Molecular detection of virulence genes associated with pathogenicity of *Gram positive* isolates obtained from respiratory tract of apparently healthy as well as sick goats

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Abstract

Aim: To know the prevalence of gram positive isolates and their virulence genes obtained from respiratory tract of apparently healthy as well as sick goats.

Material and Methods: Nasal swabs and tissue samples were screened for the presence of microbial pathogens by cultural isolation, biochemical confirmation. Further, these isolates were subjected to the virulence gene detection by PCR.

Results: Out of 144 isolates, 57 isolates of gram positive pathogens were obtained and confirmed as *Staphylococcus* spp. (43 isolates) and *Streptococcus* spp. *(14 isolates)* on the basis of Gram staining, morphology, cultural identification and biochemical characters. Five isolates (11.6%) were found to be positive for Coagulase gene; 11 isolates (25.6%) were found to be positive for *clfA* gene; and 14 isolates (32.6%) were found to be positive for *Spa* gene.

Conclusion: The presence of these genes confirmed the pathogenic potential of gram positive pathogens and their association with clinical manifestations in respiratory tract infections of goats.

Key Words: biochemical characters, PCR, respiratory tract of Goat, *Staphylococcus*, *Streptococcus*, virulence genes

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Introduction

Staphylococci and Streptococci are group of bacteria that cause a wide spectrum of clinical manifestations, such as pneumonia, wound infections, septicemia, endocarditis, mastitis and metritis in animals. It is responsible for both nosocomial and community-acquired infections [1]. These species occur as commensals on skin and mucous membranes; some may act as opportunistic pathogens causing pyogenic infections. They are gram positive cocci (1 µm) that tend to occur in irregular clusters resembling bunches of grapes. They are facultative anaerobes, fermentative and catalase positive. They are non-motile, oxidase-negative and do not form spores. Streptococcus species having similar characteristics as Staphylococcus species but are catalase negative and found in chain of different lengths; acts as commensals on the mucosae of the upper respiratory tract and lower urogenital tract [2]. Many species are saprophytic including those of veterinary importance. These both are major pathogens having wide host range and are associated with various clinical manifestations in small ruminants, viz., pneumonia, mastitis and pyogenic infections.

*clf*A gene codes for clumping factor, helps in infection process by facilitating bacterial binding via solubilised or immobilized fibrinogen. Because fibrinogen plays significant role in platelet thrombus formation, it is likely that *clf*A may be involved in bacterial platelet interactions. Therefore, it is implicated as virulence factor [3]. *spa* gene codes for the IgG binding region of the protein A and is well known for the binding ability for its immunoglobulin Fc region. *spa* has affinity with solubilised or immobilized von Willebrand factor (vWF) and identified as a novel Staphylococcal adhesin [4]. *coa* gene codes for enzyme coagulase and coagulase production is important phenotypic determinant of *S. aureus* which is associated with virulence [5]. In addition to these, it produces several other extracellular virulence factors which affect the host cell metabolism. These genes are associated with the

Table 1. Primers used for amplification of virulence genes of *Staphylococcus spp.*

Primers	Sequences(5'- 3')	Target gene	Size of amplified product(bp)	Reference
coa F	ATA GAG ATG CTG GTA CAG G	*coa*	polymorphism	5
coa R	CTT CCG ATT GTT CGA TGC			
clfA F	GGC TTC AGT GCT TGT AGG	*clfA*	980	3
clfA R	TTT TCA GGG TCA ATA TAA GC			
Spa F	CAC CTG CTG CAA ATG CTG CG	*spa*	920	4
Spa R	GGC TTG TTG TTG TCT TCC TC			

coa, codes for coagulase; *clfA*, codes for clumping factor; *spa*, codes for the IgG binding region of the protein A.

Table 2. Primers used for amplification of virulence genes of *Streptococcus spp.*

Primers	Sequences(5'- 3')	Target gene	Size of amplified product(bp)	Reference
bcaF	TAA CAG TTA TGA TAC TTC ACA GAC	*bca*	535	6
bcaR	ACG ACT TTC TTC CGT CCA CTT AGG			
ribF	CAG GAA GTG CTG TTA CGT TAA AC	*rib*	382	6
ribR	CGT CCC ATT TAG GGT TCT TCC			

bca, codes for alpha-C protein; *rib*, codes for surface Rib protein.

virulence of *S. aureus*.

rib gene codes for surface Rib protein and it has role in putative virulence. *bca* gene codes for alpha-C protein and is thought to play a role in epithelial cell adherence and invasion, and resistance to phagocytosis [6]. These genes are associated with the virulence of *Streptococcus* spp.

In the present study, the animals were screened for the respiratory tract infections caused by *Staphylococcus* spp. and *Streptococcus* spp. by cultural and molecular techniques. Along with their primary and secondary biochemical characteristics, the isolates were also examined for the presence of various virulence associated genes by PCR to study their possible role in pathogenicity.

Materials and Methods

Sample collection: During the present study, a total of 102 nasal swabs were collected from apparently healthy and sick goats from organized farms and 96 tissue samples were collected from post-mortem animals and animals slaughtered in the local meat market and transported to the laboratory on ice. All the samples were processed and screened for possible microbial pathogens.

Cultural isolation of the organisms: Bacterial isolation was done following standard technique by inoculating tissue samples and nasal swabs primarily on blood agar and plates were incubated for 24-48 hrs at 37°C. After incubation, the nature of growth and cultural characters of colonies were studied. Preliminary morphological identification was done based on Gram's staining. Cultural characteristics of the isolates were further studied on Potassium Tellurite Agar, Nutrient agar and Mannitol salt agar (HiMedia).

Biochemical identification of *Gram positive* pathogens: Primarily the isolates were characterized by KOH, Catalase, Oxidase and O-F tests [2]. Following primary biochemical tests, the isolates were characterized by various secondary biochemical tests [7]. Secondary biochemical tests include Coagulase Test, t-DNAse production, Voges-Proskeur (VP) test, CAMP reaction, Lancefield grouping and Carbohydrate fermentation test.

DNA Extraction: The template DNA from colony was prepared with minor modifications [8]. Briefly, from the culture plate, bacterial colonies were picked and suspended in 100 µl milli-Q water. The samples were boiled for 15 min, cell debris was removed by centrifugation at 5000 rpm and the supernatant was collected and used as a template DNA.

Polymerase chain reaction (PCR) for amplification of virulence associated genes: PCR was carried out with template DNA (3 µl), forward and reverse primers (1 µl), 12.5 µl of master mix (2x) and 7.5 µl DNAse Free Water in a total volume of 25 µl. The DNA was amplified using the specific cycling conditions (Table 2). PCR products were separated in 1.5% agarose gel for 90 min at 80 V, stained with ethidium bromide (1%) [added @ 0.5 µl/100ml] and detected by UV transillumination (wavelength 312 nm) [9]. Amplified genes were identified on the basis of fragment size shown in Table 1 and the cyclic conditions for PCR are explained in table 2.

Results and Discussion

After morphological, cultural and primary

Table-3. Cycling condition for PCR of virulence genes of *Staphylococcus spp.*

Primers (forward and reverse)	Cycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final extension
coa F	94°C	94°C	57°C	70°C	72°C
coa R	45 sec	20sec	15sec	15sec	2min
			Repeated for 30 cycles		
*clf*AF	94°C	94°C	57°C	72°C	72°C
*clf*AR	4min	60sec	60sec	60sec	5min
			Repeated for 38cycles		
Spa F	94°C	94°C	58°C	72°C	72°C
Spa R	2min	60sec	60sec	60sec	5min
			Repeated for 30cycles		

Table 4. Cycling condition for PCR of virulence genes of *Streptococcus spp.*

Primers (forward and reverse)	Cycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final extension
bca F	95°C	94°C	55°C	72°C	72°C
bca R	5min	30sec	30sec	2min	10min
			Repeated for 35cycles		
Rib F	95°C	94°C	55°C	72°C	72°C
Rib R	5min	30sec	30sec	2min	10min
			Repeated for 35cycles		

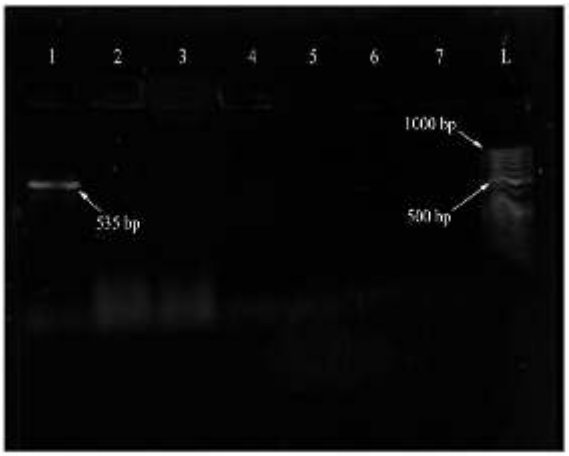

Figure-1. Agarose gel showing amplification product of *bca* gene for *Streptococcus spp.* (approximately 535bp)
Lane1: Positive field isolates
Lane 2-7: Negative field isolates
L: 100 bp-1 Kbp DNA ladder

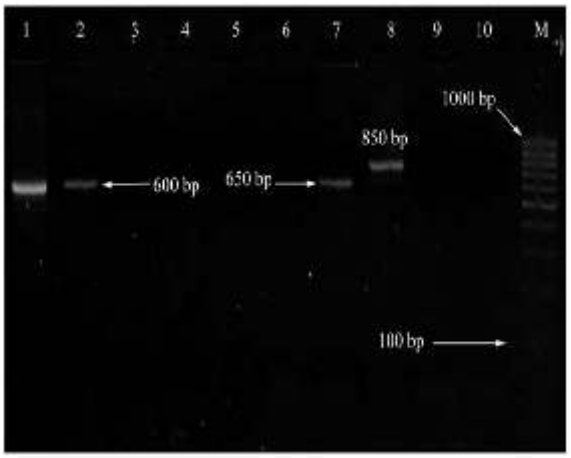

Figure-2. Agarose gel showing amplification product of *coa* gene for *Staphylococcus spp.* (approximately 850bp, 650bp and 600bp)
Lane1,2,7,8: Positive field isolates
Lane 3-6,9,10: Negative field isolates
L: 100 bp-1 Kbp DNA ladder

biochemical identification, 43 isolates of *Staphylococcus* were confirmed. Out of theses 43 isolates, 15 isolates showed yellow discoloration on Mannitol salt agar, 25 isolates showing partial hemolysis on Blood agar, 13 isolates showed golden yellow pigmentation on Nutrient agar and all isolates produced black colonies on Potassium tellurite agar. All *Staphylococcus* isolates were found to be negative for t-DNAse production and 5 isolates were positive for Coagulase production. In the present study, the prevalence of *Staphylococcus* was found to be 29.9%. The prevalence rate of the present study is in agreement with reports of different authors [10,11]. Higher prevalence of *Staphylococcus* spp. was detected by one author as 73.7% from pneumonic lung of goats [12]. Low prevalence of *Staphylococcus* spp. was detected in the previous study from pneumonic lung of sheep [13,14,15]. Lower prevalence of this species was also detected in previous studies which were from pneumonic lung of goats [16,17]. Low prevalence of

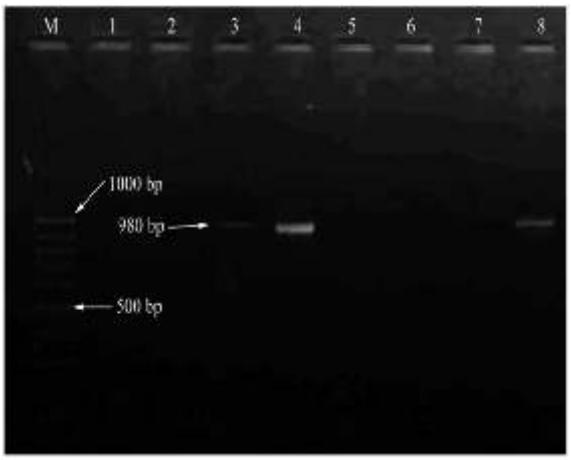

Figure-3. Agarose gel showing amplification product of *clf*A gene for *Streptococcus spp.* (approximately 980bp)
Lane 3,4,8: Positive field isolates
Lane 1,2,5,7: Negative field isolates
M: 100 bp-1 Kbp DNA ladder

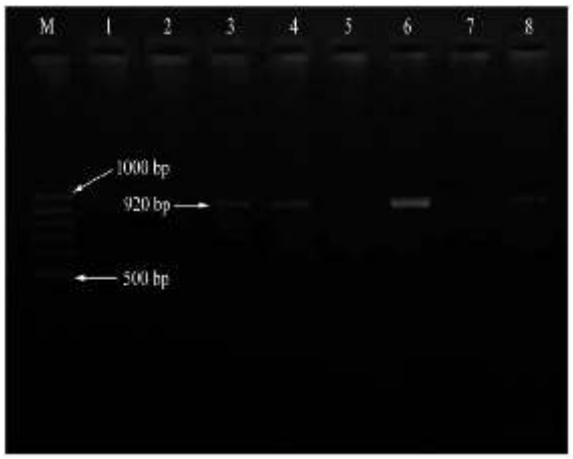

Figure-4. Agarose gel showing amplification product of *spa* gene for *Staphylococcus spp.* (approximately 920bp)
Lane 3,4,6,8: Positive field isolates
Lane 1,2,5,7: Negative field isolates
M: 100 bp-1 Kbp DNA ladder

Staphylococcus spp. was also detected from different anatomical sites of respiratory tract of goats [18].

Based on morphological, cultural and preliminary biochemical identification 14 isolates were confirmed as *Streptococcus* spp. All the isolates were subjected to cultural and secondary biochemical tests for specific identification. Out of 14 isolates, 4 isolates produced partial hemolysis, while others failed to produce hemolysis on sheep blood agar. These isolates were confirmed as Group-D *Streptococci*, on the basis of Lancefield grouping by using Latex Agglutination Test Kit (HiMedia). All the isolates revealed negative CAMP reaction. These isolates utilized inulin, trehalose, lactose and glucose, except sorbitol. In the present study, the prevalence of *Streptococcus* spp. was found 9.7%. The reports of the present study are in agreement with reports of previous study [10,19]. Low prevalence of *Streptococcus* spp. were detected in the previous studies [15,20].

The PCR assay was standardized for detection of individual virulence associated genes of *Staphylococcus* spp. namely *clf*A gene (codes for clumping factor), *spa* gene (codes for the IgG binding region of the protein A) and *coa* gene (codes for enzyme coagulase) using specific primer sequences which gave product sizes of 980 bp, 920 bp and polymorphism (850 bp, 650 bp and 600 bp), respectively. Five isolates (11.6%) were found positive for coagulase gene (Fig.2); 11 isolates (25.6%) were found positive for *clfA* gene (Fig.3); and 14 isolates (32.6%) were found positive for *Spa* gene (Fig.4). Out of 5 Coagulase positive isolates, 1 isolate revealed an amplified 850 bp product, 2 isolates revealed an amplified 650 bp product and 2 isolates revealed an amplified 600 bp product. All the coagulase positive isolates of *Staphylococcus* spp. by *in vitro* test were also found positive for coagulase gene by PCR detection. The results of the present study were in agreement with reports of previous studies who designed these oligos complementary to each end of the sequence of the *clf*A and *spa* genes which specifically amplified the product sizes of 980 bp and 920 bp, respectively [3,4]. PCR of *coa* genes was also carried out in the present study *using* specific primers resulted in polymorphism (850 bp, 650 bp and 600 bp). The findings were in contrast with the reports of previous study who used these oligos complementary to each end of the sequence of the *coa* gene which showed polymorphism and specifically amplified the product sizes of 875, 660, 603, or 547 bp *[*5]. These virulence factors might contribute for mixed respiratory infections in goats. The findings were also in contrast with the previous reports in which 86 positive samples of *Staphylococcus spp.*, were collected, out of that 42 specimens (48.8%) contained the *coa* gene, 63 specimens (73.3%) contained the *clfA* gene and 22 specimens (25.5%) contained *spa* gene (IgG Binding region) [21]. The findings were also in contrast with the previous studies were reported that all the coagulase positive isolates out of collected 92 samples of *Staphylococcus aureus*, were found to be harbour *coa*-gene and IgG binding region (*spa*-IgG) whereas, 84 (91.3%) isolates were positive for *clf*A gene [22]. The reports of present study were in contrast with previous study [23].

The PCR assay was standardized for detection of individual virulence associated genes of *Streptococcus* spp., viz., *rib* (Codes for surface Rib protein), *bca* (Codes for alpha-C protein) using primer sequences which yielded a product sizes of 382 bp and 535 bp, respectively. Out of total 14 isolates, none of the isolate was confirmed as *S. agalactiae* and none of isolates were found to be positive for *rib* gene whereas 2 isolates (14.3%) were found to be positive for *bca* gene (Fig.1). The results of the present study is in agreement with previous reports who used these oligos complementary to each end of the sequence of the *bca* gene which specifically amplified the product size of 535 bp, whereas the present results were found in contrast to the previous studies for rib gene using specific primers which amplified the product size of 382 bp [6]. The reports of present study were also found contrast with previous studies [24,25,26]. The detection of *bca* gene indicated potential anti-phagocytic and invasive properties of the isolates.

Conclusions

Therefore, although the prevalence rate of the organisms in respiratory tract infections is low, the presence of the virulence associated genes provides them considerable pathogenic potential in the cases where the host is immuno-compromised. In view of all the findings, we need to further investigate the role of other factors related to host and pathogen which assist in the progression of disease at physiological and molecular levels.

Author's Contribution

The work was carried out by TKA under the guidance of AR with the technical support of PK. PK revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

Authors declare that they have no competing interests.

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