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Evaluation of Various Methods for Genomic DNA Extraction from Pure Cultures of Lysis Resistant Campylobacters Isolated from Wild Animals

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ABSTRACT

Campylobacter is one of the important foodborne zoonotic bacterial pathogen that causes enteric disorders in animals, birds as well as in humans. The organism is fastidious in nature, requires microaerophilic environment for its growth and survival. Morphologically, it is gram- negative rods with spiral and gull wing appearance. Polymerase chain reaction (PCR) is gold standard method for the detection of *Campylobacter* from clinical and food samples. For performing PCR, extraction of DNA to be used as template is a challenging task due to lysis resistant nature of bacteria. The genomic DNA isolation was attempted from pure cultures of *Campylobacter* by three methods *viz.* Snap-chill, Salt-Tris EDTA (STE) and Columns based commercial kit method. The average concentration of extracted DNA was highest in STE method (03 -3500 ng/µl) followed by Kit method (03 -2000 ng/µl) and Snap-chill method (00-20 ng/µl). The absorbance ratio at 260 nm and 280 nm (A260/A280) was high up to 1.90 in STE method followed by up to 1.80 with column-based kit and 1.5 with snap-chill method which reflects the high purity of isolated DNA by STE and kit-based protocol. As per the results STE method and Kit method was comparable to each other but due to high cost of commercial kit, STE method is proposed to be desirable and may be used routinely for extraction of DNA of lysis resistant bacteria. The PCR results also advocate the preference of STE method over kit method while Snap-chill method was not found effective for lysis resistant *Campylobacter* isolates.

HIGHLIGHTS

- STE method yielded comparatively better results than other two methods for DNA extraction of lysis resistant thermophilic Campylobacters.
- Higher DNA yield gives better results in multiplex PCR for *Campylobacter* species identification.

Keywords: *Campylobacter*, Lysis resistant, Thermophilic, DNA extraction, PCR, STE method

Animal derived contaminated foodstuffs especially meat, milk and its products are the important sources of foodborne bacterial pathogens that primarily causes gastroenteritis in animal and human being (Rosef *et al.,* 2007). Cattle, chicken, pig, Goat, sheep and Turkey are the major food producing animals which are considered as reservoirs

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of many foodborne pathogens such as *Campylobacter* species, non-typhi serotypes of *Salmonella enteric*, Shiga toxin producing strains of *Escherichia coli* and *Listeria monocytogenes* (Heredia and Garcia, 2018). Foodborne pathogens cause millions of cases of sporadic illness and chronic complications, as well as large and challenging outbreaks in many countries and between countries (EFSA-ECDC, 2016). *Campylobacter* is now recognized as an important zoonotic foodborne bacterial pathogen that causes enteric, reproductive and neurological disorders in wild and domestic animal as well as in humans (Sahin *et al.,* 2017). Studies on urban wild birds (ducks, goose, swans and starlings) and pet animals have identified the link between the *C. jejuni* populations from wild birds and human Campylobacteriosis (Gras *et al.,* 2013). Pathogenic *Campylobacter spp*. which are known to cause the infections in animal and humans include *C. jejuni*, *C. concisus*, *C. rectus*, *C.* h*yointestinalis*, *C. insulaenigrae*, *C. sputorum*, *C. helveticus*, *C. lari*, *C. fetus*, *C. mucosalis*, *C. coli*, *C. upsaliensis*, and *C. ureolyticus* (Igwaran and Okoh, 2019). Of these, *C. jejuni* and *C. coli* are considered the most commonly reported zoonosis and are associated with diarrhoeal disease in humans and animals (Garcia-Sanchez *et al.,* 2018). *Campylobacter* infection is the leading cause of acute bacterial gastroenteritis in human with rising prevalence worldwide (WHO, 2018). *Campylobacter* is a fastidious organism generally requiring specific atmospheres and temperatures to grow, uses menaquinones as their respiratory quinones, does not ferment or oxidize carbohydrates and requires microaerophilic environment (5%O_2, 10% CO_2 and 85% N_2) for growth (Vandamme and De Ley, 1991). Thermophilic *Campylobacter* species are able to grow between 37˚C and 42˚C with an optimum temperature of 41.5˚C, but incapable of growth below 30˚C due to absence of cold shock protein genes which play a role in low-temperature adaptation (Levin, 2007). For detection of *Campylobacter* species, Polymerase chain reaction (PCR) is one of the standard methods for confirmation of bacteria either from direct clinical samples or from pure isolated cultures (Ricke *et al.,* 2019; Ferone *et al.,* 2020). DNA isolation from pure culture of *Campylobacter* is a herculean task because of its lysis resistant nature. A critical step in the application of molecular techniques such as PCR for diagnostic purposes is to obtain nucleic acid template of sufficient purity and quantity. Standard methods for isolating bacterial DNA rely on cell lysis using combinations of heat, detergents and enzymes followed by phenol/chloroform extraction of the DNA (Sambrook *et al.,* 1989; Goldenberger *et al.,* 1995). Mohran *et al.* (1998) reported that 20% of the *Campylobacter* isolates examined were resistant to lysis by boiling water. Previous studies also reported difficulties encountered in the extraction of DNA for PCR from *C. jejuni* (Nachamkin *et al.,* 1993). Englen and Kelley (2000) reported that either boiling alone or enzyme treatment is not sufficient to disrupt the cells of lysis resistant *Campylobacter*. Therefore, mechanical disruption in the presence of a guanidine-based DNA isolation reagent was used for genomic DNA extraction and PCR assay for lysis-resistant strain of *Campylobacter.* Present study was designed to assess the suitable method for DNA extraction from pure culture of *Campylobacter* for PCR assay.

MATERIALS AND METHODS

Sample collection and processing

A total of 350 faecal samples from wild animals including Mammals and Birds were collected from April 2020 to March 2021 from six Zoos/National parks two each from Chhattisgarh, Uttar Pradesh and Uttarakhand province of India. Samples were processed as per ISO 10272-1:2017(E); OIE Terrestrial manual, 2008; Singh *et al.,* 2022 and Correy *et al.,* 2003, micro-aerobically at 5% CO_2 concentration using CO_2 incubator (Eppendorf, Galaxy 170 R, New Brunswick, Germany) through pre-enrichment in buffered peptone water (Hi-Media, Mumbai, India), enrichment in Bolton broth (Oxoid, UK) supplemented with 5% lysed sheep blood and finally isolated in selective media such as Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) (Hi-Media, Mumbai, India) with CCDA selective supplement FD-135 (HiMedia, India) at 42^{o}C for 48-72h. The inoculated plates were observed for the development of characteristic colonies (1-2 mm size, circular, flat to slightly raised, sticky, spreading, and shiny gray) and one unique colony from each plate was further sub-cultured in mCCDA and incubated at the same time-temperature combination. The presumptive colonies were phenotypically identified by Gram's staining and confirmed by various biochemical reactions viz; Catalase test, Oxidase test, Hippurate hydrolysis test (HHT), Campylobacter Nitrate reduction test (CNRT), Urease test and Triple Sugar Iron (TSI)

test. Biochemically positive pure cultures (n=60) of *Campylobacter* species (n=60) were further processed for template DNA extractions to be used in the Polymerase chain reaction after confirmation by gel electrophoresis and quantification by spectrophotometry.

DNA extraction procedures

For genomic DNA extraction of lysis resistant *Campylobacter*, three methods *viz*. Snap-Chill or Heat lysis or boiling method, Bacterial DNA purification commercial Kit method (GeNei, Bangalore, India) and Salt Tris EDTA (STE) were tried. A total of 60 samples (20 samples in each method) were used for genomic DNA isolation.

Snap-chill or boiling method

Genomic DNA extraction from 20 pure cultures of lysis resistant *Campylobacter* isolates were performed by boiling method as previously described by Rawat *et al*., (2018) and Shams *et al*., (2017) with slight modifications. In this method, firstly a loopful of broth culture or 4-5 colonies from Petri-plate (Size 90x15mm; Genaxy, Solan, India) culture were taken in 100 µl distilled water containing 1.5 ml microcentrifuge Eppendorf tubes and then boiled at 100ºC for 15 minutes and sudden chilled in -20°C for 15 minutes. The mixtures were then centrifuged at 12000 rpm for 10 minutes and supernatant were taken into 1.5 ml sterilized Eppendorf tubes. The extracted DNA was visualized by gel electrophoresis and stored at -20°C for using as template DNA for performing multiplex PCR test.

Bacterial DNA purification by commercial kit method

A commercial column based Bacterial DNA purification kit (GeNei Laboratory Pvt. Ltd., Bangalore, India) was used for extraction of DNA from 20 pure cultures of lysis resistant *Campylobacter* isolates following the manufacturer's instructions and protocol provided. The extracted DNA was visualized by gel electrophoresis and stored at -20°C for using as template DNA for performing multiplex PCR test.

Salt-tris-EDTA (STE) method

DNA extraction using STE method was done as per the protocol of Yadav *et al.* (2016), Ertas *et al.* (2004) and Goldenberger *et al.* (1995) with minor modifications. Briefly, a loopful of colonies from mCCDA plate was dispensed in 400 µl phosphate-buffered saline (PBS), vortexed and centrifuged at 12000rpm for 5 min. Supernatant was discarded and the pellet was resuspended in 375 µl Salt–Tris EDTA (STE) buffer, 5µl Proteinase K (20mg/ml) and 20 µl SDS (10 per cent). The solution was mixed by vortexing and incubated at 60ºC for 3 hours with intermittent vortexing in every 30 minutes. After incubation, equal volume (400 µl) of saturated phenol (Hi-media; Mumbai, India) was added and shaken vigorously followed by centrifugation at 12000rpm for 10 min. After centrifugation the two phases were obtained, the upper aqueous and lower organic phase. The upper aqueous phase containing genomic DNA was transferred into another sterile1.5 ml eppendorf tube and precipitated by adding equal volume of absolute ethanol and 0.3 M sodium acetate and incubating at -20ºC for 1 hr or 4ºC for overnight. Then the solution is centrifuged at 12000rpm for 10 minutes and pellet was washed twice with 90% and 70% ethanol (400 µl each), respectively and each step was followed by centrifugation at 12000rpm for 5 min. DNA pellet was resuspended in 100µl nuclease free sterile distilled water or Tris-EDTA buffer and stored at -20^{0}C for using as template DNA to perform PCR.

Agarose gel electrophoresis

The extracted DNA (5 µl) with 6X gel loading buffer (GeNei, Bangalore, India)were loaded in the wells of gel prepared with 1 per cent Agarose (HiMedia Mumbai, India) in 1X TAE buffer (GeNei Laboratory Pvt. Ltd., Bangalore, India) and stained with ethidium bromide (0.5µg/ml). The DNA loaded gels were run at 85 Volts for 1 hour in electrophoresis system (Genei, Bangalore, India) and bands were visualized in Gel Imaging system (AlphaImager HP-Alpha Innotech, Germany). The extracted DNAs were quantified in Bio-Spectrometer with the help of micro cuvette (Eppendorf, Germany) and DNAs were stored at -20^0 C for using as template DNA while performing PCR.

Determination of concentration and purity

The genomic DNA isolated by three different methods as described above was quantified for concentration and

purity. The quantification was done using Bio-Spectrometer (Eppendorf, Germany) using a sample volume of 1 µl in micro-cuvette of 1 mm size. The concentration of genomic DNA was denoted in ng/µl. The ratio of absorbance at 260 (A260) and 280 nm (A280) was taken as a parameter for determining the purity of genomic DNA isolated.

Polymerase Chain Reaction

The isolated DNA from each of the three methods described above were finally subjected to *Campylobacter* genus specific PCR assay using the genus specific primers targeting CadF gene. The forward primer (CadF-F) and reverse primer (CadF-R) sequences were 5'TTGAAGGTAATTTAGATATG3' and 5'CTAATACC TAAAGTTGAAAC3'respectively (Konkel *et al.,* 1999). The PCR reaction was set using 3 µl of genomic DNA (10 ng/µl), 12.5 µl of 2X PCR Master mix (GeNei Bangalore, India) and 1 µl of each forward and reverse primer in a total reaction volume of 25 µl. The thermocyclic condition used was initial denaturation at 95ºC for 5 minutes; 32 cycles of denaturation at 94ºC for 60 seconds, annealing at 49ºC for 60 seconds and extension at 72ºC for 60 seconds followed by final extension at 72ºC for 7 minutes. A positive control and negative control were also run in the PCR reaction. Commercially procured *Campylobacter jejuni* genomic DNA (Hi Media, Mumbai, India) was used as template in positive control reaction and nuclease free water as negative control template. The expected amplicon size was approximately 400 bp. The PCR product was again checked for specific amplicon using 1.5 per cent agarose gel electrophoresis as described previously.

RESULTS

Gel electrophoresis

Gel electrophoresis of DNA samples isolated by three different methods revealed the considerable difference in terms of quality, strength and sharpness of bands (Fig. 1).

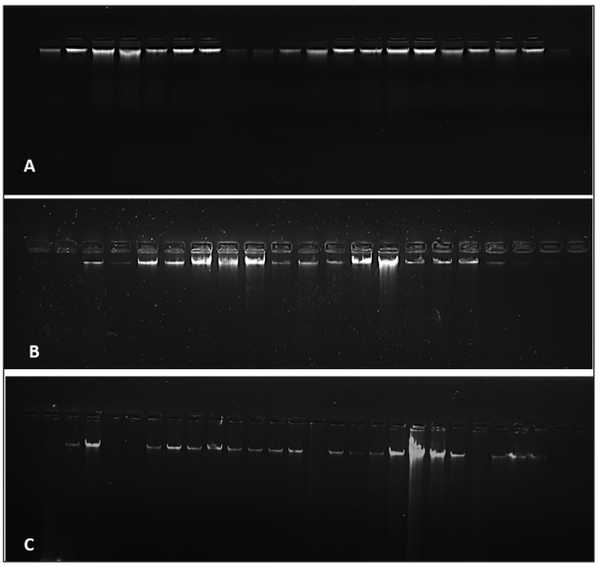

Fig. 1: Genomic DNA isolation by three methods. **(A)** STE method; **(B)** Commercial Kit method; **(C)** Snap Chill method

Concentration and purity of DNA

The average concentration of isolated DNA along with A260/A280 ratio is given in Table 1.

It was found that average concentration of extracted DNA by STE method was 03 -3500 ng/µl followed by 03 -2000 ng/µl with Kit method and 01-20 ng/µl with Snap-chill method. The absorbance ratio at 260 nm and 280 nm (A260/A280) was high up to 1.90 in STE method followed by up to 1.80 with column-based kit and 1.50 with snap-chill method which reflects the high purity of isolated DNA by STE and kit-based protocol.

Table 1: Average concentration and purity of DNA isolated by three different methods

Methods of DNA isolation	Minimum conc. of extracted DNA (ng/µl)	Maximum conc. of extracted DNA (ng/µl	Average conc. of extracted DNA (ng/µl)	Ratio of A260/ A280
STE Method	3.00	3500.00	1500.00	1.9
Kit Method	3.00	2000.00	700.00	1.8
Snap-chill method	1.00	20.00	10.0	1.5

Campylobacter specific PCR

A specific product of approximately 400 bp was obtained in all the reactions except in case of negative control. However, the concentration of amplified product varies which was reflected by the intensity of bands on gel as shown in Fig. 2. In all the cases 5 µl of PCR product was run. A very clear and intense band was observed in case of positive control, STE method and kit method whereas a very faint band was observed in case of snap chill method which indicate that the initial concentration and purity of DNA template was better in case of STE and kit method in comparison of DNA isolated by snap chill method.

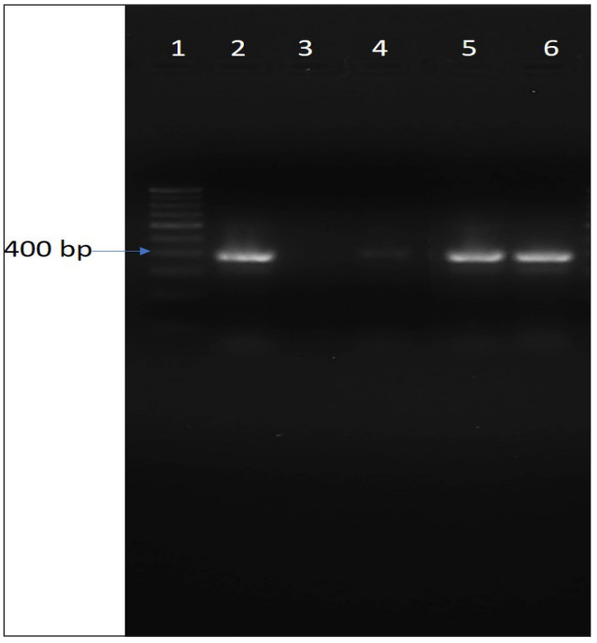

Fig. 2: Polymerase chain reaction. **Lane 1& 8:** 100 bp Ladder; **Lane 2:** Positive control; **Lane 3:** Negative control; **Lane 4 & 5:** Snap Chill method; **Lane 6:** Kit method; **Lane 7:** STE method

DISCUSSION

The thermophilic *Campylobacter* especially *C. jejuni* and *C. coli* are considered as the most common bacterial cause of gastroenteritis in humans and animals worldwide (Nguyen *et al.,* 2017). The routine detection of these thermophilic *Campylobacter* by traditional culture methods is often difficult which make it an excellent candidate for detection by polymerase chain reaction (Banowary *et al.,* 2015). However, the thermophilic and lysis resistant nature of bacteria often poses a challenge during preparation of template DNA for PCR. Simply boiling in water or snap chill method is a relatively simple method isolating bacterial DNA but for there are various studies where it has been observed that *Campylobacter* isolates were resistant to lysis by boiling water (Englen and Kelley, 2000). The direct isolation from fecal samples by boiling method is even more challenging as it contains extraneous materials like fat and digesta which make lysis more difficult and impractical. There are several reports on comparison of various method for DNA isolation in food samples as well as from blood but such studies are still lacking for the isolation of DNA from resistant lysis *Campylobacter* (Papatheodorou *et al.,* 2021). It is therefore important to optimize a standard DNA isolation protocol suitable for lysis resistant *Campylobacter* which provides the template DNA of better purity and concentration so that it can be easily detected by PCR.

As per the results STE method and Kit method was comparable to each other but due to high cost of commercial kit, STE method is proposed to be desirable and may be used routinely for extraction of DNA of lysis resistant bacteria. The PCR results also advocate the preference of STE method over kit method while Snap-chill method was not found suitable for extraction of DNA from pure culture of lysis resistant *Campylobacter* isolates. Yadav *et al.* (2016) also did not get the minimum critical yield of DNA by snap chill method to perform multiplex PCR for detection of lysis resistant Campylobacter species while Englen and Kelley (2000) in his experiment cell suspensions were held in a boiling water-bath to lyse the bacteria and the cell lysates were used as template DNA in the PCR reaction. Seven of the *C.jejuni* isolates (none belonging to the hippuricase-negative group) failed to show the expected 735-bp PCR product. Mohran *et al.,* (1998) described heat-resistant *Campylobacter* strains, suggesting that these seven isolates might be resistant to lysis by boiling in water. The thermophilic and lysis resistant nature of Campylobacters contributes to antimicrobial resistance against commonly used drugs (Singh *et al.,* 2022). Hence, the method which includes enzyme treatment along with boiling was found suitable for DNA extraction of heat-resistant *Campylobacter* strains. Our data suggest that within the *Campylobacter* population there is also a subset which does not release

PCR-detectable DNA upon boiling in water. Based on these findings, STE method with use of Proteinase K and SDS for extraction of DNA is recommended over boiling for a more sensitive and accurate detection of *Campylobacter* strains.

CONCLUSION

The DNA isolation for performing PCR from *Campylobacter* is a herculean task due to its thermophilic and lysis resistant nature. The STE methods which contain detergent, enzymes along with heat treatment has proven be a better DNA isolation protocol in terms of quality and concentration of DNA compared to simple boiling method and column based commercial kit.

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