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Kosakonia sp. PROTEOLYTIC BACTERIA ISOLATED FROM RUMEN AND RETICULUM OF ACEH CATTLE

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ABSTRACT

The aim of this study was to identify proteolytic bacteria from the ruminal and reticulum fluids of aceh cattle based on the 16S rRNA gene. Samples used were ruminal and reticulum fluids of aceh cattle slaughtered in abattoir of Aceh Besar. Samples were diluted and cultured into Skim Milk Agar medium at 39° C for 48 hours. The morphology of bacterial colonies growth in the medium was observed. Colonies resulted in the largest clear zone were isolated and used for Deoxyribonucleic Acid (DNA) isolation, 16S rRNA gene amplification and sequencing. The results showed that morphology of dominant colonies was yellowish white color, round shape, position on the agar surface. The results of phylogenetic analysis of RS1 and ReS2 isolates isolated from rumen and reticulum fluids of aceh cattle respectively had a close familial relationship and belonged to the bacterial group of Kosakonia. Sequence homology showed isolate RS1 and ReS2 are probably either new Enterobacteriaceae species or unconfirmed species**.** Halo zone produced by ruminal bacteria had a wider diameter (25 mm vs 20 mm) than that caused by reticulum bacteria). Based on the results, RS1 (bacterium in the rumen) and ReS2 (bacterium in the reticulum) belong to similar type, namely *Kosakonia* sp. with a proteolytic activity. Presumably, these bacteria originate from the rumen that enters the reticulum with degraded feed.

Key words: Aceh cattle, kosakonia, reticulum, rumen

ABSTRAK

Tujuan dari penelitian ini adalah mengidentifikasi bakteri proteolitik dari rumen dan cairan retikulum sapi aceh berdasarkan gen 16S rRNA. Sampel dikumpulkan dari rumen dan cairan retikulum sapi aceh di Rumah Potong Hewan Aceh Besar. Sampel diencerkan dan dibiakkan ke dalam media Agar Susu Skim pada suhu 39° C selama 48 jam. Morfologi koloni diamati, koloni bakteri dengan zona bening terbesar diambil untuk isolasi Deoxyribonucleic Acid (DNA), amplifikasi dan sekuensing gen 16S rRNA. Hasil penelitian menunjukkan bahwa morfologi dominan koloni berwarna putih kekuningan, berbentuk bulat, dengan posisi pada permukaan agar. Hasil analisis filogenetik isolat RS1 dalam cairan rumen dan isolat ReS2 dalam cairan retikulum sapi aceh memiliki hubungan kekerabatan yang dekat dan termasuk dalam kelompok bakteri Kosakonia. Tingkat homologi sekuens yang diperoleh menunjukkan bahwa isolat RS1 dan ReS2 mungkin merupakan spesies baru Enterobacteriaceae atau spesies yang belum dikonfirmasi. Diameter zona bening yang dihasilkan oleh bakteri rumen memiliki diameter lebih lebar (25 mm vs 20 mm) dibandingkan dengan yang disebabkan oleh bakteri retikulum.Berdasarkan hasil, RS1 (bakteri dalam rumen) dan ReS2 (bakteri dalam retikulum) memiliki jenis yang sama, yaitu Kosakonia sp. yang memiliki aktivitas proteolitik. Disimpulkan bahwa bakteri ini berasal dari rumen yang memasuki retikulum dengan pakan terdegradasi.

Kata kunci: sapi aceh, kosakonia, reticulum, rumen

INTRODUCTION

Aceh cattle are one of the local cattle that exist in Indonesia. They have a good resistance to bad environments such as feed crises, water availability crises, high fiber fodder, parasitic diseases, high temperature environments and extensive traditional grazing systems (Abdullah *et al*., 2007; Safika *et al*., 2018a), short postpartum periods and able to adapt well to a new environment (Martojo, 2003). Although the production is lower than imported livestock, the local cattle should be preserved and developed. Thus, the Indonesian germ plasma is not lost (Mohamad *et al*., 2012).

Production and health of the cattle highly depend on digestive process of feed. It is more complex than other livestock digestive processes. Based on the physiology studies of ruminant livestock, rumen and reticulum are often regarded as a single organ called reticulocorum where feed can flow from the rumen to the reticulum and vice versa. In the rumen and reticulum as much as 60-90% of incoming feed is degraded into its simple forms by microbes such as bacteria, protozoa, fungi and Archaea. In addition, microbial metabolism in the rumen is determined both by the total and the rate of degradation of carbohydrates and proteins (Colville and Bassert, 2014).

Proteolytic bacteria are found in digestive system of cattle. They are able to degrade proteins because of their extracellular enzymes. Protease catalyzes the termination of peptide bonds in proteins, into amino acids (Desiandura *et al*., 2014). The availability and ability of the bacteria in degrading proteins will affect to the amount of amino acids that enter the bloodstream (Genzebu and Tesfay, 2015).

Proteolytic bacteria found in the rumen of cattle have been identified as *Bacteroides amylophilus, Clostridium sporogenes, Bacillus licheiniformis, Prevotella ruminicola, Ruminobacter amylophilus, Clostridium bifermentans, Clostridium aminophilum, Clostridium locheadi, Butyrivibrio fibrisolvens, Streptococcus bovis, Selenomonas ruminantium,*

Lachonospira multiparus, Bacillus cereus, Bacillus sterothermophilus, Bacillus mojavensis, Bacillus megaterium, Bacillus subtilis, Tatumella, and Pseudomonas (Kamra, 2005; Alnahdi, 2012; Das and Qin, 2012; Petri *et al*., 2013; Vijayaraghavan and Vincent, 2013; Peng *et al*., 2015; Safika *et al*., 2018b).

Currently, there are no reports on types of proteolytic bacteria found in the rumen and reticulum of Aceh cattle. Meanwhile, the data is important as basic information in order to increase productivity of Aceh cattle based on digestion process efficiency. Thus, it is important to conduct a research on identification of proteolytic bacteria found in the rumen and reticulum of aceh cattle based on their 16S rRNA gene.

MATERIALS AND METHODS

Sample Collection

Samples used were ruminal content and reticulum fluids collected from 5 Aceh cattle slaughtered at the Abattoirs of Lambaro, Aceh Besar. Ruminal content was collected in triplicate namely from the left, right, and middle parts of the rumen. This aimed to make the sample homogeneous. All samples were brought in ice condition to the Laboratory of Microbiology of Faculty of Veterinary Medicine of Universitas Syiah Kuala for examination.

Isolation of Proteolytic Bacteria

Sample aliquot (1 mL) was spread into a sterile Petri dish, added with 20 mL of Skim Milk Agar medium using pour plate method, and incubated at 39° C for 48 hours. Diameter of halo zones that emerged was measured and gram staining method was performed. Pure colonies with the widest clear zone were cultured into liquid medium and incubated for 48 hours. Bacterial cell pellets was obtained by centrifuging the mixture at 7000x g for 5 minutes and used for Deoxyribonucleic Acid (DNA) extraction.

DNA Extraction

Total DNA was extracted using a Presto TM Mini gDNA Bacteria kit (Geneaid) according to protocol provided by the manufacture. The process was started by the addition of 180 µL of extraction buffer and 20 µL of Proteinase-K to a sterile microtube containing bacterial cell pellet. After an incubation step at 60° C for 10 minutes, the mixture was mixed with 200 µL of GB buffer, incubated at 70° C for 24 h, and added with 200 µL of absolute ethanol. The entire mixture was poured into a spin column (in a collection tube) and centrifuged at 14000-16000x g for 1 minute. The column was added with 400 µL of Buffer W1 and centrifuged at 14000-16000x g for 30 seconds. After discarding the flow through, the column was added with 600 µL buffer W2 and centrifuged at 14000-16000x g for 30 seconds, followed by discarding the flow through. The column was added with 30-50 µL of elution buffer, and incubated at room temperature for 3-5 min, and centrifuged at 14000-16000x g for 1 min. The pure DNA was stored -20° C until used for Polymerase Chain Reaction (PCR).

Amplification of 16S rRNA Gene

The amplification of bacterial 16S rRNA gene was done by PCR method using BactF and UniB primers. The BacF is complement to conserve region in the bacterial gene whereas UniB is complement to 16S rRNA gene of *E. coli* (Baker *et al*., 2003). The PCR condition used (25 cycles) was denaturation at 94° C for 1 minute, annealing at 50° C for 1 minute and elongation at 72° C for 2 minutes, followed by a final elongation at 72° C for 8 minutes (Safika *et al*., 2013). The product was detected using 1% agarose gel electrophoresis and 1x TAE buffer (40 mM Tris HCl, 40 mM acetate, 1.0 mM EDTA), and visualized under gel Doc (Biorad) using Gel red DNA staining.

Phylogenetic Analysis

Sequencing of the 16S rRNA gene was carried out by Macrogen Inc. (Korea) results were compared with referent genes using the Local Basic Alignment Search Tool (BLAST) program at NCBI http://www.ncbi.nlm.nih.gov while the homology of 16S rRNA gene sequences was analyzed using data in GeneBank. Phylogenetic tree was constructed using a neighbor-joining model matrix from MEGA 7.0 (Kumar *et al*., 2016), with the Maximum Composite Likelihood substitution method (Sari *et al*., 2017) and bootstrap analysis 1000 replicate data sets.

RESULTS AND DISCUSSION

Morphology of Proteolitik bacteria

Isolation of proteolytic bacteria collected from ruminal content and reticulum fluid of Aceh cattle was performed on SMA medium which is a selective medium agar for proteolytic bacteria. It aimed to isolate the desired pure strain bacteria and inhibit the growth of other undesirable bacterial strains. The colonies grown on the SMA medium are showed in Figure 1. Morphological observations showed that the colonies generally have a circular, convex, smooth shape with entire margins, yellowish white color and halo zones.

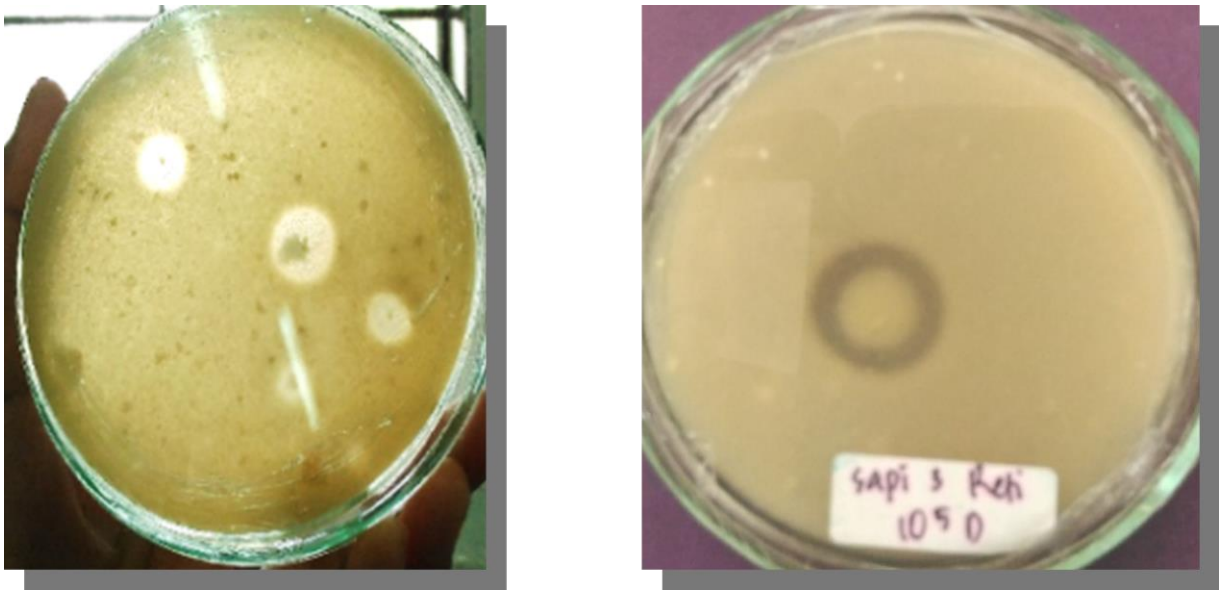
Morphological differences and halo zone diameter indicated that the bacteria belong to different species. It can also be assumed that the enzymes produced by each bacterium have different properties. Furthermore, RS1 and ReS2 were characterized by Gram staining. It showed that they are bacilli shaped-negative gram bacteria (Figure 2). RS1 and ReS2 are bacilli shaped-negative gram bacteria non-spore forming, motile rod with peritrichous flagella. While negative for indole production, the bacteria were positive for fructose, galactose, gluconate, glucose, glycerol, lactose, malate, maltose, mannitol, mannose, sorbitol, and sucrose.

Proteolytic bacteria are protease-secreting bacteria, which produce their enzyme on the growing medium. They form halo zones around the colonies because the enzyme produced is able to degrade casein in Skim Milk Agar by breaking the peptide bond CO-NH. Halo

zone that resulted in by the rumen and reticulum bacteria ranged from 10-25 mm and 0.9-20 mm, respectively. In addition, RS1 that was collected from the rumen of aceh cattle had the widest clear zone among the rumen bacteria that was about 25 mm, whereas ReS2 that collected from reticulum had the widest clear zone of 20 mm.

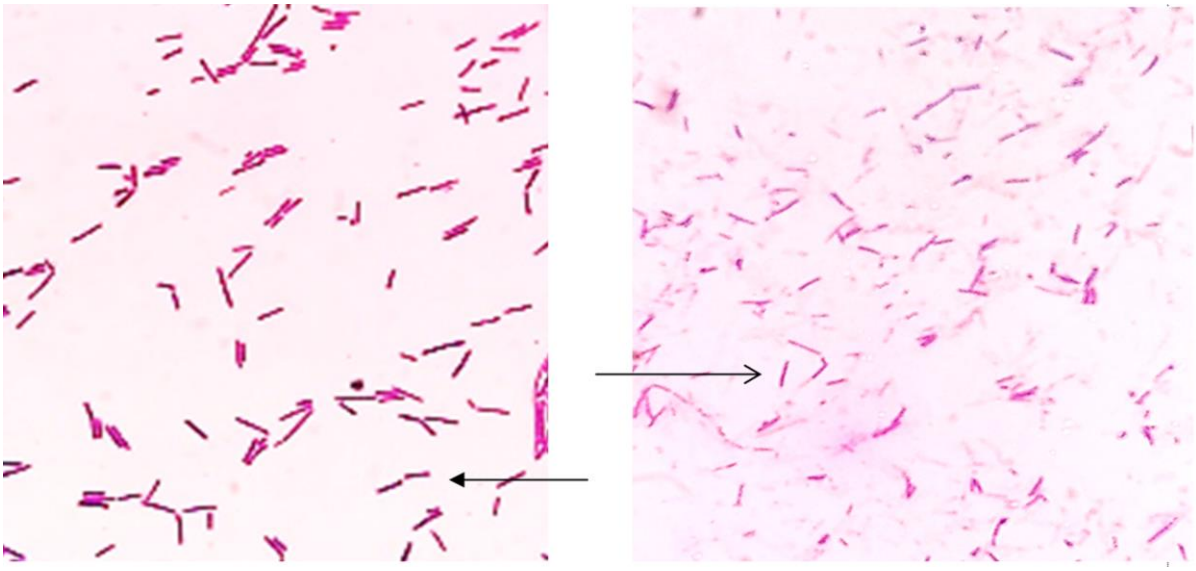
High fiber forage feeding without additional protein sources decreases the presence of proteolytic bacteria or microbes in the rumen of cattle. According to Jones *et al*. (1994) and Mcsweeney *et al*. (1999) leguminous feed contains a protein-tannin complex (polyphenols). It reduces the availability of nitrogen in the rumen and inhibits the growth of dominant bacteria in the rumen especially proteolytic bacteria due to the lack of nitrogen.

RS1 and ReS2 bacteria have similar types, namely *Kosakonia* sp., presumably these bacteria originate from the rumen and enters the reticulum with degraded feed. Based on the study, halo zone that was emerged by rumen bacteria had a wider diameter than that of caused by reticulum bacteria. Rumen is a starting place where protein is degraded by proteinase. The enzyme converts proteins into peptides and amino acids, which can be utilized directly by microflora. Furthermore, peptides and amino acids also can be degraded by peptidase and deaminase enzymes into short chain fatty acids and ammonia. Reticulo-ruminal fold is a part that connects rumen and reticulum. Thus, the bolus of food can flow from rumen to reticulum or vice versa. Feed that has not been digested in the rumen will be digested in the reticulum. Proteins consumed by ruminants are



(a) (b)

Figure 1. The colonies grown on Skim Milk Agar medium have a clear halo zone. A= Bacteria collected from rumen, b= Reticulum fluid of aceh cattle



(a) (b)

Figure. 2. Results of Gram staining test with 1000x magnification. A= RS1, bacteria collected from ruminal content of Aceh cattle, b= ReS2, bacteria collected from reticulum fluid of Aceh cattle

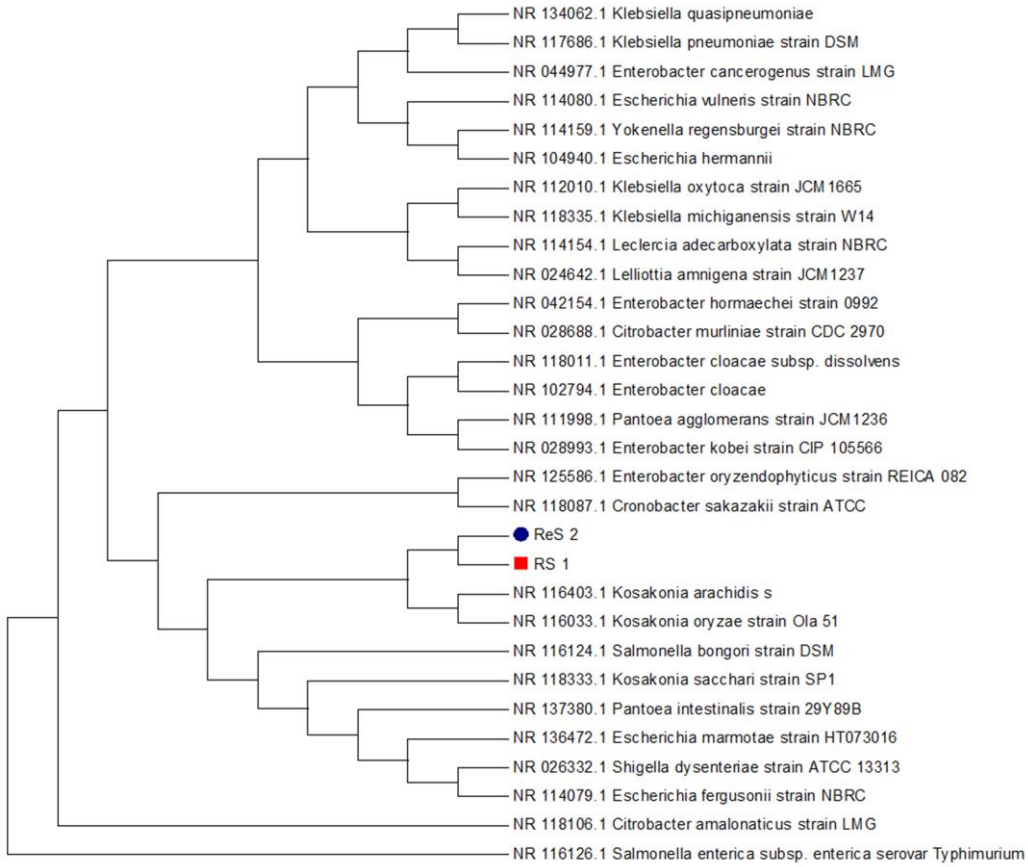


Figure 3. Phylogenetic tree of RS1 (bacterium collected from ruminal content) and ReS2 (bacterium collected from reticulum fluid). As an outgroup is *Salmonella enterica*

not entirely degraded in the rumen and will take longer time to digest all of them in it. Therefore, the undigested proteins will be brought to the reticulum for further processing (Safika *et al*., 2017).

Homology of DNA sequence and Phylogenetic Analysis

Alignment had been done using BLASTN program. Homology of the DNA sequence and DNA references in the GenBank can be determined by aligning DNA sequence with nucleotide data at the National Center for Biotechnology Information (NCBI) online via http://www.ncbi.nlm.nih.gov. (Ghasemi *et al*., 2011).

Data sequences taken from the GenBank have different sizes. DNA sequences with the same size and position of the gene is used to obtain the correct analysis. To get the correct position, alignment of the DNA was done using ClustalW followed by phylogenetic analysis using MEGA 7.0. Although Blast program showed different results, RS1 and ReS2 have a close familial relationship. Moreover, they have a close familial relationship with *Kosakonia arachidis* and *Kosakonia oryzae* (Figure 3).

Similarity score which ranged between 97%-100% indicates that the genes come from the same species. In a different way, similarity score that was less than 97% indicate that the gene comes from different species (Vandamme *et al*., 1996; Janda and Abbot, 2007). Homology analysis showed that RS1 (accesion number: MH900180) had similarity score of 96% with *Kosakonia arachidis* (accession number: NR_116403.1). Meanwhile ReS2 (accession number: MH922844) had similarities score of 91% with *Kosakonia radicincitans* strain DSM (accession number: NR 117704.1). It showed that RS1 and ReS2 are from a new species or species that cannot be confirmed. Further research is needed to determine whether the isolates are new species, such as DNA-DNA hybridization analysis, GC content and free fatty acid analysis.

Formerly, Kosakonia belonged to the Enterobacter genus but separated into a specific group based on multilocus sequence analysis of protein coding genes such as *rpoB* gene (RNA polymerase β subunit), *gyrB* (DNA gyrase gen subunit B), *infB* and *atpD* genes (Brady *et al*., 2013). It is a rod shaped-negative gram bacterium, motile with peritrichous flagella, and non-spores bacterium that can live mesophilically at optimum temperature of 28° C and pH 7. Bacteria have positive reactions to L-alanine, D-selobiose, citrate, D-fructose, D-galactose, D-glucose, glycerol, maltose, D-mannitol and D-mannose. The difference between Kosakonia and *E. cloacae* is in utilization of D-arabitol and L-fucose (Madhaiyan *et al*., 2010; Chen *et al*., 2014). Previous research showed that Enterobacter producing-cellulase is also found in aceh cattle (Sari *et al*., 2017).

Nitrogen metabolism is mainly resulted by metabolic activity of ruminal proteolytic bacteria such as Kosakonia. Although Kosakonia is known as a

nitrogen-fixing bacterium, it is capable of converting nitrogen to ammonia in the rumen. The efficiency of dietary nitrogen that escapes ruminal degradation is the major sources of protein and amino acid requirements of cattle.

CONCLUSION

Based on the results, RS1 (bacteria in the rumen) and RS2 (bacteria in the reticulum) have similar types, namely, *Kosakonia* sp. has proteolytic activity. Presumably, these bacteria originate from the rumen that enters the reticulum with degraded feed. Halo zone that was modified by rumen bacteria has a wider diameter (25 mm) than that caused by reticulum bacteria (20 mm).

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