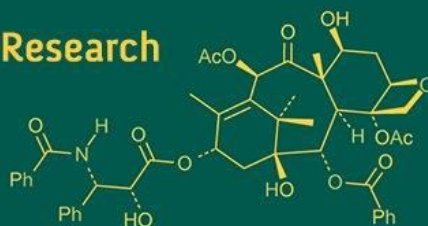


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Prevalence and antibiogram of *Campylobacter* spp. from cattle in Anand, Gujarat

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Abstract

Aim: The present study was undertaken to determine the prevalence and antimicrobial susceptibility pattern of *Campylobacter* spp. isolated from cattle faecal samples in Anand city, Gujarat, India.

Materials and Methods: A total of 200 faecal samples comprising 50 from healthy cattle, 50 from diarrhoeic cattle, 50 from healthy calves and 50 from diarrhoeic calves were collected from different cattle farms in Anand city. The samples were processed using standard microbiological culture techniques, and the presumptive isolates were further confirmed by genus-and species-specific polymerase chain reaction (PCR) using specific oligonucleotide primers targeting the 16S rRNA gene for *Campylobacter* genus, hippuricase (hipO) gene for *Campylobacter jejuni*, and aspartokinase (asp) gene for *Campylobacter coli*. The confirmed isolates were further subjected to antimicrobial susceptibility testing by the disc diffusion method.

Results: Out of the 200 samples examined, 8 (4%) samples were found positive for *Campylobacter* spp. All the isolates were recovered from diarrhoeal faecal samples, whereas none of the samples from healthy cattle and calves were found positive. Among the isolates, seven were identified as *Campylobacter jejuni* and one as *Campylobacter coli*. Antibiotic susceptibility testing revealed that all isolates were resistant to Nalidixic acid, while they showed sensitivity to Chloramphenicol, Erythromycin, Gentamicin and Streptomycin sulphate.

Conclusion: The detection of *Campylobacter* spp. in cattle faeces suggests that cattle may serve as an important reservoir and potential source of infection through manure contamination. Faecal contamination of milk during the milking process may represent a potential route for transmission of *Campylobacter* infection to humans. Therefore, strict implementation of good hygienic practices, proper sanitation during milking, and rational use of antimicrobials in livestock production are essential to reduce the risk of zoonotic transmission and antimicrobial resistance.

Keywords: Antibiotic susceptibility, *Campylobacter jejuni*, gastroenteritis, polymerase chain reaction

Introduction

Foodborne infections remain a major global public health concern. Among foodborne pathogens, *Campylobacter* has emerged as one of the leading causes of bacterial gastroenteritis worldwide. In recent years, the global burden of *Campylobacter* infections has continued to increase due to factors such as globalization of food supply, intensive livestock production, and increased surveillance systems. According to recent global estimates, *Campylobacter* species are responsible for millions of cases of diarrhoeal disease each year and remain among the most frequently reported bacterial causes of foodborne illness in many countries (WHO, 2023; CDC, 2022) [2, 5].

Campylobacter belongs to the family *Campylobacteraceae*, which also includes the genera *Helicobacter*, *Arcobacter*, and *Wolinella*. This family is classified within the epsilon subdivision of the class Proteobacteria. Advances in molecular taxonomy have expanded the genus, and currently more than 30 species of *Campylobacter* have been identified. Among them, *Campylobacter jejuni* and *Campylobacter coli* are the most significant species associated with human disease. These organisms are commonly present as commensals in livestock, poultry, and domestic animals. *C. jejuni* accounts for approximately 80-90% of human *Campylobacteriosis* cases, while *C. coli* contributes about 5-10% of infections worldwide (Man, de Steenhuijsen Pijters & Bogaert, 2021) [1].

Campylobacter species are Gram-negative, microaerophilic, spiral-shaped or curved rods measuring approximately 0.5-8.0 µm in length and 0.2-0.5 µm in width.

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Most strains possess a single polar flagellum, although some may have bipolar flagella. These bacteria exhibit a characteristic corkscrew-like motility with rapid darting movements and instantaneous directional changes. This motility allows the organism to penetrate the mucus layer and colonize the epithelial surfaces of the gastrointestinal tract (EFSA, 2022) [3].

Campylobacter organisms are widely distributed in the gastrointestinal tract of warm-blooded animals, including cattle, sheep, pigs, and poultry. In cattle, *C. jejuni* commonly colonizes the proximal small intestine and may be shed in feces, which contributes to environmental contamination. Human infections occur mainly through the foodborne route, particularly through consumption of contaminated poultry products, unpasteurized milk, or contaminated water. However, recent studies suggest that multiple food sources and environmental reservoirs may contribute significantly to human infections (EFSA & ECDC, 2023) [3].

The role of cattle in the epidemiology of *Campylobacteriosis* is not limited to contamination of milk or carcasses during slaughter. Cattle may also contribute to environmental contamination through manure disposal, slurry spreading on agricultural land, and abattoir effluents. These activities may contaminate soil, water bodies, and food products, thereby increasing the risk of transmission to humans.

Campylobacteriosis is an acute gastrointestinal infection characterized by symptoms such as diarrhoea, abdominal pain, fever, nausea, headache, and muscle pain. The incubation period generally ranges from 2 to 5 days, and the illness usually lasts about 5-7 days. Although most infections are self-limiting, some patients may develop severe post-infectious complications. One of the most serious sequelae is Guillain-Barré syndrome (GBS), an autoimmune neuropathy that can cause acute flaccid paralysis. Other complications include reactive arthritis and irritable bowel syndrome (CDC, 2022; WHO, 2023) [5, 2].

In most cases, *Campylobacter* infections are self-limiting, and antimicrobial therapy is not required. However, antibiotic treatment may be recommended in severe or prolonged cases, especially among immunocompromised patients. Macrolides such as erythromycin or azithromycin are considered the drugs of choice. However, the increasing emergence of antimicrobial resistance, particularly to fluoroquinolones and tetracyclines, among *Campylobacter* isolates has become a significant global health concern (WHO, 2023; Tang *et al.*, 2022) [2, 4]. Considering the increasing public health importance of *Campylobacter* infections and the growing problem of antimicrobial resistance, continuous surveillance of this pathogen in food-producing animals is essential. Therefore, the present study was undertaken to determine the prevalence and antibiotic resistance pattern of *Campylobacter* spp. isolated from cattle in Anand city, Gujarat, India, in order to understand their epidemiological significance and potential risk to human health.

Materials and Methods

Approximately, a total 200 faecal samples were collected aseptically and transported to the laboratory in an icebox for

microbiological analysis. Samples were processed in laboratory immediately within two hour.

Isolation and identification

Samples were processed to isolate the *Campylobacter* spp. as per the method described by Salihu *et al.* (2010) [31]. The samples were enriched into 45 ml of Blood free *Campylobacter* broth base containing CCDA selective supplement (HiMedia Laboratories, Mumbai, India). Mixed properly and incubated at microaerophilic environment (85% N₂, 5% O₂ and 10% CO₂) in water jacketed CO₂ incubator (NUAIRE, Plymouth, MN) at 42 °C for 48 hour. After enrichment culture, a loopful of enriched culture was streaked on blood free charcoal cefoperazone deoxycholate agar medium plates and incubated at microaerophilic environment at 42 °C for 48 hour. The typical dew drops like colonies were isolated on charcoal based media such as mCCDA.

Identification of *Campylobacter* species

The presumptive *Campylobacter* isolates were identified on the basis of culture characteristics, Gram staining morphology, Nitrate reduction test, Urease test, H₂S production, Hippurate and Indoxyl acetate hydrolysis.

Template DNA preparation

A loopful of overnight growth culture of *Campylobacter* spp. was suspended in 100 µl of sterilized DNase and RNase-free milliQ water (Millipore, USA) in PCR tube. After proper mixing, the tube was then kept at 95 °C for 10 min in PCR tubes. Cell debris was sedimented by centrifugation at 10,000 rpm for 5 min and 3 µl of supernatant was used as a DNA template in PCR reaction mixture.

Confirmation and species identification of isolates using polymerase chain reaction (PCR)

The biochemically identified isolates were further employed for confirmation by PCR as genus *Campylobacter*, *C. jejuni*, *C. coli* and *C. lari* by amplifying specific target gene with species specific oligonucleotide primers. The details of primers are tabulated in Table 1. The DNA amplification for each primer pair was carried out in a Applied Biosystems 2720 Thermal Cycler in 25 µl reaction containing 3 µl of DNA template, 12.5 µl mastermix (Thermo Scientific, USA) (containing 0.05 unit/µl Taq DNA Polymerase, reaction buffer, 4 mM MgCl₂, 0.4 mM of each dNTP), 10 pmole of each forward and reverse primer (10 pmole/µl), and 7.5 ml nuclease free water with positive and negative control. The control strains of *C. jejuni* and *C. coli* isolates in our department were used for positive control whereas DNase free distilled water was used for negative control. The cycling protocol for the genus confirmation was standardized to set the PCR assay as initial denaturation at 95 °C for 5 min followed by 35 cycles with denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 10 min. For hippuricase (*hipO*) gene of *Campylobacter jejuni*, cycling condition was optimized to initial denaturation at 95 °C for 5 min, followed by 35 cycles with denaturation at 95 °C for 45 s, annealing at 51 °C for 45 s and extension at 72 °C for 45 s and final extension at 72 °C for 7 min and for asperkinase A (*askA*) gene of *C. coli* cycling condition was optimized to initial denaturation at 95 °C for 5 min followed by 35 cycles with denaturation at 95 °C for 30 s, annealing

at 53 °C for 30 s and extension at 72 °C for 30 s and final extension at 72 °C for 7 min. On completion of the reaction, the amplified products were held briefly at 4 °C. Amplification of the PCR products were detected by electrophoresis in 1.5% agarose gel with ethidium bromide (10 µg/ml) in 0.5X TBE buffer (Sigma, USA) at 100 V for 40 min and documented in G: BOXF3 (SynGene, USA).

***In vitro* Antibiotic sensitivity pattern**

The antimicrobial susceptibility testing of the *Campylobacter* isolates was performed to determine their resistance or sensitivity pattern using the disc diffusion method as originally described by William Bauer *et al.* (1966) [10], with modifications according to recent Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines. *in vitro* antibiotic susceptibility testing was carried out using commercially available antibiotic discs. The isolates were tested against commonly used antimicrobial agents including Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Nalidixic acid (30 µg), Erythromycin (15 µg), Gentamicin (10 µg), Streptomycin (30 µg), and Tetracycline (30 µg). Pure isolates were inoculated into nutrient broth and incubated at 37 °C for 18-24 hours under microaerophilic conditions to obtain fresh bacterial growth. The broth cultures were adjusted to match 0.5 McFarland turbidity standard to ensure uniform inoculum density. A sterile cotton swab was then used to evenly spread the bacterial suspension on the surface of Mueller-Hinton agar plates supplemented with 5% sheep blood (HiMedia), which is recommended for susceptibility testing of *Campylobacter* species. After inoculation, the plates were allowed to dry for a few minutes, and antibiotic discs were carefully placed on the agar surface using sterile forceps within 15 minutes of inoculation. The plates were then incubated at 37 °C for 24 hours under microaerophilic conditions (5% O₂, 10% CO₂, and 85% N₂). Following incubation, the diameter of the zones of inhibition around each antibiotic disc was measured in millimeters using a ruler or caliper. The results were interpreted as Sensitive (S), Intermediate (I), or Resistant (R) according to the interpretative criteria recommended by Clinical and Laboratory Standards Institute (CLSI, 2023) or European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2024) guidelines.

Results and Discussion

Prevalence of *Campylobacter* spp.

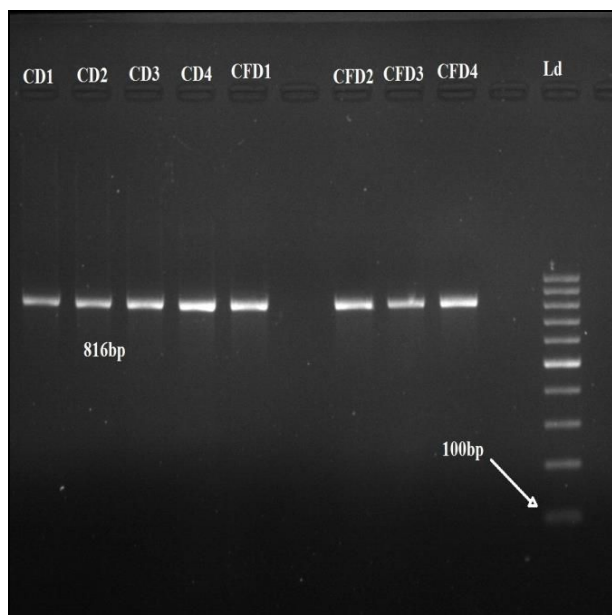
In the present study, an overall 4% prevalence of *Campylobacter* spp. was observed among the 200 samples examined. All the isolates were obtained from diarrhoeal faecal samples, while none of the samples collected from healthy cattle and calves were found positive for *Campylobacter*. According to the sample-wise analysis, the prevalence of *Campylobacter* was 8% in diarrhoeal faecal samples of cattle and 8% in diarrhoeal faecal samples of calves, whereas the prevalence in healthy cattle and calves was 0%. Species identification revealed that the prevalence of *Campylobacter jejuni* was 87.5%, while *C. coli* accounted for 12.5% of the isolates. This observation indicates that *C. jejuni* is the predominant species associated with bovine *Campylobacteriosis*, which is consistent with global reports that identify *C. jejuni* as the most frequently isolated species from animals and humans (Man *et al.*, 2021; EFSA & ECDC, 2023) [1, 3].

The findings of the present study are comparable with the results reported by Chatur (2014) [13], who observed 4% prevalence of *Campylobacter* in cattle samples. However, several other studies have reported higher prevalence rates. For example, Brown *et al.* (2004) [11], Sato *et al.* (2004) [33], Baserisalehi *et al.* (2005) [8], Saito *et al.* (2005) [30], Fernandez and Hirschfeld (2009) [18], Ghane *et al.* (2010) [19], Vilar *et al.* (2010) [38] and Eghbalia *et al.* (2011) [15] reported prevalence rates of 30%, 27.9%, 28.57%, 23.1%, 21.3%, 28.57%, 20.5% and 20%, respectively, which were considerably higher than those observed in the present study. Similarly, Tesfaye *et al.* (2005) [36], Salihu *et al.* (2009) [32] and Uaboi-Egbenni *et al.* (2012) [37] reported 12.7%, 12.9% and 19.3% prevalence, respectively, which were also higher than the prevalence observed in the present study. The variation in prevalence rates among different studies may be attributed to differences in geographical location, sample size, diagnostic methods, animal management practices, environmental conditions, and seasonal variations (WHO, 2023) [2]. In contrast to the present findings, Rajendran *et al.* (2012) [28] did not detect *Campylobacter* in any of the cattle faecal samples examined. According to sample-wise analysis, Singh *et al.* (2011) [34] reported 6.25% prevalence, which was lower than that observed in the present study. However, Hannon *et al.* (2009) [20] and Ramonaite *et al.* (2013) [29] reported 76% and 78.6% prevalence, respectively, in cattle faecal samples, which were substantially higher than the results obtained in the present investigation. Recent surveillance reports also indicate that livestock, particularly cattle and poultry, serve as important reservoirs for *Campylobacter* species and play a significant role in the transmission of infection to humans through food and environmental contamination (EFSA & ECDC, 2023) [3].

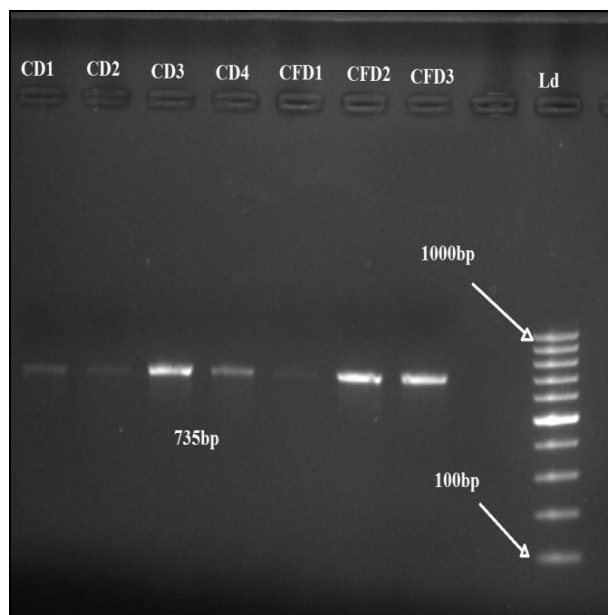
In the present study, all *Campylobacter* isolates showed 100% resistance to Nalidixic acid. Resistance to Ciprofloxacin and Tetracycline was observed in 62.5% and 25% of isolates, respectively. On the other hand, all isolates were found to be sensitive to Chloramphenicol, Erythromycin, Gentamicin and Streptomycin sulphate. Six isolates (75%) were sensitive to Tetracycline, while three isolates (37.5%) were sensitive to Ciprofloxacin. Among the tested antibiotics, Chloramphenicol, Erythromycin, Gentamicin and Streptomycin sulphate showed the highest sensitivity, indicating their potential effectiveness against *Campylobacter* isolates in the present study. Similar findings have been reported by Afset and Maeland (2001) [7], Sato *et al.* (2004) [33] and Eghbalia *et al.* (2011) [15], who also observed high sensitivity of *Campylobacter* isolates to macrolides and aminoglycosides. With respect to Ciprofloxacin resistance, the findings of the present study are comparable with the observations of Elisabetta *et al.* (2014) [16], who reported 62.76% resistance to Ciprofloxacin among *Campylobacter* isolates. However, other studies such as Parisi *et al.* (2007) [27], Englen *et al.* (2007) [17] and Wonki *et al.* (2005) [39] reported 29%, 2.5% and 5.1% resistance, respectively, which were much lower than those observed in the present study. Recent global reports have highlighted an increasing trend of antimicrobial resistance in *Campylobacter* species, particularly against fluoroquinolones and tetracyclines. This rising resistance is mainly attributed to the extensive use of antibiotics in veterinary practice and livestock production, which may contribute to the emergence of resistant strains that can be

transmitted to humans through the food chain (WHO, 2023; Tang *et al.*, 2022) [2, 4]. Therefore, continuous monitoring of antimicrobial resistance patterns in *Campylobacter* isolates

from food-producing animals is essential to ensure effective treatment strategies and to minimize the risk of transmission of resistant strains to humans.



16S rRNA gene of Genus *Campylobacter* (816 bp)



Hippuricase (*hipO*) gene specific for *C. jejuni* (735 bp)

Fig 1: Amplification of PCR products for the detection of *Campylobacter* spp.

Table 1: List of the genus and species specific primers.

Sr. No.	Gene name	Primer Sequence	Target gene	Amplicon size (bp)	Reference
1.	C412F	GGATGACACTTTTCGGAGC	<i>Campylobacter</i> genus 16S rRNA	816	Linton <i>et al.</i> , 1996 [24]
	C1228R	CATTGTAGCACGTGTGTC			
2.	Hip O1	AGCTAGCTTCGCATAATAAATTG	<i>C. jejuni</i> Hippuricase gene	735	Linton <i>et al.</i> , 1997 [23]
	Hip O2	GAAGAGGGTTTGGGTGGT			
3.	CC1	GGTATGATTTCTACAAAGCGAG	<i>C. coli</i> Asperkinase gene	500	Linton <i>et al.</i> , 1997 [23]
	CC2	ATAAAAAGACTATCGTCGCGT			

Conclusion

The findings of the present study indicate that cattle may act as an important reservoir of *Campylobacter* spp. and may contribute to the transmission of infection through faecal shedding and environmental contamination. Manure from infected animals can contaminate soil, water sources, and farm environments, thereby increasing the risk of transmission to other animals and humans. Faecal contamination of milk during the milking process represents a potential route of transmission leading to human *Campylobacteriosis*. Therefore, strict implementation of good hygienic practices during milking, proper sanitation of equipment, and maintenance of personal hygiene among dairy workers is essential to reduce the risk of contamination and protect consumers from this important zoonotic pathogen. The study also highlights the emergence of antimicrobial resistance among *Campylobacter* isolates, which is a growing global public health concern. The widespread and indiscriminate use of antibiotics in livestock production for therapeutic, prophylactic, and growth-promoting purposes may contribute to the development and spread of resistant strains. Therefore, rational use of antimicrobials in veterinary practice, implementation of antimicrobial stewardship programs, and continuous surveillance of antimicrobial resistance patterns in food-producing animals are necessary to control the spread of resistant *Campylobacter* strains and safeguard both animal and human health.

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