuged at 3000 rpm for 20 minutes. The cream and supernatant fluid were discarded. Methylene blue stain was used routinely to detect the suggestive bacterial causes. The sediment was streaked on the surface of blood agar and Edward's media (Oxoid). The inoculated plates were incubated at 37°C for 24-48 hours and examined for bacterial growth. Suspected streptococcal colonies were sub- cultured, purified and preserved in semisolid agar for further identification.

Identification of Streptococcus Species: The isolates were initially identified by characteristic morphology and catalase-negative before being subjected for identification according to Cowan [9] and Carter and Cole [10], using the following tests: hemolysis onto 7 % sheep blood agar, arginine hydrolysis, esculin hydrolysis, sodium hippurate hydrolysis, growth in 6.5%NaCL, litmus milk, gelatin liquefaction, catalase test, bile solubility and carbohydrate fermentation Viz. trehalose, raffinose, sorbitol, mannitol, salicin, lactose and inulin. Also, CAMP test [11] using *S. aureus* (ATCC 25923) was carried out.

Serological Identification of Streptococcal Species: Streptococcal grouping kit (Oxoid): a latex agglutination test for the identification of the streptococcal groups A, B, C, D, F and G was used.

Identification of Streptococci by PCR: All isolates were confirmed to be streptococci by PCR [12] using two primers for detection of streptococci and *S. agalactiae*. The sequence of the primers used to identify Streptococcus species [5] were: Str1 5'-GTA CAG TTG CTT CAG GAC GTA TC-3' and Str 2 5'-ACG TTC GAT TTC ATC ACG TTG -3'. The sequence of the primers used to identify *S. agalactiae* isolates [13] were: Sag 40 5'CGC TGA GGT TTG GTG TTT ACA 3' and Sag 445 5' CAC TCC TAC CAA CGT TCT TC 3'. DNA was extracted from the isolates [14]. Protocol for identifying *S. agalactiae* isolates was carried out according to Riffon *et al*. [13]. 50µl volume of X µl of DNA contains 100-200 ng of extracted DNA, 0.5 µl of each primer contains (50 picomol) 0.5 µl of Tag polymerase enzyme, and 5 µl 10 X pcr buffer contain 1.5 mM $MgCl_2$ and 0.2mM dNTP were added. DNA molecular weight marker (100bp) was supplied by Amers Co., Cleveland, Ohio, USA.

Antibiotic Sensitivity Testing: *S. agalactiae* isolates were tested for susceptibility to 9 different antimicrobial discs (Oxoid): ampicillin, erythromycin, clindamycin, cefotaxime, chloramphenicol, ofloxacin, penicillin G, tetracycline and vancomycin according to Finegold and Marten [15]. The result was interpreted according to National Committee for Clinical Laboratory Standards [16].

Molecular Characterization of *Streptococcus agalactiae:* Ten multidrug resistant *S. agalactiae* isolates (2 ewe isolates and 8 cow isolates) were characterized using RAPD-PCR. The Primers were designed according to Martinez *et al.* [17] and the primers sequences were: OPS11 5' AGTCGGGTGG 3' and OPS16 5' AGGGGGTTCC 3'. The protocol for RAPD was carried out according to Martinez *et al*. [17] A final volume of 50 µl volumes of 100-200ng of extracted DNA in the mixture consisted of buffer (10mM tris. HCL {pH8.3}, 50mM Kcl, 1.5mM $MgCl_2$), 0.4µM primers, 2.5U Taq and 100 µm of each of the four deoxynucleoside triphosphates were added.

RESULTS

Identification of Streptococcus Isolates by PCR: All isolates were identified by conventional methods and serologically. Also, streptococci specific PCR primers (Str1 and Str2) generated 197 bp amplicon from all streptococci isolates and the selected *S. agalactiae* specific species primers generated 405 bp amplicon as shown in Figure 1.

Incidence of Streptococcus Species among the Examined Cows' Milk Samples: In case of subclinical mastitis as shown in Table 1, Streptococcus species isolated from cows were 26 *S. agalactiae* (21.7%), 23 *S. dysgalactiae* (19.2%), 20 *S. uberis* (16.7%) and *3 S. pyogenes* (2.5%). While in clinical mastitis samples, *S. agalactiae S. dysgalactiae* S. *uberis S. pyogenes* and *S. pneumoniae* were recovered with incidence of 31.6, 28.4, 26. 3, 5.3 and 2.1 % respectively. Among California mastitis negative samples only *S. agalactiae* (2.4%), *S. dysgalactiae* (1.2%) and *S. uberis* (1.2 %) were identified.

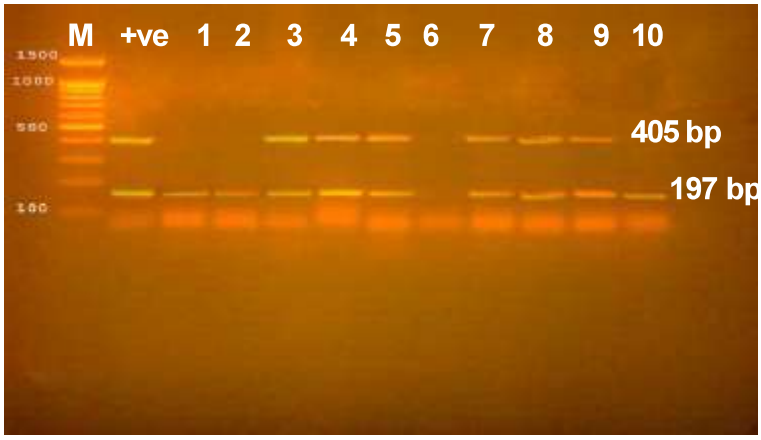

Fig. 1: Multiplex PCR amplified products among the examined streptococci isolates.
Lane (M): 100 bp marker (Axygen). Lane +ve: positive control for *S. agalactiae* standard strain (SS615). Lanes 1, 2 and 10: positive for Streptococcus species (*S. dysgalactiae, S. uberis* and *S. pyogenes* respectively). Lane 6: negative control. Lanes 3,4,5,7, 8 and 9: *S. agalactiae* isolates.

Table 1: Incidence of *Streptococcus* species among the examined cows' milk samples

Streptococcus Species	California mastitis negative samples (n=85)		Subclinical mastitis (n=120)		Clinical mastitis (n=95)		Total (n=300)	
	Positive	%	Positive	%	Positive	%	Positive	%
S. agalactiae	2	2.4	26	21.7	30	31.6	58	19.3
S. dysgalactiae	1	1.2	23	19.2	27	28.4	51	17
S. uberis	1	1.2	20	16.7	25	26.3	46	15.3
S. pyogenes	0	0	3	2.5	5	5.3	8	2.7
S. pneumoniae	0	0	0	0	2	2.1	2	0.7
Total	4	4.7	72	60	89	93.6	165	55

Table 2: Incidence of *Streptococcus* species among the examined ewes' milk samples

Streptococcus Species	California mastitis negative samples (n=65)		Subclinical mastitis (n=115)		Clinical mastitis (n=90)		Total (n=270)	
	Positive	%	Positive	%	Positive	%	Positive	%
S. agalactiae	0	0	22	19.1	33	36.7	55	20.4
S. dysgalactiae	0	0	19	16.5	24	26.7	43	15.9
S. uberis	0	0	15	13	13	14.4	28	10.4
S. pyogenes	0	0	3	2.6	4	4.4	7	2.6
S. pneumoniae	0	0	1	0.9	2	2.2	3	1.1
Total	0	0	60	52.2	76	84.4	136	50.4

Incidence of Streptococcus Species among the Examined Ewes' Milk Samples: Table 2 illustrated that streptococci isolated from subclinical mastitic ewes were 22 *S. agalactiae* (19.1%), 19 *S. dysgalactiae* (16.5%), *S. uberis* 15(13%), *S. pyogenes* 3(2.6%) and one isolate of *S. pneumoniae* (0.9%).While in clinical mastitis 33 *S. agalactiae* (36.7 %), 24 *S. dysgalactiae* (26.7%), 13 *S. uberis* (14.4%), 4 *S. pyogenes* (4.4%) and two isolates of *S. pneumoniae* (2.2 %) were detected. Streptococci could not be isolated from California mastitis negative samples collected from the examined ewes.

Results of Antibiotic Sensitivity Test of *Streptococcus agalactiae* Isolated from Cows and Ewes: Fifteen *S. agalactiae* isolates recovered from the examined cows and ewes (each) were examined for their sensitivity to 9 antimicrobial agents. Table 3 showed that most of the tested isolates were sensitive to ampicillin, penicillin,

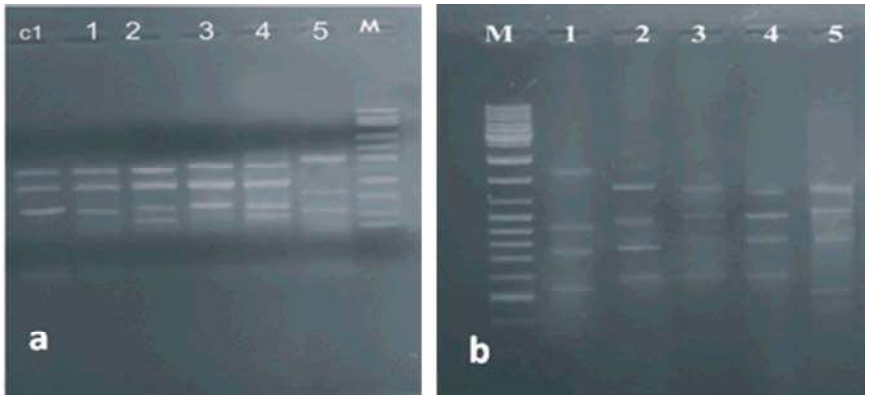

Fig. 2: RAPD –PCR profile analysis among the examined *S. agalactiae* isolates.
a): Lane (C) positive control for *S. agalactiae*, Lane (M) 100 bp marker 100-1000 DNA marker, Lanes (1, 2 and 3) cattle isolates and Lanes (4 and 5) sheep isolates. b): Lane (M) 100 bp marker 100-1000 DNA marker and Lanes (1 to 5) cattle isolates.

Table 3: *Streptococcus agalactiae* resistant isolates among cow and ewe samples

	Cow isolates (n:15)		Ewe isolates (n:15)	
Antimicrobial agents	Positive	%	Positive	%
Ampicillin	1	6.7	3	20
Cefotaxime	5	33.3	6	40
Chloramphenicol	9	60	9	60
Clindamycin	7	46.7	7	46.7
Erythromycin	6	40	7	46.7
Ofloxacin	6	40	7	46.7
Penicillin G	3	20	5	33.3
Tetracycline	8	53.3	8	53.3
Vancomycin	10	66.7	9	60

cefotaxime, erythromycin and ofloxacin. Meanwhile the examined isolates showed resistance to vancomycin, chloramphenicol and tetracycline.

Molecular Characterization of *Streptococcus agalactiae:* As shown in Figure 2 the used primers can amplify 3-4 bands from the used isolates.

Figure 2a illustrated that all tested isolates had bands between 494-428, 366-317 and 238-203 bp 2 isolates had a band between 168-123 bp and one isolates had a band at 307 bp.

From Figure 2b it is clear that the tested *S. agalactiae* isolated had amplified bands at 944-831, 723-626, 565-491 and 365-277 bp.

DISCUSSION

Mastitis, the most common infectious disease of dairy cows, is the most economically-important disease of dairy industries allover the world. Streptococci form a large group of organisms which are associated with bovine udder infections [18]. The most common pathogens cause bovine mastitis is *S. agalactiae*, *S. dysgalactiae* and *S. uberis* [19]. In the present study a total of 570 milk samples collected from cow (n=300) and ewes (n=270) were investigated for detection of streptococci. The apparently normal udder quarter samples from cows and ewes were screened for subclinical mastitis using California mastitis test.

As shown in tables 1 and 2, 120 cow milk samples (40%) and 115 ewe milk samples (42.6%) were positive for CMT. From all milk samples 0.01 ml of milk sediment was cultured on blood agar and Edward's agar. Bacterial growth was identified and recorded after 24 and 48 hour of incubation. A conventional scheme based on biochemical tests was used for identification of Streptococcus isolates from the collected milk samples of cows and ewes. Identification of streptococci are based on bacteriological examination of blood agar plates including the hemolysis, CAMP (the Christie, Atkins and Munch-Petersen) test and the lacking of ability to hydrolyze esculin [20]. Also, PCR was used for identification of streptococci (Figure 1).

Streptococcus species were isolated from cows (55 %) and ewes (50.4%) as shown in Tables 1 and 2. The organisms were isolated from cases of mastitis by other authors [21,22]. It is clear that the Streptococcus species, inhabiting the udder and causing mastitis. The transmission of mastitis from infected udder to healthy udder is through hands during milking processes and possibly flies [23]. Among sheep samples Streptococcus species were identified with a percentage of 50.4 % (Table 2). Mastitis has a major impact on both economy

and animal welfare in sheep production. It frequently causes damage to the affected glands leading to reduced milk yield and retarded growth of the lambs [24].

The most important streptococcal agents of bovine mastitis are *S. agalactiae*, *S. dysgalactiae* and *S. uberis*. *S. agalactiae* is a highly infectious pathogen that can rapidly spread among a herd from a single infected animal [19]. It is clear that, *S. agalactiae*, *S. dysgalactiae* *S. uberis, S. pyogenes* and *S. pneumoniae* were isolated from cow samples with incidence of 19.7, 17, 15.3, 2.7 and 0.7% respectively (Table 1). *S. dysgalactiae* and *S. uberis* are the most commonly isolated environmental streptococci of bovine mastitis.

As shown in Table 2 *S. agalactiae*, *S. dysgalactiae* *S. uberis, S. pyogenes* and *S. pneumoniae* were isolated from the examined sheep with incidence of 20.4, 15.9, 10.4, 2.6 and 1.1% respectively. The microbiological examination of milk sample from affected sheep quarters revealed the presence of *S. aureus*, *E. coli* and Streptococcus species [25]. *S. uberis* was isolated from sheep (10.4 %) and cows (15.3 %) as shown in Tables 1 and 2. It is known worldwide as an environmental pathogen responsible for a high proportion of cases of clinical and subclinical mastitis in lactating cows and is also the predominant organism isolated from mammary glands during the nonlactating period [26,27].

Also, Tables 1 and 2 illustrated that *S. dysgalactiae* was recorded among cow (17 %) and sheep (15.9 %) samples. It is a member of group C streptococci (GCS) can be isolated from udders of cows with mild mastitis and from blood and tissues of lambs with polyarthritis, an inflammation of the protective membrane covering the central nervous system [28,29].

S. agalactiae was isolated from cows and sheep milk samples with percentage of 19 % and 20.4% respectively as shown in Tables 1 and 2. *S. agalactiae* is a major cause of subclinical mastitis in dairy cattle and a source of economic losses for the industry [30]. For humans, *S. agalactiae* is responsible for severe invasive disease in adults and neonates, it has been estimated to have caused 16,880 human cases, including 1,650 deaths, in the United States alone in 1998 [31].

In the present investigation 30 *S. agalactiae* isolated from cows (n=15) and ewes (n=15) were tested for antibiotic resistance [16]. Gao *et al.* [32] characterized the antibiotic resistance patterns of *S. agalactiae* isolated from cows with mastitis in China. It is clear that *S. agalactiae* isolated from dairy cows and sheep was sensitive to ampicillin, penicillin and cefotaxime (Table 3). Moatamedi *et al.* [19] revealed susceptibility of streptococci to amoxi clavulanic acid, amoxicillin, sulbactam, followed by ciprofloxacin, enrofloxacin, cefotaxime then gentamicin, kanamycin and tetracycline.

Resistant to vancomycin (66.7 and 60%) chloramphenicol (60% each) tetracycline (53.4% each) and clindamycin (46.7% each) had been recorded among the examined S. *agalactiae* isolated from cows and ewes respectively (Table 3). Group B streptococcal infections are a leading cause of neonatal mortality and also affect pregnant women and the elderly. ß-lactam agents are the treatment of choice for these infections, but macrolides are useful alternative therapy for allergic patients. *S. agalactiae* is considered to be susceptible to ß- lactam antimicrobial agents, but the resistant strains to macrolides and tetracycline has been increasingly reported [33].

It is clear that 40 and 46.7 % of *S. agalactiae* isolated from cows and sheep were resistant to erythromycin respectively as shown in Table 3. The resistance mechanism of erythromycin in *S. agalactiae* depended upon target site modification and active drug efflux encoded by the *erm* and *mef* genes and caused resistance to macrolide compounds [33].

RAPD is a simple method based on the use of arbitrary primers to amplify DNA polymorphic segments and to detect the diversity among isolates. Limited information was available on the variety of *S. agalactiae* isolated from bovine milk. Previous studies on *S. agalactiae* isolates of human origin have suggested that RAPD is superior to serotyping for epidemiological evaluations of this pathogen [34]. In Martinez *et al*. [17] work,RAPD was used to study a large collection of bovine isolates from Canada and high genetic diversity was found. In the present study ten multi drug resistant *S. agalactiae* isolates (cow n=8 and ewe n=2) were typed by RAPD-PCR in comparison to the standard strain using opsll R ops16 primers [17]. As shown in Figure 2 the used primers can amplify 3-4 bands from the used isolates. RAPD typing conducted by Sukhnanand *et al*. [31] showed that *S. agalactiae* isolated from asymptomatic human and from bovines represented different subtypes; also they found one human isolate and one bovine isolate shared an identical RAPD type. A total of 35 different RAPD patterns were studied by Culebras *et al*. [33] among the 54 erythromycin-resistant *S. agalactiae* strains

It could be concluded that, *S. agalactiae* is an important cause of mastitis in cows and ewes in Egypt. *S. agalactiae* was sensitive to ampicillin, penicillin and cefotaxime so each of them is a drug of choice for treatment of *S. agalactiae* mastitic cows and ewes. The data indicated the existence of different RAPD patterns

among the multidrug resistant *S. agalactiae* isolates. Epidemiologically further study was needed to investigate the genetic diversity of *S. agalactiae* using a large number of isolates collected from different sources.

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