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RESEARCH ARTICLE
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Multidrug resistance-encoding gene in *Citrobacter freundii* isolated from healthy laying chicken in Blitar District, Indonesia

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Abstract

Background and Aim: The increasing prevalence of resistance (MDR) of Enterobacteriaceae in Indonesia has caused concern regarding human health. *Citrobacter freundii* reportedly targets the gastrointestinal tract of animals and is a common cause of foodborne diseases associated with diarrhea, peritonitis, meningitis, brain abscess, bacteremia, and urinary tract infection. This study aimed to estimate the prevalence of MDR and the presence of Class 1 integron-encoding genes in *C. freundii* isolates obtained from cloacal swabs of healthy laying chickens in Blitar district, Indonesia.

Materials and Methods: One hundred and sixty-five cloacal swab samples were collected from 33 farms in Blitar over a period of 4 months. Standard microbiological techniques such as bacterial culture in MacConkey agar, Simmons citrate agar, and triple sugar iron agar and biochemical tests such as the indole test were performed to identify the isolates. The antibiotic sensitivity patterns of *C. freundii* isolates were determined by the disk diffusion method, and MDR-encoding genes (Class 1 integron) were detected by polymerase chain reaction (PCR).

Results: Out of 165 cloacal swab samples, 7 (4.24%) were positive for *C. freundii*. *Citrobacter freundii* was highly resistant to erythromycin (71.43%) and moderately to streptomycin, tetracycline, and trimethoprim-sulfamethoxazole (all 42.86%); however, it showed low resistance to ampicillin (28.57%). All isolates were found to exhibit MDR. Only 1 (14.29%) of the seven *C. freundii* isolates harbored a Class 1 integron gene. This study revealed that Class 1 integron-encoding genes have a low prevalence in *C. freundii* isolated from healthy laying chickens in Blitar, Indonesia.

Conclusion: Poultry animals can play a role in the transmission of resistance genes to humans due to the MDR of Enterobacteriaceae, including *C. freundii* in the intestines.

Keywords: *Citrobacter freundii*, Class 1 integron gene, human health, laying chickens, multidrug resistance.

Introduction

Citrobacter freundii, a member of the Enterobacteriaceae family, is a facultative anaerobic, Gram-negative coccobacillus. It has flagella-driven motility and is commonly found in water, soil, food, and the intestines of animals and humans [1]. This species reportedly targets the gastrointestinal tract of animals and is a common cause of foodborne diseases, leading to diarrhea, peritonitis, meningitis, brain abscess, bacteremia, and urinary tract infection [2].

Livestock products are important for food security in Blitar, Indonesia, where the cost of meat has been a focus of the government, academia, and consumers. Poultry chicken and products such as eggs are the most widely consumed types of meat and livestock products in Indonesia, accounting for more than 70% of total meat consumption [3]. Thus, their products are the most common sources of contamination by infectious bacteria that can cause diseases in humans and animals [4]. *Citrobacter freundii* is a bacterium for which antibiotic treatment can be applied in the gastrointestinal tract of animals and humans [5]. Antibiotics can inhibit the growth of microorganisms, although treatment with multiple antibiotics can cause multidrug resistance (MDR) [6]. Recently, MDR has been recognized as a global public health problem [7, 8]. Multidrug resistance of *C. freundii* has also increased worldwide [9, 10].

Integrons are genetic structures expressing genes usually encoding antibiotic resistance [11]. Class 1 integrons include the characteristic features of integrons while also being mobile elements. They are widely distributed in Gram-negative bacteria found clinically and in the environment and are responsible for the dissemination of different cassette-associated antibiotic-resistance genes. The Class 1 integrons

are a highly prevalent source of the spread of MDR among Gram-negative bacteria [12, 13] and are found at high frequencies in pathogens and commensals isolated from livestock, creating a worldwide crisis in the management of bacterial infections [14]. Insufficient data have been obtained on the antimicrobial resistance of *C. freundii* isolates harboring Class 1 integron genes isolated from cloacal swabs of healthy laying chickens.

Thus, this study aimed to estimate the prevalence of MDR and the presence of Class 1 integron-encoding genes in *C. freundii* isolates obtained from cloacal swabs of healthy laying chickens in Blitar, Indonesia.

Materials and Methods

Ethical approval

Cloacal swabs were used in this study; hence, ethical approval was not necessary. Cloacal swab samples were collected from laying chicken farms, Blitar district, Indonesia, as per standard collection procedure.

Study period and location

This cross-sectional study was conducted from June to August 2019. One hundred and sixty-five cloacal swab samples were collected from laying chicken farms in Blitar district, Indonesia.

Sample isolation and identification

The samples were collected from 33 farms in Blitar, Indonesia, which included 165 cloacal swabs of healthy laying chickens. Farms were selected on the basis of purposive sampling methods. The cloacal swab samples were cultured in MacConkey agar plates (Oxoid, Cheshire, UK) and incubated at 37°C for 24 h [15]. The colonies showing positivity for lactose-fermenting bacteria were subcultured to obtain pure cultures, followed by biochemical tests (Indole test, Simmons citrate agar, and TSIA) to determine *C. freundii* [16].

Antibiotic sensitivity test

Antibiotic sensitivity testing was performed using the Kirby–Bauer disk diffusion assay on Mueller-Hinton agar medium [17]. The suspension of 3 mL of freshly prepared pure culture colonies was standardized to an equivalent of 0.5 McFarland and inoculated onto culture plates. The antibiotic disks (Oxoid) used were as follows: Erythromycin (E, 15 μg), streptomycin (S, 10 μg), tetracycline (TE, 30 μg), trimethoprim-sulfamethoxazole (SXT, 25 μg), and ampicillin (AMP, 10 μg). After the attachment of the antibiotic disks, the plates were then incubated at 37°C for 24 h. Interpretations of antibiotic resistance were performed in line with the recommendations in the Clinical and Laboratory Standards Institute guidelines. Multidrug resistance was classified for those isolates that were resistant to more than two of the different classes of antibiotics [18].

Detection of Class 1 integron-encoding gene

Multidrug-resistant (MDR) *C. freundii* was further analyzed for the presence of Class 1 integron-encoding genes using molecular polymerase chain reaction (PCR) identification techniques. Bacterial DNA of *C. freundii* isolates was extracted with the QIAamp® DNA Mini Kit (Qiagen, Germany). The primers used to detect Class 1 integron-encoding genes were hep 58 (TCATGGCTTGTTATGACTGT) and hep 59 (GTAGGGCTTATTATGCACGC). The PCR conditions with denaturation temperatures for 2 minutes at 94°C; extended denaturation at 94°C for 30 seconds; annealing at 55°C for 45 seconds; extension at 72°C for 45 seconds; final extension at 72°C for 7 minutes, this reaction was carried out for 30 cycles [19]. Polymerase chain reaction results were visualized by electrophoresis using 1.5% agarose gel (Invitrogen, USA) [20, 21].

Results

Among 165 cloacal swab samples collected from healthy laying chickens at poultry farms in Blitar, Indonesia, seven samples were positive for *C. freundii.* The prevalence of *C. freundii* in chickens was 4.24% among the 33 farms (Table-1). Five panels of different classes of antibiotics were tested against *C. freundii* isolates. The antibiotic sensitivity test indicated that *C. freundii* isolates were resistant to E at a high rate of 71.43%. Intermediate rates of resistance were recorded for S, TE, and SXT (42.86% for each), while a low rate was observed for AMP (28.57%) (Table-2 and Figure-1). This study clearly identified different levels of antibiotic resistance among *C. freundii* isolates derived from animals. The highest level of antibiotic resistance being observed for E is an indication that this antibiotic has been overused or abused in animal health care [22]. Five (71.43%) of the seven *C. freundi*i isolates were discovered to be MDR (Table-3).

Discussion

This study provides evidence that *C. freundii* of animal origin is a possible pathogen that can cause foodborne diseases. The prevalence of *C. freundii* in chickens was 4.24% among 33 farms. This is in accordance with the findings of Liu *et al.* [1], who stated that the prevalence of *C. freundii* was approximately 3%–6% among the isolates of Enterobacteriaceae in samples. This research also agreed with the previous studies recognizing *C. freundii* as an animal pathogen that causes foodborne outbreaks worldwide [23]. The identification of MDR *C. freundii,* as reported in this study, is in line with other studies on organisms that belong to the same family of Enterobacteriaceae [9]. This finding is a major public health concern. Previous

Table-1: Prevalence of *C. freundii* from laying chickens in Blitar, Indonesia.

Samples	165
C. freundii positive	7 (7/165; 4.24%)
Class 1 integron	1 (1/7; 14.29%)

C. freundii = Citrobacter freundii

Table-2: Antibiotic susceptibility profile of *C. freundii* isolated from laying chickens in Blitar, Indonesia.

Antibiotic	Resistant (%)	Intermediate (%)	Sensitive (%)
Erythromycin	5 (71.43%)	2 (28.57%)	0
Streptomycin	3 (42.86%)	0	4 (57.14%)
Tetracycline	3 (42.86%)	0	4 (57.14%)
Trimethoprim-sulfamethoxazole	3 (42.86%)	0	4 (57.14%)
Ampicillin	2 (28.57%)	1 (14.29%)	4 (57.14%)

C. freundii = Citrobacter freundii

Table-3: Prevalence of MDR and Class 1 integron in *C. freundii* isolated from laying chickens.

Location	Code of *C. freundii*	MDR	Class 1 integron by PCR
hFarm 1	L1dn	3 MDR	1
Farm 5	L5an	4 MDR	0
Farm 10	L10cn	1 R	0
Farm 11	L11dn	4 MDR	0
Farm 16	L16an	2 R	0
Farm 26	L26dn	5 MDR	0
Farm 29	L29an	5 MDR	0
Total	7	5 (71.43%)	1 (14.29%)

C. freundii = Citrobacter freundii, MDR = Multidrug resistant, R = Resistant, PCR = Polymerase chain reaction

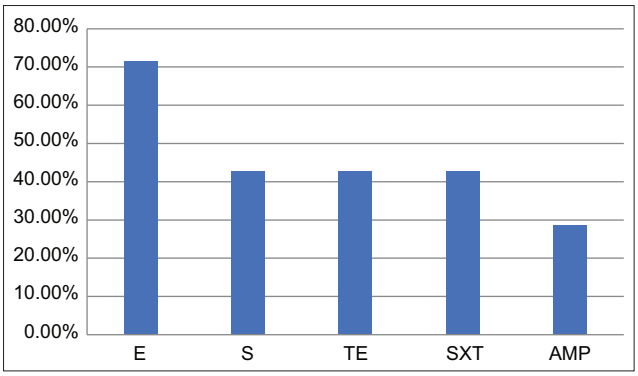

Figure-1: Antibiotic resistance pattern of *Citrobacter freundii* isolated from laying chickens. E=Erythromycin, S=Streptomycin, TE=Tetracycline, SXT=Trimethoprim-sulfamethoxazole, AMP=Ampicillin.

Figure-2: Class 1 integron gene identified from *Citrobacter freundii* using polymerase chain reaction from laying chickens in Blitar, Indonesia. MRK=Marker (100 bp); K+=Positive control; K-=Negative control; L1DN-L29an=Samples, identified amplicon size of Class 1 integron encoding gene = 200 bp.

studies from all over the world have revealed that the prevalence of MDR bacterial pathogens is now a major public health issue [24–26]. Organisms that are resistant to three or more classes of antimicrobials are called MDR [27, 28]. *In vitro* antimicrobial susceptibility testing is commonly used to characterize organisms as MDR bacteria [29].

Using a molecular method, 1 (14.29%) of the seven *C. freundii isolates* was identified to harbor a Class 1 integron-encoding gene. The amplification of such a gene from *C. freundii* in healthy layer chickens was observed at an amplicon size of 200 bp (Figure-2) in the isolates recovered from cloacal swab samples using the primers hep 58 and hep 59 [30]. The results indicate that resistance to several different antibiotics is associated with the presence of Class 1 integron. This finding is in agreement with studies that observed MDR bacteria harboring integron genes that boost their infectivity and resistance to many classes of antibiotics [31]. Other studies have detected integrons in Gram-negative bacteria, which have also increasingly been discovered in Gram-negative bacteria [32, 33]. Class 1 integron gene was also reported as one of the fastest spreading resistance genes in Gram-negative bacteria [34, 35]. Integrons are genetic elements that can possess MDR genes; their existence can obstruct the treatment of bacterial infections [36]. Integrons are classified based on differences in the gene structure of integrases [37]. Class 1 integrons have been found more frequently in Gram-negative bacteria among the different classes of integrons [14]. This study has provided genetic evidence of the presence of an integron-encoding gene in *C. freundii*, a Gram-negative bacterium. The importance of Class 1 integrons as repositories of MDR has been reported [38], and there is increasing evidence for the presence of genes encoding Class 1 integrons in poor sanitation. The presence of Class 1 integrin-encoding gene in an amplicon obtained using *hep* 58–59 primers suggested the possibility of MDR gene, which was confirmed by studying the isolated strains. These results indicate that Class 1 integron genes are present in poultry animals and their products. *Citrobacter freundii* has mostly been collected from poultry [39, 40]. This bacterium is potentially pathogenic to animals and humans, being cytotoxic and aggregative [2]. Aggregative adherence to epithelial cells has been recognized as a putative virulence factor contributing to bacterial pathogenicity [2]. The presence of integrons in MDR properties of *C. fruendi*i will add to the difficulties in treating infections by this bacterium [41–43].

Recent studies on environmental pollution have confirmed that MDR bacteria can be transmitted to humans and poultry animals through various

distribution routes [44]. The MDR bacteria involvement in Enterobacteriaceae is well established as an important part of global public health policy. This idea that poultry is a good indicator as a source for MDR bacteria from environmental pollution and argues the importance of the One Health approach because these poultry animals can significantly contribute indirectly to the transmission of resistant genes to other segments of environments [45]. Immediate efforts are required to deal with the emergence of MDR bacteria, including antimicrobial resistance in humans and poultry. Antimicrobial resistance from animals has become a public health issue, as evidence has shown the transmission of antimicrobial-resistant bacteria, including Gram-positive bacteria [46–48], or their resistance genes, between animals and humans [49]. Poultry animals can play a role in the transmission of resistance genes to humans due to the MDR of Enterobacteriaceae, including *C. freundi*i in the intestines.

Conclusion

The prevalence of *C. freundii* in healthy laying chickens was observed to be low, with an incidence of 4.23%. *Citrobacter freundii* was discovered to show resistance to multiple classes of antibiotics. A Class 1 integron-encoding gene was identified in *C. freundii* of animal origin. The finding of MDR *C. freundii* with a plasmid containing a characteristic Class 1 integron supported the concept that there is an environmental reservoir of resistance. Exploration of the molecular genetic features of *C. freundii* harboring a Class 1 integron gene in poultry may also facilitate epidemiological monitoring and source tracking of resistance determinants and their environments. This would deepen our understanding of the ecological cycle of antibiotic resistance. This may enable practical interventions to reduce the traffic of resistance genes through the use of appropriate and effective antibiotics in laying hens, good farm biosecurity practices, and proper hygiene in farms. As such, this study provides a basis for improving public health.

Authors' Contributions

MHE and FJW: Conceptualization. MHE, AMW, and DAP: Data curation. FJW and AMW: Formal analysis. MHE and FJW: Funding acquisition. AMW and DAP: Investigation. MHE and AMW: Methodology. MHE and AMW: Project administration. MHE, AMW, and DAP: Resources. MHE and FJW: Supervision. MHE and ENU: Validation. FJW and AMW: Visualization. MHE, FJW, and ENU: Writing original draft. MHE and ENU: Writing – review and editing. All authors have read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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