Original Article

Biochemical and serological characterisation of beta-haemolytic streptococci from various clinical samples in a tertiary care hospital, South India

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Abstract

Introduction: Beta-haemolytic streptococci (BHS) are one of the most frequent human pathogens capable of producing a variety of diseases ranging from pharyngitis, impetigo, to more severe and life-threatening diseases such as toxic shock syndrome, neonatal sepsis, pneumonia, meningitis and acute rheumatic fever.

Methods: This was a hospital-based prospective study which was carried out on BHS isolated from various clinical specimens submitted in microbiology laboratory from inpatients and outpatients, and these isolates were studied biochemically by pyrrolidonyl arylamidase (PYR), hippurate hydrolysis and sugar fermentation tests. Differentiation of various serogroups was done with bacitracin (0.04 U), co-trimoxazole (sulphamethoxazole-trimethoprim [23.75/1.25 mcg]) and penicillin (10 units/disc) discs (n = 220). The isolates were identified serologically by Hi Strep™ Latex agglutination test Kit (Himedia).

Results: The findings showed that the highest percentage of streptococci isolated was from throat swabs (35.5%), followed by sputum (15.9%), urine (14.1%), blood (10.5%), pus (8.6%), cerebrospinal fluid (6.4%), bronchoalveolar lavage (5.9%) and endotracheal tips (3.2%). The highest percentage of BHS belongs to Group C (74, 33.6%), followed by Group G (51, 23.2%), Group B (42, 19.1%), Group F (28, 12.7%), Group A (21, 9.5%) and Group D (4, 1.8%).

Conclusions: Rapid identification and consequent prompt treatment of patients with BHS can reduce the risk of spread and aid in the prevention of infections and their serious complications, such as rheumatic fever/rheumatic heart disease, nephritis and local or systemic infections.

Keywords: Endocarditis, enterococcus, latex agglutination test, neonatal septicaemia, *Streptococcus*

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INTRODUCTION

Beta-haemolytic streptococci (BHS) are one of the most frequent human pathogens capable of producing a variety of diseases ranging from pharyngitis, impetigo, to more severe and life-threatening diseases such as toxic shock syndrome, neonatal sepsis, pneumonia, meningitis and acute rheumatic fever.[1] Group A haemolytic streptococci (GAS) are one of the most frequent human pathogens capable of producing

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a variety of diseases. Group B BHS (GBS) cause sepsis, meningitis, osteomyelitis and respiratory tract infections in neonates.[2] Group C streptococci (GCS) and Group G streptococci (GGS) resemble GAS, but cause a number of different diseases in animals such as horses, cows, cats, dogs and pigs.[3] Although GCS and GGS had been regarded as weak pathogenic bacteria, it has been reported in recent years that these streptococci can also cause streptococcal toxic shock syndrome similar to GAS.[4] Their ability to haemolyse red blood cells to various degrees is an important basis of classification.[5]

Beta-haemolytic, bacitracin-susceptible streptococci are presumptively identified as group A. BHS that are bacitracin resistant are presumptively identified as groups B and non-A non-B streptococci.

In the present study, we tried to further classify BHS based on biochemical reactions and serotyping and also studied the antimicrobial susceptibility patterns of the different groups of isolated BHS.

MATERIAL AND METHODS

This was a hospital-based prospective study which was carried out on BHS isolated from various clinical specimens submitted in microbiology laboratory from inpatients and outpatients during 1-year period from March 2016 to March 2017. A total of 220 BHS were isolated and included during the study.

All clinical specimens were included, but for sputum samples, Bartlett's grading system was applied. Patients already on antibiotic therapy and dry throat swabs were excluded from the study. Preparation and procedure of media required for the isolation of BHS were followed as per the standard suggested protocol.[6]

For primary isolation, all the samples were streaked onto double-layered blood agar plates, and the inoculated plates were incubated at 37°C in an incubator. The colonies were observed for beta haemolysis after 24 h–48 h of incubation. Gram stain, catalase test and oxidase test were performed for the primary identification of BHS.

Pyrrolidonyl arylamidase (PYR) test plays an important role in the differentiation and detection of Lancefield group A from other non-A non-B serological groups.[7] The test requires PYR agar (HiMedia, catalogue number M1489-500G), and the media was prepared according to the standard suggested protocol.

Presumptively identified beta-haemolytic colonies were subjected to antibiotic susceptibility test on double-layered blood agar plates by Kirby–Bauer disc diffusion method as per the Clinical and Laboratory Standards Institute (CLSI) guidelines 2012,[8] and the antibiotic discs used were bacitracin (0.04 U), co-trimoxazole (sulphamethoxazole-trimethoprim [23.75/1.25 μg]) and penicillin (10 units/disc) for the differentiation of various serogroups from the isolated BHS. The plates were examined after overnight incubation. Zone diameter was measured in millimetres using callipers or a ruler. They were then compared with the CLSI published guidelines. The strains were designated sensitive or resistant according to the CLSI guidelines, 2012.[8-11]

The biochemical tests which helped in the presumptive differentiation of various serogroups of BHS were PYR test, hippurate hydrolysis, bile aesculin, 6.5% NaCl, methyl red and Voges–Proskauer (VP) tests, arginine hydrolysis and fermentation of sugars such as glucose, lactose, sorbitol, trehalose, salicin and ribose.[6]

Definitive identification and confirmation of Lancefield's group was done using Hi Strep™ Latex agglutination Kit [Figures 1-5].

Figure 1: Beta-haemolysis on double-layered agar

Figure 2: Identification of Group A streptococcus by pyrrolidonyl arylamidase test with appearance of red-coloured colonies

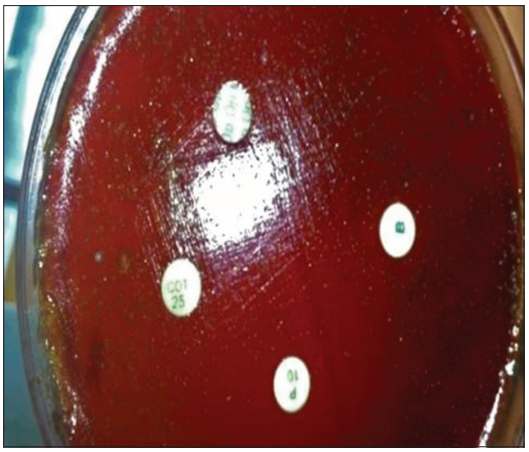

Figure 3: Antibiotic susceptibility test with bacitracin, co-trimoxazole and penicillin discs

Latex agglutination test

Latex particles in the Hi Strep™ Latex Test Kit were individually sensitised with rabbit antibodies specific to one of the streptococcal carbohydrate antigens of groups A, B, C, D, F and G. Streptococcal colonies from culture plates were incubated in the enzyme solution to extract the antigen. The extract/antigen was tested on a reaction card against six suspensions of antibody-coated latex particles each specific to one of the groups A, B, C, D, F and G. In the presence of homologous antigen, particles in one of the suspensions will aggregate to give visible agglutination in contrast to the other suspensions which will remain unagglutinated. Interpretation of the results was done based on the strength of agglutination.[12]

Figure 4: Biochemical identification of different serogroups

Figure 5: Definitive identification of various serological groups by Hi Strep™ Latex Agglutination Test Kit (A, B, C, D, F and G)

RESULTS

Streptococci are significant human pathogens causing a wide spectrum of disease, ranging from uncomplicated infections to serious life-threatening diseases. In this study, we concentrated on 220 BHS to know their prevalence in the pathogenesis of various clinical diseases. Other isolates such as Gram-positive cocci (*Staphylococcus, Pneumococcus*, etc.), Gram-negative bacilli (members of family *Enterobacteriaceae*), Gram-positive bacilli and other isolates were not included in the present study.

All BHS when observed individually under microscope were arranged in pairs and in short chains, and all were catalase and oxidase negative.

The highest percentage of streptococci isolated was from throat swabs – 35.5%, followed by sputum – 15.9%, urine – 14.1%, blood – 10.4%, pus – 8.6%, cerebrospinal fluid (CSF) – 6.7%, bronchoalveolar lavage (BAL) – 5.9% and endotracheal tips (ET tips) – 3.1% [Figure 6]. There was a male preponderance of 67.3% among the isolated 220 BHS [Figure 7]. Biochemical tests and antibiotic susceptibility were performed for all the BHS isolates [Table 1].

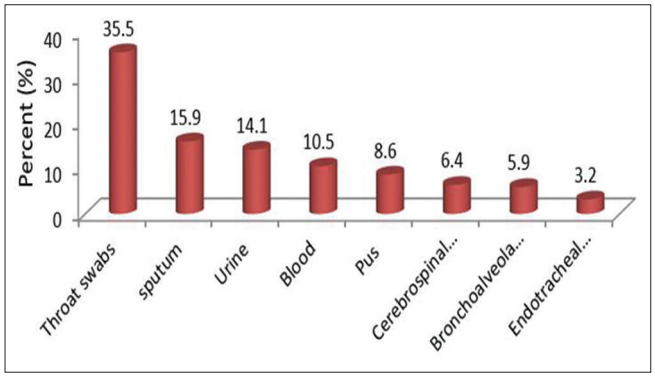

Figure 6: Percentage of distribution of BHS in various clinical samples (*n*=220). BHS=Beta-haemolytic streptococci; CSF= Cerebrospinal fluid; BALF= Bronchoalveolar lavage fluid

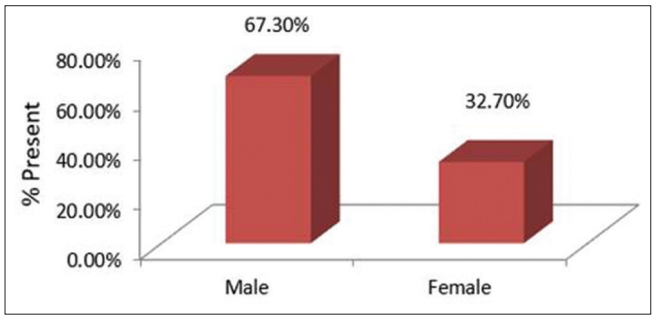

Figure 7: Gender-wise distribution of isolated beta-haemolytic streptococci (*n* = 220)

Table 1: Classification of serogroups of beta-haemolytic streptococci (*n*=220) based on bacitracin, co-trimoxazole and penicillin-susceptibility as per the Clinical and Laboratory Standards Institute guidelines (2012)[8]

Serogroup	Antibiotic disc and concentration					
	Bacitracin (0.04 U/disc)		Co-trimoxazole SXT/TMP (23.75/1.25 μg)		Penicillin (10 U/disc)	
	S	R	S	R	S	R
Group A	21	-	-	21	21	-
Group B		42	-	42	42	-
Group D	-	4	4	-	4	-
Non-A non-B	153		153	-	153	-

SXT/TMP=Sulfphamethoxazole/trimethoprim; S=Sensitive; R=Resistant

Among the 220 BHS isolates in this study, we found that 174 (79.1%) isolates were sensitive to bacitracin and 46 (20.9%) were resistant to bacitracin, with the highest percentage of sensitivity being non-A non-B group 153 (87.9%), followed by GAS 21 (12.1%). Among the 153 isolates of non-A non-B group, GCS 74 (48.4%), followed by GGS 51 (33.3%) and Group F streptococci (GFS) 28 (12.7%). Among the 46 (20.9%) isolates which were resistant to bacitracin, 42 (91.3%) were GBS and 4 (8.7%) were Group D streptococci (GDS) [Table 2].

Table 2: Bacitracin susceptibility among 220 beta-haemolytic streptococci isolates

Name of the serogroup	No.	Number of isolates and percentage of sensitivity and resistance Bacitracin (0.04 U)	
		S (174 [79.1%])	R (46 [20.9%])
Group A	21	21 (12.1)	-
Group B	42	-	42 (91.3)
Group D	4	-	4 (8.7)
Non-A non-B	153	153 (87.9)	-
Group C	74	74 (48.4)	-
Group F	28	28 (18.3)	-
Group G	51	51 (33.3)	-

S=Sensitive; R=Resistant

Of the 220 isolates in the study, 157 (71.4%) were sensitive to co-trimoxazole, with the highest percentage of sensitivity being in non-A non-B group 153 (97.5%), followed by GDS 4 (2.5%). GCS 74 (48.4%) of non-A non-B group has shown the highest percentage of sensitivity to co-trimoxazole, followed by GGS 51 (33.3%) and GFS 28 (18.3%) isolates of non-A non-B group. Sixty-three (43.8%) isolates have shown resistance to co-trimoxazole, of which, GBS 42 contributed 66.6% followed by GAS 21 (33.3%) [Table 3].

Hence, all bacitracin-sensitive isolates are not Group A. Hence, biochemical tests were performed for better identification of Groups C, F, G etc., as these are equally invasive as Group A, which is the highlight point in our study. We have also performed PYR test which has

Table 3: Co-trimoxazole susceptibility among 220 beta-haemolytic streptococci isolates

Name of the serogroup	No.	Number of isolates and percentage of sensitivity and resistance Co-trimoxazole SXT/TMP (23.8/1.3 μg)	
		S (157 [71.4%])	R (63 [28.6%])
Group A	21	-	21 (33.3)
Group B	42	-	42 (66.7)
Group D	4	4 (2.5)	-
Non-A non-B	153	153 (97.5)	-
Group C	74	74 (48.4)	-
Group F	28	28 (18.3)	-
Group G	51	51 (33.3)	-

S=Sensitive; R=Resistant; SXT=Sulfphamethoxazole; TMP=Trimethoprim

confirmed the isolate as GAS, as it is the only BHS that gives positive PYR reaction and helps to differentiate from other BHS groups.

Twenty-one bacitracin-resistant isolates were PYR positive and were presumptively identified as GAS. Forty-two isolates were positive for hippurate hydrolysis and VP test, and hence presumptively identified as GBS. Four isolates were bile aesculin positive and did not grow in 6.5% NaCl. These isolates were presumptively identified as GDS other than *Enterococci*. A total of 153 isolates showed variable biochemical patterns, and hence presumptively identified as non-A non-B streptococci based on variable sugar fermentation tests (ribose, salicin, trehalose and sorbitol) and arginine dehydrolase test [Table 4].

The group specific BHS which were presumptively identified by antibiotic susceptibility tests and biochemical tests, were then identified serologically based on latex agglutination test as a definitive identification method. It has been shown that the highest percentage of streptococci belongs to GCS showing 33.6%, followed by GGS 23.2%, GBS 19.1%, GFS 12.7%, GAS 9.5% and GDS 1.8% [Figure 8].

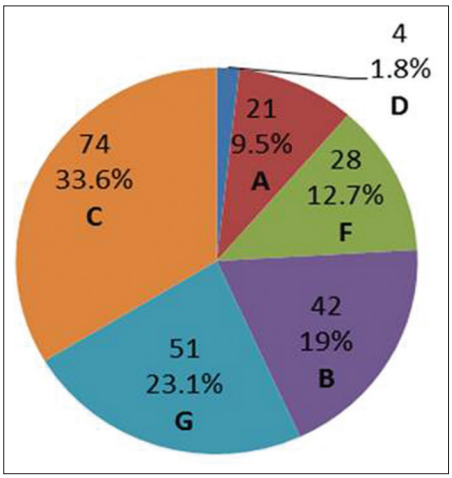

Figure 8: Distribution of various serogroups by latex agglutination test (*n* = 220).
A,B,C,D,F,G=Serogroup

Among the 74 isolates of GCS, 46 isolates had fermented salicin, ribose, glucose, lactose and sorbitol with acid production and were speciated as *Streptococcus dysgalactiae*. Twenty-eight isolates fermented ribose, glucose, lactose and sorbitol and did not ferment salicin. These isolates were speciated as *Streptococcus equisimilis*.

The highest percentage of streptococci isolated was from throat swabs 35.5%, followed by sputum 15.9%,

Table 4: Biochemical differentiation of serogroups among isolated beta-haemolytic streptococci (*n*=220)

Serogroup	Number of isolates	Biochemical test	
		Positive	Negative
Group A	21	PYR, fermented glucose, lactose, salicin and trehalose with acid production	Ribose, sorbitol, 6.5% NaCl, B/E, Hippurate, MR, VP, arginine dehydrolase
Group B	42	Hippurate, VP, fermented glucose, lactose, trehalose and ribose salicin with acid production	No growth in 6.5% NaCl, B/E, sorbitol, MR, arginine dehydrolase and PYR
Group D	4	B/E, fermented glucose and lactose with acid production	6.5% NaCl did not ferment ribose, sorbitol, trehalose, salicin. Hippurate, MR, VP, arginine dehydrolase, PYR
Group non-A non-B (n=153)			
Group C	74	Fermented ribose, glucose, lactose and trehalose with acid production. Forty-six among 74 isolates fermented salicin and sorbitol with acid production	Twenty-eight isolates among 74 isolates did not ferment salicin and sorbitol No growth in 6.5% NaCl and B/E Hippurate, PYR MR, VP, arginine dehydrolase
Group F	28	Fermented ribose, glucose with acid production	Salicin, trehalose, lactose, sorbitol, MR, VP, 6.5% NaCl, B/E, hippurate and PYR
Group G	51	Fermented ribose, glucose, lactose and trehalose with acid production	Did not ferment salicin, sorbitol. No growth in 6.5% NaCl, B/E MR, VP, arginine dehydrolase Hippurate and PYR

NaCl=Sodium chloride; B/E=Bile aesculin; PYR=Pyrrolidonyl arylamidase; MR=Methyl red; VP=Voges-Proskauer's test

urine 14.1%, blood 10.5%, pus 8.6%, CSF 6.4%, BAL 5.9% and ET tips 3.2% [Table 5].

Table 5: Sample-wise distribution of various serogroups based on latex agglutination test

Lancefield group	Throat swabs	Sputum	Pus	BAL	CSF	Urine	Blood	ET tips	Total
Group A	6	4	1	1	2	3	3	1	21
Group B	15	7	2	6	4	3	3	2	42
Group C	25	11	8	2	5	14	7	2	74
Group D	4	0	0	0	0	0	0	0	4
Group F	8	3	4	2	2	3	5	1	28
Group G	20	10	4	2	1	8	5	1	51
Total	78	35	19	13	14	31	23	7	220

BAL=Bronchoalveolar lavage; CSF=Cerebrospinal fluid; ET=Endotracheal tip

DISCUSSION

The classification of BHS which was initially based on haemolytic and biochemical properties now requires serogrouping for definitive identification. This serotype analysis, first described by Lancefield, was based on the polysaccharide antigen present in the streptococcal cell wall. Lancefield demonstrated distinctive antigenic differences in the cell wall carbohydrates of the BHS.

Lancefield GAS and GBS are considered the major pathogenic BHS; other non-group A or B BHS that are frequently normal inhabitants of the oropharynx, skin and gastrointestinal and genitourinary tracts are also capable of causing significant disease.[13] These include groups C, G, F and L, of which the most common are groups G and C.[14] Several studies have reported an increasing incidence of disease due to beta-haemolytic GCS and GGS, which have surpassed GAS as the leading cause of invasive streptococcal infection in some medical centres.[15-17]

In this study, 220 BHS were distinctively categorised into specific groups using Hi Strep™ Latex Agglutination Test Kit. Coagglutination is, therefore, a suitable method to be used routinely in the place of bacitracin screening. Use of coagglutination eliminates the problems of standardisation and interpretation inherent in disc diffusion tests such as the bacitracin test.[18]

By latex agglutination test, our findings of GAS are similar to those of another study.[19] Different studies have quoted rates ranging from 2.8% to 40%.[20,21]

Our finding are saimiliar to another study[22] on serotyping of BHS and their distribution in clinical specimens, in which 300 BHS were isolated from various clinical specimens and have been serogrouped, in which BHS have been recovered from 10.6% of throat cultures, 8.7% of urine cultures and 7% of other cultures, of which GAS have been mostly isolated from throat cultures with a percentage of 71.9, whereas the mostly isolated serogroup from urinary specimens was GDS (73.7%). GBS have been isolated in 21.1% and 22.7% ratios from urinary tract and other specimens, respectively.

In the present study, among 220 BHS, GAS were isolated from 21 samples, the highest number being throat swabs 6, followed by sputum 4, three samples from each blood and urine and one sample each from pus, ET tips and BAL, indicating that GAS are well-known causative agents of upper respiratory tract infections responsible for post-streptococcal sequelae including rheumatic fever and acute glomerulonephritis.

GBS have been isolated from 42 samples, the highest number being throat swabs 15, followed by sputum – 7, BAL fluid 6, CSF 4, three samples each from blood and urine and two samples each from pus and ET tips. The presence of GBS as a causative agent in urinary tract infections, septicaemia and neonatal sepsis has been reported.[23]

GCS and GGS are normal inhabitants of human nasopharynx, skin and genital tract. They are now emerging as an important cause of invasive infections, and their large colony-forming strains resemble GAS in terms of virulence. Two milk-borne epidemics of pharyngitis due to GCS followed by acute glomerulonephritis have been reported.[24]

In our study, GCS 74 streptococci accounted for the highest number of isolation which included throat swabs 25, followed by urine 14, sputum 11, pus 8, blood 7, CSF 5 and two samples each from BAL fluid and ET tips. GGS have been isolated from 51 samples which included throat swabs 20, sputum 10, urine 8, blood 5, pus 4, BAL 2 and one each isolate from CSF and ET tips.

In recent years, GGS have been reported with increasing frequency as the cause of a variety of human infections, such as pharyngitis, cellulitis, meningitis, endocarditis and sepsis.[25,26]

The frequency of GCS and GGS isolates from acute pharyngitis from different regions ranges from 0.7% to 6.2% and 3.7% to 5.1%, respectively.[23,24] The rate of GGS- and GCS-positive throat cultures varies from 1% to 25% in various studies.[25,26]

The role of GGS and GCS in pyoderma, uterine, pulmonary and throat infections has been highlighted.[27] *S. equisimilis* is reported to have caused pharyngitis with

secondary bacteraemia. A fatal case of pharyngitis followed by bilateral cavitatory pneumonia with empyema, bacteraemia and arthritis[28,29] was reported by Stamm and Cobbs.[30]

In view of the high rate of isolation of GCS from throat swabs in our study, all BHS from throat swab cultures must be grouped and the clinical practice of ignoring GGS and GCS, unless isolated from sterile fluids, must be abandoned as they are equally invasive as GAS. Rapid diagnosis of pharyngeal carriage of GCS and GGS strains, which leads to glomerulonephritis, toxic shock syndrome and rheumatic fever, may prevent unnecessary death and disability.[31,32]

The GDS are normal inhabitants of the human gastrointestinal tract and may spread from this site to cause bacteraemia, cholecystitis and wound infections. In our study, we have isolated only four GDS from throat swabs which are considered to be a part of normal flora.

The oropharynx, gastrointestinal tract and vagina are potential sources for GFS in bacteraemia and suppurative infections. In one study, GFS which have been isolated from the urinary tract were considered the possible contaminants.[33,34]

In this study, we have isolated 28 isolates of GFS which included throat swabs 8, blood 5, pus 4, sputum 3, BAL fluid 2, CSF 2 and one isolate from ET tips, indicating that GFS may also cause serious infections which may lead to life-threatening complications. Reports of GFS in infections of the central nervous system and meninges are numerous.[35] In recent years, GFS have been recognised as a cause of purulent pleuropulmonary disease, notably empyema thoracis.[36]

Knowledge of the drug susceptibility patterns of BHS and the prevalence of the Lancefield groups in specific geographic regions would assist clinicians in appropriate patient management and therapeutic regimen. Rapid identification and consequent prompt treatment of patients with BHS can reduce the risk of spread and aid in the prevention of infections and their serious complications, such as rheumatic fever/rheumatic heart disease, nephritis and local or systemic infections.

Serogrouping and typing of BHS is done only in a few laboratories; others report beta-haemolytic streptococcal isolates only as Group A or non-Group A, which would fail to identify these potentially pathogenic organisms. Hence, clinical laboratories should be encouraged to perform group identification of all BHS to evaluate the role of these organisms in invasive and non-invasive infections.

In the present study, we have observed that the GAS was outnumbered by the other beta-haemolytic groups such as Groups B, C, D, F and G. GAS was around 10% and other beta-haemolytic groups were 90%. It suggests that the diagnostic laboratory whenever isolating beta-haemolytic *Streptococcus* should go for further specification by biochemical test or serological identification to know the prevalence of circulating serogroups in the community so that active therapeutic intervention can be made possible.

In view of the high rate of isolation of GCS from throat in our study, we conclude that all BHS from throat cultures must be grouped and the clinical practice of ignoring GGS and GCS, unless isolated from sterile fluids, must be abandoned as they are equally invasive as GAS.

Our institute which is a tertiary care centre situated in urban area is a nodal point for all the surrounding villages. All village dwellers are often exposed to animals they rear and pets. Other than GAS are considered as primary animal pathogens.

Culture isolates were not correlated with clinical signs and symptoms. Thus, we cannot conclude whether these isolates are pathogenic, commensal or contaminant. To conclude, epidemiological surveillance of BHS is mandatory to know the prevalence of serogroups in the community.

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Conflicts of interest
There are no conflicts of interest.

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