Screening and Rapid Identification of Campylobacter Spp. DNA by FlaA PCR
Based Method on Chicken and Human Fecal Samples in Egypt

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Abstract: Due to culturability requirements encountered by the conventional isolation of Campylobacter spp., rapid molecular techniques for its direct identification from clinical samples are useful. In this study, Campylobacter spp. DNA from human stool and chicken fecal samples were detected by flagellin gene (flaA) PCR. A total of 297 samples consisting of 163 adult human stools (102 from diarrheic patients and 61 from healthy persons) and 134 chicken feces were subjected to flaA PCR. Ten reference strains of Campylobacter spp. were included in this study as positive controls. Thirteen stool samples (7.98%) from the human fecal samples and 39 chicken fecal samples (29.1%) yielded the genus specific 1.7 Kb amplicon of Campylobacter spp. Eight (7.84%) diarrheic human stool specimens out of 102 samples and 5 (8.2%) apparently healthy human stool specimens out of 61 samples were positive by flaA PCR assay. All the Campylobacter reference strains examined giving the specific amplicon of 1.7 Kb. The existence of Campylobacter spp. DNA detected by flaA PCR in poultry and human samples taken from locations of Egypt highlights the zoonotic potential of Campylobacter. To the best of our knowledge, this is the first report in Egypt that uses flaA PCR as a rapid screening method for the direct detection of Campylobacter spp. from human and chicken feces.

Key words: Campylobacter, human stool, poultry feces, flaA PCR

INTRODUCTION
Campylobacters are considered one of the most frequent causes of foodborne bacterial infections in developing as well as developed countries (Allos, 2001). Zoonotic infections with Campylobacter spp. (campylobacteriosis) are usually the result of the consumption of contaminated poultry meat that has not been properly prepared or cross contamination between raw poultry and hands of food preparers, kitchen utensils, other foods, cutting boards, etc. (Guyard-Nicodème et al., 2013).

The identification of Campylobacter in chicken feces is crucial especially before slaughtering since the bacterium is carried in the alimentary tract of live birds, and contaminated fecal material may contaminate broiler carcasses during processing (Oosterom et al., 1983). In the countries where slaughtering and evisceration processes of poultry were manual, the chance for presence of large numbers of Campylobacter spp. in poultry meat was high (Parkar et al., 2013).

The isolation and identification of thermophilic Campylobacter from clinical samples by conventional culture methods and biochemical identification tests are laborious, time consuming, and expensive (Erdt, et al., 1991). Also, direct identification of pathogens by PCR in environmental samples such as feces, provides an improved method for the detection of this foodborne pathogen (Fode-Vaughan et al., 2001). The use of PCR-based methods for the rapid identification of Campylobacter may provide a more reliable method to detect broiler flocks contaminated with this pathogen before the birds enter processing facilities (Persson and Olsen, 2005).

Although there are many reports on the usage of flaA PCR as a valuable tool for the detection of Campylobacter spp. from human and chicken feces (Linton et al., 1997; Lawson et al., 1998; Fitzgerald et al., 2001; Al Amri et al., 2007), data on direct PCR identification of Campylobacter from fecal samples in many developing countries such as Egypt is still lacking.

The overall aim of this study was to assess the applicability of flaA PCR for rapid detection of Campylobacter spp. directly from human and chicken feces and to investigate the role of chicken as a potential source for human infections with Campylobacter in the examined area.

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MATERIALS AND METHODS

Sample collection: A total of 297 samples consisting of 163 human fecal samples (102 from diarrheic patients and 61 from healthy persons) and 134 chicken fecal samples were included in this study. The diarrheic stool samples were taken from four private clinical laboratories in the district of Mansoura City, Egypt. All the diarrheic patients (62 male and 40 female) were between 18 and 50 years of age with foul-smelling diarrhea, fever and abdominal disturbances. Stool samples from healthy persons (36 female and 25 male) were collected from rural households that raised chickens on a small scale near Mansoura City, Egypt. A detailed questionnaire was taken from diarrheic patients and healthy persons with information about their health status, previous exposure to diarrhea and contact with live birds. Before stool sample collection, verbal consent was taken from patients in the private clinical laboratories that provided the diarrheic samples, and also from healthy persons.

Chicken fecal samples were randomly collected from birds in five broiler flocks located in the district of Mansoura City, Egypt. Broiler flocks were located within the same proximity from which human stool samples were collected and the five flocks were of 10,000-20,000 birds with an average age of 28-35 days at the time of sample collection.

Fecal samples were collected in sterile sample vials during the period from November 2013 to March 2014, transferred within 2 h from collection to the laboratory of Hygiene and Zoonoses Department, Faculty of Veterinary Medicine, Mansoura University and stored at -20°C until DNA extraction.

DNA extraction from clinical samples: DNA was extracted from clinical samples by suspending 2 g of human or chicken fecal sample in 3 ml of phosphate buffered saline (PBS) in a closed plastic tube and then mixing by vortexing for 1 min at room temperature. From this suspension, 200 µl was transferred to a 1.5 ml sterile eppendorf tube and DNA was extracted using the QiAamp DNA stool extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions.

Reference strains: The following type strains were used as reference strains: C. jejuni ATCC 33560 (from bovine feces), C. fetus subsp. fetus ATCC 27374, C. lari ATCC 35221 (from Herring gull cloacal swab) and C. coli ATCC 86119 (from poultry). C. jejuni PPMQ2b and PPMQ2b were isolated from processed broiler carcasses (Hinton et al., 2004). All other isolates including C. jejuni 86605 (poultry), 48100 (poultry), 1997-8 (human) and 1999-1 (human) were provided by Dr. Richard Meinersmann of the Agricultural Research Service, Russell Research Center, Athens, GA.

Growth of reference strains and DNA extraction: Stock cultures of Campylobacter spp. reference strains were plated onto fresh blood agar plates (Remel®, Lenexa, KS) and incubated in a GasPak Jar with a CampyPak Plus Hydrogen and carbon dioxide with Integral Palladium Catalyst to set microaerophilic conditions for 48 h at 37°C (Hinton, 2006). After incubation, harvesting of cultures was done by pouring 9 ml of 0.1% Difco Bacto Peptone solution to the surface of the plates and using sterile bacterial cell spreaders to remove bacterial growth from the agar surface. The extraction of DNA from the bacterial culture of reference strains was done using PureLink Genomic DNA extraction Kit (Invitrogen Life Technologies, Carlsbad, CA).

PCR: The primer pairs used were flaAF (5'-GGATTTCGATTAAACCAAAATGTTGC-3') and flaAR (5'-CTGTAGTTAATCTTTAAAAACATTITG-3'), which generate a 1.7 Kb amplicon (Nachamkin et al., 1993). PCR was performed in a final volume of 25 µl PCR mixture consisting of 12.5 µl of 2X PCR Master Mix (Promega, Madison, WI), 1 µM of each primer, and 2.5 µl template DNA. Following an initial denaturation for 5 minutes at 94°C, products were amplified by 30 cycles of denaturation for 30 sec at 94°C, annealing for 60 sec at 55°C, and elongation for 90 sec at 72°C with a final extension step of 7 minutes at 72°C. Positive DNA detection for each sample was identified by its specific bp DNA bands on 2% agarose gel, stained with ethidium bromide and evaluated under UV transilluminator. A positive control consisting of DNA extracted from C. jejuni 86605 and also a negative control (nuclease free water instead of DNA template) were included in each PCR run.

RESULTS AND DISCUSSION

The PCR based methods used for direct detection of Campylobacter isolates recovered from different sources are required for infection control and also provides a useful aid in the study of risk assessment of Campylobacter and its zoonotic potential. By using flaA PCR to screen human and chicken feces for the presence of Campylobacter spp., DNA, 7.98% of the human stool samples (13/183) yielded the 1.7 kb amplicon for Campylobacter genus identification (Fig. 1) where 8 samples originated from 102 diarrheic stool specimens and 5 were from 61 healthy specimens. The presence of Campylobacter in chicken feces was higher than that from human stool samples. Approximately 29% (39/134) of the extracted chicken fecal samples were positive for the 1.7 Kb fragment targeting flaA (Fig. 2) and all the reference strains including C. jejuni, C. coli, C. fetus and C. lari were positive by flaA PCR (Fig. 3).

Findings from this study confirmed that the genus specific 1.7 Kb fragment targeting flaA is well identified in samples containing this bacterium. These results are
Fig. 1: PCR identification of *Campylobacter* spp. DNA from human stool samples. Lane M: 100 bp DNA ladder. Lane 1, 2, 3, 4 and 5: DNA extracts from diarrheic human stool samples. Lane 6, 7 and 8: DNA extracts from healthy persons stool samples.

Fig. 2: PCR identification of *Campylobacter* from chicken fecal samples. Lane M: 100 bp DNA ladder. Lane 1, 2, 3, 4, 5, 6 and 7: DNA extracts from chicken fecal samples. Lane 8: *C. jejuni* 86605 (Control Positive).

consistent with many studies that detected *Campylobacter* spp. in feces by flaA PCR (Nachamkin et al., 1993; Linton et al., 1997; Lawson et al., 1998; Fitzgerald et al., 2001). It was also observed that the flaA PCR-based assay in this study could identify *Campylobacter* spp. DNA from human and chicken feces, although these clinical samples might contain PCR inhibitors such as DNases, polysaccharides, and proteases (Wilson, 1997). However, DNA extraction by the QIAamp DNA stool extraction kit provided an effective tool for destruction of PCR inhibitors that interfere with DNA amplification. The usefulness in the QIAamp DNA
stool extraction kit has been previously determined by McCrist et al. (2002) in a study of a comparative assessment of DNA extraction kits from feces. This study confirmed that the QIAamp DNA stool extraction kit was the most effective extraction method among those tested for DNA extraction. Moreover, many researchers are obtaining the desired effect and satisfactory results by using QIAamp DNA stool extraction kit that sufficiently removed PCR inhibitors in fecal samples that interfered with PCR (Inglis and Kalischuk, 2003; LaGier et al., 2004).

It is not surprising that Campylobacter spp. was detected by flaA PCR from the stool of diarrheic patients; however, the identification of Campylobacter from stool of apparently healthy persons may be attributed to contact with infected animals, rearing of animals in personal homes and unhygienic conditions. Healthy carriers of Campylobacter was also observed in a study by Coker et al. (2002) who found that the recovery of Campylobacter organisms from apparently healthy children was common in developing countries. Also, the development of pre-immunity against Campylobacter spp. might lead to the occurrence of asymptomatic infection (Allos, 2001).

It was also clearly noticed that all the stool samples from healthy participants that were positive for flaA PCR for Campylobacter DNA were taken from those individuals living in rural areas who had direct or indirect contact with live birds. This finding strengthens the notion that poultry play an important role as a reservoir for human campylobacteriosis in Egypt. There is a close association between human and live birds in this area, and freshly slaughtered poultry comprises a significant portion of the diets of most Egyptians. The carcasses of these broilers may become contaminated by fecal bacteria during slaughter (USDA, 2001), and if the carcasses are not properly cleaned and sanitized, Campylobacter may survive for up to one week in humid, wet environments of the poultry carcasses (Corry and Al-Ataby, 2001).

In conclusion, the findings from this study indicate that screening of clinical samples from both human and chicken with a rapid and accurate molecular approaches such as flaA PCR is beneficial in implementation of appropriate control measures that will reduce the number of cases of human campylobacteriosis associated with the consumption of contaminated poultry and other food products. The presence of Campylobacter circulated in the examined area that was detected in human stool and chicken fecal samples indicates that further study to isolate and characterize the isolates by genotyping to identify which species circulated between poultry and human is needed. This information will aid in identifying potential source of campylobacteriosis in human. The rapid detection of Campylobacter spp. within a few hours provided a valuable tool which can be used for epidemiological surveillance and prevention strategies especially in developing countries such as Egypt.

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Fig. 3: PCR identification of Campylobacter from chicken fecal samples. Lane M: 100 bp DNA ladder. Lane 1, 2, 3, 7, 15 and 16: DNA extracts from chicken fecal samples. Lane 4: C. coli ATCC 86119. Lane 5: C. jejuni ATCC 33560. Lane 6: C. jejuni 86605. Lane 8: C. jejuni PPMQ2b. Lane 9: C. jejuni PPMQ3b. Lane 10: C. jejuni 48100. Lane 11: C. fetus subsp. fetus ATCC 27374. Lane 12: C. lari ATCC 35221. Lane 13: C. jejuni 1997-8. Lane 14: C. jejuni 1999-1. Lane -ve: Control Negative.
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Conflicts of interest
Authors declared no conflict of interest.

REFERENCES
Al Amri, A., A.C. Senok, A.Y. Ismaeel, A.E. Al-Mahmeed
and G.A. Botta, 2007. Multiplex PCR for direct
identification of Campylobacter spp. in human and
Coker, A.O., R.D. Isokpehi, B.N. Thomas, K.O. Amisu
and C.L. OBI, 2002. Human campylobacteriosis in
developing countries. Emerg. Infect. Dis., 8: 237-
244.
Corry, J.E. and H.I. Aatabay, 2001. Poultry as a source of
Campylobacter and related organisms. J. Appl.
Microbiol., 90: 965-114S.
Endtz, H.P., G.J. Ruijs, A.H. Zwijnderman, T.V.
Comparison of six media, including a semisolid
agar, for the isolation of various Campylobacter
29: 1007-1010.
Use of pulsed-field gel electrophoresis and flagellin
gene typing in identifying clonal groups of
Campylobacter jejuni and Campylobacter coli in
farm and clinical environments. Appl. Environ.
Fode-Vaughan, K.A., C.F. Wimpee, C.C. Remsen and
environmental samples by direct PCR without DNA
extraction. Biotechniques, 31: 598-607.
Guyard-Nicodème, M., O. Tresse, E. Houard, F. Jugiau,
C. Courtillon, K. El Manaa, M. Laisneya and M.
Chemaly, 2013. Characterization of Campylobacter
spp. transferred from naturally contaminated
chicken legs to cooked chicken slices via a cutting
Hinton, A. Jr., 2006. Comparison of growth of
campylobacteriaceae on media supplemented with
organic acids and on commercially available media.
Hinton, A. Jr., J.A. Cason, M.E. Hume and K.D. Ingram,
2004. Use of MIDI-Fatty acid methyl ester analysis to
monitor the transmission of Campylobacter
during commercial poultry processing. J. Food Prot.,
67: 1610-1616.
direct detection of Campylobacter species in bovine
LaGier, M.J., L.A. Joseph, T.V. Passaretti, K.A. Musser,
and N.M. Crino, 2004. A real-time multiplexed PCR
assay for rapid detection and differentiation of
Campylobacter jejuni and Campylobacter coli. Mol.
Cell Probes, 18: 275-282.
genome of “Candidatus Campylobacter hominis” a
novel uncultivated species, are found in the
gastrointestinal tract of healthy humans. Microbiol.,
144: 2063-2071.
PCR detection, identification to species level, and
fingerprinting of Campylobacter jejuni and
Campylobacter coli direct from diarrheic samples.
comparison of five methods for extraction of
bacterial DNA from human faecal samples. J.
Microbiol. Methods, 50: 131-139.
Flagellin gene typing of Campylobacter jejuni by
restriction fragment length polymorphism analysis.
Oosterom, J., S. Notermans, H. Karman and G.B.
Engels, 1983. Origin and prevalence of
Campylobacter jejuni in poultry-processing. J. Food
Parkar, S.F.D., D. Sachdev, N. deSouza, A. Kamble, G.
Suresh, H. Munot, D. Hanagal, Y. Shouche and B.
Kapadnis, 2013. Prevalence, seasonality and
antibiotic susceptibility of thermophilic
Campylobacter spp. in ceca and carcasses of
poultry birds in the “live- bird market”. Afr. J.
identification of Campylobacter coli and
Campylobacter jejuni from pure cultures and
directly on stool samples. J. Med. Microbiol., 54:
1043-1047.
U.S. Department of Agriculture, Foreign Agricultural
Service, USDA, 2001. Egypt poultry and products,
Annual GAIN Report EG1020, Washington, D.C.
Wilson, I.G., 1987. Inhibition and facilitation of nucleic
acid amplification. Appl. Environ. Microbiol., 63:
3741-3751.