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Molecular diagnosis of Campylobacteriosis in diarrhoeic poultry

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Abstract - *The present investigation was undertaken to isolate and identify Campylobacter spp. from diarrhoeic poultry by conventional culture method and the molecular confirmation in clinical samples by PCR. A total of 60 samples including 33 caecal contents and 27 cloacal swabs were collected and subjected for the isolation and identification of Campylobacter spp..All the samples were subjected to molecular confirmation by PCR using the genus specific 16S rRNA gene. Out of 60 samples, 11 were positive for Campylobacterby culture method and 15 were positive in PCR.*

***Key Words*: *Campylobacter* spp, PCR, *16S rRNA* gene, culture method**

1. INTRODUCTION

In India, the poultry industry is a rapidly developing sector and is being modernised progressively with better organisation when compared to other livestock sectors. It also gives better employment opportunity which can help to reduce poverty.

Though, the poultry industry is rising by its economic importance, they are vulnerable to various factors which adversely affect the poultry production *viz.,* manage mental and environmental factors. It also includes bacterial, viral and parasitic infestations. Of the various economically graving diseases affecting the poultry sector, campylobacteriosis has a special significance.

Campylobacteriosis is one of the threatening gastro-intestinal infections in poultry. It is caused by the bacteria belonging to *Campylobacter* spp.. Poultry and poultry products are the major reservoirs of *Campylobacter* spp. in and around the world. Primary cause of *Campylobacter* infection in human is from the consumption of contaminated poultry meat.

The bacteria can be found in the excrements and caecal contents of the infected birds. It causes distension of the jejunum and disseminated haemorrhagic enteritis. Poultry act as the major risk factor for the transmission of disease to other birds, animals and human beings via environmental contamination, litter, contaminated water as well as undercooked meat.

Though isolation and identification is the confirmatory method for diagnosis of campylobacteriosis, it is time consuming and is usually complicated by reduced viability of the organism. Conventional culture methods for *Campylobacter* detection include the utilisation of selective pre-enrichment media and plating onto selective media. Suspected colonies are normally affirmed of their character in the light of biochemical tests which are highly time consuming.

Hence, they are replaced by molecular diagnostic technique like Polymerase chain reaction (PCR), whichis more reliable, sensitive, risk free and specific. The detection of the pathogen in the clinical samples by PCR might conquer the intricate time consuming procedures.

Polymerase chain reactionis used for the demonstration of *Campylobacter* in faeces of animals and meat samples (Olsen *et al*., 2001; Bang *et al*., 2003; Lund *et al*., 2004). Amplification of *16S rRNA* and sequencing are required to confirm *Campylobacter* infections in clinically suspected cases orinlatentinfections (Vanniasinkam *etal*., 1999).

Considering the above facts, the present study was designed with the following objectives:

1. Isolation and identification of *Campylobacter* spp. from diarrhoeic poultry by conventional culture method

2. Molecular detection of *Campylobacter* in clinical samples by PCR.

2. LITERATURE SURVEY

Campylobacteriosis is a contagious disease of poultry affecting the gastro-intestinal tract and caused by *Campylobacter jejuni* (*C. jejuni*)and *Campylobacter coli*(*C. coli*) under the family *Campylobacteriaceae.* It causes bloody diarrhoea and severe entritis in poultry.

Hald*et al.* (2000) tested 88 Danish broiler flocks after slaughtering and reported *Campylobacter*in 52 per cent of cloacal swab samples and 24 per cent of neck skin samples. *Campylobacterjejuni* was found in 87 per cent of the samples, while eight carried *C.coli*.

Aydin*et al.* (2001) reported 100 per cent prevalence of *C.jejuni*in 40 cloacal swabs from domestic geese in Turkey.

Vashinand Stoyanchev(2005) investigated the occurrence of *Campylobacter* spp. In Japanese quail in Bulgaria and presence of the organism was found in 80 per cent of the 30 caecal samples and 16.7 per cent of the 30 liver samples. The isolation rates of *C.jejuni* and *C.coli* were 89.7 and 10.3 per cent, respectively.

Baserisalehi*et al.* (2007) analysed 126 faecal samples from domestic animals and poultry in India and 120 from Iran. Seventy and thirty-seven isolates of *Campylobacter* spp. were obtained from India and Iran, respectively.

Mena *et al*. (2008) screened 164 samples of poultry for the identification of *Campylobacter* spp.. A total of 99 *Campylobacter* strains were isolated and further identified by agar diffusion technique.

Feng*et al.* (2009) collected 120 cloacal samples from healthy red-crowned cranes in China and *Campylobacter* spp. was detected in 26 (21.67 per cent) samples.

Adziteye*et al.* (2011) collected 40 large intestinal contents from domestic pekin ducks in Malaysia. *Campylobacter*spp.was present in 67.5 per cent of the samples, among which 55.6 and 44.4 per cent were*C.jejuni* and *C.coli*, respectively.

Nadeem *et al*. (2015) reported a drastic increase incampylobacteriosis in children inNorth America, Europe and Australia and found that poultry was the significant repository and wellspring of the disease.

The occurrence of *Campylobacter* spp. in poultry meat preparations was investigated and identified that it was significantly lower than previous surveys. Chicken meat was more contaminated than turkey meat (Ziad *et al.,* 2016).

The presence of *C.jejuni*in apparently healthy market chicken in Bangalore was investigated by Pillai (1989). Sixty-seven isolates consisting of biotype i (32), biotype ii (26) and biotype iii (9) were obtained and caecum showed the highest colonisation followed by large and small intestines.

Chattopadhyay *et al*. (2001) examined 160 intestinal contents from chicken and ducks in and around Calcutta. Sixty-three samples were found to be contaminated with *C.jejuni* (88.9 per cent).

Bandekar *et al*. (2005) screened hot dressed poultry carcasses from Pune and Mumbai and reported that 95 per cent of the 40 samples were contaminated with *Campylobacterjejuni, C.coli, Campylobacter foetus* (*C. foetus*) and *Campylobacterlari*(*C. lari).*

A study was conducted by Rajkumar *et al.* (2010) in small scale poultry dressing units of Bareilly region of Northern India. *Campylobacter jejuni*(71.42 per cent) and *C.coli*(28.47 per cent) were isolated from 300 poultry breast skin samples.

Kumar (2011) collected 50 each of chicken caecum, quail caecum and chicken meat from Bareilly region of Uttar Pradesh, India and recorded an overall prevalence rate of 18, 18 and 12 per cent, respectively. Out of the 18 per cent positive chicken and quail caecum samples, six and eight isolates were positive for *C.jejuni* and three and one isolates were positive for *C.coli*, respectively. All the positive chicken meat samples were contaminated by *C.jejuni*.

Rajendran *et al*. (2012) examined 25 chicken faecal samples from Vellore, South India. They found that *Campylobacter* spp. was present in 16 of them and all were characterised as *C. jejuni*.

Joby (2016) determined the critical control points of *Campylobacter* spp. in chicken egg production chain from the retail markets, from a total of 450 samples from different sources by isolation and subsequent confirmation using multiplex PCR assay. It was observed that cloaca of birds was the critical point of contamination.

Jacob*et al.*(2017) screened 50 cloacal swabs of chicken from backyard poultry in Kerala by conventional culture method and isolated*Campylobacter* spp. from 14 samples.

Savitha (2017) tested chicken meat in summer and monsoon season for the presence of *Campylobacter* spp. and identified that 42.94 per cent of the samples revealed presence of the organism during the monsoon season. During summer, 3.51 per cent of *C. jejuni* and 8.77 per cent of *C.coli* were isolated.

Beery *et al.*(1988) enumerated *Campylobacter* in intestine of eight-day-old chicks by transmission electron microscopy and revealed the presence of the organism in the lumina of crypts without attachment to crypt microvilli.

Chantarapanont *et al.*(2003) developed a method to determine the survival of *C. jejuni* at specific sites on chicken skin using confocal scanning laser microscopy.

Skirrow (1977) made a specific medium for isolating *Campylobacter* from faecal samples utilising antibiotics supplemented blood agar. The antibiotics include vancomycin, polymixinBand trimethoprim. The incubation was done under microaerophilic conditions with five per cent oxygen, 10 per cent carbon dioxide and 85 per cent hydrogen at 43°C overnight.

Bolton and Robertson (1982) developedPreston medium along with blood for isolating *C. jejuni* and *C. coli*by incorporating the antibioticspolymixin, rifampicin, trimethoprim and actidione.Microaerophilic conditions were provided at 43°C under six per cent oxygen, ten per cent carbon dioxide and 84 per cent hydrogen for 48 h. This medium was found to be more selective and superior than Skirrow's medium for isolation of *Campylobacter* from all kind of samples.

Bolton and Coates (1983) used Columbia agar with five per cent horse blood for the isolation of *Campylobacter*spp.. Microaerophilic conditions were created using anaerobic jar and it was found that five to ten per cent oxygen and one to ten per cent carbon dioxide were required for optimum growth of the organism.

First selective medium for *Campylobacter*, without blood, containing charcoal known as charcoal-cefazolin-sodium deoxycholate agar (CCD agar) along with the selective suppliments *viz*., cefazolin (10 mg/L) and 0.1 per cent sodium deoxycholate was developed by Bolton *et al.* (1984).

The selectivity of CCD agar could be increasedusing cefoperazone (32 mg/L). Maximum isolation rate was obtained at 48 h. incubation period for both the modified CCD agar and Preston agar. But,the isolation rate of modified CCD agar was greater at 42°Cthanat 37°C (Bolton *et al*.,1984).

Karmali *et al.* (1986) designed a charcoal based selective medium for isolating *Campylobacter* spp.from faecal samples by incorporating activated charcoal, haematin, sodium pyruvate, cefoperazone, vancomycin and cyclo heximide in Columbia agar base. Incubation was done in anaerobic jars at 43°C under seven per cent oxygen, 10 per cent carbon dioxide, 25 per cent nitrogen and 58 per cent hydrogen. The medium was more selective than Skirrow's medium and charcoal was found to be the best substitute for blood in the medium.

Jeffrey *et al.* (2000) designed an aerobic enrichment medium by incorporating rifampin, trimethoprim, cephalothin, polymixin B and amphotericin B for isolation of*C.jejuni*.

Martin *et al.* (2002) evaluated the efficacyof amphotericin B (10mg/L), anantifungal agent in the selective medium by replacing cycloheximide. Both these agents inhibited fungal growth adequately but were not able to eliminate bacteria other than *Campylobacter*.

Oyarzabal *et al*. (2005) reported that*Campylobacter* spp. could be isolated from rinses of poultry carcass employing modified charcoal cefoperazone deoxycholate agar (mCCDA) and modified campy-cefex (MCC agar). They were more selective and less costly. Modified campy-cefex agar was prepared by using amphotericin B in place of cycloheximide. The lysed horse blood in campy-cefex agar was replaced with laked horse blood.

Adzitey *et al.* (2011) swabbed the large intestine of pekin ducks on mCCDA supplemented with *Campylobacter* growth supplements (ferrous sulphate, sodium metabisulphite and sodium pyruvate). Incubation was done at 42°C for 48 h. under microaerophilic conditions.The isolation rate of *Campylobacter*was relatively better in this medium.

Chon *et al.* (2012) utilised mCCDA by supplementing polymixinB. The medium was found to have superior selectivity showing higher isolation rate for *Campylobacter* and reduced growth rate for competing organisms.

Teramura *et al*. (2015) developed a new chromogenic medium based on mCCDA for improved *Campylobacter* isolation. This medium was supplemented with sodium cefoxitin and granular charcoal. It inhibited expanded-spectrum beta-lactamase (ESBL)-producing bacteria, a common contaminant in the isolation of *Campylobacter* spp. from poultry samples and it also enhanced the viewability by producing purple-coloured colonies.

According to OIE (2017), fresh faeces/caecal droppings or cloacal swabs are the best samples for the isolation of *Campylobacter* in mCCDA incubated at 42°C.

Different morphological forms of *Campylobacter* were observed by Lai-King *et al.*(1985) from different parts of *Campylobacter* colonies. They concluded that spiral shaped cells were located at the periphery and were actively growing or young cells, while coccoid at the center were older cells.

Thomas *et al*. (2002) assessed the morphological characteristics of *C*. *jejuni* and *C. coli* from National Collection of Type Cultures (NCTC), using scanning electron microscopy and light microscopy under stimulated aquatic conditions at 10°C. Spiral or gull wing shaped organisms were observed.

Tangwatcharin *et al.* (2006) observed *Campylobacter*in light microscopy as spiral shaped cells.

Gram negative coma or gull wing shaped organisms were identified as *Campylobacter* spp. byMushi *et al.*(2014).

Joby (2016) and Savitha (2017) reported *Campylobacter* spp. as spiral shaped cells, on Gram's staining.

Joby (2016) incubated *Campylobacter* spp. in polymixin supplemented mCCDA plate under micro aerophilic incubation at 42°C for 48 h. Greyish, round with one to two millimeter diameter , flat to slightly raised, spreading type, shiny, moistened colonies with or without metallic sheen were observed. On prolonged period of incubation, the colonies turned greyish and sticky.

According to OIE (2017),*Campylobacter* coloniesare slightly pink, round, convex, smooth and

shiny, with a regular edge on Skirrow's or other blood-containing agars. On mCCDA, the colonies were greyish, flat and moistened and spreading type. A metallic sheen may be present in most of them.

Savitha (2017) reported that colonies of *Campylobacter* spp. were shiny with a moistened appearance on polymixin supplemented CCDA agar plates.

Karmali *et al.* (1981) examined three groups of catalase positive *Campylobacter* spp. by phase contrast microscopy and differentiated catalase positive isolates with respect to their morphology.

Krausse and Ullmann (1985) screened a total of 56 strains of *C. jejuni* from diarrhoeic patients and characterised by hydrolysis of hippurate, reduction of nitrate and nitrite, activity of deoxyribonuclease and hydrolysis of tween 40, 60 and 80.

Taylor *et al.* (1989) characterised *Campylobacter* spp.from humans by biochemical tests *viz*., catalase, oxidase, hippurate hydrolysis test, resistance to nalidixic acid and sensitivity to cephalothin.

Hanninen (1989) developed a rapid agar diffusion method for the detection of DNase production by*Campylobacter* spp..

Steinbrueckneret *al.*(1999) selected catalase and oxidase positive isolates to identify*C. jejuni* and *C.coli* from human stool samples for molecular confirmation by PCR.

Ma *et al.* (2007) reported the influence of pH of TSI medium for the detection of hydrogen sulphide production by*Campylobacter* spp.. It wasidentified that medium with alkaline pH contributed for the fastest detection of *Campylobacter* spp..

Joby (2016) and Savitha (2017) identified catalase and oxidase positive isolates of *Campylobacter* spp. by further biochemical reactions and mPCR.

Various biochemical tests used in the identification of *Campylobacter* spp. were catalase test, oxidase test, TSI test, hippurate hydrolysis test and indoxyl acetate test. *Campylobacter jejuni* could be differentiated from other *Campylobacter* spp. on the basis of a positive hippurate hydrolysis test(OIE, 2017).

Monfort *et al.* (1994) developed monoclonal antibody based ELISA for detecting the flagellar antigen of *C.jejuni*and *C.coli* from canine faecal samples. This assay had a specificity of 94.4 per cent.

Ang *et al.*(2007) developed anELISA to detect antibodies (IgG and IgM)against *Campylobacter*in human beings.

Jakubczak *et al.*(2007) evaluated the utility ofELISAto detect anti-*Campylobacter*-antibodies by testingserum samples from 145 patients with gastro-enteritis.Seven different heat-stable antigens and OMP (45kDa) of *C. jejuni* were used.

Carmen *et al.* (2013) reported that EIA provided rapid detection of *Campylobacter* antigen and could be used as an alternative method for isolation and identification of *C. jejuni* and *C. coli*.

Thomas and Ignatious (2014) evaluated immune chromatographic assay, (ICA) and EIA for the detection of 38 frozen *Campylobacter* spp..Thirty-seven samples were positive in both the assays .*One C. lari*-positive sample was also identified by ICA.

Giesendorf *et al*. (1992) reported the utility of PCRto identify *Campylobacter* spp. in chicken products using primers specific for*16S rRNA*genes. Out of the 45 samples, 80 per cent were positive by PCR.

The first application of PCR for the specific detection of *C.jejuni* and *C.coli*from environmental water samples in United States was described by Oyofo and Rollins (1993). *Fla*agene was demonstrated to be specific and sensitive for identifying the two species.

Genus specific PCR assay was designed by Linton *et al.* (1996) amplifying *16S rRNA* to generate an amplicon of 816 bp from *Campylobacter* species.

Vanniasinkam *et al*. (1999) reported that*16S rRNA* gene amplification was reliable for genus specific detection of *Campylobacter* from clinical materials.

A multiplex PCR was developed for the genes *16S rRNA*, *map*a and *ceu*ecorresponding to the genus *Campylobacter*, the species *jejuni* and the species *coli*. Amplicons were produced at 857 bp, 589 bp and 462 bp, respectively (Denis *et al.*, 1999).

Konkel *et al.* (1999) developed a PCR assay for the conserved virulence gene *cad*f in *Campylobacter*. Amplicon of 400bp was produced in 93.5 per cent of the *C.jejuni* and *C.coli* isolates tested with the primers.

The species specific identification of *Campylobacter* spp. using*16S rRNA*gene sequence was evaluated (Gregor*et al*.,2003).They screened135 *Campylobacter* strains from National Culture Collections and found that*16S rRNA* sequence analysis was an effective and rapid method for the detection of the organism.

Real-Time PCR assay wasperformed by Lund *et al.* (2004) for detecting different species of *Campylobacter*from faecal swab samples of chicken. The *16S rRNA* gene sequences of *Campylobacter* were used for the test. No statistically significant differences were observed in the performance between culture and PCR.

Rozynek *et al.* (2005) found that all of the 53 *C.jejuni* and 39 *C.coli*isolated from chicken carcasses carried *cad*f virulence genes.

Multiplex PCR using *fla*a, *cad*f, *cdt*b, and *iam* genes for the simultaneous detection of *C.jejuni*and *C.coli*was analysed bySelwet and Galbas (2012). Out of the 100 samples, 70 per cent of the *Campylobacter*isolates had *cad*f and *fla*a genes.

Seventeen culturally positive *Campylobacter* spp. from the cloacal swab of chicken were subjected to multiplex PCR and identified that all of them carried the conserved *16S rRNA* and *cad*F genes (Joby, 2016).

All the positive samples of *Campylobacter* spp. from 94 chicken meat samples were subjected to multiplex PCR by Savitha (2017) and confirmed that the isolates carried genus specific *16S rRNA* gene.

3. MATERIALS AND METHODS

All the reagents used in the study were of molecular biology grade, procured from Sigma Aldrich (USA), Merck GeNei (Bangalore), Sisco Research Laboratories (SRL) private limited and MBI Fermentas. The culture media were procured from Hi Media laboratories, Mumbai.The glassware werefrom Borosil and plasticware from Tarson. The facilities in the Department of Veterinary Microbiology and Central Instruments Laboratory, College of Veterinary and Animal Sciences, Mannuthy, were utilised for the study.

Cloacal swabs from diarrhoeic poultry were collected from University Veterinary Hospital, Mannuthy, University Poultry and Duck Farm (UPDF), Mannuthyas well as from organised poultry farms in Thrissur district. Cloacal swabs and caecal contents were also collected from poultry, brought for disease diagnosis to the Department of Veterinary Pathology and Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy.

For isolation of *Campylobacter*

1. Cloacal swab samples and caecal contents
2. Cary-Blair medium
3. Modified mCCDA with CAT (Cefoperazone, Amphotericin B and Teicoplanin) selective supplement and *Campylobacter* supplement V Colony characteristics of *Campylobacter* on mCCDA were studied as per OIE (2017).

3.1 Morphology and staining characters

The morphology and staining characters of the organisms were studied by Gram's staining technique.

3.1.1Biochemical characters

All the procedures were followed as per OIE (2017)

Materials:-

1. Hydrogen peroxide (three per cent) for catalase test
2. Oxidase disc for oxidase test
3. Hippurate disc for hippurate hydrolysis test
4. Indoxyl Acetate disc for indoxyl acetate hydrolysis test
5. Triple Sugar Iron (TSI) agar for Hydrogen Sulphide production (H_2S) test
6. DNase agar for DNA hydrolysis test
7. Mueller-Hinton agar supplemented with fiveper cent sheep blood were usedfor disc diffusion test
8. Concavity slide for motility test

Methods:-

1. Catalase test

A loopful of bacterial growth was taken from the surface of the colonies avoiding the agar medium. The bacterial cells were placed on a clean microscopic slide and a drop of three per cent hydrogen peroxide was added. An effervescence of nascent oxygen gas, within a few seconds, indicated a positive reaction.

2. Oxidase test

Oxidase disc was used and the test organism was looped out from the plate and applied on the disc with a glass rod. The development of purple colour in 10sec. and 60sec. indicated strong positive and weak positive reaction, respectively.

3. Hippurate hydrolysis test

Aseptically placed the hippurate disc in brain heart infusion broth inoculated with the organism. Incubated at 42℃ for 48 h.The cells were sedimented by centrifugation at 10,000 rpm for 10 minutes. Added two millilitreof ferric chloride reagent to two millilitreof supernatant from the centrifuged culture tube. Shaken well and observed the persistence of precipitate formed even after 10 min.

4. Indoxyl Acetate Hydrolysis test

An absorbent filter paper disc was soaked with indoxyl acetate (10per cent w/v) (HiMedia) and air dried. Loopful growth of colonies from mCCDA was applied on

the test disc and a drop of sterile distilled water was added. Appearance of a blue green-colour within five to ten minutesindicated a positive result.

5. Triple sugar iron agar test

Inoculated the test organism into TSI agar slant and incubated at 37°C. examined daily for up to seven days for growth, colour change and production of gas.

6. DNA hydrolysis test

A loopful of 48h. old culture grown at 42°C under microaerophilic condition was used to inoculate heavily a circular area of about five millimetre in diameter. The plates were examined after one, two and three days of incubation at 42°C, under microaerophilic conditions. A clear colourless or pinkish zone around the inoculum was considered positive. No change around the inoculum was taken as negative.

7. Sensitivity to nalidixic acid and resistance to cephalothin

Disc diffusion method was employed for susceptibility testing on Mueller-Hinton agar supplemented with fiveper cent sheep blood. A sterile swab was charged with the culture suspension and was dispersed over the surface of agar. Sterile tweezers were used to place the nalidixic acid (30µg) and cephalothin (30µg) discs (HiMedia) on the surface of the agar plate ensuring that they were widely spaced. Incubated at 37°C overnight. The next day, zones of inhibition were examined around the antibiotic discs and sensitivity and resistance were recorded.

8. Motility test

A drop of overnight broth culture of suspected colony was tested by hanging drop method. Samples showing cork-screw darting type motility, typical of genus *Campylobacter* was considered positive presumptively.

3.1.2Growth characteristics

1. Aerobic growth test

Suspected isolates were streaked on duplicate mCCDAplates and incubated under aerobic conditions at 37°C. The isolates showing growth was discarded. This test differentiates *Campylobacter* spp.from *Arcobacter*spp., wherein the latter organisms are reported to show growth.

2. Growth at 25°C and 42°C

A loopful of growth was inoculated on to duplicate mCCDA plates and incubated one plate at 25°C and the other at 42°C for upto three days in a microaerophilic atmosphere. A positive reaction was indicated by the development of typical *Campylobacter*colonies after incubation at 42°C.

3.2 DETECTION OF *Campylobacter*IN CLINICAL SAMPLES

The template DNA was extracted using DNA extraction kit (HiPurA™ Multi-Sample DNAPurification Kit). The caecal contents and cloacal swabs were thoroughly ground and placed in a twomillilitremicro-centrifuge tube. Then, 180 µL re suspension buffer was added followed by 20 µL proteinase K. It was then mixed by vortexing and incubated at 55°C for three hours until completely lysed. It was vortexed occasionally during incubation. Then, 200 µLlysissolution was added and mixed thoroughly by vortexing for 15 seconds. The samples were incubated at 70°C for 10 minutes followed by addition of 200 µL absolute alcohol. This was mixed thoroughly by gentle pipetting. The mixture was pipetted in to a Miniprep spin column placed in a two millilitre collection tube. It was centrifuged at 10,000 rpmfor oneminute. Then, the flow-through and collection tubes were discarded. The spin column was placed into a new two millilitre collection tube and added 500 µL pre-wash solution. Centrifugation was done for one minute at 10,000 rpm. Then, discarded the flow-through, the same collection tubewas reused and added 500 µL diluted wash solution. Centrifugation was done for three minutesat 16,000 rpm. The spin column was placed into a new two milliliter collection tube. The flow-through and collection tube were discarded and eluted the DNA by adding 200 µL elution buffer to the center of the spin column membrane. This was incubated for one minute at room temperature. It was then centrifuged for one minute at 10,000 rpm.

The concentration of DNA was measured using Nano drop (Thermo Scientific). The purity of the extracted DNA was checked by measuring the ratio of absorbance(OD of DNA preparation at 260 and 280 nm).

1. PCR Master Mix (Thermo Scientific)
2. Nuclease free water
3. Primers (Sigma Aldrich)
4. PCR grade water
5. DNA template

The primers obtained in lyophilised form were reconstituted in DNAse free water to a concentration of 200 pM/µL and the tubes were spun briefly. The stock solution was distributed into 10 µL aliquots and stored at -20°C until use. The working solution was made by diluting the stock solution 10 fold to obtain a concentration of 20 pM/µL, before using for PCR.

The PCR was conducted using the primers specific for *Campylobacter16S rRNA*

Table 1. Primers for amplification of *Campylobacter 16SrRNA*

Primers	Sequence	Amplicon Size
F	5'-GGAGGATGACACTTTTCGGAGCG-3'	816bp
R	5'-TCGCGGTATTGCGTCTCATTGTA-3'	

4. RESULTS

Among the samples collected from 60 birds, materials from 11 birds exhibited colonies suggestive of *Campylobacter* on mCCDA plates after 48h.

The samples streaked on mCCDA plates were observed for growth after 48h.incubation at 42℃ under microaerophilic condition.

The colonies were shiny, greyish, opaque, moistened, round, slightly raised, and spreading type with metallic sheen (Fig.1.). On prolonged incubation, the suspected colonies appeared greyish to white and sticky.

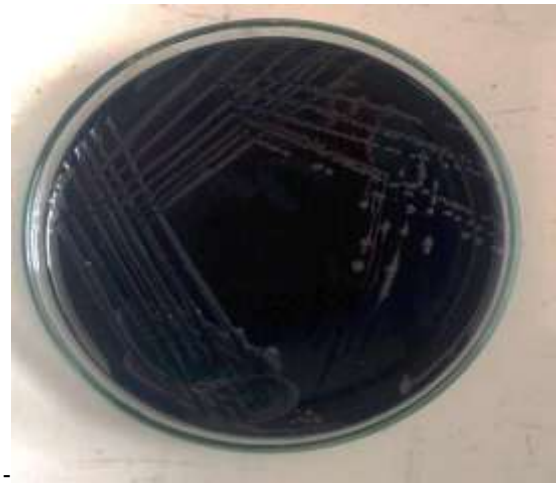

Fig. 1. Colonies of *Campylobacter* spp. on mCCDA

4.1 Morphology and staining characters

On Gram's staining, from the fresh culture plates, the isolates appeared as Gram negative, spiral shaped or 'S' shaped organisms. In some cultures, cocco-bacillary organisms were observed which were arranged individually(Fig.2.). Cultural characters and Gram's reaction of the isolates are illustrated in table 5.

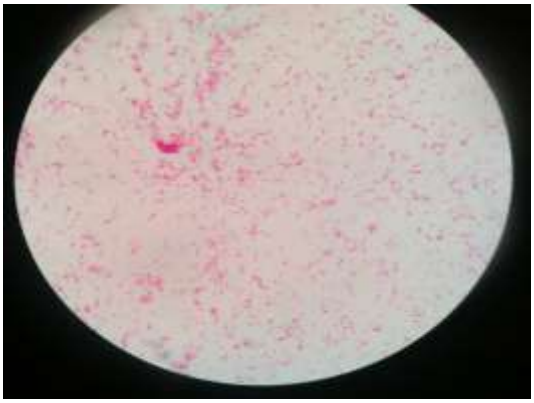

Fig. 2. Gram's staining reaction of *Campylobacter*

Out of the 60 samples, 11revealed colonies suggestive of *Campylobacter*. Among these, four were Gram negative cocco-bacilli and five of them showed spiral appearance. S shaped morphology was observed for two isolates. The isolates from samples CC2, CC3, CC7, CC8, CD6, CC12, CC13, CC15, CSC4, CSC8 and CC17produced shiny, greyish, opaque, round, slightly raised, and spreading type, moistened with metallic sheen colonies on mCCDA plates. The isolates were designated with the sample number from which isolations were made.They were motile and showed positive reaction for catalase

On hippurate hydrolysis test, all of them produced deep purple colour except CC8 and CC15. All the suspected colonies produced bluish green colour on indoxyl acetate hydrolysis test(Fig. 6)and all were negative for TSI test (Fig.7). Of the 11 isolates, CC2, CC3, CC8, CSC4, CSC8 and CC17 presented clear zone of clearance on DNA hydrolysis test, whereas CD6, CC12, CC13 and CC15 showed slightly clear zone and CC7 showed slightly pinkish zone of clearance (Fig. 8).All the 11 isolates showed sensitivity to cephalothin and resistance to nalidixic acid (Fig. 9).The results of biochemical tests are shown in table 6.In the light of all biochemical reactions, it was confirmed that all the 11 isolates were *Campylobacter* spp. The isolates CC8 and CC15 did not produce any colour in hippurate hydrolysis test. Based on this, they were confirmed as *C. coli* and the rest (nine) were identified as *C. jejuni*.

Fig. 3. Catalase test (positive)

Fig. 4. Oxidase test (positive)

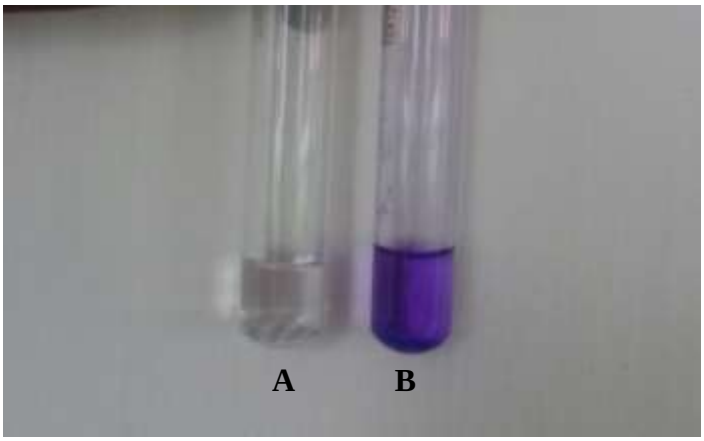

Fig. 5. Hippurate hydrolysis test

A :- Negative B:- Positive

Fig. 6. Indoxyl acetate test

A :- Negative B:- Positive

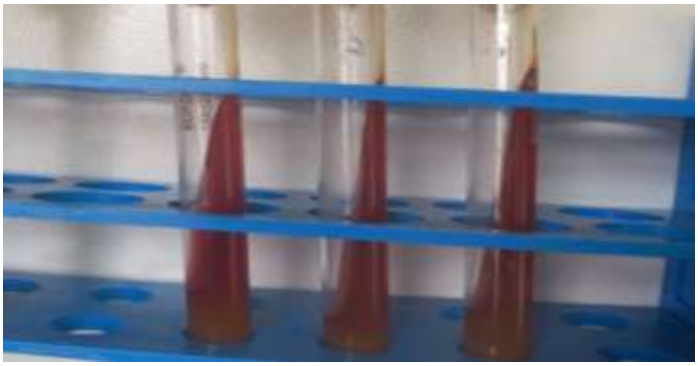

Fig. 7. TSI test (Negative)

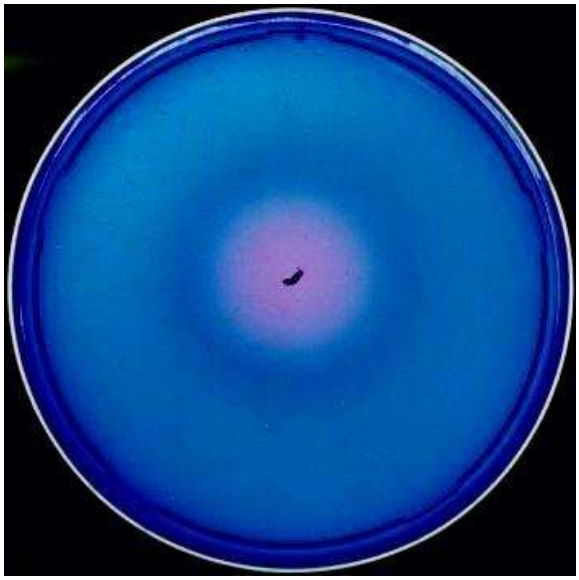

Fig. 8. DNase test indicating clear zone

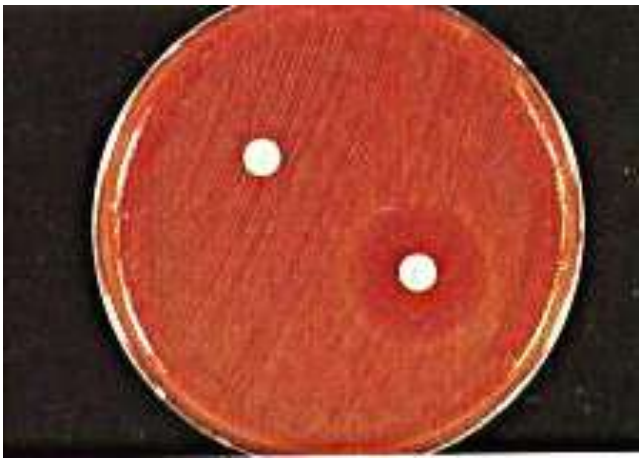

Fig. 9. Resistance to nalidixic acid and cephalothin (Representation)

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