Molecular Identification Beta Hemolysis Isolates Species from Contact Lens Cleaner Residual Solution

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Abstract. A contact lens cleaner solution should not be left in place for storage and reuse for subsequent storage. The cleaner that has been used does not have enough disinfectant power, even becomes a place of microorganism growth so the risk of infection increases. The study aims to identify the bacterial isolates of the beta hemolysis contained in the residual cleaning fluid the lens contact. The sample in this research was taken from all students in the STIKES Mandala Waluya Kendari who wear contact lenses as many as 10 people. The suspension of bacteria obtained is then planted on media BA (Blood agar) with the streak plate method. Isolates showing beta hemolysis then identified using amplification gene 16S rRNA using primers universal primers 27 F/1495 R, the results of the amplification electrophoresis with agarose of 1.5% and subsequent sequencing on the gene 16S rRNA. Based on the results of this study show the 10 isolates, namely W1, W2, W3, W4, W5, W6, W7, W8, W9, and W10 showed positive results which are characterized by the growth of bacteria on BA medium and there is one isolate of beta hemolysis namely sample W6. Molecular identification has shown that isolates W6 shared a 93% percent identity with the species *Bacillus* sp.

Keywords: Contact Lens · Beta hemoliysis · Molecular Identification

1 Introduction

A Contact lens is a tool that laid on the surface of the cornea to resolve the refractive disorder. Currently, contact lens users in Indonesia increased by more than 15% per year. With the increasing number of users, contact lenses complications are also increasing. As much as 4–10% of contact lens users experience complications from mild irritation to the blind [1]. A contact lens solution has an expiration period from 2–6 months after the bottle is opened. When it has expired, the contact lens solution should not be re-used [2].

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Pathogenic bacteria can be found in the contact lens solution that causes eye infection, increase developing keratitis, ulcer of the cornea until blindness. This is because most users using contact lens cleaning solution, not by following procedures like lazy to do the cleaning of the place and replace the cleaning solution [3].

In general, the microorganisms found in contact lens cleaning solution namely Acanthamoeba, Pseudomonas, *Staphylococcus* [4]. Murugan [5] found microorganisms in the contact lens cleaning solution, from 40 samples 22.5% contained bacteria that are normal flora, and 35.0% are potential pathogens bacteria. One of the media used to cultivate and isolate bacteria pathogens is Blood Agar (BA). BA is a growth medium for bacteria that can distinguish pathogenic bacteria based on the effect of bacteria hemolytic exotoxin on red blood cells. Three types of hemolysis that is alpha hemolysis, beta hemolysis, and gamma hemolysis. Alpha hemolysis is bacteria that showed a decline in the hemoglobin of red blood cells around the colony so that the perimeter of the bacteria will appear the color green or brown in the media. Beta hemolysis is bacteria that showed perfect lysis with the appearance of transparent color around bacteria in the medium. Gamma hemolysis bacteria is bacteria that showed a lack of signs hemolysis existing in the media. Hemolysis caused by bacteria is always connected with the ability of germs to cause infection [6].

Habiburrahman research results [7] identified contact lens cleaning fluid samples by bacterial culture, gram staining and Biochemical tests, which obtained *Bacillus subtilis* (22%), *P. aeruginosa* (11%), *S. aureus* (8%), *Serratia.sp* (7%), *Klebsiella. Sp* (7%) and *E.coli* (2%). The same study was also conducted by Agrimunury [8] and Murugan [5] using bacteriological testing, while Mardhiah [9] and Ardiyanti & Amelia [10] tested the inhibitory power of contact lens fluid on the growth of *S. aureus* and *P. aeuroginosa.*

Therefore this study, tested with a diagnosis of the presence of pathogenic bacteria using the PCR (*Polymerase Chain Reaction*) method and proceed with sequencing method, to find out more about pathogenic bacterial species, because of the advantages of this sequencing method is able to offer accurate data through homology testing which is better for existing characters, and provides many character states because the difference in the rate of change of nucleotide bases in different loci is large, and is proven to produce a more natural kinship relationship (natural).

Identification proses of pathogenic bacteria can be done utilizing observation of the organism both in morphology and physiology. Morphology observation includes colonies form, colony structure, cell shape, cell size, flagellum shape, and endospore staining from bacteria. Physiology observation includes biochemical tests [11]. Besides, bacteria identification can also be performed utilizing genetically identification, i.e. by isolating the DNA of the bacterial chromosome, and then continue with the Polymerase Chain reaction method (PCR). PCR results further identified by agarose gel electrophoresis to understand the size of the DNA. The results of the electrophoresis will show the characteristics of the DNA. Nucleotide sequences subsequently were sequenced by the dideoxy Sanger method [12].

2 Methods

2.1 Bacterial Culture

Bacteria suspension originating from the contact lens cleaner residual solution was cultured on BA media using the streak plate method. Then incubated at a temperature of 37 °C for 24 h. After incubated, hemolysis occurred and the blood was observed. Isolates are said to be positive if there are growth and hemolysis occurred in BA media such as beta hemolysis, alpha hemolysis, or gamma hemolysis. While isolates are said to be negative if there is no growth in BA media. Isolates that meet the criteria beta hemolysis will be followed for the molecular identification.

2.2 Molecular Identification of Beta Hemolysis Isolates

Beta hemolysis isolates were identified using the 16S rRNA gene. Isolates were extracted by taking 2 ose pure culture of bacteria, then added 100 μL of distilled water. Vortex briefly the sample then centrifuged for 30 s [13]. PCR amplification for Fragments 16S rRNA genes was attempted with universal primers. Primer forward using the 27f (5′-AGAGTTTAGTCCTGGCTCAG-3′) and primer reverse using 1495r (5′-ACGGCTACCTTGTTAGGACTT-3′) are complementary to the ends of the gene 16S rRNA of all strains. PCR products generated an amplicon of approximately 1500 bp. PCR conditions included a pre-denaturation at 94 °C (3 min), denaturation at 94 °C for 60 s, primer annealing at 52 °C for 60 s, extension at 72 °C for 60 s during 30 cycles and a final extension at 72 °C for 5 min. PCR completion reaction mixtures were stored at 4 °C until electrophoresis was performed. PCR products were analyzed by agarose gel 1.5% gel agarose in the current of 100 V for 30 min. The gels were stained with floro safe and DNA bands were visualized under UV light using a UV transilluminator. PCR products were sequenced by the chain termination method using a Genetic Analyzer. The sequencing results are entered in the algorithm program namely BLAST in NCBI, for searching homologs sequence in the Genebank. Phylogenetic trees constructed using the program MEGA6. Database search and comparison using a database BLAST [14].

3 Results and Discussion

3.1 Duration Users Using Contact Lens

Based on the results observations that have been made on STIKES Mandala Waluya college students, obtained data of respondents based on the duration users using contact lenses is as follows:

Table 1 shows duration college students using contact lenses ranged from 3 to 6 months. The range is 2 people use contact lenses for 2 months, 2 people use contact lenses for 3 months, 3 people use contact lenses for 4 months, 3 people use contact lenses submarine 5 months and 2 people use contact lenses for 6 months. The longer duration using contact lenses, the lower level of cleanliness and comfort using contact lenses [15].

Table 1. Distribution of respondents based on duration users using contact lenses.

No.	Sample Code	Duration
1.	W1	4 month
2.	W2	5 month
3.	W3	6 month
4.	W4	4 month
5.	W5	3 month
6.	W6	6 month
7.	W7	5 month
8.	W8	3 month
9.	W9	4 month
10.	W10	5 month

Table 2. Results of the examination contact lens cleaner residual solution with the culture method.

No.	Sampel Code	Culture (±)
1.	W1	Positive (+)
2.	W2	Positive (+)
3.	W3	Positive (+)
4.	W4	Positive (+)
5.	W5	Positive (+)
6.	W6	Positive (+)
7.	W7	Positive (+)
8.	W8	Positive (+)
9.	W9	Positive (+)
10.	W10	Positive (+)

3.2 Results Bacterial Isolation from Contact Lens Cleaner Residual Solution

Based on the results of laboratory tests to 10 samples in BA media obtained data hemolysis on each sample as follow:

Table 2 results obtained a positive culture of all the samples grown on blood agar medium. This shows that the 10 samples from contact lens cleaner residual solution positively contaminated by bacteria. According to Sinaga and Whadini [15], the level of compliance in maintaining the cleanliness of the lenses or lens care was a risk factor for microorganisms colonizes on the lens. Where more than 91% of contact lens users do not show compliance. One of the compliance must be done by contact lens users is care for the additional contact lenses equipment example contact lens cleaner solution. A

contact lens cleaner solution contains a disinfectant that can kill pathogens, but contact lens cleaner solution should not be left in place for storage and reuse for subsequent use. This can cause contact lens cleaner solution does not have enough power to kill bacteria, even become a place of growth of microorganisms that increases the risk of infection. Washing hands using soap and clean water before wearing contact lenses and when will take it off are also often ignored by users of contact lenses. This can increase the risk of contamination pathogenic bacteria and parasites in contact lenses and contact lens cleaner solution fluid [2].

On 10 isolates observed in this study showed different hemolysis from each sample. In Table 2 showed 5 isolates namely isolate W1, W4, W7, W8, W10 shows gamma hemolysis, and 4 isolates namely isolate W2, W3, W5, W9 show alpha hemolysis, and 1 isolate namely isolate W6 shows beta hemolysis. The difference hemolysis that occurs can be caused due to the type of contact lens cleaner solution used by the respondents is different, also how they care for contact lenses which do also affect.

3.3 Results Molecular Identification Beta Hemolysis Isolates from Contact Lens Cleaner Residual Solution

A sample that chooses for molecular identification is isolate showing beta hemolysis when culture inspection. The sample that shows beta hemolysis is toxic because it can lyse blood cells hospes. This is by following research Pratiwi [16], which states that pathogenic bacteria can lyse the erythrocyte so in the blood media plate will be visible hemolysis zone around the colony. Hemolysis is excoprotein which has enzymatic and toxin activity so the bacteria that form hemolysis are pathogenic [17]. The first stage molecular identification is DNA isolation that obtains DNA of the bacterial isolates targets to be used as a DNA template in the PCR stage. An amplicon of the gene 16S rRNA is subsequently visualized by gel electrophoresis. From the results of the visualization appear band with 1500 bp in all samples, indicating that the gene 16S rRNA has been amplified well (Fig. 1).

Based on the results PCR examination by using primers 27 F and primer 1495 R, has amplicon size 1500 bp, which is the area of bacteria genes marker. From electrophoresis

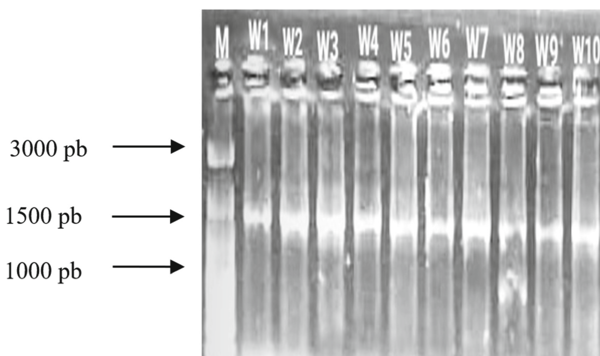

Fig. 1. Amplification Products of 16S rRNA using Universal Primers. Each sample is1500 bp.

result, DNA bands formed on the wells W1, W2, W3, W4, W5, W6, W7, W8, W9, W10. DNA bands show varying in thickness from one sample to another, depending on the amount of DNA that was amplified [18].

Sequence 16S rRNA coding gene results from beta hemolysis isolate tracked their homology against the sequences of 16S rRNA belonging to the other bacteria in the GeneBank through the BLAST program. BLAST result is stored in the Fasta format, then processed using the program Clustal W and MEGA 7. Table 3 shows the presentation of percent identity <97%. This indicates isolate W6 is a different species with all the isolates in Table 3. If the presentation of the homology of the species above 97% then the species can be expressed as the same species [19].

16S rRNA sequence from BLAST program is then used to construct phylogenetic trees. Phylogenetic tree construction aims to see the kinship between organisms sample sequence with the organism comparison sequence based on the evolutionary relationship. Phylogenetic trees constructed using neighbor-joining tree method. According to Dharmayanti [20], neighbor-joining tree method chooses sequences that when combined will give the best estimate branch length most closely reflects real distance among the sequences. Phylogenetic trees were tested statistically using bootstrap method as much as 1000 replicates. Hall [21] states the value of the bootstrap as much as 100 to 1000 replications are used to estimate the phylogenetic tree confidence level. Also, Ubaidullah and Sutrisno [22] stated the greater bootstrap value is used, the higher phylogenetic tree topology confidence level that its reconstruction results based on character distribution data which is influenced by random effects.

Table 3. BLAST result.

No	Species Name	Percent Identity	Accession
1	Uncultured *Lysinibacillus* sp. clone 1 16S ribosomal RNA gene	93.22%	MN736508.1
2	*Lysinibacilluspakistanensis* strain GDLB-21 16S ribosomal RNA gene	93.81%	MK791672.1
3	*Lysinibacillusmacroides* strain AVS1 16S ribosomal RNA gene	93.16%	MG493189.1
4	*Lysinibacillus* sp. strain K46 16S ribosomal RNA gene	94.55%	KX821649.1
5	*Lysinibacillus* sp. CH-37 16S ribosomal RNA gene	93.86%	KR148987.1
6	*Lysinibacillus*sp. S-4 16S ribosomal RNA gene,	93.86%	KP279288.1
7	*Bacillus* sp. F8 16S ribosomal RNA gene	93.86%	JQ991003.1
8	*Lysinibacillus* sp. xfqu3 16S ribosomal RNA gene	93.16%	GQ480504.1
9	*Bacillus* sp. Cb9 16S ribosomal RNA gene	93.81%	KT449784.1
10	*Lysinibacillus* sp. mkx-31 16S ribosomal RNA gene	91.87%	KU159214.1

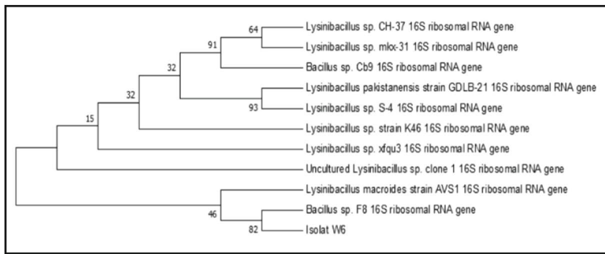

Fig. 2. Phylogenetic trees are construct based on 16S rRNA sequences. Construction using neighbor joining method with bootstrap 1000 replication.

Bootstrap value on the branching in figure shows the value of the branching accuracy in phylogenetic trees. Values below <70% indicate branching which is formed can still be changed with other phylogenetic trees preparation methods. This is in line with Horiike et al. [23] and Coenye & Vandamme [24] opinions, the value bootstrap 95% or more has means branching topology is very accurate, consistent or nothing will change although done with other phylogenetic trees preparation methods.

In Fig. 2 beta hemolysis isolate W6 within one clade with *Lysinibacillus* sp. strain K46 and *Bacillus*sp species. However, isolates W6 closer with *Bacillus* sp. compared with *Lysinibacillus* sp. *Lysinibacillus* sp. is a gram-positive bacteria. Organisms in this genus were previously considered as genus Bacillus members, but the taxonomy status of these microorganisms converted into Lysinibacillus genus in 2007 [25]. Compared to Bacillus, Lysinibacillus peptidoglycan walls contains lysine and aspartic acid that diagnose as amino acids, with different mesodiaminopimelik acids in Bacillus genus [26]. Bacteria *Lysinibacillus* sp. and *Bacillus* sp. shares the same nature that exists everywhere and it is not considered can cause serious illness. But both of these species can cause serious infections in humans. Bacteremia is one of the most common conditions of systemic infection caused by species of Bacillus hile the species *Lysinibacillus*, *L. sphaericus* cause bacteremia cases in 12 of 469 (2%) in children that suffering from cancer or those who are undergoing surgery on the Children Cancer Hospital in Italy [27].

4 Conclusion

Based on the PCR results showed that the isolated bacteria from contact lens cleaner solution successfully amplified and have amplicon size 1500 bp. The results of the phylogenetic analysis based on the gene 16S rRNA shows that the bacterium is closely related to Bacillus sp.

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