First report of phenotypic and genotypic (bla$_{OXA-61}$) beta-lactam resistance in *Campylobacter jejuni* from broilers in Indonesia

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Abstract

Background and Aim: *Campylobacter* is a zoonotic bacterium that is a major source of foodborne diseases. In humans, most cases of campylobacteriosis are caused by *Campylobacter jejuni*. Poultry is the main reservoir of *Campylobacter* for humans, because *Campylobacter* is part of the normal flora of the digestive tract of poultry. Antimicrobial resistance to several antibiotics in *Campylobacter* isolated from humans and food animals has increased rapidly. Beta-lactam is an antibiotic with a high prevalence of resistance in *Campylobacter*. This study aimed to investigate phenotypic and genotypic (bla$_{OXA-61}$) beta-lactam resistance in *C. jejuni* from broilers in Indonesia.

Materials and Methods: A total of 100 samples of broiler intestinal contents were obtained from 10 broiler farms in Pasuruan Regency, Indonesia. *Campylobacter jejuni* was identified using conventional and polymerase chain reaction (PCR)-based methods. Phenotypic detection of beta-lactam resistance was performed using an antimicrobial susceptibility test with antibiotic disks of aztreonam, ampicillin, and amoxicillin-clavulanic acid. Genotypic detection by PCR was performed using the bla$_{OXA-61}$ gene, which encodes beta-lactamase.

Results: *Campylobacter jejuni* was identified in 23% of the samples. Phenotypically, 100% (23/23) and 73.9% (17/23) *C. jejuni* isolates had high resistance to aztreonam and ampicillin, respectively, but all isolates were susceptible to amoxicillin–clavulanic acid. Genotypically, all isolates carried bla$_{OXA-61}$, indicated by the presence of a 372-bp PCR product.

Conclusion: *Campylobacter jejuni* is highly resistant to beta-lactams and is a serious threat to human health. Resistance to beta-lactams should be monitored because beta-lactamase genes can be transferred between bacteria. Public awareness must also be increased on the importance of using antibiotics rationally in humans and animals.

Keywords: antimicrobial resistance, beta-lactam, bla$_{OXA-61}$, *Campylobacter jejuni*, human health.

Introduction

Campylobacter is a zoonotic pathogen that is a major source of foodborne bacterial illness worldwide [1]. In humans, campylobacteriosis is caused by *Campylobacter jejuni*, whereas the remainder is mostly caused by *Campylobacter coli* [2]. Recently, the incidence and prevalence of campylobacteriosis have been increasing in both developing and developed countries [1]. According to the Centers for Disease Control and Prevention, campylobacteriosis affects >1.5 million people (excluding undiagnosed and unreported cases) in the United States every year [3]. Campylobacteriosis is generally self-limiting with fever, stomach cramps, and bloody diarrhea. However, campylobacteriosis can become chronic and cause reactive arthritis, Guillain-Barré syndrome, Miller-Fisher syndrome, urinary tract infection, irritable bowel, sepsis, and certain neuropathies. [4].

Poultry is the main source of *Campylobacter* transmission to humans, because it is a part of the normal flora in the digestive tract of poultry [5]. *Campylobacter* spp., especially *C. jejuni*, can be found in high numbers in ceca broilers at the farmer level and on carcasses in poultry slaughterhouses and meat markets [6]. *Campylobacter* can be transmitted from poultry through activities that may expose humans to poultry excrement, or by handling and consuming contaminated meat [7]. In Indonesia, 61.9% of chicken meat was reported to contain *Campylobacter* and most isolates (41.07%) were *C. jejuni* [8]. In South Asia, the incidence of antimicrobial

resistance in *Campylobacter* isolated from humans and food animals has increased during the past decade, especially in countries with widespread use of antibiotics in livestock [9–11]. *Campylobacter* spp. are highly resistant to beta-lactam antibiotics [11]. *Campylobacter* strains that produce beta-lactamase were more resistant to ticarcillin, amoxicillin, and ampicillin than beta-lactamase-negative strains. This is attributed to the bla_{OXA-61} gene, which encodes Cj0299-a putative periplasmic amino acid class D beta-lactamase [12]. The overuse of antibiotics in human and animal populations has increased the number of antimicrobial-resistant bacteria [13]. Antimicrobial resistance poses an additional risk because infection with antibiotic-resistant *Campylobacter* leads to longer hospitalizations, higher rates of treatment failure, and increased morbidity and mortality [14].

In Indonesia, information regarding antimicrobial resistance in *C. jejuni* is limited; therefore, this study aimed to investigate phenotypic and genotypic (bla_{OXA-61}) beta-lactam resistance in *C. jejuni* from broilers. An antimicrobial susceptibility test was used for phenotype detection, and polymerase chain reaction (PCR) for bla_{OXA-61} was used for genotype detection.

Materials and Methods

Ethical approval

Animal ethical approval was obtained from the Ethical Clearance Committee of the Faculty of Veterinary Medicine, Universitas Wijaya Kusuma Surabaya, Indonesia (ethics no.: 86-KKE/2022).

Study period and location

The study was conducted from October 2022 to December 2022 at the Balai Besar Veteriner Wates Yogyakarta and the Institute of Tropical Disease Airlangga University.

Sample collection

A total of 100 samples of broiler intestinal contents were obtained from ten broiler farms in Pasuruan Regency, Indonesia. Sampling was performed by slaughtering broilers and dissecting the abdomen to obtain the contents of the small intestine. Samples were placed in sterile plastic and stored in a cool box on the way to the laboratory.

Isolation and identification of *C. jejuni* strains

Bacterial enrichment

The sample was placed in a dark bottle containing 40 mL of Bolton Broth (Oxoid AM7526, England), supplemented with 5% lysed sheep blood, Preston supplements (Oxoid SR0117, England), ferrous sulfate, sodium metabisulfite, and sodium pyruvate. The sample was incubated at 37°C for 4 h and then at 42°C for 24 h under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) [15].

Isolation and identification

Campylobacter isolation was performed using a modified BAM 2001 method [15]. One loop pellet was streaked on modified charcoal cefoperazone deoxycholate selective medium containing charcoal cefoperazone deoxycholate selective supplement (Oxoid SR 155E, England) and incubated at 42°C for 48 h under microaerophilic conditions. Isolates were confirmed as *Campylobacter* by microscopic examination, Gram staining, and biochemical tests for catalase and oxidase.

Genotypic detection of *C. jejuni* strains (hipO gene) and beta-lactam resistance (bla_{OXA-61} gene)

DNA extraction

QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and QIAprep Spin Miniprep Kit (Qiagen) were used to extract genomic and plasmid DNA, respectively, according to the manufacturer's recommendations.

Oligonucleotides primers

Polymerase chain reaction primers were synthesized by Integrated DNA Technologies (Iowa) (Table-1) [16, 17].

Polymerase chain reaction assay

The PCR master mix contained 5 µL DNA template, 1 µL each primer, 0.5 µL nuclease-free water, and 12.5 µL PCR master mix (Promega, USA), containing Taq DNA polymerase, dNTPs, $MgCl_2$, and reaction buffer. The final volume of the reaction mixture was 20 µL.

The PCR products were separated by electrophoresis in 1.5% agarose gel (Invitrogen, USA), supplemented with RedSafe Nucleic Acid Staining Solution gel dye (Intron, South Korea). The size of the PCR products was evaluated using a 100-bp DNA marker. The gel was electrophoresed for 30 min at a constant voltage of 100 V and visualized under ultraviolet light.

Phenotypic detection of beta-lactam resistance using an antibiotic sensitivity test

All *C. jejuni* isolates were analyzed for antimicrobial resistance on Mueller–Hinton agar plates (Oxoid CM 0337b, England), supplemented with 5% defibrinated sheep blood, containing ampicillin (10 µg), aztreonam (30 µg), and amoxicillin–clavulanic acid (30 µg) disks. The medium was incubated at 42°C for 24 h under microaerophilic conditions [18]. The bacterial susceptibility to antibiotics was interpreted by measuring the inhibition zone and using the Clinical Laboratory Standard Institute table as a standard [19].

Results and Discussion

In this study, samples were collected from the small intestine, especially from parts of the jejunum, because the results for *C. jejuni* isolation were good. *Campylobacter jejuni* mainly colonizes the ceca and small intestine [20]. *Campylobacter jejuni* can colonize the intestinal tract, especially at the junction between the ceca, small intestine (jejunum), and large intestine, where their number is high ($\geq 10^8$ Colony-forming unit/g) [21].

Table-1: Primer sequences, target genes, amplicon sizes, and cycling conditions.

Target gene	Primer Sequences	Size (bp)	Primary denaturation	Amplification (35 cycles)			Final Extension	Reference
				Secondary denaturation	Annealing	Extension		
hipO	F-ACTTCTTTATTGCTTGCTGC R-GCCACAACAAGTAAAGAAGC	323	95°C 0.5 min	95°C 0.5 min	59°C 0.5 min	72°C 0.5 min	72°C 7 min	[16]
bla_{OXA-61}	F-AGAGTATAATACAAGCG R-TAGTGAGTTGTCAAGCC	372	95°C 5 min	95°C 50 s	54°C 30 s	72°C 1 min	72°C 7 min	[17]

Based on the results of isolation, morphological identification, and biochemical tests (catalase and oxidase tests), *Campylobacter* was detected in 44/100 samples. Polymerase chain reaction analysis showed that 23% (23/100) isolates were positive for *C. jejuni* (323-bp product) (Table-2 and Figure-1). Moreover, 52.3% (23/44) *Campylobacter* isolates were *C. jejuni*, which is most commonly found in poultry, whereas *C. coli* is predominant in pigs [12].

In Indonesia, the prevalence of *C. jejuni* in poultry is 23%. The prevalence rates were similar to our study in Jordan 17% [22], Ecuador 18.9% [23], Benin (West Africa) 23.4% [24], and India 24% [25]. Polymerase chain reaction after selective enrichment is recommended for identifying *Campylobacter* in drinking water, dairy products, aquatic habitats, and poultry products. In other countries, prevalence rates vary, which is probably influenced by variations in sampling design and testing technique [25].

Based on antimicrobial susceptibility tests, *C. jejuni* isolates had the highest level of resistance to aztreonam 100% (23/23) and a lower level of resistance to ampicillin 73.9% (17/23). However, all isolates (23/23) were susceptible to amoxicillin-clavulanic acid (Figure-2).

In this study, all *C. jejuni* isolates were resistant to aztreonam (100%), which is consistent with results from Lebanon [12] and Jordan [26]. Most *C. jejuni* are resistant to ampicillin (74%), which is the most commonly used antibiotic in poultry [27]. This result is similar to studies in South China 70.7% [28], but lower than the incidence in several countries with a range of 81.8%–100% [26, 29, 30]. The combination of amoxicillin-clavulanic acid is the most efficient against *C. jejuni* based on the results of this study, because all *C. jejuni* isolates were susceptible to amoxicillin-clavulanic acid. These results are similar to studies in the Lebanon [12] and United Kingdom [31]. In contrast, a study in Tunisia showed that *C. jejuni* was 22.7% resistant to amoxicillin-clavulanic acid [29].

This study focuses on discovering the gene (bla_{OXA-61}) in *C. jejuni*, a new gene discovered in Indonesia. Polymerase chain reaction detected the bla_{OXA-61} gene (372-bp PCR product) in all 23 *C. jejuni* isolates (Figure-3). Six *C. jejuni* isolates that were susceptible to ampicillin also carried the bla_{OXA-61} gene. Most *C. jejuni* strains can produce beta-lactamase, which inactivates beta-lactam by hydrolyzing the lactam ring [32]. Beta-lactamase is divided into four classes according to Ambler's classification: class A (KPC and most extended-spectrum β-lactamase), class B (MBL), class C (AmpC beta-lactamase), and class D (OXA). The bla_{OXA-61} gene belongs to class D and is widely encoded in integrons [33]. Class D beta-lactamase OXA-61 is the most frequently identified in *Campylobacter* [34].

In this study, all *C. jejuni* isolates were phenotypically resistant to aztreonam and genotypically possessed the bla_{OXA-61} gene, indicating a strong correlation between the two. Consistently,

Table-2: Results of isolation, identification, and PCR test to detect *Campylobacter jejuni*.

Total samples	Isolation (culture on mCDDA)		Identification (microscopy, Gram stain, biochemical tests)		PCR test hipO gene	
	No of positive		No of positive		No of positive	
100	No	%	No	%	No	%
	44	44	44	44	23	23
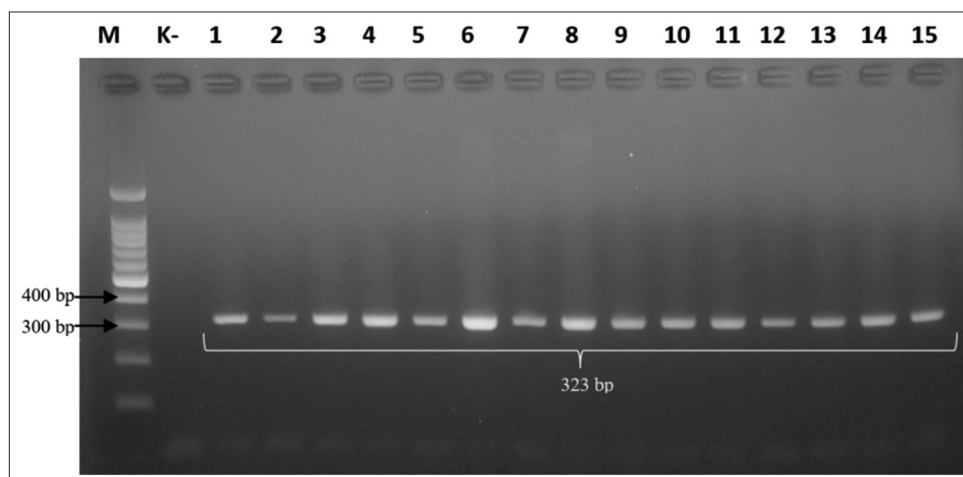
Figure-1: Gel electrophoresis results for detection of *Campylobacter jejuni* using the hipO gene (323-bp). Note: Lanes: M, 100-bp marker; K-, control negative; 1-15, representative of *Campylobacter jejuni* isolates.
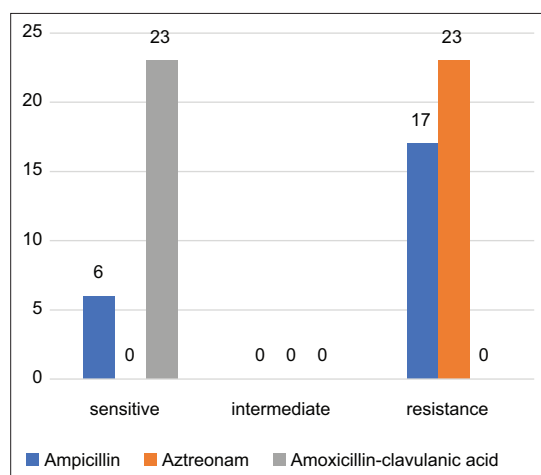
Figure-2: Results of the antimicrobial susceptibility test of *Campylobacter jejuni* isolate.

class D beta-lactamase, which consists only of OXA, can hydrolyze cephalosporins and aztreonam and has carbapenemase activity [35]. The ability to hydrolyze monobactam (aztreonam) is shared by class A and class C, but not class B, beta-lactamases [33, 36].

Molecular investigation of antimicrobial resistance in the tested isolates showed a strong correlation between the antibiotic resistance phenotype and the genotypes and mutations coding for antibiotic resistance [18]. In this study, all isolates that were phenotypically resistant to ampicillin were detected by the bla_{OXA-61} gene, which encodes antimicrobial resistance to beta-lactams. Most ampicillin-resistant *Campylobacter* isolates from poultry carried the bla_{OXA-61} gene, according to other studies conducted in the UK [31] and Brazil [37].

However, despite the strong correlation between antimicrobial resistance to beta-lactams and bla_{OXA-61}, 6 ampicillin-susceptible *C. jejuni* isolates possessed this gene. Our results are supported by a report in which 59% of ampicillin-susceptible isolates carried bla_{OXA-61} [38], indicating that bla_{OXA-61} is poorly expressed in ampicillin-susceptible isolates, and therefore, less beta-lactamase is produced compared with resistant isolates [39]. This could be because there is a relationship between ampicillin susceptibility and the presence of G-T transversion in the bla_{OXA-61} promoter, only isolates that are resistant to ampicillin have G-T transversion [38]. Alfredson and Korolik [34] (GenBank accession number AY587956) identified bla_{OXA-61} in ampicillin-resistant *C. jejuni* isolates with G-T transversion. A large-scale study is warranted to evaluate the association between G-T transversion and high-level bla_{OXA-61}-mediated beta-lactam resistance in *C. jejuni* isolates [38].

CmeABC efflux pumps contribute to beta-lactamase resistance and could explain the ampicillin susceptibility of *C. jejuni* strains carrying bla_{OXA-61} [12]. In a study, ampicillin susceptibility increased 32-fold after CmeB insertion mutagenesis in *C. jejuni* strain 81-176136 and other strains [40]. In another study utilizing the NCTC 11168 strain, the CmeB mutant was four times more susceptible to ampicillin than parental strain, and cmeB overexpression increased ampicillin resistance four folds [41].

In this study, all *C. jejuni* isolates susceptible to amoxicillin–clavulanic acid carried bla_{OXA-61}, indicating that this combination strongly inhibits beta-lactamase and can be used as an alternative to
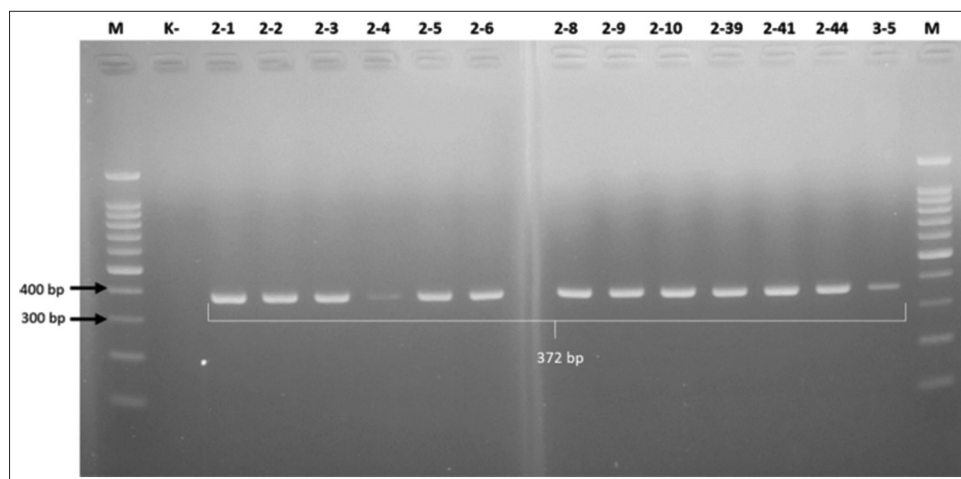
Figure-3: Gel electrophoresis results for detection of beta-lactam resistance using the bla$_{OXA-61}$ gene in *Campylobacter jejuni* isolate (372-bp). Note: Lanes: M, 100-bp marker; K-, control negative; 2-1 – 3-5, representative of *Campylobacter jejuni* isolates.

fluoroquinolone, tetracycline, and erythromycin, which *C. jejuni* is resistant to [31, 39]. In *C. jejuni*, the expression of beta-lactamase, which causes resistance to amoxicillin, ampicillin, and ticarcillin, can be inhibited with tazobactam, clavulanic acid, and sulbactam [12].

Antimicrobial resistance in pathogenic microorganisms, especially *C. jejuni*, is a global health challenge. According to the Centers for Disease Control and Prevention, drug-resistant strains of *Campylobacter* infect >300,000 individuals each year, making treatment a major health challenge and economic burden. Antimicrobial resistance in bacteria can be generated by antibiotic overuse by humans, antibiotic use in animal feed or veterinary treatment, and increased industrial waste in the environment [26, 32, 42]. Bacterial resistance to antimicrobials has evolved in a variety of ways; in most situations, bacteria exposed to antibiotics discover ways to avoid or resist antimicrobial agents [31, 40, 43, 44].

Conclusion

Our results show that *C. jejuni* has high resistance to beta-lactams and is a serious threat to human health. Beta-lactam resistance should be monitored because beta-lactamase genes can be transferred between bacteria. Public awareness of the importance of using antibiotics rationally in humans and animals must be increased. More studies are needed to understand antibiotic resistance to and develop diagnostic media for *Campylobacter* spp. To the best of our knowledge, this is the first study to genotypically identify beta-lactam resistance in *C. jejuni* from Indonesian poultry. Our findings highlight the importance of a surveillance and monitoring system and risk analysis for *C. jejuni* prevalence and resistance in poultry and other animal feeds.

Authors' Contributions

SMY, MHE, and WT: Conceptualized and supervised the study and drafted the manuscript. SMY: Data curation and formal analysis. SMY and MM: Investigation and visualization. MHE and WT: Methodology. MHE, WT, and ENU: Validation. WT and ENU: Review and editing. All authors have read, reviewed, and approved the final manuscript.

Acknowledgments

Competing Interests

The authors declare that they have no competing interests.

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