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Identification of Enteropathogenic *Campylobacters* in Poultry's Faeces by PCR and its Comparison with Culture in Zahedan (Iran)

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The present study was performed in order to identify the organisms in poultrys by culture and the PCR. This is the first step in an effort to devise strategies for control of human *Campylobacters* infection in Zahedan. One hundred and sixteen samples of rectal swabs from chickens of Zahedan (south-east Iran) poultry farms were collected and cultured on *Campylobacter* selective agar. Subsequently, identification was carried out by biochemical tests. DNA was isolated from suspensions, which were already prepared from stool samples and purified with specific kits. The PCR was done by application of species-specific primers of *C. jejuni* and *C. coli*. The results of culture in case of 11 samples were positive out of them 8 were as *C. jejuni* and 3 belonged to *C. Coli*, whereas in case of the PCR results 27 samples were positive out of them 18 isolates belonged to *C. jejuni* and 9 were found as *C. coli*. Hence, sensitivity and specificity of the PCR were found as 100 and 84.7%, respectively, therefore it is concluded that the PCR protocol applied in this study could identify all positive samples. Furthermore, detection of the species by the PCR was exactly identical with biochemical procedures, so taking into account accuracy and rapidity of the technique, it does seem that PCR is a reliable alternative for identification of *Campylobacters* in stool samples of the poultrys.

Key words: *Campylobacter jejuni*, *Campylobacter coli*, Polymerase Chain Reaction (PCR), cultural method, poultrys

INTRODUCTION

Nowadays *Campylobacter jejuni* and *Campylobacter coli* are considered as the most common cause of bacterial diarrhea and in developing countries, they are associated mainly with illness in children (Coker *et al.*, 2002; Oberhelman and Taylor, 2002).

Sporadic cases of human infection are generally attributed to the consumption of raw or undercooked poultry meat (Pearson *et al.*, 2000; Deming *et al.*, 1987) whereas outbreaks have been traced to contamination of the water supply (Pearson *et al.*, 2000; Tokumaru *et al.*, 1991; Sacks *et al.*, 1986).

Natural reservoirs for *Campylobacter jejuni* include chicken and other poultries (Stern, 1992; Skirrow, 1991) and *Campylobacter coli* is usually isolated from intestines of pigs (Pearce *et al.*, 2003).

The incidence of human *Campylobacter* enteritis in Iran has increased over past few years (Bokaeian and Dabirzaden, 1999; Bokaeian and Sultandallal, 1994). On the other hand, some difficulties in diagnosis of the species by culture (long incubation time, uncertainty of biochemical tests, live atypical strains incapable of growth on media) have induced attentions of researchers towards other alternative procedures such as the PCR (Magistrado *et al.*, 2001; D'Sullivan *et al.*, 2000).

Hence, as till now, there has been no investigation of the source(s) of human *Campylobacter* infections in Zahedan, the present study was performed in order to identify the organisms in poultries by culture and the PCR. This is the first step in an effort to devise strategies for control of human *Campylobacter* infection in Zahedan.

MATERIALS AND METHODS

A total of 116 samples in random from rectal swabs of chickens as of poultry farms in Zahedan were collected between January and June 2004. After enrichment of samples in Campy-Thio broth for 12 to 18 h, they were sub-cultured on *Campylobacter* selective agar (containing 5% defibrinated sheep blood in addition to vancomycin, polymyxin and trimethoprim). After 48 h incubation under microaerophilic condition at 42°C, colonies having *Campylobacter* characteristics (convex, gray, 2 to 4 mm in diameter) were gram stained. Furthermore, the species of *Campylobacters* were identified by application of catalase, oxidase and hyppurate hydrolysis tests plus antibiotic sensitivity tests applying 30 µg discs of cephalothin and nalidixic acid. Subsequently, for direct DNA isolation from the samples, High Pure Template preparation Kits (Roche, Germany) were employed and for this purpose enriched suspension of the samples in

Campy-Thio was centrifuged (2000 rpm for 10 min) and the supernatant was then removed. Afterward, to the precipitant lysosyme and phosphate buffer were added and kept in water bath at 37°C for 30 min, subsequently to the mixture isopropanol was added and samples were transferred to filter tube and after centrifugation, inhibitor removal buffer was put in. The samples were then recentrifuged and wash buffer was added to them, once more the specimens were spun and this time elution buffer was put in. In the final step after centrifugation, DNA of the samples were collected and with the help of UV spectrophotometer, optical density of the specimens were assessed and DNA content of each sample was found out. Furthermore, two sets of primers were employed for the PCR amplification as: (JEJ1-JEJ2) *Jejuni* species specific and (COL1-COL2) *coli* species specific primers (Gonzalez *et al.*, 1997). The sequence of primers were as following:

JEJ1 (5' CCTGCTACGGTGAAAGTTTTGC3'), JEJ2 (5' GATCTTTTTGTTTTGTGCTGC 3')
COL1 (5' ATGAAAAAATATTTAGTTTTTGCA3'),
COL2 (5' ATTTTATTATTTGTAGCAGCG3')

Concentrations of the reagents for the PCR cocktail (final volume 25 µL) were as: 100 ng of the purified DNA, KCL 50 mM, Tris-HCL (PH 8.3) 10 mM, Gelatine 0.001%, Mg Cl₂ 3 mM, dNTP 0.2 mM, primers 200 pM and Taq DNA polymerase 0.5 unit (Gonzalez *et al.*, 1997). Total PCR cycles were 30 and each cycle was as following: denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and finally extension at 72°C for 1 min (Gonzalez *et al.*, 1997). The PCR product was then mixed with loading buffer and ethidium bromide before electrophoresis, which was carried out in presence of specific ladder. Moreover, in order to control PCR, DNA extraction and purification from control strains (*Campylobacter jejuni* Lior 1, *Campylobacter jejuni* Lior 2 and *Campylobacter coli* Lior 8) were also included exactly as for tests.

RESULTS

Out of 116 cultured samples, 11 were found positive by culture (9.4% of the total samples) having the morphology of campylobacters as: curved, gram-negative bacilli. Catalase and oxidase tests of all strains were established positive and all were resistant to cephalothin and sensitive to nalidixic acid. Furthermore, hyppurate hydrolysis test for 8 samples was positive and for the rest negative, therefore biochemical tests indicated that 72% of the strains belonged to *C. jejuni* species and remaining 28% to *C. coli* species.

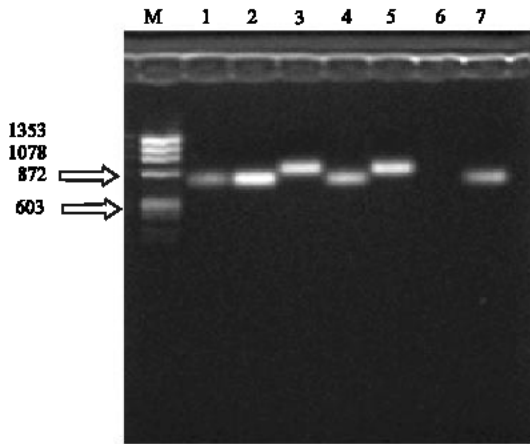


Fig. 1: Electrophoresis of the PCR product of extracted and purified DNA from samples of poultries. M: marker for molecular weight, Lanes 3 and 5 samples of the poultries having *C. coli*, Lanes 1,2, 4 and 7: samples of the poultries having *C. jejuni*, lane 6: sample devoid of *C. jejuni* or *C. coli*

Table 1: The comparison of PCR and culture in identification of *Campylobacters* in poultries' faeces

Total	Negative	Positive	Culture/PCR
18	3 FP	15 TP	Positive
178	178 TN	0 FN	Negative
196	181	15	Total

Out of 116 samples, which were subjected to DNA extraction and the PCR 27 were positive, that comprises 23.2% of the total specimens. Subsequently, 18 (66.7%) samples were amplified by *C. jejuni* specific primers and 9 (33.9%) with *C. coli* specific primers (Fig. 1).

All positive samples by culture (n = 11) were positive by the PCR too as well as other 16 specimens that were negative by culture found positive by the PCR. The remaining 89 samples were established negative by both culture and the PCR (Table 1).

DISCUSSION

Campylobacter is considered part of the normal flora of the gut of chickens but its isolation manifest massive differences. In some investigations 30 to 100% of the poultries have been regarded carriers of the bacteria as normal flora of intestinal tract (D'Sullivan *et al.*, 2000). In the other hand, Magistrado *et al.* (2001) has reported only 5.9% isolation of *Campylobacters* and in our study, the isolation rate was 9.4% by culture method. There has been no evidence for these differences but age of chicken may be an important factor. In another study

(Workman *et al.*, 2005) all of the negative chicks were under the age of 3 weeks and in our study, 80% of examined samples were from chicks between 2 to 4 weeks and all of the positive chicks were above the age of 5 weeks.

Thus investigation of older chicks may yield a different prevalence rate and give a better indication of the overall prevalence of *Campylobacter* in chicks in Zahedan.

On the other hand, there are reports of seasonal variation in shedding by animals (Workman *et al.*, 2005). Our study was done in winter and spring, thus frequent sampling throughout the year may give a better indication of the prevalence of *Campylobacter* in chickens. However in a study in Thailand the most *Campylobacter* isolated were collected during the rainy months of year (Meeyam *et al.*, 2004).

In comparison to the culture, the PCR was capable of detecting more positive samples (27 versus 11) therefore it can be concluded that PCR is a suitable substitute for diagnosis of *Campylobacters* in chickens' specimens. Other investigators have also reported that PCR detection of *Campylobacter* directly from feces may give better indication of the carriage rate in other animals (Inglis and Kalischuk, 2003)

The reasons for revealing culture negative samples in spite of being positive by the PCR in our study could be unsuitability of the used selective media for some strains, gap between sampling and culture and insufficient number of bacteria in some specimens. These factors have contributed to low *Campylobacter* isolation rates in chickens by others (Workman *et al.*, 2005; Mahendru *et al.*, 1997; Goossens *et al.*, 1984).

In this study, 72 and 28% of the isolated species belonged to *C. jejuni* and *C. coli*, this is comparable with results of similar study in Thailand (Meeyam *et al.*, 2004).

Roughly in all other studies, *C. coli* has been the most common species isolated from pigs (Workman *et al.*, 2005) but there are reports of isolation of this species from chicken meat (Workman *et al.*, 2005; Meeyam *et al.*, 2004).

Moreover the available data indicates enormous dissimilarities between species isolation from poultries for instance; in one study 99% of the infections in poultries were due to *C. jejuni* species (Ng *et al.*, 1997) while Van Looveren *et al.*, (2001) have attributed 79 and 21% of the isolations to *C. jejuni* and *C. coli*, respectively. In a similar study carried out by other researchers these figures were reported 67 and 33% correspondingly (Eyigor *et al.*, 1999) and according to Magistrado and associates' report out of 8 isolated strains of *Campylobacters* from poultries 3 belonged to *C. jejuni* and 5 to *C. coli* species (Magistrado *et al.*, 2001).

Identification of strains by biochemical tests in this study was quite identical with that of PCR. Nonetheless, we recommend PCR as an efficient alternative for culture due to its sensitivity and speed in addition to intricacy of some strains' cultivation in selective media (specially due to insufficient number of bacteria in poultries' specimens) or at least for those samples whose results by culture are negative.

In our previous study in Zahedan (Bokaeian and Dabirzadeh, 1999) *Campylobacter*s were identified in 5% of diarrheic children. As *Campylobacter* can transmit from chicken to human (Meeyam *et al.*, 2004) and based on genotyping studies there are similarities between isolates from chicken and those isolated from human (Meeyam *et al.*, 2004) health protection measures to control infectious disease in Zahedan should focus on preventing transmission from chicken meat to human.

We recommend of cooking chicken meat thoroughly and treatment with acidic juice and salt to prevent transmission if *Campylobacter*s to human.

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