Original Research

Journal of Advanced Veterinary Research
(2024) Volume 14, Issue 1, 204-208

Fluoroquinolone resistance and phylogenetic analysis of broiler *Campylobacter jejuni* isolates in Indonesia

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ARTICLE INFO

Recieved: 15 October 2023

Accepted: 15 December 2023

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Keywords:

Campylobacter jejuni
Broiler
gyrA
Phylogenetic
Human health

ABSTRACT

Consumption of poultry contaminated with *Campylobacter jejuni* is the main source of sporadic campylobacteriosis in humans, while fluoroquinolone resistance is increasing in *Campylobacter jejuni* isolated from poultry. The aim of this study was to detect the incidence of fluoroquinolone resistance and analyze phylogenetics by sequencing gyrAse subunit A from broiler *Campylobacter jejuni* isolates. The contents of 200 chicken intestines were taken from chicken farms in 4 sub-districts (Sukorejo, Pandaan, Kejayan, and Grati) in Pasuruan Regency, Indonesia. The Kirby-Bauer Diffusion Test method is used to detect fluoroquinolone resistance phenotypically. Polymerase chain reaction is used to detect fluoroquinolone resistance genotypically through detection of the *gyrA* gene. A phylogenetic tree based on *gyrA* genes was created using MEGA12. The results showed that 31 *Campylobacter jejuni* isolates had high resistance to nalidixid acid (100%), enrofloxacin (96.7%), and ciprofloxacin (93.6%). All *Campylobacter jejuni* isolates (100%) were fluoroquinolone resistant phenotypically and had the *gyrA* gene genotypically. Phylogenic analysis showed that the *Campylobacter jejuni gyrA* gene sequence isolated from broilers from different sub-districts were highly related. Sequence results from broilers with *gyrA* gene sequences from humans appear to be in the same cluster, indicating that zoonotic transmission can occur. The discovery of a high percentage of fluoroquinolone resistance genes, where fluoroquinolone is the first line drug for the treatment of diarrhea in humans, should certainly be an important issue related to human health.

Introduction

Infection with *Campylobacter jejuni*, which accounts for over 500 million cases of gastroenteritis annually, is the most common cause of diarrheal illness worldwide (Kirk *et al*., 2015). Most human cases are accompanied by gastroenteritis, with symptoms of acute watery or bloody diarrhea, vomiting, abdominal pain, dehydration, and fever (Riwu *et al*., 2020). *Campylobacter jejuni* is very pathogenic and has the ability to cause health complications such as reactive arthritis, irritable bowel syndrome, septicemia, irritable bowel, Miller Fisher syndrome (MFS) and Guillain Barre syndrome (GBS) (Chukwu *et al*., 2019).

Consuming poultry contaminated with *Campylobacter jejuni*, through consumption of eggs and meat, is the main source of sporadic campylobacteriosis in humans (Cha *et al*., 2016). Humans can also be infected through activities where humans come into contact with poultry droppings, such as cleaning poultry cages, changing cage bedding, and handling poultry, because birds excrete *Campylobacter jejuni* bacteria through their droppings (Varga *et al*., 2019). The widespread use of antibiotics in chicken farming, according to numerous research, is a factor in the rise in antimicrobial resistance (AMR) (Chang *et al*., 2015; Marshall and Levy, 2011). Enrofloxacin is an antimicrobial from the fluoroquinolone class which is often used for prophylactic or therapeutic purposes in poultry (Grabowski *et al*., 2022). In poultry, enrofloxacin is generally added to drinking water, while in pigs and cattle, treatment is limited to sick animals. As a result, *Campylobacter* isolated from chicken had the highest level of fluoroquinolone resistance (Ragimbeau *et al*., 2014). This incident is of course a matter of concern because antimicrobial resistance bacteria can be transmitted quickly to humans through food derived from poultry and cause infections that are difficult to treat (Hemeg, 2018).

Due to a surge in *Campylobacter* strains that are resistant to antibiotics, particularly fluoroquinolones, antimicrobial resistance in this bacterium has recently become a significant issue for human health (Frazão *et al*., 2020). The number of *Campylobacter* strains that are drug-resistant to ciprofloxacin has nearly doubled in the last 20 years, according to the Centers for Disease Control and Prevention, and treatment options are restricted due to the disease (CDC, 2019). Most cases of human *campy-*

ISSN: 2090-6277/2090-6269/

*lobacter*iosis are self-limited and do not require antibiotic therapy, but in rare instances the infection can proceed to bacteremia or become an extraintestinal infection and necessitates antibiotic therapy, especially in people with impaired immune systems (Marotta *et al*., 2019). In cases like this, the drug of choice that is often given to humans is fluoroquinolone, which is the last class of antibiotics. Fluoroquinolone is an antibiotic that is generally used to treat all diarrheal diseases (Baggio and Ananda-Rajah, 2021). However, excessive use of antibiotics in humans and animals has increased the resistance of these bacteria to fluoroquinolones (Varga *et al*., 2019).

Fluoroquinolone resistance in *Campylobacter jejuni* is primarily caused by the single mutation C257T in the quinolone resistance-determining region (QRDR) of the *gyrA* gene, which is connected to the single threonine-to-isoleucine mutation (Thr-86-Ile) of the *gyrA* gene in isolates from humans and animals (Sierra-Arguello *et al*., 2018). When a C to T transition occurs at codon 86 in the QRDR of the *gyrA* gene, a Thr-86-Ile substitution results, resulting in high level resistance to fluoroquinolones (Kinana *et al*., 2007). There are studies that report another substitution, namely Thr-86-Ala, in the *gyrA* QRDR, which plays a role in fluoroquinolone resistance, but provides a phenotypically lower resistance effect when compared to Thr-86-Ile (Han *et al*., 2012). Polymerase Chain Reaction (PCR) or sequenced-based methods targeting the QRDR region of *gyrA* have proven to be highly predictive for detecting *Campylobacter jejuni* strains that are phenotypically resistant to fluoroquinolones (Ragimbeau *et al*., 2014).

Information regarding antimicrobial resistance in *Campylobacter jejuni* in developing countries is very lacking, especially genotypically. Currently, there is no data on molecular antimicrobial resistance in *Campylobacter* in Indonesia. The World Health Organization has categorized fluoroquinolone as a critically important antimicrobial drug for humans, so surveillance to monitor its use and resistance needs to be implemented (WHO, 2018). To the best of the authors' knowledge, this is the first study in Indonesia which aimed to detect the incidence of fluoroquinolone resistance and analyze phylogenetics by sequencing gyrAse subunit A from broiler *Campylobacter jejuni* isolates.

Materials and methods

Ethical statement

Biosafety, Animal Use, and Ethics Committee of the Faculty of Veterinary Medicine, Wijaya Kusuma Surabaya University, approved this study under the reference ethics No: 86-KKE/2022. Verbal consent was sought from farm owners prior to interviewing.

Study area and sample collection

This study was conducted from August to October 2022. A sample of 200 was taken from 4 sub-districts (Sukorejo, Pandaan, Kejayan, and Grati) in Pasuruan Regency. Samples in the form of broiler intestinal contents were collected by slaughtering the broiler, then performing abdominal dissection to remove the contents of the small intestine. Samples were placed in sterile plastic bags, which had been prepared to prevent microbial contamination from the environment. Samples were stored in a cool box while traveling to the laboratory for research. Sample processing was carried out at the Wates Yogyakarta Veterinary Center and the Institute of Tropical Disease Airlangga University.

Microbiological analysis

A sample of 10 grams of chicken feces was put into a dark bottle containing 40 ml of Nutrient Broth No. 2 (Oxid, England) which had been added with 5% lysed sheep's blood, Preston supplement, and FBP (ferrous sulfate, sodium metabisulfite and sodium pyruvate), under microaerophilic conditions (5% O_2, 85% N_2, 10% CO_2) incubated at 37°C for 4 hours, then at 42°C for 24 hours (FDA, 2021). In this study, microaerophilic conditions were obtained by inserting bacterial cultures into a 2.5 L aerojar and adding a 2.5 L sachet CampyGen Gas Generating Kit (Oxoid, England).

The culture in the enrichment media was centrifuged first at a speed of 3500 rpm for 20 minutes. A total of 1 loop pellet was etched onto modified charcoal cefoperazone deoxycholate agar/mCCDA selective media (Oxoid, England) containing CCDA selective supplement (Oxoid, England). Using a quadrant scratching technique, scratching was done, and then the sample was incubated under microaerophilic conditions for 48 hours at 42°C. Purification of bacteria was carried out by streaking on mCCDA media. Bacterial identification is then carried out using Gram staining, catalase test, and polymerase chain reaction (PCR). Positive control *Campylobacter jejuni* strain used ATCC 33560 (Microbiologics, Minnesota).

Phenotipic fluoroquinolone resistance

The Kirby-Bauer Diffusion Test method on Mueller Hinton Agar (MHA) media (Oxoid, England) was used to detect fluoroquinolone resistance phenotypically. *Campylobacter jejuni* cultures were inoculated in physiological NaCl solution using an equalization of 0.5 Mc. Farland (1.5 x 10^8 CFU/ml). Using a sterile swab, the suspension that has been made is then spread over the surface of MHA containing 5% lysed sheep blood, then an antibiotic disk paper (Oxoid, England) is placed on the surface of the media. The antibiotic discs tested were ciprofloxacin 5 µg, nalidixic acid 30 µg, and enrofloxacin 5 µg. The media was incubated at 37°C for 24 hours under microaerophilic conditions (Gharbi *et al*., 2018). After incubation, the inhibition zone surrounding the paper disc is seen and the bacteria's sensitivity standard to antibiotics is determined using the standard interpretation table determined by the Clinical Laboratory Standard Institute 2018 (CLSI, 2018).

Genotipic fluoroquinolone resistance (gyrA)

Polymerase chain reaction was used to detect *Campylobacter jejuni* and fluoroquinolone resistance strains genotypically. Qiamp DNA small kit (Qiagen, Hilden, Germany) and QIAprep Spin small-prep Kit (Qiagen, Hilden, Germany) were used to extract DNA, respectively, according to the manufacturer's recommendations. The master mix formulation for PCR amplification consists of 5 µl DNA template, 1 µl each primer, 0.5 µl Nuclease Free Water and 12.5 µl PCR master mix containing Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffer. The final volume of the reaction mixture was 20 µl. The PCR reagent mixture is then inserted into the Select Cycler II BioPoducts thermal cycler. The *hipO* primers for detecting *Campylobacter jejuni* strains consist of forward-ACTTCTTTATTGCTTGCTGC and reverse-GCCACACAAGTAAAGAAGC, with amplification conditions consisting of primary denaturation for 0.5 minutes at 95°C, then 35 cycles of secondary denaturation at 95°C for 0.5 minutes, annealing at 59°C for 0.5 min, extension at 72°C for 0.5 min, and final extension at 72°C for 7 min (Wang *et al*., 2002). The *gyrA* primers for detecting fluoroquinolone resistance consist of forward-GAAGAATTTTATATGCTATG and reverse-TCAGTATAACGCATCGCAGC, with amplification conditions consisting of primary denaturation for 5 minutes at 95°C, then 35 cycles of secondary denaturation at 95°C for 50 seconds, annealing at 53°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 7 minutes (Reddy and Zishiri, 2017).

The results of PCR product amplification were carried out by electrophoresis in a 1.5% agarose gel to which RedSafe Nucleid Acid Staining Solution gel dye was added. Marker 100 was also inserted into the agarose gel well to determine the size of the PCR product DNA. Electrophoresis was carried out for 30 minutes with a constant voltage of 100 volts. Electrophoresis was stopped and the gel was removed for observation

under Ultra Violet (UV) light. The results obtained are DNA band patterns which show different numbers and patterns.

Deoxyribonucleic Acid (DNA) sequencing and phylogenetic analysis

The sample's PCR product produced positive electrophoresis results, and 1st Base performed DNA sequencing through PT. Genetic Science Indonesia. The Sanger dideoxy technique was used to carry out the sequencing process. The PCR products sequenced in this study were the results of *gyrA* primer amplification of four PCR products. Using the BioEdit program, consensus order was created by editing, aligning, and aligning the forward and reverse sequences. The BLASTn search engine was used on nucleotide sequences (https://www.ncbi.nlm.nih.gov/BLAST), to confirm the detected genes. After that, the sequences were analyzed, and a phylogenetic tree based on the *gyrA* genes was created using MEGA12.

Results

Microbiological analysis

Of a total of 200 samples of broiler small intestine contents, 25% (50/200) of the samples were positive for *Campylobacter* and 15.5% (31/200) of *Campylobacter jejuni* were present, as seen in Figure 1. PCR testing showed that isolates that were positive for *Campylobacter jejuni* produced a PCR product of 323 bp, as seen in Figure 2.

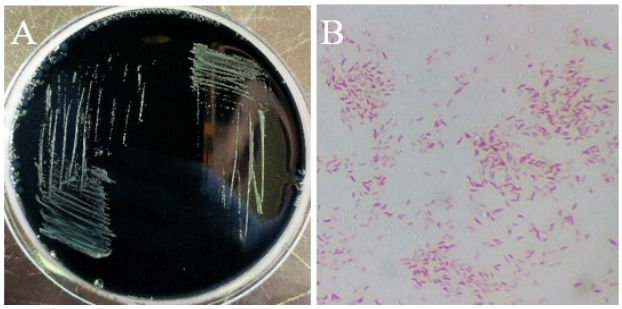

Fig. 1. A) Macroscopic and microscopic (B) *Campylobacter jejuni*.

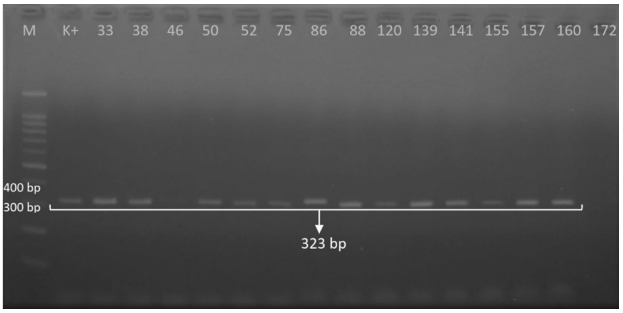

Fig. 2. Molecular identification of *Campylobacter jejuni* (*hipO* gene) by PCR. Note: M: marker; K+: positive control; 33-172: samples code.

Phenotipic fluoroquinolone resistance

The results of antimicrobial susceptibility tests on 31 *Campylobacter jejuni* isolates showed that there was high resistance to nalidixid acid 100%, enrofloxacin 96.7% (30/31), and ciprofloxacin 93.6% (29/31). All *Campylobacter jejuni* isolates (100%) had fluoroquinolone resistance when viewed phenotypically.

Genotipic fluoroquinolone resistance (gyrA)

A total of 31 *Campylobacter jejuni* isolates were tested using PCR to detect the gene encoding fluorquinolone resistance, namely *gyrA*. Of the 44 isolates, all (100%) detected the *gyrA* gene as indicated by the presence of a PCR product of 235 bp (Figure 3). The findings of this study demonstrate that the *gyrA* gene is present in all *Campylobacter jejuni* isolates that exhibit phenotypical fluoroquinolone resistance, demonstrating a close relationship between the two.

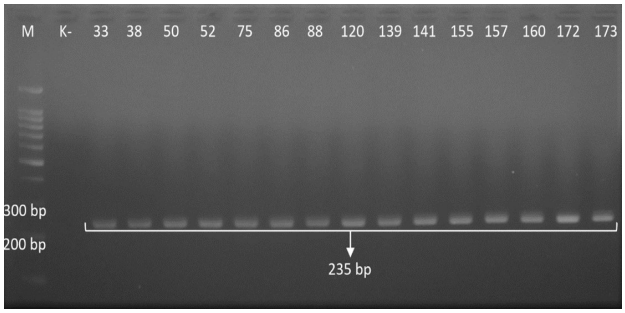

Fig. 3. Molecular identification of fluoroquinolone resistance (*gyrA* gene) from *Campylobacter jejuni* by PCR. Note: M: marker; K-: negative control; 33-173: samples code.

Deoxyribonucleic Acid (DNA) sequencing and phylogenetic analysis

The *gyrA* gene sequence was used in this study's phylogenetic analysis. The results of phylogenetic analysis revealed three clusters (Figure 4). The four isolates from this study were in the same cluster, cluster I. These four isolates came from different sub-districts in the Pasuruan Regency area. This shows the close relationship between isolates from different sub-districts.

In addition to the four isolates from this study, 21 *gyrA* gene sequences from *Campylobacter jejuni* (from several countries) were also taken from the public domain (NCBI) for analysis. The results of the phylogenetic tree show that the four isolates from Indonesia are in the same cluster (cluster I) with the *gyrA* gene from several Asian countries such as Korea, China, Thailand, India and Japan, as well as countries other than Asia such as the United States of America, Egypt, Austria, Slovenia, Spain, Ireland, and Kenya.

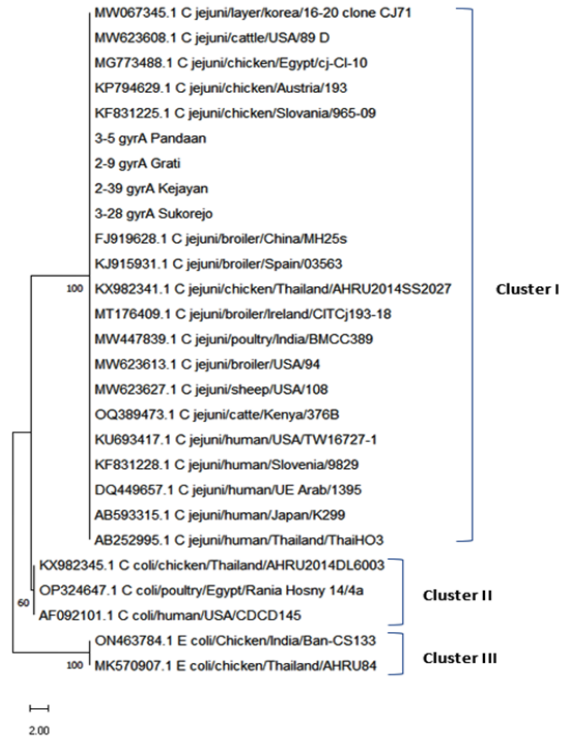

Fig. 4. Investigation of the *gyrA* gene's phylogenetic relationships using the neighbor-joining method.

Discussion

Campylobacter jejuni colonies that grow on mCCDA agar media are grayish white, round, convex, smooth and shiny (Esson *et al.*, 2016). *Cam-*

pylobacter jejuni bacteria appear red with Gram Hucker staining, have a spiral shape, wavy rods, are gram negative and catalase active (Sison *et al*., 2014). The high prevalence of *Campylobacter jejuni* compared to other Campylobacter species in broilers is similar to previous research in other countries, where *Campylobacter coli* was reported as the predominant Campylobacter species in broilers (Han *et al*., 2016; Torralbo *et al*., 2015).

In recent years, high fluoroquinolone resistance has also been reported in other Asian countries such as India (Yadav and Maherchandani, 2020), Thailand (Wangroongsarb *et al*., 2020), and China (Yang *et al*., 2023). Compared to other Gram-negative bacteria, Campylobacter can develop fluoroquinolone resistance quickly because of a single-step point mutation in the *gyrA* gene at position Thr-86-Ile (Otigbu *et al*., 2018).

High fluoroquinolone resistance is caused by the unwise use of antibiotics including fluoroquinolones as feed additives in intensive chicken farming, both for therapeutic and prophylactic purposes (Ragimbeau *et al*., 2014). The use of antimicrobials in animal food is common in Indonesia (Wijayanti *et al*., 2023). Enrofloxacin is an antimicrobial from the fluoroquinolone class which is often used because its price is relatively cheap in Indonesia (Trouchon and Lefebvre, 2016). Although in Indonesia the ban on the use of antibiotic growth promoters (AGP) in the livestock sector became effective in January 2018 referring to Minister of Agriculture Regulation No. 14/2017, however its use for therapeutic purposes is permitted provided that no residue is left in the product circulating in the community. This could be one of the causes of *Campylobacter jejuni*, which was identified from Indonesian poultry, having a high level of enrofloxacin resistance.

To combat antibiotic resistance, agricultural biosecurity must be strengthened and enforced more strictly. Antibiotics will no longer be used in the production of food in a country, but if this is not accompanied by strong biosecurity, the number of resistant bacterial isolates will still rise (Da Silva *et al*., 2023). This incident has occurred in Australia, where giving fluoroquinolones to animals is not allowed there (Collignon, 2005). The frequency of Campylobacter isolates with antimicrobial resistance was relatively low. However, several years later, fluoroquinolone-resistant Campylobacter isolates emerged in chickens in Australia, even without the use of fluoroquinolone in poultry. This fluoroquinolone resistant *Campylobacter jejuni* isolate probably came from abroad and was brought to Australian chickens via humans, vectors or wild birds (Abraham *et al*., 2020). These findings show the importance of biosecurity in efforts to overcome antimicrobial resistance

The research results show a high number of cases of *Campylobacter jejuni* which has fluoroquinolone resistance. This provides important guidance for physicians and veterinarians in providing judicious therapy for cases of campylobacteriosis in humans and animals within the same geographic area.

A total of 31 *Campylobacter jejuni* isolates were tested using PCR to detect the gene encoding fluorquinolone resistance, namely *gyrA*. Of the 44 isolates, all (100%) detected the *gyrA* gene as indicated by the presence of a PCR product of 235 bp (Figure 3). These results are comparable to the results of research conducted by other researchers. Woźniak-Biel *et al*. (2017) showed that 100% of *Campylobacter jejuni* isolated from broilers in Poland had the *gyrA* gene detected. Research in China also stated that all *Campylobacter jejuni* ciprofloxacin resistance isolates had the *gyrA* gene (Yang *et al*., 2017).

Because *Campylobacter jejuni* only needs one mutation in the quinolone resistance determining region (QRDR) of gyrAse A to confer fluoroquinolone resistance (*gyrA*) to the bacterium, the rate of identification of the *gyrA* resistance gene in this species is high (Han *et al*., 2012). This is different from *Escherichia coli* or Salmonella which require several point mutations in QRDR gyrAse A to be able to have the fluoroquinolone resistance gene (Shaheen *et al*., 2021). Fluoroquinolone-resistant mutants appear quickly and easily in both animals and humans because just one point mutation is necessary for high-level resistance (Goulart *et al*., 2023). The most prevalent mutation is Thr-86-Ile (Han *et al*., 2012). There are other mutations that also occur in *Campylobacter jejuni* gyrAse A, namely the Ala-70-Thr and Asp-90-Asn mutations, but these mutations are rare and cause intermediate resistance to fluoroquinolones (Wieczorek and Osek, 2013; Aksomaitiene *et al*., 2018). Other research also proves that the increase in fluoroquinolone resistance strains throughout the world also occurs due to clonal *Campylobacter jejuni* lineages that are resistant to fluoroquinolone (Cha *et al*., 2016).

The *gyrA* gene is the only gene encoding fluoroquinolone resistance in *Campylobacter jejuni*, although in other bacteria such as *E. coli*, mutations in gyrAse B or the *gyrB* gene also encode fluoroquinolone resistance (Chien *et al*., 2016). Several studies have proven that the gyrAse B mutation is a silent mutation in *Campylobacter jejuni* (Adiguzel *et al*., 2021; Aksomaitiene *et al*., 2018; Hakanen *et al*., 2002). There is no evidence to date that changes in the *gyrB* gene cause *Campylobacter jejuni* to become resistant to fluoroquinolones (Kinana *et al*., 2007; Yang *et al*., 2017). *Campylobacter jejuni* also does not have the *parC* gene and the parE gene in topoimerase IV, which are also genes encoding fluoroquinolone resistance, which other bacteria can have (Yadav and Maherchandani, 2020).

The isolates in this study came from broiler chickens and after phylogenetic analysis it was found that all the *Campylobacter jejuni gyrA* genes originating from chickens were in the same cluster, namely cluster I. However, the *Campylobacter jejuni gyrA* genes originating from humans and other animals such as chickens and cows also is in cluster I. This study's phylogenetic analysis using the *Campylobacter jejuni gyrA* gene reveals a high degree of resemblance across isolates from humans and animals. The similarity in the distribution of the *gyrA* gene sequence between chicken and human isolates indicates that there are cases of *Campylobacter jejuni* infection in humans that are related to the consumption of poultry food products contaminated with *Campylobacter jejuni*, or through direct contact with chickens (Yang *et al*., 2023). These findings back with earlier studies showing that chickens can infect people with *Campylobacter jejuni*. The significant degree of similarity between the *gyrA* genes of *Campylobacter jejuni* isolated from various sources, locations, and years suggests the possibility of transmission between clinical and non-clinical sources as well as between human and animal sources (Frazão *et al*., 2020).

From the phylogenetic analysis it can also be seen that the *gyrA* gene from *Campylobacter jejuni* is in a different cluster from the *gyrA* gene from *Campylobacter coli* even though it comes from the same genus, but they are still closely related. *E. coli* and other Gram-negative bacteria with the same *gyrA* gene are located in a separate cluster and are typically distantly related to the *Campylobacter jejuni gyrA* gene. In contrast to *Campylobacter*, which only needs one point mutation in the *gyrA* QRDR to generate substantial fluoroquinolone resistance, *E. coli* only exhibits resistance to nalidixic acid as a result of a single *gyrA* QRDR mutation. Additional *gyrA* mutations are necessary for fluoroquinolone high-level resistance (Ruiz, 2003). There are differences in point mutations in gyrAse A between *Escherichia coli* and *Campylobacter jejuni*, the S83L and D87N substitutions in *gyrA* are the ones most frequently identified in *E. coli* isolates (Mirzaii *et al*., 2018). It is well known that *Campylobacter jejuni* is inherently less vulnerable to fluoquinolones than *E. coli* is, maybe as a result of variations in the *gyrA* sequence (Kinana *et al*., 2007).

This research has several shortcomings, continuous surveillance was not carried out, but only at one time and in certain regions, which may not be nationally representative. This research was limited to broiler chicken isolates only and did not include isolates from humans, animals or other animal products. Further studies on *Campylobacter jejuni* to meet the "One Health" principle are needed to help provide information for future public health strategies to reduce the incidence of antimicrobial resistance (Redondo *et al*., 2019).

Given that fluoroquinolones are the first-line medication for treating diarrhea in people, the identification of a high percentage of fluoroquinolone resistance genes should undoubtedly be a significant concern for human health (von Wintersdorff *et al*., 2016). This would preclude the use of these antimicrobials for the treatment of human campylobacteriosis, only if fluoroquinolone susceptibility still exists can these antimicrobials be recommended for the indicated therapy of campylobacteriosis (Wieczorek and Osek, 2013). Intensive use of the fluoroquinolone class of drugs in poultry should be evaluated considering that fluoroquinolones are used to treat campylobacteriosis in humans (de Vries *et al*., 2018).

Conclusion

Sequence results from broilers with *gyrA* gene sequences from humans appear to be in the same cluster, indicating that zoonotic transmission can occur. The discovery of high fluoroquinolone resistance in *Campylobacter jejuni* isolates from broilers, both phenotypic and genotypic, where fluoroquinolone is the first line drug for treating diarrhea in humans must certainly be an important issue related to human health. This would rule out using these antibiotics to treat human campylobacteriosis; these antibiotics can only be suggested for use in campylobacteriosis advised therapy if fluoroquinolone susceptibility still exists. Intensive use of the fluoroquinolone class of drugs in poultry should be evaluated considering that fluoroquinolones are used to treat campylobacteriosis in humans.

Acknowledgments

This article was supported in part by the Postgraduate Research-Doctoral Dissertation Research Funding from Center for Higher Education Funding (Balai Pembiayaan Pendidikan Tinggi) and Indonesia Endowment Funds for Education (Lembaga Pengelola Dana Pendidikan), with grant number from KEPUTUSAN KEPALA PUSAT LAYANAN PENDIDIKAN Nomor: 1306/J5.2.3/BPI.06/10/2021. BPI ID Number: 202101121565; 202209091803.

Conflict of interest

The authors declare that they have no conflict of interest.

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