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Unveiling the Cultivation Dynamics of Thermophilic Campylobacters in Different Culture Media Substrate

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Abstract

Campylobacter species, known for their potential zoonotic transmission, play a significant role in instigating enteritis across a diverse array of animals, including those in captive, wild, and domestic environments, alongside avian populations. We isolated *Campylobacter jejuni* and *Campylobacter coli* strains from faecal specimens sourced from wild animals. The sample collection encompassed 521 faecal samples procured from wildlife sanctuaries, zoos, and national parks situated in Uttar Pradesh, Uttarakhand, and Chhattisgarh. Employing a meticulously aseptic approach throughout the collection and isolation process, the growth patterns of the identified *Campylobacter* strains were meticulously scrutinized using five distinct artificial culture media, classified into two groups: blood-free and blood-containing media. The prevalence of Campylobacter spp. was observed to be 11.90% (62 out of 521 samples), with *Campylobacter jejuni* accounting for 7.10% and *Campylobacter coli* for 4.80% of the isolates. Subsequent enrichment and plating on Columbia Blood Agar (CBA) supplemented with selective additives demonstrated a notably higher prevalence (11.90%) of *Campylobacter* spp. compared to alternative media such as Modified Charcoal Cefoperazone Deoxycholate Agar [mCCDA] (10.56%) and Sheep Blood Agar [BA] (8.25%). The culture of *Campylobacter* strains exhibited the lowest isolation rates on Chocolate Agar [CA] (5.76%) and Hi-chrome *Campylobacter* Agar [HCCA] (4.22%). The findings from multiplex PCR assays confirmed both the precise identification of Campylobacter species and the efficacy of each culture method employed in this study.

Keywords: *Campylobacter* spp., Culture media, Multiplex PCR, Prevalence.

Introduction:

Campylobacter species are fastidious organisms that have specific atmospheric and temperature requirements for growth, utilize menaquinones as their respiratory quinones, do not ferment or oxidize carbohydrates, and thrive in a microaerophilic (5% O2, 10% CO2, and 85% N2)environment (Penner,1988) Campylobacter strains causing human gastroenteritis are predominantly thermotolerant and capable of growing at temperatures as high as 42°C–43°C (Vandamme and De Ley, 1991).Campylobacter is a significant zoonotic foodborne bacterial pathogen that causes diarrheal diseases in both humans and animals (Garcia-Sanchez et al., 2018; WHO, 2020). *Campylobacter* is one of the most prevalent bacterial agents responsible for gastroenteritis, although the true incidence of *Campylobacter*-related gastroenteritis, particularly in low- and middle-income countries (LMIC), remains poorly understood, with estimates indicating around 3 cases per 1000 population (WHO, 2012). Transmission of the pathogen to humans can occur through various routes, including contaminated food, water, and direct contact with farm animals and pets (Elbrissiet al., 2017).

Materials and Methods:

We collected 521 faecal samples from wild animals, including mammals and birds between April 2021 and March 2022 from eight zoos, national parks, and sanctuaries located in Uttarakhand (n=3), Uttar Pradesh (n=2), and Chhattisgarh (n=3), India (Table 1).

Samples were immediately processed following the guidelines of ISO 10272-1:2017(E). The samples underwent pre-enrichment in Buffered peptone water (BPW) and then enrichment was performed using Bolton broth supplemented with 5% sterile lysed defibrinated sheep blood and FD231 supplement. The enriched samples were incubated microaerobically in a CO_2 incubator at 42°C for 48 hours. After the primary isolation of Campylobacter species, five different artificial media were assessed, and categorized into two groups: blood-free media and blood-containing media.

The blood-free media included Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) and Hi-chrome *Campylobacter* agar (HCCA). The blood-containing media consisted of Columbia blood agar (CBA), Sheep blood agar (BA), and chocolate agar (CA).

Table1: Places of faecal samples collection and description of animals

Sl. No.	Places of sample collection	No. of faecal samples collected	Ruminants	Non-Ruminants	Birds
1.	Deer Park and Wild Animal Rescue Center, NTD, Almora, Uttarakhand, India	24	6	18	0
2.	G. B. Pant High Altitude Zoo Nainital, Uttarakhand, India	32	11	18	3
3.	Jim Corbett National Park, Ramnagar, Nainital, Uttarakhand, India	138	138	0	0
4.	Kanpur Zoological Park, Nawabganj Kanpur, Uttar Pradesh, India	34	10	10	14
5.	Nawab Wajid Ali Shah Zoological Garden Lucknow, Uttar Pradesh, India	11	4	3	4
6.	State Nandanban Zoo & Safari, New Raipur, Chhattisgarh, India	76	22	48	6
7.	Peripherry of Achanakmar Sanctuary, Bilaspur, Chhattisgarh, India	99	51	33	15
8.	State Zoo, Bilaspur, Chhattisgarh, India	107	60	36	11
	Total	**521**	**302**	**166**	**53**

Following pre-enrichment and enrichment, the obtained isolates were inoculated onto mCCDA, HCCA, CBA, BA, and CA media and incubated in a CO_2 incubator maintained at 5% CO_2 and 42°C. Incubation was carried out for a period of 48-72 hours.

Suspected and well-isolated colonies were subcultured onto the same media for purification. Gram staining and standard biochemical tests, including oxidase test, catalase test, Hippurate hydrolysis test, *Campylobacter* nitrate reduction test, urease test, and H_2S production on TSI test methods, were performed for further identification of the presumed colonies (Atabay and Corry, 1997). Biochemically positive isolates were grown in Tryptone soya broth, aliquoted into cryo-vials with 20% sterile glycerol, and preserved at -80°C for future use.

Positive isolates, based on colony appearance and biochemical results, were further confirmed through multiplex PCR following DNA extraction (Shams et al., 2017). The prevalence data of *Campylobacter* spp. recovered from each culture medium were statistically compared using one-way analysis of variance followed by the least significant difference (DMRT) test. The statistical analyses were performed using SPSS version 26.

Results and Discussions:

The identification of suspected *Campylobacter* isolates was based on their colony characteristics, motility test, inability to grow in aerobic conditions, and Gram staining features. *Campylobacter* colonies exhibited small (1-2 mm), circular, flat to slightly raised, sticky, spreading, shiny grey-coloured colonies or water droplets on mCCDA, CBA, BA, and Chocolate agar plates. On HCA plates, *Campylobacter* species appeared as mauve to purple-coloured colonies. The organisms appeared as pink Gram-negative rods, spiral curved rods with comma-shaped (S) or gull-wing appearance cells. Similar colony characteristics were reported by Monika (2014) and Garhia(2017).

The recovery rate of *Campylobacter* spp. in this study was higher in CBA culture media compared to mCCDA, HCA, BA, and Chocolate agar culture methods studied (Table 2). The overall prevalence of *Campylobacter* spp. was 11.90% (62 out of 521), with *Campylobacter jejuni* accounting for 7.10% and *Campylobacter coli* for 4.80%, which aligns with the findings of Acke et al. (2008). After enrichment, plating on CBA with selective supplement resulted in a significantly higher ($P<0.05$) prevalence of 4.65% of *Campylobacter* spp., as also reported by Hutchinson and Bolton (1984). However, we observed a recovery rate of 10.56% on mCCDA, as reported by Corry and Atabay (1997), and 8.25% on BA (Byrne et al., 2001), showing no significant differences (Table 2), followed by 5.76% on CA, as also reported by Aspinall et al. (1996), and 4.22% on HCCA (Humphrey et al., 2007). Multiplex PCR results confirmed the speciation of *Campylobacter* isolates as well as the sensitivity of each culture method. Considering the majority of *Campylobacter* spp. was isolated using CBA media with a selective supplement, it can be concluded that this method is preferable for the isolation of *Campylobacter* sp. in this study.

Table 2: *Campylobacter* spp. prevalence for each culture and combined method (%)

Sl. No.	Culture Methods	*Campylobacter* species prevalence (%)
1.	mCCDA	55/521 (10.56)[d]
2.	Hi-chrome CA (HCCA)	22/521 (4.22)[a]
3.	CBA	**62/521 (11.90)[e]**
4.	BA	43/521 (8.25)[c]
5.	Chocolate Agar (CA)	30/521 (5.76)[b]
6.	Total	**62/521(11.90)**
7.	C.D.	0.075
8.	SE (m)	0.023
9.	SE (d)	0.033
10.	C.V.	0.627

* Figures having different superscripts differ significantly. (P=1.42e-12) P<0.05

It was also observed that the pre-enrichment and enrichment steps reduced transport stress and enhanced the recovery of *Campylobacter* spp. compared to direct plating or filtration onto selective media. Since CBA showed a higher recovery rate of *Campylobacter* spp. (P<0.05), it may be considered a more accurate blood-based method for assessing the actual prevalence of *Campylobacter* spp. in the sampled population. In the blood-free method, mCCDA may be relatively better for assessing the prevalence of *Campylobacter* spp. In both methods, CBA and mCCDA, hemin (Fe3+) and charcoal, respectively, act as a source of energy and oxygen-quenching agent, which are necessary for growth in a microaerophilic environment (Hutchinson and Bolton, 1984).

Conclusions:

The recovery of *Campylobacter spp*. is very tedious and time-consuming task owing to the presence of multifaceted micro-flora in faecal samples as well as fastidious and microaerophilic nature of *Campylobacter* spp. It takes 3-5 day in confirmation of a faecal sample. For isolation of *Campylobacter species* from faecal samples of wild animals pre-enrichment in PBS and enrichment in Bolton broth as well as CBA selective media were found very suitable method for accurate prevalence assessment. In India, majority outbreaks of foodborne disease go unreported, unrecognized or un-investigated and may only be noticed after major health or economic damage has occurred. In such a condition controlling the outbreaks, detection and removal of implicated foods, identification of the factors that contribute to the contamination, growth, survival and dissemination of the suspected agent, prevention of future outbreaks and strengthening of food safety policies and programmes is not possible. Hence a regular monitoring and surveillance system like European countries in needed to combat foodborne diarrhoeal diseases.

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