ORIGINAL ARTICLE

Bali Medical Journal (*Bali MedJ)* 2022, Volume 11, Number 1: 56-60
P-ISSN.2089-1180, E-ISSN: 2302-2914

Identification of PST 10 bacterial isolate with β-hemolysis characteristic isolated from pig's tonsil

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Received: 2021-12-04
Accepted: 2022-02-07
Published:2022-02-18

ABSTRACT

Background: Pig's tonsil is one of the entry points and habitat of commensal microbes and pathogenic bacteria, both Gram-positive and Gram-negative bacteria. Recently, a human meningitis outbreak has reported at Sibang Kaja, Badung-Bali. The cause of this outbreak has been suspected due to the consumption of pig-derived foods. The outbreak was known to be caused by Gram-positive β-hemolytic bacteria. Based on this fact, PST 10 bacterial isolate with similar characteristics to the outbreak caused interesting identification.
Method: PST 10 isolate was cultivated in a specific medium 5% defibrinated sheep blood agar plate. Subsequently, Gram staining, catalase, oxidase, salt tolerance (6% NaCl), and hemolysis test. This presumptive isolation was then conventionally identified by KIT API 20 STREP and molecularly using the 16S rRNA gene.
Results: PST 10 isolate was identified as *Enterococcus faecium* using KIT API 20 STREP. Furthermore, the 16S rRNA gene sequencing analysis shows that the isolate has 99.6% similarities with *Enterococcus faecalis* (MG543832). The isolate shares the same clade in the phylogenetic tree analysis with a 100% bootstrap value.
Conclusion: The high sensitivity in molecular identification mainly to distinguish close species using phenotypic approaches, PST 10 isolate was concluded as *Enterococcus faecalis*.

Keywords: KIT API 20 STREP, 16S rRNA gene, PST 10 isolate, tonsil of pig, sequencing.
Cite This Article: Suharsono, H., Suardana I.W., Putri, R.K. 2022. Identification of PST 10 bacterial isolate with β-hemolysis characteristic isolated from pig's tonsil. *Bali Medical Journal* 11(1): 56-60. DOI: 10.15562/bmj.v11i1.3180

INTRODUCTION

Pig's tonsil is one of the entry points and colonization of commensal microbes and zoonotic agents.[1] According to the study, pig's tonsil contained several Gram-positive bacteria, such as *Streptococcus* sp, *Enterococcus* sp. *Lactobacillus* sp, and *Staphylococcus* sp.[2] In 1994, *Streptococcus equi* subspecies *zooepidemicus* was identified as the causative agent of the meningitis outbreak. This outbreak resulted in the deaths of thousands of pigs in Bali. This organism was classified as Streptococcus β-hemolytic Group C (SGC), which has zoonotic potency based on its biochemical and serological tests.[3]

In general, the microorganism can be identified conventionally and supported molecularly to ensure the identification. Conventional identification involves culturing the agent in a specific medium. The physiological and biochemical characteristics of the bacteria were then analyzed after being cultured.[4] However, conventional identification was incapable of fastidious microorganisms and classifying species levels.[5] Recently, molecular-based identification methods have been developed to accommodate the limitation of conventional identification. Also, molecular-based technic has rapid speed with high sensitivity and specificity. The 16S rRNA was one of these molecular methods using the gene as a target. It can classify and characterize bacteria specifically.[6] Many organisms have been analyzed successfully using this method, like *Streptococcus equi* subspecies, *Zooepidemicus,*[7] *Escherichia coli* O157:H7,[8,9] *Pasteurella multocida,*[10] and others. Based on this fact, a study on identifying PST 10 isolate with β hemolytic characteristics originated from the pig's tonsil interest to serve.

METHODS

Cultivation of Isolates.

PST 10 isolate was cultured in a 5% defibrinated-sheep blood agar plate. Then, the cultured was incubated for 24 h at 37°C. Subsequently, Gram staining, catalase, oxidase, salt tolerance (6.5% NaCl), and ended by hemolysis tests on some suspected colonies.[11, 12]

Identification of Isolates Using Kit API 20 Strep Test

The cultured bacterial colony on the 5% defibrinated sheep blood agar plate was then transferred into a tube containing 2 ml of distilled water. Subsequently, three suspension drops were placed into each microcapsule of the strips. The suspensions were incubated at 37°C in a normal atmosphere for 4 hours, and the test results were then recorded. The suspension was incubated again at 37oC for 20 h. Furthermore, the test results 4 and 20 h were interpreted according to the manufacturer's profile index and Kit's table.[13]

DNA Extraction and PCR Amplification of 16S rRNA Gene

The universal primer B27F (5'-AGAGTTTGATCCTGGCTCAG-3') and U1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify the 16S rRNA gene. A total of 36 µl reaction volume containing two µl DNA template, one µl (20 pmol/µl) of primer 27F and U1492R, 25 µl My Taq HS Red Mix, and 7 µl distilled water was used in this method. An initial DNA denaturation was performed the PCR amplification at 94°C for 5 min. The process followed 30 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 45 sec, and an extension at 72°C for 1 min. A final extension completed the amplification process at 72°C for 5 min. Furthermore, five µl of PCR product was analyzed by electrophoresis in 1% agarose.[8,9]

Sequencing and Phylogenetic Analysis

ABI Prism 3130 and 3130xl Genetic Analyzer was used to sequence the 16S rRNA gene through the Institute for sequencing service providers at PT Genetika Science, Jakarta. The sequencing process used similar primers with the previous PCR reactions. The sequence results were edited and corrected with the MEGA 5.2 version software (https://www.megasoftware.net/).[9] The edited gene sequences were compared automatically using the BLAST program against the sequences of bacteria available in databanks (www.ncbi.nlm.nih.gov). The neighbor-joining algorithm method was then used to construct the phylogenetic tree.[14]

RESULTS

The cultivation of PST 10 isolate is characterized by white colonies with 1.5 mm x 2 mm diameter and coccus Gram-positive. The catalase and oxidase test results were negative, while the salt tolerance test (6.5% NaCl) was positive, with β hemolysis in the blood agar medium.

Table 1 showed PST 10 isolate positive Acetoin production (VP), Aesculin hydrolysis (ESC), Pyrrolidonylaryl-amidase (PYRA), â-galactosidase (âGAL), Alkaline phosphatase (PAL), Leucine arylamidase (LAP), Arginine dihydrolase (ADH), Ribose fermentation (RIB), Arabinose fermentation (ARA), Mannitol fermentation (MAN), Sorbitol fermentation (SOR), Lactose fermentation (LAC), Trehalose fermentation (TRC), Raffinose fermentation (RAF), Starch fermentation (AMD), Glycogen fermentation (GLYG), and positive â-Haemolysis (â-HAEM). Biochemically analysis showed PST 10 isolate was confirmed as *Enterococcus faecium*.

Furthermore, the molecular method as a confirmation of biochemical test was started by amplifying the 16S rRNA gene before DNA sequencing of the isolate was performed. Figure 1 presents the amplification result of the 16S rRNA gene on 1% agarose.

Figure 1 showed that the 16S rRNA gene was successfully amplified, characterized by 1500 bp of PCR product. The PCR product proceeded to be sequenced. The nucleotide sequences of sequencing were aligned with other sequences deposited in Genbank. Several nucleotides were found similar or different, and those were used to analyze the genetic distance of PST 10 isolate among other strains (Table 2).

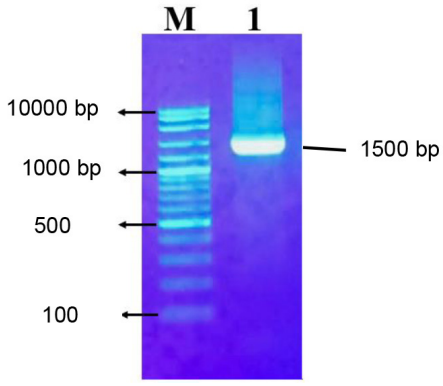

Figure 1. The 16S rRNA gene-amplification result of PST 10 on 1% agarose. M: marker; 1: PST 10 isolate.

Table 1. Identification of PST 10 isolate using Kit API 20 Strep.

Test	VP	HIP	ESC	PYRA	áGAL	âGUR	âGAL	PAL	LAP	ADH
Results	+	-	+	+	-	-	+	+	+	+
Test	RIB	ARA	MAN	SOR	LAC	TRE	INU	RAF	AMD	GLYG
Results	+	+	+	+	+	+	-	+	+	+
Test	â-HAEM									
Results	+									

VP: sodium pyruvate; HIP: hippuric acid; ESC: esculin ferric citrate; PYRA: pyroglutamic acid-β-naphthylamide; αGAL: 6-bromo-2-naphthyl-αD-galactopyranoside; βGUR: naphthol ASBI-glucuronic acid; âGAL:2-naphthyl-βD-galactopyranoside; PAL: 2-naphthyl phosphate; LAP: L-leucine-β-naphthylamide; ADH: L-arginine; RIB: D-ribose; ARA: L-arabinose; MAN: D-mannitol; SOR: D-sorbitol; LAC: D-lactose; TRE: D-trehalose; RAF: D-raffinose; AMD: starch; GLYG: glycogen fermentation (GLYG); â-HAEM: â-Haemolysis.

Table 2 showed PST 10 has a close genetic distance with *Enterococcus faecalis* (MG543832). Only four out of 1000 nucleotides were different from *Enterococcus faecalis* (MG543832). The data in Table 2 proceeded to construct the phylogenetic tree. Figure 2 shows PST 10 share the same clade with *Enterococcus faecalis* (MG543832) with a 100% bootstrap value.

DISCUSSION

According to Kit API 20 Strep, PST 10 isolates have belonged to the *Enterococcus faecium* corresponding on the Profile Index Identification Table available on the Kit. The result was following the study stated that the Enterococcus genus is a group of Gram-positive bacteria D-Streptococcus. This genus exists in the form of a single coccus, paired or short-chain, negative oxidase, negative catalase, non-spore producing, and facultative anaerobes.[15] This organism grows optimally in an environment with 30-37°C, 6.5% NaCl, and pH 9.6. They are also exhibit either α, β, or γ hemolysis.

The identification was followed by molecular identification based on the 16S rRNA gene. The 16S rRNA nucleotide analysis showed PST 10 as an *Enterococcus faecalis*. This result follows a previous study that mentioned that the API 20 STREP biochemical test successfully identified the *E. faecium*. However, the PCR test with 100% specificity identified *Enterococcus faecalis*.[16] This shows that 16S rRNA sequencing analysis is the answer to the need for a more precise and accurate diagnostic method in microbiology to complement conventional biochemical microbiologic methods with several weaknesses.[6]

The 16S rRNA gene sequencing has proven to be a precise and accurate tool in classifying microorganisms, including streptococci and enterococci.[17,18] The ribosomal RNA (rRNA) coding gene is the most conserved gene with a sustainable structure, allowing the 16S rRNA to be used in Polymerase Chain Reaction (PCR) and sequencing analysis.[17] Previous studies recommended a concept of similarity,

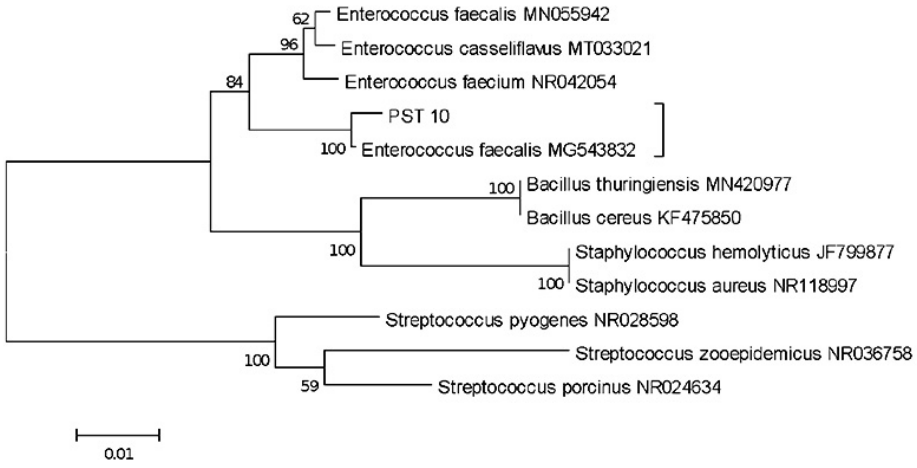

Figure 2. Phylogenetic tree of PST 10 isolates among other bacteria based on 16S rRNA gene. The phylogenetic tree was constructed using the neighbor-joining algorithm of the nucleotides sequence of 16S rRNA gene.[14] The number in the branch of phylogram indicates bootstrap value (%) by 1000-replication multiple, and scale indicates one per 1000 substitutions of nucleotides sequence of 16S rRNA gene.

Tabel 2. The pairwise distance of PST 10 isolates among other isolates deposited in Genbank.

	PST 10	*Enterococcus faecium* (NR042054)	*Enterococcus faecalis* (MN055942)	*Enterococcus faecalis* (MG543832)	*Enterococcus casseliflavus* (MT033021)	*Bacillus thuringiensis* (MN420977)	*Bacillus cereus* (KF475850)	*Streptococcus zooepidemicus* (NR036758)	*Streptococcus pyogenes* (NR028598)	*Streptococcus porcinus* (NR024634)	*Staphylococcus hemolyticus* (JF799877)	*Staphylococcus aureus* (NR118997)
PST 10												
Enterococcus faecium (NR042054)	0.028											
Enterococcus faecalis (MN055942)	0.022	0.008										
Enterococcus faecalis (MG543832)	0.004	0.024	0.021									
Enterococcus casseliflavus (MT033021)	0.027	0.007	0.004	0.025								
Bacillus thuringiensis (MN420977)	0.056	0.053	0.054	0.051	0.053							
Bacillus cereus (KF475850)	0.056	0.053	0.054	0.051	0.053	0.000						
Streptococcus zooepidemicus (NR036758)	0.122	0.109	0.110	0.117	0.112	0.125	0.125					
Streptococcus pyogenes (NR028598)	0.088	0.086	0.083	0.083	0.086	0.112	0.112	0.040				
Streptococcus porcinus (NR024634)	0.099	0.086	0.086	0.094	0.089	0.116	0.116	0.043	0.040			
Staphylococcus hemolyticus (JF799877)	0.066	0.056	0.059	0.062	0.057	0.044	0.044	0.137	0.112	0.116		
Staphylococcus aureus (NR118997)	0.066	0.056	0.059	0.062	0.057	0.044	0.044	0.137	0.112	0.116	0.000	

which include (i) The 16S rRNA gene length should be at least between 500 to 525 bp and ideally 1,300 to 1,500 bp; (ii) The criteria for species identification should have a minimum of >99% similarity and ideally >99.5%.[6] Furthermore, Bosshard also emphasized that the queries were categorized as the same species if the 16S rRNA gene sequences were more than 90% comparable or the nucleotides differ between 14-22 bp queries, or the nucleotide percentage difference is between 1-1.5%.[19]

Enterococcus faecium and *Enterococcus faecalis* are the most prevalent organisms observed in the gastrointestinal flora of warm-blooded animals, including pets, wild animals, and humans.[20,21] The bacterium can contaminate the soil and water.[16] The bacteria appeared extensively in humans and animals, so they are easily isolated from inhabited environments.[22,23] These organisms are characterized by their ability to resist harsh conditions and the presence of a "Janus face" behavior, which allows them to transform from a commensal into a causative agent of invasive infections.[24]

Enterococcus faecalis and *E. faecium* are the two most pathogenic enterococcal species with the highest resistance to desiccation and starvation. These organisms are majorly isolated from the nasal cavity and tonsil piglets. In addition, meningitis, bacteremia, and endocarditis in pigs are also all caused by the enterococcus pathogen.[25] Therefore, careful pathogen detection and early initiation of treatment are essential to prevent the spread of agent.[26]

CONCLUSION

Due to their high sensitivity of molecular identification mainly to distinguish close species using phenotypic approaches, PST 10 isolate with β-hemolysis activity that originated from the surrounding area of human meningitis in Badung Regency was concluded *Enterococcus faecalis*.

FUNDING

The authors are grateful to the Udayana research institution and community service for funding in the form of "the Competitions Grant" with agreement Letter No: B/2049/UN14.4.A/PT.01.05/2020 March 10, 2020.

ETHIC CONSIDERATION

This study has been approved by Faculty of Veterinary Animal Ethics Committees Universitas Udayana.

ABBREVIATIONS

PST 10: Punggul sample tonsil no.10. MEGA: Molecular Evolution and Genetic Analysis.

AUTHOR CONTRIBUTION

All authors have contributed to all processes in this research including preparation, data gathering and analysis, drafting, and approval for publication of this manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the publication of this article

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