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Molecular detection of the *Escherichia coli heme-utilization gene A* virulence factor in *E. coli* isolated from the feces of horses in Sumbawa island, Indonesia

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Abstract

The transmission of *Escherichia coli* (*E. coli*) containing virulent genes from animals to humans and the environment poses significant public health challenges. This study aimed to detect the virulence factor of the *E. coli heme-utilization gene A* (*chuA*) in *E. coli* isolated from the feces of apparently healthy horses in the island of Sumbawa, Indonesia. The study utilized 52 fecal samples from a total horse population of 283, calculated using the disease detection formula. Fresh feces were collected immediately after excretion and placed in buffered peptone water for subsequent analysis. The samples were then isolated on eosin methylene blue media and identified using biochemical tests. Identified *E. coli* strains were further examined for detecting the *chuA* gene using polymerase chain reaction techniques. The *E. coli* was successfully isolated and identified in 11 (21.15%) of the 52 collected fecal samples. Polymerase chain reaction analysis detected the *chuA* gene in 8 (15.38%) *E. coli* isolates at 279 bp on gel electrophoresis. The close interaction between horses and humans in the island of Sumbawa, Indonesia, may facilitate the spread of *E. coli*. Thus, surveillance is needed to employ a One Health approach to monitor *E. coli* strains encoding the *chuA* gene and other virulence factors to control their dissemination.

Introduction

Sumbawa island, located in West Nusa Tenggara province, Indonesia, is rich in horse populations. As of 2020, the horse population in Sumbawa regency numbered 14,378.[1] Historically, horses in Sumbawa island have been utilized by the local community as companion animals, beasts of burden, transportation tools, sports, and for their meat. The use of horses as working animals and means of transport increases the interaction between humans and horses, potentially enhancing the transmission of diseases. Horses, as companion animals, are considered potential reservoirs of microbial agents that can attack to various hosts, including humans.[2] One such microbe is *Escherichia coli*, with several strains exhibiting anti-microbial resistance (AMR) and virulence factors that can be transmitted through direct or indirect contact with humans and animals.[3]

Escherichia coli is a bacterial species with diverse characteristics, naturally present in the gastrointestinal tracts of humans and animals. Pathogenic forms of *E. coli* arise due to virulence genes associated with pathogenicity, which are absent in non-pathogenic E. coli strains and can cause various diarrheal diseases in both animal and human hosts.[4] Virulence gene-encoding *E. coli* has been frequently found in horses, domestic animals, and wildlife. Diarrhea-causing *E. coli* has been classified into six phenotypes based on pathogenicity, including enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E. coli*, diffusely adherent *E. coli*), and Shiga toxin-producing *E. coli* (STEC), with STEC including the enterohemorrhagic *E. coli* sub-pathotype.[5]

Based on virulence genes, *E. coli* is divided into eight phylogenetic groups, including A, B1, B2, C, D, E, F, and clade I.[6] Groups A or B1 consist of commensal *E. coli* strains. Groups A, B1, and D typically include pathogenic *E. coli* strains causing intestinal infections, while extra-intestinal *E. coli* strains belong to groups B2 or D.[6] Epidemiological studies indicate that virulence gene-encoding *E. coli* strains can be transmitted from animals to humans through clonal transfer of virulent *E. coli* strains *via* direct and indirect contacts, or consumption of food contaminated with animal feces.

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Healthy horses can carry *E. coli* with virulence genes and interact with humans in Sumbawa Island, potentially transmitting these *E. coli* strains and releasing them into the environment. One such *E. coli* strain with virulence genes, which poses a transmission risk to humans and the environment from healthy working horses, is *E. coli* harboring the *E. coli heme-utilization gene A* (*chuA*). The *chuA* gene is widely distributed, along with *eefC* and *fitA* genes, among adherent-invasive *E. coli*. It is rarely found in commensal and diarrheagenic *E. coli* strains.[7] Recent research has documented that *E. coli* 0157:H7 encoding the *chuA* gene has been found in healthy workhorses in Sumbawa island through quantitative polymerase chain reaction (PCR) analysis.[8] Previous report has also identified *E. coli* with the genetic marker *chuA* in both animals and humans. For instance, *chuA*-marked *E. coli* was found in 7 out of 50 cattle fecal samples and 5 out of 29 goat fecal samples analyzed.[9] Another study revealed that 30.00% of *E. coli* strains isolated from the environment, approximately 70.00% of the isolated *E. coli* strains were of human origin, carried the *chuA* gene from 304 *E. coli* strains analyzed.[10]

This study aimed to detect the virulence factor of the *chuA* gene in *E.coli* isolated from the feces of apparently healthy horses in Sumbawa island, Indonesia. The *E. coli* encoding the *chuA* gene has the potential to spread from animals to humans, posing public health risks. The sequencing results of *E. coli* encoding the *chuA* gene can be used as DNA barcodes to quickly detect and identify invasive *E. coli*, contributing to the development of superior Sumbawa horses.

Materials and Methods

Study design. This research is an observational descriptive study conducted from July to August 2024, focusing on horse breeders in Sumbawa island, specifically in the Alas district of the province of West Nusa Tenggara, Indonesia. The study aimed to observe and detect the presence of the *chuA* gene virulence factor in *E. coli* isolated from the feces of healthy working horses. The horse population in Alas district was recorded at 283 as of 2020.[1] The sample size of horse feces used in this study was determined based on the disease detection formula established by Thrusfield.[11] Based on the formula of disease detection by Thrusfield with a 95.00% confidence level and an assumed minimum expected prevalence of 6.00%, the required sample size was calculated to be 49 fecal samples from working horses out of a total population of 283 horses in Alas district, being necessary to detect the presence of the *chuA* gene in *E. coli*.[11] However, this study utilized 52 fecal samples to enhance the accuracy and validity of the research. The horses (*Equus caballus*) involved in this study did not undergo any direct interventions that could cause pain, suffering, or harm during the research process. Fresh fecal samples were collected from the ground for bacterial culture under the supervision of a veterinarian from the Faculty of Veterinary Medicine at Universitas Pendidikan Mandalika, Mataram, Indonesia with proposal number 110/E5/PG.02.00.PL/2024, June 11, 2024.

Isolation and identification of *E. coli.* The fecal samples used in this study were fresh horse feces, recently excreted; they were collected and placed in a buffered peptone water medium. Each sample was properly labeled and documented. During transportation, all sample specimens were stored in a coolbox and promptly delivered to the Tropical Disease Center Laboratory of Airlangga University, Surabaya, Indonesia, for the culture of *E. coli* and detection of the *chuA* gene using PCR. The fecal samples were inoculated onto the eosin methylene blue (Merck, Darmstadt, Germany) media and incubated at 37.00 ˚C for 24 hr to promote the growth of *E.coli*. The *E. coli* colonies that developed were subsequently identified through Gram staining and biochemical tests. The biochemical tests performed included sulfide indole motility , and the fermentation of lactose, glucose, fructose, and mannitol, following the procedures outlined in the Basic Laboratory Procedures in Clinical Bacteriology.[12]

DNA extraction in *E. coli.* Molecular detection of the *chuA* gene in *E. coli* began with the extraction of *E. coli* DNA, and performed using the QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany). The concentration of the extracted DNA of *E. coli* was measured by a nanodrop spectrophotometer.

Detection of *chuA* gene in *E. coli.* The molecular detection of the *chuA* gene in *E. coli* was conducted using a PCR assay with specific primers. The PCR reagents were prepared in a total volume of 20.00 µL, consisting of 12.50 µL GoTaq (Promega, Madison, USA) PCR mix, 1.00 µL forward primer, 1.00 µL reverse primer, 2.50 µL nuclease-free water, and 3.00 µL DNA, following the procedure described by Sadeq *et al*.[13] The specific forward and reverse primers as F: GACGAACCAACGGTCAGGAT, R: TCGCCAGTACCAAAGACA with an amplicon of 297 were used in this study according to Clermont *et al* .[6] The PCR conditions used were as follows: Pre-denaturation at 94.00 ˚C for 5 min, denaturation at 94.00 ˚C for 30 sec, annealing at 50.00 ˚C for 30 sec, extension at 72.00 ˚C for 30 sec, and a final extension at 72.00 ˚C for 30 sec, conducted over 35 cycles. The PCR amplicon of the *chuA* gene was electrophoresed on a 2.00% agarose gel. The electrophoresis results were visualized by the Bio-Rad Gel Imager (Hercules, USA). The data obtained from the research were analyzed descriptively, including electrophoresis images of the *chuA* gene in *E. coli*. The data from the research were descriptively analyzed through electrophoresis images of *E. coli* encoding the *chuA* gene. Phylogenetic tree analysis was conducted to assess the relationship of the *chuA* gene from *E. coli* isolates with

various *E. coli* sequences in the registered data of the GenBank® in National Center for Biotechnology Information (NCBI). This was performed using the BLAST Neighbor-Joining tree method online on the NCBI website with Query ID of Query_3359765. The sequencing results of *E. coli* encoding the *chuA* gene were also used to create DNA barcoding using the DNA Barcode Generator from Bio-Rad available at https://biorad-ads.com/DNABarcodeWeb/.

Results

Based on Gram staining, morphological characteristics on Eosin methylene blue media, and biochemical tests conducted on 52 fecal samples from working horses in Alas district, Sumbawa island, Indonesia, it was revealed that 11 (21.15%) fecal samples were identified as *E. coli*. The *E. coli* isolates exhibited macroscopic morphological characteristics, with colonies appearing round and metallic green in color. Gram staining showed that the bacteria were rod-shaped and stained red. The biochemical tests indicated that *E. coli* was catalase-positive and oxidase-negative. Additionally, *E. coli* tested positive for indole production and sulfide indole motility, and it fermented sugars, such as maltose, glucose, lactose, and mannitol. The *E. coli* isolates in this study also showed a positive urea test, a negative Simmon's citrate test, and a positive triple sugar iron agar test.

Detection of the virulence factor *chuA* gene in the 11 *E. coli* isolates identified from horse feces in Sumbawa island revealed that eight (15.38%) *E. coli* isolates from the 52 samples encoded the *chuA* gene. These were found in samples No. 2, 3, 4, 5, 6, 7, 8, and 9. The *E. coli* isolates encoding the *chuA* gene were located at the 279 bp position on the agarose electrophoresis gel for each sample (Fig. 1).

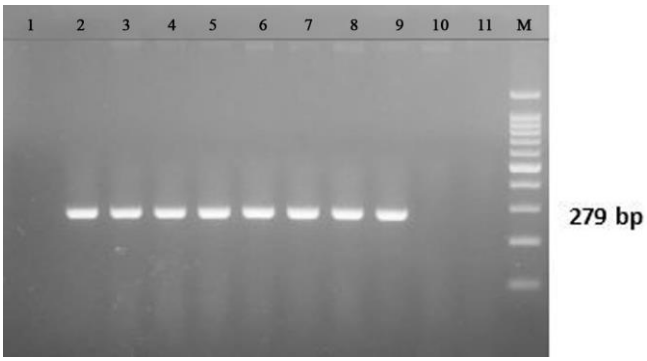

Fig. 1. Detection of *Escherichia coli heme-utilization gene A* (*chuA*) by polymerase chain reaction. A 2.00% agarose gel electrophoresis shows that eight samples (No. 2, 3, 4, 5, 6, 7, 8, and 9) of *E. coli* encode the *chuA* gene (279 bp). The M represents the DNA marker, and the samples are in lanes 1-11.

The phylogenetic analysis of the *E. coli* isolates tested positive for the *chuA* gene in this study (IcIQuery_3359), when compared to the *E. coli* sequences available in GenBank® using the Neighbor-Joining method on the NCBI website, revealed that the *E. coli* encoding the *chuA* gene from Sumbawa horse feces is closely related to the *E. coli* strains (O157 strain TR01, strain APEC37, ETEC6335, DETEC-C31, O157 strain Santai, strain RHBD04-C01, and RHB09-C24). In contrast, it branches separately from *E. coli* ATCC 25922 (Fig. 2).

DNA barcoding of the *E. coli* sequences positive for the *chuA* gene from Sumbawa horse feces was generated using the DNA Barcode Generator from Bio-Rad (Fig. 3). The DNA barcode in Figure 3 shows 248 nucleotides forming the barcode, with a predominance of thymine bases.

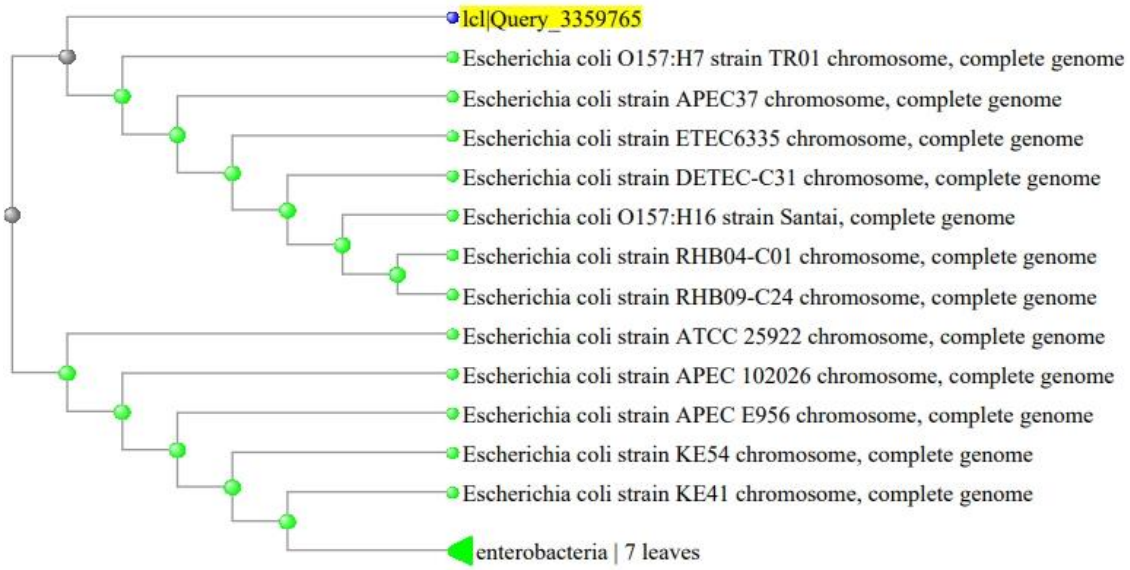

Fig. 2. Phylogenetic analysis of the *Escherichia coli heme-utilization gene A* in *E. coli* from horse feces (IcIQuery_3359 is a sample of *E. coli* highlighted in yellow) compared to the *E. coli* sequences from GenBank® data on NCBI (round green).

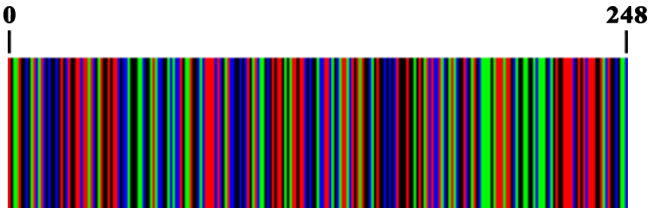

Fig. 3. The DNA Barcode of *Escherichia coli* encodes the *E. coli heme-utilization gene A*. The DNA barcode shows 248 nucleotides forming the barcode, with a predominance of thymine bases (represented in red).

Discussions

The study identified *E. coli* in 21.15% of the 52 fecal samples collected from working horses in Sumbawa island, West Nusa Tenggara province, Indonesia. These findings are in line with previous studies on the identification of *E. coli* in large animals within the same province. Earlier studies reported that *E. coli* had been isolated from Bali cattle feces in local farms.[14] This occurrence highlights that *E. coli* naturally resides in the gastrointestinal tracts of both humans and animals but can also infect other organs in both species. Several strains of *E.coli* in humans have been linked to the intestinal and extra-intestinal diseases, such as septicemia, diarrhea, and urinary tract infections.[15] In animals, *E. coli* has been collected from the reproductive tracts of cattle of Bali, with a prevalence of 25.00% in West Nusa Tenggara province.[16] These findings suggest that *E. coli* strains harboring virulence genes, like *chuA*, may have the potential to infect other organs and be transmitted to animals, humans, and the environment.

The *chuA* gene in *E. coli* from horse feces in Sumbawa island, identified as a virulence factor, was detected in 15.38% of the samples, with a band position of 279 bp on agarose gel electrophoresis, being consistent with the target gene reference by Clermont *et al.*, that identified *E. coli* of human origin.[6] In that study, it was reported that *E. coli* O157:H7 produced verotoxin and encoded the *chuA* gene in 100% of the cases. The electrophoresis results of the *chuA* gene in this study closely resembled those of previous research, which detected the *chuA* gene in fecal and urine samples from human patients in 297 bp on the electrophoresis gel at AL-Diwaniyah Teaching Hospital, Al-Qādisiyyah, Iraq.[17]

The detection of *E. coli* encoding the *chuA* gene in this study indicates that such strains can exist in animals that do not exhibit clinical symptoms or illness. Previous research has shown that virulent genes, like *chuA*, *vjaA*, *arpA*, and *TspE4.C2*, have been found in the feces of goats and cattle in Salakpra Wildlife Sanctuary Kanchanaburi, Thailand.[18] This evidence suggests that *E. coli* encoding the *chuA* gene as a virulence factor is not only found in humans but also animals, as demonstrated in this study in working horses in Sumbawa island, Indonesia.

From an epidemiological perspective, the existence of *E. coli* encoding the *chuA* gene in the working horses in Sumbawa island can be associated with the environment, agent, and host status. Field observations revealed that environmental factors related to livestock management, particularly the minimal application of biosecurity measures, play a significant role. Previous research on *E. coli* contamination in cattle in West Nusa Tenggara province indicated that contamination in the reproductive tract of cattle could be attributed to poor environmental conditions, particularly post-partum, with many farmers in the region being inexperienced and practicing conventional farming methods.[16] The potential for *E. coli* contamination in animals may originate from water sources or wastewater. This environmental contamination hypothesis is supported by phylogenetic analysis, which found that *E. coli* encoding the *chuA* gene from the samples is closely related to the *E. coli* O157:H7 strain TR01, as recorded in GenBank® with the accession code of CP033605.1, which was isolated from a slaughterhouse and its wastewater in Türkiye.[19] The environmental contamination factor is further reinforced by phylogenetic analysis, which also revealed that *E. coli* from horse fecal samples is related to the *E. coli* strain APEC37. According to the GenBank® data with the accession code of CP127303.1, *E. coli* strain APEC37 is a pathogenic strain isolated from livestock farms in Russia.

Considering the host and agent factors, where the sampled horses were healthy working animals showing no signs of illness, it is evident that *E. coli* encoding the *chuA* gene can reside in apparently healthy animals. This finding aligns with the hypothesis that *E. coli* can inhabit the intestines without causing clinical symptoms yet acting as an etiological agent of extra-intestinal infections, with no clear distinction between *E. coli* types.[20] This hypothesis is also supported by a study in Baghdad, Iraq, which reported that livestock can act as reservoirs for ECO157:H7 without displaying clinical symptoms.[21] Additionally, it is consistent with the assertion that the *chuA* gene is widely distributed among Adherent-invasive *E. coli*.[7]

The presence of *E. coli* encoding the *chuA* gene in fecal samples from Sumbawa horses in this study may be attributed to the interaction between horses, humans, and the environment, facilitating the horizontally transfer of gene of *chuA*-encoding *E. coli* within the colonies. Horizontal gene transfer has been recognized as a contributing factor to bacterial evolution. All extra-intestinal pathogenic *E. coli* strains possess pathogenicity islands.[22] This is consistent with a study reported that 30.00% of *E. coli* strains isolated from the environment and approximately 70.00% of *E. coli* strains isolated from human sources carry the *chuA* gene.[10]

The presence of *E. coli* encoding the *chuA* gene in fecal samples from healthy working horses in Sumbawa island highlights the potential for this bacterium to spread to

humans and the environment. The *E. coli* ability to survive in feed, water, soil, and feces has significant implications for its persistence in horse populations and its potential to contaminate water supplies and crops. Therefore, surveillance based on the One Health approach is needed to monitor the presence of *E. coli* encoding the *chuA* and other virulence genes. As a consideration, previous research has indicated that effective measures to reduce or eliminate *E. coli* O157:H7 in cattle could not only decrease foodborne diseases but also reduce the risk of transmission of this organism to the environment.[23]

This study provides baseline data for controlling the spread of *E. coli* encoding virulence genes that could impact human, animal, and environmental health. Based on the phylogenetic analysis in this study, which found that *E. coli* encoding the *chuA* gene from Sumbawa horse fecal samples is related to the *E. coli* encoding *AMR* genes in GenBank® data with the accession code of CP055736.1, future research could explore the relationship between *E. coli* encoding the *chuA* gene and AMR in horses in Sumbawa island. The *E. coli* encoding *AMR* genes was isolated from different species of livestock (cattle, pigs, and sheep) in southcentral England, being associated with antimicrobial use.[24]

The spread of invasive *E. coli* can be prevented through DNA barcoding to differentiate invasive from non-invasive *E. coli*. This provides baseline data regarding the presence of invasive *E. coli* in horse farms in Sumbawa island, enabling the prevention of its spread from the source to ensure the health and superiority of Sumbawa horses.

The *E. coli* harboring the *chuA* virulence gene was detected in 8 (15.38%) out of 52 fecal samples from apparently healthy horses in Sumbawa island, Indonesia. The detection of *E. coli* with the *chuA* virulence gene in horses, combined with the close interaction between horses and humans in Sumbawa island, suggests a potential increase in the spread of this bacterium. Therefore, surveillance based on a One Health approach is essential to monitor the presence of *E. coli* strains encoding the *chuA* and other virulence genes to control the dissemination of these virulent *E. coli* strains.

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Conflict of interest

In this study, there was no conflict of interest.

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