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Prevalence and molecular heterogeneity analysis of *Campylobacter jejuni* and *Campylobacter coli* isolated from human, poultry and cattle, in Pantnagar, India

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ABSTRACT

Thermophilic campylobacters are the leading cause of food-borne bacterial gastroenteritis worldwide. Reports regarding the prevalence of campylobacters in India are very few and no report on the use of molecular typing tools is available for this important pathogen. In the present study, a total of 612 stool/fecal samples collected from humans (n = 260), poultry (n = 239) and cattle (n = 113) were examined for the presence of thermophilic campylobacters by direct plating on modified charcoal cefoperazone deoxycholate agar plates, and employing conventional morphological and biochemical tests. Of these, only 43 samples showed positive *Campylobacter* colonies. Further, genus and species level identification and confirmation by multiplex PCR revealed the isolates from human (4) and cattle (1) to be *Campylobacter jejuni*, whereas, out of 38 isolates from poultry, 29 (76.32%) and 9 (23.68%) were *C. coli* and *C. jejuni,* respectively. The genetic diversity of the isolates studied by *fla*A-RFLP typing, using *Dde*I restriction enzyme, revealed the presence of 11 and 7 fla-types among the 14 *C. jejuni* and 29 *C. coli* isolates, respectively. Dendrogram analysis showed that one of the *C. jejuni* isolates from poultry shared 100% genetic similarity with the human isolate. The prevalence rate in human, poultry and cattle was estimated to be 1.54, 15.89 and 0.88%, respectively, with a comparatively high

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prevalence of *C. coli* in poultry. This study appears to be the first of its kind from India, on the application of multiplex PCR and *fla*A-RFLP typing of *Campylobacter* isolates obtained from a variety of sources.

Key words: *Campylobacter*, *C. jejuni*, *C. coli, fla*A-RFLP typing

Introduction

Thermophilic campylobacters are the leading cause of food-borne bacterial gastroenteritis worldwide (MOORE et al., 2005; BEHRINGER et al., 2011; SILVA et al., 2011; MDEGELA et al., 2011). The number of reported cases of campylobacteriosis is high in developed countries, while, in developing countries, due to the absence of regular surveillance programmes the disease remains underreported (COKER et al., 2002; WORKMAN et al., 2006). Among thermophilic campylobacters, *Campylobacter jejuni* and *C. coli* account for 85-90% and 5-10% of the infections in humans (MOORE et al., 2005; MESSENS et al., 2009; MAĆKIW et al., 2011). Transmission in humans occurs mainly by handling or consumption of foods of animal/poultry origin. Poultry and their products are considered the major source for human infection (WAGENAAR et al., 2008; RIZAL et al., 2010; HERMANS et al., 2011; MDEGELA et al., 2011; FINDIK et al., 2011). The majority of human infections occur sporadically, characterized by self limiting diarrhea for 2-5 days, but secondary complications, such as Gullian-Barre syndrome and reactive arthritis, have also been reported (MOORE et al., 2005; MESSENS et al., 2009).

Campylobacteriosis may be diagnosed by isolation and identification of the organism, and by using recent molecular techniques (MOORE et al., 2005; SILVA et al., 2011).Though species level identification of the isolates can be done by biochemical tests, the close relationship between the species make the tests unreliable, requiring the use of polymerase chain reaction (PCR) based methods for species differentiation (PERSSON and OLSEN, 2005). The intra-species variability among the isolates can be characterized by molecular typing techniques, which are valuable tools in tracking the routes of transmission during epidemiological investigations (ACKE et al., 2010; BEHRINGER et al., 2011). A wide range of molecular typing methods have been developed for genotyping of *Campylobacter* isolates *viz.,* RFLP, RAPD, PFGE, ALFP, REP-PCR, MLST and others. The most common genotyping methods employed for thermophilic campylobacters are PFGE, RAPD and PCR-RFLP techniques (FINDIK et al., 2011). The *fla*-RFLP typing method has been reported to be rapid, and the most valuable and cost effective method, which can be employed in epidemiological studies involving a small number of isolates (MOORE et al., 2005; ZORMAN et al., 2006; MÜLLER et al., 2011).

In view of limited information available on the isolation, identification and typing of *Campylobacter* spp. in developing countries such as India (RIZAL et al., 2010), the present study was undertaken to study the prevalence of thermophilic campylobacters among human, poultry and cattle in and around the Pantnagar region, Uttarakhand, India. Both

conventional cultural methods and molecular tools of multiplex PCR were employed for identification of campylobacters at genus as well as species level. Additionally, molecular typing of the isolates by the *fla*A-RFLP typing method was also done to explore Molecular Heterogeneity among the isolates obtained from both animals/poultry and humans.

Materials and methods

Isolation and identification of thermophilic Campylobacters. A total number of 612 samples consisting of 260 human stools (adult males 143 and 117 females), 239 poultry droppings and 113 samples of cattle dung were collected in sterile sample collection vials, kept at 4 °C and processed for isolation of campylobacters within one hour of collection. The human stools samples were collected from apparently healthy people residing within the G.B. Pant University of Agriculture and Technology university campus, Pantnagar, Uttarakhand, India. A loop-full content of each sample was directly streaked on modified charcoal cefoperazone deoxycholate agar (mCCDA) media and then incubated under microaerobic conditions at 42 °C for 48 h. The agar plates showing characteristic small (1-2 mm), circular, flat to slightly raised, grey colored colonies were considered as putative campylobacters. The isolates were subjected to Gram's staining, motility and biochemical tests, such as catalase, oxidase, urease, nitrate reduction and indoxyl acetate hydrolysis, for identification of campylobacters. Further, species level differentiation of the isolates was performed using a hippurate hydrolysis test and susceptibility to cephalothin and nalidixic acid by the disc diffusion method (TENOVER and FENNELL, 1992).

DNA extraction. Genomic DNA from each presumptive isolate was extracted using a HiPurA Bacterial Genomic DNA Purification kit (HiMedia, Mumbai, India) according to the manufacturer's instructions and stored at -20 °C until used.

Multiplex-polymerase chain reaction (mPCR). A multiplex PCR (mPCR), targeting 16S *rRNA* (genus specific), *mapA* (*C. jejuni* specific) and *ceuE* (*C. coli* specific) genes, was applied for confirmatory identification of the 43 isolates, both at genus and species level, as described previously by DENIS et al. (1999). The amplicons were analyzed in 1.5% agarose gel and documented.

FlaA-RFLP typing and dendrogram analysis. The *fla*A-RFLP typing of all the 43 *Campylobacter* isolates was carried out as per the method described in CAMPYNET (http://campynet.vetinst.dk/Fla.htm). The *fla*A gene (1.7 kb) of each *Campylobacter* isolate was amplified by PCR and subjected to restriction enzyme digestion with the *Dde*I enzyme (Fermentas). The digested fragments were electrophoresed in 2% agarose gel and documented. The *fla*A-RFLP profiles thus generated were analyzed using Alpha imager analysis tools, and each different pattern obtained was considered as a fla-type. Dendrograms were constructed for *C. jejnui* and *C. coli* isolates based on pair-wise binary band matching patterns, using a NTSYS-PC 2.11V (Numerical Taxonomy and

Multivariate Analysis System, Exter Publishing Ltd.) software program employing the Dice similarity coefficient and the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) methods (LLORENS et al., 2006; FINDIK et al., 2011).

Results

Out of 612 samples screened for the presence of campylobacters, 43 samples yielded characteristic bacterial colonies on mCCDA plates after 48 hours of incubation. They revealed characteristic Gram negative, 'spiral' or 'S' shaped morphology and typical corkscrew motility, as observed by the hanging drop method. During the course of biochemical testing, all the isolates were found positive for catalase, oxidase, nitrate and indoxyl acetate hydrolysis tests. None of the isolates revealed positive reaction for urease activity. Out of the 43 isolates, 17 were identified as *C. jejuni* and 26 isolates as *C. coli* species, based on the hippurate hydrolysis test and all were found to be sensitive to nalidixic acid and resistant to cephalothin. Among these 43 *Campylobacter* isolates, four were from human (all females), one from cattle and the remaining 38 isolates were from poultry samples.

In multiplex PCR (mPCR), all the 43 *Campylobacter* isolates yielded the genus specific (16S *rRNA*) 857 bp amplicon, and 14 isolates showed the *C. jejuni* specific 589 bp (*mapA*) while 29 produced the *C. coli* specific 462 bp (*ceuE*) amplicon (Fig. 1). All the human (4) and cattle (1) isolates were of *C. jejuni* species. Out of the 38 poultry isolates, 9 (23.68%) were identified as *C. jejuni* and 29 (76.32%) as *C. coli* species.

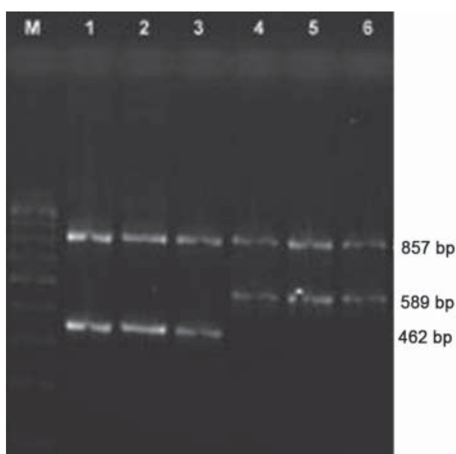

Fig. 1. Multiplex PCR amplicons on 1.5% agarose gel. Lane M: 100 bp ladder; Lane: 1, 2, 3 - *C. coli*; Lane: 4, 5, 6 - *C. jejuni*

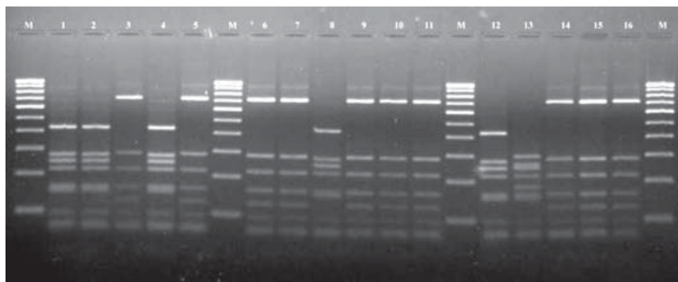

Fig. 2. PCR-RFLP profiles of *C. coli* isolates generated using *Dde*I in *fla*A typing. Lane M: 100 bp ladder; Lane: 1-16 *C. coli* isolates

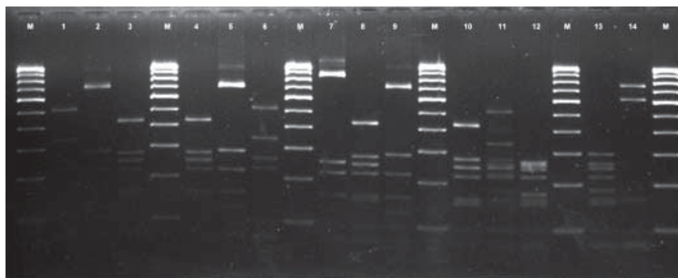

Fig. 3. PCR-RFLP profiles of *C. coli* and a *C. jejuni* isolates generated using *Dde*I in *fla*A typing. Lane M: 100 bp ladder; Lane: 1-13 *C. coli* isolates, Lane: 14 *C. jejuni*

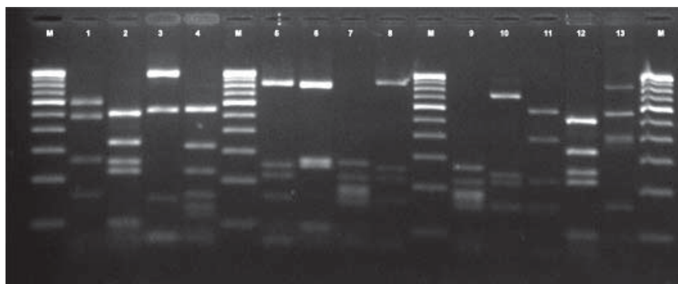

Fig. 4. PCR-RFLP profiles of *C. jejuni* isolates generated using *Dde*I in *fla*A typing
Lane M: 100 bp ladder; Lane: 1-13 *C. jejuni* isolates

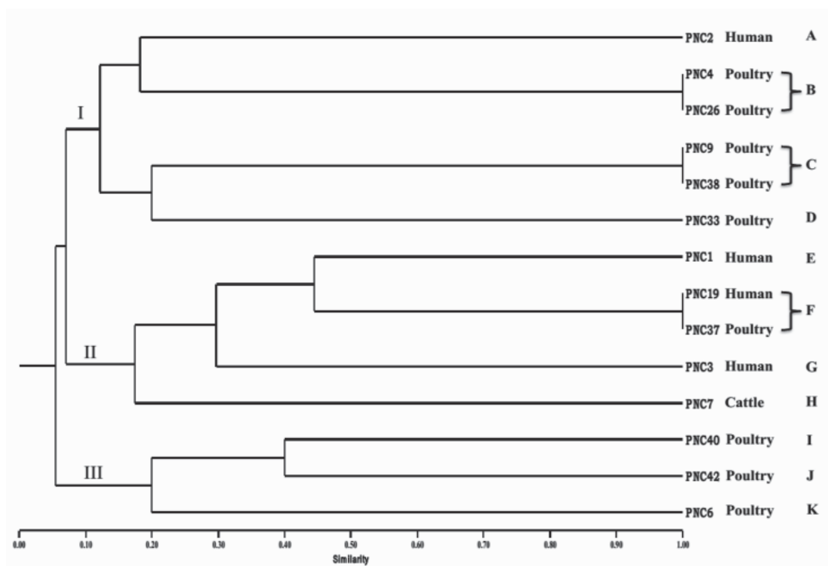

Fig. 5. Dendrogram of *fla*A typing of 14 *C. jejuni* isolates

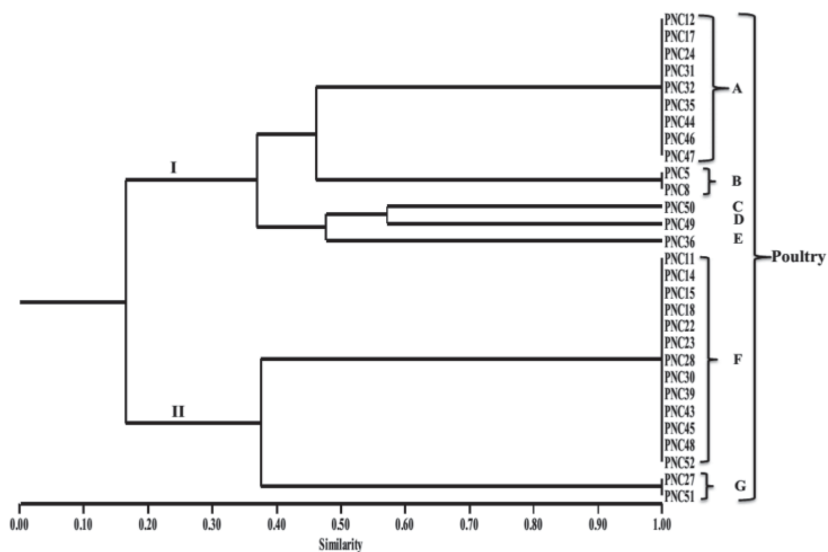

Fig. 6. Dendrogram of *fla*A typing of 29 *C. coli* isolates

Molecular typing of these 43 *Campylobacter* isolates using the *fla*A-RFLP typing technique revealed 11 and 7 fla-types among 14 *C. jejuni* and 29 *C. coli* isolates, respectively (Fig. 2, 3, 4). Dendrogram analysis showed that the 11 fla-types (A-K) of the *C. jejuni* were found to be distributed into three clusters, with clusters I and II having four fla-types each, and cluster III having three fla-types. A poultry isolate in cluster II was found to share 100% similarity with that of a human isolate, producing a single fla-type (F). The cattle isolate was also observed to cluster with three human and one poultry isolates in cluster II (Fig. 5). Similarly, the seven fla-types (A-G) of *C. coli* were distributed into two clusters, with five fla-types in cluster I and two in cluster II (Fig. 6). Among the *C. coli* isolates obtained from poultry, fla-type F was the dominant clone (44.8%) followed by A (31.0%).

Discussion

Campylobacters are zoonotic pathogens, transmitted to humans mainly through food of animal origin. Numerous studies on *Campylobacter* prevalence in humans, poultry and cattle have been carried out worldwide (ANDERSON et al., 2012; GARIN et al., 2012). Most of these were from developed nations and very few reports are available from developing countries, including India (RIZAL et al., 2010; RAJENDRAN et al., 2012). Based on conventional cultural methods, 43 isolates were identified as campylobacters, which were further confirmed by multiplex PCR. The mPCR based assay has been reported to be more consistent and confirmatory compared to conventional methods such as hippurate hydrolysis test which has been found to exhibit inconsistent results at species level identification of campylobacters (AL-AMRI et al., 2007; HERMANS et al., 2011). In the present study, based on hippurate hydrolysis testing, 17 isolates were identified as *C. jejuni* and 26 as *C. coli* species, while mPCR identified 14 *C. jejuni* and 29 *C. coli* isolates among the total of 43 isolates. Four of the *Campylobacter* isolates found positive by hippurate hydrolysis test were later identified as *C. coli* by mPCR, and one isolate which was negative by hippurate hydrolysis, was found to be *C. jejuni* through mPCR. Similarly, DENIS et al. (1999) identified 220 isolates as *C. jejuni* and 74 as *C. coli* out of a total of 294 isolates, using the hippurate hydrolysis test, but using mPCR only 66 isolates were found to be *C. jejuni* and the rest belonged to the *C. coli* species. JAMSHIDI et al. (2008) reported that although the hippurate test is rapid, it is not reliable, as the amino acids and peptides from the culture media could produce false positive reactions. Additionally, the occurrence of hippurate negative strains of *C. jejuni* has also been reported (ALLOS, 2001; ON and JORDAN, 2003), which could have been the reason for the misidentification of the lone hippurate negative isolate, identified as *C. coli* by conventional cultural methods.

The present study revealed a prevalence rate of 1.54, 15.89 and 0.88% for thermophilic campylobacters among human, poultry and cattle, respectively. Similar prevalence rates

of campylobacters were also recorded by RIZAL et al. (2010) and RAJENDRAN et al. (2012) from India. Out of the four isolates obtained from humans, all were from apparently healthy adult females, indicating that the organism could have caused asymptomatic infection resulting from development of pre-immunity (ALLOS, 2001). However, no thermophilic *Campylobacter* could be isolated from the 143 males, which was contradictory to the reports by PEARSON and HEALING (1992) and MOORE et al. (2005), who reported that *Campylobacter* infection is common among the male gender. Several studies have reported that *C. jejuni* are more frequently associated with poultry than *C. coli* (MENA et al., 2008; RAJENDRAN et al., 2012). However, in the present study, among the 38 poultry isolates only 23.68% belonged to the *C. jejuni* species, whilst the remaining 76.32% isolates were *C. coli*. Similar findings were also reported by PADUNGTOD and KANEENE (2005), ZORMAN et al. (2006) and HENRY et al. (2011). The reason for this difference in the prevalence rates of *C. jejuni* and *C. coli* among poultry is unknown, however the impact of differences in isolation procedures and geographic differences has been suggested (ZORMAN et al., 2006). Among cattle, a very low *Campylobacter* prevalence of only 0.88% was obtained during the present study. RAJENDRAN et al. (2012) also reported that no *Campylobacter* spp. could be detected among 589 cows, 2 buffaloes, 11 bullocks and 25 goats. They suggested that this could be due to the low infection burden in these animals, or better methods of recovery of the organism, such as an enrichment step, might be required.

The *fla*A-RFLP typing, being simple, fast, cheap and reliable, and having high discriminatory power, is a widely used method for subtyping of *Campylobacter* isolates (ACKE et al., 2010; BEHRINGER et al., 2011). Using the standard *fla*A-RFLP typing method described in CAMPYNET, all the isolates could be typed (100% typeability) with identification of a total of 11 and 7 fla-types among 14 *C. jejuni* and 29 *C. coli* isolates, respectively. The *C. jejuni* isolates obtained were genetically more diverse compared to that of *C. coli,* on the basis of the number of fla-types obtained. This diversity could be due to the fact that they all were obtained from different sources, including humans, poultry and cattle, or due to the genetic instability of campylobacters, as suggested by different authors (AQUINO et al., 2010; MÜLLER et al., 2011). Interestingly in this study, one *C. jejuni* isolate from poultry was found to share 100% similarity to that of a human isolate, indicating that they both might possibly have had a common source of infection or the human could have become infected with the same clone of *C. jejuni* circulating among poultry in this region. Among *C. coli* isolates obtained from poultry, the predominance of clones F and A indicates that they all might have been acquired from a common source or the persistent contamination of the flock (ZWEIFEL et al., 2008), but the presence of other fla-types of *C. coli* suggests varied sources of infection (GÜRTLER et al., 2005).

In conclusion, multiplex PCR was found to be more reliable than the conventional cultural methods in species level identification and differentiation of *Campylobacter* isolates. The *fla*A-RFLP typing also gave promising results in genotyping the *Campylobacter* isolates obtained in the present study. This study appears to be the first of its kind from India on the application of multiplex PCR and *fla*A-RFLP typing of *Campylobacter* isolates. Further extensive epidemiological studies with a larger number of samples, obtained from a variety of sources and different geographical areas, need to be carried to discover the prevalence, magnitude and importance of *Campylobacter* infection in animals and humans in this country.

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SAŽETAK

Termofilni kampilobakteri vodeći su uzrok bakterijskog gastroenteritisa diljem svijeta koji se prenose hranom. Izvješća o njihovoj prevalenciji u Indiji su rijetka, a potpuno nedostaju izvješća o upotrebi molekularnih metoda za njihovu tipizaciju. U ovom je istraživanju bilo pretraženo ukupno 612 uzoraka i to 260 uzoraka stolice ljudi, 239 uzoraka izmeta peradi te 113 uzoraka izmeta goveda na prisutnost termofilnih kampilobaktera izravnim nasađivanjem na preinačen cefoperazon deoksikolatni agar s drvenim ugljenom. Uzročnik je bio identificiran uobičajenim testovima za određivanje morfologije i biokemijskih osobina. Kolonije kampilobaktera dokazane su samo u 43 pretražena uzorka. Daljnjim postupkom identifikacije na razini roda i vrste te potvrde višestrukom lančanom reakcijom polimerazom dokazano je da su četiri izolata iz ljudi i jedan iz goveda pripadali vrsti *Campylobacter jejuni*, dok je od 38 izolata iz peradi njih 29 (76,32 %) pripadalo vrsti *C. coli*, a 9 (23,68 %) vrsti *C. jejuni*. Istraživanjem genetske raznolikosti izolata na osnovi polimorfizma dužine restrikcijskog fragmenta gena *fla*A uporabom restrikcijskog enzima *Dde*I dokazano je 11 fla tipova među 14 izolata vrste *C. jejuni* i sedam fla tipova među 29 izolata *C. coli*. Analiza dendrograma je pokazala da je jedan izolat *C. jejuni* iz peradi bio identičan izolatu iz ljudi. Procijenjena prevalencija u ljudi iznosila je 1,54, u peradi 15,89, a u goveda 0,88 %, s relativno velikom prevalencijom vrste *C. coli* u peradi. Ovo je prvo istraživanje takve vrste u Indiji.

Ključne riječ: *Campylobacter jejuni*, *Campylobacter coli, fla*A-RFLP tipizacija