Characterization of *Campylobacter jejuni* isolated from poultry

मुर्गियो से प्राप्त *कैम्पिलोबैक्टर जेजुनी* का चरित्रण

RAHUL YADAV
M.V.Sc.

THESIS

DOCTOR OF PHILOSOPHY

(Veterinary Microbiology)

।पशुधनं नित्यं सर्वलोकोपकारकम्।

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Department of Veterinary Microbiology and Biotechnology
College of Veterinary and Animal Science, Bikaner
Rajasthan University of Veterinary and Animal Sciences
Bikaner – 334001

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Submitted to the
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By

RAHUL YADAV

M.V.Sc

2017

Rajasthan University of Veterinary and Animal Sciences, Bikaner
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This is to certify that this thesis entitled **"Characterization of *Campylobacter jejuni* isolated from poultry"**submitted for the degree of **Doctor of Philosophy** in the subject of **Veterinary Microbiology** embodies bonafide research work carried out by **Mr. Rahul Yadav** under my guidance and supervision and that no part of this thesis has been submitted for any other degree. The assistance and help received during the course of investigation have been fully acknowledged. The draft of the thesis was also approved by the advisory committee on ……....

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ABBREVIATIONS

%	=	Percentage
bp	=	Base pair
CP	=	Capsular Polysaccharide
DNA	=	Deoxyribonucleic acid
EDTA	=	Ethylene diaminetetraacetic acid
ERIC-PCR	=	Enterobacterial repetitive intergenic consensus PCR
h	=	Hour/hours
lb	=	Pounds
mcg	=	Microgram
mg	=	Milligram
min	=	Minutes
ml	=	Milliliter
MLST	=	Multilocus sequence typing
mM	=	Micromolar
ng	=	Nanogram
nm	=	Nanometre
ºC	=	Degree Celsius
OD	=	Optical density
PAGE	=	Polyacrylamide Gel Electrophoresis
PBS	=	Phosphate buffer saline
PCR	=	Polymerase Chain Reaction
PFGE	=	Pulsed field gel electrophoresis
RAPD	=	Random amplified polymorphic DNA
REP-PCR	=	Repetitive element sequence-based PCR
RE	=	Restriction Endonuclease
RFLP	=	Restriction Fragment Length Polymorphism
RNA	=	Ribonucleic acid
rRNA	=	Ribosomal Ribonucleic Acid
SDS	=	Sodium dodecyl sulphate
TBE	=	Tris borate EDTA
TE	=	Tris EDTA
UV	=	Ultraviolet
V	=	Volt
μg	=	Microgram
μl	=	Microliter
μm	=	Micrometer

INTRODUCTION

1. INTRODUCTION

Foodborne diseases have been an issue for all societies since the beginning of humanity and are an important cause of morbidity and mortality. Although, the types, severity and impacts of these illnesses have changed through the ages across the regions, still food borne diseases are an important cause and a significant impediment to socioeconomic development worldwide through countries and communities. To study, the global impact of food borne disease agents WHO prepared a Food borne Disease Burden Epidemiology Reference Group (FERG). For the global estimates FERG identified, thirty-one food borne hazards which included, 11 diarrhoeal disease agents (1 virus, 7 bacteria, 3 protozoa), 7 invasive infectious disease agents (1 virus, 5 bacteria, 1 protozoon), 10 helminthes and 3 chemicals. Together, these 31 global hazards caused 600 million food borne illnesses and 420,000 deaths in 2010; of these 230,000 deaths were caused by food borne diarrhoeal disease agents (WHO, 2015). The most frequent food borne diarrhoeal disease agents identified were *Norovirus* and *Campylobacter* spp.

Campylobacter spp. are second most emerging bacterial zoonotic pathogen after *Salmonella* (Bereswill and Kist, 2003; Andrzejewska *et al.,* 2011; Silva *et al.,* 2011; Epps *et al.,* 2013). Humans are susceptible to infection with multiple *Campylobacter* spp*.;* the major human pathogens are *Campylobacter jejuni*, *Campylobacter coli* (enteritis) and *Campylobacter fetus* (septicemia). Additional species, such as *Campylobacter lari, Campylobacter upsaliensis,* and *Campylobacter hyointestinalis* have been less commonly detected (ERS-USDA, 2000; CDC, 2008; FDA, 2009; Silva *et al.,* 2011; Epps *et al.,* 2013).

Campylobacters received serious attention as a causative agent of diarrhoea only since 1973 (Butzler *et al.,* 1973). But, its growing incidence has made it a notifiable food borne disease in Austria, Denmark, Finland, Germany, Italy, Sweden and Norway (Taylor and Chang, 1991; Ikram *et al.,* 1994).

Campylobacter gastroenteritis has also been recognized as the major cause for Guillain-Barre's syndrome (GBS), which may lead to either short-term or lengthy paralysis, reactive arthritis (ReA), and irritable bowel syndrome (Rollins and Joseph, 2001). The Miller Fisher syndrome (MFS), a GBS variant, is also associated with preceding *Campylobacter* infection (Rozynek *et al.,* 2005' Al-Mahmeed *et al.,* 2006).

A total of 16 species and six subspecies are now categorized in the genus *Campylobacter* and 12 of these species are associated with human diseases (Vandamme *et al.,* 1991). Of these *C. jejuni, C. coli* and *C. fetus* infections are found worldwide. *C. jejuni* and to a lesser extent *C. coli* are the species most often encountered as cause of acute enterocolitis in man (Nielsen *et al.,* 1997; Wooldridge and Ketley, 1997; Anon, 1999). They are distinguished from most other *Campylobacter* spp. by their high optimum growth temperature (42°C) and are thus called as thermophilic campylobacters. Out of the four most common thermophilic campylobacter responsible for gastroenteritis i.e. *C. jejuni*, *C. coli*, *C. lari* and *C. upsalinesis*; *C. jejuni* is responsible for the majority (80-90%) of these infections and a leading food borne pathogen followed by *C. coli* (Biswas *et al.,* 2011).

C. jejuni are small (0.2–0.8 μm wide and 0.5–6.0 μm long) gram-negative, spirally curved, motile by unipolar or bipolar flagella, microaerophillic, non-spore forming organism belonging to the family *Campylobacteraceae* (Wieczorek and Osek, 2013). *C. jejuni* can infect cattle, sheep, chickens, turkeys, dogs, cats, mink, ferrets, pigs, non-human primates and other species. However, chicken is considered to be a major reservoir. This organism efficiently colonize caecal mucosal crypts of the gastrointestinal tract of chickens and they remain asymptomatic carriers until they reach slaughter age (Moore *et al.,* 2005; Lee and Newell, 2006; Coward *et al.,* 2008; Bolton, 2015).

Colonization rates in chickens are age-related. Intestinal colonization of chicks less than seven days old is rarely detected (Moore *et al.,* 2005). Most

flocks are negative until 2-3 weeks of age. Once *C. jejuni* colonization occurs in a flock, transmission, via coprophagy, is extremely rapid and complete flock can become colonized within 72 hours and once established, it is very difficult to eliminate (Newell and Wagenaar, 2000). High flock size, environmental water supplies, litter, insects, wild birds, rodents, fecal contact, personnel and other animals, increase the risk of colonization and dissemination (Sahin *et al.,* 2001).

C. jejuni can persist for long times in chilled and frozen products (FAO/WHO, 2009). It is able to persist at 4°C and remain viable for 3 weeks in feces and 5 weeks in urine (Calnek *et al.,* 1991; Lee *et al.,* 1998). Survivability of *C. jejuni* at refrigeration and freezing temperatures is of relevance to food safety and public health (Chan *et al.,* 2001).

Identification of *Campylobacter* at genus or species level does not help in understanding the epidemiology of the disease (Nachamkin *et al.,* 1993). There is a continuous cycle of transmission of the bacterium from one to another host. More importantly humans are commonly infected *via* various animal and poultry sources throughout the year (Aydin *et al.,* 2007). Thus, typing of *Campylobacter* is important for differentiating and identifying various strains molecularly (Khoshbakht *et al.,* 2013). Two typing methods, Restriction fragment length polymorphism (RFLP) of Polymerase chain reaction (PCR) amplified product of *fla*A gene by restriction enzymes (*Dde*I, *Hinf*I, *Eco*rI, *Pst*I and *Alu*I etc.) and Repetitive extragenic palindromic PCR (REP-PCR) have proved to be useful in epidemiological investigations of *Campylobacteriosis* (Dingle *et al.,* 2001; Fitzgerald *et al.,* 2001; Gondo *et al.,* 2006).

Major virulence attribute of *C. jejuni are* adhesion, invasion, presence of lipoologisacchrides responsible for evading host defense mechanism and production of cytotoxins (Konkel *et al.,* 1999; Bang *et al.,* 2003; Datta *et al.,* 2003; Fouts *et al.,* 2005).

Adherance is governed by flagellum, lipooligosaccharides, the major outer membrane protein, and pili (Konkel *et al.,* 2001). The important adherence

factors are; *Campylobacter* adhesion protein A (*Cap*A) a autotransporter (Flanagan *et al.,* 2009), factor for *Campylobacter* adhesion to fibronectin (*cad*F) (Rizal *et al.,* 2010), heat shock proteins (*Dna*J), thermoregulation protein (Rozynek *et al.,* 2005), major outer membrane protein (MOMP) also called *PorA* (Flanagan *et al.,* 2009) and a surface exposed lipoprotein loosely attached to the bacterial cell surface (*Jlp*A) (Jin *et al.,* 2001).

Invasion associated marker (*Iam*) is associated with *C. jejuni's* potential for colonization of multiple hosts (Al-Mahmeed *et al.,* 2006; Wieczorek *et al.,* 2012). The *Cia* proteins are released after contact of *C. jejuni* with host cells or unidentified serum factors (Ghorbanalizadgan *et al.,* 2014).

Lipooligosaccharide (LOS) plays a crucial role in immune avoidance and is important for serum resistance (Yang *et al.,* 2014). *Campylobacter* cytotoxin consists of three subunits (*Cdt*A, *Cdt*B and *Cdt*C) and is lethal for host enterocytes (Purdy *et al.,* 2000; Lara-Tejero and Galan, 2001; Ghorbanalizadgan *et al.,* 2014).

Differences in the presence of pathogenicity associated genes have been reported depending on the source and region of sampling (Datta *et al.,* 2003; Rizal *et al.,* 2010; Thomas *et al.,* 2014).

Over the past few decades, there has been an increase in the emergence of antibiotic-resistant *C. jejuni* effecting the clinical management of *Campylobacter* enteritis (El-Baky *et al.,* 2014; Ghimire *et al.,* 2014). Intensive rearing of poultry and use of antibiotics such as fluoroquinolone as feed additives, growth enhancer and therapeutics has resulted in emergence of quinolone-resistant campylobacters (Wieczorek and Osek, 2013). The resistant bacteria can be transmitted through contaminated poultry meat and eggs into humans (El-Baky *et al.,* 2014). Chickens have also been described as a source of antibiotic resistant bacterial strains in humans in northern India (Chatur *et al.,* 2014). Thus, US Food and Drug Administration have withdrawn use of fluoroquinolone from poultry since 2005 (Nelson *et al.,* 2007). Monitoring drug resistance pattern among the *Campylobacter* isolates not only gives vital clues to

the clinician regarding the judicious therapeutic regime to be adopted against individual cases, but also an important tool to devise a comprehensive chemoprophylactic and chemotherapeutic drug schedule within a geographical area (Siddiqui *et al.,* 2015).

Looking into the increasing incidence of Campylobacteriosis and its resistance to commonly used antibiotics the present study was designed with following objectives:

1. Isolation, identification and typing of *C. jejuni* isolates from poultry.
2. Characterization of *C. jejuni* in terms of its properties and virulence associated factors.
3. To study antibiotic resistance profile of *C. jejuni* isolates.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Campylobacter belongs to *Epsilon* subdivision of *Proteobacteria* and family *Campylobacteraceae*. This family includes three closely related genera i.e. *Campylobacter*, *Arcobacter* and *Sulfospirillum* (Vandamme, 2000; On, 2001). *Campylobacter* infection is a leading cause of human bacterial gastroenteritis in the United States, with more than 1.3 million Campylobacteriosis cases reported in 2012 (CDC, 2013). *Campylobacter jejuni* (*C. jejuni*) exists as a commensal organism in the gastrointestinal tract of chickens where its colonization rate is 60-80% more as compared to that of domesticated animals such as cattle, swine, sheep and dog (Datta *et al.,* 2003; EFSA, 2010). Thus contaminated poultry meat is considered as an important source for Campylobacteriosis (Ertas *et al.,* 2004; Wieczorek, 2010). By 2 to 3 weeks of age most commercial chickens become commensally colonized with as many as 10^8 colony forming units (CFU) of *C. jejuni* per gram of caecal contents (Sahin *et al.,* 2002). Consequently, *Campylobacter* organisms are frequently recovered from processed broiler carcasses (Stern *et al.,* 2006).

The ability of this organism to cause disease is dependent on various virulence factors: (i) intestinal colonization by ingested organisms and the production of bacterial cytotoxin leading to diarrhoea, (ii) bacterial invasion of intestinal cells, resulting in damage to the mucosal surface cells of the jejunum, ileum and colon (iii) extra intestinal translocation, in which the organisms cross the intestinal epithelium and migrate *via* the lymphatic system to various extra intestinal sites (Chansiripornchai and Sasipreeyajan, 2009). They primarily share either a commensal or parasitic relationship with their human or animal hosts (Vandamme, 2000). The infection is usually self-limiting causing acute enteritis ranging from loose feces to dysentery, systemic illnesses, depression, poor weight gain, anaemia and jaundice. Macroscopic lesions of Campylobacteriosis include intestinal hemorrhage and distension, mucoid or watery intestinal content, swelling of kidney and spleen and necrotic lesions on liver (Songer and

Post, 2005). However, Campylobacteriosis can also lead to more severe, occasionally long-term, sequelae, such as Guillain-Barre Syndrome (Tang *et al.,* 2009). The genus *Campylobacter* comprises 16 species and 6 subspecies (On, 2001; Rozynek *et al.,* 2005). The most recognized species within the *Campylobacter* genus are *C. jejuni* and *C. coli* (Butzler *et al.,* 1983; Skirrow, 2006) which are considered as major gastrointestinal pathogens causing enteric infections in millions of cases in adults and children worldwide each year (Allos, 2001; Gibreel and Taylor 2006). *C. jejuni* is now recognized as one of the leading (>95%) causes of bacterial foodborne disease in many developed countries (Park, 2002).

A well-recognized problem associated with identification of *Campylobacter* species is the lack of effective discriminating biochemical tests, therefore now day's genetic tools are becoming more and more popular. Different molecular genetics based methods have been developed that have the potential to replace classical subtyping methods. Polymerase chain reactions (PCR)-based assays have facilitated identification of *C. jejuni* (Linton *et al.,* 1997; Burnett *et al.,* 2002; Ertas *et al.,* 2004; Tang *et al.,* 2009). Genetic diversity within *C. jejuni* has also been well established and it has been suggested that this reflects the ubiquitous nature of these organisms in the environment (Burnett *et al.,* 2002).

The literature available on *C. jejuni* has been reviewed under following heads and subheads:

2.1 *Campylobacter* overview

a. Historical perspective

b. Phenotypic characterization of *Campylobacter jejuni*

2.2 Genotyping of *Campylobacter jejuni*

2.3 Molecular Typing of *Campylobacter jejuni*

a. Repetitive extragenic palindromic (REP) PCR

b. *fla*A typing

c. Random amplified polymorphic DNA (RAPD)

2.4 Virulence associated genes of *C. jejuni*:

a. Adherence (*cad*F, *cap*A, *jlp*A, *por*A and *dna*J)

b. Lipo-oligosacchrides (*wla*N and *waa*C)

c. Motility (*fla*A, *fla*B and *flg*R)

d. Invasion (*iam*AB, *cia*B, *pld*A)

e. Toxin (*cdt*A, *cdt*B and *cdt*C)

2.5 Antibiotic resistance determinants

a. Fluoroquinolone resistance

b. Macrolide resistance

c. Tetracycline resistance

d. Aminoglycoside resistance

e. Efflux pumps and Integrons

2.1 *Campylobacter* overview

a. Historical perspective

Campylobacter was first identified by Theodor Escherich in 1886, who described non-culturable corkscrew-shaped bacteria in stool samples of children with diarrhoea (Escherich, 1886; Kist, 1986). These infections were named cholera infantum (*Samie et al.,* 2007) or summer complaint (Condran and Jennifer, 2008). The first *Campylobacter* spp. isolated was *C. fetus* (classified initially as *Vibrio fetus*) in 1906 by McFadyean and Stockman from uterine mucous of aborted ewes (Butzler, 2004; Skirrow, 2006). The reason behind the reclassification of *Campylobacter* organisms was that these organisms had genetic differences as compared to other species of Genus *Vibrio* in terms of G+C content, requirement for microaerobic growth conditions, non-fermentative metabolism, elevated optimum growth temperature requirement and lack of saccharolytic enzymes (Sebald and Veron, 1963; Walker *et al.,* 1986). The genus *Campylobacter* was first named in 1963 by Sebald and Veron (Moore *et al.,* 2005).

However, *Campylobacter* did not gain much recognition until the 1970's when it was shown to be highly prevalent in humans and first isolation of thermophilic *Campylobacter* as an enteric pathogen was carried out by a Belgian

team in 1972 by filtering stools samples of female patients with diarrhoea (Dekeyser *et al.,* 1972; Butzler *et al.,* 1973). The improvement in isolation methods and development of selective growth media for cultivation of *Campylobacter* from fecal samples of patients with enteric symptoms resulted in establishing *Campylobacter* spp*.* as a common human pathogen (Karmali *et al.,* 1981; Bolton *et al.,* 1984).

b. Phenotypic characterization of *Campylobacter jejuni*

Campylobacter are Gram negative, helically curved short or "S" shaped rods 1.5 to 6 mm long and 0.2 to 0.5 mm wide (Ketley, 1997). The organism is motile via unipolar or bipolar flagella having typical cork-screw type motion. Growth of *C. jejuni* is optimal under microaerophillic conditions, requiring 5-10% carbon dioxide at 42°C (Walker *et al.,* 1986; Vandamme, 2000).

Park (2002), Lee and Newell, (2006) and Murphy *et al.* (2006) reported that greatest challenges for *C. jejuni* occurs outside their habitat i.e. intestinal milieu, where they are exposed to six main environmental stressors that affect their viability: desiccation, osmotic stress (salt), pH (acidity and alkalinity), temperature stress (high and low), oxidative stress, and starvation.

C. jejuni survive for more than 4h at 27˚C and 60–62% relative humidity on some food surfaces (De Cesare *et al.,* 2003). Stern and Kazmi (1989) observed reduction in population of *Campylobacter* spp. after freeze–thawing. However, freezing does not eliminate the pathogen from contaminated foods (Lee *et al.,* 1998). Hazeleger *et al.* (1995) noticed the longest survival of *C. jejuni* cells at 4˚C and the optimal pH range was found to be 6.5–7.5 (Chaveerach *et al.,* 2003; Keener *et al.*, 2004). Jacobs-Reitsma (2000) and Kelana and Griffiths (2003) observed the impact of incubation temperature on NaCl tolerance of *Campylobacter* spp. and reported that the organism can tolerate high salt concentrations at 4°C than at 25°C.

Campylobacter cells are generally found to be transformed to coccoid or spherical cell shapes when harvested at late exponential to stationary phase or under oxidative stress or nutritive deficiency (Hazeleger *et al.,* 1995; Chaveerach

et al., 2003). After a prolonged deficiency a transitional stage so-called "Viable but nonculturable" (VBNC) occurs. The bulk of such culture remains motionless and in coccoid cell forms incapable for reproduction and thus cannot be recovered by standard laboratory culture methods. But still such VBNC forms are stable and metabolically active to cause infection if taken up by host animals (Karmali *et al.,* 1981; Moran and Upton, 1987; Rollins and Colwell, 1986). VBNC forms were cultivable after passage through the intestinal tract of chickens (Tholozan *et al.,* 1999). However, the concept of bacteria adopting a VBNC state as a survival strategy in adverse environmental conditions has not gained universal acceptance (Weichart, 1999).

Campylobacter are very sensitive to dryness and culturing of *Campylobacter* from a dry poultry associated samples (chick pad, clean pine shaving and eggshell halves) is greatly diminished during a relatively short period of time (Doyle and Roman, 1982; Cox *et al.,* 2009).

The colony morphology of thermophilic *Campylobacter* has been reported to be of two major types by Shane and Montrose (1985). First types of colonies are flat, mucoid, or wet in appearance and other type is round, convex or raised and has discrete margins. Spreading along the streak line or swarming on the agar is also commonly observed (Kaplan and Weissfeld, 1994). Allos (1998) reported that colony morphology depends on age and water content of the selected culture medium. As the incubation time increases the transparent dew drop like colonies turns to be thickened and appear grey to yellowish grey or even tan or slightly pink and orange in color.

Many enzymes such as superoxide dismutases (SODs), catalases, peroxidases, glutathione synthetases and glutathione reductases play important role in protection against oxygen toxicity and enable the cells to survive under stress conditions (Persson and Olsen, 2005). Wassenaar and Newell (2006) reported that production of catalase by many *Campylobacter* helps in reduction of the toxic byproducts such as hydrogen peroxide (H_2O_2) in their environment.

The sensitivity of *Campylobacter* spp. to oxygen and oxidizing radicals led to development of several selective media containing one or more oxygen scavengers, such as blood, charcoal, ferrous iron pyruvate etc., and selective agents, particularly antibiotics. Most methods involve a pre-enrichment in a liquid medium, before plating on agar. Preston agar has the best isolation rates for majority of the *Campylobacter* specimens tested (Bolton and Robertson, 1982; Bolton *et al*., 1983). Bolton *et al.* (1984) also designed charcoal cefoperazone deoxycholate agar (CCDA), a blood-free medium that could be used to isolate *Campylobacter* strains and is now one of the most preferred medium. The most recent standard method (ISO, 2006) for detection and isolation is direct plating method for enumeration of *Campylobacter*s on mCCDA as selective agar.

Campylobacter hydrolyse hippurate and indoxy acetate, reduce nitrate but not nitrites and don't produce H_2S gas on TSI (triple sugar iron) agar. These tests can be used to distinguish amongst different *Campylobacter* species (Cowan and Steel, 1993; Persson and Olsen, 2005; Sun, 2007). Hippurate hydrolysis is the only phenotypic test differentiating *C. jejuni* and *C. coli*. The rapid tube hippurate test was first described by Hwang and Ederer (1975) and modified for use with *Campylobacter* by Harvey (1980). Vandamme (2000) and Ghorbanalizadgan *et al.* (2014) found that Hippurate hydrolysis used to differentiate *C. jejuni* and *C. coli* did not always correlate with the genetic classification as some *C. jejuni* strains are also negative for this test. Most *C. jejuni* strains hydrolyse hippurate to glycine and benzoic acid while other *Campylobacter* species lack this trait (Burnett *et al.,* 2002). In support, Totten *et al.* (1987) isolated 98 *C. jejuni* (52 hippurate-positive strains and 46 hippurate-negative strains) and further performed phenotypic characterization and genetic classification. All hippurate-positive strains were classified as *C. jejuni*. Of the hippurate-negative strains, 20% were *C. jejuni*, 78% were *C. coli*, and 2% were *C. laridis* on genetic confirmation by PCR.

Several alternative and rapid methods have been developed for detecting and confirming *Campylobacter* spp. e.g. fluorescence in situ hybridization (Lehtola *et al.*, 2006), latex agglutination (Wilma *et al.*, 1992) and physical

enrichment method (filtration) that permits the separation of *Campylobacter* from other organisms present in the food matrix.

In the past, phenotypic differences between isolates were used to develop subtyping schemes. The disadvantage of phenotypic subtyping in general is that it depends on expression of a characteristic phenotype, which can be influenced by culture conditions and laboratory environment etc. and also relatively high percentage of strains are untypable due to lack of phenotypic expression (Wilson *et al.*, 2010).

2.2 Genotyping of *Campylobacter jejuni*

The *Campylobacter* genome has G+C content of 30-50% and is of small size (1.619Mb) as compared to *Escherichia coli* (4.6Mb) (Taylor *et al.,* 1992). It codes 1,654 proteins (94.3 % coding region) and 54 stable RNA species. The relatively small genome of *Campylobacter* may be responsible for its inability to ferment or oxidize carbohydrates (Griffiths and Park, 1990). Linton *et al.* (1997) and Kulkarni *et al.* (2002) described 16S rRNA gene ribotyping that has been used extensively for rapid detection and identification of many bacterial taxa, including *Campylobacter* species. Inglis and Kalischuk (2003) and Man *et al.* (2010) reported that, for *Campylobacter*, this method is less useful because of sequence similarity amongst *Campylobacter* species and lack of discrimination between *C. jejuni*, *C. coli* and some *C*. *lari* strains, although it enables specific identification of *Campylobacter* genus.

This led to development of PCR (conventional and quantitative) strategies based on species-specific genes that have been used in species identification i.e. *hip*O (Wang *et al.,* 2002; Persson and Olsen, 2005), *cad*F (Khoshbakht *et al.*, 2013), *map*A (Best *et al.,* 2003), *ceu*E (Houng *et al.,* 2001), 23S rDNA (Eyers *et al.,* 1993), *ipx*A (Klena *et al.,* 2004) and *cdt*A (Asakura *et al.,* 2008).

Amplification of hippurase gene (*hip*O) is a specific identification marker for *C. jejuni* especially for certain strains that fail to express hippurate hydrolysis phenotypically, which were otherwise misclassified as *C. coli* (Totten *et al.* 1987; On and Jordan; 2003; Vashin *et al.* 2012; Aldraghi, 2014).

Asperatokinase gene (*asp*) gene amplification has been reported to be highly specific for identification of *C. coli* and can also be used in multiplex PCR for discrimination of *Campylobacter* strains (Linton *et al.,* 1997; Amri *et al.,* 2007).

Amri *et al.* (2007) designed for the first time a combination of virulence and species-specific genes in a multiplex protocol comprising three genes: *cad*F (genus-specific virulence gene) and *hip*O and *asp* (species specific for *C. jejuni* and *C. coli*, respectively) for identification of *Campylobacter*. The protocol and the primers used for their identification have been studied independently and reported by other workers (Linton *et al.*, 1997; Nayak *et al.*, 2005). The *cad*F gene, a putative virulence gene associated with adhesion, is 100% conserved among all the isolates from diverse sources (Datta *et al.*, 2003; Rozynek *et al.*, 2005).

Stephen and Jordan (2003) examined the sensitivity and specificity of 11 PCR assays for the species identification of *Campylobacter* of 111 strains of *C. jejuni*, *C. coli* and *C. lari* but found none of the tests to be 100% sensitive or specific. Rajagunalan *et al.* (2014) carried out multiplex PCR (mPCR), targeting 16S rRNA (genus specific), *map*A (*C. jejuni* specific) and *ceu*E (*C. coli* specific) genes for identification of the 43 isolates, both at genus and species level and could successfully differentiate *C. jejuni* from *C. coli*.

Advantages and disadvantages of phenotypic techniques like biotyping, serotyping, and multilocus enzyme electrophoresis and currently available technologies for genotypic subtyping of *Campylobacter* spp., their merits and demerits have been described by several researcher (Wassenaar and Newell, 2000; Sheppard *et al.*, 2009; Boxrud, 2010; Guerin *et al.,* 2010; Eberle and Kiess, 2012).

2.3 Molecular Typing of *Campylobacter jejuni*:

a. Repetitive extragenic palindromic (REP) PCR:

Various classes of short repetitive element sequences have been described in diverse prokaryotic genomes. The sequence consists of a highly

conserved inverted repeat present in high copy number and is dispersed around the chromosomes. Currently, three types of such repetitive elements have been described i.e. repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC) and the BOX elements. REP elements are 35–40-bp long, ERIC elements are the 124–127-bp long and BOX elements are 154-bp sequences (Stern *et al.*, 1984; Lupski and Weinstock, 1992). REP and ERIC elements have mostly been described from Gram Negative bacteria and BOX elements have been described from Gram positive bacteria (Hulton *et al.,* 1991; Martin *et al.,* 1992). Consensus primers to each of these elements have been used in PCR to amplify regions between neighboring repetitive elements. This generates unambiguous DNA fingerprints that enable discrimination amongst the bacterial isolates (Gilson *et al.*, 1984).

In REP-PCR, primers targeting REP and ERIC consensus sequences have inosine base at the non-conserved positions (Lupski and Weinstock, 1992). Validation of the "Diversi-Lab automated REP-PCR system" for strain typing has been carried out successfully and convincingly showed its usefulness for molecular epidemiology (Healy *et al.*, 2005).

Usefulness of REP-PCR for *C. jejuni* was reported by Hiett *et al.* (2006) for subtyping the *Campylobacter* spp. from different sources *i.e*. chicken, turkey, mice, wild birds, humans, ducks, hogs and calf and found this technique to be good is classifying isolates into spatially and temporarily relevant epidemiological groups. Prapas *et al.* (2012) recorded discriminatory index of 0.8917 of REP-PCR, and could classify all the isolates used in the study into 9 subgroups; thus reporting it to be an effective screening tool in large epidemiological studies.

b. *fla*A typing:

The flagellin gene *fla*A in *Campylobacter* spp*.* has significant sequence heterogeneity, and its analysis is a good epidemiologic marker. Flagellin gene locus of *C. jejuni* contains two flagellin genes (*fla*A and *fla*B), which are arranged in tandem and are separated by approximately 170 nucleotides. Both are highly conserved with variable regions interspersed between them (Khoshbakht *et al.*,

2013). This locus is suitable for restriction fragment length polymorphism (RFLP) analysis of PCR products. The conserved regions in this locus are also partially conserved in species other than *C. jejuni* making it suitable for typing other *Campylobacter spp.*

*fla*A-RFLP typing has been reported to be rapid and cost effective in epidemiological studies of *C. jejuni* (Acik and Cetinkaya, 2005; Moore *et al.*, 2005; Zorman *et al.*, 2006; Muller *et al.*, 2011). The level of discrimination in this technique is dependent on the restriction endonuclease chosen for the RFLP analysis (Owen and Leeton, 1999).

Various restriction enzymes have been widely used for RFLP analysis of *fla*A genes, namely *Dde*I (Nachamkin *et al.,* 1996), *Hinf*I (Santesteban *et al.*, 1996) and *eco*RI/*Pst*I (Alm *et al.*, 1993). Low discrimination by *Hinf*I has been reported previously by different workers (Ayling *et al.*, 1996; Santesteban *et al.*, 1996; Owen and Leeton*,* 1999). However, *Dde*I has repeatedly been confirmed as more discriminatory than *Hinf*I, *Pst*I or *Eco*RI, while, *Alu*I has been found to produce too many small bands which were practically difficult to analyze for inter-laboratory standardization (Wassenaar and Newell, 2000; Petersen and Newell, 2001). But, Nielsen *et al.* (2000) reported that differences in the *Alu*I profiles of the *fla*A genes are epidemiologically important. Fitzgerald *et al.* (2001) carried out RFLP analysis of the *fla*A (1.45kb) gene on 302 *C. jejuni* and 13 *C. coli* isolates with set of different restrictions endonuclease enzymes and found 35 different profiles by *Dde*I and 26 different digest profiles by e*co*RI and *Pst*I respectively.

In other study Ertas *et al.* (2009) performed RFLP of *fla*A (1.7 kb) gene with *Alu*I, *Dde*I and *Hinf*I+*Dde*I restriction enzyme combination in 28 *C. jejuni* isolates of poultry and found six different profiles. Digestion with *Pst*I, *Hinf*I and *Eco*RI generated seven, five and two different profiles, respectively and *Pst*I gave best discrimination level when compared to other enzymes. Similarly, Rajagunalan *et al.* (2014) carried out *fla*A-RFLP typing of 43 *Campylobacter* isolates and found 11 and seven *fla*A-types among 14 *C. jejuni* and 29 *C. coli*

isolates, respectively. All the 11 *fla*A-types (A-K) of the *C. jejuni* were found to be distributed into three epidemiological clusters, with cluster I and II having four *fla*A-types each, and cluster III having three *fla*A-types.

c. Random amplified polymorphic DNA (RAPD):

RAPD is not widely used in characterization of *Campylobacter* because of its poor reproducibility. In some cases minor differences in band patterns and weak band patterns observed from outbreak samples yielded poor discriminatory results (Nielsen *et al.,* 2000; Wassenaar and Newell, 2000). The analysis of random amplified polymorphic DNA was carried out from whole DNA of purified *Campylobacter* isolates using a single 10-bp primer. The PCR products when visualized by simple gel electrophoresis resulted in highly diverse DNA banding patterns (Hernandez *et al.,* 1995; Misawa *et al.,* 2000).

2.4 Virulence associated genes of *C. jejuni*

Campylobacter spp. are reported to have different virulence factors responsible for colonization of host intestinal mucosa leading to enteric disease in host with invasive and toxin producing capabilities (Bereswill and Kist, 2003).

a. Adherence (*cad*F, *cap*A, *jlp*A, *por*A and *dna*J)

Campylobacter fibronectin (*Cad*F) is a highly conserved 37-kDa outer membrane protein bound to extracellular matrix (ECM) (Konkel *et al.,* 1999; Rozynek *et al.,* 2005). *Cad*F promotes adherence of *Campylobacters* to cell cultures and are involved in chicken colonization (Konkel *et al.,* 1997; Flanagan *et al.,* 2009). In a study *C. jejuni Cad*F deletion mutants had 60% reduction in binding to immobilized fibronectin and simultaneously had reduced adherence (50%) to intestinal human cells (INT 407) as compared to wild-type *C. jejuni* strain (Monteville *et al.*, 2003). Studies conducted on newly hatched chickens revealed that wildtype *C. jejuni* isolates could readily colonize the cecum of chickens, whereas a *Cad*F null mutants failed to colonize the cecum, indicating the essentiality of *Cad*F to establish colonization in newly hatched leghorn chickens (Ziprin *et al.*, 1999). Additional work by Ziprin *et al.* (2001) has shown

that chickens challenged with the *Cad*F null mutation provided protection from a subsequent attack of wild-type *C. jejuni*.

Campylobacter adhesion protein A (*Cap*A) is an autotransporter and was for the first time identified during *in silico* analysis. Expression of the functional *cap*A protein is dependent upon frameshifts within a homopolymeric nucleotide tract located near the 5'end of coding region (Flanagan *et al.,* 2009). Fouts *et al.* (2005) demonstrated conservation of the characterized and putative *C. jejuni* adhesins in *C. jejuni* NCTC 11168 and RM1221 strains, and three other *Campylobacter* species. *C. jejuni Cap*A deletion mutant exhibited 47% reduction in binding of *Campylobacter* to chicken LMH epithelial cells in comparison to wild-type isolate. Ashgar *et al.* (2007) reported that a *Cap*A knockout reduced the binding of *C. jejuni* to human Caco-2 colorectal adenocarcinoma cells by approximately 30% and such mutants failed to colonize and persist in Rhode Island Red chickens and concluded its role to be during the initial steps of adherence process.

Jin *et al.* (2001) identified a 42.3 kDa surface exposed lipoprotein termed *Jlp*A (jejuni lipoprotein A) having role in binding to HEp-2 cells. Earlier studies suggested the presence of *Jlp*A in the bacterial inner membrane loosely attached with the outer membrane (Flanagan *et al.,* 2009). Adherence to HEp-2 cells by a *Jlp*A null mutant reduced binding of such *Campylobacter* by 18-19.4% when compared to wild-type *C. jejuni*; however, no difference in invasion was observed (Jin *et al.*, 2001). Jin *et al.* (2003) found *Jlp*A to interact with heat shock protein (Hsp) 90-α on the surface on HEp-2cells. The adherence of *Jlp*A to Hsp 90-α led to the activation of NF-kB and p38 MAP kinase which suggested the role of *Jlp*A in triggering inflammatory and immune responses in host cells following *C. jejuni* infection (Jin *et al.*, 2003; Scott *et al.*, 2009). Furthermore, *Jlp*A gene has been shown to be upregulated in response to human mucin along with other *C. jejuni* genes suggesting its role in pathogenicity (Tu *et al.*, 2008). However, in contrast Novik *et al.* (2010) reported minimal reduction in invasion with a *Jlp*A mutant when compared to the wild-type strain of *C. jejuni* in T84 cells. Similarly Flanagan

et al. (2009) revealed no reduction in invasion of a *Jlp*A mutant on chicken epithelial cell line.

Once the bacteria have adhered to the host cells, different genes come into action such as heat shock proteins (Hsp) which help in thermotolerance and are also involved in colonization of the intestinal tract of chickens. In an experiment, severely retarded growth of *C. jejuni, Dna*J (Hsp gene) mutants at 46°C was found and simultaneously such mutants could not colonize the newly hatched chickens (Konkel *et al.,* 1998; Hermans *et al.,* 2011).

The role of another important protein *Por*A (43 kDa) was noticed by Flanagan *et al.* (2009) in *Campylobacter* infection when mutations in *Por*A proved lethal to the bacterium. Iovine (2013) studied the expression of the *PorA*, (major outer membrane protein or MOMP) in acquiring antibiotic resistance by inducing inability of the antibiotic to reach its target through it.

Studies based on prevalence of various virulence factors related to adherence, *cad*f gene has been reported to be found maximum in almost 100% of the isolates (Wieczorek, 2010; Chae *et al.*, 2012; Khoshbakht *et al.*, 2013; Cho *et al.,* 2014) followed by *jlp*A and *dna*J gene in 80-100% isolates (Datta *et al.*, 2003; Biswas *et al., 2*011) respectively. Comparatively, *cap*A and *por*A genes have been reported to be in lesser number of isolates (Rizal *et al.*, 2010; Andrzejewska *et al.*, 2011).

Although, no study has been done with null mutants, having simultaneous deletion for all the adherence related genes. But, null mutants with deletion of individual adherence related genes have clearly shown their effect on adherence.

b. Lipo-oligosacchrides (*wla*N and *waa*C)

LPS (lipopolysaccharide) is a major surface component of gram-negative bacteria and an endotoxin associated with virulence (Ketley, 1997). Unlike most other gram-negative enteric pathogens, *C. jejuni* do not express lipopolysaccharide (LPS) but possessed low molecular weight (LMW) 3-10 sugar residues or carbohydrate chain termed as Lipo-oligosacchrides (Logan and

Trust, 1984). Lipo-oligosacchrides (LOS) lack an O-polysaccharide chain, has greater structural diversity in the outer core and different from LPS. In a study mutation in LOS biosynthesis genes affected serum resistance, adherence and invasion (Fry *et al.,* 2000).

LOS is required for growth, natural transformation (Marsden *et al.,* 2009) and pathogenesis because mutations in LOS biosynthesis genes altered *C. jejuni* invasion of human intestinal epithelial cell lines (Fry *et al.,* 2000; Javed *et al.,* 2012). Stephenson *et al.* (2013) studied the structure of LOS and found differences in sialic acid incorporation in it. This incorporation modulated the host immune responses and the severity of disease. It has two main components: a hydrophobic lipid A anchor and an oligosaccharide consisting of a conserved inner core and a variable outer core. LOS is believed to play an important role in adherence, invasion and colonization.

LOS activates TLR4 and produce inflammatory response through cytokine production and phagocytosis (Huizinga *et al.,* 2012; Stephenson *et al.,* 2013). In addition, LOS mimics eukaryotic glycoproteins and glycolipids (Houliston *et al.,* 2011) and thus have role in the generation of autoreactive antibodies responsible for Guillain-Barre's syndrome (GBS). The similarity in the structure of human gangliosides and LOS of *C. jejuni* has been predicted to be one of the many reasons for *Campylobacter* associated Guillain-Barre's and Miller-Fisher's syndrome neuropathies.

The *wla* gene cluster contains genes coding for the biosynthesis of LOS molecules and genes for regulating protein glycosylation whereas *waa*C encodes for heptosyltransferase I and attaches the first heptose (HEp-I) to *Kdo* (Karlyshev *et al.,* 2005). The LOS gene loci from multiple *C. jejuni* strains have been sequenced and grouped into 19 different LOS classes based on the gene content by Gilbert *et al.* (2008).

In a study Gilbert *et al.* (2002) found that there are at least five distinct mechanisms which enable *C. jejuni* to vary the structure of the LOS molecule that it produces. These were 1) different genes present within the LOS

biosynthesis locus, 2) phase variation due to slipped-strand mispairing in regions containing homopolymeric nucleotide runs, 3) a single base deletion or insertion leading to gene inactivation, 4) a point mutation leading to gene inactivation and 5) mutations which alter the acceptor specificity of glycosyltransferases.

Datta *et al.* (2003) isolated 111 *Campylobacter* strains (56 from human clinical samples, 21 from poultry meat, 21 from broiler feces and 13 from bovine feces) and *wla*N gene was found in 25% of the isolates from human sample followed by 23.8% isolates from poultry meat, 4.7% in broiler feces and 7.7% in bovine feces. Chae *et al.* (2012) isolated 43 *C. jejuni* strains from poultry carcass and found that 40 (90%) of the isolates were positive for *wla*N gene by producing amplicon of 330bp. Khoshbakht *et al.* (2013) isolated *48 C. jejuni* strains from chicken feces for detection of *wla*N gene which is detected among 82.22% of the isolates. Cho *et al.* (2014) detected 25.2% prevalence rate of *C. jejuni* from dog fecal samples and found that *wla*N gene was found in all isolates.

c. Motility (*fla*A, *fla*B and *flg*R)

Campylobacter jejuni contains one or two polar flagella which are important colonization factors. The flagellar filament is made up of multimers of protein flagellin attached by hook protein to a basal structure embedded in the cell membrane (Nachamkin *et al.*, 1993). Ghorbanalizadgan *et al.* (2014) reported two subunits of flagellin i.e. *fla*A (major flagellin) and *fla*B (minor flagellin) which were subjected to antigenic variation as well as phase variation and found to mediate motility, colonization and invasion of gastrointestinal tract.

The *fla*A gene seems to be highly conserved among *Campylobacter* isolates and its transcription is independent of *fla*B. *fla*A gene expression is governed by promotor σ28 (Neal-McKinney *et al.,* 2010). The *fla*B gene expression is governed by σ54 promotor (Wegmuller *et al.,* 1993; Hermans *et al.,* 2011). Experiments with mutants have shown that *fla*A but not *fla*B is essential for colonization of chickens (Jones *et al.*, 2004) but both are needed for full motility.

However, Wassenaar *et al.* (1994) found some strains of *C. jejuni* that expressed only *fla*A, and were fully motile in the absence of *fla*B expression. The role of the flagellin B is not yet clearly known. Wassenaar *et al.* (1995) suggested that the second flagellin gene may be donor to *fla*A, to compensate for mutations or to increase the immunogenic repertoire of *C. jejuni* strain. However, a mutation in *fla*B appeared least significant as compared to structurally normal flagellum (Guerry, 2007).

Environmental and chemotactic stimuli such as pH, growth temperature and the concentration of certain inorganic nutrients modulated *fla*A and *fla*B promoter activity (Alm *et al.*, 1993). *fla*A promoter is up-regulated by lower pH, bovine bile, deoxycholate, L-fucose, high osmolarity and chemotactic effectors such as aspartate, glutamate, citrate, fumarate, ketoglutarate and succinate (Allen *et al.*, 2001; Ding *et al.*, 2005).

The expression of *fla*A gene is regulated by a two-component system composed of the sensor kinase *flg*S and the response regulator *flg*R. Chickens exposed to the *flg*R mutants showed delayed colonization. Moreover, reinfection of *Campylobacter*-negative chickens was not observed. Since bird-to-bird transmission in flocks is generally considered to be very rapid, *flg*S/*flg*R system is required for initial colonization of the cecum of chicks. *flgR gene* regulates flagellar expression by controlling flagellar biosynthesis, phase variation through slip strand mutagenesis in its poly(A/T) tracts within *flg*R gene. Chickens exposed to the *flg*R mutants showed delayed colonization (Neal-McKinney *et al.,* 2010; Wilson *et al.,* 2010; Silva *et al.,* 2011). Phase variation of flagellar motility *via flg*R is a phenomenon specific to *C. jejuni* that is absent in other *Campylobacter* species (Hendrixson, 2006).

The flagellar apparatus functions as a type III secretion apparatus for the *Campylobacter* invasion antigens (*Cia* proteins) important for *in vitro* cell invasion (Konkel *et al.*, 2004) and chick colonization (Ziprin *et al.*, 2001) and secretion is enhanced upon exposure to chicken mucus (Biswas *et al.*, 2007). A correlation has been demonstrated between chicken colonization potential and *in vitro*

secretion of *Cia* proteins (Biswas *et al.*, 2007). The *fla*A gene is responsible for the expression of adherence, colonization of the gastrointestinal tract and invasion of the host cells (Jain *et al.*, 2008).

d. Invasion (*iam*AB, *cia*B, *pld*A)

Campylobacter related pathogenesis depends on the ability of the organism to invade the epithelial cells of the host gastrointestinal tract. One of the markers found to be involved in invasion by *Campylobacter* is invasion associated marker (*Iam*) which helps in colonization of multiple hosts (Al-Mahmeed *et al.,* 2006; Wieczorek *et al.,* 2012). Invasion associated marker (*iam*) of *Campylobacter* strains was found to be associated with adherence and invasion of HEp-2 cells *in vitro* (Carvalho *et al.*, 2001). Invasion associated marker (*iam*) is 1.6 kb genetic marker having ABC transporter (*iam*A) gene and integral membrane protein (*iam*B) gene have been found to be associated with adherence and invasion of HEp-2 cells *in vitro* (Carvalho *et al.,* 2001). *cia*B gene is required for secretion of other *Campylobacter* invasion antigens (*Cia* proteins) (Konkel *et al.,* 1999). Different genes like *iam*AB, *cia*B and *pld*A are involved in bacterial invasion of cultured epithelial cells (Talukder *et al.,* 2008; Ghorbanalizadgan *et al.*, 2014; Yang *et al.*, 2014). Antiserum generated against a recombinant *Cia*B protein was used to study the translocation of *Cia*B into the cytoplasm of the host cell indicating it to be a true effector molecule facilitating invasion (Konkel *et al.*, 1999; Croinin and Backert, 2012). Konkel *et al.* (1999) got 8 proteins in the supernatant fluids of media in which C*. jejuni* was co-cultured with INT 407. The proteins were designated *Cia*A-*Cia*H and ranged in size from 10.8-12.8 kDa. However, a *Cia*B null mutant contained no secreted proteins in the supernatant fluid (Konkel *et al.*, 1999; Talukder *et al.*, 2008). Studies by Rivera-Amill *et al.* (2001) demonstrated the role of bile salts (deoxycholate, cholate, chenodeoxycholate) in induction of the expression of *cia* genes. In addition, a component of fetal bovine serum indorsed synthesis and secretion of the *Cia* proteins. *In vivo* studies have revealed that a *Cia*B null mutant failed to colonize the cecum of chickens. Moreover, the initial exposure of chicks to the *Cia*B null mutant followed by challenge with a *C. jejuni* parental strain did not

provide significant protection against colonization in chicks (Ziprin *et al.*, 2001). Newborn piglets infected with the wild-type *C. jejuni* developed diarrhea within 24 hours of post-infection but *Cia*B null mutant infection did not cause diarrhea until 72 hours post-inoculation (Konkel *et al.*, 2001). *C. jejuni* colonization in multiple hosts widens and facilitates the interactive impact of major animal sources such as chicken and human in the transmission of *Campylobacter* (Al-Mahmeed *et al.*, 2006; Wieczorek *et al.*, 2012).

The *pld*A gene codes for phospholipase A protein having homology with *Escherichia coli* outer membrane phospholipase (Grant *et al.,* 1997). *Pld*A encoded protein is localized in outer membrane thus is involved in maintenance of the functional integrity of the surface exposed adhesions in *Campylobacter* strains (Ziprin *et al.*, 2001).

Ziprin *et al.* (1999) demonstrated different mutants of *cad*F and *pld*A genes which were impaired in their ability to colonize in cecum, indicating their prominent role in successful colonization.

e. Toxin (*cdt*A, *cdt*B and *cdt*C)

Campylobacter produce cytolethal distending toxin (CDT) which causes progressive cellular distension ultimately leading to cell death. CDT is a complex coded by three linked genes i.e. *cdt*A, *cdt*B, and *cdt*C responsible for three subunits of the toxin that in turn act together to block cell division by performing cell cycle arrest (Ge *et al.,* 2008). Expression of these is necessary for toxin production and release of active tripartite holotoxin (Konkel *et al.*, 2001). Out of all the three components, *Cdt*B has been found as the active component of holotoxin localized in the host nucleus. It cleaves dsDNA molecules during the G1 and G2 phase. *Cdt*B also cause cell distention and irreversible cell-cycle arrest (Al-Mahmeed *et al.*, 2006). The genes *cdt*A and *cdt*C usually bind to the cell surface and help in the delivery of active subunit *Cdt*B to cause DNA damage (Ge *et al.,* 2008). CDT elicit IL-8 production also in intestinal epithelial cells (Hickey *et al.,* 2000), an important inflammatory cytokine released by the intestinal epithelium leading to epithelial damage and diarrhoea. Datta *et al.*

(2003); Dipineto *et al.* (2011); Chae *et al.* (2012) and Cho *et al.* (2014) detected 100% of the *C. jejuni* isolates from various sources of poultry to possess distending cytotoxic activity with variable prevalence of *cdt*A, *cdt*B and *cdt*C genes respectively.

According to Martinez *et al.* (2006), all *C. jejuni* strains possess *cdt* genes. However, there are exceptions of rare isolates which mutate and do not show activity of such genes. They also sequenced and characterized CDT-negative isolates, and observed the presence of *cdt*A, *cdt*B and *cdt*C pseudo-genes with deletions in their sequences. Asakura *et al.* (2007) observed the difficulty in identification of *cdt* genes due to mutations such as nucleotide deletion, insertion or substitution, and suggested the involvement of mutations in toxin activity. Purdy *et al.* (2000) reported *C. jejuni* isogenic CDT mutants showing reduced invasiveness in a mouse model of enteric infection. CDT is usually expressed in those *C. jejuni* strains which are colonizing their natural host, chicken but they don't generate CDT-neutralizing antibodies. Thus, toxin might provide a way to either avoid host immune-response mechanisms or redirect them towards immune tolerance or asymptomatic infections (Abuoun *et al.,* 2005; Muller *et al.,* 2006; Ghorbanalizadgan *et al.,* 2014).

2.5 Antibiotic resistance determinants

Antimicrobial resistance is one of the biggest challenges of the present world. *Campylobacter* species are resistant to a number of antibiotics, including cefoperazone, cephalothin, bacitracin, vancomycin, rifampicin and trimethoprim (Allos, 2001), which are used in selective media for isolation. The antibiotic resistance can be because of any one or combination of mechanism i.e. (1) modification of the antibiotic's target and/or its expression e.g. DNA gyrase mutations (2) inability of the antibiotic to reach its target i.e. expression of the major outer membrane protein or MOMP (3) efflux of the antibiotic i.e. multidrug efflux pumps such as *Cme*ABC (4) modification or inactivation of the antibiotic i.e. β-lactamase production (Iovine, 2013). Acquired antimicrobial resistance phenotypes most often develop *via* conjugative transfer of plasmids (Mathew *et*

al., 2003). Plasmids carry class I integrons, which are mobile genetic elements important in horizontal gene transfer containing elements necessary for acquisition, site-specific recombination, proliferation of bacterial multidrug resistance and expression of foreign DNA (Nemergut *et al.,* 2004). Antibiotic use could be a selective force responsible for the accumulation of resistance genes in integrons. They move to plasmids and other accessory elements, and then from their ancestral bacteria into the commensal and pathogenic bacteria of mammals (Mazel, 2007). To monitor antimicrobial resistance in foodborne pathogens, CDC, in collaboration with the FDA Center for Veterinary Medicine, the Agricultural Research Service of the US Department of Agriculture, and selected state health departments, launched National Antimicrobial Resistance Monitoring System (NARMS). In 1997, NARMS began surveillance for antimicrobial-resistant *Campylobacter* species (Nelson *et al.*, 2007). The resistance of *C. jejuni* against various antibiotic classes has been reviewed as follows:

a. Fluoroquinolone resistance

Fluoroquinolone-resistant *Campylobacter* species in humans were first reported in late 1980's and have been documented in numerous countries (Engberg *et al.*, 2001). Rapid increase in the prevalence of fluoroquinolone resistance among human *Campylobacter* isolates became a cause of concern during the late 1990's (Gupta *et al.*, 2004). Molecular epidemiological studies provided further support for the causal link between chicken consumption and fluoroquinolone-resistant *Campylobacter* infections. Strains of *Campylobacter* found in the meat of chickens were found to be identical to those responsible for human infections (Lipsitch *et al.,* 2002). The rates of fluoroquinolone resistance were highest in commercial free-range broilers, at >95%, but were lower in industrial broiler (16%) and lowest (8%) in poultry from family farms (Bester *et al.*, 2012). World Health Organization (WHO) has classified such drugs as 'critically important in human medicine' and suggested efforts for reducing fluoroquinolone and another antibiotic class known as the modern cephalosporin for use in farm animals (WHO, 2011). Several countries have reported that fluoroquinolone-resistant *Campylobacter* persists even after withdrawal of fluoroquinolone

antibiotic from poultry flocks for several years (Luo *et al.*, 2005; Nelson *et al.*, 2007; Price *et al.*, 2007) suggesting little/no fitness cost of fluoroquinolone-resistance in *Campylobacter* (Zhang *et al.*, 2003; Price *et al.*, 2007).

In *Campylobacter*, there are two well-described mechanisms for fluoroquinolone resistance: (1) inactivation of the target of fluoroquinolones (2) efflux of fluoroquinolones. These two mechanisms work together synergistically. Generally, two intracellular enzymatic targets of fluoroquinolones are DNA gyrase encoded by *gyr*A and *gyr*B and the structurally related topoisomerase IV encoded by *par*C and *par*E (Drlica and Zhao, 1997; Luangtongkum *et al.*, 2009). Fluoroquinolones reportedly form a stable complex with these enzymes and trap them onto DNA, leading to decreased DNA replication, transcription, and ultimately to cell death (Bachoual *et al.*, 2001). The genes encoding topoisomerase IV (*par*C/*par*E) are also found to be involved in fluoroquinolones resistance in Gram-negative bacteria. However, several studies have shown lack of *par*C and *par*E genes in *C. jejuni* and *C. coli* strains respectively (Parkhill *et al.*, 2000; Cooper *et al.*, 2002; Luo *et al.*, 2003). In *Campylobacter*, resistance to fluoroquinolones is mainly found to be mediated by point mutations in the quinolone resistance-determining region (QRDR) of DNA gyrase A (*gyr*A) (Payot *et al.*, 2006; Zhang *et al.,* 2008) with most common Thr-86-Ile mutation (Chatur *et al.*, 2014). Unlike fluoroquinolones resistance in other enteric organisms e.g. *Salmonella* and *Escherichia coli*, where acquisition of high-level of fluoroquinolones resistance requires stepwise accumulation of point mutations in *gyr*A and *par*C, a single point mutation in the QRDR of *gyr*A was found sufficient for substantialy reducing susceptibility of *Campylobacter* to fluoroquinolones (luo *et al.,* 2005; Payot *et al.*, 2006; Luangtongkum *et al.*, 2009). McDermott *et al.* (2002) studied single mutation in *gyr*A that lead to high-level of resistance against nalidixic acid and fluoroquinolones (ciprofloxacin minimum inhibitory concentration, MIC >16 g/ml). The most frequently observed mutation in fluoroquinolone-resistant isolates of *Campylobacter* was C257T change in the *gyr*A gene, leading to Thr-86-Ile substitution in the gyrase, conferring high-level resistance to fluoroquinolones (Payot *et al.*, 2006). In another study

Campylobacter having Thr-86-Ile mutation in *gyr*A were more fit in a chicken model (Luo *et al.,* 2005). Interestingly, the less common Thr-86-Ala mutation conferred resistance only against nalidixic acid (Boven *et al.*, 2003). The less common Asp-90-Asn and Ala-70-Thr mutations in *gyr*A conferred intermediate-level resistance against ciprofloxacin (MIC 6-16g/ml) (Piddock *et al.*, 2003). Ge *et al.* (2005) reported double point mutations of the *gyr*A gene together with Asp85Tyr, or Asp90Asn, or Pro104Ser. Other reported resistance-associated mutations include T-86-K, A-70-T and D-90-N, which were less common and did not confer fluoroquinolone resistance as high as that observed with Thr-86-Ile mutation (Engberg *et al.*, 2001). The missense mutations in *gyr*B gene also have been reported which does not confer fluoroquinolones resistance (Bachoual *et al.*, 2001 and Payot *et al.*, 2003 and Chatur *et al.*, 2014). In addition to the mutations in *gyr*A, *Campylobacter* multidrug efflux (*cme*) pump, *cme*ABC, also contributes to fluoroquinolone resistance through reduction of accumulation of the antibiotics in *Campylobacter* cells (Lin *et al.*, 2002; Luo *et al.*, 2003; Ge *et al.*, 2005). Thus, *cme*ABC functions synergistically with the *gyr*A mutations in mediating fluoroquinolone resistance. Inactivation of the *Cme*ABC efflux pump by insertional inactivation of *cme*B or with efflux pump inhibitors led to increased susceptibility to different antibiotics, including those to which *Campylobacter* were intrinsically resistant, indicating role of *cme*ABC in both intrinsic and acquired resistance of *Campylobacter* (Lin *et al.*, 2002; Pumbwe and Piddock, 2002; Akiba *et al.*, 2006). Furthermore, it was found that when the efflux pump was blocked, the minimum inhibitory concentration (MIC) values for ciprofloxacin were reduced to the level of susceptible strains even with mutations in the *gyr*A (Luo *et al.*, 2003).

b. Macrolide resistance

Macrolide group of antibiotics are the drug of choice for Campylobacteriosis. Macrolide group include clarithromycin, azithromycin, telithromycin, tylosin and tilmicosin. The main mechanisms of resistance to macrolides in *Campylobacter* are target modification, efflux pumps and alterations in membrane permeability. The first two mechanisms act

synergistically in conferring high-level macrolide resistance (Lin *et al.*, 2007). A fourth mechanism for macrolide resistance is enzymatic modification of macrolides, which has not been described in *Campylobacter* (Payot *et al.*, 2006). Macrolides interrupt protein synthesis in bacterial ribosome by targeting the 50S subunit inhibiting bacterial RNA-dependent protein synthesis (Yao and Moellering, 2003; Poehlsgaard and Douthwaite, 2005). Point mutations in the peptidyl encoding region in domain V of the 23S rRNA gene at positions 2074 (A2074C, A2074G) and 2075 (A2075G) of adenine residues (*rrn*B operon) confer high-level macrolide resistance (erythromycin MIC > 128 mg/L) in *C. jejuni* and *C. coli* (Mamelli *et al.*, 2005; Lin *et al.*, 2007; Egger *et al.*, 2012) with 2075 substitution being more common (Gibreel and Taylor, 2006). *C. jejuni* and *C. coli* carry three copies of 23S rRNA gene (Parkhill *et al.*, 2000; Fouts *et al.*, 2005) all of which are usually mutated in macrolide-resistant strains. However, some strains with lower MICs to macrolides have been found to have only two mutated gene copies suggesting gene dosage effect (Gibreel *et al.*, 2007). Strains harboring single mutations in 23S rRNA have not been reported. Several modifications such as modifications in ribosomal proteins L4 and L22 have been shown to be associated with low level resistance to the macrolides. However, the exact role of L4 and L22 modifications is not known (Payot *et al.*, 2006; Cagliero *et al.*, 2006). Efflux is another common mechanism causing macrolide resistance in *Campylobacter* where at least eight different efflux systems have been identified contributing to high-level of macrolide resistance in some *Campylobacter* isolates (Corcoran *et al.*, 2006). The *Cme*ABC multidrug efflux pump works in synergy with 23S rRNA gene specific mutations, in absence of any other factor affecting resistance (Payot *et al.*, 2006; Cagliero *et al.*, 2006). It was found that even in the highly resistant *Campylobacter* strains (erythromycin MIC >128 µg/ml)) with 23S rRNA gene specific mutations, inactivation of *Cme*ABC could significantly (2-4 fold) reduce the resistance level to macrolides (Cagliero *et al.*, 2006). Also, inactivation of *Cme*ABC leads to restoration of susceptibility against erythromycin in isolates having low level of erythromycin

resistance (MICs 8-16 mg/L), suggesting involvement of *Cme*ABC in the intrinsic resistance of *Campylobacter* (Lin *et al.*, 2007).

A third mechanism for macrolide resistance is altered membrane permeability mediated by expression of major outer membrane protein *i.e*. porin (MOMP), which is chromosomally encoded by *por*A (Pumbwe *et al.*, 2004). In *C. jejuni* and *C. coli*, MOMP form a small cation-selective pore that restricts the entry of most antibiotics with a molecular weight of greater than 360MW such as the macrolides (MW > 700) (Page *et al.*, 1989). Lin *et al.* (2002) found the sub therapeutic use of tylosin in chickens, given continuously in feed having significant impact on therapeutic use macrolides. Ladely *et al.* (2007) described relatively low prevalence of macrolide resistance in *Campylobacter* as compared with fluoroquinolones resistance due to low rate of emergence and requirement of prolonged antibiotic exposure to select macrolide-resistance mutants and significant fitness cost of erythromycin-resistant mutants. Such efforts made macrolides the drug of choice to treat Campylobacteriosis.

c. Tetracycline resistance

High level of tetracycline resistance was observed in isolates of *C. jejuni* and *C. coli*, due to the presence of previously described *Campylobacter* tetracycline resistance determinants encoded by the *tet*O gene (Ng *et al.*, 2001). Although many *tet* genes are found in plasmid as well as chromosome of various gram positive and grams negative organism but *tet*O is highly prevalent in *Campylobacter* species (Dasti *et al.*, 2007). According to NARMS (2010) 43% of *C. jejuni* and 49% of *C. coli* isolates were tetracycline-resistant making this class of antibiotic of little use in veterinary or human *Campylobacter* mediated disease. Previous studies have described significant role of plasmids in tetracycline resistance however, resistance in the majority of *Campylobacter* isolates examined was due to chromosomally encoded *tet*O, including those that harbored plasmids (Pratt and Korolik, 2005; Abdi-Hachesoo *et al.*, 2014). Recombination between a *tet*O harboring plasmid and the chromosome or the integration of such a plasmid into the chromosome might have led to emerging

resistant strains (Lee *et al.*, 1994). Although interspecies conjugative transfer of drug-resistant plasmids was reported with *Campylobacter* but conjugation was most successfully at the intra-species level (Dasti *et al.*, 2007). Known mechanisms of tetracycline resistance in *Campylobacter* are alteration of tetracycline's ribosomal target and efflux pumps (Iovine, 2013). Although high-level of resistance to tetracyclines could be mediated by *tet*O gene alone or in combination with efflux demonstrated by the increase in tetracycline MIC when efflux pumps were genetically activated (Gibreel *et al.*, 2004). Abdi *et al.* (2014) detected high prevalence of *tet*O resistance gene along with new detection of *tet*A resistance gene in *Campylobacter* spp. isolated from poultry carcasses in Iran which revealed an extensive tetracycline resistance.

d. Aminoglycoside resistance

Compared to fluoroquinolones, macrolides and tetracyclines, *Campylobacter* resistance to other antibiotics has received less attention. Aminoglycosides resistance was more commonly prevalent in *C. coli* than *C. jejuni*. Aminoglycosides (e.g. gentamicin, streptomycin, and kanamycin) mainly act by binding to decoding region in the A site of the bacterial ribosomal 30S subunit (Taylor and Chang, 1991). Enzymatic modification was found to be the most common type of aminoglycoside resistance. Over 50 different enzymes have been identified. Enzymatic modification results in high-level of resistance. The genes encoding aminoglycoside modifying enzymes are usually found on plasmids and transposons (Jana and Deb, 2006). Most enzyme-mediated resistance in gram-negative is due to multiple genes (Llano-Sotelo *et al.*, 2002). There are three types of aminoglycoside modifying mechanisms namely: 1) N-Acetyltransferases (AAC)-catalyzes acetyl CoA-dependent acetylation of an amino group. 2) O-Adenyltransferases (ANT)-catalyzes ATP-dependent adenylation of hydroxyl group. 3) O-Phosphotransferases (APH)–catalyzes ATP-dependent phosphorylation of a hydroxyl group. The aminoglycosides usually interfere with the translocation of the nascent peptide chain from the ribosomal A site to the P site leading to premature termination and also interfere with proof-

reading that causes incorporation of incorrect amino acids and dysfunctional protein (Herman, 2007).

f. Efflux Pumps and Integron

The *Campylobacter* multidrug efflux pump (*Cme*) plays an important role in multidrug resistance in *C. jejuni*, to heavy metals, broad range of antibiotics and other antimicrobial agents (Lin *et al.*, 2003). *Cme* is coded by operon *cme*ABC and consists of a periplasmic protein (*Cme*A), an inner membrane efflux transporter (*Cme*B) and an outer membrane protein (*Cme*C). It is believed that the three members function together and form a membrane channel for the extrusion of antimicrobials and other toxic compounds in *Campylobacter*. Expression of *cme*ABC in *C. jejuni* is modulated by *Cme*R, which functions as a transcriptional repressor (Hermans *et al.*, 2011). Inactivation of the *Cme*ABC efflux pump by insertional inactivation of *Cme*B or with efflux pump inhibitors leads to increased susceptibility of *Campylobacter* to different antibiotics, including those to which *Campylobacter* were intrinsically resistant (Lin *et al.*, 2002; Pumbwe and Piddock, 2002). Cagliero *et al.* (2006) reported high degree of genetic variation in the *Cme*B gene in *C. jejuni* and *C. coli*.

Integrons and mobile genetic elements, such as transposons and insertional sequences are important players for the transmission and spread of antibiotic resistance genes in bacteria (Mazel, 2007). Class I integrons, which are the most common integrons associated with antibiotic resistance, were reported in both *C. jejuni* and *C. coli* and were found to carry aminoglycoside resistance genes (*aad*A2 and *aac*A4) (Lee *et al.*, 2002; O'Halloran *et al.*, 2004). Lee *et al.* (2002) also examined class 1 integron associated torbramycin-gentamicin resistance among environmental *C. jejuni* from broiler chicken houses. The entire positive isolates contained *aac*A4 inserted into the integron and all of them were isolated originally from drinking water lines for the chickens.

Looking in to the above facts the present study was framed to evaluate the status of Campylobacteriosis in Bikaner region.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Sampling

A total of 370 cloacal swab samples were collected in two phases from local poultry farms in and around Bikaner in sterilized container and processed. In the 1st phase of sampling form October-2014 to March-2015, a total of 220 cloacal swabs and in the 2nd phase from October 2015-February 2016, 150 cloacal swabs were processed for isolation of thermophilic *Campylobacter spp.* in the study area (Table 1).

Table 1: Details of sample collection

Sampling phase	Type of sample	Poultry		Total
		Layers	Broilers	
Phase 1	Cloacal swab	135	85	220
Phase 2	Cloacal swab	90	60	150
TOTAL		**225**	**145**	**370**

3.2 Isolation and identification

Samples were processed for isolation of *Campylobacter* spp. as per method described in OIE Terrestrial Manual, chapter 2.9.3 (2008).

3.2.1 Pre-enrichment

Briefly, the swabs collected were inoculated in 5ml *Campylobacter* enrichment broth (Preston enrichment broth base, Himedia) supplemented with *Campylobacter* supplement IV (Himedia) (Appendix 1) and 7% lysed horse blood for pre enrichment. The inoculated tubes were incubated under microaerophillic conditions (reduced O_2 level and 5% CO_2) at 42ºC for 48 h. Incubation at 42^0C helps in selective enrichment of thermophilic campylobacters.

3.2.2 Plating on selective medium

A loopful of inoculated broth was streaked onto modified charcoal cefoperazone deoxycholate agar (mCCDA) (Himedia) supplemented with *Campylobacter* supplement V (Himedia) (Appendix I) and incubated under microaerophillic conditions at 42ºC for 48 h. The characteristic greyish, flat and moistened colonies growing along the streak were further; Gram stained to observe for typical spiral or curved slender rods and hanging drop method was used to observe motility.

Colonies, showing typical *Campylobacter* spp*.* morphology, were further subjected to primary biochemical tests i.e*.* catalase test and oxidase test (Appendix 1). All the catalase and oxidase positive isolates were stored by dispensing lawn of colonies from selective agar to 1ml brain heart infusion broth with 15-20% glycerol in 1.5 ml cryo-vials and stored at-80ºC till further use.

3.2.3 Genotypic confirmation of the isolates

Correct identification of *Campylobacter spp*. is cumbersome. Although mCCDA is a selective medium but it can also lead to growth of environmental contaminants (Kiess *et al.,* 2010; Smith *et al.,* 2015). The widely used biochemical tests might mislead, therefore, genotypic methods were used for identification of the isolates.

a. DNA extraction

DNA extraction was done as per the protocol of Ertas *et al.* (2004) with some modifications. Briefly, stored isolates were taken from the cryo-vials and inoculated on mCCDA plates and incubated for 24-48 h under microaerophillic condition such that a proper lawn of isolate was formed on the culture plates. The lawn of culture from agar plate was transferred to 2 ml eppendorf tubes, containing 400 µl, phosphate-buffered saline (PBS) (Appendix-II), vortexed and centrifuged at 12000 rpm for 5 min. Supernatant was discarded and the pellet was re-suspended in 375 µl Salt-Tris EDTA (STE) buffer (Appendix-II). The suspension was incubated at 55ºC for 4 h, with intermittent vortexing every 30

min. After incubation, equal volume of phenol was added to the suspension, shaken vigorously by hand for 5 min and then centrifuged at 12000 rpm for 10 min. The upper phase was transferred into another eppendorf tube. Genomic DNA was precipitated with absolute ethanol and 0.3 M sodium acetate (Appendix-II) at -20ºC for 1 h or 4ºC overnight. The mixture was then centrifuged at 12000rpm for 10 min and the upper phase was discarded. The pellet was washed twice with 90% and 70% ethanol, respectively; each step was followed by 5 min of centrifugation. DNA pellet was suspended in 100µl sterile distilled water or Tris-EDTA buffer (Appendix-II).

The integrity of the DNA was checked by routine Agarose gel electrophoresis on 0.8 percent agarose gels in 1X TBE buffer (Appendix-II). Quantification of the eluted DNA was done using spectrophotometric method. 260 and 280 nm and A_{260}/A_{280} ratio of around 1.9 (1.85-1.95) indicated good quality DNA.

DNA concentration was estimated (Sambrook *et al.*, 1989) by using following formula:

$$\text{Amount of DNA } (\mu g/\mu l) = \frac{(OD)\ 260 \times 50 \times \text{dilution factor}}{1000}$$

Quantified DNA was diluted to a final concentration of 25 mg/µl in TE buffer for PCR.

b. Confirmation of *Campylobacter* isolates on the basis of genus and species specific primers sequences

The genus *Campylobacter* was confirmed on the basis of a 16S rRNA gene based PCR (Linton *et al.,* 1996; Tang *et al.,* 2009). For this reaction mixture of 25µl volume (Appendix-II) was prepared using Promega, Go Taq PCR Core System-I Kit. The primer sequence used in the PCR assays is given below:

C412F: 5’ GGATGACACTTTTCGGAGC 3’

C1288R: 5’ CATTGTAGCACGTGTGTC 3’

Amplification was carried out in 'Eppendorf Mastercycler gradient' with standard PCR cycles. Annealing temperatures for the PCR was kept 55^0C. Amplified PCR products were detected by electrophoresis in 1.2% agarose gels with ethidium bromide (0.5µg/ml) in 1X TBE buffer (Appendix-II).The gel was then visualized under UVP gel documentation system (BioDoc-It Imaging System).

3.2.4 Speciation of the *Campylobacter* isolates

a. Secondary biochemical tests

The isolates once confirmed to be *Campylobacter* spp. were subjected to an array of secondary biochemical tests and genotyping method for species identification as per the method described by Fitzgerald and Nachamkin (2007); Lastovica and Allos (2008) and OIE Terrestrial Manual, chapter 2.9.3 (2008). The secondary biochemical tests used were urease test, nitrate reduction test, hippurate hydrolysis, indoxyl acetate hydrolysis and H_2S production on triple sugar iron agar (Appendix I). The biochemical characteristics of different species of *Campylobacter* genus are given in table 2. Hippurate hydrolysis test helped in differentiating *C. jejuni* from *C. coli* as *C. jejuni* hydrolyses sodium hippurate whereas *C. coli* does not.

Table 2: Biochemical characteristics of *Campylobacter* species

Sr. No.	*Campylobacter* Species	Catalase	Oxidase	Hippurate hydrolysis	Indoxyl acetate hydrolysis	H_2S productio	Urease	Nitrate reduction	Growth at 25°C	Growth at 42°C
1.	*C. jejuni* subsp. *jejuni*	+	+	+	+	-	-	+	-	+
2.	*C. jejuni* subsp. *doyley*	V	+	V	+	-	-	-	-	-
3.	*C. coli*	+	+	-	+	V	-	+	-	+
4.	*C. fetus* subsp. *fetus*	+	+	-	-	-	-	+	+	V
5.	*C. upsaliensis*	-	+	-	+	-	-	+	-	V
6.	*C. lari*	+	+	-	-	-	V	+	V	+
7.	*C. hyointestinalis*	+	+	-	-	-	+	+	V	+
8.	*C. sputorum* bv *sputorum*	-	+	-	-	-	-	+	-	V
9.	*C. rectus*	V	+	-	-	-	-	+	-	V

b. Genotypic method for speciation

Identification of species was done by PCR based amplification of *hip*O gene and *asp* gene for *C. jejuni* and *C. coli*, respectively, using standard conditions (Linton *et al.,* 1997; Amri *et al.,* 2007). The PCR protocol followed was same as for genus identification. The primer sequences and annealing temperatures used in the PCR assay are summarized in table 3.

Table 3: PCR primers and conditions for confirmation of *Campylobacter C. jejuni* and *C. coli*

Target species	Sequence 5'-3'	Annealing Temp. (˚C)	Target size (bp)	Reference
*C. jejuni (hip*O gene)	*HIP*O F: GAAGAGGGTTTGGGTGGTG *HIP*O R: AGCTAGCTTCGCATAATAACTTG	64	735	Linton et al., 1997; Amri et al., 2007
C. coli (*asp* gene	CC 1: GGTATGATTTCTACAAAGCGAG CC 2: ATAAAAAGACTATCGTCGCGT	60	500	

3.3 Genotypic characterization of *C. jejuni* virulence associated genes:

a. Motility (*fla*A, *fla*B and *flg*R)
b. Adherence (*cad*F, *cap*A, *jlp*A, *por*A and *dna*J)
c. Invasion (*iam*AB, *cia*B and *pld*A)
d. Lipo-oligosacchrides (*wla*N and *waa*C)
e. Toxin (*cdt*A, *cdt*B and *cdt*C)

Presence of all the virulence associated genes was done using primer sets as reported earlier or designed for the present study. Standard protocols for PCR amplification were used (Appendix II). The primer sets used and the annealing temperature are given in table 4.

3.4 Primer designing

The primers were designed by primer 3 tool for *iam*AB, *aph*3, *cme*RABC genes and further confirmed by nucleotide BLAST (basic local alignment search tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and *in silico* PCR amplification was

carried out to evaluate the primers designed using http://insilico.ehu.eus/PCR/ tool.

Table 4: PCR primers and conditions for detection of virulence associated genes

Sr. No	Marker name	Primer sequence	Annealing temp	Product size	References
1.	*cad*F	F- TTGAAGGTAATTTAGATATG R- CTAATACCTAAAGTTGAAAC	45	400	Konkel *et al.* (1999)
2.	*cap*A	F-TGAATCGAAGTGGAAAAATAGAAG R- CCCATTTTTGTATCTTCATAACCT	60	1351	Flanagan *et al.* (2009)
3.	*jlp*A	F- TCTCAGGACTCTGGAATAAAGATTG R-GTGTGCTATAGTCACTAACAGGGATG	60	868	Flanagan *et al.* (2009)
4.	*por*A	F- CAATTTGACTATAATGCTGCTGATG R- ATGCTGAGAAGTTAAGTTTTGGAGA	50	932	Chae *et al.* (2012)
5.	*dna*J	F- AAGGCTTTGGCTCATC R- CTTTTTGTTCATCGTT	46	720	Datta *et al.* (2003)
6.	LOS-wlaN	F- TGCTGGGTATACAAAGGTTGTG R- AATTTTGGATATGGGTGGGG	60	330	Muller *et al.* (2006)
7.	*waa*C	F- TAATGAAAATAGCAATTGTTCGT R-GATACAAAAATCACTTTTATCGA	42	1029	Khoshbakht *et al.* (2013)
8.	*fla*A	F- GGATTTCGTATTAACACAAATGGTGC R- CTGTAGTAATCTTAAAACATTTTG	52	1725	Nachamkin *et al.* (1993)
9.	*fla*B	F- ATAAACACCAACATCGGTGCA R- GTTACGTTGACTCATAGCATA	50	1670	Chae *et al.* (2012)
10.	*flg*R	F- GAGCGTTTAGAATGGGTGTG R- GCCAGGAATTGATGGCATAG	54	390	Wilson *et al.* (2010)
11.	*iam*AB	F- CGACTACTATGCGGATCAAG R- TTGTAAATGCTATATTTTGGG	53	601	This study
12.	*cia*B	F- TTTTTATCAGTCCTTA R- TTTCGGTATCATTAGC	42	986	Datta *et al.*(2003)
13.	*pld*A	F- AAGCTTATGCGTTTTT R- TATAAGGCTTTCTCCA	45	913	Datta *et al.* (2003)
14.	*cdt*A	F- CCTTGTGATGCAAGCAATC R-ACACTCCATTTGCTTTCTG	49	370	Talukder *et al.* (2008)
15.	*cdt*B	F- CAGAAAGCAAATGGAGTGTT R- AGCTAAAAGCGGTGGAGTAT	51	620	Talukder *et al.* (2008)
16.	*cdt*C	F- CGATGAGTTAAAACAAAAAGATA R- TTGGCATTATAGAAAATACAGTT	47	182	Talukder *et al.* (2008)

3.5 Molecular typing by repetitive extragenic palindromic-PCR (REP-PCR)

REP-PCR was conducted using single pair of primers targeting noncoding conserved sequence with multiple numbers of repeats in *C. jejuni* genome according to the method described by van Belkum *et al.* (1995) with annealing temperature of 37^0C (Appendix II). The primer pair used is given as under:

REP1 5'- TCGCTCAAAACAACGACACC -3'

The images were analyzed with *Pyelph* application (Pavel and Vasile, 2012) and further hierarchical clustering and discriminatory index of REP-Patterns was carried out respectively by using Dice + UPGMA and discriminator index calculator tool (Bikandi *et al.,* 2004).

3.6 Restriction fragment length polymorphism (RFLP) of *fla*A gene

Restriction fragment length polymorphism of *fla*A PCR products digested by *Dde*I, *Hinf*I and *Dpn*II was carried out (Nachamkin *et al.,* 1993; Owen and Leeton, 1999; Fitzgerald *et al.,* 2001; Ertas *et al.,* 2009). The restriction enzymes were used as per the recommendation of the manufacturer (New England Biolabs). Briefly, 10µl of PCR product was added with nuclease free water (5µl), 10x Buffer (2µl) and 0.2µl of restriction enzymes (*Dde*I, *Hinf*I and *Dpn*II) (0.2 µl = to 10 U/µl of Restriction enzyme). The mixture was mixed gently and incubated in water bath at 37°C for 3 h. The digest was resolved on 1.5% agarose gel prepared in 1X TBE buffer containing 0.5µg/ml of ethidium bromide (Appendix-II). 100bp DNA ladder was used as molecular marker. The digested products were electrophoresed for 1 h at 100V. The gel was then visualized under UVP gel documentation system (BioDoc-It Imaging System).

The images were analyzed with *Pyelph* application (Pavel and Vasile, 2012) and the resultant binary matrix was subjected to one of the agglomerative hierarchical clustering (UPGMA) method along with (Dice) similarity coefficient (Bikandi *et al.,* 2004). Further the discriminatory index of RFLP amplified

products was calculated using discriminator power calculator tool (Bikandi *et al.,* 2004).

3.7 Antimicrobial sensitivity test and MIC determination

Antibiotic sensitivity testing was done as per the method described by Bauer *et al.* (1966) following the guidelines of Clinical laboratory standard institute (CLSI) against 24 antibiotics of different classes with necessary modifications. As per the guidelines of CLSI, (2010) and European committee on antimicrobial susceptibility testing (EUCAST, 2012); 0.5 McFarland concentration of *Campylobacter* inoculum is to be swabbed over Muller-Hinton agar supplemented with 5% sheep blood for antibiotic sensitivity test. However, variations in standard disk diffusion method have been approved by CLSI for *Campylobacter* isolates (Beek *et al.,* 2010). Due to poor visibility of zone of inhibition on M-H plates; for the present study antibiotic sensitivity test was performed on mCCDA with *Campylobacter* supplement V (Appendix-I). On this medium the visibility of zones of inhibition was clearer. Briefly, isolates were grown in Preston enrichment broth with *Campylobacter* supplement IV (Appendix-I) at 42°C for 24 h under microaerophillic conditions. The cultures were standardized to 0.5 McFarland concentrations. The broth culture was then swabbed on mCCDA plate with the help of sterile cotton swab. The plates were allowed to dry. Antibiotic disc or antibiotic Ezy MIC™ strips as per the test were placed on the agar surface within 15 minutes of inoculation of plates. The plates were incubated in microaerophillic condition 42°C for 24 h. Zone of inhibition for an antibiotic were interpreted as per the standards defined by Clinical laboratory standards institute (CLSI, 2011).

3.8 Antibiotic discs (Hi-media)

In the present investigation various categories and generations of antibiotics according to their different mechanism of action were used in order to study the antibiogram for *C. jejuni* isolates. The antibiotics used and their concentrations per disc are given in the table 5.

Table 5: List of antibiotics used for antibiogram study against *C. jejuni* isolates obtained in the present study

Sr. No.	Class of antibiotics	Antibiotic/Symbol (interpretation zone in mm- R/I/S)	Concentration (mcg)
Cell wall synthesis inhibitor			
1	1st gen. Cephalosporin	Cephalothin/CEP^{30} (14/15-17/18)	30
2	2nd gen. Cephalosporin	Cefaclor/ CF^{30} (14/15-17/18)	30
3	3rd gen. Cephalosporin	Ceftriaxone/CTR^{30} (19/20-22/23)	30
4	4th gen. Cephalosporin	Cefepime/CPM^{30} (14/15-17/18)	30
5	Beta-lactamase resistant penicillin	Methicillin/ MET^{5} (9/10-13/14)	5
6	Broad spectrum penicillin-	Ampicillin/AMP^{10}(13/14-16/17)	10
7	Narrow spectrum penicillin	Penicillin –G/ P^{10}U) (16/-/17)	10unit
8	Glycopeptides	Vancomycin/ VA^{30} (14/15-16/17)	30
9	Polypeptide	Polymxin –B (PB^{300}U) (11/-/12)	300unit
10	Monobactams	Aztreonam, AT^{30} (15/16-21/22)	30
11	Carbapenems	Imipenem/ IPM^{10} (19/20-22/23)	10
12		Meropenem/ MRP^{10} (13/14-15/16)	10
	Protein synthesis inhibitor (30-S)		
13	1stgen.aminoglycoside	Kanamycin/ K^{30} (13/14-17/18)	30
14	2ndgen.aminoglycoside	Gentamicin/ GEN^{10} (12/13-14/15)	10
15	3rdgen.aminoglycoside	Amikacin/ AK^{30} (14/15-16/17)	30
16	Tetracycline	Tetracycline/ TE^{30} (14/15-18/19)	30
17		Minocycline/ MI^{30} (14/15-18/19)	30
	Protein synthesis inhibitor (50-S)		
18	Macrolide	Erythromycin/E^{15} (13/14-22/23)	15
19	Phenicoles	Chloramphenicol/C^{30} (12/13-17/18)	30
	DNA synthesis inhibitor		
20	1st gen quinolone	Nalidixic acid/ NA^{30} (13/14-18/19)	30
21	2nd gen quinolone	Ciprofloxacin/CIP^{5} (15/16-20/21)	5
22		Norfloxacin/ NX^{10} (12/13-16/17)	10
23	3rd gen quinolone	Ofloxacin/ OF^{5} (12/13-15/16)	5
	RNA synthesis inhibitors		
24	Rifampicin	Rifampicin/ RIF^{5} (16/17-18/19)	5

3.9 Antibiotic Ezy MIC™ Strip (Hi-Media)

The antibiotic Ezy MIC™ Strips mentioned in (Table 6) were used for the determination of minimum inhibitory concentrations.

Table 6: List of antibiotics Ezy MIC™ strips used to determine minimum inhibitory concentration (MIC) against *C. jejuni* isolates

Sr. No.	Antibiotics Ezy MIC™ strips (Symbol)	Strip content (mcg/ml)	Interpretative criteria in mcg conc. (S/I/R)
1.	Amikacin (AMK)	0.016-256	<16/32/>64
2.	Erythromycin (ERY)	0.016-256	<0.5/1-4/ >8
3.	Chloramphenicol (CHL)	0.016-256	<2 /4/ >8
4.	Ciprofloxacin (CPH)	0.016-256	<1/ 2/ >4
5.	Gentamicin (HLG)	0.016-256	<4 /8/ >16
6.	Ofloxacin (OFX)	0.002-32	<2/ 4/ >8

3.10 Multiple antibiotic resistance index (MAR) value

All Multidrug resistant isolates were evaluated for their multiple antibiotic resistance (MAR) index. In an effort for risk assessment of MDR isolates this index was calculated as per method given by Krumperman, (1983).

MAR Index of single isolate = a/b, where a -represents the number of antibiotics to which the isolate was resistant and b -represents the number of antibiotics to which the isolate was exposed.

3.11 Detection of antibiotic resistance genes

Detection of antibiotic resistance genes responsible for resistance to tetracycline, aminoglycosides, fluoroquinolones and sulphonamide was done. The primer sets used in PCR along with their annealing temperatures for detection of different genes is given in table 7. Rest of the conditions of PCR used is given in appendix II. In addition genes coding for associated resistance mechanisms i.e. efflux of antibiotics and integrons were detected using PCR. The conditions for PCR are given in appendix II. Their primers sequence, annealing

temperature and reference from which the primers were taken are mentioned in the Table 7.

Table 7: PCR primers and conditions for detection of genes related to antibiotic resistance

S. no	Antibiotic resistance gene	Gene name	Primer sequence	A. temp	Size (bp)	reference
Antibiotic Resistance genes						
1	Tetracycline resistance genes	*tet*O	F-AACTTAGGCATTCTGGCTCAC R-TCCCACTGTTCCATATCGTCA	56°C	515	Abdi-Hachesoo *et al.* (2014)
2		*tetA*	F-GTAATTCTGAGCACTGTCGC R-CTGCCTGGACAACATTGCTT	57°C	956	Wilkerson *et al.* (2004)
3		*tetB*	F-CTCAGTATTCCAAGCCTTTG R-ACTCCCCTGAGCTTGAGGGG	52°C	414	
4		*tetC*	F-GGTTGAAGGCTCTCAAGGGC R-CCTCTTGCGGGATATCGTCC	62°C	505	
5		*tetD*	F-CATCCATCCGGAAGTGATAGC R-GGATATCTCACCGCATCTGC	57°C	485	
6		*tetE*	F-TGATGATGGCACTGGTCA R-GCTGGCTGTTGCCATTA	57°C	262	
7		*tetG*	F-GCAGCGAAAGCGTATTTGCG R-TCCGAAAGCTGTCCAAGCAT	62°C	662	
8	Aminoglycoside resistance genes	*aph*3	F-TTCTAGCCACGACCAAAAAG R-CGTGAGCCATAAAGTCTAGC	56°C	363	This study
9		*strA*	F-CCAATCGCAGATAGAAGGC R-CTTGGTGATAACGGCAATTC	55°C	286	Scholz *et al.* (1989)
10		*aadA2*	F-ATTTGCTGGTTACGGTGACC R-CTTCAAGTATGACGGGCTGA	59°C	451	
11	Fluoro-quinolones resistance genes	*gyr*A	F -GAAGAATTTTATATGCTATG R-TCAGTATAACGCATCGCAGC	50°C	235	Chatur *et al.* (2014)
12		*gyr*B	F-ATGGCAGCTAGAGGAAGAGA R-GTGATCCATCAACATCCGCA	53°C	382	
13		*parC*	F-CTATGCGATGTCAGAGCTGG R-TAACAGCAGCTCGGCGTATT	59°C	285	
14	Sulphonamide resistance genes	*Sul1*	F-TGAGATCAGACGTATTGCGC R-TTGAAGGTTCGACAGCACGT	58°C	406	Aarestrup *et al.* (2003); Perreten and Boerlin (2003).
15		*Sul2*	F-GCGCTCAAGGCAGATGGCATT R-GCGTTTGATACCGGCACCCGT	70°C	225	
16		*Sul3*	F-GAGCAAGATTTTTGGAATCG R-CATCTGCAGCTAACCTAGGGCTTTGG	53°C	799	
Multidrug resistance determinants genes						
17	Efflux pump (*cme*ABC operon) genes	*cme*R ABC, Strain	F-CAATCTTCAATCAGGGGCAA R-TCGCAAAAAGAGTGCACATA	56°C	625	This study
18	Integron genes	*int*1F *int*1R`	F-CCTCCCGCACGATGATC R-TCCACGCATCGTCAGGC	55°C	280	Moura *et al.* (2007)
19		*int*2F *int*2R	F-TTATTGCTGGGATTAGGC R-ACGGCTACCCTCTGTTATC	50°C	233	
20		*int*3F *int*3R	F-AGTGGGTGGCGAATGAGTG R-TGTTCTTGTATCGGCAGGTG	50°C	600	

3.12 Sequence analysis of selected isolates for antibiotic resistance and virulence associated genes

The sequence of PCR products of *gyr*A and *iam*AB genes were sequenced (DNA Sequencing Facility, Delhi University). The sequences obtained were subjected to nucleotide BLAST (Basic Local Alignment Search tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the similarity with the already prevalent gene sequences. The sequences were also aligned using Bio edit and MEGA6 software to study the variations in the nucleotide sequences and their phylogenetic cluster analysis.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

Thermophilic *Campylobacter* spp*.* are able to colonize gastrointestinal tract of wide variety of hosts. Most common thermophilic *Campylobacter* found are *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari* and *Campylobacter upsalinesis.* Amongst the thermophilic *Campylobacter*, *C. jejuni* is responsible for the majority (80-90%) of gastrointestinal tract infections and is a leading food borne pathogen followed by *C. coli* (Biswas *et al.,* 2011). Poultry is considered to be the major reservoir for *C. jejuni*. Requirement of relatively low infective dose and the potentially serious clinical consequences suggest the significance of *Campylobacter* spp*.* as a significant health hazard. Like other bacterial infections, the pathogenicity of the *Campylobacter* is governed by various virulence factors. This study involves the molecular characterization of some of these virulence associated genes.

1. Isolation and identification of *Campylobacter jejuni* isolates

In the present study, 370 cloacal swab samples from poultry were collected and processed for isolation and detection of thermophilic *Campylobacter* (Table 1). Grayish dew-drop like colonies on mCCD agar at 42°C (but not at 25°C) (Fig. 1) were obtained in 75 clinical specimens which was suggestive of the genus *Campylobacter*. These 75 (35 and 40 from two different phases) isolates were of Gram negative spiral rods (Fig. 2) which became coccoid/pleomorphic under high oxygen tension. All the 75 isolates selected were catalase and oxidase positive (Fig. 4 and 5). When subjected to 16S rRNA ribotyping (Fig. 3), out of the 75, only 43 isolates belonged to the genus *Camplyobacter*. The remaining 32 isolates that were able to grow in mCCD agar at 42°C were discarded as environmental/commensal bacteria (Kiess *et al.* 2010; Smith *et al.,* 2015). For speciation of the 43 *Campylobacter* spp. isolates, a battery of secondary biochemical tests and genotypic methods were used. All the isolates hydrolysed hippurate and indoxyl acetate and reduced nitrate. None of the isolates was positive for urease (Fig. 6) and H_2S production (Table 8). All

these growth, biochemical and morphological characteristic were suggestive of *C. jejuni* (Kaplan and Weissfeld, 1994; Allos, 1998).

Table 8: Biochemical test results of *Campylobacter* species

Sr. No.	Tests for phenotypic identification	Results	Remarks
1.	Gram staining	Gram negative	Spiral shaped
2.	Catalase	Positive	Appearance of gas bubble
3.	Oxidase	Positive	Violet color appeared
4.	Hippurate hydrolysis	Positive	Dark purple/blue color appeared
5.	Indoxyl acetate hydrolysis	Positive	Dark blue color appeared
6.	H_2S production	Negative	No change in media
7	Urease	Negative	Yellow color persist, not changed
8.	Nitrate reduction	Positive	Red color developed with precipitation
9.	Growth at 42°C	Positive	Greyish, dew drop like colonies on mCCDA
10.	Growth at 25°C	Negative	Absence of growth

These 43 *Campylobacter* isolates were further subjected to species specific PCR targeting hippurase (*hip*O) gene for *C. jejuni* and asperkinase (*asp*) gene for *C. coli.* All the 43 *Campylobacter* isolates tested were positive for *hip*O gene and negative for presence of *asp* gene and thus, were confirmed to be *C. jejuni* (Fig. 3). *hip*O gene is highly conserved in *C. jejuni* and is absent in other *Campylobacter* species (Amri *et al.,* 2007). Similarly, *asp* gene is only present in *C. coli* (Linton *et al.,* 1997). Thus, these genes are most widely used genes for the identification of *C. jejuni* and *C. coli* respectively.

Campylobacter spp. was detected in 11.23% (43/370) of the collected specimens; all the isolates were *C. jejuni*. Similar, prevalence was also detected by other workers such as 8% by Shweta *et al.* (2009) from poultry and eggs, 13.9% by Rizal *et al.* (2010) from chicken intestine and human stool and 4.9% by Cortez *et al.* (2006) from poultry feces, feather and carcass.

Fig. 1: Growth on mCCD agar plate (greyish dew drop like colonies)

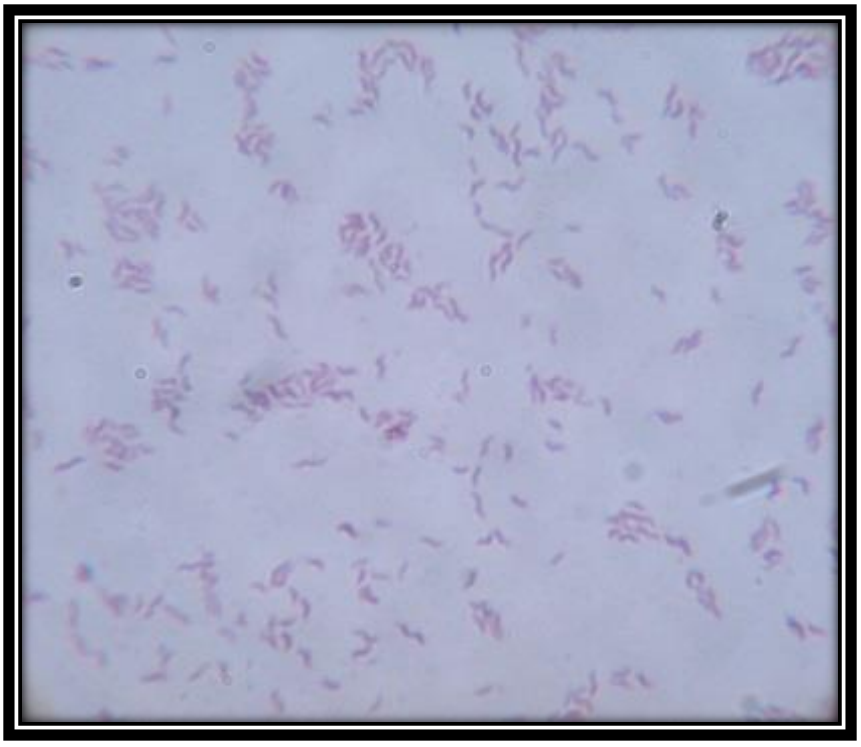

Fig. 2: Gram’s staining (typical ‘S’ shaped curved rods)

Fig. 3: Molecular confirmation of *Campylobacter jejuni* isolates by genus specific 16srRNA gene (816bp) and species specific *hip*O gene (735bp)

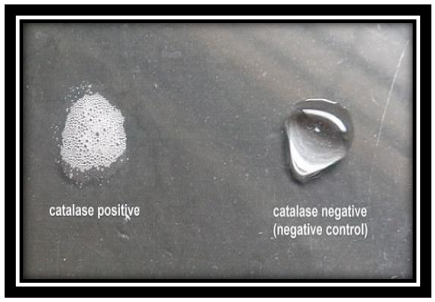

Fig. 4: Catalase test (+ve reaction)

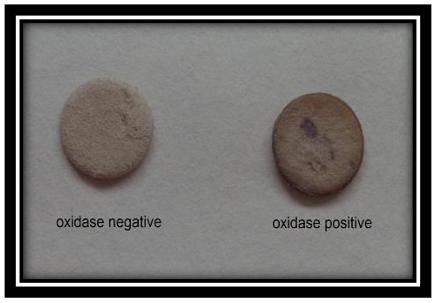

Fig. 5: Oxidase test (+ve reaction)

Fig. 6: Urease test (-ve reaction)

Similar to our observation absence or low prevalence of *C. coli* then *C. jejuni* was detected by Feizabadi *et al.* (2007), Chae *et al.* (2012), Ghorbanalizadgan *et al.* (2014), Ramonaite *et al.* (2014) and Vaishnavi *et al.* (2015). Whereas, Wieczorek (2010) detected higher (54.5%) prevalence of *C. coli* than *C. jejuni* (45.5%) isolates from poultry carcass. Marinou *et al.* (2012) also isolated 14 *C. coli* isolates from 860 samples of chicken carcass and didn't confirm any of the isolates to be *C. jejuni*.

2. Molecular typing of *C. jejuni* by REP-PCR

REP-PCR is a DNA amplification-based method that targets known, conserved, repetitive DNA sequences that are usually present in bacterial genomes in multiple copies (Lupski and Weinstock, 1992). REP-PCR was first, described by van Belkum *et al.* (1995) and involves PCR-mediated genomic fingerprinting using interspersed repetitive sequences. REP-PCR has high discriminatory index. Being a rapid, cost-effective and efficient method, it is suitable for molecular epidemiology studies.

In the present investigation, 12 different REP patterns with band size ranging from 80 to 700bp in different arrangements (Fig. 7) were recorded amongst 43 isolates (Table 9) with a discriminatory index (D.I.) of 0.9181 by Dice+UPGMA method (Bikandi *et al.,* 2004). Discriminatory power of above 0.5 is considered reliable for discrimination of the isolates. Amongst the REP patterns, REP8 was most common with 7 isolates followed by REP5, REP10 and REP12 with five isolates each. REP3, REP6 and REP7 had four isolates each and other remaining REP-patterns had less than four isolates. REP4 and REP9 patterns were represented by single isolates only. The maximum number of four bands were produced by REP11 and REP12 that ranged from 80-700bp. The assay was considered reliable for performing molecular epidemiology of C*. jejuni* from various sources. During phylogenetic cluster analysis of *C. jejuni* REP-patterns, all 12 REP-patterns could be divided into three clusters at 80% of genetic similarity (Fig. 8). First cluster comprised maximum number of 20 isolates and six REP-patterns (REP2, REP5, REP6, REP9, REP10 and REP12), while second

cluster comprised of ten isolates and three REP patterns (REP1, REP3 and REP7) and third cluster had 13 isolates and three REP-patterns (REP4, REP8, and REP12) (Table 10). The cluster analysis showed that isolates from two different phase of sampling were randomly distributed indicating persistence of similar *C. jejuni* strains in the environment over a long period of time.

Table 9: Banding pattern of different *C. jejuni* isolates in REP-PCR

Sr. No.	REP pattern	Band size	Isolate number	Number of isolates
1	REP1	200	C30,C42	2
2	REP2	300	C4,C6,C33	3
3	REP3	400	C16,C24,C34,C39	4
4	REP4	100,250	C14	1
5	REP5	100,300	C5,C20,C32,C36,C40	5
6	REP6	150,300	C2,C11,C22,C31	4
7	REP7	200,500	C23,C25,C27,C37	4
8	REP8	250,700	C1,C9,C12,C17,C28,C38,C43	7
9	REP9	300,600	C7	1
10	REP10	100,300,600	C3,C13,C19,C26,C41	5
11	REP11	80,100,300,600	C10,C21	2
12	REP12	100,250,300,700	C8,C15,C18,C29,C35	5
Number of strains:		**43**		
Number of types:		**12**		
Discriminatory index:		**0.9181**		

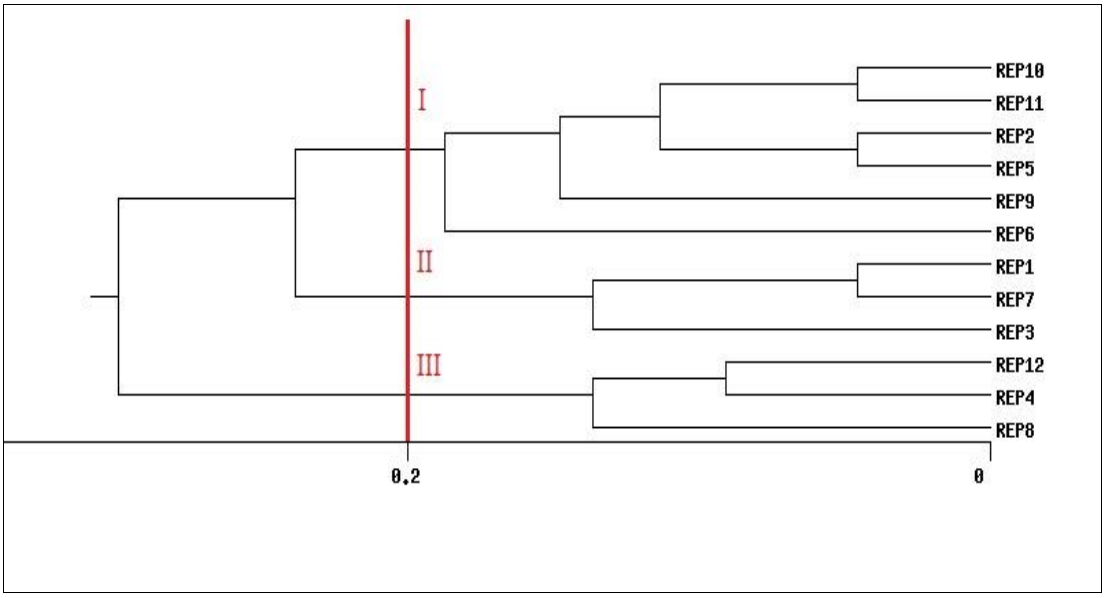

Fig.8: Dendrogram of REP-PCR patterns of *C. jejuni*

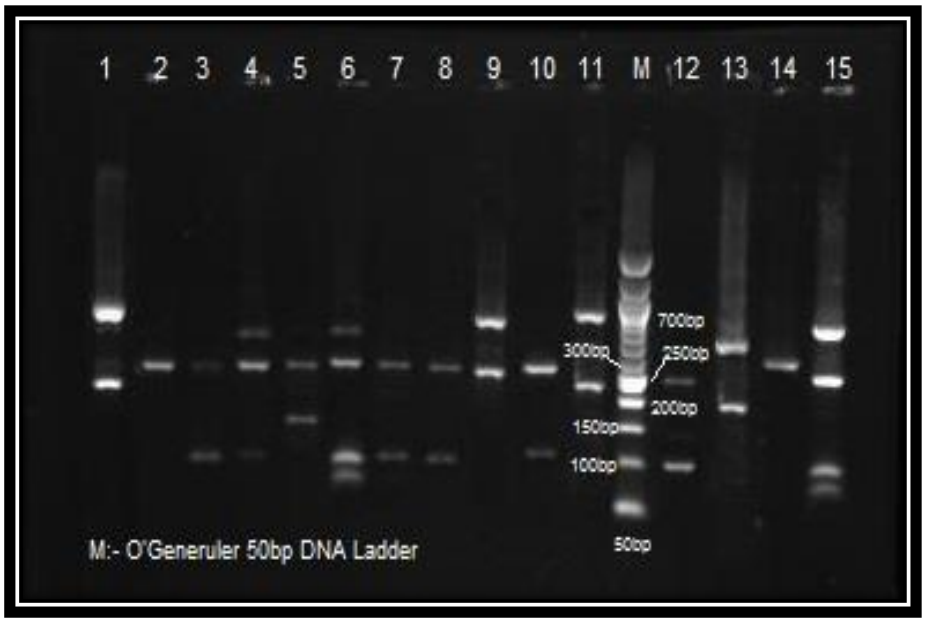

Fig. 7: Detection of various REP-patterns of *C. jejuni*

Table 10: Cluster analysis of *C. jejuni* REP-PCR

Sr. No.	Clusters	REP pattern	Isolate number	Number of isolates
1	Cluster I	REP2,REP5,REP6,REP9 REP10,REP12	C2,C3,C4,C5,C6,C7,C10,C11 C13,C19,C20,C21,C22,C26, C31,C32,C33,C36,C40,C41	20
2	Cluster II	REP1,REP3,REP7	C16,C23,C24,C25,C27,C30, C34,C37, C39,C42	10
3	Cluster III	REP4,REP8,REP12	C1,C8,C9,C12,C14,C15,C17, C18,C28, C29,C35,C38,C43	13

Similar to our observations, Hiett *et al.* (2006) obtained four to eight bands ranging from 400 to 5000bp from a set of 48 *Campylobacter* isolates from various sources and geographical regions. In their study discriminatory index (DI) of REP-PCR was determined to be 0.8364 at 90% similarity values. Isolates recovered from fecal samples were closely related (>90% similarity) to isolates recovered from the processed carcasses. Likewise, Prapas *et al.* (2012) also identified 9 subgroups amongst 12 isolates with discriminatory power of 0.8917. Behringer *et al.* (2011) detected 100% typeability amongst 100 *Campylobacter* isolates (*C. jejuni* and *C. coli*) producing 29 distinct profiles, with five of the profiles identified in both species. Twelve types contained only one isolate, whereas three types (h, k and m) had more than 10 isolates each with random distribution of the isolates irrespective of strain, source and area. REP-PCR technology has advanced to the point of eliminating agarose slab gels by employing microfluidic devices for resolution of fluorescent labeled amplicons (Healy *et al.,* 2005) minimizing gel-to-gel and lab-to-lab variations, maximizing reproducibility and incorporation of bioinformatics tool for online data collection and analysis (Hiett *et al.,* 2006).

3. PCR-RFLP analysis of *fla*A gene sequence

The flagellin gene locus of *C. jejuni* contains *fla*A gene which is arranged in tandem, is highly conserved and has variable regions (Khoshbakht *et al.*, 2013), therefore making it suitable for RFLP analysis. In the present study, PCR amplification of *fla*A gene sequence was performed for all the 43 isolates of *C.*

jejuni producing 1725bp amplicons. The amplicons were further subjected to restriction endonuclease digestion with *ddeI*, *hinfI* and *dpnII*.

3.1 PCR- RFLP of *fla*A gene digested with *Dde*I enzyme

To find the nucleotide polymorphism in the *fla*A gene, RFLP analysis of *fla*A gene was carried out with nucleotide site specific restriction endonuclease *Dde*I (3'..C^TA..5' and 5' AT^C..3'). Restriction enzyme digestion produced 15 different *DdeI*-RFLP patterns with band size ranging from 200 to 1100 bp and discriminatory index of 0.9258 (Fig. 9). The discriminatory index of *DdeI*-RFLP was high for *C. jejuni* isolates. On the basis of observed discriminatory index, both *DdeI*-RFLP (D.I.-0.9258) and REP-PCR (D.I.-0.9181) were efficient in discriminating *C. jejuni* isolates. Out of the total 15 *DdeI*-RFLP types, *Dde*9 was the most common type having 9 isolates followed by *Dde*4 having 4 isolates while other remaining *Dde* types containing less than four isolates (Table 11). The number of bands produced varied from one to nine with ranging from 80bp to 1100bp.

Table 11: RFLP analysis of *fla*A gene sequence digested by *Dde*I

Sr. No.	*Dde* pattern	Band size	Isolate number	Number of isolates
1	*Dde*1	200	C5	1
2	*Dde*2	80,180	C40,C43	2
3	*Dde*3	100,180,250	C7,C35	2
4	*Dde*4	180,200,1100	C3	1
5	*Dde*5	180,220,1100	C6,C8,C9	3
6	*Dde*6	100,180,220,350	C26,C27,C29,C31	4
7	*Dde*7	100,180,220,900	C33,C34	2
8	*Dde*8	100,180,220,280,600	C28,C30,C32	3
9	*Dde*9	100,180,220,300,600	C1,C4,C12,C13,C14, C17,C18,C20,C21	9
10	*Dde*10	100,180,220,280,700	C10,C11,C15,C38	4
11	*Dde*11	100,220,280,350,500	C22,C23,C24	3
12	*Dde*12	80,100,180,200,250,350	C16,C25,C39	3
13	*Dde*13	80,100,180,220,900,1000	C2,C19,C36	3
14	*Dde*14	100,120,180,200,250,350,500	C41,C42	2
15	*Dde*15	100,180,220,300,350,550,700,800 ,1100	C37	1
Number of strains: 43 **Number of types: 15** **Discriminatory power: 0.9258**				

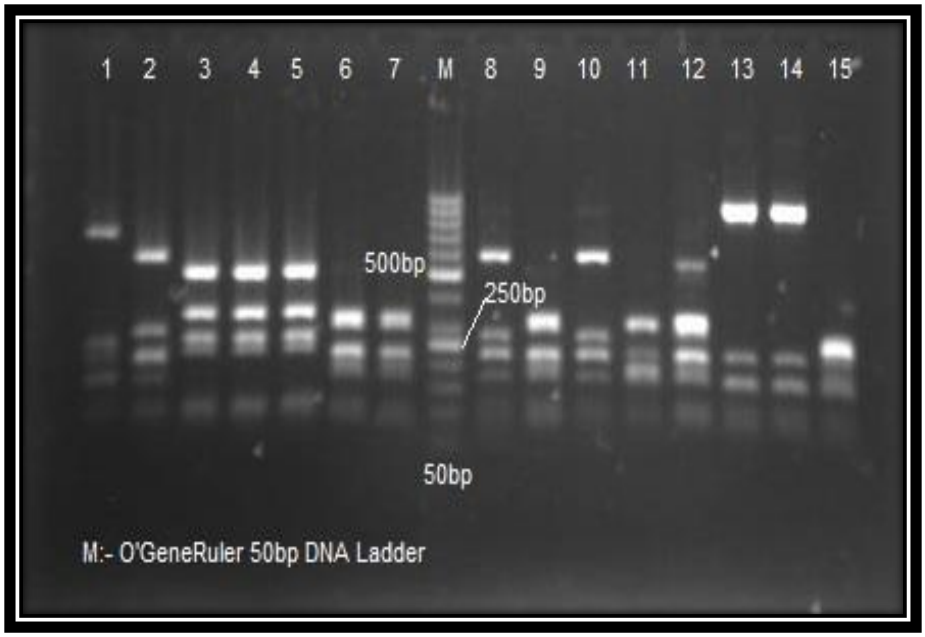

Fig. 9: Distribution of *Dde*-RFLP patterns of *fla*A gene amplicon of *C. jejuni*

During phylogenetic cluster analysis of *C. jejuni*, all 15 *DdeI*-RFLP patterns grouped in six clusters (Fig. 10). First and second cluster had a single isolate (C5 and C37), with a single *Dde* pattern, *Dde*1 and *Dde*15 respectively. Third cluster comprised of maximum 28 isolates with seven *Dde* patterns (*Dde*6, *Dde*7, *Dde*8, *Dde*9, *Dde*10, *Dde*11 and *Dde*13), fourth cluster included seven isolates and three *Dde* patterns (*Dde*3, *Dde*12 and *Dde*14), fifth cluster had two isolates and single *Dde* pattern and sixth cluster possessed four isolates and two *Dde* patterns (Table 12).

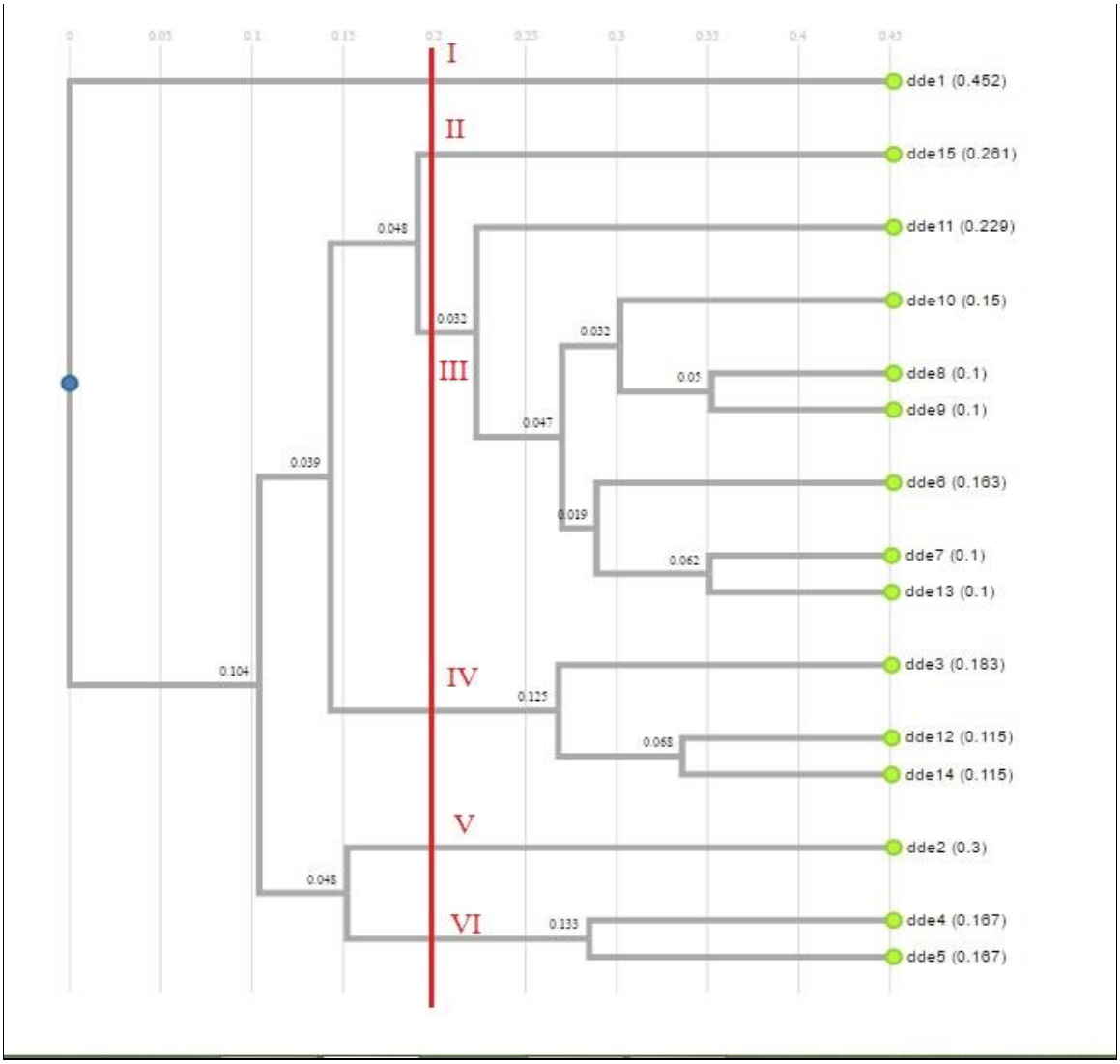

Fig. 10: Dendrogram of *Dde*I-based RFLP patterns of *C. jejuni fla*A gene

Table 12: Cluster analysis of *C. jejuni* on the basis of *Dde*-RFLP patterns

Sr. No.	clusters	(*fla*A-RFLP) *Dde* pattern	Isolate number	Number of isolates
1	Cluster I	*Dde*1	C5	1
2	Cluster II	*Dde*15	C37	1
3	Cluster III	*Dde*6,*Dde*7,*Dde*8, *Dde*9,*Dde*10,*Dde*1 1*Dde*13	C1,C2,C4,C10,C11,C12,C13,C14,C 15,C17,C18,C19,C20,C21,C22,C23, C24,C26,C27,C28,C29,C30,C31,C3 2,C33,C34,C36,C38	28
4	Cluster IV	*Dde*3,*Dde*12, *Dde*14	C7,C16,C25,C35,C39,C41,C42	7
5	Cluster V	*Dde*2	C40,C43	2
6	Cluster VI	*Dde*4,*Dde*5	C3,C6,C8,C9	4

3.2 PCR- RFLP of *fla*A gene digested with *Hinf*I enzyme

RFLP analysis of *Hinf*I (5'..G^ATC..3' and 3'..CTA^G..5') digested *fla*A gene, produced six different patterns with a discriminatory index of 0.6977 (Fig. 11, Table13) and hence was less efficient as compared to REP-PCR and *Dde*I digested PCR- RFLP of *fla*A gene.

Table 13: RFLP analysis of *fla*A gene sequence digested by *Hinf*I

Sr. No.	RFLP pattern	Band size	Isolate number	Number of isolates
1	*Hinf*1	100	C5	1
2	*Hinf*2	120,500,1000	C1,C4,C12,C13,C14,C17,C18,C26,C27C28, C29,C30,C31,C33,C34,C35,C36,C39,C42	19
3	*Hinf*3	150,500,1100	C3,C6,C8,C9,C10,C11,C15,C16,C20, C21,C38,C43	12
4	*Hinf*4	100,150,200,500,800	C2,C7,C19,C22,C23,C24,C25,C40,C41	9
5	*Hinf*5	120,150,200,350,1000	C37	1
6	*Hinf*6	150,200,300,500,1000	C32	1
Number of unrelated strains: 43 **Number of types: 6** **Discriminatory power: 0.6977**				

Out of the total six *Hinf*-RFLP patterns obtained by digestion with *Hinf*I enzyme, *Hinf*2 was the most common pattern observed (Table 13). The number of bands produced varied from one to five with size ranging from 100bp to 1000bp. *Hinf*-RFLP based phylogenetic cluster analysis of *C. jejuni* revealed three clusters (on the basis of 80% genetic similarity) (Fig. 12). First cluster had a single isolate *i.e*. C5 and only single *Hinf*1 pattern. Second cluster comprised of maximum 22 isolates and three *Hinf* patterns (*Hinf*3, *Hinf*4 and *Hinf*6) and third cluster included 20 isolates and two *Hinf* patterns (*Hinf*2 and *Hinf*5) (Table 14).

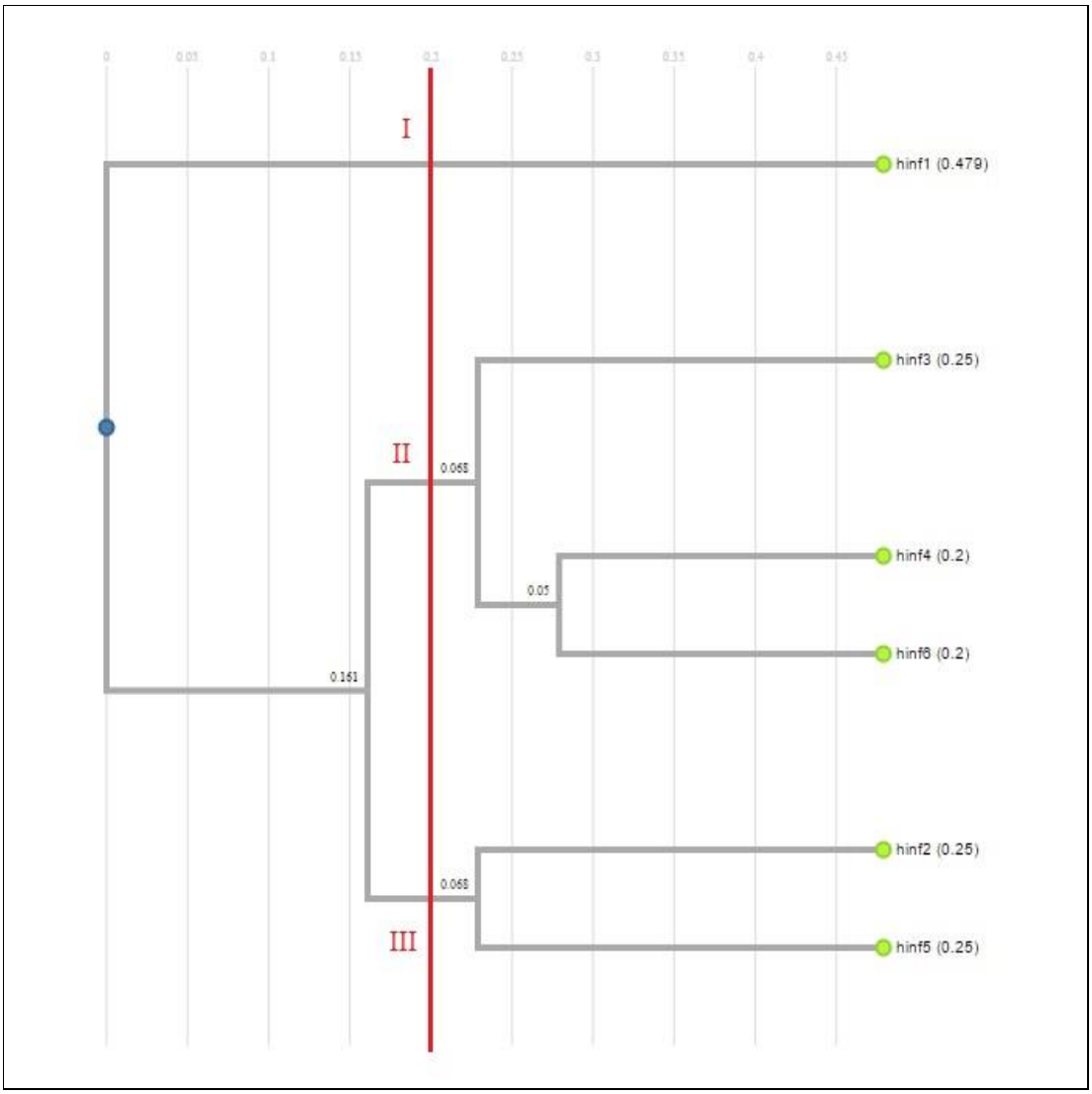

Fig. 12: Dendrogram of *Hinf*I-based RFLP patterns of *C. jejuni fla*A gene

Table 14: Cluster analysis of *C. jejuni* on the basis of *Hinf*-RFLP patterns

Sr. No.	clusters	(*fla*A-RFLP) *Hin*f pattern	Isolate number	Number of isolates
1	Cluster I	*Hinf*1	C5	1
2	Cluster II	*Hinf*3, *Hinf*4, *Hinf*6	C2,C3,C6,C7,C8,C9,C10,C11,C15 C16,C19,C20,C21,C22,C23,C24, C25, C32,C38,C43,C40,C41	22
3	Cluster III	*Hinf*2, *Hinf*5	C1,C4,C12,C13,C14,C17,C18,C26 C27,C28,C29,C30,C31,C33,C34, C35,C36, C37,C39,C42	20

3.3 PCR- RFLP of *fla*A gene digested with *Dpn*II enzyme

*Dpn*II-based RFLP analysis of *fla*A gene revealed seven different *Dpn*-RFLP patterns with discriminatory index of 0.8427 (Fig. 13, Table15), and had slightly lower discrimination as compared to REP-PCR and *Dde*I-based PCR-RFLP analysis.

Table 15: RFLP analysis of *fla*A gene sequence digested by *Dpn*II

Sr. No.	RFLP pattern	Band size	Isolate number	Number of isolates
1	*Dpn*1	250,500,700	C4,C5,C10,C11,C15,C17,C19, C20	8
2	*Dpn*2	200,250,500,800	C1,C12,C13,C18,C21,C23,C26, C28,C30,C33,C34,C37	12
3	*Dpn*3	75,250,500,900	C2,C7,C8,C22,C24,C25,C31	7
4	*Dpn*4	150,250,500,700,900,1300	C3,C9,C32,C40,C43	5
5	*Dpn*5	150,250,500,900	C6,C14,C16,C41,C42	5
6	*Dpn*6	250,500,900	C27,C29	2
7	*Dpn*7	150,500,700	C35,C36,C38,C39	4
Number of unrelated strains: 43 **Number of types: 07** **Discriminatory power: 0.8427**				

Out of the total six *Dpn*-RFLP pattern obtained by digestion with *Dpn*II enzyme, *Dpn*2 was the most common pattern which was observed in 12 isolates followed by *Dpn*1 having eight and *Dpn*3 with seven isolates. Remaining *Dpn* patterns had less than five isolates. The number of bands produced varied from

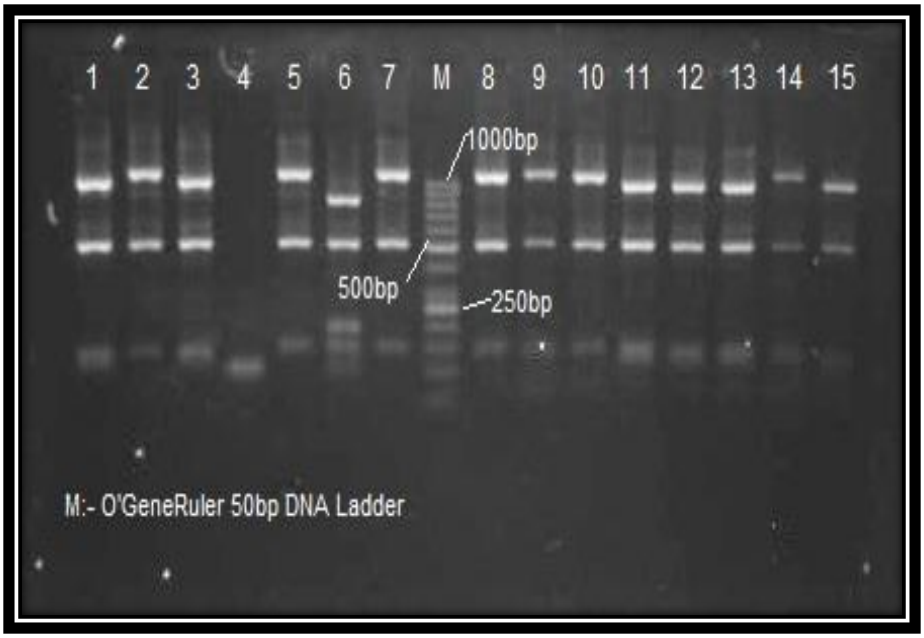

Fig. 11: Distribution of *Hinf*-RFLP patterns of *fla*A gene amplicon of *C. jejuni*

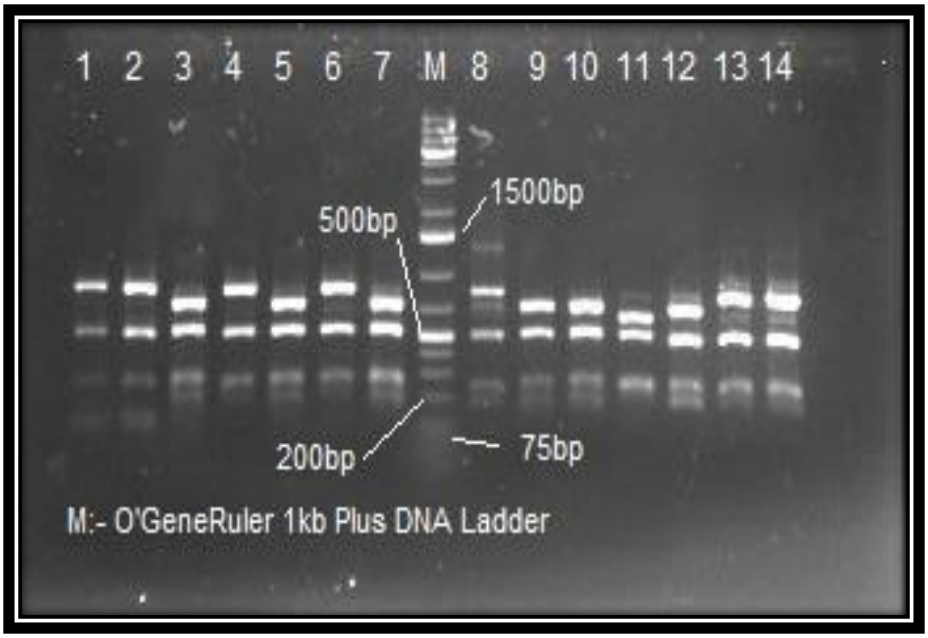

Fig. 13: Distribution of *Dpn*-RFLP patterns of *fla*A gene amplicon of *C. jejuni*

three to six with amplicons size ranging from 75bp to 1300bp. *Dpn*II-based RFLP analysis of *C. jejuni* revealed seven patterns which were divided into five clusters on the basis of 80% genetic similarity (Fig. 14). First cluster had 12 isolate with a single *Dpn*2 pattern. Second cluster comprised of five isolates and single *Dpn*4 pattern, third cluster had maximum 14 isolates and 3 *Dpn* patterns (*Dpn*3, *Dpn*5 and *Dpn*6), fourth cluster possessed eight isolates and single *Dpn*1 pattern and fifth cluster comprised four isolates and single *Dpn*7 pattern (Table 16).

Table 16: Cluster analysis of *C. jejuni* on the basis of *Dpn*-RFLP patterns

Sr. No.	Clusters	*Dpn* pattern	Isolate number	Number of isolates
1	Cluster I	*Dpn*2	C1,C12,C13,C18,C21,C23,C26,C28, C30,C33,C34,C37	12
2	Cluster II	*Dpn*4	C3,C9,C32,C40,C43	5
3	Cluster III	*Dpn*3,*Dpn*5, *Dpn*6	C2,C6,C7,C8,C14,C16,C22,C24,C25, C27,C29,C31,C41,C42	14
4	Cluster IV	*Dpn*1	C4,C5,C10,C11,C15,C17,C19,C20	8
5	Cluster V	*Dpn*7	C35,C36,C38,C39	4

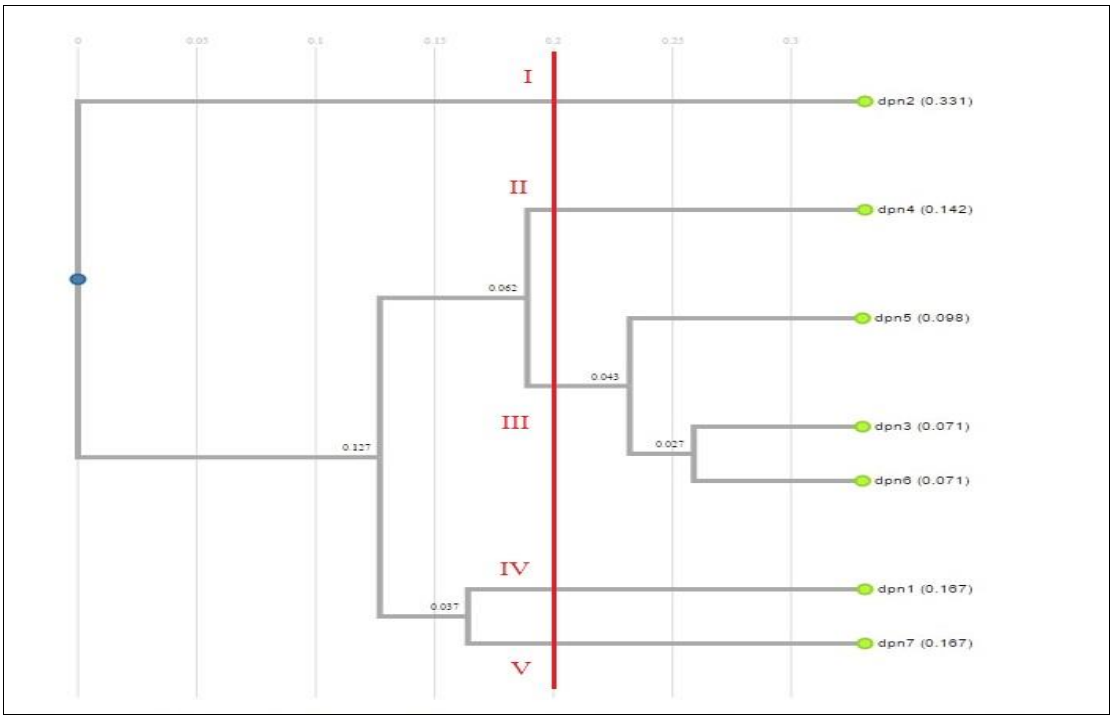

Fig. 14: Dendrogram of *Dpn*II-based RFLP patterns of *C. jejuni fla*A gene

Dde-based RFLP (D.I.-0.9258) was most efficient in discriminating *C. jejuni* isolates compared to *Hinf*-RFLP (D.I.-0.6977) and *Dpn*-RFLP (D.I.-0.8427). Hiett *et al.* (2006) also got similar results. Likewise, Rivoal *et al.* (1999), Khoshbakht *et al.* (2014) and Rajagunalan *et al.* (2014) also obtained high number of RFLP patterns when *flaA* PCR products were subjected to *Dde*I digestion. Fitzgerald *et al.* (2001) also observed that *Dde*I enzyme (35 band patterns) was more discriminatory as compared to *Hinf*I, *Eco*RI and *Pst*I (26 band patterns) for *C jejuni* from wide range of animal hosts. Aydin *et al.* (2007) also was of the opinion that *Hinf*I alone didn't have good discriminatory power, but discrimination level could be enhanced by combining *Dde*I with *Hinf*I (Harrington *et al.*, 2003). In contrast to our observation, Ertas *et al.* (2009) carried out *fla*A-RFLP with five different restriction enzymes (*Alu*I, *Dde*I, *Hinf*I, *Eco*RI and *Pst*I) alone or in combination and found that RFLP patterns of *Pst*I were more discriminatory for *C. jejuni*.

4. Characterization of virulence-associated factors of *Campylobacter jejuni*

Presence of virulence-associated genes of *C. jejuni* viz. bacterial adherence to intestinal mucosa (*cad*F, *cap*A, *jlp*A, *por*A and *dna*J), Lipo-oligosacchrides (*wla*N and *waa*C), Motility (*fla*A, *fla*B and *flg*R), invasion (*iam*AB, *cia*B, *pld*A) and ability to produce toxin (*cdt*A, *cdt*B and *cdt*C) were evaluated by PCR amplification of respective gene.

4.1 Adherence (*cad*F, *cap*A, *jlp*A, *por*A and *dna*J)

Campylobacter adhesion protein *Cad*F is a highly conserved 37-kDa outer membrane protein bound to the extracellular matrix (ECM) protein (fibronectin) of the bacteria (Konkel *et al.,* 1999). Previous studies have suggested that *Cad*F deletion mutants had 60% reduction in binding to immobilized fibronectin, and reduced adherence (50%) to intestinal human cells (INT 407) compared to wild-type *C. jejuni* strain (Flanagan *et al.,* 2009). Studies conducted on newly hatched chickens revealed that wild type isolates of *C. jejuni* can readily colonize the cecum of chickens, whereas a *Cad*F null mutant failed to colonize the caecum,

indicating that *Cad*F is required to establish colonization in newly hatched leghorn chickens (Ziprin *et al.*, 1999). *Campylobacter* adhesion protein A (*Cap*A) is an autotransporter and expression of this functional protein is dependent upon frameshifts within a homopolymeric nucleotide tract located near the 5' end of the *cap*A coding region. *cap*A gene knockout reduced the binding of *C. jejuni* to human Caco-2 colorectal adenocarcinoma cells by approximately 30% (Ashgar *et al.,* 2007). This particular protein (*Cap*A) contributes during the initial steps of adherence process but that it is not required for colonization in the broiler chickens (Ashgar *et al.,* 2007). *jlp*A gene is a newly identified adhesion that plays a role in the adherence of *C. jejuni* to HEp-2 epithelial cells (Jin *et al.,* 2011). The adherence of both insertion and deletion mutants of *Jlp*A to HEp-2 epithelial cells was reduced compared with wild type strain. The major outer membrane protein (MOMP) of *C. jejuni* encoded by *por*A gene is a common antigen with the potential to provide broad protection to bacteria. There are three conformational forms of MOMP: folded monomer (35 kDa), denatured monomer (45 kDa), and the native trimer (120 to 140 kDa). Only the folded monomer and the native trimer have pore-forming activities (Zhang *et al.,* 2000). MOMP is involved in ion transport across the bacterial cell wall and adhesion of the bacterium to the intestinal mucosa (Moser *et al.,* 1997). Heat shock protein encoded by *dna*J gene, serve vital roles in normal cell function and thermoregulation. Thermoregulation plays an important role in virulence gene expression in pathogenic bacteria (Lindquist and Craig, 1988). *Dna*J mutants *C. jejuni* isolates had severely retarted growth at 46°C indicating that *dna*J plays an important role in *C. jejuni* thermo tolerance and colonization in chicken, which have comparatively high body temperature (Konkel *et al.,* 1998; Hermans *et al.,* 2011).

*cad*F and *por*A genes were detected respectively in 97.67% and 93.02% of the isolates (Fig. 15). Chae *et al.* (2012) also found 93% of the *C. jejuni* isolates from poultry to possess *por*A gene. Likewise, *jlp*A, *dna*J and *cap*A gene were detected respectively in 90.69%, 88.37% and 51.16% of the isolates (Fig. 15, Table 19). Set of all the five genes, were detected in 44.18% of the isolates (Table 17 and 18).

Prevalence of various virulence factors related to adherence, *cad*f gene has been reported to be found maximum in almost 100% of the isolates (Wieczorek, 2010; Chae *et al.*, 2012; Khoshbakht *et al.*, 2013; Cho *et al.,* 2014) followed by *jlp*A and *dna*J gene in 80-100% isolates (Datta *et al.*, 2003; Biswas *et al., 2*011) respectively. Comparatively, *cap*A gene has been reported to be in lesser number of isolates (Rizal *et al.*, 2010; Andrzejewska *et al.*, 2011).

4.2 Lipo-oligosacchrides (*wla*N and *waa*C)

Lipooligosaccharide (LOS) is a major cell-surface structure expressed by *C. jejuni.* It is an important constituent of the bacterial outer membrane, and acts as a barrier, and maintains cellular integrity (Raetz and Whitfield. 2002). The *Campylobacter* LOS molecule consists of a lipid A moiety and a non-repeating unit of inner and outer core oligosaccharides (OS) (Aspinall *et al.,* 1995; Karlyshev *et al.,* 2005). The outer core of LOS mimics GM1 ganglioside (Oldfield *et al*., 2002; Szymanski, *et al*., 2003). Such molecular mimicry forms a strategy for the avoidance of host immune defenses by *C. jejuni* and therefore has association with the neuropathies Guillain-Barré syndrome and Miller Fisher syndrome (Allos, 1997). It has two main components: a hydrophobic lipid A anchor and an oligosaccharide consisting of a conserved inner core and a variable outer core. LOS is believed to play an important role in adherence, invasion and colonization. The *wla* gene cluster contains genes coding for the biosynthesis of LOS molecules and genes for regulating protein glycosylation whereas *waa*C encodes for heptosyltransferase I and attaches the first heptose (HEp-I) to *Kdo* (Karlyshev *et al.,* 2005). The LOS gene loci from multiple *C. jejuni* strains have been sequenced and grouped into 19 different LOS classes based on the gene content by Gilbert *et al.* (2008).

In the present study, *wla*N gene was detected in 88.37% of the isolates with an amplicons size of 330bp (Fig. 16, Table 19) whereas *waa*C gene was detected in 65.11% of the isolates with single amplicon size of 1029bp (Fig. 16). Dual presence of both the genes, were detected in 60.46% of the isolates (Table 17 and 18) similar to previous observations of Datta *et al.* (2003). Contrary, Chae

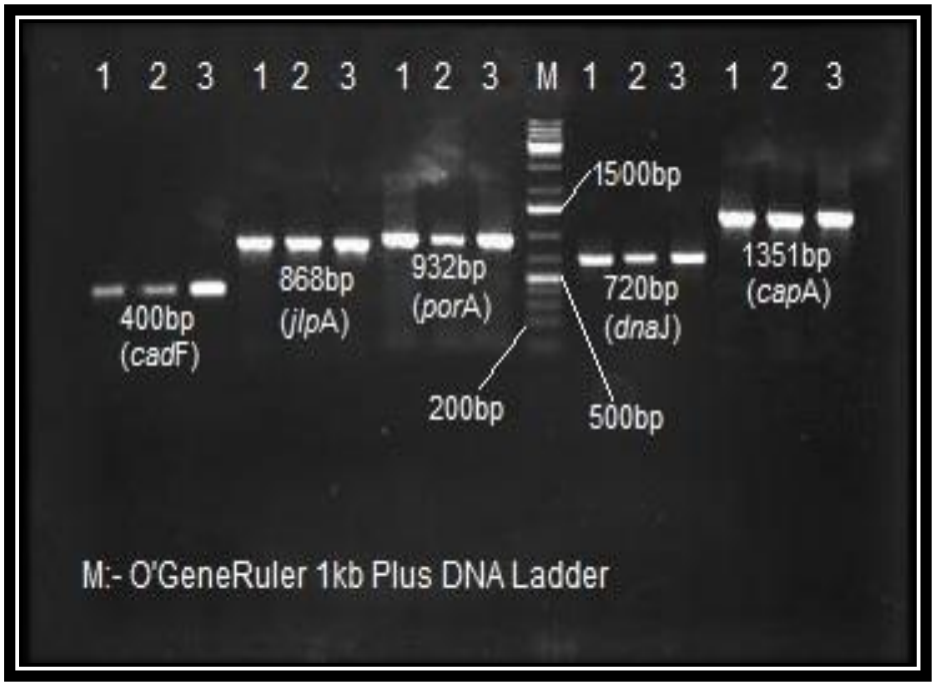

Fig. 15: Detection of adherence associated virulence genes (*cad*F, *jlp*A, *por*A, *dna*J and *cap*A) among *C. jejuni* isolates

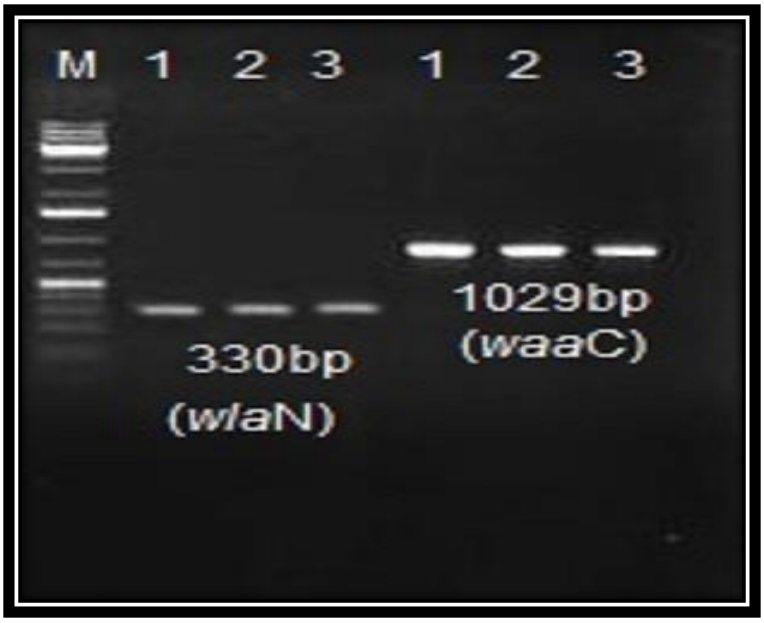

Fig. 16: Detection of Lipo-oligosacchrides virulence genes (*wla*N and *waa*C) among *C. jejuni* isolates

et al. (2012) reported that 90% of the isolates were positive for *wla*N gene (330bp). Likewise, Khoshbakht *et al.* (2013) detected *wla*N gene in 82.22% and Cho *et al.* (2014) in 100% *C. jejuni* isolates.

4.3 Motility (*fla*A, *fla*B and *flg*R)

Like other bacteria, flagellin is an immuno-dominant protein of *C. jejuni* and recognized as a major virulence factor in colonizing the host. Flagellin consists of two subunits encoded by *fla*A (encoding the major flagellin) and *fla*B (encoding a minor flagellin), both are subjected to antigenic and phase variation and mediate motility, colonization, and invasion of gastrointestinal tract (Ghorbanalizadgan *et al.,* 2014). Since *fla*A is expressed at a higher level, the flagellums consist normally of *fla*A. Expression of the two genes is differentially regulated. Some strains of *C. jejuni* strain express only *fla*A, and are fully motile, while *fla*B is not expressed. However, bacteria expressing *fla*B have been isolated and these are less motile than *fla*A expressed flagella. The function of the flagellin B in flagella is not yet known. It has been suggested that the second flagellin gene may serve as a donor, of which parts would be introduced into *fla*A to compensate for mutations (Wassenaar *et al.,* 1995). The *flg*R gene in *C. jejuni* is responsible for regulating flagellar expression and phase variation through slip strand mutagenesis in its poly (A/T) tracts (Wilson *et al.,* 2010).

Of the total 43 isolates, *fla*A was detected in 100% isolates with an amplicon size of 1725bp (Fig. 17, Table 19). Previous studies also detected *fla*A gene in 100% of the *C. jejuni* isolates from various sources of poultry (Datta *et al.*, 2003; Ertas *et al.,* 2004; Rizal *et al.*, 2010; Wieczorek, 2010; Andrzejewska *et al.,* 2011; Yang *et al.,* 2014; Cho *et al.,* 2014). The *fla*B gene was detected in 72.09% of the isolates with an amplicon of 1670bp (Fig. 17) (Table 17 and 18) contrary to what has been reported by, Chae *et al.* (2012) (100%). The *flg*R gene was detected in 69.76% isolates with an amplicon size of 390bp (Fig. 17). Modi *et al.* (2015) detected *flg*R gene in all the isolates. 55.81% of the isolates from present study had all three genes.

The *fla*A gene coding for flagella in addition to motility also functions as a type III secretion apparatus for the *Campylobacter* invasion antigens (*Cia* proteins) important for in vitro cell invasion (Konkel *et al.*, 2004) and chick colonization (Ziprin *et al.*, 2001). Thus presence of *fla*A gene in 100 percent isolates is indicative that all the isolates had pathogenic potential.

4.4 Invasion (*iam*AB, *cia*B and *pld*A)

Campylobacter related pathogenesis depends on the ability of the organism to invade the epithelial cells of the host gastrointestinal tract. One of the markers found to be involved in invasion by *Campylobacter* is invasion associated marker (*Iam*) which helps in colonization of multiple hosts (Al-Mahmeed *et al.,* 2006; Wieczorek *et al.,* 2012). Invasion associated marker (*Iam*) is 1.6 kb genetic marker having ABC transporter (*iam*A) gene and integral membrane protein (*iam*B) gene and have been found to be associated with adherence and invasion of HEp-2 cells *in vitro* (Carvalho *et al.,* 2001). *cia*B gene is required for secretion of other *Campylobacter* invasion antigens (*Cia* proteins) (Konkel *et al.,* 1999). *Cia*B protein is translocated into the cytoplasm of host cells, suggesting that it is a true effector molecule facilitating invasion (Ghorbanalizadgan *et al.,* 2014). The *pld*A gene encodes for phospholipase A protein having homology with *Escherichia coli* outer membrane phospholipase (Grant *et al.,* 1997). *Pld*A encoded protein is localized in outer membrane thus is involved in maintenance of the functional integrity of the surface exposed adhesions in *Campylobacter* strains (Ziprin *et al.*, 2001). *cia*B, and *pld*A genes have been found to be responsible for invasion (Talukder *et al.,* 2008; Yang *et al.,* 2014).

In the present study the *iam*AB (600bp) gene was amplified from 88.37% of the isolates (Fig. 17, Table 19) whereas *cia*B (986bp) and *pld*A (913bp) genes were detected in relatively lesser number of isolates viz. 34.88% and 46.51%, respectively (Fig.17). Four isolates (9.30%) were having all the three genes (Table 17 and 18). Similar observations were reported by Chansiripornchai and Sasipreeyajan, (2009). *pld*A gene was detected only in 13.33% of the isolates by

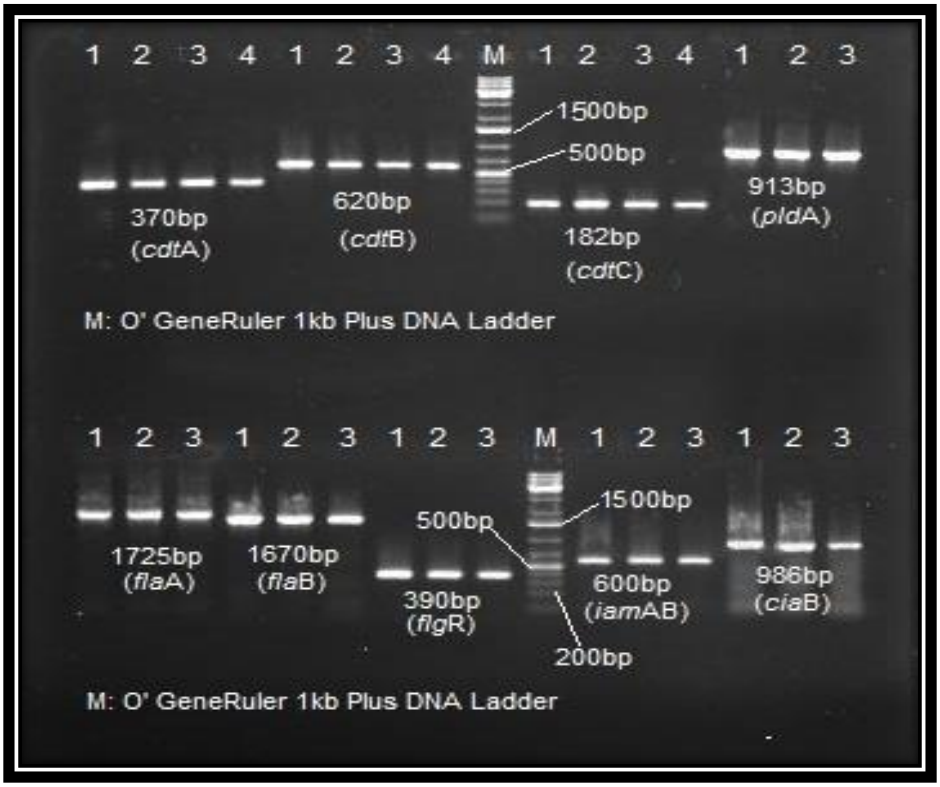

Fig. 17: Detection of flagellar (*fla*A, *fla*B and *flg*R), invasion (i*am*AB, *cia*B and *pld*A) and toxins (*cdt*A, *cdt*B and *cdt*C) associated virulence genes among *C. jejuni* isolates

Rizal *et al.* (2010). Contrary Biswas *et al*. (2011) reported high prevalence of *pld*A (92.16%) and *cia*B (91.18%) genes. Chae *et al.* (2012) and Cho *et al.* (2014) also detected 100% prevalence of *iam*A, *cia*B and *pld*A gene in all isolates. Yang *et al.* (2014) also reported that *pld*A, *iam*A and *cia*B genes were expressed by in 87.5%, 84.7% and 77.8% of the *C. jejuni* isolates from duck, respectively.

Although, all the three genes studied have been reported to be involved in invasion using *in vitro* models but involvement of other factors in invasion cannot be negated (Chansiripornchai and Sasipreeyajan, 2009). Therefore, presence of *cia*B and *pld*A in lesser number of isolates cannot lead to the conclusion that the *Campylobacter jejuni* isolated in the present study have lesser potential to invade the gastrointestinal tract of the host.

4.4.1 Sequence analysis of *iam*AB gene

For further characterization, PCR products of *iam*AB gene were sequenced at DNA Sequencing facility, Delhi University. One of the isolate, C4 from 1st phase of sampling and two isolates (C22 and C23) from IInd phase of sampling were selected for sequencing. Sequences obtained were subjected to nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and sequence similarity of our sequences with other *iam*AB sequences available in the public domain was determined. In order to analyze the point mutations, query sequence were aligned with other sequences (obtained in BLAST) using Bio-Edit and MEGA6 software. Bioinformatics analysis of partial *iam*AB gene revealed 5 nucleotide variations in C4 isolate *viz.* A166G, C255T, C261T, A385G and G426T. A noteworthy difference was present among isolates from two phases of sampling.

For phylogenetic analysis, in addition to three isolates from the current study, we selected 15 sequences (from across the world) of *iam*AB gene from the public domain. The phylogenetic tree constructed using these 18 sequences revealed three major clusters (Fig. 18). All three isolates under study grouped under a separate cluster (cluster III). Though, sequences of *iam*AB gene for other isolates from India are unavailable in the public domain but separation of the

isolates into an entirely different cluster suggests their unique genetic character. The only single poultry isolate (originating from UK) available in the public domain did not cluster together with the isolates under study; rather it grouped under a separate cluster (cluster II). Cluster I and cluster II represented *C. jejuni* isolates from US, Canada, Finland, UK and most of them belonged to humans. Taken together, we first time sequenced *iam*AB gene of *C. jejuni* isolates originating from India and phylogenetic analysis based on it suggest their unique genetic makeup.

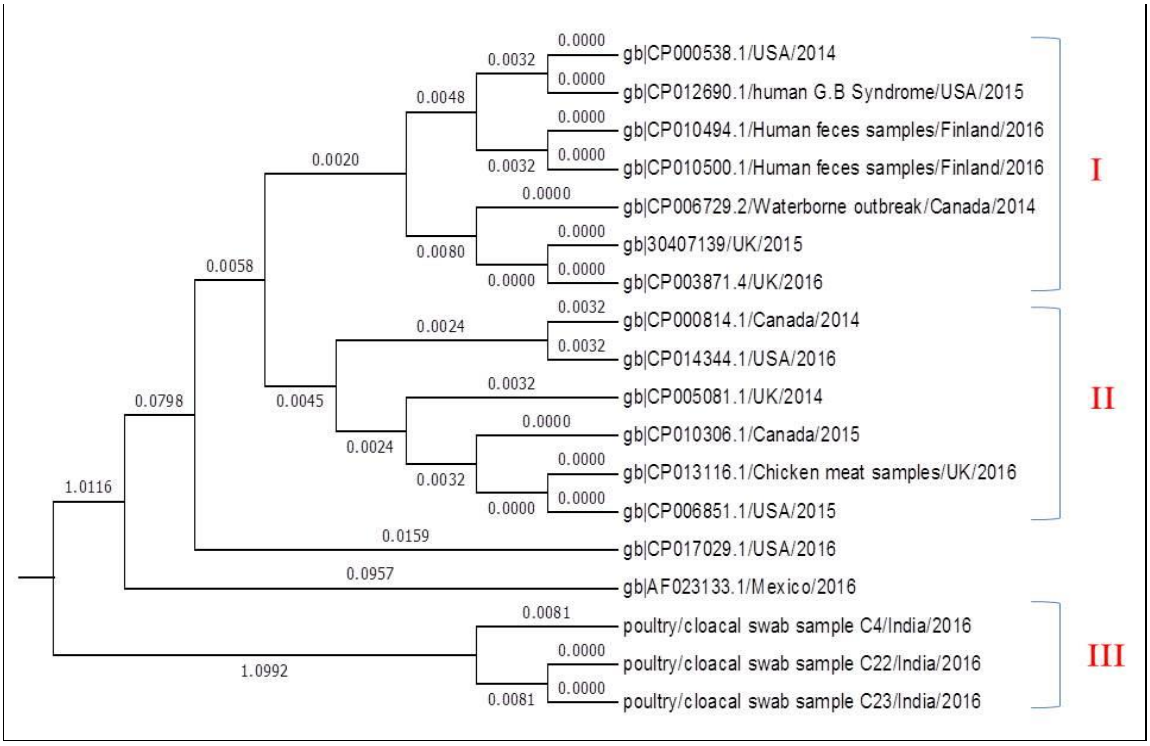

Fig. 18: Phylogenetic analysis of *iam*AB gene sequences

4.5 Toxin (*cdt*A, *cdt*B and *cdt*C)

Campylobacter produce cytolethal distending toxin (CDT) which causes progressive cellular distension ultimately leading to cell death. CDT is a complex coded by three linked genes viz. *cdt*A, *cdt*B, and *cdt*C responsible for three subunits of the toxin that in turn act together to block cell division by performing cell cycle arrest (Ge *et al.,* 2008). C*dt*A and *Cdt*C bind to the cell surface to deliver the active subunit *Cdt*B inside host cell. *Cdt*B uses its DNase-I-like activity to cleave dsDNA molecules of the host during G1 and G2 phase.

Table 17: Detection of virulence associated genes among *C. jejuni* isolates

Isolates	Adherance					Lipo-oligosacchrides		Flagellin			Invasion			Toxins		
	***cad*F**	***cap*A**	***jlp*A**	***por*A**	***dna*J**	***wla*N**	***waa*C**	***fla*A**	***fla*B**	***flg*R**	***iam*AB**	***cia*B**	***pld*A**	***cdt*A**	***cdt*B**	***cdt*C**
C1	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+
C2	+	-	+	-	+	+	+	+	-	+	-	+	-	+	+	+
C3	+	-	+	+	+	-	+	+	-	+	+	-	+	+	+	+
C4	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+
C5	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+
C6	+	-	-	+	+	-	+	+	-	+	+	+	-	+	+	+
C7	+	+	+	+	+	+	-	+	+	+	+	-	+	+	-	+
C8	+	-	+	+	+	+	+	+	-	+	+	+	-	+	+	+
C9	+	-	+	+	+	+	+	+	-	+	+	-	-	+	+	+
C10	+	-	+	-	+	+	+	+	+	+	+	-	-	+	+	+
C11	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
C12	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
C13	-	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+
C14	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
C15	+	-	+	-	-	-	+	+	+	+	+	-	-	+	+	+
C16	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+
C17	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+
C18	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
C19	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
C20	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
C21	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+

Table 18: Detection of virulence associated genes among *C. jejuni* isolates

Isolates	Adherance					Lipo-oligosaccharides		Flagellin			Invasion			Toxins		
	***cad*F**	***cap*A**	***jlp*A**	***por*A**	***dna*J**	***wla*N**	***waa*C**	***fla*A**	***fla*B**	***flg*R**	***iam*AB**	***cia*B**	***pld*A**	***cdt*A**	***cdt*B**	***cdt*C**
C22	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
C23	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+
C24	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
C25	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
C26	+	+	-	+	+	+	-	+	-	-	-	-	-	+	+	+
C27	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
C28	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+
C29	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+
C30	+	-	+	+	+	+	+	+	+	-	+	-	-	+	+	+
C31	+	+	+	+	+	+	-	+	-	-	+	-	+	+	-	+
C32	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+
C33	+	-	+	+	+	+	-	+	+	-	+	+	-	+	+	+
C34	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	+
C35	+	-	+	+	+	-	-	+	+	-	-	-	+	+	-	+
C36	+	-	+	+	-	+	+	+	+	+	+	-	-	+	+	+
C37	+	+	+	+	+	+	+	+	-	-	+	-	+	-	-	+
C38	+	+	-	+	-	+	-	+	-	-	-	-	-	+	-	+
C39	+	+	+	+	+	+	-	+	-	+	+	+	+	-	+	+
C40	+	-	+	+	-	+	-	+	-	-	+	-	-	+	-	-
C41	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
C42	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+
C43	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+

In the present study *cdt*A (370bp) *cdt*B (620bp) and *cdt*C (180bp) genes were detected respectively in 93.02%, 86.04% and 97.67% of the isolates (Fig. 17, Table19). All the three genes were detected in 81.39% of the isolates (Table 18 and 19). Our results corroborated with previous findings by Datta *et al.* (2003), Dipineto *et al.* (2011) and Chae *et al.* (2012) who investigated *C. jejuni* isolates from human, poultry and bovine (>90% detection). According to Martinez *et al.* (2006), all *C. jejuni* strains possess *cdt* genes. However, there may be cases where the detection of *cdt* genes may be difficult because of mutations such as nucleotide deletion, insertion or substitution (Asakura *et al.,* 2007). CDT is usually expressed in those *C. jejuni* strains which are colonizing their natural host, chicken but they don't generate CDT-neutralizing antibodies. Thus, toxin might provide a way to either avoid host immune-response mechanisms or redirect them towards immune tolerance or asymptomatic infections (Abuoun *et al.,* 2005; Muller *et al.,* 2006; Ghorbanalizadgan *et al.,* 2014).

Table 19: Prevalence of virulence associated genes among *C. jejuni* isolates

Sr. No.	Virulence factors	Genes	Amplicon size (bp)	Positive (%), Total isolates (43)
1	Adherence	*cad*F	400	42 (97.67%)
2		*cap*A	1351	22 (51.16%)
3		*jlp*A	868	39 (90.69%)
4		*por*A	932	40 (93.02%)
5		*dna*J	720	38 (88.37%)
6	Lipo-oligosacchrides	*wla*N	330	38 (88.37%)
7		*waa*C	1029	28 (65.11%)
8	Flagellar	*fla*A	1725	43 (100%)
9		*fla*B	1670	31 (72.09%)
10		*flg*R	390	30 (69.76%)
11	Invasion	*iam*AB	600	38 (88.37%)
12		*cia*B	986	15 (34.88%)
13		*pld*A	913	20 (46.51%)
14	Toxins	*cdt*A	370	40 (93.02%)
15		*cdt*B	620	37 (86.04%)
16		*cdt*C	180	42 (97.67%)

5. Virulotyping of *Campylobacter jejuni*

Like other bacteria, *C jejuni* also produce a variety of extracellular, intracellular and cell wall associated virulence factors viz. adherence factors, flagellin, lipo-oligosacchrides, invasion factor and toxins governed by their corresponding genes. In the present study, all 43 isolates were subjected to detection of combination of 16 various virulence factors associated genes (*cad*F, *cap*A, *jlp*A, *por*A, *dna*J, *wla*N, *waa*C, *fla*A, *fla*B, *flg*R, *iam*AB, *cia*B, *pld*A, *cdt*A, *cdt*B, *cdt*C) (Virulotyping) where 33 virulotypes (V1-V33) were identified (Table 21). Maximum 15 virulence genes were detected in V32 (isolate C12 and C22) and V33 (isolate C11, C14, C20, C24 and C27). Lowest numbers of virulence-associated genes were detected in V1 (C38) and V2 (C40). The discriminatory power of virulotyping was calculated as 0.9812 using Dice + UPGMA tool (Bikandi *et al.,* 2004). Frequency of the detection of individual virulence associated genes was used to construct phylogenetic tree by using Dice + UPGMA tool (Bikandi *et al.,* 2004). All the virulotypes divided into seven clusters at 80% genetic similarity (Table. 21 and Fig. 19).

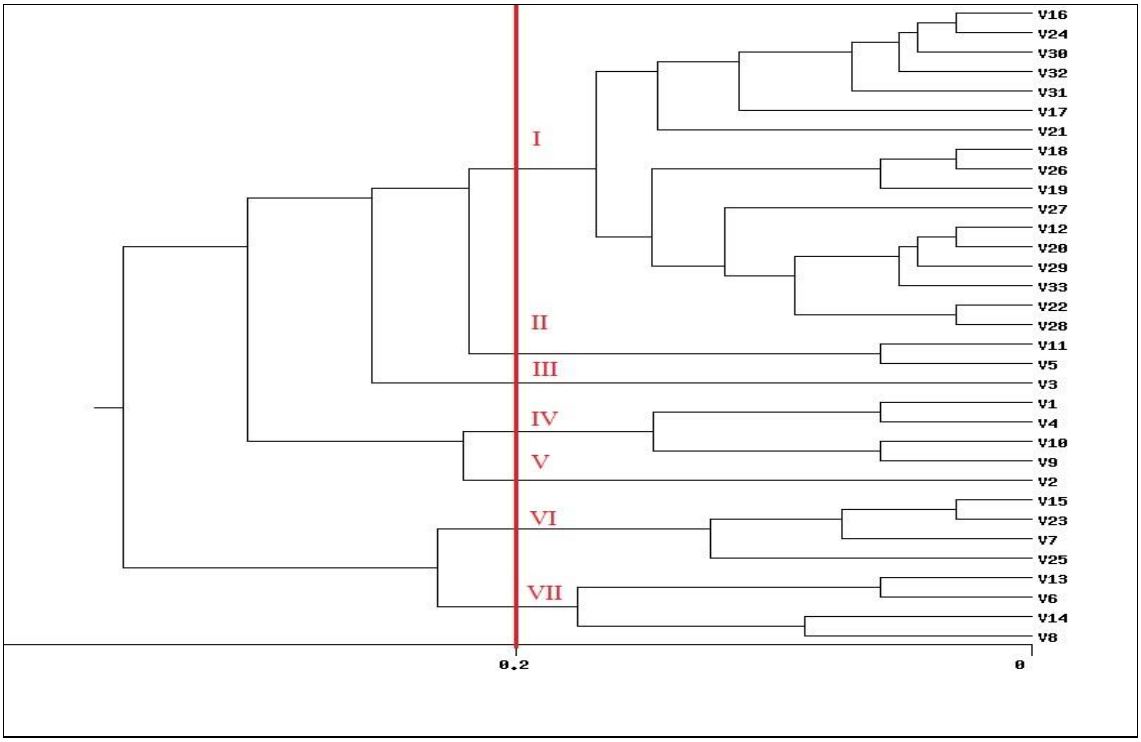

Fig. 19: Phylogenetic cluster analysis of *C. jejuni* virulotype

Table 20: Virulotypes of the *Campylobacter jejuni* isolate

S. No	V pattern	Isolate number	Number of isolates	Virulence genes (16)	Number of genes
1	V1	C38	1	*cad*F,*cap*A,*por*A,*wla*N,*fla*A,*cdt*A,*cdt*C	7
2	V2	C40	1	*cad*F,*jlp*A,*por*A,*wla*N,*fla*A,*iam*AB,*cdt*A	7
3	V3	C35	1	*cad*F,*jlp*A,*por*A,*dna*J,*fla*A,*fla*B,*pld*A,*cdt*A,*cdt*C	9
4	V4	C26	1	*cad*F,*cap*A,*por*A,*dna*J,*wla*N,*fla*A,*cdt*A,*cdt*B,*cdt*C	9
5	V5	C13	1	*jlp*A,*por*A,*dna*J,*fla*A,*fla*B,*iam*AB,*cia*B,*cdt*A,*cdt*B,*cdt*C	10
6	V6	C15	1	*cad*F,*jlp*A,*waa*C,*fla*A,*fla*B,*flg*R,*iam*AB,*cdt*A,*cdt*B,*cdt*C	10
7	V7	C2	1	*cad*F,*jlp*A,*dna*J,*wla*N,*waa*C,*fla*A,*flg*R,*cia*B,*cdt*A,*cdt*B,*cdt*C	11
8	V8	C6	1	*cad*F,*por*A,*dna*J,*waa*C,*fla*A,*flg*R,*iam*AB,*cia*B,*cdt*A,*cdt*B,*cdt*C	11
9	V9	C31	1	*cad*F,*cap*A,*jlp*A,*por*A,*dna*J,*wla*N,*fla*A,*iam*AB,*pld*A,*cdt*A,*cdt*C	11
10	V10	C37	1	*cad*F,*cap*A,*jlp*A,*por*A,*dna*J,*wla*N,*waa*C,*fla*A,*iam*AB,*pld*A,*cdt*C	11
11	V11	C33	1	*cad*F,*jlp*A,*por*A,*dna*J,*wla*N,*fla*A,*fla*B,*iam*AB,*cia*B,*cdt*A,*cdt*B,*cdt*C	12
12	V12	C10	1	*cad*F,*jlp*A,*dna*J,*wla*N,*waa*C,*fla*A,*fla*B,*flg*R,*iam*AB,*cdt*A,*cdt*B,*cdt*C	12
13	V13	C36	1	*cad*F,*jlp*A,*por*A,*wla*N,*waa*C,*fla*A,*fla*B,*flg*R,*iam*AB,*cdt*A,*cdt*B,*cdt*C	12
14	V14	C3	1	*cad*F,*jlp*A,*por*A,*dna*J,*waa*C,*fla*A,*flg*R,*iam*AB,*pld*A,*cdt*A,*cdt*B,*cdt*C	12
15	V15	C9	1	*cad*F,*jlp*A,*por*A,*dna*J,*wla*N,*waa*C,*fla*A,*flg*R,*iam*AB,*cdt*A,*cdt*B,*cdt*C	12
16	V16	C30	1	*cad*F,*jlp*A,*por*A,*dna*J,*wla*N,*waa*C,*fla*A,*fla*B,*iam*AB,*cdt*A,*cdt*B,*cdt*C	12
17	V17	C43	1	*cad*F,*cap*A,*jlp*A,*por*A,*dna*J,*wla*N,*waa*C,*fla*A,*iam*AB,*cia*B,*cdt*B,*cdt*C	12
18	V18	C4,C32	2	*cad*F,*jlp*A,*por*A,*dna*J,*wla*N,*fla*A,*fla*B,*flg*R,*iam*AB,*pld*A,*cdt*A,*cdt*B,*cdt*C	13
19	V19	C28	1	*cad*F,*por*A,*dna*J,*wla*N,*fla*A,*fla*B,*flg*R,*iam*AB,*cia*B,*pld*A,*cdt*A,*cdt*B,*cdt*C	13
20	V20	C23	1	*cad*F,*jlp*A,*por*A,*dna*J,*wla*N,*waa*C,*fla*A,*fla*B,*flg*R,*iam*AB,*cdt*A,*cdt*B,*cdt*C	13
21	V21	C39	1	*cad*F,*cap*A,*jlp*A,*por*A,*dna*J,*wla*N,*fla*A,*flg*R,*iam*AB,*cia*B,*pld*A,*cdt*B,*cdt*C	13
22	V22	C7	1	*cad*F,*cap*A,*jlp*A,*por*A,*dna*J,*wla*N,*fla*A,*fla*B,*flg*R,*iam*AB,*pld*A,*cdt*A,*cdt*C	13
23	V23	C8	1	*cad*F,*jlp*A,*por*A,*dna*J,*wla*N,*waa*C,*fla*A,*flg*R,*iam*AB,*cia*B,*cdt*A,*cdt*B,*cdt*C	13
24	V24	C34	1	*cad*F,*cap*A,*jlp*A,*por*A,*dna*J,*wla*N,*waa*C,*fla*A,*fla*B,*iam*AB,*cdt*A,*cdt*B,*cdt*C	13
25	V25	C1	1	*cad*F,*jlp*A,*por*A,*dna*J,*wla*N,*waa*C,*fla*A,*fla*B,*flg*R,*cia*B,*pld*A,*cdt*A,*cdt*B,*cdt*C	14
26	V26	C5	1	*cad*F,*jlp*A,*por*A,*dna*J,*wla*N,*fla*A,*fla*B,*flg*R,*iam*AB,*cia*B,*pld*A,*cdt*A,*cdt*B,*cdt*C	14
27	V27	C16	1	*cad*F,*cap*A,*jlp*A,*por*A,*wla*N,*fla*A,*fla*B,*flg*R,*iam*AB,*cia*B,*pld*A,*cdt*A,*cdt*B,*cdt*C	14
28	V28	C42	1	*cad*F,*cap*A,*jlp*A,*por*A,*dna*J,*wla*N,*fla*A,*fla*B,*flg*R,*iam*AB,*pld*A,*cdt*A,*cdt*B,*cdt*C	14
29	V29	C19,C25	2	*cad*F,*jlp*A,*por*A,*dna*J,*wla*N,*waa*C,*fla*A,*fla*B,*flg*R,*iam*AB,*pld*A,*cdt*A,*cdt*B,*cdt*C	14
30	V30	C18,C21 C41	3	*cad*F,*cap*A,*jlp*A,*por*A,*dna*J,*wla*N,*waa*C,*fla*A,*fla*B,*flg*R,*iam*AB,*cdt*A,*cdt*B,*cdt*C	14
31	V31	C17,C29	2	*cad*F,*cap*A,*jlp*A,*por*A,*dna*J,*wla*N,*waa*C,*fla*A,*fla*B,*iam*AB,*cia*B,*cdt*A,*cdt*B,*cdt*C	14
32	V32	C12,C22	2	*cad*F,*cap*A,*jlp*A,*por*A,*dna*J,*wla*N,*waa*C,*fla*A,*fla*B,*flg*R,*iam*AB,*cia*B,*cdt*A,*cdt*B,*cdt*C	15
33	V33	C11,C14 C20,C24 C27	5	*cad*F,*cap*A,*jlp*A,*por*A,*dna*J,*wla*N,*waa*C,*fla*A,*fla*B,*flg*R,*iam*AB,*pld*A,*cdt*A,*cdt*B,*cdt*C	15
Number of unrelated strains: 43 **Number of types: 33** **Discriminatory power: 0.9812**					

Table 21: Virulotype-based cluster analysis of *C. jejuni* isolates

S. No	Clusters	Virulotype pattern	Isolate number	Number of isolates
1	Cluster I	V12,V16,V17,V18,V19,V21 V20,V22,V24,V26,V27, V28,V29,V30,V31,V32, V33	C4,C5,C7,C10,C11,C12,C14 C16,C17,C18,C19,C20,C21, C22,C23,C24,C25,C27,C28, C29,C30,C32,C34, C41,C42	25
2	Cluster II	V5,V11	C13,C33	2
3	Cluster III	V3	C35	1
4	Cluster IV	V1,V4,V9,V10	C26,C31,C37,C38,C39,C43	6
5	Cluster V	V2	C40	1
6	Cluster VI	V7,V15,V23,V25	C1,C2,C8,C9	4
7	Cluster VII	V6,V8,V13,V14	C3,C6,C15,C36	4

6. Determination of Antibiotic resistance pattern and MIC

Indiscriminate use of antibiotics over the last few decades in veterinary and medical practice has resulted in development of antibiotic resistant bacterial strains. Therefore, it is important to study drug resistance in human and animals. All the 43 *C. jejuni* isolates were evaluated for their susceptibility towards 24 different antibiotics (Table 22). The antibiotics used belonged to β-lactam antibiotics, aminoglycosides, carbepenems, cephalosporins, chloramphenicol, fluoroquinolones, glycopeptides, macrolides, monobactam, polypeptide, tetracyclines and rifampicin groups. The diameter of zone of inhibition was recorded as per the guidelines of clinical laboratory standards institute (CLSI). The MIC was defined as the lowest concentration of an antimicrobial agent that completely inhibited visible growth on agar surface. Since, CLSI recommendations do not include specific break points for defining resistance in *Campylobacter* spp.*;* the criteria adopted in our study were those described for *Enterobacteriaceae* (El-Baky *et al.,* 2014). According to zone of inhibition, the isolates were grouped as sensitive, intermediate and resistant (Fig. 20 and Table 22).

Highest (100%) sensitivity was observed for polymxin-B followed by chloramphenicol (97.67%), gentamicin (95.35%), amikacin (88.37%), aztreonam

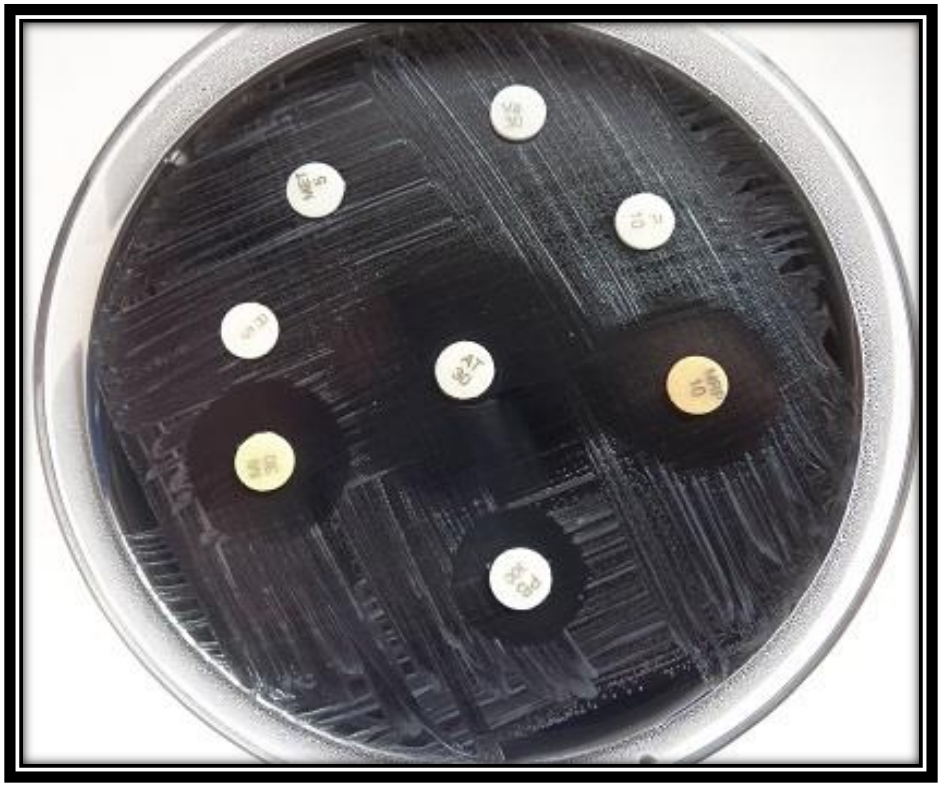

Fig. 20: Antibiotic sensitivity test of *C. jejuni* on mCCDA plate

(83.72%), meropenem and imepenem (76.74%), kanamycin (72.09%), ceftriaxone (65.12%), erythromycin and ampicillin (53.49%). Isolates were 100% resistant to Penicillin-G, methicillin and rifampcin. Relatively lower level of resistance was detected against cephalothin (95.35%), vancomycin (93.02%), ciprofloxacin (90.70%), ofloxacin (79.07%), nalidixic acid (74.42%) and norfloxacin (72.09%). High resistance was reported against β-lactam antibiotics. Similarly, a very high resistance against fluoroquinolones group of antibiotics (ciprofloxacin, ofloxacin, nalidixic acid and norfloxacin) was also seen.

Table 22: Antibiogram of *C. jejuni* isolates against various antibiotics

Sr. No	Class of Drug	Antibiotic	Sensitive (%)	Intermediate (%)	Resistant (%)
Cell wall synthesis inhibitor					
1	**Cephalosporins**	**Cephalothin**	1 (2.33%)	1 (2.33%)	41 (95.35%)
2		**Cefaclor**	11 (25.58%)	7 (16.28%)	25 (58.14%)
3		**Ceftriaxone**	28 (65.12%)	5 (11.63%)	10 (23.26%)
4		**Cefepime**	11 (25.58%)	10 (23.26%)	22 (51.16%)
5	**Penicillins**	**Ampicillin**	23 (53.49%)	8 (18.60%)	12 (27.91%)
6		**Penicillin-G**	0 (0.00%)	0 (0.00%)	43 (100.00%)
7		**Methicillin**	0 (0.00%)	0 (0.00%)	43 (100.00%)
8	**Glycopeptides**	**Vancomycin**	2 (4.65%)	1 (2.33%)	40 (93.02%)
9	**Polypeptide**	**Polymxin–B**	43 (100.00%)	0 (0.00%)	0 (0.00%)
10	**Monobactams**	**Aztreonam**	36 (83.72%)	7 (16.28%)	0 (0.00%)
11	**Carbapenems**	**Imipenem**	33 (76.74%)	7 (16.28%)	3 (6.98%)
12		**Meropenem**	33 (76.74%)	9 (20.93 %)	1 (2.33%)
Protein synthesis inhibitor (30-S)					
13	**Aminoglycoside**	**Kanamycin**	31 (72.09%)	7 (16.28%)	5 (11.63%)
14		**Gentamicin**	41 (95.35%)	2 (4.65%)	0 (0.00%)
15		**Amikacin**	38 (88.37%)	1 (2.33%)	4 (9.30%)
16	**Tetracycline**	**Tetracycline**	17 (39.53%)	13 (30.23%)	13 (30.23%)
17		**Minocycline**	2 (4.65%)	28 (65.12%)	13 (30.23%)
Protein synthesis inhibitor (50-S)					
18	**Macrolide**	**Erythromycin**	23 (53.49%)	14 (32.56%)	6 (13.95%)
19	**Phenicoles**	**Chloramphenicol**	42 (97.67%)	1 (2.33%)	0 (0.00%)
DNA synthesis inhibitor					
20	**Quinolones**	**Nalidixic acid**	0 (0.00%)	11 (25.58%)	32 (74.42%)
21		**Ciprofloxacin**	0 (0.00%)	4 (9.30%)	39 (90.70%)
22		**Norfloxacin**	4 (9.30%)	8 (18.60 %)	31 (72.09%)
23		**Ofloxacin**	6 (13.95%)	3 (6.98%)	34 (79.07%)
RNA synthesis inhibitor					
24	**Rifampicin**	**Rifampicin**	0 (0.00%)	0 (0.00%)	43 (100.00%)

The antibiotic resistance patterns indicate that *C. jejuni* isolates have evolved themselves for resistance to quinolone group of antibiotics. The mutations responsible for such isolates have been described later. But, these results clearly indicate that there has been use of quinolone group of antibiotics leading to evolution of such mutants. Since such mutants are more stable as compared to sensitive isolates quinolone group of antibiotics should be discontinued as far as Campylobacter infections are concerned (Luangtongkum *et al.* 2009).

Resistance against multiple antibiotics was observed by multiple antibiotic resistances (MAR) index which is an epidemiological tool used to assess the risk analysis of environment for bacterial contamination and acquisition of drug resistance through use of multiple antibiotics. If MAR index is greater than 0.2; it implies that strains of such bacteria originated from an environment where several antibiotics have been used (Krumperman, 1983). The average MAR index of the 43 isolates under study was 0.45 (Table 23).

Table 23: Detection of multiple antibiotic resistance index (MAR) value among *C. jejuni* isolates

Sr. No.	MAR Index Value Type (MAR)	Isolate I.D.	No. of Isolate	No. of antibiotic, which the isolate was resistant	Total no of antibiotics	MAR Index Value	Significance
1.	MAR1	C1,C18,C29,C30	4	7	24	0.29	43(100%) isolates had 0.2 or more than 0.2 MAR index value with high risk potential source of spread MDR
2.	MAR2	C25,C28	2	8	24	0.33	
3.	MAR3	C7,C12,C14,C16,C22,C23,C26, C31,C42	9	9	24	0.38	
4.	MAR4	C24,C27,C36,C39,C40,C41	6	10	24	0.42	
5.	MAR5	C4,C5,C21,C37,C43	5	11	24	0.46	
6.	MAR6	C2,C3,C8,C13,C17,C20,C32, C34	8	12	24	0.50	
7.	MAR7	C6,C9,C11,C19,C38	5	13	24	0.54	
8.	MAR8	C35	1	14	24	0.58	
9.	MAR9	C10,C15,C33	3	15	24	0.63	
TOTAL			**43**			**19.17**	
AVERAGE MAR VALUE						**0.45**	

Among 43 isolates nine different MAR values were observed. Three isolates (C10, C15 and C33) had highest 0.63 MAR index. Four isolates (C1, C18, C29 and C30) were resistant to seven antibiotics (minimum) and had MAR index more than 0.2. Nine isolates had MAR index of 0.38. In agreement to our observation, Ghimire *et al.* (2014) detected 77.8% of the isolates with MAR index value >0.2. The results indicate that the poultry from which samples were collected had recent use of antibiotics.

Antimicrobial resistance is increasing day-by-day and is a major problem for diseases management so it is very necessary to know the exact dose of antibiotic which can be used for avoiding indiscriminate or misuse of antibiotic for disease management. Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of antibiotics that will inhibit the visible growth of a microorganism after overnight incubation minimum inhibitory concentrations (MICs) are considered the `gold standard' for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. MICs are used in diagnostic laboratories to confirm unusual resistance, to give a definitive answer when a borderline result is obtained by other methods of testing, or when disc diffusion methods are not appropriate. In the present investigation, all 43 isolates were subjected to MIC determination for six antibiotics by Ezy MIC™ Strip method (Fig. 21). The MIC value for various antibiotics is shown in the Table 24.

Among all isolates, highest MIC value of erythromycin (12mcg/ml) was detected in C23 and C36 isolates and lowest MIC value (2mcg/ml) was found in C31 isolate. The C33 isolate did not form any zone of inhibition so maximum concentration of the strip (256mcg/ml) was considered its MIC value. Highest MIC value of gentamicin (6mcg/ml) was detected in C28 and C30 isolate and lowest MIC value (0.38mcg/ml) was found in C37. Likewise, highest MIC value of chloramphenicol (16mcg/ml) was detected in C25, C30 and C35 isolate and lowest MIC value (3mcg/ml) was found in C3. For amikacin, highest MIC (32 mcg/ml) was detected in C13, C28 and C30 isolate and lowest MIC value (0.5 mcg/ml) in C37. The isolate C30 have highest MIC values for gentamicin,

chloramphenicol and amikacin while C37 have lowest MIC values for gentamicin and amikacin. None of the isolate formed any zone of inhibition for ciprofloxacin and ofloxacin, considered them to be 100% resistant. Erythromycin has highest average MIC value of 5.74mcg/ml followed by chloramphenicol (4.80mcg/ml), amikacin (0.86mcg/ml) and gentamicin (0.23mcg/ml). On the basis of their average MIC value, isolates were detected as sensitive for amikacin and gentamycin, intermediate for erythromycin and chloramphenicol and resistant for ciprofloxacin and ofloxacin.

Similar to our observation Miflin *et al.* (2007) and Bordon *et al*. (2009) recorded resistance for tetracycline in 18.4-19.2% isolates and for ampicillin in 17.6-26% isolates from humans and poultry by disk diffusion and MIC methods. Also, Saenz *et al*. (2000), Bordon *et al*. (2009), Wieczorek (2010) and Wieczorek *et al.* (2012) observed highest resistance (>80% isolates) to quinolone antibiotics. While, 100% isolate susceptibility was detected for gentamicin and erythromycin by Wieczorek *et al.* (2012) and El-Baky *et al.* (2014).

Similarly, Oporto *et al.* (2009) revealed that all *C. jejuni* strains from poultry were susceptible to erythromycin, chloramphenicol, streptomycin, gentamicin and meropenem and multi drug resistance was detected in 21% of the isolates by MIC. Contrarily, Akwuobuv *et al.* (2010) recorded high resistance rates to cephalothin (84%), ampicillin (58%), and low resistance to ofloxacin (5%) and ciprofloxacin (5%) while all the 64 isolate were sensitive to gentamicin. Likewise, Liao *et al.* (2012) detected lower MIC values for both ciprofloxacin and levofloxacin.

Fig. 21: MIC determination of antibiotics against *C. jejuni* by Ezy MIC™ strips

Table 24: MIC of *C. jejuni* isolates for different antibiotics

Sr. No	Source of Isolate	Antibiotic with MIC value in mcg/ml					
		Erythromycin ERY (0.016- 256 mcg/ml)	Gentamicin HLG (0.016- 256 mcg/ml)	Chloramphenicol CHL (0.016-256 mcg/ml)	Amikacin A MK (0.016-256 mcg/ml)	Ciprofloxacin CPH (0.016-256 mcg/ml)	Ofloxacin OFX (0.002-32 mcg/ml)
1	C1	3	0.75	4	2	R	R
2	C2	3	1.5	4	1.5	R	R
3	C3	8	1	3[b]	2	R	R
4	C4	4	1.5	4	1.5	R	R
5	C5	4	1	4	1.5	R	R
6	C6	8	1	4	2	R	R
7	C7	4	1	8	1.5	R	R
8	C8	3	1.5	4	2	R	R
9	C9	6	1	4	1.5	R	R
10	C10	8	0.75	8	1.5	R	R
11	C11	6	1	6	2	R	R
12	C12	6	1	6	4	R	R
13	C13	4	2	6	32[a]	R	R
14	C14	6	1	6	4	R	R
15	C15	8	0.75	8	1.5	R	R
16	C16	6	1	8	1.5	R	R
17	C17	4	1.5	4	4	R	R
18	C18	3	0.75	4	2	R	R
19	C19	8	0.75	8	1.5	R	R
20	C20	6	1	6	4	R	R
21	C21	6	1	6	4	R	R
22	C22	8	0.75	6	8	R	R
23	C23	12[a]	1.5	4	1.5	R	R
24	C24	6	1	4	2	R	R
25	C25	8	0.5	16[a]	16	R	R
26	C26	8	0.5	4	16	R	R
27	C27	6	1.5	4	1.5	R	R
28	C28	8	6[a]	12	32[a]	R	R
29	C29	6	1.5	4	1.5	R	R
30	C30	8	6[a]	16[a]	32[a]	R	R
31	C31	2[b]	4	4	12	R	R
32	C32	4	0.5	4	1.5	R	R
33	C33	R	1	8	1	R	R
34	C34	3	1.5	8	8	R	R
35	C35	3	1.5	16[a]	12	R	R
36	C36	12[a]	1	8	2	R	R
37	C37	6	0.38[b]	8	0.5[b]	R	R
38	C38	8	1.5	6	1.5	R	R
39	C39	4	0.5	4	1.5	R	R
40	C40	3	0.75	4	1.5	R	R
41	C41	4	0.75	4	2	R	R
42	C42	6	0.5	6	1.5	R	R
43	C43	8	1	8	2	R	R
		Average value of MIC of each antibiotic for total isolates					
Total	**43**	**5.74**	**0.23**	**4.80**	**0.86**	**-**	**-**

Superscript: a – Highest value of MIC
b- Lowest value of MIC

Campylobacter species are intrinsically resistant to a number of antibiotics, including cefoperazone, cephalothin, bacitracin, vancomycin, rifampin and trimethoprim (Allos, 2001), some of these are utilized in selective media for isolation. Resistance may be chromosomal or plasmid-borne, and represent a combination of endogenous and acquired genes viz. (i) Modification of the antibiotic's target and/or its expression i.e. DNA gyrase mutations (ii) Inability of the antibiotic to reach its target i.e. expression of the major outer membrane protein or MOMP (iii) Efflux of the antibiotic *i.e.* multidrug efflux pumps such as *cme*ABC (iv) Modification or inactivation of the antibiotic i.e. β-lactamase production (Iovine, 2013).

In the present study high resistance was observed towards fluoroquinolones antibiotics. The resistance to fluoroquinolones is mainly mediated *via* point mutations in the quinolone resistance-determining region (QRDR) of DNA gyrase A (*gyr*A) (Payot *et al.*, 2006; Zhang *et al.,* 2008) with the Thr-86-Ile mutation being most common (Chatur *et al.*, 2014). In addition to the mutations in *gyr*A, *Campylobacter* multidrug efflux (*cme*) pump, *cme*ABC, also contributes to fluoroquinolone resistance by reducing the accumulation of the agents in *Campylobacter* cells (Lin *et al.*, 2002; Luo *et al.*, 2003; Ge *et al.*, 2005).

7. Molecular detection of antibiotic resistance genes in *C. jejuni* isolates

In the present study, PCR amplification of 16 antibiotic resistance-associated genes viz. tetracycline resistance (*tet*O, *tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*G), aminoglycoside resistance (*aph*3, *str*A, *aad*A2), fluoroquinolones resistance (*gyr*A, *gyr*B, *par*C), sulphonamide resistance (*sul*1, *sul*2, *sul*3) and four antibiotic resistance determinants genes such as efflux pump (*cme*RABC), integron (*int*1, *int*2, *int*3) was carried out for all the *C. jejuni* isolates as per the standard procedures. However, we were able to successfully amplify only seven genes (*tet*O, *aph*3, *gyr*A, *gyr*B, *cme*RABC, *int*1 and *int*2) (Table 25).

Table 25: Detection of antibiotic resistance genes and other determinants amongst *C. jejuni* isolates

ANTIBIOTIC RESISTANCE AND ITS DETERMINANTS GENES							
Isolate No.	**Tetracycline**	**Aminoglycosides**	**Fluoro-quinolones**		**Multidrug Efflux Pump**	**Integron**	
	***tet*O**	***aph*3**	***gyr*A**	***gyr*B**	***cme*RABC**	***int*1**	***int*2**
C1	+	+	+	+	+	-	-
C2	+	-	+	+	+	-	-
C3	+	+	+	+	-	-	-
C4	+	+	+	+	+	-	-
C5	+	+	+	+	-	-	-
C6	+	+	+	+	-	-	-
C7	-	-	+	+	-	-	-
C8	+	+	+	+	+	-	+
C9	+	+	-	+	+	-	-
C10	+	+	+	+	+	-	-
C11	+	+	+	+	-	-	-
C12	-	+	+	+	-	-	-
C13	+	+	+	+	-	-	-
C14	+	+	-	+	-	+	-
C15	+	-	+	+	-	-	-
C16	-	+	+	+	-	+	-
C17	+	+	+	+	+	-	-
C18	+	+	+	+	+	+	-
C19	+	+	+	+	+	+	-
C20	+	+	+	+	+	+	-
C21	+	+	+	+	+	+	-
C22	+	-	+	+	+	-	-
C23	+	+	+	+	+	-	+
C24	+	+	+	+	+	-	+
C25	+	+	+	+	+	-	-
C26	+	+	+	+	+	+	-
C27	+	+	+	+	+	+	-
C28	+	+	+	+	+	+	-
C29	-	+	+	+	+	-	-
C30	+	+	+	+	+	-	-
C31	-	-	+	+	-	+	-
C32	+	-	+	+	+	-	-
C33	+	-	+	+	+	-	-
C34	-	+	+	+	+	-	-
C35	-	-	+	+	+	-	-
C36	+	+	+	+	+	-	-
C37	-	-	+	+	+	-	-
C38	+	+	+	+	-	-	-
C39	-	+	+	+	+	+	-
C40	-	-	+	+	+	+	-
C41	-	+	+	+	+	+	-
C42	+	+	+	+	+	-	-
C43	+	-	+	+	+	-	-
Total +ve	**32 (74.41%)**	**32 (74.41%)**	**41 (95.34%)**	**43 (100%)**	**31 (72.09%)**	**13 (30.23%)**	**3 (6.97%)**
Total -ve	**11 (25.58%)**	**11 (25.58%)**	**2 (4.65%)**	**0**	**12 (27.90%)**	**30 (69.76%)**	**40 (93.02%)**
total	**43 (100%)**	**43 (100%)**	**43 (100%)**	**43 (100%)**	**43 (100%)**	**43 (100%)**	**43 (100%)**

The *tet*O (515bp) and *aph*3 (363bp) genes were amplified from majority of the isolates (74.41%), (Fig. 22 and 23) very much similar to the finding of Abdi-Hachesoo *et al.* (2014). Pratt and Korolik (2005) detected *tet*O gene in 100% isolates. Contrarily, Vaishnavi *et al*. (2015) detected *tet*O genes in only 50% of the *C. jejuni* isolates. Although many *tet* (*tet*A, *tet*B, *tet*C, *tet*E, *tet*g, *tet*O) genes are found in plasmid as well as chromosome of various gram positive and grams negative organism but *tet*O is highly prevalent in *Campylobacter* species (Dasti *et al.*, 2007).

*gyr*B gene which codes for proteins responsible for fluoroquinolones resistance was detected in 100% of the isolates by producing amplicon size of 382bp followed by *gyr*A gene in 41 (95.34%) isolates by producing amplicon size of 235bp (Fig. 22). In agreement Chatur *et al.* (2014) also detected *gyr*A and *gyr*B gene in 100% of the isolates. This increasing trend of fluoroquinolone resistance in our and previous studies indicate the need of interventions to limit spread of resistant isolates. Multiple mechanisms associated with antibiotic resistance have been identified in *Campylobacter*, but target mutations and drug efflux are most relevant to the resistance to fluoroquinolones and macrolides (Luangtongkum *et al.,* 2009). Complete gene cassette for efflux pumps i.e*. cme*RABC gene (625bp) was present in 31 (72.09%) isolates (Fig. 24). *Cme*ABC is also a major player in the efflux of bile acids and plays a critical role infacilitating *Campylobacter* colonization of the intestinal tract (Lin *et al.,* 2003). Out of three integron genes only two were amplified; *int*1 (280bp) in 30.23% isolates (Fig. 25) and *int*2 (233bp) in 6.97% of the isolates. These elements are not common in *Campylobacter* and do not considered to play a major role in the horizontal transfer of antibiotic resistance in *Campylobacter*. However, studies by Lee *et al.* (2002) and O'Halloran *et al.* (2004) suggested the integrons-associated antibiotic resistance (aminoglycoside resistance genes (*aad*A2 and *aac*A4), in *C. jejuni* and *C. coli.*

We also performed *gyr*A gene sequence based phylogenetic analysis. In addition to nine isolates from the current study, we selected 27 sequences (from

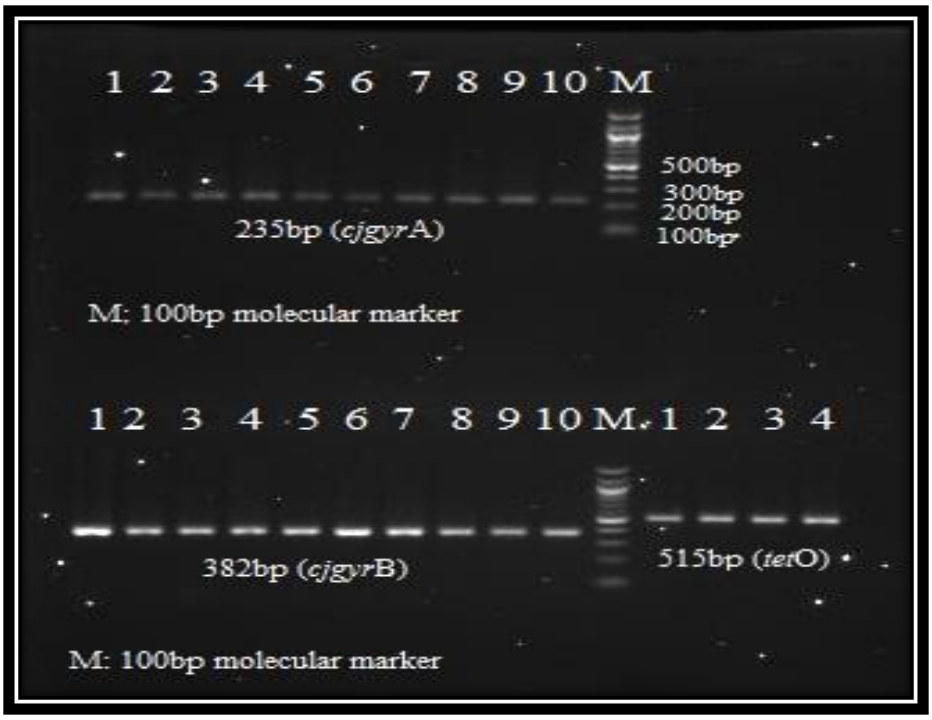

Fig. 22: Detection of fluoroquinolones resistance (*gyr*A and *gyr*B) and tetracycline resistance (*tet*O) genes among *C. jejuni* isolates

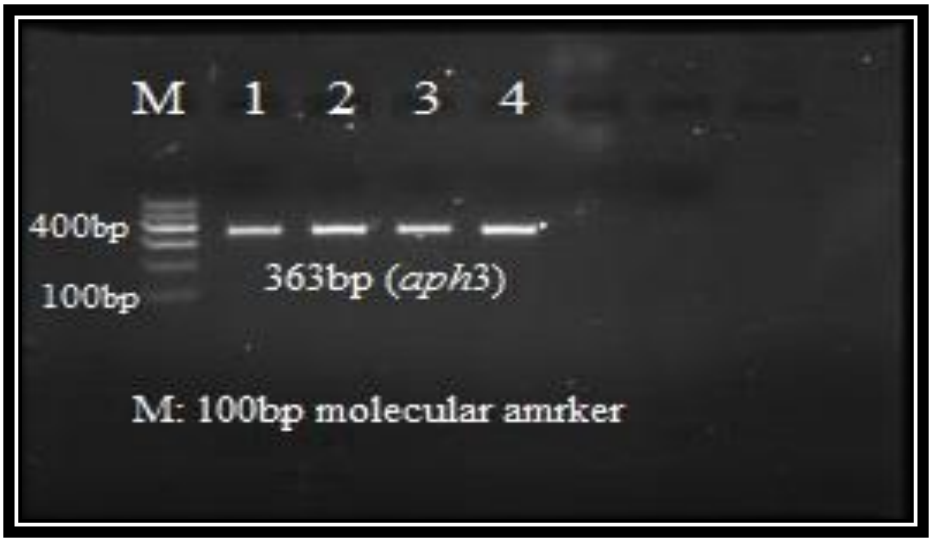

Fig. 23: Detection of aminoglycosides resistance (*aph*3) gene among *C. jejuni* isolates

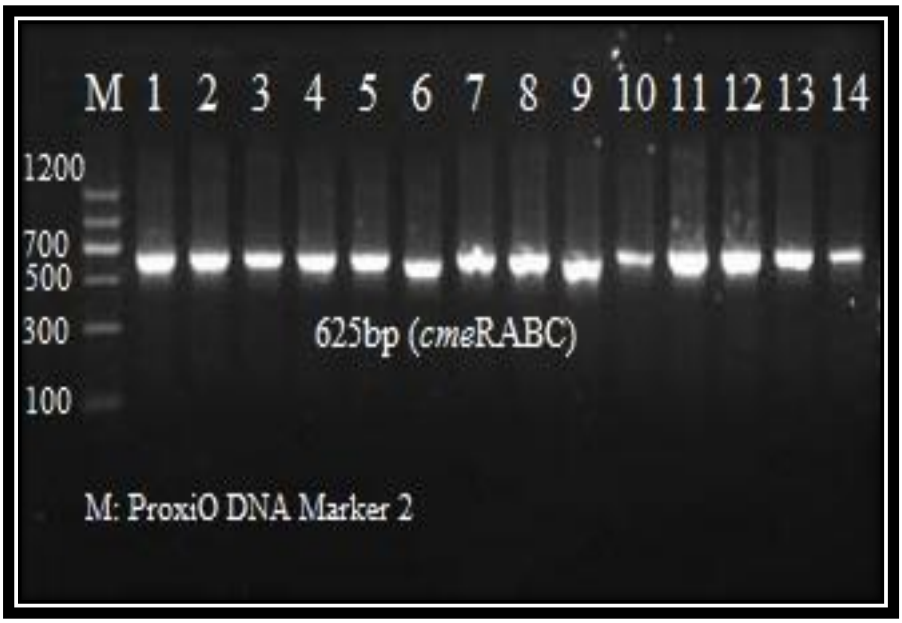

Fig. 24: Detection of multidrug efflux operon (*cme*RABC) gene among *C. jejuni* isolates

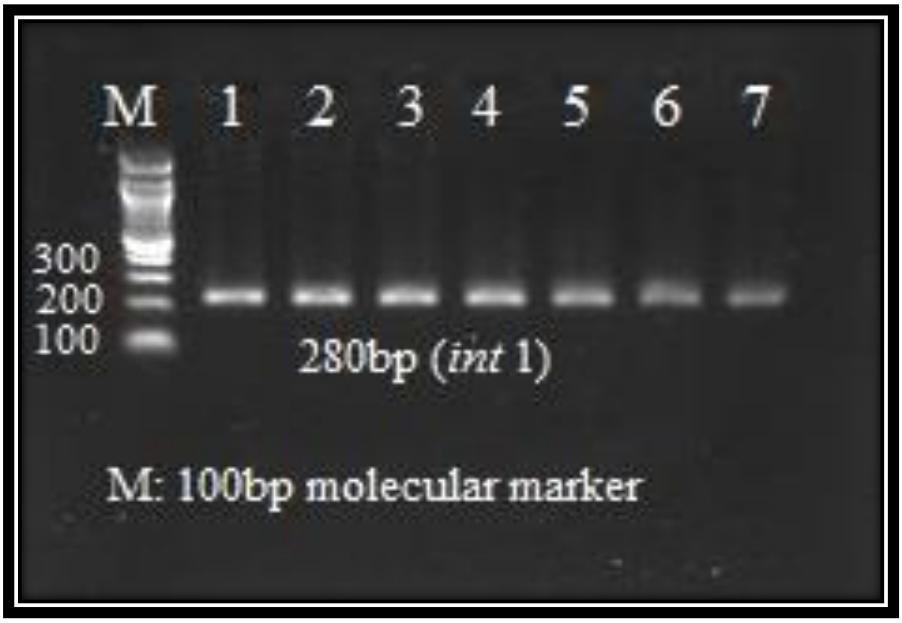

Fig. 25: Detection of integron 1 (*int*1) gene among *C. jejuni* isolates

across the world) of *gyr*A gene from the public domain. The phylogenetic analysis revealed three major clusters (Fig. 26). Five isolates from the present study grouped along with poultry and human isolates from Europe (Austria, Slovenia, Germany, Serbia, Bosnia and Herzegovina), New Zealand and Japan. Rest four isolates (C22, C25, C31, and C32) were grouped under separate cluster (cluster II) that has majority of isolates form USA. The previously reported *gyr*A gene sequences from India were grouped in separate cluster (Cluster III) and didn't represent close homology with the isolates from this study. Taken together *gyr*A gene-based phylogeny represented close homology of our *C. jejuni* isolates with isolates from Europe.

In conclusion, all the thermophilic *Campylobacter spp.* isolated from poultry in the study region were *C. jejuni*. Biochemical tests and genotyping revealed no differences as far as characterization is concerned. Molecular epidemiological tools i.e. RFLP of *fla*A gene and REP-PCR revealed that RFLP of *fla*A gene using *Dde*I and REP-PCR with primer sequences used in the study had comparable discriminatory index. Bacterial adherence associated virulence genes, LOS, flagellin, invasion and cytolethal distending toxin and antibiotic resistance genes were detected at variable rates *viz.* from 50% to 100% among various isolates, therefore represented genetic variability. *iam*AB gene-based phylogenetic analysis suggested unique genetic character of the isolates under study (grouped in entirely different separate cluster). *gyr*A gene-based phylogenetic analysis revealed close homology with isolates from Europe, rather than isolates from India. High MAR values and MDR status of all the isolates is suggestive of excessive use of antibiotics and is a point of concern.

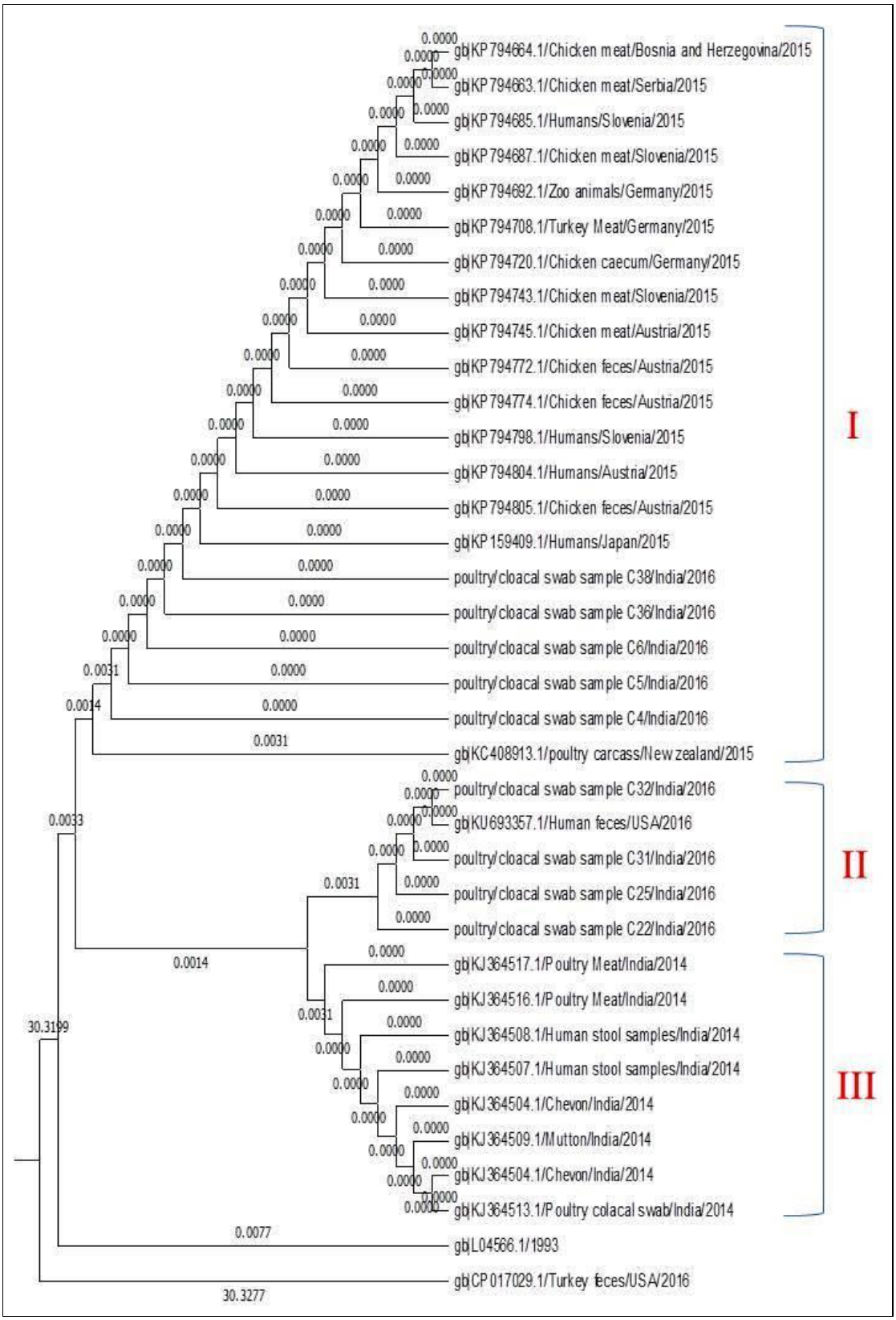

Fig. 26: Phylogenetic analysis of *gyr*A gene sequence

SUMMARY

5. SUMMARY

Campylobacter jejuni is an emerging zoonotic pathogen responsible for gastroenteritis (Campylobacteriosis) in humans and poultry. The spectrum of disease may range from mild, self-limiting, non-inflammatory diarrhea to severe inflammatory bloody diarrhea with pyrexia, abdominal cramps, bacteraemia and faecal leukocytes. Campylobacterosis has become the most often reported zoonotic disease estimated to affect over 2.4 million peoples with economic loss of approximately $1.2 billion annually. Since environmental factors may influence changes in biochemical and phenotypic properties of the organisms hence, modern diagnostic tools are preferentially required.

In the present study, 370 cloacal swabs were collected in two phases (October 2014 to February 2016) from poultry and subjected for isolation and detection of *Campylobacter.* Based on corckscrew type motility, growth at 42°C, characteristic 'S' shaped morphology, biochemical tests, 16S rRNA-based ribotyping and amplification and sequence analysis of *hip*O gene, all the 43 isolates were confirmed as *C. jejuni.*

REP-PCR of *fla*A gene of *C. jejuni* revealed 12 different REP patterns with a discriminatory index (D.I.) of 0.9181. *Dde*-based RFLP (*Dde*-RFLP) analysis of *fla*A gene revealed 15 patterns with a discriminatory index of 0.9258. However, *Hinf*I enzyme-based RFLP analysis of *fla*A gene revealed six different patterns with a discriminatory index of 0.6977, less efficient in differentiating *C. jejuni* isolates. Likewise, *Dpn*II-based RFLP analysis of *fla*A gene revealed 7 different patterns with a discriminatory index of 0.9258 and hence served most efficient in discriminating *C. jejuni* isolates.

Bacterial adherence associated virulence genes i.e*. cad*F, *por*A, *jlp*A, *dna*J and *cap*A were detected in 97.67%, 93.02%, 90.69%, 88.37% and 51.16% of the isolates respectively. Set of all the five genes studied were found only in 44.18% of the isolates. Lipooligosaccharide (LOS) genes i.e*. wla*N and *waa*C were detected in 88.37% and 65.11% of the isolates.

Of the total 43 isolates, *fla*A gene was detected in 100% followed by *fla*B in 72.09% and *flg*R in (69.76%) isolates. Only 55.81% of the isolates possessed all three genes combination. Among cytolethal distending toxin (CDT) genes, *cdt*C was detected in 97.67% of the isolates whereas *cdt*A and *ctd*B in 93.02% 86.04% of the isolates. Set of all three genes were present in 81.39% of the isolates.

Invasion associated marker (*iam*AB) gene was amplified from 88.37% of the isolates whereas *cia*B and *pld*A genes were detected in 34.88% and 46.51% of the isolates respectively. Set of all three genes were detected in 9% of the isolates. Partial nucleotide sequence analysis of *iam*AB gene revealed three major clusters. All three isolates under study grouped under a separate cluster (cluster III). Though, sequences of *iam*AB gene from other isolates from India are unavailable in the public domain but separation of the isolates into an entirely different cluster suggests their unique genetic character.

Total 33 virulotypes (V) were generated from 16 virulence-associated genes. Maximum 15 set of virulence genes were detected in isolate C12 and C22 (V32) and isolate C11, C14, C20, C24 and C27 (V33). The discriminatory power of virulotyping was calculated as 0.9812. All the virulotypes could be divided into seven clusters on the basis of 80% genetic similarity.

Over the last few decades, the intensive use of antibiotics in food animals has created a selective pressure for selection of resistant strains which can be easily transmitted to humans *via* food chain which lead to colonization of resistant clones of bacterial species in human gut. Highest (100%) sensitivity was observed against polymxin-B followed by chloramphenicol (97.67%), gentamicin (95.35%), amikacin (88.37%), aztreonam (83.72%), meropenem and imepenem (76.74%), kanamycin (72.09%), ceftriaxone (65.12%), erythromycin and ampicillin (53.49%). penicillin-G, methicillin and rifampicin were found to be 100% resistant followed by cephalothin (95.35%), vancomycin (93.02%), ciprofloxacin (90.70%), ofloxacin (79.07%), nalidixic acid (74.42%) and norfloxacin (72.09%). High burden of residual β-lactam antibiotics in the environment would have

resulted in resistance against this group of antibiotics. Similarly, a very high resistance against fluoroquinolones group of antibiotics (ciprofloxacin, ofloxacin, nalidixic acid and norfloxacin) was also seen against *C. jejuni* isolates.

All the *C. jejuni* isolates were considered to be multidrug resistant (MDR) as they had average MAR value of 0.45. MAR value of more than 0.2 MAR value indicates recent use of antibiotics. The MIC values were calculated for six antibiotics. Erythromycin had highest average MIC value 5.74 mcg/ml followed by chloramphenicol (4.80mcg/ml), amikacin (0.86mcg/ml) and gentamicin (0.23mcg/ml). MIC for ciprofloxacin and ofloxacin could not be calculated as no zone was observed.

Of the 35 antibiotic resistance genes and four antibiotic resistance determinants genes, we were able to successfully amplify only seven genes (*tet*O, *aph*3, *gyr*A, *gyr*B, *cme*RABC, *int*1 and *int*2). The *tet*O and *aph*3 genes were detected in majority of the isolates 32 (74.41%). Complete gene cassette for efflux pumps i.e. *cme*RABC gene (625bp) was present in 31 (72.09%) isolates. Out of three integron genes only two i.e. *int*1 in 13 (30.23%) isolates and *int*2 in 3 (6.97%) of the isolates was found. Likewise, fluoroquinolones resistance *gyr*B gene was detected in all isolates followed by *gyr*A gene in 41 (95.34%) of the isolates. *gyr*A gene sequence based phylogenetic analysis, of nine isolates from the current study, had mutations. The phylogenetic analysis of this gene with sequences of other isolates present in the public domain revealed three major clusters. Five isolates from the present study grouped along with poultry and human isolates from Europe (Austria, Slovenia, Germany, Serbia, Bosnia and Herzegovina), New Zealand and Japan. Rest four isolates (C22, C25, C31, and C32) grouped under separate cluster (cluster II) that had isolates form USA. The previously reported *gyr*A gene sequences from India formed a separate cluster (Cluster III) and didn't represent close homology with the isolates from this study.

ABSTRACT (ENGLISH AND HINDI)

Characterization of *Campylobacter jejuni* isolated from poultry

Ph.D. Thesis

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Major Advisor : Prof. S. Maherchandani

ABSTRACT

The present study was aimed for isolation and identification of *Campylobacter jejuni* from cloacal swabs of poultry from Bikaner, Rajasthan. Of the 370 cloacal swabs *C. jejuni* was detected in 11.62% (43/370) isolates using genus specific (16S rRNA) and species specific (*hip*O) primers. Morphological and biochemical characters such as spiral or 'S' shaped Gram negative curved rods, corkscrew type motility and ability to grow under microaerophillic conditions (5%CO_2) at 42°C further confirmed their identity as *C. jejuni*. On the basis of discriminatory index, as compared to REP-PCR, *Dde*-restriction enzyme-based RFLP (*Dde*-RFLP) was found better in discriminating *C. jejuni* isolates. Among virulence genes, *cad*F, *por*A, *jlp*A, *dna*J and *cap*A were detected in 97.67%, 93.02%, 90.69%, 88.37% and 51.16% of the isolates respectively. The *wla*N and *waa*C genes were detected in 88.37% and 65.11% of the isolates respectively. Flagellin gene *fla*A was detected in 100% isolates followed by *fla*B (72.09%) and *flg*R (69.76%). Likewise, cytolethal distending toxin (CDT) genes *viz. cdt*C, *cdt*A and *cdt*B, genes were detected in 97.67%, 93.02% and 86.04% isolates, respectively. Invasion associated genes viz*. iam*AB, *pld*A and *cia*B were detected respectively in 88.37%, 46.51% and 34.88% isolates. Further, we first time sequenced *iam*AB gene of *C. jejuni* isolates originated from India. *iam*AB gene-based phylogenetic analysis of *C. jejuni* isolates from India revealed their unique genetic makeup. On the basis of detection of 16 various virulence associated genes, 33 virulotypes were detected with a discriminatory index of 0.9812. A variable degree of resistance and susceptibility was observed against polymxin-B, chloramphenicol, gentamicin, amikacin, penicillin-G, methicillin, cephalothin, vancomycin, ciprofloxacin, ofloxacin, nalidixic acid and norfloxacin. The average MAR value of 0.45 suggested multidrug resistant (MDR) phenotypes. Erythromycin had highest average MIC value (5.74mcg/ml) followed by chloramphenicol (4.80mcg/ml), amikacin (0.86mcg/ml) and gentamicin (0.23mcg/ml). None of the isolate formed any zone of inhibition for ciprofloxacin and ofloxacin. Tetracycline resistance gene (*tet*O) and aminoglycosides resistance gene (*aph*3) genes were amplified from majority of the isolates (74.41%). Complete gene cassette for efflux pumps i.e*. cme*RABC gene was detected in 72.09% isolates. Out of three integron genes only two were amplified *int*1 in 30.23% isolates and *int*2 in 6.97% of the isolates. Likewise, fluoroquinolones resistance *gyr*B gene was detected in all the isolates followed by *gyr*A gene (95.34%). *gyr*A gene-based phylogenetic analysis represented close homology of *C. jejuni* isolates of this study with isolates from Europe.

मुर्गियो से प्राप्त कैम्पिलोबैक्टर जेजुनी का चरित्रं

विद्यावाचस्पति शोध ग्रन्थ
पशु सूक्ष्मजीव विज्ञान एवम बायोटेक्नोलॉजी विभाग,
पशु चिकित्सा एवम पशु विज्ञान महाविद्यालय,
राजस्थान पशु चिकित्सा एवम पशु विज्ञान विश्वविद्यालय
बीकानेर (334001) राजस्थान

शोध कर्ता : **राहुल यादव**
मुख्य उपादेष्टा : **प्रोफेसर.एस. मेहरचन्दानी**

अनुशेपण

वर्तमान अध्ययन को बीकानेर, राजस्थान से पोल्ट्री क्लोयकल स्वेब नमूनों से *कैम्पिलोबैक्टर जेजुनी* के अलगाव और पहचान के उद्देश्य से किया गया हैं | 370 क्लोयकल स्वेबों में से जीनस विशिष्ट16) *एसआरआरएनए* (और प्रजाति विशिष्ट) *हिप*औ(प्राइमरों के उपयोग से *सी. जेजुनी* (370/43) %11.62वियोजकों में पाया गया | रूपात्मक और जैव रासायनिक पात्रों जैसे कि ऐसे सर्पिल या 'एस' आकार के ग्राम नकारात्मक घुमावदार छड़,पेंचकश प्रकार की गतिशीलता और 42 डिग्री सेल्सियस पर माइक्रोएरोफिलिक (5% सी औ$_2$) स्तिथि के तहत् विकसित होने की क्षमता होने से उनकी *कैम्पिलोबैक्टर जेजुनि* के रूप में पहचान की पुष्टि की गई। विभेदक सूचकांक के आधार पर, *डीडीई*-प्रतिबंध एंजाइम आधारित *आर.एॅफ़.एल.पी,* रैप-पीसीआर की तुलना में *सी. जेजुनी* वियोजकों को विभेदक करने में बेहतर पाया गया। डाह जीन के बीच,*कैड* एॅफ़, *पोर* ए, *जेएलपी* ए, *डीएनए* जे और *कैप* ए क्रमशः 97.67%, 93.02%, 90.69%, 88.37% और 51.16% वियोजकों में पाए गई। *डब्लूएलए* एन और *डब्लूएए* सी जीन क्रमशः 88.37% और 65.11% वियोजकों में पाए गई | फ्लेजिलिन जीन *फला*-ए 100% वियोजकों में पाए जाने का पीछा करते हुए *फला*-बी जीन 72.09% और *एॅफ़एलजी* आर 69.76% वियोजकों में पाए गई | इसी प्रकार साईंटोलिथल डिसटेनडिंग विष जीनो अर्थात *सीडीटी* सी, *सीडीटी* ए और *सीडीटी* बी क्रमशः 97.67%, 93.02% और 86.04% वियोजकों में पाए गई | आक्रमण जुड़े जीन अर्थात *आईएएम* एबी, *पीएलडी* ए और *सीआईए* बी क्रमश: 88.37%, 46.51% और 34.88% वियोजकों में पाए गई | इसके अलावा, हम पहली बार भारतवर्ष से उत्पन्न वियोजकों के *आईएम्ए* एबी जीन का अनुक्रम किया। *आईएम्ए* एबी जीन आधारित वंशावली विश्लेषण से भारतवर्ष के वियोजकों के अद्वितीय आनुवंशिकी श्रृंगार का पता चला। 16 विभिन्न डाह जुड़े जीन का पता लगाने के आधार पर, वियोजकों को 0.9812 विभेदक सूचकांक के साथ 33 डाह समूहों में बांटा गया | पोलीमिक्सिन बी, क्लोरमफेनीकोल, जेंटामाइसिन, एमिकासिन, पेनीसिलीन-जी, मेथीसिलीन, सिफलोथिन, वैनकोमाइसिन, सिप्रोफ्लोक्साक्सिन, ऑफ़लोक्सासिन, नैलीडीक्शिक एसिड और नौरफ्लोक्सासिन के खिलाफ परिवर्तनशील प्रतिरोध और संवेदनशीलता देखी गई | 0.45 की औसत मार मूल्य ने बहुऔषध प्रतिरोधी (एमडीआर) फिनोटाइप्स का सुझाव दिया। ऐरीथ्रोमाइसिन के सर्वाधिक औसत एम.आई.सी मुल्य (5.74 एमसीजी/एमएल) का पीछा करते हुए क्लोरमफेनीकोल की (4.80 एमसीजी/एमएल), एमिकासिन की (0.86 एमसीजी/एमएल) और जेंटामाइसिन की (0.23 एमसीजी/एमएल) औसत एम.आई.सी मुल्य आंकी गयी | किसी भी वियोजक ने सिप्रोफ्लोक्साक्सिन और ऑफ़लोक्सासिन के लिए कोई निषेध छेत्र नही दर्शाया | टेट्रासाइक्लिन प्रतिरोध जीन (*टीईटी* औ) और एमिनोग्लाईकोसाइड्स प्रतिरोध जीन (*एपीएच* 3) वियोजनों के बहुमत (74.41%) से परिलक्षित कर रहे थे | इफ्लक्स पम्पस का सम्पूर्ण जीन कैसेट जैसे कि *सीएमइआर* एबीसी (625बीपी) जीन 72.09% वियोजकों में पाई गयी | तीन इंटीग्रोन जीनों में से केवल *आईएनटी* 1 30.23% में और *आईएनटी* 2 6.97% वियोजकों में पाई गयी | *जी.वाय.आर* ए जीन आधारित वंशावली में इस अध्ययन के *सी. जेजुनी* वियोजनों ने यूरोप के वियोजनों के निकट अनुरूपता का प्रतिनिधित्व किया।

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APPENDIX

8. APPENDIX- I

A. Staining

Gram stain: The composition of Gram stain is:

1. Crystal violet: 2gm of crystal violet (90% dye) was dissolved in 100ml ethyl or methyl alcohol.

2. Gram iodine solution: Weigh1gm of iodine crystal and 2gm of potassium iodide and dissolved in 300ml distilled water.

3. Decolorizer: 40ml acetone mixed with 120ml ethyl alcohol (95%).

4. Counter stain: 1gm saffranin dye(99%) was dissolved in 100ml distilled water.

Heat fixed smear on staining tray was flooded with crystal violet for 1 minute and gently rinsed with tap water. Then, Gram iodine poured over the smear for 1 minute and rinse with tap water. Decolorized using 95% ethyl alcohol or acetone for 5 to 10 seconds and immediately rinsed with water. After that, counter stained by saffranin for 1 minute and rinsed with tap water. The slide was dried properly and examined under oil immersion microscope.

B. Bacteriological Media

1. *Campylobacter* Enrichment Broth (Preston enrichment broth base)

Ingredients	Gms / Litre
Peptic digest of animal tissue	10.000
Beef extract	10.000
Sodium chloride	5.000
Final pH (at 25°C)	7.5±0.2

12.5 grams of media dissolved in 470 ml distilled water and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Further, cooled to room temperature and added 25 ml sterile lysed horse blood and 5ml reconstituted *Campylobacter* Supplement IV aseptically. Mixed well and dispense as desired.

2. *Campylobacter* Selective Supplement IV

Ingredients	Concentration
Polymyxin B sulphate	2500IU
Rifampicin	5mg
Trimethoprim	5mg
Cycloheximide	50mg

Rehydrate the contents of 1 vial aseptically with 5 ml of 50% aqueous acetone for 500ml Preston enrichment broth.

3. Blood Free *Campylobacter* Selectivity Agar (modified charcoal cefoperazone deoxycholate agar)

Ingredients	Gms / Litre
Meat extract B #	10.000
Peptone	10.000
Casein enzymichydrolysate	3.000
Sodium chloride	5.000
Sodium deoxycholate	1.000
Ferrous sulphate	0.250
Sodium pyruvate	0.250
Charcoal, bacteriological	4.000
Agar	12.000
Final pH (at 25°C)	7.4±0.2

Blood Free *Campylobacter* Selectivity Agar Base (Himedia) also known as modified charcoal cefoperazone deoxycholate agar (mCCDA) prepared by adding 22.75gm of blood free *Campylobacter* selectivity agar (Himedia) into 400ml of distilled water and dissolved completely by heating. Further, sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes and cooled to 45-50°C. Aseptically rehydrated contents of 1 vial of *Campylobacter*Supplement V was added to it and poured into sterile petri plates.

4. *Campylobacter* Supplement V

Ingredients	Concentration
Cefoperazone	16mg

Campylobacter supplement V was prepared by rehydrating 1 vial aseptically with 2ml of distilled water for 500 ml of agar.

5. Oxidase reagent

100 mg Oxidase reagent (Tetramethylene-p-phenylenediaminedihydrochloride) was dissolved in 10 ml glass distilled water. The oxidase discs was prepared by saturation of Whatman AA filter Paper (0.64 cm diameter) discs of oxidase reagent solution and allowed to air dry and stored in amber colored bottle contain desiccant at 4°C.

6. Catalase test

Transfer a small amount of bacterial colony to 3% H2O2 on a surface of clean, dry glass slide using a sterile loop. A positive result is the rapid evolution of oxygen (within 5-10 sec.) as evidenced by bubbling.

7. Christensen Urea Agar Base

Ingredients	Gms / Litre
Peptic digest of animal tissue	1.000
Dextrose	1.000
Sodium chloride	5.000
Disodium phosphate	1.200
Monopotassium phosphate	0.800
Phenol red	0.012
Agar	15.000
Final pH (at 25°C)	6.8±0.2

Amount of 24.01 grams of media was dissolved in 950 ml distilled water by gently heating and sterilized by autoclaving at 10 lbs pressure (115°C) for 20 minutes. Further, cooled to 50°C and added 50 ml of sterile 40% Urea Solution aseptically. Then, dispensed into sterile tubes and allow setting in the slanting position.

8. 40% Urea Solution (5ml per vial)

Ingredients	Concentration
Urea	2g
Distilled water	5ml
Final pH (at 25°C)	8.0±0.2

Preformed 5ml per vial Urea solution was added 5 ml in 95 ml sterile, cooled (45-50ºC) Christensen Urea Agar aseptically.

9. Nitrate Broth

Ingredients	Gms / Litre
Peptic digest of animal tissue	5.000
Meat extract	3.000
Potassium nitrate	1.000
Sodium chloride	30.000
Final pH (at 25°C)	7.0±0.2

Amount of 39 grams nitrate broth was dissolved in 1000 ml distilled water by gently heating. Then, dispensed in tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

10. Nitrate test reagent

Solution A :Suphanilic acid soluction 0.8% w/v (Hi-media)

Solution B :∝ - Naphthylamine solution (Hi-media)

11. Sodium hippurate solution (1%)

1 gram sodium hippurate was added to 99 ml distilled water and gently mixed.

12. Ninhydrin solution (3.5%)

3.5 gram of Ninhydrin was added to 96.5 ml distilled water and gently mixed.

13. Indoxyl acetate hydrolysis

10 gm of Indoxyl acetate was poured in to 90ml acetone and mixed well. Indoxyl acetate disks was prepared by saturation of Whatman AA filter Paper (0.64 cm diameter) disk in 10% (w/v) solution of indoxyl acetate in acetone and allowed to air dry and stored in amber colored bottle contain desiccant at 4°C.

14. Triple Sugar-Iron Agar Medium

Ingredients	**Gms / Litre**
Beef extract	3.000
Peptone	20.000
Yeast extract	3.000
Lactose	10.000
Sucrose	10.000
Dextrose monohydrate	1.000
Ferrous sulphate	0.200
Sodium chloride	5.000
Sodium thiosulphate	0.300
Phenol red	0.024

Amount of 64.42 grams media dissolved in1000 ml distilled water by heating gently.Further, dispensed into test tubes andsterilized by at 10lbs pressure (115°C) for 30 minutes. Then, allowed to set in sloped form with a butt about 2.5cm long.

C. Horse blood: 5-10 ml of horse blood was collected aseptically in a sterilized test tube having anticoagulant and lysed by 10X RBC lysis buffer (Appendix- II).

9. APPENDIX- II

1. Phosphate buffer saline (1 %)

Solution A :	Sodium diphosphate	1.4 gm
	Distilled water	1000 ml
Solution B :	Sodium dihydrogen orthophosphate	1.4 gm
	Distilled water	1000 ml

An amount of 84.1 ml of solution A and 15.9 ml of solution B were mixed and 8.5g sodium chloride was added. The volume was made to 1000 ml with distilled water and autoclave it at 15 lbs (121°C) for 15 min.

2. Tris (0.5M)

In 800 ml of distilled water 4.44 g of TrisHcl and 2.65 g of Tris base/ buffer was dissolved and the pH was adjusted to desired value. The volume was then adjusted to 1000 ml, dispensed in aliquots and sterilised by autoclaving.

3. TE (Tris EDTA) Buffer

20ml of 0.5 M Tris (pH,8.0) was added to 200 µl of 0.5 M EDTA pH, 8.0 and the volume was made up to 100 ml.

4. EDTA (0.5 M), pH- 8.0

186.1 g of Ethylene diaminetetraacetic acid was poured in 800 ml of distilled water and shake vigorously on a magnetic stirrer for several hours. The pH was adjusted to 8.0 with 1.0 N NaOH, dispensed into aliquots and sterilized by autoclaving.

5. Proteinase K solution

10 mg proteinase K was dissolved in 1 ml of distilled water and stored at 20°C.

6. SDS buffer 10% (w/v)

100 gm of sodium dodecyl sulfate/sulphate was mixed with 800ml of distilled water and final volume was make upto 1lt.

7. 1M NaCl

58.44g of NaCl dissolved in 800ml of distilled water and final volume was made upto **1 liter**.

8. Salt Tris EDTA buffer (STE Buffer)

- 100mM Nacl (5ml of 1M Nacl),

- 50mM TrisHcl (4ml of 0.5M TrisHcl),
- 1mm EDTA (0.4 ml 1M EDTA),
- 5ml (20mg/ml) Protinase K,
- 20ml (10%) SDS,
- finalpH. 8.0

9. Agarose solution (1.2% and 0.8%)

To prepare 1.2% agarose, 1.2gm of agarose was dissolved in 100 ml of 1 × TBE.To prepare 0.8% agarose solution, 0.8gm of agarose was dissolved in 100 ml of 1 × TBE.

10. Alcohol (70%)

70% alcohol was prepared by mixing 70 ml of absolute alcohol with 30 ml of double glass distilled water.

11. Ethidium bromide solution

Stock solution was prepared by using 5 mg ethidium bromide per ml of TBE working solution. Working solution was prepared in the concentration range of 0.5-1 mg/ml using TBE working solution.

12. TBE (Tris Borate EDTA) Buffer, pH 8.2

Stock solution (5X)

54.0 gmof Tris base, 27.5 gm of Boric acid and 20ml ofDisodium EDTA (0.5M) was mixed in 800ml of Distilled water and final volume was made up to 1000ml.

Working solution (0.5X) and (1X)

100 ml stock solution of TBE buffer (5X) was dissolved in 900ml of triple distilled water to prepare working solution of 0.5x TBE buffer. Likewise, 100 ml stock solution of TBE buffer (5X) was dissolved in 400ml of triple distilled water to prepare the working solution of 1x TBE buffer.

13. RBC Lysing Buffer10X (Ammonium chloride lyse)

8.02gm of NH_4Cl (ammonium chloride),0.84gm of $NaHCO_3$ (sodium bicarbonate) and 0.37gm of EDTA was mixed in 80 ml of Distilled water and final volume was made up to 100ml. Store at 4^oC for six months. Dilute 10ml 10X concentrate with 90 ml Millipore water for 1X working RBC lysing buffer.

14. PCR Master mixand Standard PCR conditions

The PCRmaster mix of 25.0 µl volume was prepared using Promega, Go Taq PCR Core System-I Kit by mixing:

De-ionised water	11.3µl
5x *Taq* Buffer A containing	5.0 µl
$MgCl_2$ (25mM)	2.5 µl
Primer-1 (1 µM/µl)	1.0 µl
Primer-2 (1 µM/µl)	1.0 µl
dNTP-mix (10mM)	1.0 µl
*Taq*DNA polymerase (5U/µl)	0.2 µl
DNA (25ng/ µl)	3.0 µl

Amplification was carried out in 'EppendorfMastercycler gradient' as followsusing below tabulated cycling parameters:

Cycle	**Step**	**Temperature (^oC)**	**Time (seconds)**
Cycle 1	Denaturation	95	60 s
Cycle 2-35	Denaturation	95	60 s
	Primer annealing	variable	60 s
	Primer extension	72	60 s
Cycle 36	Final extension	72	10 min
	Hold	4	infinite

Amplification of the PCR products were detected by electrophoresis in 1-1.5% agarose gels with ethidium bromide (0.5µg/ml) in 1X TBE buffer for 50-60 min at 100 V. The gel was then visualized under UVP gel documentation system (BioDoc-It Imaging System).