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Phenotypic and genotypic of gram-positive coccus β-hemolysis bacteria isolates from tonsil and nasal of pig and antimicrobial susceptibility test against penicillin G and tetracycline

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ABSTRACT

Background: The nasal cavity of a pig serves as an entry point and a habitat for the colonization of commensal microbes and pathogenic bacteria such as bacterial zoonosis. This study aimed to identify the phenotypic and genotypic of Gram-positive β-hemolytic organisms collected from nasal and tonsil swabs of pigs that were collected from the abattoir surrounding outbreak area, complete with testing sensitivity test of the bacterial isolates to Penicillin G and Tetracycline antibiotics.
Material and Methods: Totaling 18 isolates consisting of 9 isolates from nasal and 9 isolates from tonsil of coccus β-Hemolysis bacteria originating from 60 samples were used. These isolates were then conventionally identified, then molecularly using 16S rRNA gene analysis. The sensitivity test was carried out by the Kirby Bauer method following the standards of the Clinical and Laboratory Standards Institute (CLSI).
Results: The results of the phenotypic identification (biochemical test) revealed that 9 out of 18 isolates were identified as Enterococcus spp. Furthermore, the confirmation of isolates by molecular analysis i.e. the 16S rRNA gene showed consistency with the biochemical test, and there was confirmed as Enterococcus faecium and Enterococcus faecalis. The sensitivity test showed as many as 9 isolates resistant to Penicillin G and Tetracycline, 5 isolates were resistant, 1 isolate was sensitive and 3 were intermediates. Moreover, the results of the sensitivity test to Penicillin G and Tetracycline, 5 out of 9 isolates showed resistance to more than one antibiotic.
Conclusion: The phenotypic and genotypic of Gram-Positive Coccus β-Hemolysis bacteria isolates from the tonsil and nasal of pigs showed as Enterococcus faecium and Enterococcus faecalis. The use of antibiotics such as Penicillin G and Tetracycline is not recommended for the treatment of this agent resulting in most of the agents being resistant.

Keywords: *β-Hemolysis, antibiotic sensitivity test, Nasal and tonsil of pig, Penicillin G, Tetracycline*.
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INTRODUCTION

Bacterial diseases infecting pigs generally can attack various organ systems, such as the respiratory tract. Among some of the normal flora bacteria found in the upper respiratory tract of pigs, especially the nasal and tonsils, several bacteria can become pathogenic.[1] Pathogenic bacteria in pigs such as *Enterococcus spp, Streptococcus, etc.* generally, show α, β, and γ hemolysis on blood agar media. Naomi et al., identified 3 out of 24 samples (12.5%) showed α hemolysis, and also 3 out of 24 samples (12.5%) showed β-hemolysis, but none of the isolates showed γ-hemolysis.[2] Another study conducted by Yanti et al. showed as many as 36.4% of isolates were α hemolysis, 54.6% were β-hemolysis, and 9% were γ-hemolysis from all isolates of Streptococcus sp tested.[3] In general, bacteria producing β-hemolysis in blood agar are generally more pathogenic than other hemolysis patterns. It is because more bacterial species produce toxins or toxins that can destroy red blood cells.[3,4]

Bacterial diseases can be treated using antibiotics. The use of antibiotics Tetracycline and Penicillin G are often used in livestock. Besides having a positive impact, unwittingly, the use of antibiotics that are not following procedures can cause sensitivity or resistance problems. It is the main route for the development of resistant bacteria in the environment. The problem is that these resistant genes are transferred from the environment to humans.[5] The food chain acts as a route of transmission of antibiotic-resistant bacteria between animals and the human population. The presence of antibiotic residues in meat when consumed by humans will harm health because bacterial immunity to these antibiotics will appear and the effect will appear as resistance or

reduced human immunity to disease.[5]

The problem of antimicrobial resistance against pathogenic bacteria will continue. Wardoyo et al. identified an increase in the susceptibility of *E. coli* to 10/16 antibiotics. The prevalence of extended-spectrum beta-lactamase (ESBL)-producing *E. coli* in group A was 50% and in the group, B was 20.9% with a significantly different ($p<0.05$).[6] Suardana et al. also reported all isolates of Gram-positive α hemolysis resistant to Penicillin G and 11.1% resistant to Tetracyclin.[7,8] This study aimed to identify the phenotypic and genotypic of Gram-positive β-hemolytic organisms collected from nasal and tonsil swabs of pigs that were collected from the abattoir surrounding outbreak area, complete with testing sensitivity test of the bacterial isolates to Penicillin G and Tetracycline antibiotics.

METHODS

Phenotypic Identification of Isolates

Cultivation of Isolates on Blood Agar Media

The cultivation of bacterial isolates used research isolates stock Pinatih *et al*[2,9] that were then grown on blood agar media. Bacterial isolates were taken using a sterile tube and then scratched on the surface of the blood media. Thus, 1 sachet of anaerobic Kit was added and incubated at 37°C for 24 hours.

Gram Staining

Gram staining was carried out by coloring the preparations that had been fixed using crystal violet, dried and then washed with running water, then dropped with Lugol's liquid and left for 1 minute. It was washed again with running water, then dripped with acetone alcohol and dried, then washed with running water. Furthermore, it was stained with safranin staining for 30 seconds, then washed again with running water. The staining results could be seen under a 1000x magnification microscope with the help of immersion oil.[9,10]

Catalase Test

The catalase test was conducted by taking 20 µl of the colony culture that had been grown on Brain Heart Infusion (BHI) media and placing the colonies on a glass object that was then dripped with 20 l of H2O2. A positive result was indicated by the formation of bubbles.[9,10]

Oxidase Test

Colonies were taken from the Nutrient Agar (NA) slanted agar media using a sterile loop. It was inscribed on the oxidase strip paper. The color change on the oxidase paper was observed. The interpretation of the positive oxidase test results was indicated by the formation of a purple color.[9,10]

Salt Tolerance Test

This test began with the inoculation of bacterial isolates in Brain Heart Infusion (BHI) media containing 6.5% NaCl. Hereafter, the media was incubated for 24 hours at 37°C. After being incubated for 24 hours, if it showed positive results on BHI media, there would be a color change from clear yellow to cloudy that was visually observed.[9,10]

Genotypic Identification of Isolates

DNA isolation

DNA from all isolates was extracted using the Presto™ Mini gDNA Bacteria Kit (GBB100/101, GBB300/301) according to the manufacturer's procedure.[11,12]

PCR Test of The 16S rRNA gene of Isolates

The PCR reaction was made in 34 µl volume with the composition 7 µl dH2O, 25 µl My Taq Red, 1 µl Primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 1 µl primer 1492R (5'-GGTTACCTTGTTACGACTT-3'), and 2 µl template DNA. Thermal cycler conditions were treated with initial parameters at 94°C for five minutes, denatured at 94°C for a minute, annealing at 45°C for 45 seconds, 72°C extension for one minute and one minute, the cycle was repeated 30 times, and post-extension at 72°C for 5 minutes. Each primer was calibrated first to determine the appropriate annealing temperature.[7]

Sequencing and Phylogenetic Analysis

DNA sequencing was conducted in Jakarta through PT Genetika Science Indonesia. The sequencing results were analyzed using the MEGA X program. The nucleotide sequences were aligned with Clustal W from the MEGA X program. The data analyzed were confirmation of the sequencing results with the Basic Local Alignment Search Tool (BLAST), and phylogenetic analysis with nucleotides and amino acids available at GenBank. Kinship relationships (genetic and phylogenetic distance estimates) were analyzed using the Neighbor-Joining method.[13]

Antibiotic Sensitivity Test

Isolates incubated for 24 hours into BHI were vortexed to homogenize. The bacterial suspension was then equated for clarity with the standard turbidity of McFarland's solution no. 0.5. Then, it was vortexed again to mix. After being clear, the colonies were taken using a sterile cotton swab and then rubbed evenly on Mueller Hinton Agar media. After that, the antibiotic disk to be tested (Penicillin and Tetracycline) was placed on Mueller Hinton Agar media and incubated for 24 hours at 37°C. Observations on Mueller Hinton Agar media were conducted by measuring the killing zone formed around the antibiotic disk.[6]

RESULT

Phenotypic Identification of Isolates

Phenotypic identification is based on observations of colony morphology, microscopic observations, and biochemical tests[4] This research used existing isolates and then they were grown on blood agar media. 18 isolates were obtained. The results of cultivation on blood agar media from 18 isolates revealed that 9 isolates showed α-hemolysis and 9 other isolates showed β-hemolysis which can be seen in Table 1.

The shape of bacteria in all isolates was coccus and clustered which could be seen through a microscope and interpreted in Table 2.

The biochemical tests conducted in this research included the catalase test, oxidase test, and salt tolerance test which can be seen in Table 3.

Based on the data in Table 3, the results of the catalase test revealed that the 9 isolates of nasal origin showed negative results, while the tonsil isolates showed 1 positive and 2 negatives. The oxidase test results from tonsil and nasal nine isolates showed negative results and the salt tolerance test results showed positive results.

Table 1. Results of tonsil and nasal bacterial isolates cultivation on blood agar media

Tonsil Isolates	Result	Nasal Isolates	Results
TMT 1	α	TMN 1	α
TMT 2	α	TMN 2	α
TMT 3	α	TMN 3	β
TMT 4	α	TMN 4	β
TMT 5	α	TMN 5	α
TMT 6	β	TMN 6	β
TMT 7	β	TMN 7	β
TMT 8	β	TMN 8	β
TMT 11	α	TMN 9	β

Table 2. Gram staining results of β-hemolysis bacteria

Code Isolates	Isolates	Gram Staining	Shape
TMT 6	Tonsil	+	Coccus
TMT 7	Tonsil	+	Coccus
TMT 8	Tonsil	+	Coccus
TMN 3	Nasal	+	Coccus
TMN 4	Nasal	+	Coccus
TMN 6	Nasal	+	Coccus
TMN 7	Nasal	+	Coccus
TMN 8	Nasal	+	Coccus
TMN 9	Nasal	+	Coccus

Table 3. Test results of catalase, oxidase, and salt tolerance of β-hemolysis bacteria

Code Isolates	Isolates	Catalase	Oxidase	Salt tolerance
TMT 6	Tonsil	-	-	+
TMT 7	Tonsil	-	-	+
TMT 8	Tonsil	+	-	+
TMN 3	Nasal	-	-	+
TMN 4	Nasal	-	-	+
TMN 6	Nasal	-	-	+
TMN 7	Nasal	-	-	+
TMN 8	Nasal	-	-	+
TMN 9	Nasal	-	-	+

Genotypic Identification of Isolates

Genotypic identification was conducted using molecular analysis, through 16S rRNA gene sequencing using the Polymerase Chain Reactions (PCR) method. The result of the identification in the form of electrophoresis result is shown in Figure 1.

The data analyzed were confirmation of the sequencing results with the Basic Local Alignment Search Tool (BLAST) program, and then phylogenetic analysis using nucleotides and amino acids available in GenBank. The data from the sequences were used as a reference in downloading secondary data at GenBank. Downloaded secondary data were accompanied by a description of the access code (accession number) and Percent Identity. The nucleotide sequences were aligned with Clustal W from the MEGA X program.[13] The similarity percentage of the 16s rRNA gene to several isolates in Genbank is shown in Table 4. And their pairwise distance of isolates is shown in Table 5.

Based on the data in Table 4 and Table 5, the TMT 7 isolate had a similarity value of 98.36% with the Enterococcus faecalis strain (MT611645) and the difference value was 7 out of 1000 nucleotides. The TMT 8 isolate had a similarity value of 95.65% with the Enterococcus faecalis strain (HM480367) and the difference value was 0 out of 1000 nucleotides. The TMN 8 isolate had a similarity value of 98.17% with the Enterococcus faecium strain (MW330396) and the difference value was 11 out of 1000 nucleotides. The TMN 9 isolate had a similarity value of 95.65% with the Enterococcus faecalis strain (HM480367) and the difference value was 8 out of 1000 nucleotides. Based on Janda and Abbott[14] the criteria used in species identification are considered to be similar if the sequence similarity level is >99% or ideally >99.5%. For a match to the next closest species with a distance score of <0.5%, other traits, including phenotype, should be considered at the final stage of species identification. The data in Table 5 is analyzed to construct a phylogenetic tree and the results are shown in Figure 2.

According to the data in Figure 2, the phylogenetic tree shows that the isolates TMN 3 and TMN 7 were in the same clan with a bootstrap value of 65%. The isolates of TMN 9 and Enterococcus faecalis MT611645 were in the same clan with a bootstrap value of 99%. TMT 7 isolates formed their clan. Yet, it still has a close relationship with TMN 9 and Enterococcus faecalis MT611645 with a bootstrap value of 73%. These results were close to the previous study by Pinatih who found the PSN 2 and PSN 9 Gram-positive isolates with β-hemolysis activity from pig nasal swabs were identified as *Enterococcus faecium*.[9]

The Antibiotic Sensitivity Test of Isolates

The illustration of the sensitivity pattern of the isolates from tonsil and nasal to Penicillin and Tetracycline antibiotics on Muller Hinton Agar media can be seen in Figure 3. The complete result data of the 9 isolates tested can be seen in Table 6.

DISCUSSION

Based on the results of the cultivation of bacterial isolates on blood agar media, colonies showing β-hemolysis pattern were taken for further Gram staining. Gram staining is a method to differentiate bacteria into Gram-negative bacteria or Gram-positive bacteria and to see the shape of these bacteria. Gram-negative bacteria are bacteria that do not retain methyl purple dye in the Gram staining method. Therefore, these bacteria will be pink in color while Gram-positive bacteria will retain methyl purple dye during the

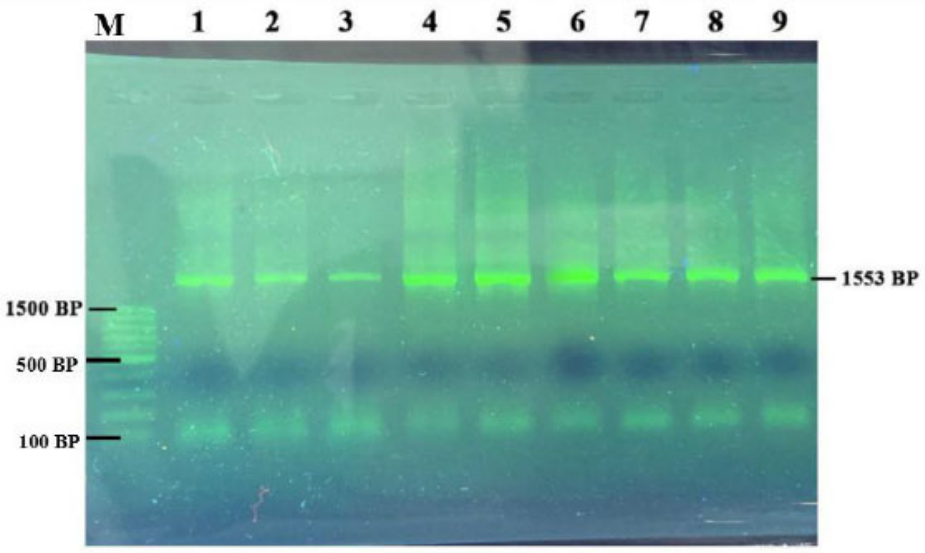

Figure 1. The PCR electrophoresis results of all isolates used primers B27F and U1492R. 1% agarose electrophoresis visualized on UV, the marker was a 100-bp ladder. A single DNA band from each sample at a position of about 1553 bp. Notes: 1. TMT 6; 2: TMT 7; 3: TMT 8; 4: TMN 3; 5: TMN 4; 6: TMN 6; 7: TMN 7; 8: TMN 8; 9: TMN 9.

Table 4. The results of the similarity percentage of the 16s rRNA gene to several isolates in Genbank

No	Code Isolates	Percent Identity	Species of bacteria	Accession Numbers
1	TMT 6	90.54%	Enterococcus faecalis	MT611645
2	TMT 7	98.36%	Enterococcus faecalis	MT611645
3	TMT 8	99.91%	Enterococcus faecalis	MT611645
4	TMN 3	93.04%	Enterococcus faecium	MN533909
5	TMN 4	98.64%	Enterococcus faecalis	MT611645
6	TMN 6	93.81%	Enterococcus faecalis	KX752885
7	TMN 7	95.65%	Enterococcus faecalis	HM480367
8	TMN 8	98.17%	Enterococcus faecium	MW330396
9	TMN 9	98.80%	Enterococcus faecalis	MT611645

Description: Percent Identity and Accession Number obtained from the NCBI (National Center for Biotechnology Information) website.

Gram staining process. The difference in classification between the two types of bacteria is based on differences in the structure of the bacterial cell wall.[4]

In blood agar, β -hemolysis is a complete lysis of red blood cells and hemoglobin to produce a clear zone around the colony. Gram-positive coccus bacteria that produce β-hemolysis on blood agar media were the *Enterococcus spp. group; Enterococcus faecalis, Enterococcus avium, Enterococcus casseliflavus, Enterococcus cecorum, Enterococcus durans, Enterococcus flavescens, Enterococcus gallinarum, Enterococcus malodoratus, and Enterococcus raffinosus*.[17]

The principle of the oxidase test is to determine whether an organism has a cytochrome oxidase enzyme that will later be tested using oxidase strip paper. This color change is caused by cytochrome oxidase oxidizing the oxidase reagent solution.[18] The test reagent, Tetra-methyl-p-phenylenediamine dihydrochloride (TMPD) plays a role as an artificial electron acceptor for the oxidase enzyme. The reagent is oxidized to form a purple-colored compound.[18]

The salt tolerance test aims to determine the ability of an organism to grow in the preparation of 6.5% NaCl. Positive results are indicated by a change in color from clear yellow to cloudy which is visually observed.[19] Several species such as Pediococcus, Leuconostoc, and β -hemolytic Streptococcus can grow in BHI media. Enterococcus and Aerococcus species usually give positive results after 24 hours of incubation at 37°C.[20]

Furthermore, analysis of the 16S rRNA gene in bacterial identification has a high level of accuracy and effectiveness and a short time in the identification process, especially when compared to conventional methods.[7] Its large size (1,500 bp) is also considered advantageous for informatics purposes.[16] The results of the molecular test confirmation using the DNA sequencing method for the 9 isolates were confirmed as *Enterococcus spp. Enterococcus spp. Enterococcus spp*. is a genus of lactic acid bacteria in the phylum Firmicutes. *Enterococcus spp.* is a Gram-positive coccus that often appears in pairs (diplococci) or short chains, can grow on nonselective media such as blood agar, grows well in 6.5% NaCl, and is catalase negative and oxidase negative. These bacteria are normal flora of the Gastrointestinal (GI) in mammals and warm-blooded animals, the urinary tract, and can also colonize the oral cavity.[21] *Enterococcus spp*. infections can be in the form of urinary tract infections, and opportunistic infections including intra-abdominal infections, septicemia, and endocarditis.[22]

The limitation of 16S rRNA sequences in the identification of bacteria showed that in some genera, there were areas of 'blindspots', where the 16S rRNA sequences did not have sufficient differences for the identification of certain species. In these circumstances, alternative targets should be investigated. In addition, if an individual wants to compare strains for epidemiological purposes or to detect strains possessing specific virulence factors, analysis of the 16S rRNA gene is also inadequate because it does not have sufficient variation, and this region does not encode virulence factors.[23]

The results of the biochemical tests conducted were directly proportional to the results of molecular confirmation. However, one of the nine isolates was found to be positive in the catalase test. *Enterococcus spp*. generally produces catalase-negative, but some of these bacteria can produce peroxidases that

Table 5. Pairwise distance analysis results of all isolates and others obtained from GenBank

	TMT_6	TMT_7	TMT_8	TMN_3	TMN_4	TMN_6	TMN_7	TMN_8	TMN_9	Enterococcus_faecalis_MT611645	Enterococcus_faecium_MN533909	Enterococcus_faecalis_KX752885	Enterococcus_faecalis_HM480367	Enterococcus_faecium_MW330396	Streptococcus_zooepidemicus_NR_036758	Streptococcus_pyogenes_NR_028598	Streptococcus_porcinus_NR_024634	Streptococcus_agalactiae_NR_040821	Staphylococcus_haemolyticus_D83367	Staphylococcus aureus_NR_119997
TMT_6																				
TMT_7	0.152																			
TMT_8	0.142	0.006																		
TMN_3	0.359	0.056	0.046																	
TMN_4	0.131	0.011	0.003	0.032																
TMN_6	0.136	0.031	0.029	0.027	0.035															
TMN_7	0.189	0.050	0.043	0.026	0.049	0.056														
TMN_8	0.176	0.020	0.012	0.025	0.012	0.025	0.029													
TMN_9	0.153	0.009	0.007	0.052	0.017	0.044	0.057	0.025												
Enterococcus faecalis MT611645	0.145	0.007	0.003	0.052	0.012	0.037	0.052	0.018	0.006											
Enterococcus faecium MN533909	0.148	0.017	0.014	0.044	0.018	0.034	0.047	0.011	0.032	0.036										
Enterococcus faecalis KX752885	0.139	0.009	0.001	0.048	0.006	0.030	0.041	0.012	0.013	0.016	0.039									
Enterococcus faecalis HM480367	0.141	0.008	0.000	0.046	0.006	0.030	0.043	0.012	0.008	0.009	0.025	0.007								
Enterococcus faecium MW330396	0.147	0.017	0.013	0.044	0.017	0.033	0.046	0.011	0.032	0.038	0.017	0.041	0.029							
Streptococcus zooepidemicus NR 036758	0.200	0.091	0.075	0.109	0.074	0.092	0.113	0.083	0.114	0.116	0.109	0.111	0.105	0.112						
Streptococcus pyogenes NR_028598	0.178	0.076	0.063	0.097	0.055	0.074	0.089	0.063	0.113	0.111	0.107	0.107	0.103	0.106	0.046					
Streptococcus porcinus NR_024634	0.182	0.079	0.065	0.100	0.060	0.075	0.092	0.071	0.114	0.121	0.113	0.112	0.098	0.117	0.035	0.045				
Streptococcus agalactiae NR_040821	0.175	0.073	0.061	0.097	0.055	0.072	0.084	0.064	0.107	0.111	0.099	0.103	0.090	0.109	0.043	0.038	0.023			
Staphylococcus haemolyticus D83367	0.163	0.056	0.055	0.052	0.057	0.058	0.072	0.029	0.082	0.086	0.076	0.089	0.078	0.079	0.129	0.122	0.134	0.127		
Staphylococcus aureus NR_119997	0.163	0.054	0.055	0.050	0.058	0.058	0.072	0.029	0.082	0.086	0.074	0.088	0.075	0.091	0.126	0.123	0.134	0.125	0.009	

slowly catalyze the breakdown of H_2O_2 and the test may appear weakly positive. The enzyme responsible for this catalase activity has not been demonstrated and is often associated with pseudo-catalase.[24]

The results of the sensitivity test toward Penicillin antibiotics showed that 9 isolates were resistant to Penicillin G (100%). It was also found in the research of Mohanty *et al.*[25], Enterococcus spp. was resistant to Penicillin G (100%) and in the research of Di Rosa *et al.*, who found Enterococcus faecalis showed resistance to β -lactam antibiotics (95%).[26] According to Munita and Arias[27], resistance to penicillin is caused by the formation of an enzyme that destroys penicillin, such as the β -lactamase enzyme. This enzyme will cause the opening of the β-lactam ring on the penicillin so that it destroys the antimicrobial activity.

Enterococcus faecalis can enter the Viable but Non-Culturable (VBNC) phase. In this state, the bacteria lose the ability to grow and develop but remain alive and are pathogenic. In this VBNC condition, *Enterococcus faecalis* can be elongated, coco bacillary shaped with an uneven surface, there is an increase in the production of Penicillin Binding Protein (PBP) when produced in large quantities, and they can cause resistance to penicillin.[28] The initial stage of the action of this antibiotic starts from the binding of the drug to the bacterial cell receptor, namely to the penicillin-binding proteins (PBPs). After the drug is attached to one or more receptors, the transpeptidation reaction will be inhibited and further peptidoglycan synthesis will be inhibited. The further stage is the inactivation and loss of inhibitors of autolytic enzymes in the cell wall. As a result, the activation of lytic enzymes will cause bacterial lysis.[28]

Tetracycline is an antibiotic. It is often used by farmers because it is a broad-spectrum antibiotic and can inhibit bacterial protein synthesis.[29] The results showed that 5 of 9 isolates were resistant to Tetracycline, 1 was sensitive and 3 were intermediates. Tetracycline binds to the 30S subunit of the microbial ribosome. Furthermore, it will inhibit the synthesis through the inhibition of the attachment of aminoacyl-tRNA. As a result, there will be an inhibition in the introduction of newly formed amino acids in the peptide chain. Tetracycline resistance occurs due to inhibition of the translational and transcriptional stages of genetic material.[30] The intermediate value of Tetracycline may be due to the transfer of plasmids from resistant bacteria to sensitive bacteria. This can occur when bacteria that were originally sensitive are exposed to drugs.[31] The widespread and irrational use of Tetracycline in the community can cause exposure to pathogenic bacteria by antibiotics to become resistant.[32]

The distribution of antibiotic sensitivity and resistant strains varies between countries.[33] The pattern of use of antibiotics in animal husbandry varies across regions and in developing countries, even antibiotics that have been banned in other countries including developed countries are still used in most developing countries.[34] The choice of antibiotics and patterns of antimicrobial use reflect varying geographic distributions across continents influenced by animal species, intensive or extensive agriculture, agricultural purposes (commercial/industrial/domestic), and lack of a clear legislative or policy framework on antibiotic use.[35–37]

The results of the sensitivity test toward Penicillin and Tetracycline antibiotics in this research revealed that 5 of 9 isolates showed a pattern of resistance to more than 1 antibiotic. Multi-Drug Resistance (MDR) is a term for a bacterium that is resistant to 2 or more antibiotics. Many bacteria exhibit multiple drug resistance, including Staphylococci, Enterococci, Gonococci, Streptococci, and Salmonella, as well as many other Gram-negative bacteria and Mycobacterium tuberculosis. Some resistant bacteria can transfer DNA copies of their resistance mechanisms to other bacteria. This process is called horizontal gene transfer. Horizontal gene transfer (HGT) is the transfer of genetic information between organisms, a process that includes the spread of antibiotic resistance genes between bacteria (except genes from parent to offspring) and triggers the evolution of pathogens. Once transferred, pathogenic genes continue to evolve, often resulting in bacteria with greater resistance. There are several mechanisms for horizontal gene

transfer, namely transformation. It is a genetic change in cells resulting from the introduction, absorption, and expression of foreign genetic material (DNA or RNA). Transduction is the process in which bacterial DNA is transferred from one bacterium to another by a virus (bacteriophage). Conjugation is a process that involves the transfer of DNA through a plasmid from a donor cell to a recombinant recipient cell during cell-to-cell contact.[38]

CONCLUSION

The results of the phenotypic identification (biochemical test) in this research reveal 9 out of 18 isolates show Enterococcus spp. Furthermore, after confirmation by genotypic identification (molecular analysis) with the 16S rRNA gene, the results are consistent and confirmed as Enterococcus faecium and Enterococcus faecalis. The sensitivity pattern of 9 isolates showed resistance to Penicillin G and the sensitivity pattern to Tetracyclines showed 5 isolates were resistant, one isolate was sensitive and 3 isolates were intermediates. Hereafter, the result of the antibiotic sensitivity test of Penicillin and Tetracycline, 5 of 9 isolates show a pattern of resistance to more than one antibiotic.

CONFLICT OF INTEREST

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

ETHICAL CONSIDERATION

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

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AUTHOR CONTRIBUTIONS

All authors have contributed to all processes in this research including preparation, data gathering and analysis,

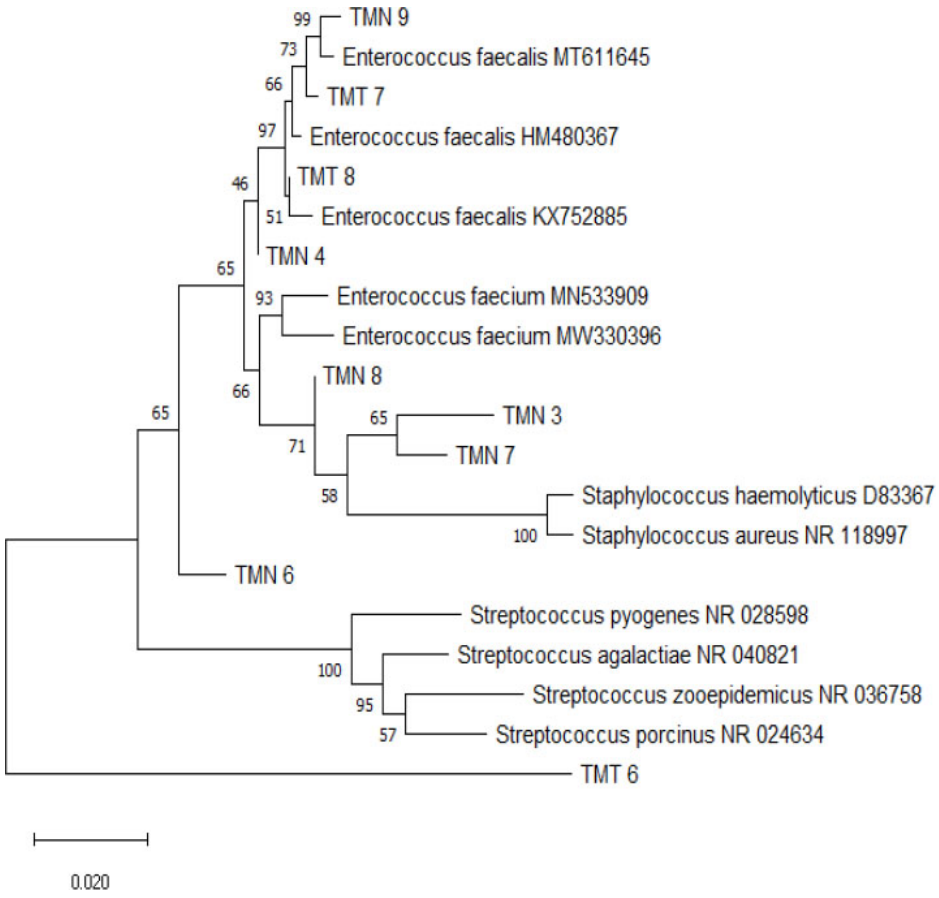

Figure 2. The results of the phylogenetic analysis of all isolates and others are based on the 16S rRNA gene sequence. Phylogenetic trees used a neighbor-joining tree[15] nucleotide sequence of the 16S rRNA gene. The numbers on the phylogram branches show the bootstrap value (%) with 1000 multiple replications, and the scale shows one per 1000 nucleotide sequence substitution of the 16S rRNA gene.

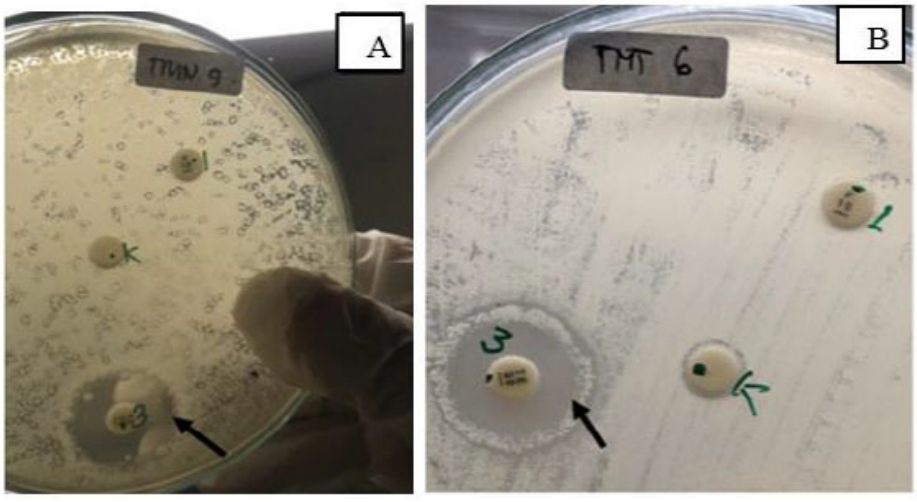

Figure 3. The results of the antibiotic sensitivity test were isolated on Muller Hinton media agar. Description: A= isolated TMN 9; B= isolated TMT 6; K = negative control; 1=Penicillin (10 units); 3= Tetracyclines (30 µg); The arrow indicates the killing zone.

Table 6. Test results of sensitivity to the antibiotics Penicillin and Tetracycline

No	Code Isolates	Isolates	Antibiotics	Diameter Zone (mm)			Result
				1	2	Average	
1	TMT 6	Tonsil	Penicillin G	0	0	0	R
			Tetracycline	15	17	16	I
2	TMT 7	Tonsil	Penicillin G	0	0	0	R
			Tetracycline	14	12	13	R
3	TMT 8	Tonsil	Penicillin G	0	0	0	R
			Tetracycline	11	17	14	R
4	TMN 3	Nasal	Penicillin G	0	0	0	R
			Tetracycline	16	15	15.5	I
5	TMN 4	Nasal	Penicillin G	0	0	0	R
			Tetracycline	13	11	12	R
6	TMN 6	Nasal	Penicillin G	0	0	0	R
			Tetracycline	14	13	13.5	R
7	TMN 7	Nasal	Penicillin G	0	0	0	R
			Tetracycline	10	12	11	R
8	TMN 8	Nasal	Penicillin G	0	0	0	R
			Tetracycline	20	19	19.5	S
9	TMN 9	Nasal	Penicillin G	0	0	0	R
			Tetracycline	12	19	15.5	I

Description: R: Resistant, S: Sensitive, I: Intermediate. Determination of resistance, sensitivity, and intermediates based on clinical and laboratory standards institute (CLSI).[16]

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