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Escherichia fergusonii identified in preputial swabs from healthy Aceh cattle by phylogenetic 16S rRNA analysis

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ABSTRACT

Aims: This study aimed to assess the risk of reproductive tract contamination in Aceh cattle by *Escherichia fergusonii* as revealed by 16S rRNA gene sequencing of preputial swab samples.
Methodology and results: Preputial swabs taken from 50 breeding bulls at the Indrapuri Breeding and Forage Center of Aceh Cattle, Banda Aceh, Indonesia, were examined for the presence of bacteria. Samples were streaked on MacConkey agar and incubated under aerobic conditions at 37 °C for 24 h. Smooth, yellow- or rose-colored colonies were selected for their characteristic appearance and subjected to further analysis. Genetic identification was based on 16S rRNA gene sequencing and PCR analysis. We conducted a 16S rRNA sequence similarity search with GenBank using BLAST and constructed neighbour-joining dendrograms using MEGA. From among closely related species of the genus *Enterobacteriaceae*, we identified the enteric bacterium *E. fergusonii* as having the highest sequence similarity.
Conclusion, significance and impact of study: We concluded that the *E. fergusonii* bacterium positively presence in preputial swab samples of clinically healthy Aceh cattle population. Accordingly, it is potentially allowing the bacterium to be spread during natural mating or semen collection processing for artificial insemination in cattle breeding farm.

Keywords: Aceh cattle, *Escherichia fergusonii*, 16S rRNA gene, sequencing

INTRODUCTION

Aceh cattle are one of the most important domestic cattle breeds in Indonesia, and they occupy an economically prominent position in the livestock industry of many regions. In rural areas, Aceh cattle not only provide meat and milk but also are a principal animal for draft work. Thus, attention must be focused on animal pathogen–caused diseases to ensure that a sufficient supply of Aceh cattle and their products are available. Ultimately, improvement of cattle reproduction may be expected to significantly increase the economy and living standards of many rural communities throughout the world.

Accordingly, we focused our research on pathogenic bacteria that pose a potential risk to animal health by causing widespread disease, particularly in cattle breeding farms. As contagious diseases may affect the reproductive performance of cattle, microbial preputial infections have been a major concern for cattle breeders. The preputial bacterial load plays a critical role in the transfer of serious disease during the breeding season because this form of transmission may act as a vehicle for a broad range of undesirable pathogens. Bacterial contamination of the preputial orifice by extraneous flora and true pathogens directly from soil, bedding and manure may occur routinely. In support of this hypothesis, several authors have suggested that there may be multiple potential sources for preputial infection owing to the ubiquitous nature of the contributory bacteria (Joshi *et al*., 2006; Silva *et al*., 2013; Meena *et al*., 2015; Rahmi *et al*., 2015).

The preputial orifice of animals is likely a major source of diverse bacterial species that lead to disease and the risk of microbial spread during the collection of semen destined for use in artificial insemination. Numerous bacterial agents have been isolated worldwide from semen specimens of cattle and other domestic animals. Recently, bacterial organisms such as *Micrococcus*

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luteus, *Pseudomonas aeruginosa*, *S. epidermidis* and *Staphylococcus intermedius* have been detected in frozen bovine semen (Abro *et al*., 2016). Sannat *et al*. (2015) described bacterial loads found in fresh and frozen semen from different breeds of cattle and buffalo. In Iranian bovine and buffalo semen, Dehkordi *et al*. (2014) identified *Brucella abortus* and *Brucella melitensis*, which are highly contagious zoonotic pathogens of humans and the leading pathogenic bacterial causes of abortion in ruminants. Azawi and Ismaeel (2012) detected aerobic bacteria in *Awassi ram* semen. Rana *et al*. (2012) assessed the bacterial diversity in fresh bubaline semen and isolated *Micrococcus* spp., *Bacillus* spp., *Rhodococcus equi*, *Stapyhococcus auricularis*, *Moraxella bovis*, *S. chromogenes*, *S. simulans* and *Enterobacter* spp. Patel and Patel (2012) found both Gram-positive and Gram-negative bacteria in the frozen semen of Holstein cattle bulls. Gandhi *et al*. (2008) isolated the Gram-negative bacterium *Moraxella bovis*, an opportunistic pathogen that can cause infectious bovine keratoconjunctivitis, from frozen bovine semen of clinically healthy Jersey and Jersey-cross bulls.

Escherichia fergusonii, a member of Enterobacteriaceae, is globally distributed and associated with a wide variety of intestinal and extra-intestinal infections in both humans and animals. For example, in a case study from Switzerland, Funke *et al*. (1993) reported the potential pathogenicity of *E. fergusonii* after they isolated the strain from the feces, gallbladder fluid and a superficial wound of a 69-year-old male patient with pancreatic carcinoma and cholangiosepsis. In a study of patients at an Italian hospital, Savini *et al*. (2009) described *E. fergusonii* as contributing to the bacterial commensal flora of the human enteric tract. Isolates of *E. fergusonii* were also obtained from farm animals in the United Kingdom (Wragg *et al*., 2009) and from swine and poultry in South Korea (Rayamajhi *et al*., 2011). More recently, in Egypt, Gaafar *et al*. (2015) found *E. fergusonii* to be an emerging bacterial pathogen of freshwater fish and the probable cause of a fatal disease outbreak in Nile tilapia (*Oreochromis niloticus*).

Several investigators have specifically referred to *E. fergusonii* as a potential emerging pathogen. Herràez *et al*. (2005) reported that *E. fergusonii* infection caused fatal fibrinonecrotic typhlitis in two ostrich specimens (*Struthio camelus*) that had developed clinical signs of hemorrhagic diarrhea, prostration and anorexia. In another case in Canada, a goat developed chronic diarrhea and became emaciated, and *E. fergusonii* was isolated from the feces and several internal organs, namely, kidney, lung, liver and intestine (Hariharan *et al*. (2007). Recently, in an investigation of non-human primates in Africa, Glover *et al*. (2017) identified *E. fergusonii* as a possible emerging pathogen of zoonotic importance.

In sum, *E. fergusonii* has been found in many environments, including the aquatic milieu (Maheux *et al*., 2014), that may potentially allow the bacterium to spread; in this context, *E. fergusonii* poses a substantial risk to veterinary health in cattle breeding farms. For example, a case of *E. fergusonii* infection in a pregnant cow was associated with clinical signs of acute pneumonia, hyperthermia, tachypnea and eventual death (Rimoldi and Moeller Jr., 2013). To avoid contagion, careful evaluation of reproductive tract infection—particularly in the preputial area—is indicated. Therefore, our present investigation was designed to analyze preputial swab samples from clinically healthy Aceh cattle for the presence of *E. fergusonii*. Furthermore, we used genotypic characterization of the bacterial 16S rRNA gene by polymerase chain reaction (PCR) amplification and subsequent phylogenetic analysis to elucidate the taxonomic position of *E. fergusonii* in the Escherichiaceae group.

MATERIALS AND METHODS

Specimen collection

Preputial swab samples were obtained under sterile hygienic conditions from 50 healthy Aceh bulls, two to three years of age, maintained at the local breeding center, the Indrapuri Breeding and Forage Center of Aceh Cattle, Indrapuri District, Banda Aceh, Indonesia. The external genitalia of male Aceh cattle were cleaned with sterile gauze moistened with 0.9% sodium chloride. Preputial secretions were collected on sterile cotton swabs, transferred to sterile tubes and kept in boxes at an isothermal temperature of 8 °C and transported to the Laboratory of Research, Faculty of Veterinary Medicine of Syiah Kuala University.

Isolation of preputial bacterium

Samples from preputial swabs were streaked on Petri dishes that contained MacConkey agar (Difco Laboratories, Detroit, MI, USA) and incubated under aerobic conditions at 37 °C for 24 h. Bacteria were isolated and identified as described (Silva *et al*., 2013). Characteristic smooth, yellow- and rose-colored colonies were selected for further identification. Colonies were restreaked on the same medium to obtain pure cultures. Isolated colonies were identified morphologically by Gram staining (Chai *et al*., 2017) and by biochemical tests (Bakar *et al*., 2017). Indole production, methyl red, Voges Proskauer, citrate, sulfic indole motility, mannitol, triple sugar iron agar, sucrose, and glugose tests were carried out.

DNA extraction

Total DNA was extracted separately using the gDNA PrestoTM Bacteria Mini kit (Geneaid) with slight modification. Purified total DNA (50 µL, ~200 µg/mL) was eluted and used as the template for PCR assays as described by Sari *et al*. (2017).

Amplification of the 16S rRNA gene via PCR

The bacteria-specific primers for the 16S rRNA gene (Baker *et al*., 2003) used for detecting the preputial

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bacteria in the Aceh cattle population were as follows: forward: 5′-AGAGTTTGATC(A/C)TGGCTCAG-3′; reverse: 5′-GGTTAC(G/C)TTGTTACCTGCCGGA-3′. Expected amplicon size was 1500 bp. 16S rRNA was amplified through PCR: Each reaction mixture (25 µL total) contained 10 pmol of each primer, 30 ng of template DNA and 12.5 µL of Master mix (KAPA Biosystems, Boston, MA, USA). The PCR amplification program was as follows: initial denaturation at 95 °C, 5 min, followed by 30 cycles consisting of denaturation at 95 °C, 1 min, annealing at 50 °C, 30 sec, and elongation at 72 °C, 2 min, with a final extension at 72 °C, 10 min. The PCR product was identified by electrophoresis through 1.0% agarose in 1× TAE buffer (40 mM Tris-HCl, 40 mM acetate, 1.0 mM EDTA, pH 8.3) and analysis with the Gel Doc XR+ System (Bio-Rad).

Identification of the bacterium by 16S rRNA gene sequencing

Genetic identification was based on 16S rRNA gene sequencing and PCR analysis. The 16S rRNA analysis was performed on nine isolates from Banda Aceh bulls by sequencing >1400 nucleotides of the 16S rRNA gene. DNA was extracted with a Presto™ Mini gDNA Bacteria kit (Geneaid) and amplified using the universal primers BacF: 5′-TTTTACTGTTTTCGTAACAGTTTT-3′, and UniB: 5′-ACGCCACCGAGC-3′, ±1500 bp. This process amplified 16S rRNA gene that have length about 1500 bp. Cycle sequencing was performed with the KAPA 2G Fast ReadyMix PCR kit with dye and reaction products were sequenced by Macrogen Inc., Korea, using the Dye Terminator (3'-dye labeled dideoxynucleotide triphosphate). The amplified products were checked for purity and size by 1.2% (w/v) agarose gel electrophoresis in TAE buffer (pH 8.3). The 16S rRNA gene sequences determined in this study were aligned with sequences from GenBank using the CrustalW program in MEGA version 5.0. The multiple sequence alignment was edited by hand and used to derive a neighbor-joining tree with 1000 bootstraps (Castro *et al*., 2010; Mitra and Roy, 2010; Sujatha *et al*., 2012; Ntushelo, 2013; Sari *et al*., 2017).

Phylogenetic analysis

The sequencing results were compared using the Basic Local Alignment Search Tool (BLAST) program from NCBI (http://www.ncbi.nlm.nih.gov) and 16S rRNA gene sequence homology analysis using GenBank data. A phylogenetic tree was constructed using distance matrices by the neighbor-joining model of the MEGA 6.1 program (Tamura *et al*., 2011) with the substitution method Maximum Composite Likelihood (Tamura and Nei, 1993). The node reproducibility for tree topology was estimated by bootstrap analysis with 1000 replicate datasets. A *Bacillus* sp. was included as an outgroup for phylogenetic analysis.

RESULTS

Of 50 bovine preputial specimens analyzed, 9 (18%) yielded bacterial isolates. The bacterium formed smooth, yellow- and rose-colored colonies that were raised and irregularly shaped. Morphologically, the oval bacterium was classified as a Gram-negative rod based on Gram staining. The biochemical reactions of the bacterium are listed in Table 1. Homology analysis (Table 2) revealed that most isolated sequences were closely related to *E. fergusonii* strain ATCC 35469 with 99% identity to the sequences of the Enterobacteriaceae group available in GenBank. Based on the results of the phylogenetic tree, *E. fergusonii* was identified as the nearest phylogenetic relative of the bacterium, and we found that a small number of sequences formed a new cluster. The bacterium isolate, 8a, showed the highest similarities with five other enteric bacteria, namely, *Enterobacter massiliensis*, *Citrobacter youngae* strain, *Escherichia albertii* strain Albert, *Shigella fexnerii* and *Shigella boydii*. Comparative 16S rRNA gene sequence analysis demonstrated that the bacterium was a member of an RNA group affiliated with the Enterobacteriaceae group. The bacterium was 99% similar to the Enterobacteriaceae group as shown in the phylogenetic tree in Figure 1.

Table 1: Biochemical reactions of *E. fergusonii* isolated from preputial swabs of Aceh cattle.

No.	Test	Reaction
1	Indole production	+
2	Methyl red	+
3	Voges Proskauer	–
4	Citrate (Simmons)	–
5	Sulfic indole motility	–
6	Mannitol	–
7	Triple sugar iron agar	Acid/gas
8	Sucrose	–
9	Glucose	–

DISCUSSION

The bacterial load in animal reproductive organs is unique, and its complexity may be increased by preputial or vaginal bacteria that may ultimately determine overall animal health. It is therefore advisable to investigate these bacteria to understand the underlying causes of reproductive organ disorders. Each animal appears to harbor a unique bacterial community (Silva *et al.*, 2013; Meena *et al*., 2015). For example, *C. fetus*, *C. fetus* subsp. *venerealis*, and *C. fetus* subsp. *fetus* were isolated from prepuces of buffalo bulls (Joshi *et al*., 2006). Silva *et al*. (2013) identified *Staphylococcus intermedius* and *Proteus mirabilis*, which are aerobic bacterial microbiota that have been most frequently isolated from preputial and vaginal specimens of owl monkeys (*Aotus azarai infulatus*).

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Table 2: List of the sequences that showed similarity with the *Enterobacteriaceae* group.

Source of 16S ribosomal RNA gene	Strain	Accession Number	Sequence
Escherichia fergusonii	ATCC 35469	NR 074902.1	complete
Escherichia fergusonii	NBRC 102419	NR 114079.1	partial
Enterobacter massiliensis	JC163	NR 125600.1	partial
Citrobacter youngae	GTC 1314	NR 041527.1	partial
Escherichia fergusonii	ATCC 35469	NR 027549.1	partial
Escherichia albertii	Albert 19982	NR 025569.1	partial
Shigella flexneri	ATCC 29903	NR 026331.1	partial
Escherichia coli	U 5/41	NR 024570.1	partial

Values for the following parameters are identical for all sequences: Max Score and Total Score: 754; Query cover: 99%; E value: 0.0; Identity: 99%.

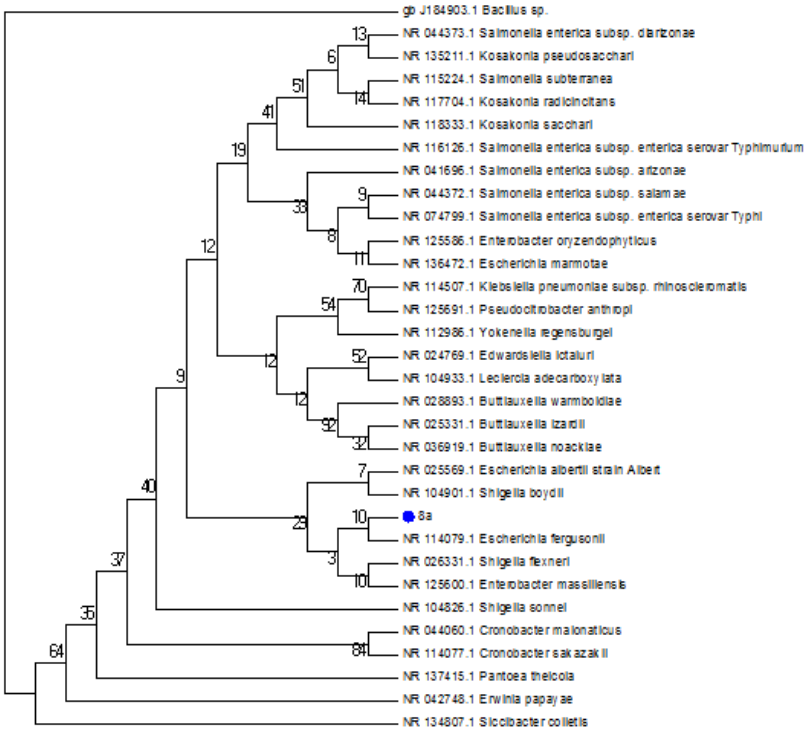

Figure 1: Phylogenetic tree of enteric bacteria constructed using the 16S rRNA gene.

Furthermore, *Staphylococcus aureus* was detected by Rahmi *et al*. (2015) in the prepuces and vaginas of horses (*Equus caballus*). Indeed, preputial washing reduces the presence of the bacterial load in the ejaculates of Murrah buffalo bulls (Meena *et al*., 2015).

Although *E. fergusonii* is considered a part of bacterial commensal flora in the enteric tract, the bacterium may colonize other organs to became virulent when conditions become conducive for growth and multiplication, which supports its role as an opportunistic pathogenic bacterium. Indeed, it is well known from veterinary reports that, under immunosuppressive conditions, transient microorganisms or resident microbiota that originated from the digestive tract may cause disease in the reproductive tract. For example, many commensal microorganisms are known to infect the reproductive tract and cause conditions like epididymitis, prostatitis, seminal vesiculitis, testicular degeneration, urethral inflammation, ampulitis, posthitis, orchitis and balanitis (Monleon *et al*., 2008; Lisboa *et al*., 2009; Rajiah *et al*., 2012; Altarac, 2015; Delcaru *et al*., 2016). Notably, these maladies often affect the prepuce and may result in male infertility.

In particular, *E. fergusonii* may act as pathogens under certain circumstances, and they are cited as responsible for several emerging bacterial diseases in animals and humans (Funke *et al*., 1993; Savini *et al*., 2009). We describe herein that the preputial colonization

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of cattle by potential emerging opportunistic veterinary- and human-pathogenic bacteria such as *E. fergusonii* may result in the contamination from the environment. This phenomenon can cause serious and even fatal infections in otherwise healthy hosts. Rimoldi and Moeller Jr. (2013) described that *E. fergusonii* may function as a fatal pulmonary pathogen and cause acute pneumonia, leading to death in cattle. Previously, Weiss *et al*. (2011) observed a similar phenomenon in the horse with *E. fergusonii* possibly emerging as an opportunistic pathogen that caused enteritis and septicemia. In the chicken, Oh *et al*. (2012) found that *E. fergusonii* produced heat-labile enterotoxin. Moreover, Gokhale *et al*. (2014) reported that *E. fergusonii* caused a chronic low-grade endophthalmitis after cataract surgery. In the aquatic environment, Gaafar *et al*. (2015) reported that *E. fergusonii* caused pathological lesions, mortality and morbidity in tilapia.

Infection with *E. fergusonii* increases not only the risk of disease but also the associated phenomenon of increased resistance to antibiotics both in humans and animals. Lagacé-Wiens *et al*. (2010) isolated *E. fergusonii* from urine samples of a patient with cystitis, and their analyses demonstrated a high level of bacterial multidrug resistance to cephalosporins, fluoroquinolones, sulfonamides, monobactams and aminopenicillins. This result was similar to previous findings by Savini *et al*. (2008) of multidrug resistance of *E. fergusonii* recovered from a patient with clinical signs of acute cystitis. Ramayajhi *et al*. (2011) described the prevalence of β-lactam resistance of *E. fergusonii* in swine and poultry. Multidrug-resistant and virulent *E. fergusonii* were also detected by Forgetta *et al*. (2012), who demonstrated the presence of antibiotic resistance genes in chromosomes and plasmids of *E. fergusonii* isolated from broiler chickens. In light of the available data, we argue that the resistance of *E. fergusonii* to multiple classes of antibiotics has undoubtedly resulted in treatment failures and prolonged illnesses and a higher risk of invasive disease.

Various bacteria have now been recognized as important emerging pathogens in the reproductive tract, and several have been demonstrated to cause disease in both humans (Lisboa *et al*., 2009; Rajiah *et al*., 2012; Altarac, 2015; Delcaru *et al*., 2016) and animals (Monleon *et al*., 2008; Oh *et al*., 2012; Rana *et al*., 2012). In humans, various types of infections related to balanitis and infectious balanoposthitis have been reported in circumcised or uncircumcised males (Lisboa *et al*., 2009; Rajiah *et al*., 2012). *Escherichia coli* is able to adhere to and colonize the perineum and urethra and cause urinary tract infections (Altarac, 2015). Delcaru *et al*. (2016) described that bacterial biofilm formation, which is commonly caused by Gram-negative bacteria, can lead to urinary tract infection and prostatitis syndromes. In animals, Monleon *et al*. (2008) found that both *Staphylococcus aureus* and *E. coli* have been implicated as causes of orchitis and epididymo-orchitis in poultry broiler breeders. *Enterobacter* spp., a recognized cause of coliform mastitis in neonatal and calf diarrhea, was recovered by Rana *et al*. (2012) in fresh bubaline semen.

An increasing preputial bacterial load leads to contamination that affects semen quality as manifested by toxic effects on spermatozoa. Bacteriospermia in semen can lead to changes in the integrity and viability of spermatozoa and affect fertilization by direct adherence of the bacteria to individual sperm cells. Bacteria may also have indirect effects in semen. For example, Morrell (2006) described that toxins released by bacteria can impair spermatozoa motility. Thus, preputial hygiene is a primary consideration in the prevention of pathogenic bacterial transmission through artificial insemination or natural mating conditions. Because the majority of bacteria present in semen come from the prepuce, Joshi *et al*. (2006) advised that, during semen-collecting procedures, bull preputial cavities be systematically cleaned.

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