

Research Article

Study on Antimicrobial Action of Citric Acid on *Campylobacter Jejuni* in Reared Chicken Carcass

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Abstract

A total of 200 chicken samples (breast and thigh muscles) obtained from retail broilers at different localities in Egypt. All samples were transported as soon as possible to the lab in Animal Reproduction Research Institute and subjected to bacteriological examination for *C. jejuni*. All samples examined by conventional methods for isolation and identification of *Campylobacter*. Samples subjected to standard phenotypic identification of *C. jejuni* by PCR using specific primers of hippuricase gene. The high incidence of *C. jejune* breast meat samples was (20%) then thigh samples (14%). The high incidence of *C.*

jeune in breast samples in Giza (22 %) followed by Cairo (18%). The high incidence of *C. jejune* was in Giza (16 %), and Cairo (12%). All 68 *C. jejuni* isolates yielded the genus specific (*16S rRNA*) 63 isolates *C. jejuni* specific 323 bp. Amplicons 555 bp and 495 bp were confirmed to belong to *C. jejuni* cytolethal distending toxins showed at 555 bp for *CdtC* and at 495 bp for *CdtB*. The virulence genes of *C. jejuni* (*FlaA*, *virB11*) detected at 855 bp and 494 bp respectively. PCR analysis of *Campylobacter* virulence markers can be utilized as a simple and rapid tool especially when used as profile analysis for a control strategy. The effect of the citric acid was

correlated with the level of pH, in chicken muscles in BHI broth at 4°C after 4 hours of experiment. *C. jejune* (55.9%) strains was reduction after 30 min of experiment, (76.5%) after one hour, (89.7%) after 2 hours , (97%) after 3 hours and 100% completely reduction after 4 hours at pH changed from 2.5 to 3.5. at the end of experiment. The experiment of inhibitory effect of citric acid in chicken muscles in brain heart infusion (BHI) broth was done on positive muscle samples and pH measurements.

Finally, citric acid and their active may be used as an alternative to use of antibiotic performance. Further research is needed regarding understanding mechanism of action, identifying means to standardize the effects of other plant extract.

Keywords: Campylobacter jejune; Citric acid; Chicken meat

1. Introduction

Campylobacter jejuni is related to the poultry digestive system and to foodborne infection [1]. *Campylobacter jejuni* is considered the dominant species in chicken [2]. A limited number of studies have reported possible negative health implications in chickens caused by *C. jejuni* colonization of the gut, therefore this bacterium is considered to have a commensal relationship with chicken [3].

Campylobacteriosis is a significant enterocolitis of people, frequently acquired through consumption of undercooked poultry meat contaminated with *C. jejuni* [4]. Most human cases of campylobacteriosis are attributed to contaminated poultry [5]. Campylobacter infections in humans are usually

characterized by self-limiting watery/bloody diarrhea, abdominal cramps, nausea and fever bacteremia and other extra intestinal complications may develop [6].

Broiler flocks affected with *C jejuni*, the prevalence can vary from 0% to 100% depending on season (lowest in fall and winter and highest in summer). *C jejuni* has been found in all areas of poultry production [4]. However, Lynch et al. (2011) [7] , reported that most *Campylobacter* were isolated from beef chicken (16%). Efforts have been made across the supply chain; including farms, processing plants, retailers, and through educating the public. Collectively, results suggest that *Campylobacter* is often present in broilers in a poor health condition, therefore suggesting that the mortality rate could be an efficiency marker for farm management practices and biosecurity [8].

Environmental contamination with bird droppings is probably the most common source of infection for dissemination of *C jejuni*. *Campylobacter* can be transmitted vertically, either on the surface of eggs or by transovarial transmission [9]. Non-chlorinated water shallow well should be regarded as a possible source. Houseflies can be a transmission source for flocks; equipment and footwear contaminated with feces from an infected source as a vehicle of transmission. Young chicks are easily colonized when exposed to *C jejuni* and can excrete the organism in the feces for their lifetimes [10].

Contamination of chicken meat with *Campylobacter jejuni* needs hygienic measures for decontamination in poultry processing. The treatment of chicken meats with plant-derived compound as citric acid, which is

recognized as a safe , advisable for minimizing the initial level of *Jejuni* and extending the shelf life of chicken parts by reducing total microbial number [11]. Therefore, the aim of this work was studied the effect of different dilution of citric acid on the survival of *C. jejuni* inoculated experimentally in chicken meat samples.

2. Materials and Methods

2.1 Samples

A total of 200 chicken samples (breast and thigh) obtained from retail broilers at different localities in Egypt. All collected samples were transported as soon as possible to *Campylobacter* lab in Animal Reproduction Research Inst.El-Haram and subjected to bacteriological investigation for *C.jejuni*.

Locality	Total No. of Chicken Samples		No. of Chicken Samples
	Breast muscles	Thigh muscles	
Cairo	100	50	50
Giza	100	50	50
Total	200	100	100

Table (A): Number of chicken meat samples for isolation of *C. jejuni*.

2.2 Bacteriological examination

2.2.1 Isolation and identification of *Campylobacter*:

A loopful of samples was plated on semisolid Thioglycollate broth (Oxoid) and incubated in microaerophilic atmosphere (5% O₂, 10% CO₂ and 85% N₂) at 37°C and 42°C for 48 -72hrs.

Microscopic examination for the incubated samples was done for detection of *Campylobacter* microorganisms under phase contrast microscope to examine the characteristic motility. All The isolates were subjected to Gram's staining and biochemical

tests, such as catalase, oxidase, urease, nitrate reduction and indole acetate hydrolysis, hippurate hydrolysis test and susceptibility to Cephalothin and nalidixic acid by the disc diffusion method Holt et al, (1994).

2.3 Molecular characterization of *Campylobacter*

2.3.1 Oligonucleotide primers used in qPCR: Five pairs of primers were supplied from (Metabion). They have specific sequence and amplify specific products as shown in Table (2).

Target gene	Primer sequence (5'-3')	Length of amplified product	Reference
<i>C. jejuni hipO</i>	ACTTCTTTATTGCTTGCTGC	323 bp	Wang et al., 2002
	GCCACAACAAGTAAAGAAGC		
<i>FlaA</i>	AATAAAAATGCTGATAAACAGGTG	855 bp	Datta et al., 2003
	TACCGAACCAATGTCTGCTCTGATT		
<i>virB11</i>	TCTTGTGAGTTGCCTTACCCCTTTT	494 bp	-
	CCTGCGTGTCTGTGTTATTTACCC		
<i>CdtB</i>	GTTAAAATCCCCTGCTATCAACCA	495 bp	Bang et al., 2003
	GTTGGCACTTGGAATTTGCAAGGC		
<i>cdtC</i>	TGGATGATAGCAGGGGATTTTAAC	555 bp	-
	TTGCACATAACCAAAAGGAAG		

Table (B): Oligonucleotide primers sequences.

2.3.2 Extraction of DNA: according to QIAamp DNA mini kit: Twenty μ l QIAGEN protease were pipetted into the bottom of a 1.5 ml micro centrifuge tube. 200 μ l of the sample was added. 200 μ l buffer AL were added to the sample, mixed by pulse vortexing for 15 seconds. The mixture was incubated at 56°C for 10 min.

The 1.5 ml micro centrifuge tube was centrifuged to remove drops from the inside of the lid. 200 μ l ethanol (96%) were added to the sample, and mixed again by pulse vortexing for 15 seconds. After mixing, the 1.5 ml micro centrifuge tube was briefly centrifuged to remove drops from the inside of the lid.

The mixture from step 6 was carefully applied to the QIAamp mini spin column (in a 2ml collecting tube) without wetting the rim. The cap was closed, and centrifuged at 8000 rpm for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was

discarded. The QIAamp mini spin column was carefully opened and 500 μ l buffer AW1 was added without wetting the rim. The cap was closed, and centrifuged at 8000 rpm for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. The QIAamp mini spin column was carefully opened and 500 μ l buffer AW2 was added without wetting the rim.

The cap was closed, and centrifuged at full speed for 3 min. The QIAamp mini spin column was placed in a new 2 ml collection tube and the old collection tube was discarded with the filtrate.

Centrifugation at full speed for 1 min was done. The QIAamp mini spin column was placed in a clean 1.5 ml micro centrifuge tube, and the collection tube containing the filtrate was discarded. The QIAamp mini spin column was carefully opened and 100 μ l buffer AE were added. The QIAamp mini spin

column was Incubated at room temperature (15-25°C) for 1 min, and then centrifuged at 8000 rpm for 1 min [12].

2.3.3 Preparation of duplex PCR master: Mix for each of (*C. jejuni* hipO), (*cdtB* and *cdtC*) and (*virB11* and *flaA*) genes according to GoTaq® Hot Start Green Master Mix.

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension	Reference
<i>HipO</i> and <i>glyA</i>	94°C 6 min.	95°C 30 sec.	55°C 30 sec.	72°C 30 sec.	35	72°C 10 min.	Wang et al., 2002
<i>FlaA</i> and <i>virB11</i>	94°C 5 min.	94°C 30 sec.	53°C 45 sec.	72°C 45 sec.	35	72°C 10 min.	Datta et al., 2003
<i>cdtB</i> and <i>cdtC</i>	94°C 5 min.	94°C 30 sec.	42°C 45 sec.	72°C 30 sec.	35	72°C 10 min.	Bang et al., 2003

Table (C): Cycling conditions of the different primers during PCR.

2.3.4 Agarose gel electrophoreses [13]: Electrophoresis grade Agarose (1.5 g) was prepared in 100 ml TBE buffer in a sterile flask, 0.5µg/ml Ethidium bromide was added and mixed thoroughly. Twenty µl of each PCR product samples, negative control and positive control were loaded to the gel. The gel was photographed by a gel documentation system and the data was analyzed.

2.4 Preparation of *C. jejuni* inoculum

Campylobacter jejuni strains were maintained at 70°C in BHI broth containing 15% glycerol. During propagation and growth, all plates were incubated at 37°C in sealed gas jars under microaerobic conditions (5% O₂, 10 % CO₂, and 85% N₂). Cultures were prepared by growing the strains for 3 to 4 days on blood agar base (Oxoid) containing 5% sheep blood. One loopful of each culture was subsequently streaked onto blood agar base plates, which were incubated for 24 h. The cells were harvested with 2 ml of phosphate-buffered saline (Oxoid). The inoculum

was diluted based on the observation that the optical density at 600 nm, the inoculum was diluted to approximately 7 logs CFU/ml in either chicken muscle was ready for use [14].

2.5 Experiment by using Citric acid

2.5.1 Preparation of chicken muscles: Chicken breast and thigh muscles were obtained from retail broilers and stored at low temperatures (5 and 10 degrees C) *C. jejuni* remained viable in chicken juice for a remarkably longer period of time.

2.5.2 Inhibitory effect of citric acid in chicken meat and BHI broth:

Four-milliliter of the inoculum prepared in chicken muscles or BHI broth were transferred into sterile tubes, and 100 ml from a 20% stock solution lemon juice (citric acid) was pipetted into the tubes, resulting in an organic acid concentration of 0.5%. The tubes were immediately stored at 4°C. After 0, 10, 20, 30 minutes. 1, 2, 3 and 4 h of storage, samples were collected to determine

populations of *C. jejuni*. Two replicate experiments were conducted.

2.5.3 PH measurement: The values of pH for all chicken muscles with citric acid were measured after

5 to 10 min and after 1, 2, 3 and 4 h of experiment.

3. Results

3.1 Identification of *Campylobacter jejuni* by culture method

Localities	Number of examined samples	Total Positive <i>Campylobacter jejuni</i> %	Positive <i>C. jejune</i> in breast %	Positive <i>C. jejune</i> in thigh%
Cairo	100	30 30%	18 18%	12 12%
Giza	100	38 38%	22 22%	16 16%
Total	200	68 34%	40 20%	28 14%

Table 1: Occurrence of *C. jejuni* in chicken samples (breast and thigh meat) by conventional method.

Characteristics	<i>C. jejuni</i>
Oxidase	+
Catalase	+
Nitrate reduction	+
Urease	--
Hippurate hydrolysis	+
Growth at:	
37°C	+
43°C	+
Growth at 1% glycine	+
Susceptibility to:	
Nalidixic acid	S
Cephalothin	R

Table 2: Biochemical tests to detect *C. jejuni*.

3.2 Molecular typing of *Campylobacter*

Sixty eight positive samples for *C. jejuni* were examined by Multiplex PCR for detection of *C. jejuni*

and virulence strains of *C. jejuni* and cytolethal distending toxins (Figures 1, 2 and 3).

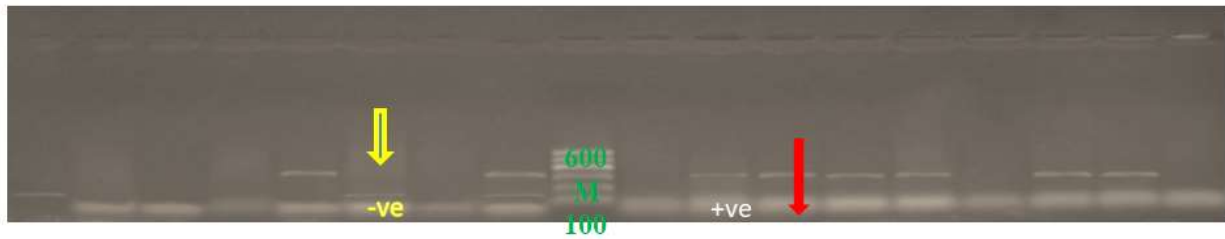


Figure 1: Multiplex PCR on 1.5% agarose gels. +ve= positive -ve= negative. *Campylobacter* isolates yielded the genus specific (*16S rRNA*) *C. jejuni* specific 323 bp Lane M: 100-600 bp ladders; Lane: 1, 2, 4, 5, 6,7,11 positive *C. jejuni* at 323 bp.

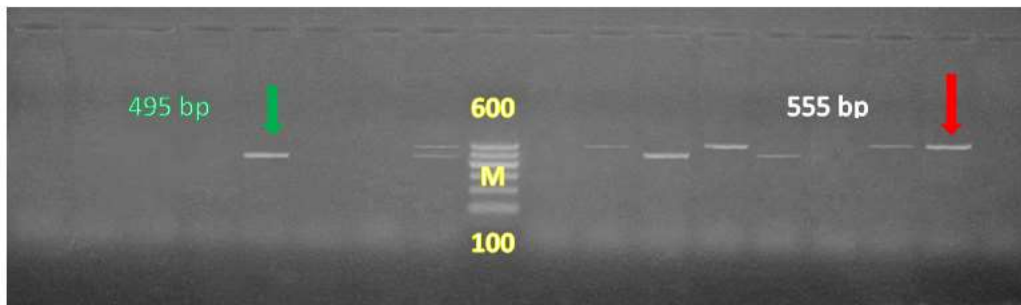


Figure 2: *C.jejuni* cytolethal distending toxins showed at 555 bp for *CdtC* and at 495 bp for *CdtB* Lane M: 100 bp – 600 bp ladder; Lane: 2, 4, 7 *C. jejuni* *CdtC* at 555bp; Lane: 1, 5, 6, 11 *C.jejuni* *CdtB* at 495b.

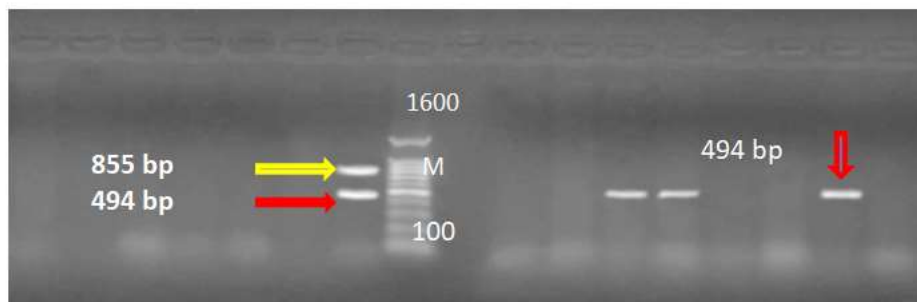


Figure3: The virulence strains of *C. jejuni* *virB11* showed at 494 bp and *FlaA* at 855 bp Lane M: 100 bp –1600 bp ladder; Lane: 1, 4, 5 *C. jejuni* *virB11* at 494bp.

3.3 Inhibitory effect of citric acid in chicken muscles

Type of samples	Positive samples	15 min	30 min	One hr.	2 hrs.	3 hrs.	4 hrs.
Breast meat	40	15 37.5%	23 57.5%	30 75%	35 87.5%	38 95%	40 100%
Thigh meat	28	12 42.9%	15 53.6%	22 78.6%	26 92.9%	28 100%	28 100%
Total	68	27 39.7%	38 55.9%	52 76.5%	61 89.7%	66 97%	68 100%

Table 3: Relative reduction of *C. jejuni* strains exposed to Citric acid in BHI at 4 ° C.

Type of examined samples	Total Positive <i>C. jejuni</i>	After 5 min. of Exp. pH	After 10 min. of Exp. pH	After 1 hrs. of Exp. pH	After 2 hrs. of Exp. pH	After 3 hrs. of Exp. pH
Chicken muscles	68	2.5	2.7	3	3.3	3.5

Table 4: pH measurements exposed to Citric acid in BHI at 4 ° C.

4. Discussion

Consumption of contaminated chicken meat products have been identified as major sources of *C. jejuni* for humans [15]. Chicken carcass contaminated with *C. jejuni* during processing by faecal [16, 17]. The cross-contamination between slaughtered flocks and processing of chicken leads to higher contamination with *C. jejuni* [18]. To reduce human exposure to *C. jejuni* is by controlling the pathogen at the farm level in the chicken flocks, by effective control measures.

Numerous control strategies have been tested to reduce *C. jejuni* contamination in chicken, such as farm biosecurity, vaccination, therapy, and the use of feed additives [19]. Non-antibiotic feed additives, such as organic acids and essential oils, were tested for their action on *C. jejuni*, showing varying efficacy.

Essential oil extracts, such as Eucalyptus, Valencia orange and cinnamon, are very potent against *C. jejuni* in vitro [20]. Organic acids such as formic acid, lactic and acetic acid are bactericidal against *C. jejuni* in vitro and are also effective at some degree in vivo [21].

The high incidence of *C. jejuni* in chicken breast muscles samples was (20%) followed by chicken thigh muscles samples (14%) (Table 1). The high incidence of *C. jejuni* in chicken breast meat samples in Giza (22 %) and Cairo (18%). But, *C. jejune* in chicken thigh samples was high incidence in Giza (16 %) and Cairo (12%) Table 1. *C. jejuni* carcass contamination level of 4.7 log CFU/carcass [22, 23]. *C. jejuni* caecal was contaminated the carcass during slaughtered and evisceration processing [24].

Conventional techniques used for the detection and identification of *Campylobacter* species are laborious, time-consuming and problems of contamination are often encountered [25, 26]. Polymerase Chain Reaction (PCR) technique is widely used of all molecular techniques, it is highly sensitive, specific and rapid for the detection of food-borne pathogens [27]. All the 68 *Campylobacter* isolates yielded the genus specific (*16S rRNA*) 63 isolates *C. jejuni* specific 323 bp in Figure 1. Amplicons 555 bp and 495 bp were confirmed to belong to *C. jejuni* cytolethal distending toxins showed at 555 bp for *CdtC* and at 495 bp for *CdtB* of the genus *Campylobacter* (Figure 2).

Therefore, the isolates obtained were further confirmed by PCR analysis whereas most of the strains contained the CDT genes. These results detected by Rajagunalan et al., (2014) [28]. Virulence gene markers were identified by multiplex PCR. The results showed that isolates of *C. jejuni* contained the *flaA* gene sequences. The virulence genes of *C. jejuni* (*FlaA*, *virB11*) have shown at 855 bp and 494 bp respectively (Figure 3). The *virB11* gene was present only in (14.7%) of the analyzed isolates. These results agreed with Wieczorek and Osek (2011) [29]. PCR analysis detection of *Campylobacter* virulence markers can be utilized as a simple and rapid tool to recover from different sources, especially when used as profile analysis for a control strategy [30].

The present study analyzed the diversity of *C. jejuni* population in chicken to monitor the contamination process throughout the farm, slaughter, and post chilling phases. There are several studies in literature assessing the use of plant extracts, as antimicrobials,

antioxidants, or digestibility enhancers in animal feeds. Dietary organic acids have been considered as antibacterial nature. Chemically, organic acids used in food animal production described as simple monocarboxylic acids (e.g., formic, acetic, propionic, and butyric acids) or carboxylic acids (e.g., lactic, malic, tartaric, and citric acids) [31].

The effect of the citric acid was correlated with the level of pH, in chicken meat (Table 3). The result was in a greater reduction of *C. jejuni* in breast and thigh meat in BHI broth at 4°C after 4 hours of experiment. As of the 68 *C. jejuni* strains tested, only *C. jejune* 38 (55.9%) strains was reduction after 30 min of experiment, 52 (76.5%) required one hour for reduction, 61 (89.7%) after 2 hours, 66 (97%) after 3 hours and 100% completely reduction after 4 hours. Survival of *C. jejuni* in the presence of citric acid observed for stresses at pH 3.5 (Table 4). These findings confirm that citric acid influences *C. jejuni* [32].

In (Table, 4) found that the antibacterial effect of citric acid on *C. jejune* on chicken meat. Lowering pH by adding citric acid onto the chicken meat surface, observed a rapid rise in pH within a few minutes, which to some extent neutralized the antibacterial effect with an acceptable taste of the prepared meat.

Perko-Ma'kela' et al. (2000) [33] found that low pH is necessary to obtain a rapid effect for reduction of *C. jejuni* population in broiler meat reported positive scores with regard to taste and texture. Citric acid is disrupting the outer membrane and initiate cell lysis with an increase in permeability of *C. jejuni* [34].

Finally, this study shows that citric acid and their active principles may be used as an alternative to the use of antibiotic performance. Further research is needed regarding understanding mechanism of action, identifying means to standardize the effects, improving delivery methods, and increasing their in vivo efficacy.

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