Biological Forum – An International Journal **14(2): 1060-1063(2022)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Comparative Studies on Growth Performance of Thermophilic Campylobacters Isolated from Wild Animals on different Culture Media

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(Received 19 March 2022, Accepted 21 May, 2022)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: Campylobacter species are one of the important foodborne zoonotic bacterial pathogens triggering enteritis in domestic, wild captive and non-captive animals as well as birds that spreads through different environmental sources as water, feed, meat and milk. In the present study comparative isolation and identification of *Campylobacter jejuni* and *Campylobacter coli* was attempted from faecal samples of wild animals during the year 2020-2021. A total of 430 faecal samples were collected from zoos/wild life sanctuaries and national parks of Uttar Pradesh, Uttarakhand and Chhattisgarh states. Since thermophilic *Campylobacters*- target bacteria are microaerophilic and fastidious in nature, require specific temperature and nutrition for its optimum growth, hence to find out the suitable selective media, present study was designed. After aseptic collection, processing and primary isolation, growth of identified *Campylobacters* were assessed on five artificial media, broadly categorized into blood free and blood containing media. The overall positivity of *Campylobacter spp*. was 59 of 430 (13.72%) with *Campylobacter jejuni* (7.67 per cent) and *Campylobacter coli* (6.05 per cent). After enrichment, plating on CBA with selective supplement yielded a significantly higher (P<0.05) prevalence (4.65%) of *Campylobacter species.* No significant differences could be observed in mCCDA (3.72%) and BA (3.48%). Least isolation observed on CA (2.0%) and HCCA (1.16%). Multiplex PCR results confirmed speciation as well as sensitivity of each culture methods. As the majority of *Campylobacter spp.* were isolated by CBA with selective supplement. Hence, this may be the method of choice for isolation of *Campylobacter* species because of presence of hemin in sheep blood as oxygen quenching agent.

Keywords: *Campylobacter*; Foodborne pathogens; Culture media; Multiplex PCR; Prevalence; mCCDA.

INTRODUCTION

Along with *Salmonella, Campylobacter* is one of the important zoonotic foodborne bacterial pathogen that causes diarrhoeal disease in human and animals (WHO, 2020; Garcia *et al*., 2018). *Campylobacter* is one of the most frequently occurring bacterial agents of gastroenteritis. The true incidence of gastroenteritis due to *Campylobacter* spp. is poorly known, particularly in Low and Middle Income Country (LMIC); studies in 3 per 1000 population (WHO, 2012). The pathogen can be transmitted to human via food, water and through contact with farm animals and pets (Elbrissi *et al*., 2017). The *Campylobacter* organism is characterized by possessing Gram-negative, spiral, non-spore forming rods that form spherical or coccoid bodies in older cultures (Penner, 1988). They are between 0.2 to 0.9 microns wide and 0.5 to 5 microns long, are motile and usually move with a polar unsheathed flagellum at one or both ends, and are microaerobic with a respiratory-type metabolism, although there are some that grow aerobically or anaerobically (Fitzgerald and Nachamkin, 2011). *Campylobacter* is a fastidious organism generally requiring specific atmospheres and temperatures to grow, uses menaquinones as their respiratory quinones, does not ferment or oxidize carbohydrates but requires microaerophilic environment (5% O_2, 10% CO_2 and 85% N_2) for growth and isolates causing human gastroenteritis are primarily of the thermotolerant variety which can also grow at 42°C–

43°C (Vandamme and De Ley, 1991). Present study is based on the assessment of different artificial media for *Campylobacter* isolation and identification.

MATERIALS METHODS

Sample collection and culture. A total of 430 faecal samples of wild animals including mammals and birds were collected during April 2020 to March 2021 from six Zoos and National parks/Sanctuary of Uttarakhand (n=2), Uttar Pradesh (n=2) and Chhattisgarh (n=2), province of India (Table 1). All samples were processed immediately in the department of Veterinary Public Health and Epidemiology, College of Veterinary and Animal Sciences, GBPUA&T, Pantnagar, Uttarakhand, India as per ISO 10272-1:2017(E) guidelines and put for pre-enrichment in Buffered peptone water for overnight at 42°C in CO_2 incubator with 5 % CO_2 supply, after that enrichment of samples were done in Bolton broth (Hi-Media, Mumbai, India) supplemented with 5% sterile lysed defibrinated sheep blood and FD231 supplement (Hi-Media, Mumbai, India) to be incubated micro-aerobically in CO_2 incubator at 42°C for 48 hours. After primary isolation of *Campylobacter* species five artificial media were assessed, which were broadly categorized into two group- blood free and blood containing media.

Table 1: List of places from where faecal samples were collected.

Sr. No.	Place from where sample collected	No. of faecal samples collected
1.	MrigVihar and wild animal rescue center, NTD, Almora, Uttarakhand, India	24
2.	G. B. Pant High Altitude Nainital Zoo, Uttarakhand, India	32
3.	Jim Carbett National Park, Ramnagar, Nainital, Uttarakhand, India	87
4.	State Nandanban Zoo & Safari, New Raipur Madhya Pradesh, India	76
5.	NawabWajid Ali Shah Zoological Garden Lucknow, Uttar Pradesh	11
6.	Kanpur Zoological Park, Nawabganj Kanpur, Uttar Pradesh, India	34
7.	Peripherry of Achanakmar Sanctuary, Bilaspur, Chhattisgarh, India	59
8.	State Zoo, Bilaspur, Chhattisgarh, India	107
	Total	**430**

Culture Media. Blood free media were Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) and Hi-chrome *Campylobacter* agar (HCCA) while blood based media were Columbia blood agar (CBA), Sheep blood agar (BA) and chocolate agar (CA).

For mCCDA agar media, CCDA selective supplement (FD135, Hi-Media, Mumbai, India) was added to the basal agar as per the manufacturer's instructions. Briefly, 22.74 gm of basal medium was suspended in 500 ml of distilled water and dissolve uniformly by boiling, sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes and the media were cooled to 45-55°C, then added aseptically rehydrated contents of 1 vial of CCDA selective supplement (FD 135), mixed well and poured into sterile Petri plates (90x15 mm, Genaxy, Solan, Himachal Pradesh, India). For preparation of HCCA agar medium, 1 vial of a selective supplement FD078 (Hi-Media) was added to 29.77 gm HCCDA basal medium in 500 ml distilled water and further were processed same as mCCDA media prepared.

For Columbia blood agar (CBA) media, 44.0 gm of CBA basal medium was added in 1000 ml of distilled water, dissolve properly by heating and sterilized by autoclaving, cool down the medium to 45-55°C, 5% v/v sterile defibrinated sheep blood was added, then rehydrated content of Campylobacter growth supplement (FD009) was added and poured about 25 ml into each sterile Petri plate (size 90 × 15 mm, Genaxy, Solan, H. P., India).

For, Sheep blood agar (BA) media, 10 per cent defibrinated sheep blood and one vial rehydrated content of Campylobacter growth supplement (FD009) were added in 14.0 gm of Nutrient agar (Hi-media) in 500 ml distilled water. Cooling, sterilizing and pouring into sterile Petri plates were done same as in CBA media.

For making Chocolate Agar media, all steps will be same as in Blood Agar media except in place of autoclaving; media were heated at 80°C for 10 minutes with constant agitation. After sterility testing of all prepared media were kept at 4° C and used within 3-4 days.

Culture on media

After pre-enrichment and enrichment, the obtained isolates were inoculated in mCCDA, HCA, CBA, BA and CA media and incubated in CO_2 incubator (New Brunswick, Germany) which were maintained 5 per cent CO_2 supply and 42°C temperatures. The incubation was done for 48-72 hrs period. Suspected and well-isolated colonies were subcultured onto the same media for purification which were checked by Gram's staining and all presumed colonies were further identified by standard biochemical tests as oxidase test, catalase test, Hippurate hydrolysis test, Campylobacter nitrate reduction test, urease test and H_2S production on TSI test methods (Atabay and Corry, 1997). Biochemically

positive isolates were grown in Tryptone soya broth and aliquoted in 4.5 ml cryo-vials with 20 per cent sterile glycerol and preserved in -80°C for future use.

Campylobacter speciation. On the basis of colonies appearance, staining properties and biochemical results, positive isolates were further confirmed through multiplex PCR (Shams *et al*., 2017) after DNA was extraction (Ertas *et al.*, 2004).

Statistical Analysis. The prevalence data of *Campylobacter spp*. recovered from each culture media was statistically compared by one way analysis of variance followed by least significance difference (DMRT). The analyses were carried out by using SPSS version 26 statistical programme.

RESULTS AND DISCUSSIONS

All the suspected *Campylobacter* isolates were identified on the basis of their colonies characteristics, motility test, inability to grow in aerobic condition at 36°C temperature and Gram's staining features. Campylobacters colonies were small (1-2 mm), circular, flat to slightly raised, sticky, spreading, shiny grey coloured or water droplets on mCCDA, CBA, BA and Chocolate agar plates while in HCA plates *Campylobacter* species appear mauve to purple coloured colonies. The organisms appeared as pink coloured Gram-negative rod, spiral curved rods with comma shaped (S) or gull wing appearance cells. The similar colonies characteristics were also recorded by Monika (2014), and Garhia, (2017). The recovery rate of *Campylobacter spp*. in this study was higher (Table 2) in CBA culture media than that of mCCDA, HCA, BA and Chocolate agar culture method studied. The overall positivity of *Campylobacter spp*. was 13.72% (59/430) with *Campylobacter jejuni* (7.67 %) and *Campylobacter coli* (6.05 %) agreeing with findings of Acke *et al.* (2009). After enrichment, plating on CBA with selective supplement yielded a significantly higher ($P<0.05$) prevalence as 4.65% of *Campylobacter species.* The similar observations were also reported by Hutchinson and Bolton (1984). However, we could observe 3.72% recovery on mCCDA as reported by Roma (2019); Ansari (2021); Corry and Atabay (1997); and 3.48% on BA (Byrne *et al.*, 2001) showing none significant differences (Table 3) and they are followed by CA (2.0%) as also reported by Aspinall *et al.* (1996) and on 1.16% HCCA (Humphrey *et al.*, 2007). Multiplex PCR results confirmed its speciation as well as sensitivity of each culture methods. As the majority of *Campylobacter* spp. were isolated by CBA media with selective supplement. It was also found that pre-enrichment and an enrichment step reduces the transport stress and enhances the recovery of *Campylobacter* spp than either direct plating or filtration on to selective media. Since CBA showed higher recovery rate of Campylobacter spp ($P<0.05$), hence it might be more accurate blood based assessment method for the actual prevalence of Campylobacter spp. in the sampled population. In blood free method mCCDA would be comparatively better for assessment of *Campylobacter* spp prevalence. In both the method - CBA and mCCDA, hemin (Fe^{3+}) and charcoal respectively act as source of energy and oxygen quenching agent, which is needed for growth and microaerophilic environment.

Table 2: Campylobacter species isolated by five culture methods.

Campylobacter species identified			
Culture methods	***Campylobacter jejuni* (%)**	***Campylobacter coli*(%)**	**Total Campylobacter species isolated(%)**
1-mCCDA	9^{d}	7^{c}	16
2-Hichrome CA	3^{b}	2^{b}	05
3- CBA	11^{e}	9^{d}	20
4-BA	8^{c}	7^{c}	15
5-Chocolate Agar	2^{a}	1^{a}	03
Combined method	33/59 (55.9)	26/59(44.0)	59/59 (100)
	33/430 (7.67)	26/430 (6.05)	59/430 (13.72)

Figures having different superscript differ significantly.

Table 3: Campylobacter spp. prevalence for each culture method and combined method (%).

Sr. No.	Culture Methods	Campylobacter spp. prevalence (%)
1.	mCCDA	$16/430\ (3.72)^{c}$
2.	Hi-chrome CA	$05/430\ (1.16)^{a}$
3.	CBA	$20/430\ (4.65)^{d}$
4.	BA	$15/430\ (3.48)^{c}$
5.	Chocolate Agar (CA)	$03/150\ (2.0)^{b}$
	Total	**59/430(13.72)**

Figures having different superscript differ significantly.

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 PBS and enrichment in Bolton broth as well as CBA selective media were found very suitable method for accurate prevalence assessment. In case of unavailability of sheep blood facility charcoal based mCCDA media can be used. 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 in India.

Acknowledgements. The author thank to all Chief conservator of Forest of Uttar Pradesh, Uttarakhand and Chhattisgarh state for providing permission for collection of faecal samples from their wild animals of concern zoo/ National reserve forests/ Sanctuary. The author is very thankful to the Director, ICAR-VPKAS, Almora for permitting me study leave to do the research work. The author is very thankful to the P.I. of project (ICAR funded)- "Outreach programme on Zoonotic diseases" for providing financial help for the present study. He also thanks the supporting staffs and lab technician for providing invaluable support and kind help during completion of the part of my Ph. D. research work.

Conflict of Interest. None.

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How to cite this article: Nawal Kishor Singh, A.K. Upadhyay, Maansi, Rajesh Kumar, Aman Kamboj, T.K. Ambwani, Himani Sharma, Namita Shukla and Ajay Kumar (2022). Comparative Studies on Growth Performance of Thermophilic Campylobacters Isolated from Wild Animals on Different Culture Media. *Biological Forum – An International Journal*, *14*(2): 1060-1063.