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Incidence and Antimicrobial Resistance of Campylobacter Species Isolated from Poultry Eggshell Samples

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CAMPYLOBACTER species (Campylobacter spp.) are imperative foodborne pathogens regarding to the consumption of contaminated poultry products. An existing survey was performed to determine the incidence and antimicrobial resistance profile of Campylobacter spp in egg samples from 9 poultry species. Four-hundred and fifty poultry eggshell samples were collected and assessed by bacterial culture. Campylobacter spp were identified by PCR. Antimicrobial resistance profile was assessed by the disk diffusion. Eighty-four of 450 (18.67%) eggshell samples were contaminated with Campylobacter species. The uppermost and lowermost rates of contamination with Campylobacter spp were found in local chicken (30%) and chicken (8%) eggshells. Incidence of *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) amongst the isolated Campylobacter spp. were 53.57% and 46.43%, respectively. *C. jejuni* displayed the uppermost incidence of resistance toward tetracycline (66.66%), nalidixic acid (62.20%) and ciprofloxacin (48.89%). *C. coli* bacteria displayed the uppermost incidence of resistance toward tetracycline (46.15%), nalidixic acid (41.03%) and ciprofloxacin (30.77%). Poultry eggshells, particularly local chicken and partridge were potential sources of transmission of Campylobacter spp. to human. Well-cooking of eggs and monitor the hygienic circumstances of aviculture can reduce the risk of Campylobacter in eggshells. Further surveys are essential to assess other roles of the Campylobacter spp in poultry eggshell samples.

Keywords: Incidence, Campylobacter species, Poultry, Eggshell, Antibiotic resistance.

Introduction

Table eggs are among the most valuable and popular foodstuff consumed by people all over the world owing to its lower cost compared to other sources of protein [1]. It is the complete source of protein and indispensable amino acids, vitamins, particularly B12, A, D, E, K and riboflavin and finally zinc, folic acid, and pantothenic acid [1, 2]. Nevertheless, the opportunity of occurrence of diverse kinds of microbial contamination owing to close contact of eggshell with the environment is disquieting issue regarding its consumption [3-5].

Campylobacter spp are Gram-negative and microaerophile bacteria measured as the most mutual cause of acute gastroenteritis. The most imperative Campylobacter spp. accompanying with human illness are *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) [6, 7]. Campylobacteriosis in human is acknowledged with abdominal cramping, fever and diarrhea [6, 7]. Plain cases are mostly faced with severe diarrhea associated with blood and leukocytes and occasionally may develop post infection complications associated with Guillain Barré or Miller Fischer Syndrome branded by ataxia, areflexia, immune-mediated neuropathies, opthalmoplegia and death [6, 7].

Campylobacter spp. are principally resist to numerous kinds of antimicrobial agents including penicillins, quinolones, macrolides, cephalosporins and tetracyclines [8]. Thus, higher load of cost for a longer period of time should perform to treat the cases of campylobacteriosis [8].

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According to the high risk of transmission of Campylobacter spp. through poultry products, particularly egg [6-8] and absence of epidemiological survey in this field in Iran, an existing research was performed to assess the incidence and antimicrobial resistance profile of Campylobacter spp. bacteria isolated from diverse kinds of poultry meat samples.

Materials and Methods

Samples

From January 2018 to January 2019, 450 egg samples were collected from diverse poultry species including turkey (n=50), chicken (n=50), quail (n=50), duck (n=50), partridge (n=50), goose (n=50), pheasant (n=50), local chicken (n=50) and horned chicken (n=50). The eggs were purchased from shopping centers of the North of Iran (Mazandaran province). Eggs were then transferred to the laboratory using ice-packs at 4℃.

Isolation of Campylobacter spp.

The eggs were kept for 12 hours at room temperature and then broken. A total of 10 g of the macerated shells were added to 100 ml of Bolton broth Base supplemented with 25 ml of defibrinated horse blood along with the following antibiotic combination: 20 mg/L of cefoperazone, 20mg/L of vancomycin, 20g/L of trimethoprim, 10mg/L of amphotericin B. Media were incubated at 42 ℃ during 24 hours in a microaerophilic circumstances [9]. The Identification test was performed immediately to confirm the characteristics of Campylobacter colonies. Identification of the isolates was conducted based on method described by Nachamkin [10]. One colony from each suspected medium was subjected to standard Biochemical tests including Gram-staining, production of catalase (3% H2O2) and oxidase and Hippurate hydrolysis, urease activity, indoxyl acetate hydrolysis, and susceptibility to cephalothin [10].

PCR detection of Campylobacter spp.

Campylobacter isolates were cultured on nutrient broth (Merck, Germany) and further incubated at 42 °C for 24 h. Principles of producing factory of DNA extraction kit (Cinnagen, Iran) were applied for DNA extraction. Extracted DNA samples were subjected to quantification by NanoDrop device (NanoDrop, Thermo Scientific, Waltham, USA), qualification (2% agarose gel) and purity checking (A260/A280). PCR was conducted rendering beforehand documents (Table 1) [11, 12]. Thermo-cycler device (Flexrcycler^2, Germany) was used for detection of Campylobacter spp. *C. jejuni* and *C. coli*. Fifteen microliters of the PCR products were electrophoresed using 1.5% agarose gel. Runs were comprised a negative control (PCR grade water) and positive controls (*C. jejuni* ATCC 33291 and *C. coli* ATCC 33559).

TABLE 1. PCR circumstances applied for detection of Campylobacter spp. C. jejuni and C. coli [11, 12].

Target gene	Primer sequence (5'-3')	PCR product (bp)	PCR Volume (50µL)	PCR programs
16SrRNA (Campylobacter genus)	F: ATCTAATGGCTTAACCATTAAAC R: GGACGGTAACTAGTTTAGTAT T	857	5 µL PCR buffer 10X 2 mM $Mgcl_2$ 150 µM dNTP (Fermentas) 0.75 µM of each primers F & R 1.5 U Taq DNA polymerase (Fermentas) 3 µL DNA template	1 cycle: 94 ^{0C} ---------- 1 min. 35 cycle: 94 ^{0C} ---------- 30 s 60 ^{0C} ---------- 30 s 72 ^{0C} ---------- 40 s 1 cycle: 72 ^{0C} ---------- 3 min
MapA (*C. jejuni*)	F: CTATTTTATTTTTGAGTGCTTGTG R: GCTTTATTTGCCATTTGTTTTATTA	589		
CeuE (*C. coli*)	F: AATTGAAAATTGCTCCAACTATG R: TGATTTTATTATTTGTAGCAGCG	462		

Antibiotic resistance test

Phenotypic profile of antibiotic resistance of Campylobactee spp. isolates were examined by disk diffusion test. Mueller–Hinton agar media (Merck, Germany) supplemented with 5% defibrinated sheep blood were applied for this goal. Protocols of the Clinical and Laboratory Standards Institute (CLSI) were applied for this goal [13]. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol was applied for this goal. Diverse antibiotic disks (Oxoid, UK) including ampicillin (10 µg/disk), amoxicillin (30 µg/disk), cephalothin (30 µg/disk), colistin (10 µg), chloramphenicol (30 µg/disk), ciprofloxacin (5 µg/disk), enrofloxacin (5 µg/disk), erythromycin (15 µg/disk), gentamicin (10 µg/disk), neomycin (30 µg/disk), nalidixic acid (30 µg), streptomycin (30 µg/disk), and tetracycline (30 µg/disk) were applied for this goal. Isolates on media and also antibiotic disks were incubated at 42 ℃ during 24 hours in a microaerophilic circumstances. After that, the dimeter of growth inhibition zone was measured and interpreted rendering the CLSI. Plates contained bacteria and also antibiotic agents were incubated at 42 °C for 48 h in a microaerophilic circumstances.

Statistical examination

Data gotten from the experimentations were classified in the Excel software. SPSS/21.0 software was accompanied for statistical examination. Chi-square and Fisher's exact two-tailed tests were applied to measure any noteworthy association. Statistical signification was determined at a P value < 0.05.

Results

Incidence of Campylobacter spp.

Table 2 shows the incidence of Campylobacter spp., *C. jejuni* and *C. coli* amongst examined poultry egg samples. Eighty-four out of 450 (18.67%) poultry eggshell samples were contaminated with Campylobacter spp. Local chicken eggshell (30%) had the uppermost incidence of Campylobacter spp., while chicken eggshell (8%) had the lowermost. Incidence of *C. jejuni* and *C. coli* amongst the isolated Campylobacter spp. were 53.57% and 46.43%, respectively. The uppermost and lowermost rates of contamination with *C. jejuni* was found in local chicken (66.66%) and partridge (66.66%) and duck (38.46%) eggshell samples. The uppermost and lowermost rates of contamination with *C. coli* was found in duck (61.54%) and partridge (33.33%) and local chicken (33.33%) eggshell samples. Statistical remarkable variance was gotten amid kinds of eggshell samples and incidence of Campylobacter spp. (P<0.05). Furthermore, statistical remarkable variance was gotten amid the incidence of *C. jejuni* and *C. coli* bacteria (P<0.05).

TABLE 2. Incidence of isolated Campylobacter spp to examined poultry egg samples as well as incidence of C. jejuni and C. coli to Campylobacter spp.

Types of egg samples	N. of samples collected	N. of isolates positive for bacteria		
		Campylobacter spp. No (%)	C. jejuni No (%)	C. coli No (%)
Chicken	50	4 (8)	2 (50)	2 (50)
Local chicken	50	15 (30)	10 (66.66)	5 (33.33)
Goose	50	11 (22)	5 (45.45)	6 (54.54)
Duck	50	13 (26)	5 (38.46)	8 (61.54)
Horned chicken	50	10 (20)	6 (60)	4 (40)
Turkey	50	12 (24)	7 (58.33)	5 (41.67)
Pheasant	50	6 (12)	3 (50)	3 (50)
Partridge	50	6 (12)	4 (66.66)	2 (33.33)
Quail	50	7 (14)	3 (42.86)	4 (57.14)
Total	450	84 (18.67)	45 (53.57)	39 (46.43)

Antibiotic resistance pattern

Table 3 signifies the incidence of resistance of *C. jejuni* and *C. coli* toward 12 diverse antibiotic agents. *C. jejuni* isolates exhibited the uppermost incidence of resistance toward tetracycline (66.66%), nalidixic acid (62.20%) and ciprofloxacin (48.89%). *C. coli* isolates exhibited the uppermost incidence of resistance toward tetracycline (46.15%), nalidixic acid (41.03%) and ciprofloxacin (30.77%). Examined bacteria had complete susceptibility toward gentamicin and chloramphenicol (100%). Some strains exhibited simultaneous resistance toward more than one antibiotic agent.

TABLE 3. Incidence of resistance of C. jejuni (n= 45) and C. coli (n= 39) toward 12 diverse antibiotic agents.

Antimicrobial Agent	N. of isolates resistant to each antibiotic	
	C. jejuni number (%)	C. coli number (%)
Amoxicillin	7 (15.56)	-
Ampicillin	9 (20)	7 (17.95)
Nalidixic acid	28 (62.22)	16 (41.03)
Ciprofloxacin	22 (48.89)	12 (30.77)
Enrofloxacin	15 (33.33)	8 (20.51)
Streptomycin	3 (6.67)	1 (2.56)
Gentamycin	-	-
Neomycin	7 (15.56)	-
Erythromycin	6 (13.33)	-
Chloramphenicol	-	-
Tetracycline	30 (66.66)	18 (46.15)
Colistin	8 (17.78)	4 (10.26)

Discussion

Yearly, an assessed 75 million diseases, 320,000 hospitalizations, and 5,000 deaths are caused by food-borne diseases in the United States [14]. Amid these cases, 31 recognized pathogens cause 9.5 million diseases, 55,000 hospitalizations, and 1200 deaths [15]. Campylobacter spp. are imperative pathogens involved in gastrointestinal, foodborne and nosocomial infections universally [15]. The annual number of described cases of foodborne diseases by Campylobacter spp in the European Union was 225,000 in 2011, but the actual number of cases could be as high as 10 million yearly [16]. The financial charge of campylobacteriosis was around €2.5 billion in the United States in 2008 [16].

An existing survey was performed to assess the incidence and antibiotic resistance of Campylobacter spp. bacteria recovered from poultry eggshell samples. Total incidence of Campylobacter spp. was 18.67% in which *C. jejuni* and *C. coli* were identified in 53.57% and 46.43%, respectively of positive strains.

Similar surveys have been conducted in the field of Campylobacter spp. in poultry products, particularly eggshells. Mezher et al. [17] conveyed that the incidence of Campylobacter species in chicken samples were 6.80% in which 27.27% of isolates were identified as *C. jejuni*. Whole incidence of *C. jejuni* amongst the poultry samples collected from Iraq [18], Iran [19], Pakistan [20], India [21], Korea [22], and China [23] was 10%, 6.84%, 40%, 26.30%, 36.30%, and finally 1.82% to 56.00%, respectively. Boost incidence of Campylobacter species was also reported in poultry samples collected from European countries (29-41%) [24-27]. Incidence of Campylobacter spp. in poultry eggshell in Italy [28] and Japan [29] was 66.66% and 27.00 to 36.00%, respectively. Jonaidi-Jafari et al. [30] conveyed that the incidence of Campylobacter spp. amongst the chicken, duck, goose, ostrich, partridge, quail and turkey eggshell samples was 7.00%, 5.00%, 3.30%, 2.50%, 4.20%, 5.00% and 3.80%, respectively. They conveyed that the incidence of *C. jejuni* and *C. coli* was 6.30% and 1.30%, respectively. Modirrousta et al. [9]

conveyed that the incidence of Campylobacter spp. in chicken eggshell samples were 31.60% in which *C. jejuni* and *C. coli* were the most commonly identified species.

Campylobacter spp. had considerable incidence of resistance toward some kinds of routinely applied antibiotic agents, particularly tetracycline, nalidixic acid and ciprofloxacin. Unlawful and imprecise antibiotic prescription particularly in poultry farms is may be the chief reason for the boost incidence of resistance in the Campylobacter spp. Boost incidence of resistance of Campylobacter spp. toward tetracycline, nalidixic acid and ciprofloxacin antibiotic agents was also conveyed from Turkey [31], Russia [32], Poland [33], Kenya [34] and Grenada [35]. Adzitey et al. (2012) [36] revealed that the *C. jejuni* bacteria isolated from poultry products in Malaysia had the higher incidence of resistance toward antibiotics than *C. coli* which was similar to our outcomes. They showed that incidence of resistance of *C. jejuni* isolates toward ampicillin, cefotaxime, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, nalidixic acid, norfloxacin, streptomycin, Suphamethoxazole/trimethoprim and tetracycline was 81%, 20%, 51%, 99%, 7%, 76%, 1%, 5%, 84%, 80%, 50%, 96% and 96%, respectively. Similar antibiotic resistance profile of Campylobacter spp. recovered from eggshell samples was also described from United States [37] and Kenya [34].

C. jejuni and *C. coli* bacteria were also previously recovered from turkey, chicken, quail, duck, partridge, goose, pheasant, local chicken and horned chicken eggshell samples [38, 39]. Moreover, findings displayed that local chicken and partridge eggshell samples had the uppermost incidence of Campylobacter spp which was similar to previously published works [30, 40]. The uppermost incidence of resistance was toward tetracycline, nalidixic acid and ciprofloxacin which was similar to previous reports [41, 42]. *C. jejuni* isolates had the higher incidence of resistance than *C. coli* which was comparable to other reports [43, 44].

Conclusion

Our survey signified that poultry eggshell samples were contaminated with antibiotic resistance Campylobacter spp. which pose an imperative public health hazard. Higher incidence to *C. jejuni* than *C. coli* were recovered from eggshell of all tested poultry species; local chicken and partridge eggshell samples had the uppermost incidence of isolation. The highest incidence of resistance was to tetracycline, nalidixic acid and ciprofloxacin. Therefore, we recommended that full boiling of poultry egg before consumption and monitor the prescription of antibiotic can diminish from the occurrence of resistant-Campylobacter foodborne diseases. However, further surveys are essential to found more details about the impact of Campylobacter spp. in poultry eggshells.

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