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Prevalence and Characterization of *Streptococcus pyogenes* Isolates From Buffalo Milk, Cattle Milk and Human Milkers. With M Protein Serotyping

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A TOTAL of 440 raw milk samples (apparently normal) were collected from different animal farms at Sohag governorate (368from cows and 72 from buffaloes) and 55 throat swap samples collected from human milkers. All the *Streptococcus pyogenes* (*S.pyogenes)* isolates were identified by bacitracin sensitivity test, PCRdetection targeting *spy1258* gene, and positive group A (GAS) Lancefield agglutination kit. The isolates from different sources had been typed serologicaly for M protein typing. Five different serotypes, M1, M2, M3, M4, and M6, were found to be indicated in different *S.pyogenes* isolates, while 30 isolates found to be untypeable. Some isolates were underwent Polymerase Chain Reaction (*spy1258*) for confirmation and comparison between animal and human isolated strains. In addition antibiogram resistance was determined for eight different antibiotics. The mechanisms of the resistance were identified phenotypically by the disc diffusion method. Resistance testing indicated highly resistance for mostly used antibiotics as erythromycin and clindamycin and found to be complete or inter mediate sensitive to penicillin-G, cefuroxime, ampicillin /sulbactam and cefoperazone. *S. pyogenes* are not only associated with human infection only but it could be transmitted to animals . According to M protein serotyping M6 is the most predominant among animal and human isolates.

Keywords: Beta haemolytic streptococci, Group (A) streptococci, Reverse zoonosis.

Introduction

Different species of streptococci are responsible for a variety of many bacterial diseases in both animals and humans. Some examples of those conditions in human like arthritis, meningitis, neonatal sepsis, and pneumonia. While in animals they causes mainly mastitis (clinical and sub clinical) [1]. *S.pyogenes* is identified as group (A) streptococci (GAS). It has (N- acetyl glucosamine) linked with rhamanose polymer [2]. *S.pyogenes* could cause infection in humans through colonization after adhesion to mucosal surface epithelial cells lined to the host upper respiratory system [3]. Also pathogenicity of *S.pyogenes* is clear in other mild infectious conditions as in pharyngitis and mild skin infections. As in severe conditions like in Streptococcal toxic shock syndrome and in necrotizing fasciitis. Rate of infection annually found to be more than 600 million infections [4]. Many virulence factors were found to be associated with GAS infection severity. It includes [Streptolysins (O-S)- Streptokinase-Streptodornase- M protein-Hyaluronidase- Hyaluronic acid in the capsule-cysteine protease- super- antigen proteins and several phage-encoded exotoxins]. The M-like protein is referring to surface protein which structurally resembles to the M protein [5]. Virulence factors found to be distributed equally within *S.pyogenes* structure as some are encoded within chromosomes. While others found on amobile genetic elements. Detection the presence

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of those virulence factors considered as a simple method of a rapid clinical diagnosis [6]. M protein considered as the most important virulence factor in which could be used in classification of *S.pyogenes* serotypically. [7]

The aim of our study is the identification of *S.pyogenes* isolates from the di□erent sources . To detect the antibiogram susceptibility of this organism. Furthermore this study aimed to classify the isolates serotypically attempting to find the relation and the possibility of reverse zoonosis between animal isolates and human isolates depending on the M protein serotyping.

Material and Methods

This study was conducted among samples collected from cows and buffalos milk (440) and throat swabs from milkers(55) in Egypt-Sohag governorate.

California Mastitis test (CMT) .Was performed by the method recommended by (APHA, 1992). The mastitis indicator kit had been obtained from [Impfstoff work Friesoythe Gmbh-Germany].

Collection of samples [8]. Within the period from January 2018 to June 2019, A total of 440 raw milk samples (mastitic and apparently normal) were collected from different animal farms at Sohag governorate ,Egypt (368from cows and 72 from buffaloes) and 55 throat swap samples collected from human milkers. As a result, 75 isolates of animal source and 25 isolates of human source were identified as β-haemolytic streptococci on sheep blood agar and by Gram staining (Fig.1), blood haemolysis (Fig. 2), Lancefield grouping agglutination strep-check kit (Fig.3) and a negative catalase production test . After the identification of the isolates they were cultured on the brain heart infusion broth and stored in 50% glycerol stock in -80º until being used. A reference strains (ATCC19615) had been obtained from the Microbiological Resource Canters MIRCENS (Faculty of Agriculture- Ain Shams University-Egypt) which used as positive control strain.

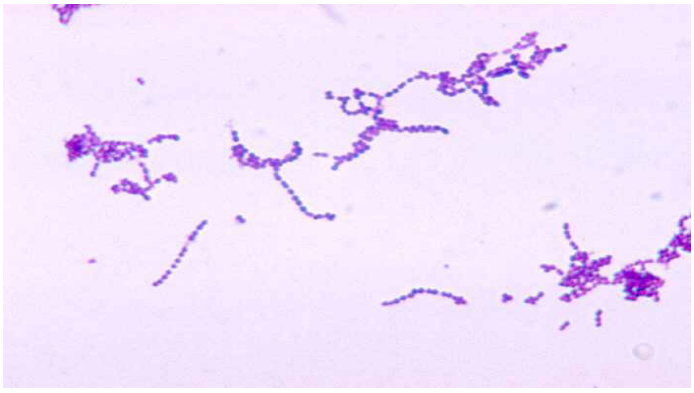

Fig. 1. *S. pyogenes* with Gram stain.

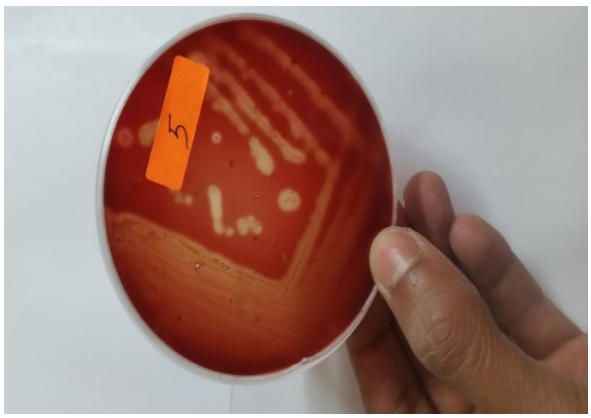

Fig.2. ß- Hemolytic strains of *S. pyogenes* on blood agar.

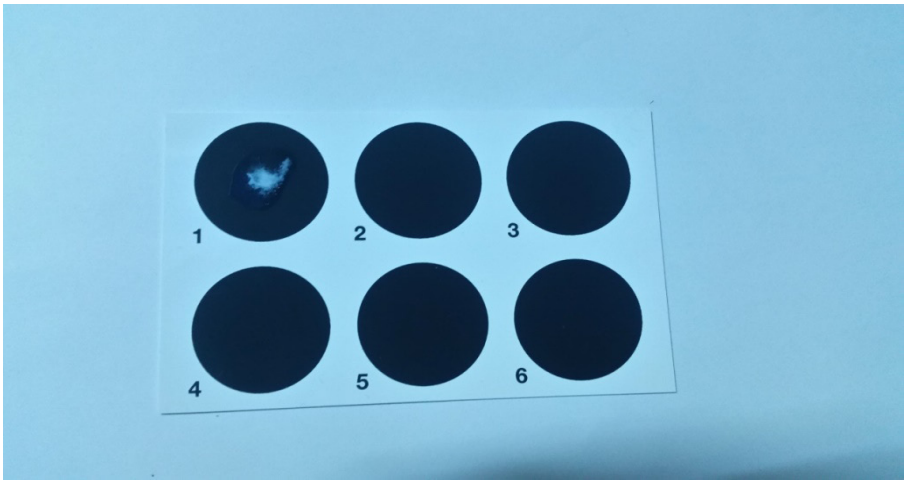

Fig. 3. Positive *S. pyogenes* agglutination with STREP-CHECK KIT.

Identification of *Streptococcus pyogenes* Isolates Phenotypically. Bacitracin sensitivity test had been performed in Abraham and Sistla method [9]. With adjusting the optical density (OD) of the overnight incubated culture to about 10^6 CFU/ml then spreaded with asterile swab on a blood agar plate and followed by bacitracin disc placing on the each plate center. After the overnight incubation the inhibition zone diameter was measured with a suitable measuring tool.

Antimicrobial (antibiogram) Sensitivity of the Isolates. All the identified *S.pyogenes* isolates from both animal and human origin in addition to the standard strains (ATTCC19615) had been subjected to antibiogram sensitivity test by using the method of (Kirby-Bauer) antibiotic disc diffusion [10] .with the standers of CLSI 2015 [11]. in this study eight different antibiotics had been used which are bacitracin, penicillin G, tetracycline, erythromycin, clindamycin ,cefuroxime, amoxicillin /sulbactam and cefoperazone (Fig.4).

Isolates Serotyping. In acapillary tubes glass aspecific antisera to different M-protein types (UMN laboratory) (M1-M2-M3-M4-M6) were used to determine strains serotype according to Johnson et al [12].

Molecular Detection of *S. pyogenes*. The chromosomal DNA Extraction and detection of *Spy1258* gene using PCR. Using bacterial specific DNA isolation kit DNA was extracted [Gene JET Genomic DNA Purification kit (k0721)] under the instructions of the manufacturer. For detection of *S. pyogenes* using *Spy1258* primers listed in Table 1. Preparing a 25 μl of the reaction volume by mixing 12.5 μl of master mix [My Red TaqMix – Bioline - United King] . A1 μl (0.5 μM) from each of forward and reverse primers of the tested gene and 3 μl of DNA template and nuclease free water. Negative control was prepared by using nuclease free water. (PCR) reactions were performed using Cycler 003 PCR Machine [A & E Lab (UK)]. With the conditions of 2 min of initial denaturation at 94°C. 35 cycles of denaturation (30 s at 94°C). annealing (30 s) extension (60 s at 72°C). With final extension at 72°C for 10 min. (PCR) products were analyzed by electrophoresis using 1% agarose gel. Finally stained with ethidium bromide to be visualized under UV light tool (Fig.5).

Results

California Mastitis test. Out of 368 of examined apparently normal cows quarter milk samples 175 (47.55%) reacted positively to CMT, while 193(52.45%) quarter milk samples reacted negatively to CMT (Fig.6).

Out of 72 of examined apparently normal cows quarter milk samples 25(34.72%) reacted positively to CMT, while 47 (65.28%) quarter milk samples reacted negatively to CMT (Fig.7).

Collection, Isolation and Identification of different sources Isolates. In this study out of 440 animal milk samples, 75 isolates were identified and out of 55 human throat swap isolates 25 isolates were identified as *Streptococcus pyogenes* (Fig.3). The isolated colonies founded to be small grey beta-hemolytic (Fig. 2). Negative catalase production test using tube method . Examination under light microscope reveal that the isolated colonies contain violet ovoidal and coccidal

bacteria which was arranged in pairs or chains (Fig. 1).Also founded that out of 495 animal and human specimens, 100 (20.2%) isolates had been identified using bacitracin sensitivity test, positive Lancefield's latex agglutination kit (fig 3). Positive (PCR) product for *spy1258* gene (Fig.5) & (Table1). According to the sources of *S. pyogenes* isolates founded that 75 (17%) isolates were from milk (Fig. 8) and 25 (45.5%) isolates were from milkers throat swabs (Fig. 9).

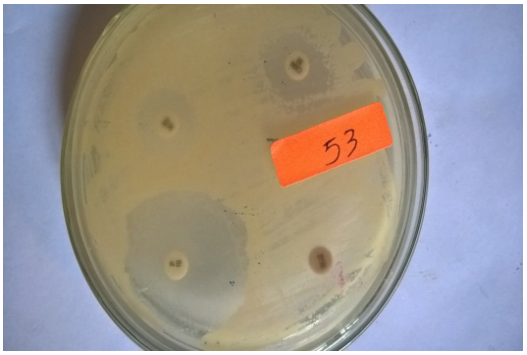

Fig. 4. Sensitivity test of *S. pyogenes* isolates according to the diameter of inhibition zone.

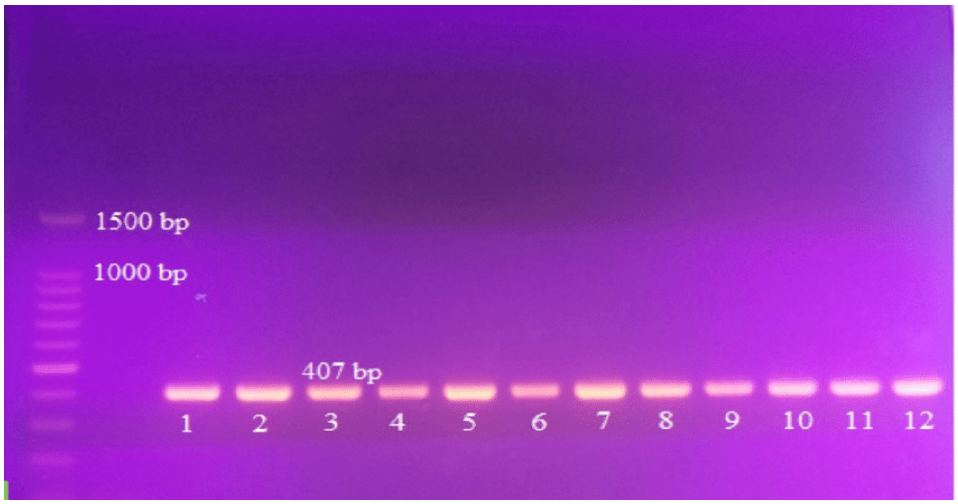

Fig. 5. Gel-electrophoresis for PCR product of SPY1258 gene.

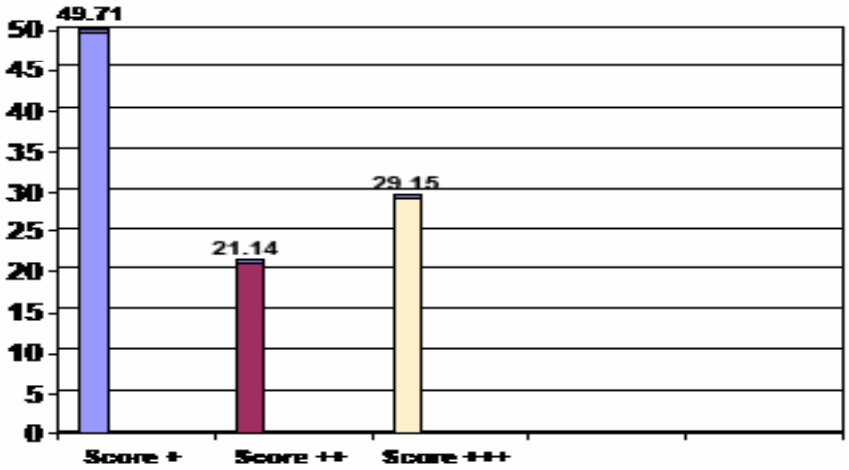

Fig. 6. Illustration of mastitis scores by using CMT in cow quarter milk samples.

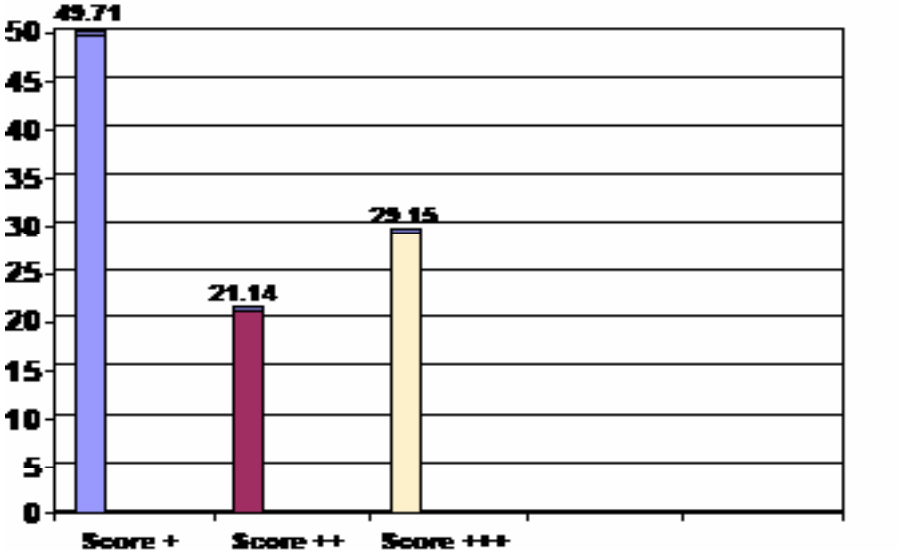

Fig. 7. Illustration of mastitis scores by using CMT in cow quarter milk samples.

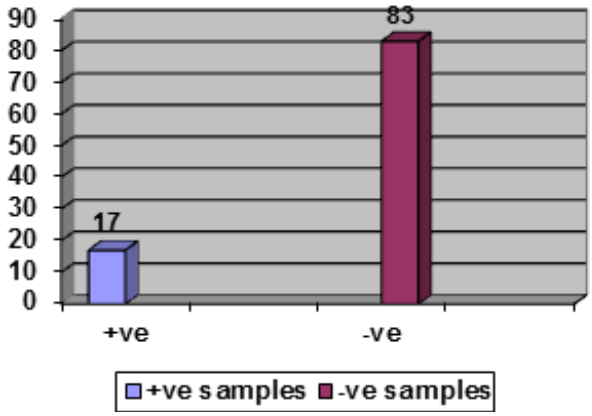

Fig. 8. Prevalence of *S. pyogenes* in examined quarter milk samples.

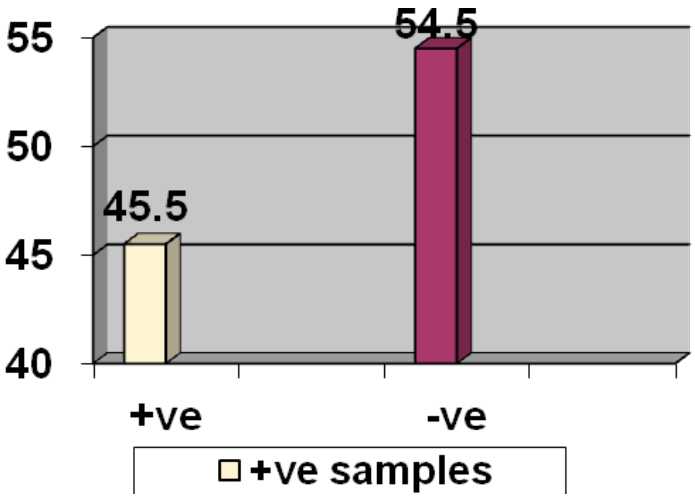

Fig. 9. Illustrated Prevalence of *S. Pyogenes* in examined throat swap samples.

TABLE 1. (PCR) primers Sequences of (SPY1258) gene.

Gene	The Primer	Sequence	Product length
(SPY1258)	SPY1258 (F)	5' AAAGACCGCCTTAACCACCT3'	407bp
	SPY1258 (R)	5' TGCCAAGGTAAACTTCTAAAGCA 3'	

Serological M Protein Typing. From the milk samples 61 (81.3%) were classified into five serotypes; however, the remaining 14 (18.7%) founded to be untypeable. M6 was the most commonly identified serotype 29 (38.67%) followed by M1 10 (13.3%) and M3 8 (10.67%) serotypes. M2 and M4 7 (9.33%) for each were founded to be the least identified serotypes.

From the milkers throat swabs samples 23 (92%) were classified into five serotypes. However the remaining 2 (8%) founded to be untypeable. M6 was the most commonly identified serotype 10 (40%). Followed by M1 and M4 4 (16%) for each, M3 3 (12%) and M2 2 (8%) serotypes (Table2).

From the obtained results we found no significant difference had been detected in the distribution of M protein serotypes between isolates obtained from bovine milk and milkers throat swabs as a sources.

Antibiogram(antibiotic) Sensitivity Testing. Antibiotics sensitivity was determined by disc diffusion method under the standards conducted by CLSI [11]. In our study different isolates were classified into sensitive, intermediate or resistant. *S. pyogenes* pattern of sensitivity shown that all the *S. pyogenes* isolates were sensitive to penicillin G, cefuroxime, ampicillin /sulbactam and cefoperazone and resistant to erythromycin and clindamycin. No significant difference was found between animal and human isolates resistance pattern regarding to isolates source.

Discussions

In this study Out of 368 of examined apparently normal cows quarter milk samples 175 (47.55%) reacted positively to CMT, while 193(52.46%) quarter milk samples reacted negatively to CMT .Out of 72 of examined apparently normal cows quarter milk samples 25(34.72%) reacted positively to CMT, while 47 (65.28%) quarter milk samples reacted negatively to CMT .

S. pyogenes is a beta-haemolytic(GAS) which is associated with human upper respiratory tract and other soft tissues infections [13–14]. We can identified *S. pyogenes* isolates by using bacitracin sensitivity test [15]. Also other more sensitive methods for identification had been used as (GAS) latex agglutination kit and (PCR) for (*spy 1258*) gene [9, 16]. In our study by using the most recent applicable techniques 75 isolates out of 440 (17%) milk samples were identified as *S. pyogenes*. avdout of 55 human throat swabs samples 25 (45.5%) found to be identified as *S. pyogenes*. The results showed higher incidence rate of *S. pyogenes* infections comparing with the incidence rate detected in India that only 160 *S. pyogenes* isolates had been detected among 34065 clinical samples (0.0047%) [9]. Despite of *S. pyogenes* annual incidence of infections is varied greatly. Ranged between (0.029) and (2.84%) per (100,000) populations [17].

In our study results antibiotic sensitivity testing reveal high sensitivity (100%) of all isolates for amoxicillin/clavulanic acid and penicillin G. A similar observation had been reported in Pakistan [18] . While lower sensitivity level (81%) had been reported in Egypt [19].

Ceftriaxone sensitivity was observed in all the isolates as mentioned by Camara et al [20]. And Vil.lasenor et al [21] reported intermediate sensitivity for ceftriaxone. Variable results were observed in Egypt and Iran where lower resistance rate had been observed (50% and 12%) respectively [19, 22]. Also in our study we found complete resistance to erythromycin and clindamycin. The increased level of bacterial resistance to different antibiotics reveals the modification patterns of bacterial sensitivity for the mostly and frequently used antibiotics [23].

TABLE 2. M protein serotyping of bovine and human isolates.

M protein serotype	Bovine samples	%	Milkers samples	%	total	%
M1	10	13.3%	4	16%	14	14%
M2	7	9.33%	2	8%	9	9%
M3	8	10.67%	3	12%	11	11%
M4	7	9.33%	4	16%	11	11%
M6	29	38.67%	10	40%	39	39%
Un-typeable	14	18.67%	2	8%	16	16%
Total	75		25		100	

In this study M protein serotyping shown that a higher prevalence rate of M6 serotype was identified in 40% of all the *S. pyogenes* isolates (animal and human isolates) as it was the first Gram-positive surface protein to be completely sequenced. In contrast to other observation which had been reported in a previous study in UK stated that M1 is the most predominant [24]. In our study no significant difference between percentage of M protein serotypes percentage in animals and human isolates as in animals isolates was (13.3% - 9.33% - 10.67 % - 9.33 % - 18.67%) for (M1-M2-M3-M4-M6) respectively. While in human isolates is (16 % - 8% - 12% - 16% - 40%) for (M1-M2-M3-M4-M6) respectively.

Conclusions

S. pyogenes are not only associated with human infection only but it could be transmitted to animals causing sub-acute mastitis and re-secreted in milk with reverse zoonosis mechanism causing spread of infection to other human hosts. *S. pyogenes* is found to be resistant to many common used antibiotics and its resistance is developed day by day. According to M protein serotyping M6 is the most predominant among animal and human isolates which may refers to a common source of infection.

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Authors contributions

All authors had been contribute to this work equally.

Conflicts of Interest

No conflicts of interest for publication of this paper.

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انتشار وتوصيف الميكروب السبحى المتقيح المعزول من لبن الجاموس ولبن الماشية والحلابين مع نمذجة بروتين إم.

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[٢] قسم الميكروبيولوجيا والمناعة - كلية الطب البيطرى - جامعة القاهرة - القاهرة - مصر.

[٣] قسم الرقابة الصحية على الألبان - كلية الطب البيطرى - جامعة القاهرة - القاهرة - مصر.

يعتبر الميكروب السبحى المتقيح (المجموعة أ)واحد من أكثر الميكروبات الممرضة شيوعا للإنسان. تقدر نسبة البشر الطبيعيين الذين يحملون الميكروب بحوالي (5-51%), خصوصا فى الجهاز التنفسى بدون ظهور علامات المرض. بإعتباره من الميكروبات التى تتواجد بشكل طبيعي داخل الجسم الميكروب السبحى المتقيح حيث يمكنه ان يسبب العدوي عندما يحدث اختلال للجهاز المناعى او تتواجد القدرة للميكروب على اختراق مستويات الحماية المختلفة لأنسجة الجسم. فى القرن الماضى اصبح هذا الميكروب مهددا للعديد من البشر حيث ان الاصابة به تسبب حمى ما بعد الولادة (حمى النفاس) والحمى القرمزية والتهاب الحلق. قد يصاب المرضى أيضًا بمضاعفات ما بعد المكورات السبحية المناعية ، مثل الحمى الروماتيزمية الحادة والتهاب الكلى الحاد ، وذلك بعد العدوى الحادة التي تسببها المكورات السبحية المتقيحة.فى هذه الدراسة تم جمع 044 عينة من اللبن الخام (طبيعي ظاهريا) من مزارع حيوانية مختلفة في محافظة سوهاج (863 من الأبقار و 27 من الجاموس) و 55 عينة مسحة من الحلق جمعت من الحلابين. تم التعرف على جميع عزلات الميكروب السبحى المتقيح عن طريق اختبار حساسية nicarticab ، واكتشاف RCP للجين (8521yps)، واختبار (dlefiecnaL). تم تصنيف العزلات المأخوذة من مصادر مختلفة من حيث التصنيف المصلي لنوع البروتين M. تم تحديد خمسة أنماط مصلية مختلفة ، 1M ، 2M ، 3M ، 4M ، 6M في عزلات مختلفة من senegoyp.S ، بينما وجد أن 03 عزلة غيرخاضعة للتصنيف. خضعت بعض العزلات لتفاعل البلمرة المتسلسل (8521yps) للتأكيد والمقارنة بين السلالات المعزولة الحيوانية والبشرية. بالإضافة إلى ذلك ، تم تحديد مقاومة المضادات الحيوية لثمانية مضادات حيوية مختلفة. تم تحديد آليات المقاومة ظاهريًا بواسطة طريقة توزيع القرص. أشار اختبار المقاومة إلى وجود مقاومة عالية للمضادات الحيوية المستخدمة في الغالب مثل الإريثروميسين والكليندامايسين ووجد أنها كاملة أو متوسطة الحساسيةة للبنسلين-جي ، سيفوروكسيم ، أمبيسيلين / سولباكتام وسيفوبيرازون.

الكلمات الدالة: الميكروبات السبحية من المجموعة أ ، الأمراض الحيوانية عكسية المنشأ ، الميكروبات السبحية الحالة للدم من النوع بيتا.