p-ISSN 2615-787X e-ISSN 2615-790X
Trop. Anim. Sci. J.
Vol. 44 No. 3: 255-376, September 2021

Tropical Animal Science Journal

Published by Faculty of Animal Science, IPB University (Bogor Agricultural University), associated with Animal Scientist's Society of Indonesia

p-ISSN 2615-787X e-ISSN 2615-790X

Tropical Animal Science Journal

Accredited by Directorate General of Strengthening for Research and Development,
Ministry of Research, Technology and Higher Education of the Republic of Indonesia
No: 30/E/KPT/2018

Vol. 44 No. 3, September 2021

PUBLISHER
Faculty of Animal Science, IPB University (Bogor Agricultural University) associated with Animal Scientist's Society of Indonesia

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Tropical Animal Scince Journal (Trop. Anim. Sci. J.) previously Media Peternakan is a scientific journal covering broad aspects of tropical animal sciences. Started from 2018, the title is changed from Media Peternakan in order to develop and expand the distribution as well as increase the visibility of the journal. The journal is published FOUR times a year in March, June, September, and December by Faculty of Animal Science, IPB University (Bogor Agricultural University), associated with Animal Scientist's Society of Indonesia. Tropical Animal Scince Journal is a peer reviewed journal that has been indexed and abstracted in Elsevier products (Scopus, Reaxys), Clarivate Analytics product (Emerging Sources Citation Index), Scimago Journal Rank, ASEAN Citation Index, DOAJ, Dimensions-Digital Science, CABI, EBSCO, Science and Technology Index (SINTA), Google Scholar, and other scientific databases. The journal also uses Similarity Check to prevent any suspected plagiarism in the manuscripts.

SECRETARIAT OF TROPICAL ANIMAL SCIENCE JOURNAL
Faculty of Animal Science Building, IPB University (Bogor Agricultural University)
Jalan Agatis, Kampus IPB Dramaga, Bogor 16680, Indonesia
Phone/Fax: +62-251-8421692
e-mail: mediapeternakan@apps.ipb.ac.id; mediapeternakan@yahoo.co.id
website: http://journal.ipb.ac.id/index.php/tasj

Subscription Rate (Not including shipping and handling):
IDR 100.000/ edition and IDR 350.000/ year (Indonesian subscribers); 50 US$ (Foreign subscribers)
Bank account: Bank Rakyat Indonesia KCP KAMPUS IPB No. 0595-01-005208-53-3, o.b. Media Peternakan, SWIFT CODE: BRINIDJA

Tropical Animal Science Journal
p-ISSN 2615-787X e-ISSN 2615-790X

Vol. 44 No. 3, September 2021

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Accredited by Directorate General of Strengthening for Research and Development,
Ministry of Research, Technology and Higher Education of the Republic of Indonesia
No: 30/E/KPT/2018

HISTORY

Tropical Animal Science Journal (Trop. Anim. Sci. J.) (p-ISSN 2615-787X and e-ISSN 2615-790X) previously Media Peternakan (published from 1967-2017 with p-ISSN 0126-0476 and e-ISSN 2087-4634) is a scientific journal covering broad aspects of tropical animal sciences. Started from 2018, the title is changed from Media Peternakan in order to develop and expand the distribution as well as increase the visibility of the journal. The journal is published FOUR times a year in March, June, September, and December started from the year 2020 by Faculty of Animal Science, IPB University (Bogor Agricultural University), associated with Animal Scientist's Society of Indonesia.

The first edition with the new title was published in April 2018 edition (Vol 41 No 1 2018), while the previous edition (up to 2017 edition) still use Media Peternakan as the title. The online version of Tropical Animal Scince Journal could be accessed in the new website (http://journal.ipb.ac.id/index.php/tasj) and the previous editions are available in the old website (http://medpet.journal.ipb.ac.id/).

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p-ISSN 2615-787X e-ISSN 2615-790X
Accredited by Directorate General of Strengthening for Research and Development No: 30/E/KPT/2018

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DOI: https://doi.org/10.5398/tasj.2021.44.3.363
Available online at http://journal.ipb.ac.id/index.php/tasj

Detection of Class 1 Integron Encoding Gene in Multidrug Resistance (MDR) *Citrobacter freundii* Isolated from Healthy Broiler Chicken

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(Received 01-09-2020; Revised 18-01-2021; Accepted 16-02-2021)

ABSTRACT

This study was aimed to find out that broiler chicken farms have problems with antibiotic resistance *Citrobacter freundii* and determined the prevalence and class 1 integron encoding gene. Multidrug resistance *Citrobacter freundii* was collected from broiler chicken among one hundred and sixty cloacal swab samples from 32 farms in Blitar for 3 months. The method of bacterial inoculation used MacConkey agar and biochemical test was conducted by IMViC and TSIA test. *Citrobacter freundii* for antibiotic sensitivity pattern was tested by disk diffusion, and the multidrug resistance encoding gene was tested by PCR. This study exposed 160 samples, and 13.75% (22/160) samples were positive of *Citrobacter freundii*. The antibiotic sensitivity pattern showed high resistances against ampicillin and erythromycin (77.27%), tetracycline (59.09%), trimetropim-sulfamethoxazole (50%), and streptomycin (22.72%). Isolates that were detected as multidrug resistance were continued with PCR testing to prove the existence of a class 1 integron encoding gene. Multidrug resistance *Citrobacter freundii* isolated from broiler chicken farms in Blitar were 81.82% (18/22), and were indicated that five were positive Class 1 Integron encoding gene. The results of this study showed that the prevalence and distribution of multidrug resistance *Citrobacter freundii* were high, so it can cause the spread of antimicrobial resistance to public health. Class 1 integron encoding gene was found 22.72% from multidrug resistance *Citrobacter freundii* by PCR. It was concluded that broiler chicken farms need assessment management to reduce and avoid multidrug resistance bacteria in animals and human. Therefore, the use of appropriate antibiotics is a good step to reduce the incidence of MDR in poultry.

Keywords: broiler chicken; Citrobacter freundii; Class 1 integron encoding gene; multidrug resistance; public health

INTRODUCTION

Antibiotic is commonly used to prevent bacteria infection in poultry (Mehdi *et al.*, 2018). Poultry management applied more than one antibiotic to control bacteria (Roth *et al.*, 2019). Multi antibiotics uses for treatment caused resistance to bacteria and became a global public health problem (Prestinaci *et al.*, 2015). Antibiotics have been used for the treatment and prevention of disease in animals. Food of animal origin contains large amounts of antibiotic residues due to high antibiotic treatment, and this residue can be transmitted from animal products to humans (Landers *et al.*, 2012). Antibiotic resistance was found and detected at the Class 1 integrons encoding gene in poultry (Asgharpour *et al.*, 2018).

Integrons are gene to express encoding for antibiotic resistance (Kheiri *et al*, 2016). Integrons are discovered on the transposons and contain inserted gene cassettes. The characterization of integrons is to harbor gene cassettes coding for resistance to antimicrobial (Domingues *et al.*, 2012; Permatasari *et al.*, 2020). Class 1 integrons are frequently reported with high prevalence in Gram negative bacteria, including *Citrobacter freundii*. The class 1 integrons are found to be pathogen and made a worldwide significant bacterial infection in livestock (Karimi *et al.*, 2020; Hidayatullah *et al.*, 2020).

Enterobacteriaceae is frequently found in poultry (Projahn *et al.*, 2018), especially *Citrobacter freundii*. *Citrobacter freundii* is Gram negative, coccobacilli, motile using flagella and facultative anaerobic bacteria and commonly found in food, soil, water, and the intestines of animals and humans (Liu *et al.*, 2018). *Citrobacter freundii* existed in gastrointestinal and distributed food borne diseases of animals (Bai *et al.*, 2012; Prota *et al.*, 2015). Antibiotics inhibited the growth of bacteria, and treatment of multiple drugs caused resistance in bacteria infection. Multidrug resistance has been the global public problem (Liu *et al.*, 2018; Ansharieta *et al.*, 2020). Therefore, a detection method is needed at the molecular level to prove the spread of multidrug resistance (MDR) in poultry farms. The use of the Class 1 integron gene coding for the detection of MDR in *C. fruendii* on poultry is a new step in the right direction.

This study was designed to evaluate the class 1 integron in broiler poultry farms and assessment of the multidrug resistance encoding gene amongst *Citrobacter freundii*. This study was designed to determine the possibility of emerge multidrug resistance caused by class 1 integron from bacteria in poultry farms.

MATERIALS AND METHODS

Ethical Clearance

Cloacal swabs were used in this study. Hence ethical clearance was not necessary. Cloacal swabs were collected from broiler chicken farms in Blitar, East Java province, Indonesia.

Sample Isolation and Identification

This cross-sectional study was conducted between June and August 2019. One hundred and sixty cloacal swab samples were collected from broiler chicken farms in Blitar, Indonesia. The Sampling of the farm was based on less maintenance, sanitation, cleanliness, and hygiene management (Effendi *et al.*, 2018). The cloacal swab samples were cultured and incubated in MacConkey agar plates (Oxoid, Cheshire, UK) at 37°C for 24 h (Al Humam, 2016). The positive of *Citrobacter freundii* were indicated by lactose fermenting in MacConkey agar and colonies of bacteria obtain acquire pure subcultures (Liu *et al.*, 2017). Colonies of *Citrobacter freundii* were confirmed by biochemistry test. Indol test, Simmon Citrate Agar and TSIA were biochemistry tests to identify genus and species of *Citrobacter freundii* (Janda *et al.*, 1994; Nayar *et al.*, 2014).

Antibiotic Sensitivity Test

Kirby-Bauer disc diffusion assay on medium Mueller-Hilton agar was done for testing antibiotic sensitivity (Nassar *et al.*, 2019; Effendi *et al.*, 2019). Erythromycin 15 µg, streptomycin (10 µg), tetracycline (30 µg), trimethoprim-sulfamethoxazole (25 µg), and ampicillin (10 µg) were antibiotics used to represent the antibiotic resistance according to Clinical and Laboratory Standards Institute recommendation. Antimicrobial susceptibility testing was carried out by measurement of inhibitory zone diameter formed at 37°C for 24 hours. (Clinical and Laboratory Standards Institute, 2017).

Detection of Class 1 Integron Encoding Gene

Citrobacter freundii was revealed multidrug resistance using PCR method and analysis subtypes Class I Integron encoding gene identification. DNA isolation was done with the QIAamp® DNA mini kit (QIAGEN, Germany). The primers encoding the gene Class 1 Integron were hep 58 (TCATGGCTTGTTATGACTGT) and hep 59 (GTAGGGCTTATTATGCACGC). We used GoTaq Green mastermix (Promega, USA) and PCR condition 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 is carried out for 30 cycles. PCR results were confirmed by electrophoresis using 2% agarose gel (Invitrogen, USA), the amplicon using primer hep 58 and hep 59 was 200 bp (Singh *et al.*, 2017).

RESULTS

Cloacal swab samples were collected from 160 chickens in broiler poultry farms in Blitar and the appearance of multidrug resistance (MDR) *Citrobacter freundii* was detected. The sample was obtained 13.75% (22/160) *Citrobacter freundii* and 81.82% (18/22) multidrug resistance cases in broiler poultry farms.

The antibiotic sensitivity test showed that the highest multidrug resistance *Citrobacter freundii* to ampicillin and erythromycin (77.27%), tetracycline (59.09%), trimetropim-sulfamethoxazole (50.00%), and streptomycin (22.72%) (Table 1). The most multidrug resistance showing sensitive to antibiotic is streptomycin (77.27%). Antibiotics are commonly used to affect sensitive bacteria, but if used inappropriately, they can lead to antibiotic resistance (Singh *et al.*, 2017).

The amplification Class 1 Integron of *Citrobacter freundii* from 22 cloacal swab samples of broiler poultry was positive for 200 bp using the primer hep 58 and hep 59 (Nagachinta *et al.*, 2009). The results indicated that five were positive Class 1 Integron encoding genes (Table 2).

The results showed that several resistance antibiotics of *Citrobacter freundii* are related to the appearance of the Class 1 Integron. Figure 1 shows *Citrobacter freundii* are resistance to several antibiotics. Figure 2 shows that multidrug resistance to antibiotics. The multidrug resistance *Citrobacter freundii* by antibiotic susceptibility testing was confirmed 18/22 (81.82%), and Class 1 Integron encoding gene positive by PCR testing was showed 5/22 (22.72%) (Figure 3).

DISCUSSION

The study discovered contamination of *Citrobacter freundii* caused pathogen and foodborne diseases in broiler farms (Aminharati *et al.*, 2019). The multidrug resistance to several antibiotics was reported from isolated 160 cloacal swab samples. The major public health problem has many cases especially higher prevalence of

Table 1. Antibiotic susceptibility profile of *Citrobacter freundii* in broiler chicken farms in Blitar, Indonesia

Antibiotics	Resistance (%)	Intermediate (%)	Sensitive (%)
Ampicillin (AMP)	17 (77.27%)	1 (4.55%)	4 (18.18%)
Erythromycin (E)	17 (77.27%)	3 (13.64%)	2 (9.09%)
Tetracycline (TE)	13 (59.09%)	0	9 (40.91%)
Trimethrophim-sulfamethoxazole (SXT)	11 (50.00%)	0	11 (50.00%)
Streptomycin (S)	5 (22.73%)	0	17 (77.27%)

Table 2. Samples result of multidrug resistant (MDR) and Class 1 Integron cases on *Citrobacter freundii*

Locations	Sample of *Citrobacter freundii*	Positive *Citrobacter freundii*	Positive MDR	Positive Class 1 Integron by PCR
Farm 3	B3an, **B3bn**, *B3cn*	3 (13.64%)	2 (9.09%)	1 (4.55%)
Farm 4	B4bn, B4dn	2 (9.09%)	0	0
Farm 7	**B7cn**	1 (4.55%)	1 (4.55%)	0
Farm 8	**B8an, B8en**	2 (9.09%)	2 (9.09%)	0
Farm 11	*B11en*	1 (4.55%)	1 (4.55%)	1 (4.55%)
Farm 14	*B14an, B14bn*	2 (9.09%)	2 (9.09%)	2 (9.09%)
Farm 26	**B26bn**	1 (4.55%)	1 (4.55%)	0
Farm 27	**B27an, B27en**	2 (9.09%)	2 (9.09%)	0
Farm 28	**B28cn**, B28dn	2 (9.09%)	1 (4.55%)	0
Farm 30	**B30an, B30dn, B30en**	3 (13.64%)	3 (13.64%)	0
Farm 31	**B31an,** *B31cn*, **B31en**	3 (13.64%)	3 (13.64%)	1 (4.55%)

Note: Bold code= positive result of MDR *Citrobacter freundii;* Italic code= positive result of Class 1 Integron gene.

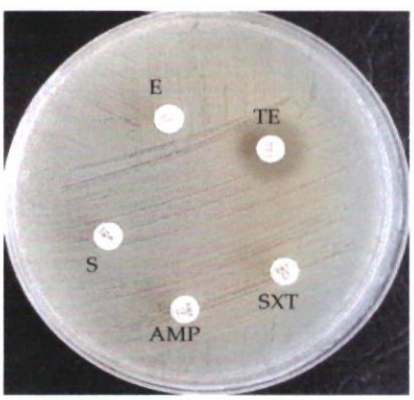

Figure 1. Antibiotic susceptibility profile of *Citrobacter freundii* from broiler chicken farms in Blitar, Indonesia with result was multidrug resistant (MDR). The antibiotic were Ampicillin (AMP), Erytrhromycin (E), Tetracycline (TE), Trimethrophim-sulfamethoxazole (SXT), and Streptomycyn (S). The samples were incubated at 37°C for 24 hours.
Note: AMP, E, SXT, and S were resistant to *Citrobacter freundii*; TE was sensitive. Therefore, this isolate was MDR due to more than three antibiotics resistant.

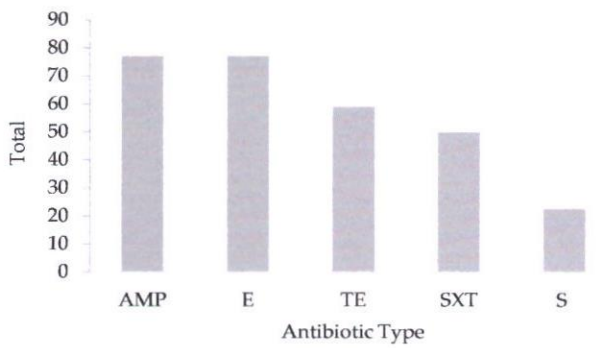

Figure 2. Precentage of antibiotic resistance on *Citrobacter freundii*. AMP (Ampicillin), E (Erytrhromycin), TE (Tetracycline), SXT (Trimethrophim-sulfamethoxazole), and S (Streptomycyn).

multidrug resistant bacterial pathogens. Integrons are related to multidrug resistance in many cases of bacterial diseases (Akrami *et al.*, 2019; Krauland *et al.*, 2009). Integrons were recognized and detected first time in Gram-negative bacteria (Deng *et al.*, 2015; Domingues *et al.*, 2012; Pormohammad *et al.*, 2019).

Organisms that are resistant to three or more classes of antimicrobials are referred to as multidrug resistant (MDR) (Magiorakos *et al.*, 2012; Wibisono *et al.*, 2020). One method frequently used by various researchers to characterize organisms as MDR is based on the results of *in vitro* antimicrobial susceptibility testing (Kallen *et al.*, 2010). The most commonly used definition is for Gram-negative bacteria that are resistant to three or more classes of antimicrobials (Gould, 2008, Kristianingtyas *et al.*, 2020). The variability of this definition is provided in the comprehensive MDR review (Falagas *et al.*, 2006), which is used by some researchers as a reference that a large number of studies do not propose a specific definition for MDR.

There were 18 MDR of *Citrobacter freundii* isolates in this study, shown in Table 2, although 5 isolates contain Class 1 integrons gene of *Citrobacter freundii* in this study. Other isolates are shown negative of Class 1 integrons gene because globally pathogens of Class 1 integrons discovered on the chromosomes of environmental bacteria. Four classes of integron have been described, each of which codes for a distinct but related integrase enzyme. Class 1 integrons are the most widely studied (Rosser & Young, 1999). Class 1 integrons are bounding in conjugative plasmids, transposons conjugative plasmids, and spreading by lateral gene transfer. Therefore, Class 1 integrons have overspread to almost all species of Gram-negative pathogens. Class 1 integron is a major competence in the global spread of multidrug resistance and important to recover management of the farm to decrease the prevalence of antibiotic resistance and improving the balance of healthy life.

Consumption of antibiotics increased antibiotic resistance containing important MDR organisms in poultry. These MDR organisms can be transmitted to humans

Figure 3. Class 1 Integron gene of *Citrobacter freundii* from broiler chicken farms in Blitar, Indonesia. MRK (marker), K+ (positive control) was used Escherichia coli from patients of multidrug resistance (MDR), K- (negative control) was used *Pseudomonas aeruginosa* ATCC 27853. A) *B3cn, B11en, B14an, B14bn* were positive result of Class 1 Integron; B) *B31cn* was positive result of Class 1 Integron.

through direct contact or consumption, and *C. freundii* causes economic losses and levels of food contamination. Therefore antibiotic-resistant genes have the potential to spread to other populations. The abundant use of antibiotics in poultry farming has been linked to treatment failure and the development of antibiotic resistance itself.

Integrons are genes encoding multidrug resistance and reduction of treatment for bacterial infections (Mostafa *et al.*, 2015). The classification of integrons is based on differences in the gene structure of integrases (Cury *et al.*, 2015). The spread of Class 1 integrons have been found commonly in Gram-negative bacteria and showed the existence of Class 1 integrons of *Citrobacter freundii* in this study. The appearance result of amplification class 1 integron from positive isolates were 22.72%. The amplification using primer hep 58 and hep 59 for 200bp represent class 1 integron of *Citrobacter freundii*. Multidrug resistance was reported to be spread by Class 1 integron genes in Gram-negative bacteria.

The multidrug resistance nature of this isolate can be explained by the fact that it is mediated by plasmids carrying multiresistant genes and by transposons, and by integrons that are easily transferred to other bacteria, not necessarily of the same species (Widodo *et al.*, 2020). Bacteria with various resistance to antibiotics are widespread in animals and the environment (Kwoji *et al.*, 2019, Riwu *et al.*, 2020). Recent surveys from China (Gao *et al.*, 2015), Thailand (Runcharoen *et al.*, 2017), and Indonesia (Effendi *et al.*, 2018), have illustrated an alarming trend regarding resistance among broad-spectrum beta lactamase-producing organisms (ESBLs), which are also multidrug-resistant isolated from animals and the environment (Wibisono *et al.*, 2020).

Farm animals are frequently found to be infected by pathogenic bacteria and the infection can be transfered from animals to humans, especially *Citrobacter freundii* (Zhou *et al.*, 2019; Liu *et al.*, 2017). The treatment to pathogenic bacteria used large amounts of antibiotics to reduce attack infection and caused resistance to multiple drugs. Multidrug resistance bacteria are most important to observation concern because influence public health problem.

CONCLUSION

Citrobacter freundii was discovered in broiler chicken. This study showed that multidrug resistance *Citrobacter freundii* was confirmed 18/22 (81.82%), and Class 1 Integron encoding gene positive by PCR testing was 5/22 (22.72%). The result revealed multidrug resistance Gram negative bacteria emerge in poultry farms and need evaluation management plans to prevent the transfer of multidrug resistance bacteria from poultry to humans and the environment.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

ACKNOWLEDGEMENT

This research was in part funded by the Direktorat Riset dan Pengabdian Masyarakat, Deputi Bidang Penguatan Riset dan Pengembangan Kementerian Riset dan Teknologi/ Badan Riset dan Inovasi Nasional, Indonesia in fiscal year 2020 with grant number: 756/ UN3.14/PT/2020.

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