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Original article

Detection and Identification of *Streptococcus* pyogenes from ENT Patients by Different Methods

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ABSTRACT:
Streptococcus pyogenes (*S. pyogenes*) is a Gram-positive bacterium tainting the upper respiratory tract, such as the tonsils and pharynx, and it also induces post-infection diseases such as rheumatic fever and glomerulonephritis. The study aim was to compare between different methods for detection and identification of *S. pyogenes* from Ear, nose and throat (ENT) patients. A total of 10 (ten) *S. pyogenes* strains were analyzed, these were recovered from throat cultures of 150 patients with (ENT). Identification of bacteria carry out depending on the conventional biochemical tests, Strepto System 9R, using universal PCR primer, followed by RFLP, and finally amplification of the specific genes which included spy 1258 and dnase B. The molecular methods shows, that all *S. pyogenes* isolates contains dnase B and Spy 1258 genes and found to be absent in the non pyogenic streptococci.

KEYWORDS: PCR; Group A *Streptococcus*; RFLP; dnaseB; spy1258; 16S rRNA.

INTRODUCTION

Streptococcus pyogenes (*S. pyogenes)* is a Gram-positive bacterium tainting the upper respiratory tract, such as the tonsils and pharynx, and it also induces post-infection diseases such as rheumatic fever and glomerulonephritis[5]. Infection caused by group A *streptococcus* (GAS) may contribute to a spectrum of diseases such as superficial infection of the upper respiratory tract and of the skin resulting to pharyngitis and impetigo, respectively[2].

The spreading of GAS strains and the virulence factors related with the various strains are not stable over time[13]. *S. pyogenes* is distinguished from other Streptococcus by its ability to completely lyse red cells (So called beta hemolysis) on sheep blood agar, in contrast to other *Streptococcus* that only exhibit partial or no lysis of red cells. In addition the presence of pyrrolidonylary lamidase in GAS has been used for diagnostic purposes[9].

Polymerase chain reaction (PCR) methods have the necessary means to detect small quantities of pathogen (even dead ones). There are various schemes to PCR amplification of bacterial DNA in clinical samples. The first depending on usage of species-specific primers. This method misses the ability to detect bacterial infection definitely[18]. The second approach involves amplification of sequences persist in all

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bacteria based on universal sequences common in bacteria[12]. A number of primer systems for 16S bacterial rRNA detection have been accomplished but they differ in focus to examinant clinical material and pathogens[8].

Dnase B gene seems to be both existing in all *S.pyogenes* and also unique to this organism[20].
Liu et al. (2005) described Spy 1258 gene that is uniquely lay out in the *S.pyogenes*. Application of PCR primer formed from this gene facilitated amplification of DNA fragment from *S.pyogenes* only, but not from other species of the genus Streptococcus and common bacteria[9].
Amplification and sequence analysis of the 16S rRNA gene can be applied to determine and identify bacteria in clinical samples[6].

The discovery of PCR and DNA sequencing, comparisons of the gene sequences of bacterial species have revealed that the 16S rRNA gene is highly conserved within a species and among species of the same genus and thus can be used as the new “gold standard” for species-level identification of bacteria[16,21]. rRNA gene RFLP analysis, or ribotyping, has been expandly used to characterize bacteria for epidemiologic and taxonomic purposes[1].

The rRNA gene Restraction Fragments Length Polymorphisam (RFLP) analysis, or ribotyping, has been expandly used to qualify bacteria for epidemiologic and taxonomic determination[3]. Numerous studies revealed that ribotyping was a stable, reproducible, and widely applicable typing system for determining the molecular epidemiology of genetically diverse bacteria[11].
The main goal of this study to compare between different methods for detection and identification of *S.pyogenes* from (ENT) patients.

MATERIAL AND METHODS

BACTERIAL STRAINS

A total of 10 *S.pyogenes* strains were analyzed. These were recovered from throat cultures of 150 patients with (ENT). A cross sectional study during 26/10/2013- 26/1/2014 in Karbala city, Iraq.

CONVENTIONAL CULTURES

For each patient, throat swabs obtained were transported to the Karbala child's hospital lab by using the transport media, then inoculated on MacConkeys, 5% blood and chocolate agar after that incubated at 37˚C in 5% CO_2 atmosphere. Gram stain was performed on all specimens and all samples subjected to the conventional methods of Streptococcus identification, which including: hemolysis and catalase test.

BACITRACIN SENSITIVITY

IDENTIFICATION BY STREPTO SYSTEM 9R

(Ref 72540-79540)

STREPTO SYSTEM 9R allows the identification of streptococci by biochemical tests performed in wells containing culture media with specific substrates. The combination of positive and negative reactions allows to build up a code number which identifies bacteria by using the Codes Table that provided with this system.

DNA EXTRACTION

Chromosomal DNA was extracted from fresh overnight culture grown in a nutrient broth at 37°C, using a GenEluteTM Bacterial Genomic DNA Kit (Sigma).

PCR AMPLIFICATION

Single plex was used in this study, 5µl of master mix (Gene All, Korea), 3µl of each primer, 5 µl of template DNA and 4 µl of deionised water mixed in 20 µl of total reaction volume.
Amplification reactions were performed in an automated thermocycler (Clever Scientific/UK) apparatus.

Table 1. Sequences, production size and references of primers used for PCR amplification of isolates.

Genes	Primer sequence 5'-------------------------------------→ 3'	Product size	References
16 rRNA	(U1)F5'CCAGCAGCCGCGG TAATACG-3', (U2)R5'-ATC G G TTACC T TG TTACGACTTC3'	996 bp	(Kalghatgi *et al.*, 2008; Lu *et al.*, 2000; Nilsson *et al.*, 2003; Rahmani *et al.*, 2006)[7,10,15,17]
Spy1258	1F:5 AAAGACCGCCTTAACCACCT 3 1R:5 TGGCAAGGTAAACTTCTAAAGCA 3	407 bp	(Dunne *et al.*, 2013; Liu et al., 2005; Schabauer *et al.*, 2014)[4,9,19]
dnas B	F 5 TGA TTC CAA GAG CTG TCG TG 3 R 5 TGG TGT AGC CAT TAG CTG TGT T 3		(Slinger et al., 2011)[20]

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THERMAL CYCLING CONDITION

Amplification was performed in an automated thermocycler, programmed as the follow.

16S rRNA gene

Cycle	Temperature	Time
1 cycle	94 °C	10 sec
	94 °C	20 sec
35 cycle	58 °C	20 sec
	72 °C	1 min
1 cycle	72 °C	10 min
	4 °C	Hold

Spy1258 gene

Cycle	Temperature	Time
1 cycle	95 °C	5 min
	95 °C	30 sec
35cycle	64 °C	30 sec
	72 °C	45 sec
1 cycle	72 °C	2 min
	4 °C	Hold

Dnase B Base

Cycle	Temperature	Time
1 cycle	95 °C	5 min
	95 °C	10 sec
40 cycle	60 °C	10 sec
	72 °C	10 sec
1 cycle	72 °C	10 min
	4 °C	Hold

RESTRICTION ENDONUCLEASE DIGESTION

Ten microlitter of each PCR product (16S rRNA gene) was digested with 3U/µl of HaeIII enzyme. After incubation for 2 hours at 37 °C, the digested DNA was electrophoresed on 2% agarose gel[14].

RESULTS AND DISCUSSION

IDENTIFICATION OF *S. PYOGENES* USING CONVENTIONAL PHENOTYPIC TESTS

10 (ten) different strains of *Streptococcus pyogenes* with pathogenic characters were collected from ENT infectious were characterized by preliminary tests, cultivating on selective media and standard biochemical tests. Based on the conventional biochemical tests, the organism was characterized as *Streptococcus pyogenes*. The results were shown in table 2.

Table 2. Identification of Streptococcus using Conventional Phenotypic tests.

Isolate no.	Growthon on			Hemolysis type	Catalase test	Gram stain and shap cell	Bacitracin disk test
	MaCconky	Blood	Choclet				
1,2, 7	-ve	+ve	+ve	β	-ve	G^{+ve} cocci	Resistant
3,4, 5, 6, 8, 9, 10, 11,12,13	-ve	+ve	+ve	β	-ve	G^{+ve} cocci	Sensitive

IDENTIFICATION OF STREPTOCOCCUS ISOLATES BY STREPTO SYSTEM 9R

According to the Strepto System 9R, the strains of Streptococcus were identified. The results were shown in table 3 and fig. 1.

Isolates no.	Codic no.	Microorganism
1	350	*E.faecalis*
2	340	*E.faecalis*
3,4, 5, 6, 8, 9, 10, 11, 12, 13	102	*S.pyogenes*
7	010	*S.salivarius*

Figure 1. Results of Strepto System 9R, right plat A and B S. faecalis, left plate A and B *S. pyogenes*

MOLECULAR CHARACTERIZATION BY 16Sr RNAGENE

Amplification of the 16S rRNA gene of *S. pyogenes* isolates with primer designated U1and U2 showed these all DNA samples generated a PCR product of the expected size (996 bp) and a nonspecific band of 160 bp was produced (Figure 2).

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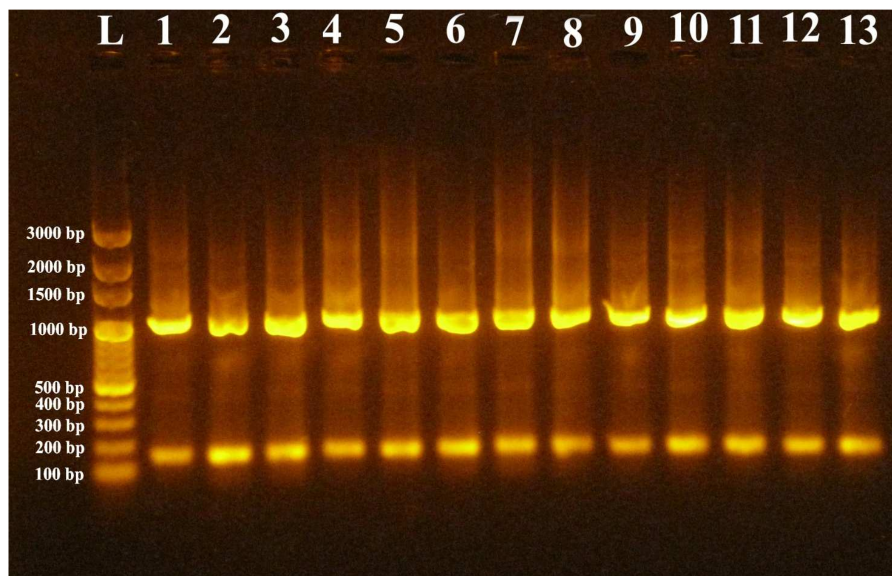

Figure 2. Electrophoresis on 0.8 agarose gel of 16S r RNA gene PCR products: lane L DNA ladder, containing molecular size standards (base pair), lanes 1and 2, *S. faecalis*, lanes 3,4,5,6,8,9,10,11,12 and 13 *S.pyogenes* , lane 7 *S.salivarius*.

The size of amplicons was about 996 bp. A band of approximately 160 bp is also seen, this band may be the result of non specific amplification.

Using universal PCR primer, followed by RFLP considered as simply and sensitively method for detection and identification of bacteria[7] hence by using PCR primer that is targeted at conserved region of 16 S rRNA genes, it is possible detecting DNA of almost any bacteria and identification of the bacterium is done by restriction endonuclease digestion[10] or nucleotide sequencing of the PCR product (Harris & Hartly, 2003)[22]. The advantage of amplification of conserved region in different bacteria by the universal primer compared with using universal PCR technique of DNA sequencing or probe hybridization that used one set of primer instated of used several sets of primer for detection and identification[17].

This study aimed to establish an alternative method of identifying bacteria by means of universal PCR followed by RFLP and compare it with conventional method of culture and identify by strepto 9 reaction systems. Thus The PCR products were digested with HeaIII, *S. pyogenes* produced four bands at 460bp,330bp,135bp and 95bp, while *S. faecalis* produced two bands at 460bp and 135bp, and *S. salivarius* produced two bands at 460bp and 200bp (Fig. 3 & 4) .

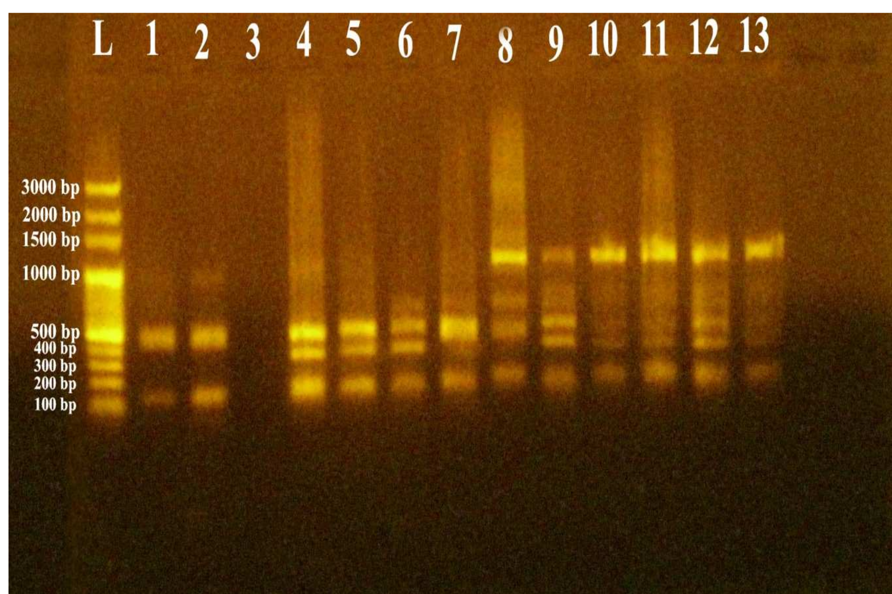

Figure 3. Electrophoresis on 0.8 agarose gel of 16S r RNA gene PCR products RFLP : lane L DNA ladder , containing molecular size standards (base pair), lanes 1 and 2, *S.faecalis*, lanes 3,4,5,6,8,9,10,11,12 and 13 *S. pyogenes*, lane 7, *S. salivarius*.

The PCR product from different species of Streptococcus genus had different restriction patterns, while PCR products from different isolates of the same species had a similar restriction pattern.

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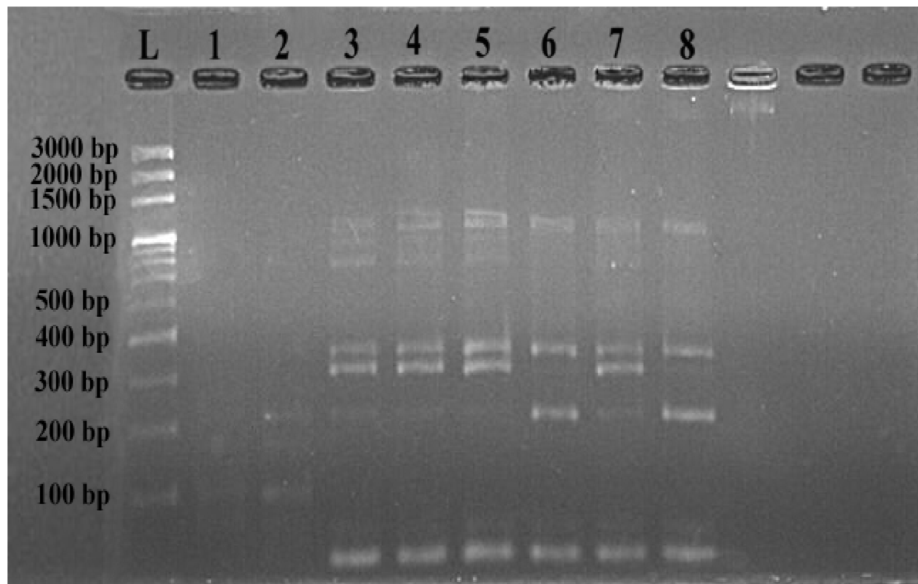

Figure 4. Electrophoresis on 2% agarose gel of 16S r RNA gene PCR products RFLP: lane L DNA ladder, containing molecular size standards (base pair), lanes 1 to 8 *S. Pyogenes*.

Rahmani et al. (2006) was showed there have no change in RFLP patterns of local (Iranian) isolates in comparison with ATCC standard bacteria, thus when compare resulted in recent study with resulted from pervious study, were concluded the RFLP pattern of examined bacteria under study was the same RFLP pattern of ATCC also[17].

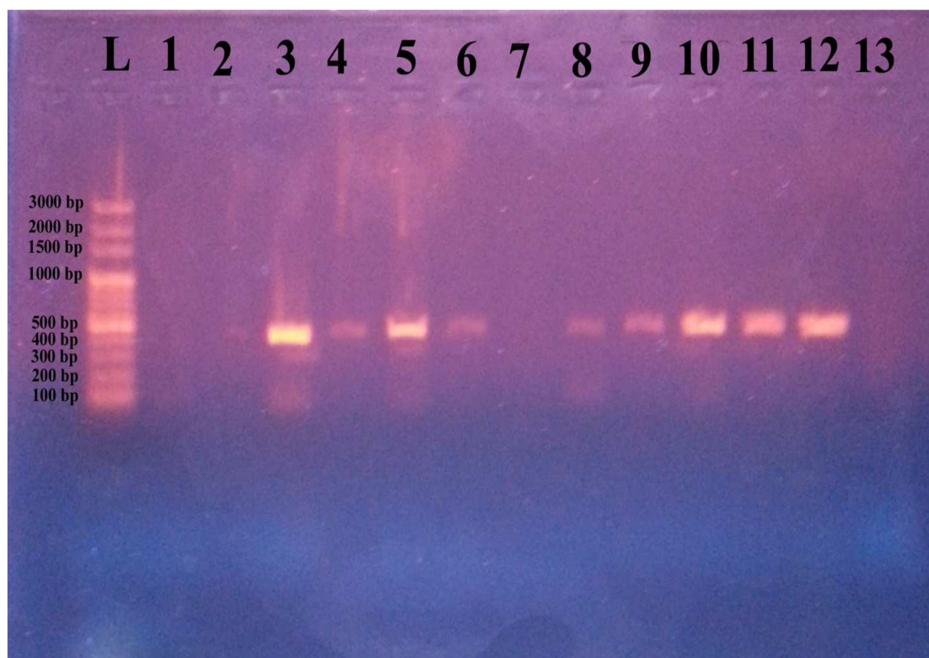

Figure 5. Electrophoresis on 0.8 % agarose gel examination of *S.pyogenes*–specific PCR products generated with primers spy 1258 F/R. lane L DNA ladder, containing molecular size standards (base pair), lanes 1 and 2, *S. faecalis,* lanes 3,4,5,6,8,9,10,11,12 and 13 *S.pyogenes*, lane 7 *S.salivarius*. The size of amplicone about 450 bp .

Using spy1258F and spy1258R in PCR was observed that a specific DNA fragment of the expected size (450bp) was generated from all S *.pyogenes* except one isolate was documented as *S. pyogenes* by biochemical tests and strepto 9 reaction before, but not from non pyogenes *streptococci* (Fig. 5).

This result agreed with each of Liu et al. (2005), Dunne et al. (2013) and Schabauer et al. (2014). All of these studies showed the gene Spy 1258, specific for *S.pyogenes* only, but not from other species of the genus Streptococcus and common bacteria[9,4,19].

When specific primers of the dnase B gene used in PCR amplification, a specific fragment of the 140bp amplicon was generated from all *S. pyogenes* isolates except one isolate, *S. salivarius* and *S. faecalis* didn't generate a PCR product (Fig. 6).

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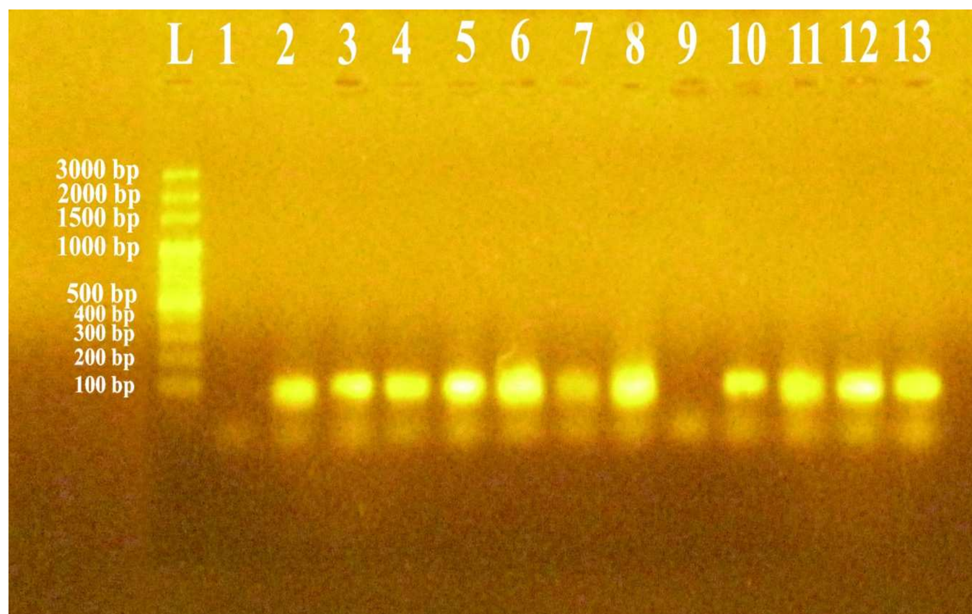

Figure 6. Electrophoresis on 0.8 % Agarose gel of *S. pyogenes* –specific PCR products generated from dnase B gene. lane L: DNA ladder, containing molecular size standards (base pair), lanes 1 and 9 *S. faecalis*, lanes 2, 3, 4, 5, 6, 8, 10, 11,12 and 13 *S. pyogenes*, lane 7 *S. salivarius*. The size of amplicone about 140 bp.

This result confirms a dnase B gene found only in *S. pyogenes* and agreed to Slinger et al. (2011) study, which showed no amplicons that could lead to false positive GAS results[20].

REFERENCES

1.Bernish, B., and van de Rijn, I. Characterization of a two-component system in Streptococcus pyogenes which is involved in regulation of hyaluronic acid production. Journal of Biological Chemistry 1999;274(8):4786-4793. [Original source]

2.Bisno, A., and Stevens, D. *Streptococcus pyogenes* (Including streptococcal toxic shock syndrome and necrotizing fasciitis). Principles and practice of infectious diseases 2000;2:2101-2117.

3.Bruneau, S., De Montclos, H., Drouet, E., and Denoyel, G. rRNA gene restriction patterns of Streptococcus pyogenes: epidemiological applications and relation to serotypes. Journal of clinical microbiology 1994; 32(12):2953-2958. [Pub Med]

4.Dunne, E. M., Marshall, J. L., Baker, C. A., Manning, J., Gonis, G., Danchin, M. H., Smeesters, P. R., Satzke, C., and Steer, A. C. Detection of group a streptococcal pharyngitis by quantitative PCR. BMC infectious diseases 2013;13(1):312. [Pub Med], [Pubmed Central]

5.Hasegawa, T., Minami, M., Okamoto, A., Tatsuno, I., Isaka, M., and Ohta, M. Characterization of a virulence-associated and cell-wall-located DNase of Streptococcus pyogenes. Microbiology 2010;156(1):184-190. [Pub Med]

6.Jenkins, C., Ling, C. L., Ciesielczuk, H. L., Lockwood, J., Hopkins, S., McHugh, T. D., Gillespie, S. H., and Kibbler, C. C. Detection and identification of bacteria in clinical samples by 16S rRNA gene sequencing: comparison of two different approaches in clinical practice. Journal of medical microbiology 2012; 61(Pt 4), 483-488. [Pub Med]

7.Kalghatgi, A., Praharaj, A., Sahni, A., Pradhan, D., Kumaravelu, S., Prasad, P., and Nagendra, A. Detection of bacterial pathogens in cerebrospinal fluid using restriction fragment length polymorphism. Medical Journal Armed Forces India 2008;64(1):29-32. [Original source]

8.Ley, B., Linton, C., Bennett, D., Jalal, H., Foot, A., and Millar, M. Detection of bacteraemia in patients with fever and neutropenia using 16S rRNA gene amplification by polymerase chain reaction. European Journal of Clinical Microbiology and Infectious Diseases 1998;17(4):247-253. [Pub Med]

9.Liu, D., Hollingshead, S., Swiatlo, E., Lawrence, M. L., and Austin, F. W. Rapid identification of Streptococcus pyogenes with PCR primers from a putative transcriptional regulator gene. Research in microbiology 2005;156(4):564-567. [Pub Med], [CrossRef],[CAS], [Web of Science®]

10.Lu, J.-J., Perng, C.-L., Lee, S.-Y., and Wan, C.C. Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. Journal of clinical microbiology 2000;38(6):2076-2080.[Pub Med], [Pub Medcentral]

11.Maslow, J. N., Mulligan, M. E., and Arbeit, R. D. Molecular epidemiology: application of contemporary techniques to the typing of microorganisms. Clinical Infectious Diseases 1993;153-162. [Pub Med]

12.McCabe, KM., Khan, G., Zhang, Y.-H., Mason, E. O., and McCabe, E. R. Amplification of bacterial DNA using highly conserved sequences: automated analysis and potential for molecular triage of sepsis. Pediatrics 1995; 95(2):165-169. [Pub Med]

ISSN NO- 2230 – 7885
CODEN JPBSCT
NLM Title: J Pharm Biomed Sci.

13.Meisal, R., Andreasson, I. K., Høiby, E. A., Aaberge, I. S., Michaelsen, T. E., and Caugant, D. A. Streptococcus pyogenes isolates causing severe infections in Norway in 2006 to 2007: emm types, multilocus sequence types, and superantigen profiles. Journal of clinical microbiology 2010;48(3):842-851. [Pub Med]
14.Nandi, S., Ganguly, N. K., Kumar, R., Bakshi, D. K., Sagar, V., and Chakraborti, A. Genotyping of group A streptococcus by various molecular methods. Indian Journal of Medical Research 2008;127(1):71. [Pub Med]
15.Nilsson, W. B., Paranjype, R. N., DePaola, A., and Strom, M. S. Sequence polymorphism of the 16S rRNA gene of Vibrio vulnificus is a possible indicator of strain virulence. Journal of clinical microbiology 2003;41(1):442-446. [Pub Med]
16.Olsen, G. J., and Woese, C. R. (1993). Ribosomal RNA: a key to phylogeny. The FASEB journal 1993;7(1):113-123. [Pub Med]
17.Rahmani, S., Forozandeh, M., Mosavi, M., and Rezaee, A. Detection of bacteria by amplifying the 16S rRNA gene with universal primers and RFLP. Medical Journal of The Islamic Republic of Iran (MJIRI) 2006;19(4): 333-338. [Original source]
18.Sauer, P., Gallo, J., Kesselová, M., Kolar, M., and Koukalová, D. Universal primers for detection of common bacterial pathogens causing prosthetic joint infection. BIOMEDICAL PAPERS-PALACKY UNIVERSITY IN OLOMOUC 2005;149(2):285-288. [Original source]
19.Schabauer, L., Wenning, M., Huber, I., and Ehling-Schulz, M. Novel physico-chemical diagnostic tools for high throughput identification of bovine mastitis associated gram-positive, catalase-negative cocci. BMC veterinary research 2014;10(1):156. [BMC], [Cross ref]
20.Slinger, R., Goldfarb, D., Rajakumar, D., Moldovan, I., Barrowman, N., Tam, R., and Chan, F. Rapid PCR detection of group A streptococcus from flocked throat swabs: a retrospective clinical study. Ann Clin Microbiol Antimicrob 2011;10(1):33. [Pub Med]
21.Woo, P. C., Fung, A. M., Lau, S. K., Wong, S. S., and Yuen, K.Y. Group G beta-hemolytic streptococcal bacteraemia characterized by 16S ribosomal RNA gene sequencing. Journal of clinical microbiology 2001;39(9):3147-3155.[Pub Med]
22. Harris K. A., Hartley J. C. Development of broad-range 16S rDNA PCR for use in the routine diagnostic clinical microbiology service. J Med Microbiol 2003;52:685–691 10.1099/jmm.0.05213-0 [PubMed], [Cross Ref]

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