Phenotypic and Genotypic Detection of *Campylobacter jejuni* at Local Chicken and Chicken Meat

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Abstract. The Objective of this study was to identify the existence of *Campylobacter jejuni* based on phenotypic and genotypic characteristic in local chicken and chicken meats. Samples of local chicken intestine and meat were tested for the bacterial existence. Phenotypic examination was carried out by means of cultivation followed by gram staining and biochemical tests. Genotypic examination was conducted by polymerase chain reaction (PCR) using genus specific16S rRNA gene at 816 bp and membrane-associated protein A (*mapA*) gene at 589 bp as *Campylobacter jejuni* species-specific gene. The result of phenotypic detection revealed the existence of *Campylobacter spp* as gram negative, curved rod shape, oxidase positive, urease negative and motile. Genotypic examination also indicated the existence of bacteria using both primers. However, no *Campylobacter jejuni* detected from meat of the chickens. The results suggest that the method of PCR using a primer detecting species-specific gene of *Campylobacter jejuni* gives a rapid and accurate detection of the bacteria as compared to that using phenotypic and biochemical test. Identification of *Campylobacter spp* from chicken meats should be improved with enrichment method and sample collection.

Key Words: *Campylobacter jejuni*, *mapA* gene, local chicken

Introduction

Demands of meat and other animal food products increase continuously in line with economic growth and population growth and increased public awareness of the importance of nutritional value for health. In addition to nutritious food, people also expect safe food. Safe food means not contaminated by microbiological, chemical and physical contaminations. Food poisoning in Indonesia are mostly caused by microbial pathogens (Kandun, 2000).

In the current era of globalization, farm products are required to be able to compete not only domestically but mainly to capture the international market. Domestic and foreign consumers nowadays increasingly demand requirements of higher quality. Products are also required to be residue free both biological materials such as microbial pathogens, and chemicals, pesticides, heavy metals, antibiotics, hormones and drugs (BSN, 2000).

Data revealing cases of food poisoning due to bacterial infection has not been revealed so that there are many cases of poisoning and infection but it cannot trace the cause. By knowing the cause or infection, the incidence of infection in humans through animal food can be prevented by doing more adequate food sanitation and decontamination.

Campylobacter jejuni and *Campylobacter coli* are leading bacterial causes of human gastroenteritis in the United States and other industrialized nations. These organisms frequently colonize avian hosts, including commercial poultry, but are also found in the gastrointestinal tract of other warm-blooded animals, including swine, sheep, and cattle (Gharst et al., 2006). *Campylobacter spp* are still some of the most important enteropathogen world wide.The major route of infection in humans is through consumption of contaminated poultry meat, probably because of contamination of chicken carcasses with *Campylobacter* and frequency of poultry consumption (Kamberovic et al., 2007).

Campylobacteriosis is the name of the illness caused by *C. jejuni*. It is also often known as *Campylobacter* enteritis or gastroenteritis. *Campylobacter* rarely causes death or spectacular outbreaks of food poisoning, so these organisms do not trigger the same degree of concern as Escherichia *coli* O157:H7 or

Salmonella. Nevertheless, *C*. *jejuni* is one of the most common causes of bacterial enteritis in humans and may lead to serious complications, such as Guillain Barré syndrome or mucosa-associated lymphoid tissue (Yuki, 1998; Lecuit et al., 2004). Danish study indicated that the risk of death was significantly increased after infection with *Campylobacter*, especially in patients older than 55 years . The main source of *Campylobacter* infections highlighted in epidemiological studies is consumption of contaminated food, particularly raw or insufficiently cooked poultry products (Evans et al., 2006).

In Indonesia, the presence of these bacteria in the digestive tract and feces in local chicken and broilers has not been much revealed yet. Likewise, its presence in meat in slaughterhouses, traditional markets, supermarkets or meat products has not been much concerned. This condition is guessed because the high cost of *Campylobacter* identification and the long time of identification. In some countries the handling of sanitation and hygiene is adequate enough, but the prevalence of this bacterial contamination in chicken meat is still high.

The purpose of this research is to identify the presence of *Campylobacter jejuni* bacteria in phenotype and genotype tests in local chicken and chicken meats as many reported its existence in broilers in another country. Isolates obtained can be used to determine the characteristics of *Campylobacter* from local chicken and chicken meats such as resistance and the presence of virulence genes. In addition by knowing the existence and level of bacterial contamination *Campylobacter jejuni* from animal source and origin foods of animal it is expected to do early action in reducing these pathogens contaminants and preventing the society away from foodborne pathogen.

Materials and Methods

Sample Collection

Samples of 3 local chicken cecal, 2 broiler cecal and 3 chicken meats were taken from the location of chicken slaughtering in Mataram. In addition, sample taking was also conducted on 66 chicken meats and 11 traditional markets in Yogyakarta. Samples were examined for the presence of *Campylobacter spp* in Bio-medical Research Unit RSU Mataram (Mataram General Hospital), and Microbiology laboratory, Faculty of Veterinary Medicine, Gadjah Mada University.

Phenotypic Test of *Campylobacter spp*

Samples of meat and intestinal contents, each was weighed as much as 0.5 grams and then diluted up 5 ml of 0.9% NaCI. A total of 100 µl suspension was cultured on media in order to be selective for *Campylobacter* that has been supplemented by antibiotic and growth supplements (Oxoid) and 5% defibrinated sheep blood. Culture was also carried out in agar *Campylobacter* blood-free media (CCDA) which was supplemented with antibiotics. Culture was done by leveling suspension on the surface of a Petri dish. Cultures Petri dish was incubated inverted at 37°C for 48 hours in a micro-aerobic situation, with 5% O_2 and 10% CO_2 atmosphere.

Bacteria Gram and Biochemistry Examinations

The object glass that has been cleaned with alcohol was given 1 ose leveled bacterial suspension, wind-dried and fixed with spirtus flames. After it became cold, violet crystal solution was then given (gram A) by 2-3 drops and then aged for 1 minute, washed with flowing water and then wind-dried. Next, it was sprinkled with lugol solution/mordan (gram B) for 1 minute, washed with flowing water and wind-dried. After that, it was given acetone alcohol solution (gram C) for 30 seconds, washed with flowing water and then wind-dried. The last, it was given safranin solution (gram D) for 2 minutes, and then washed with flowing water and wind-dried. Observations were conducted using a 1000-time magnification microscope with immersion oil. Positive gram bacteria were violet, while the negative gram bacteria were red. Cell measurements were performed using objective micrometer and ocular micrometer. After knowing the shape of bacteria was curved rod, like comma, or letter S and had negative gram nature then was followed by biochemical test. Bacterial isolates obtained were identified by biochemical tests include catalase, oxidation, urease, and motility (Al Mahmeed et al., 2006). *Campylobacter spp* isolates obtained was

stored in brain heart infusion broth which was added with 5% defibrinated sheep blood and 15% glycerol at a temperature of -20°C to be characterized further (Rivoal et al., 2005).

Genotypic Test of *Campylobacter spp*

DNA extraction

Pure cultures of *Campylobacter sp* were extracted using Trizol-LS method, in way the sample was extracted in Eppendorf tube. Trizol-LS (Invitrogen) of 750 µl was added and vigorously shaken 10 times and then vortex for 2 minutes, incubated for 5 minutes at room temperature. 200 µl of chloroform was added and shake vigorously 10 times, incubated for 10 minutes and then rotated 14,000 rpm for 15 minutes. Water phase was separated into a new eppendrof tube for RNA isolation, whereas the organic phase and inter-phase for the isolation of DNA and proteins. Absolute ethanol was added to the organic phase and inter-phase phase back and forth 10 times, incubated for 3 minutes, next rotated 14,000 rpm for 5 minutes and separated its supematan for proteins examination. Whereas pellets/DNA sediment was washed 2 times by adding 1 ml of 0.1 M Na citrate in 10% ethanol, each washing was incubated 30 minutes, then rotated 14,000 rpm for 5 minutes. The pellets were washed again with 1 ml of 75% ethanol and then incubated 20 minutes in which every 5 minutes was turned back and forth, rotated 14,000 rpm for 5 minutes. DNA in the form of pellets (sediment) was wind-dried for 5 - 10 minutes, then resuspensi with 50 µl of NaOH mM.

DNA amplification

In determining the level of *Campylobacter* genus in this study used the genus-specific primer-base sequence based on 16S rRNA gene, with the forward primer C412F: (5'GGATGACATTTTCGGAGC-3') and reverse primers C 1228R: (5'-CATTGTAGCACGTGTGTC-3') (The Midland Certified Reagen Company Inc. of Midland, Texas) with result size 816 base pairs (Inglish and Kalischuk, 2003). While for identifying *Campylobacter jejuni* was based on species-specific gene with its target genes are mapA genes. Primer used for amplification was forward MdmapAl: (5'-CTATTTTATTTTTGAGTG CTTGTG-3') and reverse MDmapA2:(5'-GCTTT ATTTGCCATTTGTTTTATTA-3') (The Midland Certified Reagen Company Inc. of Midland, Texas), with gene size 589 base pairs (Stucki et al. 1995). Amplification using Cycler machine (Biorad, USA) with reaction conditions as the temperature predenaturation 95°C for 5 minutes, denaturation 94°C for 30 seconds, annealing 45°C for 60 seconds, extension 72°C for 60 seconds consisting of 35 cycles and temperature Postwell 72°C for 5 minutes.

DNA electrophoresis.

Electrophoresis used 2% of agarose gel using a Mini Sub-Marine DNA Cell device. Running buffer used was TBE buffer (Tris boric acid EDTA) Ix which was diluted from TBE 10x. For base length standard was used 100 bp DNA ladder. Voltage used was 98 to 100 volts, with a constant voltage for approximately 45 minutes. Gel from electrophoresis and then analyzed by using GelDOC (Biorad).

Data obtained in the form of phenotypic and biochemical tests including bacteria shape, gram, catalase, oxidation, urease, H_2S, citrat, motility, Indol, and others were analyzed descriptively. Similarly, the existence of a genus-specific gene 16S rRNA genus-specific and specific gene of *Campylobacter jejuni* from PCR product in form of band was analyzed descriptively.

Results and Discussion

Phenotypic Test

Phenotypic characterization was characterized by difference conditions observed directly such as colony and cell morphology, gram properties, the influence of temperature and oxygen on the growth and sensitivity to antibiotics (Osborn and Smith, 2005). From the research results on local chicken intestine could be observed a colony of bacteria in the blood agar media supplemented with antibiotics. The identified colonies were small, round, clear, transparent and shiny. Colonies of bacteria and the source of local chicken intestine which have been purified and made gram staining showed that these bacteria have negative gram and curved rod-shaped with observation of microscope magnification 1000 times (Fig. 1). Based on phenotypic and biochemical properties of bacteria were such as curved rod-shaped, negative gram, positive

motility, positive oxidase, negative catalase, negative urease, growing in microaerophilic atmosphere (Table 1). The detected bacteria based on those characteristics showed common characteristics associated with the *Campylobacter spp* bacteria. *Campylobacter spp* bacteria had characteristics of coma rod-shaped, like letter S or the wings of birds, negative gram, motil with flagella, uni or bipolar, positive oxidase and positive catalase (Sauerwein et al., 1993). In addition *Campylobacter spp* can grow well in microaerophilic atmosphere of 5% O_2, 8-10% CO_2 85% (Pratt and Karolik, 2005).

In the digestive tract, especially local chicken intestine could be identified as *Campylobacter spp* found in broilers. This is possible because the bacterium has a considerable amount in the intestine. Natural reservoirs for *Campylobacter* include chicken and other poultry, wilds, pigs, dogs, cats, sheeps and cows. *Campylobacter* species have also been recovered from feces of exotic pets such as turtles. *Campylobacter* isolation were obtained for chicken 94,2% (Workman et al., 2005). The prevalence of *Campylobacter* in broiler chickens range 6-100%, ducks 3-100%, turkeys 16-76%, and ostrichs 19% (Saleha, 2003). Chicken intestine, especially Ceca which is colonized with *Campylobacter* with high concentrations, 10^9 organisms per g of cecal contents without symptoms usually the entire flock is colonized once an infection becomes established in a poultry house (Jacobs-Reitsma et al., 1995).

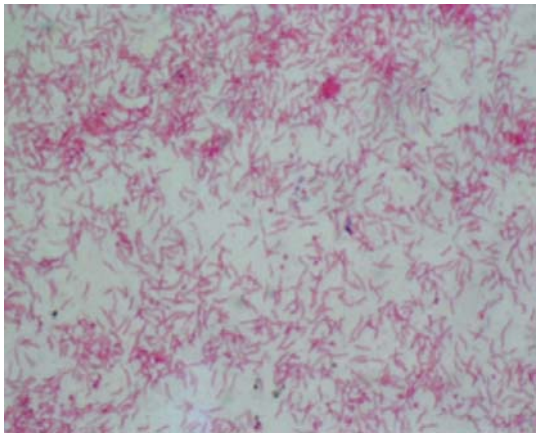

Figure 1. Shape of *Campylobacter spp* from local chicken isolate

At the same media and the same procedure, isolation of *Campylobacter spp* was done from 66 chicken meats at 11 traditional market in Yogyakarta. The results of isolation in chicken meat was identified bacteria with phenotypic and biochemical characteristics of white colonies, slimy, spread, large rod shape, negative gram, negative catalase, negative oxidase, and nonmotile. Biochemical and phenotypes characteristic of these bacteria were not in accordance with the properties of *Campylobacter spp* bacteria but much closer to *Kiebsiella spp* bacteria (Table 2). The genus of *Klebsiella* was negative gram, non-motil, rod-shaped, including in the *Enterobacteriaceae* family, producing *lysine decarboxylase*, negative catalase and negative oxidase (Podschun and Ullmann, 1998). In chicken meats examined it was not found the presence of *Campylobacter spp* bacteria, this was suspected that the bacteria were not present in the sample, another possibility, the bacteria were in the samples but did not live because the bacteria can die in the excessive oxygen conditions. *Campylobacter spp* is microaerophilic organism which requires oxygen in a small degree, and is sensitive to environmental stress such as oxygen 21%, hot, dry, disinfectants and acidity (Maff, 1993). Microaerophilic conditions required by *Campylobacter jejuni* is the oxygen content of 3-5% and carbon dioxide from 2 to 10% for optimal growth (Saleha, 2003).

Another possibility, bacteria were in meat samples but could not be cultured because these bacteria were so few in the sample that being covered by other bacteria, so the effort was required to inhibit the growth of other bacteria. Besides, it needed bacteria enrichment with selective broth media for *Campylobacter* bacteria and the sample so that the growth and development of bacteria become more optimal and bacteria will be more easily identified. The isolation process required appropriate selection of medium, i.e. by conditioning the medium of microorganism growth like the natural habitat, to achieve the optimum growth conditions (Atlas and Bartha, 1993). Several methods of *Campylobacter sp* isolation were performed by enrichment.

Table 1. Results of biochemistry and phenotype tests of *Campylobacter spp* at intestine of local chicken and broiler

No.	Bacteria properties	Local chicken intestine 1	Local chicken intestine 2	Local chicken intestine 3	Broiler intestine 1	Broiler intestine 2
1.	Colony	Small, grey, clear, shiny	Small, grey, clear, shiny	Big, white, grey, shiny	Small, grey, clear, shiny	Small, grey, clear, shiny
2.	Morphology	Curved rod	Curved rod	Big rod, straight	Curved rod	Curved rod
3.	Gram	-	-	+	-	-
4.	Katalase	+	+	+	+	+
5.	Oxidase	+	+	+	+	+
6.	Lactose	-	-	-	-	-
7.	H_2S	-	-	+	-	-
8.	Indol	-	-	-	-	-
9.	Voges Proskauer	-	-	-	-	-
10.	motility	+	+	-	+	+
11.	Urease	-	-	+	-	-
	Result	*Campylobacter spp*	*Campylobacter spp*	*Bacillus spp*	*Campylobacter spp*	*Campylobacter spp*

Table 2. Results of biochemistry and phenotype tests of suspected bacteria colony in chicken meats

No.	Bacteria Properties	Samples		
		Chicken Meat 1	Chicken Meat 2	Chicken Meat 3
1.	colony	White, slimy, spread	White, slimy, spread	White, slimy, spread
2.	Morphology	Big rod	Big rod	Big rod
3.	Gram	-	-	-
4.	Katalase	-	-	-
5.	Oksidase	-	-	-
6.	Lactose	+	+	+
7.	Citrate	+	+	+
8.	Indol	-	-	-
9.	Methyl red	-	-	-
10.	Voges Proskauer	-	-	-
11.	Motility	-	-	-
12.	Urease	+	+	+
		Klebsiella spp	*Klebsiella spp*	*Klebsiella spp*

Enrichment medium consisting nutrient broth with 5% lysed horse blood, *Campylobacter* growth supplement (sodium pyruvate, sodium metabisulfate, ferrous sulfate) and *Campylobacter* selective supplement (polymyxin, rifampicin, trimethropim, cycloheximide). Samples were incubated for 24 hours at 37-42^{o}C to allow recovery of injured cells (Bates and Phillips, 2005).

Genotypic test

Genotypic test conducted by examining the PCR results showed the existence of genus-specific gene (16S rRNA) at position 816 bp and species-specific genes (mapA) at position 589 bp. The PCR test results showed that the detected bacteria included in the *Campylobacter* genus and *Campylobacter jejuni* species (Fig. 2). *Campylobacter jejuni* is carried by most of the animal reservoirs and it's the predominant species isolated from chicken and cattle. However, some *Campylobacter* species tend to be associated with particular animal hosts. *C. coli, C. hyointestinalis* and C. mucosalis are usuallay isolated from intestines of pigs. *C. upsaliensis* and *C. helveticus* are predominantly associated with dogs and cats (Workman et al., 2005). In determining the *Campylobacter* genus was using primers-specific genus based on 16S rRNA by the size of 816 base pairs. While to

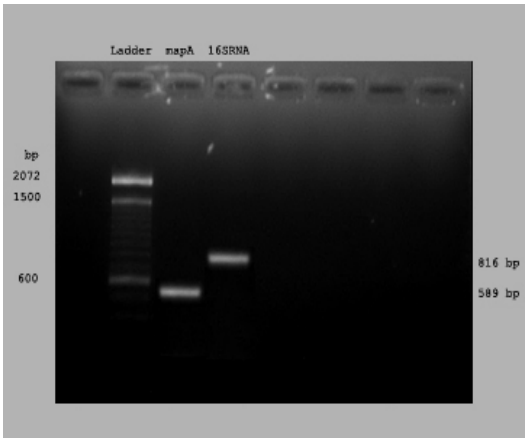

Figure 2. PCR results observed after electropho resis show the presence of genus Specific gene of *Campylobacter* (16S rRNA) at position 816 bp and species-specifif gene of *C. jejuni* (mapA gene) at position 589 bp at chicken intestine.

identify *Campylobacter jejuni* on the species level was based on species-specific gene with its target genes are *mapA* genes with gene size 589 base pairs (Stucki et al., 1995; Inglish and Kalischuk, 2003). With detection method based on the genotype with genus-specific gene and *Campylobacter* species, this can help to facilitate detecting *Campylobacter* till species level, besides this method is faster and more accurate. In many clinical laboratories, the determination of *Campylobacter* frequently comes to the genus level only because the determination till the species level is still difficult and there is a mistake. Thus, it required a simple method in order to detect and differentiate between multiple types of *Campylobacter* species based on genetic (Wegmüller et al. 1993).

The study necessary to be revealed further is related to the presence of these bacteria in chicken meat and its ability to invade and produce toxins as found in many cases of human infection. It is suspected that *Campylobacter spp* from local chicken source contains virulence genes related to invasive and produces toxins that can cause gastroenteritis in humans. In the case of *Campylobacteriosis* in Mexico 64 and 109 (58%) cases of infection caused by *C. jejuni* is positively iam gene (invasion-associated marker), this marker gene is associated with the attachment and invasion (Carvalho et al., 2001) in Bahrain and 92 *C. Jejuni* isolates that were collected and the cases of *Campylobacteriosis* in humans contains a combination of *iam* and *cdtB* gene (cytolethal distending toxin B) as much as 31% (Al Mahmeed et al., 2006).

Conclusions

Based on phenotypic and genotypic tests based on the genus-specific gene 16S rRNA and species-specific gene *mapA* (membrane-associated protein A), bacteria found in local chicken and broiler intestine is *Campylobacter jejuni*. The presence of these bacteria in the chicken intestines can be the first indication of the possibility of infection process from chicken to humans. Detection by mapA gene-based PCR in *Campylobacter jejuni* is easier and accurate compared with phenotypic and biochemical methods. Further research needs to be done related to the presence of *Campylobacter spp* virulence genes of chicken sources. Identification of *Campylobacter spp* from chicken meats should be improved with enrichment method and sample collection.

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