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Phylogenetic Tree 16S rRNA Gene of *Acinetobacter soli* Isolated from the Prepuce of Aceh Cattle

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Abstract

BACKGROUND: In the pre-seed area of healthy Aceh cattle, it is possible to be contaminated with pathogenic bacteria that can interfere with the reproductive system. This study is needed to identify these pathogenic bacteria using a molecular approach, in an effort to prevent infection.

AIM: The aim of the present study was to construct phylogenetic tree relationships of *Acinetobacter soli* identified in the preputial area of Aceh cattle by molecular analysis using 16S rRNA gene sequencing.

MATERIALS AND METHODS: A total of 75 preputial specimens were obtained from Indrapuri's Breeding and Forages Center of Aceh Cattles, Indrapuri district, Banda Aceh, Indonesia. The samples were processed for culture using standard conventional methods. The extraction of genomic DNA and the amplification of the 16S rRNA gene were assayed using polymerase chain reaction. A phylogenetic tree was constructed using distance matrices using the neighbor-joining model of the molecular evolutionary genetic analysis software 6.1 software.

RESULTS: The results showed that of 75 preputial swab samples, 18 (24%) were positive for *A. soli* isolates. There was a 100% sequence similarity to *A. soli* prototype strain B1 and a 99% similarity to *Acinetobacter parvus* prototype strain LUH4616, *Acinetobacter baylyi* strain B2, *A. venetianus* strain ATCC 31012, as well as a 99% similarity to *Acinetobacter baumannii* strain DSM 30007, the strain ATCC 19606, and the strain JCM 6841, respectively. We concluded that *A. soli*-positive presentation in the preparation of Aceh cattle has 100% sequence similarity of 16S rRNA with *A. soli* strain B1.

CONCLUSIONS: The conclusion of this study is that, based on the construction of a phylogenetic tree, it shows that 24% of the bacterial isolate is related to *A. soli*. It is essential to conduct a regular survey for bacterial contamination and to increase worker awareness and education about hygiene standards.

Introduction

Acinetobacter belongs to the *Moraxellaceae* family, which is receiving increasing attention as significant pathogenic and opportunistic pathogens. *Acinetobacter* species have been associated with serious infections implicated in outbreaks of pneumonia, skin and soft-tissue infections, wound infections, urinary tract infections, meningitis, and bloodstream infections [1], [2], [3]. *Acinetobacter baumannii* has been isolated from several sources, such as goat, camel, and sheep raw meat in Iran [4], [5], commercial raw meat of chicken, turkey, and pork in Switzerland [6]. Interestingly, Kumsa *et al*. [7] reported that *Acinetobacter soli* was prevalent in *Linognathus vituli* of infested cattle. In human, *A. soli* infection is prevalent in many parts of the world, such as in Brazil [1], Ethiopia [7], Cuba [8], and some parts of Asia with diverse distributions in different countries including Taiwan [9], China [10], and Japan [2], [3].

The posterior area could act as a vehicle for a wide range of undesirable pathogens. Routinely, bacterial contamination of the preputial orifice by extraneous flora, as well as true pathogens, can occur at any time from bedding, manure, and soil. Infectious disturbance may affect the reproductive performance of the cattle's. In support of this hypothesis, various authors explained that due to the ubiquitousness of the bacteria, there are multiple potential sources of preparation infection [11], [12], [13], [14]. In confirmation of our previous study of *Escherichia fergusonii* [13] and *Staphylococcus* species [14], both bacteria were found in the preputial swabs of Aceh cattle. In addition, studies demonstrated that the cellulolytic *Enterobacter* [15] and cellulolytic *Bacillus* [16] were successfully isolated from rumen of Aceh cattle. Due to the clinical impact of the fact, pathogenic bacteria are coupled with increasing resistance of *A. soli* to the main antimicrobial drugs [2], [3], [8], [9], [10], attention should be focused seriously on the surveillance of *A. soli,* particularly in cattle's farm breeding.

Sequence analysis of the 16S rRNA gene has been helpful in understanding the composition of the bacterial communities. Various authors argue that the 16S rRNA sequence is an essential part of the description

of an organism because 16S rRNA gene is present in all bacteria to understand the genetic relationships among all bacteria species [17], [18], [19], [20], [21]. Figueroa Castro *et al.* [22] explained that 16S rRNA gene sequencing could successfully identify *Pediococcus parvulus*, a Gram-positive bacterium, as a cause of mastitic testicular cancer infection in men. Based on the 16S rRNA gene sequence, Mitra and Roy [23] identified a novel soil bacterium capable of degrading trichloroethylene. Gram-positive bacteria isolated from tannery waste were identified using 16S rRNA gene sequencing analysis and phylogenetic tree construction showing *Bacillus thuringiensis* sp. [24]. In the study of Ntushelo [25], they rearranged all information linked to bacterial classification using sequence analysis of the 16S ribosomal RNA as a tool for studying bacterial diversity. Recently, Puteri *et al.* [26] demonstrated that the ecological diversity of the cattle microorganism consortium was dominated by bacteria. Thus, the aim of the present study was to construct phylogenetic tree relationships of *A. soli* isolated from preputial area of Aceh cattle by mean of the molecular analysis using 16S rRNA gene sequencing. To the best of our knowledge, the first molecular study of *A. soli* reported from cattle in Indonesia.

Materials and Methods

Specimen collection

A total of 75 preputial specimens were obtained under sterile hygienic condition from the local breeding center at breeding bulls maintained at the Indrapuri's Breeding and Forages Center of Aceh Cattles, Indrapuri District, Banda Aceh, Indonesia. The cattle used in this research were male cattle more than 2-3 years old and in good clinical health as discribed by Hambal *et al*. [14]. The external genitalia of male Aceh cattle were cleaned with sterile gauze moistened with 0.9% sodium chloride. Preputial secretion was collected on sterile cotton swabs and contained in sterile tubes, kept in boxes with an isothermal temperature of 8°C, and brought to the Laboratory of Research, Faculty of Veterinary Medicine of Universitas Syiah Kuala as recommended by Balqis *et al*. [13]. The study was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine at Universitas Syiah Kuala, Aceh, Indonesia (Approval Number 014/KEPH-C/III/2017).

Bacterial isolates

The preputial swab samples were streaked in Petri dishes containing MacConkey agar (Difco Laboratories, Detroit, MI, USA) and incubated under aerobic conditions at 37°C for 24 h. Samples were processed for culture by standard conventional methods described by Da Silva *et al.* [27]. Colonies showing the characteristic of a transparent and smooth appearance on the MacConkey agar were considered for further identification. A single colony was isolated and purified by subculturing on the same medium to obtain pure culture. The isolate was morphologically identified by Gram staining, cell and colony morphology, catalase, oxidase, and motility tests [28] and biochemical tests such as lactose metabolism were performed [29].

DNA extraction

Extraction of genomic DNA and amplification of the 16S rRNA gene were assayed as explained by Safika *et al.* [16]. Total DNA was extracted separately using a commercially available Presto™ Mini gDNA Bacterial kit (Geneaid), according to the manufacturer's instructions. A volume of 50 μl of pure total DNA was eluted. Eluted DNA (concentration of approximately 30 μg/ml) was used as a template for the polymerase chain reaction (PCR) amplification following the protocol.

PCR

Total DNA was used as a template in PCR to amplify 16S rRNA. Primers used for detecting the preputial bacteria of Aceh's cattle were bacteria-specific primers [30]. The forward primer sequence was 5' AGAGTTTGATC(A/C) TGGCTCAG 3' and the reverse primer sequence was 5' GGTTAC(G/C) TTGTTACCTGCCGGA 3'. Subsequently, the 16S rRNA gene was amplified by PCR using the Master Mix (Fermentas). A total of 25 μL of reaction mixture consisted of 10 pmol of each primer, 30 ng/ul of template DNA, and 12.5 μL of Master Mix (Fermentas). PCR amplification used the following program: Initial denaturation at 95°C for 5 min, 30 cycles, 1 min 95°C, 30 s of annealing at 50°C, and 2 min of elongation at 72°C, with a final extension at 72°C for 10 min. PCR product was determined by electrophoresis analysis, through 1.0% agarose and ×I TAE buffer (40 mM Tris-HCl, 40 mM acetate, and 1.0 mM EDTA) under GelDoc (BioRad) by [13], [14], [15], [16].

Phylogenetic analysis

Sequencing results were compared using the basic local alignment search tool program at NCBI http://www.ncbi.nlm.nih.gov and 16S rRNA gene sequence analysis using GenBank data. A phylogenetic tree was constructed using distance matrices using the neighboring model of the molecular evolutionary genetic analysis software (MEGA 6.1) software [31], with the substitution method Maximum Composite Likelihood [32]. The reproducibility of the node for tree topology was estimated by bootstrap analysis based on 1000× data sets.

Results and Discussion

Results

The present research was conducted to assess the occurrence of *A. soli* in the prepuce of Aceh cattle. Of 75 preputial swab samples, 18 (24%) were positive for *A. soli* isolates. All isolates had very similar phenotypic characteristics. The colonies grew well with the characteristic of a transparent and smooth appearance in MacConkey agar media. The bacterial colonies were approximately 2 mm in diameter, smooth, convex, circular and had margins after 24 h of incubation at 37℃. The isolates were oxidase negative, Gram-negative rod, catalase positive, and non-motile. Table 1 represents the sequences similarity among *Acinetobacter* spp. Based on the results of the phylogenetic tree constructed using MEGA 6.1, isolate 4A is related to *A. soli* strain B1 (Figure 1). Sequence similarity was found 100% with *A. soli* prototype strain B1 and 99% similarity to *Acinetobacter parvus* prototype strain LUH4616, *Acinetobacter baylyi* strain B2, *A. venetianus* strain ATCC 31012, *A. baumannii* strain DSM 30007, the strain ATCC 19606, and the strain JCM 6841, respectively.

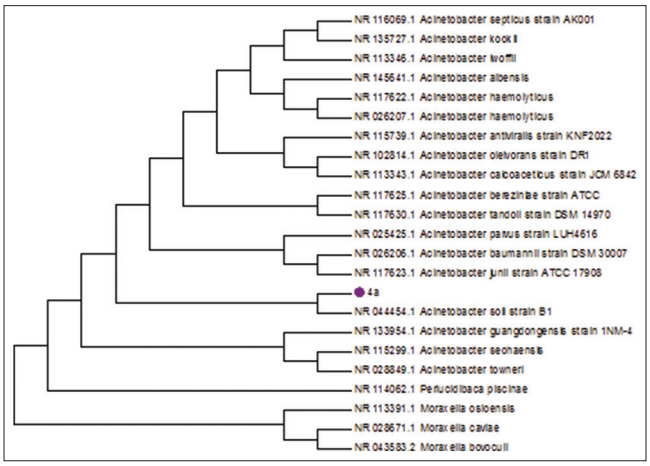

Figure 1: Phylogenetic tree relationships of Acinetobacter soli with bootstrap ×1000

Acinetobacter species are receiving increasing attention as significant opportunistic pathogens for many serious underlying diseases. This study demonstrated that the pre-employment of cattle constitutes a reservoir for *A. soli*. The presence of *A. soli* in the prepuce of Aceh cattle may represent additional concern for of veterinarians, public health as it can spread to the cattle population, particularly on breeding farms during mating season. In this study, we agree and support those of Kumsa *et al.* [7] who found that opportunistic *A. soli* is prevalent in *L. vituli* of domestic cattle. The findings of *A. soli* isolate in lice and keds may serve as a reservoir for transferring the opportunistic pathogen to cattle and provide a vector for the spread of the bacteria in both environment and animal. Within this context, the previous study noted that the opportunistic *E. fergusonii* and *Staphylococcus* species bacteria are prevalent in the preputial area of Aceh cattle [13], [14].

The study of Rafei *et al*. [33] in Lebanon has reported that A. soli was succesfully identified by partial sequencing of the rpoB gene from lettuce. Similar to the findings of Choi *et al*. [34] successfully identified *A. soli* from life environment surface samples using 16S rRNA and *rpoB* identification methods in Korea. Quiñones *et al.* [8] reported that the high prevalence of bla_{NDM-1} in *A. soli* was identified in a patient hospitalized in Cuba by multiplex PCR. In another previous study conducted in Korea, researchers reported the isolation of *A. soli* from forest soil samples using different methods such as phenotypic, fatty acids, G+C content, 16S-rRNA *gyrB,* and DNA-DNA hybridization method [35].

A. soli was detected in various biological samples of human patients. For instance, Chen *et al.* [10] reported that *A. soli* was successfully cultivated from sputum sample of a male patient. Blood samples from a man who had pelvic fracture, intestinal perforation, and mesenteric injury after traffic accident investigated by Kitanaka *et al.* [3] who showed that the bacterial culture contained *A. soli*. Furthermore, Endo *et al.* [2] described that *A. soli* bacteria identified in blood cultures from patients with systemic inflammatory response syndrome were distributed in high frequency among *Acinetobacter* spp. isolates between January 2007 and April 2012 in Japan. Similarly, Lauderdale *et al.* [9] reported that *A. soli* was isolated from sputum sample of female patient in Taiwan. Quiones *et al.* [8] stated that the sputum, cerebrospinal fluid, catheter, surgical wounds, skin and soft tissue, blood, and respiratory samples obtained from hospitalized patients were infected with *A. soli* 0.2% among *Acinetobacter* spp. *A. soli* had previously been isolated from neonates with sepsis symptoms of sepsis admitted to an intensive care unit in Brazil [1].

The bacterial load in animal reproductive organs is unique and can be more complex due to the composition of the prepuce. Vaginal bacteria may determine the state of healthiness of the animals. It is, therefore, crucial to investigate the bacteria to understand the underlying causes of reproductive organ disorders. Each animal appears to have a unique bacterial

Table 1: List of the sequences that showed similarity among *Acinetobacter* spp.

Source of the 16S ribosomal RNA gene	Strain	Accession number	Max score	Total score	Query cover	Similarity	Sequence
Acinetobacter soli	B1	NR 044454.1	1208	2249	100%	100%	partial
Acinetobacter parvus	LUH4616	NR 025425.1	1203	2239	100%	99%	partial
Acinetobacter baylyi	B2	NR 115042.2	1201	2162	99%	99%	complete
Acinetobacter venetianus	ATCC 31012	NR 042049.1	1192	2088	98%	99%	complete
Acinetobacter baumannii	DSM 30007	NR 117677.1	1175	2098	98%	99%	partial
Acinetobacter baumannii	ATCC 19606	NR 117620.1	1175	2098	98%	99%	complete
Acinetobacter baumannii	JCM 6841	NR 113237.1	1175	2098	98%	99%	partial
Acinetobacter baumannii	DSM 30007	NR 026206.1	1175	2098	98%	99%	partial

Values are identical for all sequences at E-value: 0.0.

community [11], [27]. *Campylobacter fetus*, *Campylobacter fetus* subsp. *venerealis*, and *Campylobacter fetus* subsp. *fetus* have isolated from prepuce of buffalo bulls [36]. Da Silva *et al.* [27] identified aerobic bacterial microbiota that were *Staphylococcus intermedius* and *Proteus mirabilis* in the preputial and vaginal orifices of owl monkeys (*Aotus azarai infulatus*). Furthermore, *Staphylococcus aureus* bacterium was detected by Rahmi *et al*. [12] in preputial and vaginal of horses (*Equus caballus*). However, the effect of preputial washing reduced the presence of bacteria load in the ejaculates of Murrah buffalo bulls [11]. We concluded that *A. soli* positively present in preputial area of Aceh cattle that have 100% sequence similarity of 16S rRNA gene with *A. soli* strain B1. It is essential to conduct a regular survey for bacterial contamination and to increase worker awareness and education about hygiene standards. *A. soli* possible influences on animal or human health and can be dangerous because it can cause bloodstream infection, and also, it is a new neonatal pathogen that has the potential to cause outbreaks [1].

Conclusions

As much as, 24% of the bacterial isolates from the preputial area of healthy Aceh cattle are indicated to be *A. soli* species, based on analysis of 16S rRNA gene sequences. The phylogenetic tree shown that this bacterial isolate was related to *A. soli*. It was clear that we concluded *A. soli* positively presence in preputial of Aceh cattle. It is very important to carry out routine surveys for bacterial contamination and increase worker attention and education for hygienic standards.

Data Availability

The data used to support the findings of this study are available from the corresponding author on request.

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