Print ISSN : 0972-8813
e-ISSN : 2582-2780

[Vol. 21(2) May-August 2023]

Pantnagar Journal of Research

(Formerly International Journal of Basic and Applied Agricultural Research ISSN : 2349-8765)

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PANTNAGAR JOURNAL OF RESEARCH

Vol. 21(2) May-August, 2023

CONTENTS

The growth potential of thermophilic Campylobacters on various culture media

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ABSTRACT: Campylobacter species represent a crucial group of zoonotic bacterial pathogens responsible for causing enteritis in various animals, including domestic, captive, wild, and non-captive animals, as well as birds. The objective of the current study was to comparatively isolate and identify *Campylobacter jejuni* and *Campylobacter coli* from faecal samples of wild animals. A total of 521 faecal samples were obtained from zoos, wildlife sanctuaries, and national parks located in the states of Uttar Pradesh, Uttarakhand, and Chhattisgarh. Following aseptic collection, processing, and primary isolation, the growth of identified Campylobacters was evaluated using five different artificial media, classified into two categories: blood-free and blood-containing media. The overall prevalence of Campylobacter spp. was found to be 11.90% (62 out of 521 samples), with *Campylobacter jejuni* accounting for 7.10% and *Campylobacter coli* for 4.80%. After enrichment, plating on Columbia Blood Agar (CBA) supplemented with selective supplements resulted in a significantly higher prevalence (11.90%) of Campylobacter spp. compared to other media such as Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) (10.56%) and Sheep blood agar (BA) (8.25%). The lowest isolation rate was observed on Chocolate Agar (CA) (5.76%) and Hi-chrome *Campylobacter* agar (HCCA) (4.22%). The results of multiplex PCR confirmed the identification of Campylobacter species as well as the sensitivity of each culture method.

Key words: *Campylobacter*, culture media, Multiplex PCR, prevalence

Campylobacter bacteria are fastidious organisms 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 environment (5% O_2, 10% CO_2, and 85% N_2) (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 (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 (Elbrissi *et al.*, 2017).

MATERIALS AND METHODS

A total of 521 faecal samples from wild animals, including mammals and birds, were collected 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).

All 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

Table 1: Places of faecal samples collection and description of animals

Sl. No.	Places of sample collection	No. of faecal samples collected	Ruminant	Non-Ruminant	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 and 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

hours. After the primary isolation of Campylobacter species, five different artificial media were assessed, 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). 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% CO2 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 test. The statistical analyses were performed using SPSS version 26.

RESULTS AND DISCUSSION

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 HCCA 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 11.90% of Campylobacter spp., as also reported by Hutchinson and Bolton (1984). However, we observed a recovery rate of 10.56% on mCCDA, as

Table 2: Campylobacter spp. prevalence for each culture method 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 superscript differ significantly. (P=1.42e-12) P<0.05

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. 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 selective supplement, it can be concluded that this method is preferable for the isolation of Campylobacter spp. in this study.

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).

CONCLUSION

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 PBW 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.

ACKNOWLEDGEMENTS

The author expresses gratitude to the Chief Conservators of Forest of Uttar Pradesh, Uttarakhand, and Chhattisgarh for granting permission to collect faecal samples from the wildlife in their respective zoos, national reserve forests, and sanctuaries. The author extends sincere thanks to the Director of ICAR-VPKAS, Almora, for granting study leave to conduct this research.

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Received: July 14, 2023
Accepted: August 14, 2023