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Detection of Thermophilic *Campylobacter* sp. in Unpacked Broiler Carcasses in Retail Markets of Afyonkarahisar and Confirmation *C. jejuni* Isolates Using PCR

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Abstract: The aim of this present study is to detect thermophilic *Campylobacter* species in chicken carcasses sold unpackaged in Afyonkarahisar and to confirm *C. jejuni* isolates by using a PCR test based on the *ceuE* gene. A total of 210 chicken carcasses were collected from various markets from May till the end of October in 2004. Thermophilic *Campylobacter* sp. was isolated from 141 (67%) of 210 chicken carcass samples by classical culture method. The identification of 419 thermophilic *Campylobacter* sp. isolates obtained from chicken carcass samples were; 57.04% (239) *C. jejuni*, 40.57% (170) *C. coli* and 2.39% (10) *C. lari*. PCR method was applied to 101 of 239 *C. jejuni* isolates obtained from the chicken carcasses (n = 101). According to it 62 (61.38%) of 101 isolates were confirmed by PCR. About 28 (45.16%) of 62 isolates were confirmed to be *C. jejuni* were detected to be resistant to nalidixic acid.

Key words: Thermophilic *Campylobacter*, chicken carcasses, PCR, *C. jejuni*, Turkey

INTRODUCTION

Campylobacter sp. are microorganisms found in the intestinal flora of all domestic and wild animals including poultry and in sea animals in nature (Blaser *et al*., 1983). Thermophilic *Campylobacter* sp. (*C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*) displaying dissimilarity to other *Campylobacter* sp. with respect to growth temperatures may cause serious gastroenteritis as well as cases ended in death in human (Bryan and Doyle, 1995).

Campylobacteriosis was reported to be a zoonotic disease affecting all age groups prevalently in the world and causing acute gastroenteritis in humans (Stern and Line, 1992; Federighi *et al*., 1999). In the studies, chicken meat was reported to be frequently contaminated with intestinal content during slaughter procedure and *C. jejuni* was also reported to be detected almost all poultry meat sold at markets (Willis and Murray, 1997; Atabay and Corry, 1997). It was reported that intestinal content contamination to chicken carcasses (Jones *et al*., 1991; Izat *et al*., 1988), cross contamination during food preparation stage, insufficient cooking and lack of hygiene were playing important roles in thermophilic *Campylobacter* infections (Oosterom *et al*., 1983; Annan-Prah and Janc, 1988a, b).

Poultry meat and their products were reported to be the leading high risk groups of food in the infections caused by thermophilic *Campylobacter* sp. (Schorr *et al*., 1994; Atanassova and Ring, 1999; Solomon and Hoover, 1999; Effler *et al*., 2001). Atanassova and Ring (1999) and Willis and Murray (1997) reported that contamination level of raw poultry meat and their products may vary between 0 and 100%.

Antibiotic susceptibility test, an important identification test of *Campylobacter* sp., has been reported to be insufficient in the identification of antibiotic resistant species increasing recently. Additionally, the necessity for the specific identification studies by PCR to be complementary to phenotypical identifications and to facilitate the differentiation of analogous species has been emphasized since the hippurate hydrolysis test used for the distinction of *C. jejuni* from *C. coli* may cause mistakes resulting from subjective assessments (Rautelin *et al*., 1999; Wilson *et al*., 2000; Houng *et al*., 2001; Bolton *et al*., 2002).

MATERIALS AND METHODS

The samples, a total of 35 chicken carcasses, used in this study were collected during a period of 6 months every 1st and 3rd week of each month from May to

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October 2004. Chicken carcasses belonging to 6 firms were bought from 10 markets where unpacked chicken carcasses were commercially sold. Carcass weights were determined to vary between 1.2 and 1.9 kg.

The samples from the chicken carcasses were analyzed immediately after they were brought to the laboratory in the carrying bowls under aseptic conditions kept in the cold chain. The isolation and identification of thermophilic *Campylobacter* sp. from the samples were made according to ISO (The International Organization for Standardization) method (Anonymous, 1995). ATCC 33291 *Campylobacter jejuni* (Oxoid 1400 L) was used as control strain. Chicken carcass samples (n = 210) were put into sterile rins bags. Then they were rubbed and homogenized in 225 mL Preston selective enrichment broth (Oxoid CM 67, Oxoid SR 232E, Oxoid SR 0117E, Oxoid SR 048) for 60-120 sec. Washing liquids were incubated in microaerofilic conditions (Campgen Oxoid CN 25) for 24 h at 42°C (Lillard, 1988; Lee *et al*., 1994; Cloak *et al*., 2001; Jorgensen *et al*., 2002; Josefsen *et al*., 2003). Enrichment liquid was cultered on *Campylobacter* blood-free selective Agar (CCD Agar-Oxoid CM 739, Oxoid SR 155). Then, it was incubated in microaerofilic conditions between 48 and 72 h at 42°C. Following the incubation, 5 of the suspected colonies, which were flat, grey colored and in moist appearance, growing in the Charcoal Cefoperazone Deoxycholata Agar were selected and cultured into the blood agar (Oxoid CM 331, 5% sheep blood) and they were incubated in microaerofilic conditions for 24 h at 42°C. Gram staining, activity, catalase, oxidase, sodium hippurate hydrolysis, growing at 25°C tests, nalidixic acid and ceplalothin susceptibility tests on Mueller Hinton Agar were applied to the suspected colonies growing in the blood agar. Also, H_2S producing in TSI Agar was controlled. *C. jejuni* isolates, stored in *Brucella broth*, containing 15% glycerol, at -70°C were used to PCR procedure. Isolates were incubated in microaerofilic conditions for 48 h at 42°C onto mCCDA. Boiling method (Mohran *et al*., 1998; Englen and Cray, 2002; Sails *et al*., 2003) was used to DNA extraction. Isolates suspended in 1000 μL sterile distilled water were kept in water bath for 10 min at 94°C and centrifuged for 5 minutes at 10.000 g. The obtained supernatant was used as target DNA. The primer pairs used in the study, forming ceuE gene sequence suggested by Gonzalez *et al*. (1997), contained JEJ1 (5'CCTGCTACGGTGAAAGTTTTGC3') and JEJ2 (5'GATCTTTTTGTTTTGTGCTGC3') oligonucleotide arrays.

To form a total volume of 25 μL, the PCR procedure consisted of 50 mM KCL, 10 mM Tris-HCl (10× PCR buffer) (pH 8.3), 1.5 μL $MgCl_2$, 0.5 μL dNTP mix, 0.25, 0.2 μL Ampli Taq DNA polymerase and extracted 2 μL target DNA from each primer. The samples were amplified in the thermal cycler (Biometra) over 30 cycles after the mixture was covered with mineral oil. Each cycle was occured by heating at 94°C for 3 min, denaturation at 94°C for 30 sec, primer attachment at 57°C for 30 sec and primer extension at 72°C for 1 min. Additionally, 1.5% agarose gel was used for PCR amplification products. Primer extension was made at 72°C for 1 min following the last cycle. *ceuE* gene belongs to *C. jejuni* isolates showed 793 bp band on electrophoresis.

RESULTS

Using classical culture method thermophilic *Campylobacter* was isolated from 141 (67.1%) of 210 chicken carcass samples collected from May to October 2004 and *C. jejuni* was isolated from 101 of 141carcasses. Thermophilic *Campylobacter* contamination rate in chicken carcasses were detected as 71.4, 80, 54.2 and 45.7% between May and June, July and August in September and October, respectively.

About 419 thermophilic *Campylobacter* sp. isolates were obtained from a total of 210 chicken carcass samples. Of the obtained 419 thermophilic *Campylobacter* sp. isolate 57.04% (239) was detected as *C. jejuni*, 40.57% (170) was detected as *C. coli* and 2.39% (10) was detected as *C. lari*. PCR procedure was applied to 101 of the obtained 239 *C. jejuni* isolates. According to i 62 (61.38%) of the 101 isolate, identified both using classical culture method and PCR. About 28 (45.16%) of 62 isolates were detected to be resistant to nalidixic acid.

DISCUSSION

The samples, 35 chicken carcasses per month, used in this study were collected during a period of 6 months (from May to October 2004). Chicken carcasses were bought from 10 markets selling unpackaged carcasses in Afyonkarahisar. Thermophilic *Campylobacter* sp. was isolated from 141 (67.1%) of carcass samples. Poultry carcasses were reported to be contaminated with thermophilic *Campylobacter* sp. at rates changing between 84 and 100% in the other studies concerning the same topic (Aho and Hirn, 1988; Jones *et al*., 1991).

It has been considered that in a study carried out by Atanassova and Ring (1999) between the years 1995 and 1997, using swab sampling methods during November and December was effective in finding low thermophilic *Campylobacter* sp. rates. Although, Cloak *et al*. (2001) and Atanassova and Ring (1999) study during the same period, the results of Cloak *et al*. (2001) are different from

the results of Atanassova and Ring (1999) due to Cloak's performing enrichment procedure to the samples. The thermophilic *Campylobacter* isolation rate of the study of Uyttendaele *et al*. (1999) is lower than the rates of our study. This may be explained as follows: they used chicken carcasses at different age groups as material, chickens were slaughtered in different slaughterhouses and the carcasses were supplied from different regions. Only 6-8 weeks old chickens bred in Afyonkarahisar and slaughtered in the same slaughterhouse were used as material in this present study. Most Probable Number (MPN) method used by Dufrenne *et al*. (2001) in the isolation of thermophilic *Campylobacter* sp. is different from the method used in persent study. The researcher noted down that Preston broth was used as enrichment medium and the dominant flora present in the skin of chicken carcass and pieced chicken-meat samples dominated the number of thermophilic *Campylobacter* sp. during enrichment. However, the presence of high level of thermophilic *Campylobacter sp.* in the results of Berrang *et al*. (2001), Zorman and Mozina (2002), Uyttendaele *et al*. (1999) may be assessed as their using pieced chicken-meat or chicken internal organs as the study material instead of using complete chicken carcasses. It has been reported that chicken meat sold in the pieced form are contaminated with thermophilic *Campylobacter* sp. at higher rates than chicken carcasses (Hudson *et al*., 1999).

The cause of the difference between the identification rates of *C. jejuni, C. coli* and *C. lari* may be due to contamination rate of the used chicken flocks, the difference of thermophilic *Campylobacter* epidemiology in the region where, the chicken exist, breeding style of avian flocks in the poultry coops, age of the flock, antibiotic use and the population of rodents and wild avians living in the environment of the poultry coop. Also it may be due to slaughtering techniques, methods for preserving carcasses, selling conditions, sample type where the factor is searched, method differences used in isolation and the season. When Willis and Murray (1997) investigated, the seasonal distributions of the presence of *C. jejuni* in the chicken carcasses sold in markets, they reported that the highest isolation rate was found between May and October in temperate climates. In accordance with the results obtained from the present study, the thermophilic *Campylobacter* sp. contamination rate in the carcasses was observed to increase between May and June, reach the peak point between July and August and decrease between September and October.

A study, in which the identification of *C. jejuni* and *C. coli* was confirmed by PCR method, primers specific to *hip*O, *ceu*E and *map*A genes were detected to be used and also, as a result of typing oligonucleotide primers containing ceuE gene sequence were detected to be more specific than the others On and Jordan (2003). In another study, carried out by benefiting from the *ceu*E gene of *C. jejuni* and *C. coli,* it was reported that 275 strains by PCR method and 186 strains by classical culture method were identified to be *C. jejuni*. Besides, 16 of 83 strains, detected not to be *C. jejuni* by PCR method were detected to be *C. jejuni* by classical culture method. Depending on these results, PCR method was reported to be more reliable and sensitive (Houng *et al*., 2001). Similarly, Lawson *et al*. (1998) reported that they collected 200 feces samples for their studies on identification *Campylobacter* sp. in patients with gastroenteritis. They isolated *Campylobacter* sp. from 16 (8%) using classical culture method, 19 (9.5%) by PCR method. They also stated that 1 sample yielding positive result using classical method gave a negative result by PCR and 4 of 19 samples identified by PCR gave negative results in the classical culture. Researchers associated isolates, obtained with the classical culture method as a result of phenotypic tests, yielding negative results with real-time PCR with the template DNA quantity remaining below a determinable limit subsequent to DNA extraction and inhibition possibility of template DNA molecules preserved for a long period of time (Waage *et al*., 1999; Sails *et al*., 2003). In another study, it was reported that although direct boiling method used for DNA-extraction was quite an easy and widespread method, it brought about negative results, *Campylobacter* sp. could not be eliminated due to the chemical structure of their cell walls and an effective DNA-extraction could not be performed using the boiling method, proteinase K added to the extraction procedure for DNA extraction was a markedly effective method (Mohran *et al*., 1998). Bolton *et al*. (2002), reported that 69 of 115 samples comprising various food types yielded positive results both with the PCR-ELISA and the classical culture methods, 70 yielded positive results with the classical culture method, 71 yielded positive results with PCR-ELISA. They also stated that isolation used to identify *Campylobacter* sp. with the classical culture techniques took up long time and the phenotypic identification results required after isolation could be subjective, which led to incorrect identifications. Similar to the PCR results obtained from this present study in other studies by Lawson *et al*. (1998), Houng *et al*. (2001), Bolton *et al*. (2002), Kulkarni *et al*. (2002), Hong *et al*. (2003) and Sails *et al*. (2003), it was reported that positive results were yielded with classical culture methods and negative results were yielded with PCR method. The

researchers reported that the cause could be ascribed to the cross reactions occurring during the phenotypic identifications with the classical culture method, the extraction method of the template DNA used for PCR procedure and template DNA quantity, the negative effects of nonspecific DNA products that may occur during pure DNA preparation stage, on some PCR primers, type of the used DNA polymerase and the buffer solution reaction and such conditions could inhibit PCR (Mohran *et al*., 1998; Bolton *et al*., 2002; On and Jordan, 2003; Sails *et al*., 2003).

Similar to the other studies by Wilson *et al*. (2000), Houng *et al*. (2001), Avrain *et al*. (2003), Gupta *et al*. (2004), Ishihara *et al*. (2004) and Mayrhofer *et al*. (2004) in this present study 28 (45.16%) of 62 isolates confirmed to be *C. jejuni* as a result of PCR procedure were detected to be resistant to nalidixic acid subsequent to the antibiotic susceptibility tests included by the phenotypic identification tests applied in advance. In numerous investigations, thermophilic *Campylobacter* sp. were designated to develop resistance to the nalidixic acid in the antibiotic susceptibility tests, which were significant among phenotypic identification tests (Wilson *et al*., 2000; Houng *et al*., 2001; Avrain *et al*., 2003; Gupta *et al*., 2004; Ishihara *et al*., 2004). In a similar investigation of the of antibiotic resistance, resistance to quinolones (nalidixic acid) was reported to display a high prevalence (40.7%) (Mayrhofer *et al*., 2004).

CONCLUSION

It has been established that the majority of chicken carcasses bred, slaughtered and sold unpacked in Afyonkarahisar are contaminated with thermophilic *Campylobacter* sp. and these chicken carcasses sold unpacked may comprise potential health risk concerning public health. In this regard, implementation of the food safety programs and HACCP (Hazard analysis critical control point) systems primarily on the farms, to breeding stage in slaughterhouses and lastly in the food outlets becomes important in order to prevent *Campylobacter* infections that may emanate from chicken meat and products.

Moreover, this study displays the necessity for the management of PCR and the specific identification studies parallel with the identifications depending on the classical culture methods due to the insufficiency of the antibiotic susceptibility tests, an important identification test of the classical culture test to the identification of the antibiotic resistant species increasing lately and probability of leading to mistakes with subjective evaluations by the hippurate hydrolysis tests.

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