Molecular Diagnosis of Streptococcus Pyogene by Detection Virulence Factors Genes from Clinical Isolates

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Abstract

Background: The purpose of current study Isolation and identification of *Streptococcus pyogene* from patient, culturing bacteria, diagnosis *Strept. pyogene* infection by different methods and molecular detection by virulence factors genes *SpeA*, SpeC and *speK* and comparison between different type of methods to detection for *Streptococcus pyogene*. Methods: The clinical samples were taken during the period from October 2021 to end February 2022 for 141 patients suffering from acute tonsillitis, pharyngitis, otitis media, acute fever, and UTI patients. from both sexes the ages between 5 -60 years old. 102 samples showed bacterial growth in culture, and No growth was seen in other 39 samples from ENT unit in Marjan Teaching Hospital and Al- Sadiq Hospital in Hilla city, Babylon province, and Al- Hussiany Hospital Kerbala province. Results: The results shown differences in Sensitivity, Specificity and Accuracy between different detection methods, the standard method was molecular and when comparison with other methods shown Sensitivity 100%, Specificity 80.0 % Accuracy 81.8 % in vitik2 methods and Sensitivity 100%, Specificity 0.0 % Accuracy 9.1 % in direct detection methods. Molecular Detection of virulence genes was done through utilizing Specific PCR primers. *SpeC* found that was observed in 3 of 9 (33.33%) samples from patient's throat swab of *S. pyogenes detection* by vitek 2 system. that contain this gene with the long length in (270bp), and *SpeA* gene of *S. pyogenes* it was found 3 isolates from 9 identifications in Vitek2 (33.33%) patient's samples from throat of *S. pyogenes* has gene with the long length in (102bp). accuracy (100%) of diagnosis arised from 33.33%, 9 Specimen from throat with Spec gene and 33.33%, 9 Specimen from throat with *SpeA* gene and increase to the 100% when utilizing duplex RT PCR to detect the same bacterium. Conclusion: There is different methods for detection *Strept. pyogene*, the best one with high accuracy and specificity molecular method then Vitek2 methods and Direct detection method had very low accuracy and specificity.

Keywords:_Streptococcus pyogene, SpeA, SpeC and speK genes, Sensitivity, Specificity and Accuracy.

1. Introduction

GAS is a Gram-positive bacterium, β-hemolytic, with a low G+C % DNA content. Cocci is assembled in chains of minimum two cocci. GAS is grown on blood or Todd Hewitt broth supplemented with 0.2% yeast extract agar plates and its multiplication is favored by CO2 and anaerobia. GAS is auxotroph for 15 amino acids and its primary source of carbon is glucose (1).
These genes code for a variety of virulence factors, such as exotoxins that are often superantigens and DNases like streptodornase. It's not clear where the genes that code for toxins in phages came from, but since these genes don't seem to be involved in the phage's ability to copy itself, it's likely that they were added to the phage at a late stage in its evolution. It has been suggested that virulence factors could be acquired by phages through imprecise excision events (2), But it has not been seen that known phage-associated virulence genes can be found on the bacterial chromosome. There are some superantigen genes that are not linked to prophages (3) (4).
In each strain of S. pyogenes, the SpeB and speJ genes may be found in the central region of the bacterial chromosome. Nevertheless, although it is there and that its nucleotide sequence displays a high level of conservation, between 25 and 40 percent of these strains do not express significant amounts of the SpeB toxin. (5). In contrast, bacteriophages are responsible for encoding the SpeA, SpeC, and speH-M genes.
Several years ago, a method based on the detection of nucleic acids was devised for the direct diagnosis of S. pyogenes from clinical throat swabs. Using a single-stranded chemiluminescent nucleic acid probe, the GAS Direct test detects rRNA sequences of S. pyogenes in pharyngeal Specimens (6). A commercial polymerase chain reaction (PCR) approach employing the illumigene technology for the direct detection of S. pyogenes. (7) has just lately been given clearance by the FDA. In a study that was conducted across multiple centres, it was shown that the illumigene test had an excellent sensitivity of 99 percent and a Specificity of 99.6 percent. (8)(9).

2. Material and Methods

Collect samples

Pharyngitis swabbing was obtained with sterile cotton swabs. The swab should be used to collect as much exudates as possible from the tonsils and

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posterior pharyngeal wall, Ear swabbing, venous blood was obtained from each patient and placed in EDTA tubes was utilized to carry out blood culture, sputum and urine sample. a total of (141) throat swabs, blood, sputum, ear discharge and urine sample obtained from patients suffering from acute tonsillitis, pharyngitis, otitis media, acute fever, and UTI patients. Male and female age between (5-60) years. Taken from ENT unit in Marjan Teaching Hospital and Al- Sadiq Hospital in Hilla city, Babylon province, and Al- Hussiany Hospital Kerbala province, during the period from October 2021 to end February 2022.

Culture media prepare

The following general culture media are prepared according to standard procedures of Himedia company, and they are utilized in the following tests:

Blood agar medium

This medium used both as an enrichment medium for the culture of the bacterial isolates and as a media for determining the bacteria's capacity for blood hemolysis.

Nutrient agar medium

It is utilized for general examinations as well as the cultivation and activation of bacterial isolates.

Nutrient Broth

It is utilized for general testing, culture, and activation of bacterial isolates for antibiotic susceptibility testing using the disc diffusion method. Additionally, it is utilized for the sugar ferment test.

Streptococcus selection agar

This medium was utilized in the process of isolating bacterial isolates for the purposes of general purification.

Brain heart infusion broth with 15% glycerol.
This medium was employed as an enrichment medium for bacterial isolates that had been kept for an extended period.

Identification process with Vitek 2 System

The Vitek-2 automated system (BioMerieux, France) for identification of BHS isolates. The isolates were achieved according to manufacture instructions.

Molecular detection

Molecular detection of Strept. pyogene by conventional PCR is used to amplify DNA fragments that contain the targeted sequence of the superantigens (SpeA, SpeC,speK) genes.

3. Results and Discussion

In current study, a total of (141) throat swabs, blood, sputum, ear discharge and urine sample obtained from patients suffering from acute tonsillitis, pharyngitis, otitis media, acute fever, and UTI patients. from age (5-60) years male and female. Sample taken from ENT unit in Marjan Teaching Hospital and Al- Sadiq Hospital in Hilla city, Babylon province, and Al- Hussiany Hospital Kerbala province, during the period from October 2021 to end February 2022. All swabs were subjected to aerobic culturing on selective media and showed this result.
When comparison different type of identification methods for bacterial isolate appeared as the result showed in table (1) more accuracy method for diagnosis S. pyogenes is molecular method isolated 3 isolates only from 102 with growth, then Vitek 2 diagnosis 9 isolate of S. pyogenes from 102 and last Direct and culture method with 33 isolate of S. pyogenes from 102. Also, high significances differences through P value (0.00001**).

Table (1) Bacterial isolates according to diagnosis methods

Bacterial type	No. of Isolate			P value
	Direct and culture method	Vitik 2	molecular	
Sococcus pyogenes	33	9	3	0.00001**
Streptococcus agalactiae	4	5	0	0.09697
Streptococcus pneumonia	9	12	0	0.00381*
Streptococcus mitis	4	8	0	0.01832*
Staphylococcus aureus	52	42	0	0.00001**
Streptococcus parasanguinis	0	8	0	0.00732*
Staphylococcus oralis	0	5	0	0.3114
Streptococcus salivarius	0	6	0	0.01832*
Streptococcus sanguinis	0	8	0	0.00732*
NO growth	39	39	3	0.00001**
Total	141(100%)	141(100%)		

* Means significance differences (P ≤0.05) ** means high significances differences (P ≤0.001)

Sensitivity, Specificity, Predictive Value, and Efficiency of diagnostic tests

We can learn about the caliber of diagnostic tests through cross-tabulation tables. In order to determine whether a patient has a disease, doctors frequently order tests. Following a patient's diagnosis and testing, one of four things may happen: True Positive (TP), in which the screening test successfully identifies a patient as having the disease (i.e., the disease is detected by both the diagnostic and the screening test); Both the diagnosis and the screening test must be negative in order for the result to be considered True Negative (TN); otherwise, the result is considered False Positive (FP).

	DIAGNOSIS	
Screening	Condition Present	Condition Absent
Test Positive	True Positive (TP)	False Positive (FP)
Test Negative	False Negative (FN)	True Negative (TN)

Sensitivity, specificity, positive predictive value, negative predictive value, and efficiency are used to describe a screening test's clinical performance. The likelihood that a test will be positive when administered to a group of patients who have the condition is known as test sensitivity (Sn). It is calculated using the formula Sn = (TP/ (TP + FN)) 100 and is shown as the proportion of patients who are accurately diagnosed as suffering from the condition. In other words, sensitivity can be thought of as 1 the fraction of false negatives (10).

Sn = (TP/ (TP + FN)) × 100, (1 - the false negative rate)

Sp = (TN/ [TN + FP]) × 100, (1 - the FP rate)

The positive predictive value (PPV) or (Precision): PPV = (TP/ [TP + FP]) × 100

The negative predictive value (NPV): NPV = (TN/ [TN + FN]) × 100

The efficiency EFF or (Accuracy) = ([TP + TN]/ [TP + TN + FP + FN]) × 100

For detecting Sensitivity, Specificity and accuracy in result as shown in table (2) when depending on the molecular diagnosis as golden method, Vitek2 appear Specificity (80%) and Accuracy (81.8%) compare with standard method molecular.

Table (2) Calculations of test accuracy and precision for VITIK test in comparison to molecular diagnosis of *Streptococcus pyogenes* (n=33).

Diagnostic test	Gold standard (Molecular)			Precision	
VITEK	Positive	Negative	Total	PPV	NPV
Positive	3	6	9	33%	100%
Negative	0	24	24		
Total	3	30	33		
Sensitivity 100%					
Specificity 80.0 %					
Accuracy 81.8 %					

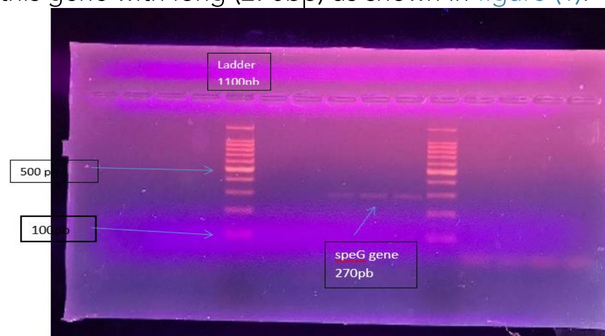
In table (3) Results shown Specificity and accuracy for direct identification method when consider molecular detection method as golden method and compare with it appear Specificity (0.0%) and Accuracy (9.1%).

Table (3) Calculations of test accuracy and precision for Culture in comparison to molecular diagnosis of *Streptococcus pyogenes* (n=33)

Diagnostic test	Gold standard (Molecular)			Precision	
Vitek	Positive	Negative	Total	PPV	NPV
Positive	3	30	33	9.1%	0.0%
Negative	0	0	0		
Total	3	30	33		
Sensitivity 100%					
Specificity 0.0 %					
Accuracy 9.1 %					

Molecular detection of *Streptococcus pyogene* virulence genes (*SpeA,SpeC*)

Molecular Detection of virulence genes genes was Achieved through the Utilization of specific PCR primers. Result shown that SpeC was spotted in 3 of 9 (33.33%) samples from patient's throat swab of S. pyogenes detection by vitek 2 system that contain this gene with long (270bp) as shown in figure (1).



Figure(1)Gel electrophoresis of PCR of Spec; molecular weight marker of ladder (270bp), samples obtained from throat swab at volt 70 for 60 min.

Besides, virulence gene of S. pyogenes SpeA codes toxin, it was found 3 isolates from 9 identification in Vitek2 (33.33%) patients samples taken from the throat this gene show long (99bp) as showed in figure (2).

Figure(2)Gel electrophoresis of PCR of SpeA; molecular weight marker of ladder (102 bp), samples obtained from throat swab. At volt 70 for 60min.

Since Strept. pyogene is one of the most important human pathogens among Gram-positive organisms and is caused a wide range of infections, it is remarkable how precisely this bacterium can be detected from a clinical sample. For the past 20 years, a variety of techniques, including the Direct approach, culture techniques, and PCR, have been used to identify Strept. pyogenes (11).

The range of the PCR sensitivity measured through serial dilutions for DNA isolated from cultures of Strept. pyogene pure cell was around 100^{-1} ng of template. But amplification can be seen in 5-mL aliquots of 10^{-5} dilution when the serially diluted cell culture aliquots were collected straight for PCR. 32 CFUs were found in 100 mL of a 10^{-5} dilution (12).

This suggests that PCR employing SCAR primers is capable of diagnosis single or small number of Strept. pyogene bacterial cells. These tests validate the PCR qualitative threshold level for amplification signal detection. The creation of SCAR primers may lessen the current range of confusion around the

identification of Strept. pyogene. Previous publications claim that Strept. pyogene is mistakenly identified because GCS and GGS express the Lancefield's group A antigen. GCS and GGS were once thought to be commensal organisms that were a natural component of the flora on the throat, skin, and other mucosal tissue. As a result, they only cause opportunistic illness in those who had preexisting risk factors. But, in healthy people, GGS is more frequently linked to a range of illnesses that resembles GAS (13).

This interspecies recombination interchange from GAS donor to (GCS-GGS) recipient may be the cause of the comparable antigen sharing between two different Streptococcus species. Integrative conjugative elements are known to mediate local gene transfer between GAS and GGS or GBS and GGS, according to modern investigations (14).

An earlier study showed how to utilize Cycler PCR to identify Strept. pyogene for throat swab samples utilizing PCR Cycler Strept. primer (15). In contrast to the SCAR primers, which detected 3 more positives (84 vs. 3) from 84 throat samples, the primer detected 3 positives (102 from cultural method) out of 141 total samples. The SCAR primers were more successful in identifying Strept. pyogene than culture-based analysis, like the PCR Cycler Strept. primer. The SCAR primers were shown to be significantly more sensitive (approximately three times) than utilizing the cultural method when comparing the effectiveness of the two techniques. The outcome implies that the SCAR primers may be particularly utilized to detect strains of Strept. pyogene. The sensitivity of SCAR primers was much higher (statistical significance $P \leq 0.05$) comparison with detection by conventional microbiology cultural method (16). Methods that depend on culture could miss very small bacterial amount. Due to the rapid proliferation of organisms in the enriched media, strains may be missed in culture analyses. Furthermore, examine for all b-hemolytic streptococci is time-consuming and risky, as it can false-negative provide results. Consequently, will be an effective tool for the quick and early diagnosis of Strept. pyogene infection by SCAR primers (17).

This result differs from that revealed by (18), who discovered that Strept. pyogene may produce the SpeA gene at a range (51%), whereas (19) discovered that the SpeA gene was percentage 16% throat samples (20) discovered that the prevalence of the SpeA gene was 72% in patients with tonsillitis, 51% in invasive cases, and 44% in upper respiratory tract cases. According to the distribution of toxin gene profiles, SpeA, which codes for the streprococcal pyrogenic exotoxin A, was substantially conserved, while SpeA and Spec were found in varying amounts. For instance, there has been conflicting information regarding the relationship between invasive diseases and the Sococcal exotoxin genes SpeA and SpeA, which are assumed to be significant in invasive disease (21).

According to research on the prevalence of the Spe genes, the great majority of GAS strains only encode SpeB, whereas SpeA and Spec are found much less frequently. It has been demonstrated that most strains associated with severe Sococcal infections can produce the SpeA toxin. The protease SpeB appears to be responsible for the significant tissue destruction seen in many patients with severe invasive illness, including toxic shock-like syndrome. Most isolates from episodes of severe invasive illness and toxic shock-like syndrome production SpeA (22).

The result of a GAS infection is dependent on several parameters, including host immunity and exotoxin generation, in addition to the strain of the organism. Because GAS has been demonstrated to induce invasive disease without the superantigen genes SpeA and Spec, bacterial traits may possibly play a pathogenic role in severe Sococcal infections (23).

References

Weckel, A., Ahamada, D., Bellais, S., Méhats, C., Plainvert, C., Longo, M., & Fouet, A. The N-terminal domain of the R28 protein promotes emm28 group A Streptococcus adhesion to host cells via direct binding to three integrins. Journal of Biological Chemistry. 2018; 293(41), 16006-16018.

Suzuha, H., Fumito, M., Michal, P., & Kazuhide, Y. Statistical analysis of automatic seed word acquisition to improve harmful expression extraction in cyberbullying detection. International Journal of Engineering and Technology Innovation. 2016; 6(2), 165-172.

Porta, K., and Rizzolo, D. Preventing group B Sococcal infections in newborns. Journal of the American Academy of Pas. 2015; 28(3), 24-29

Tokinaga, H., Xie, S. P., Deser, C., Kosaka, Y., & Okumura, Y. M. Slowdown of the Walker circulation driven by tropical Indo-Pacific warming. Nature. 2012; 491(7424), 439-443

Brosnahan, A. J., & Schlievert, P. M. Gram-positive bacterial superantigen outside-in signaling causes toxic shock syndrome. The FEBS journal. 2011; 278(23), 4649-4667.

Fakkert, I. E., Abma, E. M., Westrik, I. G., Lefrandt, J. D., Wolffenbuttel, B. H., Oosterwijk, J. C., . & Mourits, M. J. Bone mineral density and fractures after risk-reducing salpingo-oophorectomy in women at increased risk for breast and ovarian cancer. European Journal of Cancer. 2015; 51(3), 400-408

Androga, G. O., McGovern, A. M., Elliott, B., Chang, B. J., Perkins, T. T., Foster, N. F., & Riley, T. V. Evaluation of the Cepheid Xpert C. difficile/Epi and Meridian Bioscience illumi gene C. difficile Assays for Detecting Clostridium difficile Ribotype 033 Strains. Journal of Clinical Microbiology. 2015; 53(3), 973-975

Anderson, B. A. A value-driven mechanism of attentional selection. Journal of vision. 2013; 13(3), 7-7

Buchan, A., LeCleir, G. R., Gulvik, C. A., & González, J. M. Master recyclers: features and functions of bacteria associated with phytoplankton blooms. Nature Reviews Microbiology. 2014; 12(10), 686-698.

Plichta, B. and Kelvin, E. A. (2013). MUNRO'S Statistical Methods for Health Care Research. SIXTH EDITION. Wolters Kluwer Health | Lippincott

Williams & Wilkins: pp 67-68.

Nithyanand, P., Thenmozhi, R., Rathna, J., & Pandian, S. K. Inhibition of Streptococcus pyogenes biofilm formation by coral-associated actinomycetes. Current microbiology. 2010; 60(6), 454-460.

Reijtman, V., García, M. E., Mastroianni, A., Isasmendi, A., Pinheiro, J. L., Pérez, G., & Hernandez, C. Evaluation of a rapid diagnostic test for the detection of Streptococcus pyogenes in invasive infections. Revista Argentina de Microbiología. 2020; 52(4), 261-265.

Borges, A. H., O'Connor, J. L., Phillips, A. N., Baker, J. V., Vjecha, M. J., Losso, M. H.,. & INSIGHT SMART and ESPRIT Study Groups and the SILCAAT Scientific Committee. Factors associated with D-dimer levels in HIV-infected individuals. PloS one. 2014; 9(3), e90978.

Tanaka, Y., Sasaki, N., & Ohmiya, A. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. The Plant Journal. 2008; 54(4), 733-749.

Uhl, J. R., Adamson, S. C., Vetter, E. A., Schleck, C. D., Harmsen, W. S., Iverson, L. K., & Cockerill, F. R. Comparison of LightCycler PCR, rapid antigen immunoassay, and culture for detection of group A streptococci from throat swabs. Journal of clinical microbiology. 2003; 41(1), 242-249.

Kalia, A., Enright, M. C., Spratt, B. G., & Bessen, D. E. Directional gene movement from human-pathogenic to commensal-like streptococci. Infection and immunity. 2001; 69(8), 4858-4869.

Liu, L. C., Tsai, J. C., Hsueh, P. R., & Teng, L. J. Rapid differentiation between members of the anginosus group and Streptococcus dysgalactiae subsp. equisimilis within beta-hemolytic group C and G streptococci by PCR. Journal of clinical microbiology. 2006; 44(5), 1836-1838.

Jing, H.B.; Ning, B.A.; Hao, H.J.; Zheng, Y.L.; Chang, D.; Jiang, W.; Jiang, Y.Q. Epidemiological analysis of group A Streptococcus recovered from patients in China. J Med Microbiol. 2016; 55. pp:1101-7.

Hraoui, M.; Boutiba-Ben Boubaker,I.; Doloy, A.; Sami,E.; BenRedjeb, S. and Bouvet, A. Epidemiological markers ofStreptococcus pyogenes strains in Tunisia. Clinical Microbiology and Infection. 2010; 17, Issue 1, pp: 63–68.

Ekelund, K.; Darenberg, J.; Norrby-Teglund,A.; Hoffmann, S.; Bang,D.; Skinhøj,P.; and Konradsen, H. B. Variation in emm type among Group A Sococcal isolates causing invasive or noninvasive infections in a nationwide study. J Clin Microbiol. 2015; 43. pp: 3101–3109.

Watanabe,Y.;Todome,Y.;Ohkuni,H.;Sakurada,S.;Ishikawa,T.;Yu tsudo,T.;Fischetti,V.A. and Zabriskie, B. Cysteine protease activity and histamine release from the human mast cell line HMC-1 stimulated by recombinant Sococcal pyrogenic exotoxin B / Sococcal cysteine protease. Infect Immun. 2012; 70. pp: 3944–3947.

Cunningham, M. W. Pathogenesis of group A streptococcal infections. Clin Microbiol Rev.2000; 13(3), 470-511. https://doi.org/10.1128/cmr.13.3.470

Chaussee, M. S.; Liu, J.; Stevens, D. L. and Ferretti, J. J. Genetic and phenotypic diversity among isolates ofStreptococcus pyogenes from invasive infections. J. Infect. Dis. 2006; 173.pp:901–908.