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ISOLATION OF CAMPYLOBACTER FROM HUMAN STOOL SAMPLES, CONVENTIONAL CULTURE METHODS VERSUS MOLECULAR METHODS

Microbiology

Dr.Ch.Suryamani* Assistant Professor, Department of Microbiology, Andhra Medical College, Visakh apatnam,Andhra Pradesh. *corresponding author

ABSTRACT

CONTEXT: Campylobacter is an undected cause of diarrhoea especially under 5 years of age in most of the countries . Isolation of the organism is difficult,expensive and cumbersome.

AIMS: Our objective of this study was to isolate this pathogen from the stool specimens on routinely available blood containing laboratory media using the candle jar for creating the microaerophilic atmosphere in our setup.

SETTINGS AND DESIGNS: A descriptive study.

MATERIALS AND METHODS: A total of 100 stool samples were inoculated onto selective and non-selective media with and without filtration using 0.45 um membrane. The inoculated media were simultaneously incubated in microaerophilic conditions using the Candle jar at temperatures 37Oc and 420C. The culture isolates were confirmed by standard phenotypic tests. A simplex polymerase chain reaction (PCR) targeting the 16S ribosomal deoxyribonucleic acid of Campylobacter was performed on the deoxyribonucleic acid (DNA) of the culture isolates as well as on the DNA extracted from the stool filtrates.

STATISTICALANALYSIS: Data was expressed as proportion.

RESULTS: Campylobacter could be isolated in 10 out of 100 stool samples . Furthermore, we did not find any difference between the isolation using the selective and blood containing media as well as the different incubation temperatures. All the ten were confirmed phenotypically and genotypically to be Campylobacter jejuni. The PCR results corroborated with that of the culture.

CONCLUSIONS: Isolation by culture was as sensitive as that of the PCR.

KEYWORDS

Blood agar, Campyobacter, Candle jar, Culture, Polymerase chain raction.

INREODUCTION: Campylobacter jejuni and Campylobacter coli are the most commonly reported bacterial causes of acute gastroenteritis in humans in both developing and developed countries [(1)]. Campylobacters may cause a spectrum of illness in humans. The signs manifested by the patients include abdominal cramping and diarrhoea. Other extraintestinal diseases may result from Campylobacter infections include bacteraemia, endocarditis, meningitis,urinary tract infectins, Gullian-Barre syndrome, which are the acute paralyric diseases of the peripheral nervous system.

Campylobacters are curved rods that were classified as Vibrio for many years. The most common human disease caused by Campylobacter is an acute gastroenteritis. Infection occurs primarily in infants, elder people and patients with underlying disease. The organism is isolated from infants and young adults more frequently than from persons in other age groups and from males more frequently than in females. Disease is associated with fever, bloody diarrhoea, headache and severe abdominal pain[(2)]. Campylobacteriosis is a self limiting disease and antimicrobial therapy is not generally required. However timely treatment can reduce the duration and severity of the infection [(3)]. Most people who develop campylobacteriosis recover completely within 2-5 days although sometimes recovery can take upto 10 days[(4,5)].

In developed and developing countries, Campylobacter cause more cases of diarrhoea than food borne Salmonella. High incidence of Campylobacter diarrhoea, as well as it's duration and possible sequelae, makes it highly important from a socio-economic perspective[(4)]. Campylobacter infections were found to cause diarrhoeal diseases 2-7 times as frequently as infections with Salmonella species,Shigella species or are usually associated O157:h7 [(5)]. Many of the cases go undiagnosed or unreported and Campylobacteriosis is estimated to affect over 2.4 million persons every year, or 0.8% of the population.

Campylobacteriosis occurs much more frequently in the summer months than in the winter. This disease usually occurs in single, sporadic cases, but it can also occur in outbreaks. Outbreaks of Campylobacter are usually associated with unpasteurized milk or contaminated water.The organism does not usually spread from one person to another, but this can happen if the infected person is producing large volumes of diarrhoeic stools. A very small number of Campylobacter organisms(fewer than 500) can cause illness in humans[(5)]

Even though the disease resulting from an infection with Campylob acter is usually self limiting, complications like bacteremia can arise due to an inadequate therapy, sometimes sequelae can be seen in the form of Guillain-Barre's syndrome and a veriant the MillerFischer syndrome [(4-9)]. Therefore it is necessary to detect Campylobacter from diarrhoeic stool and so as to initiate prompt and appropriate antimicrobial therapy which can reduce the duration and severity of infection.[(10-11)]

Campyobacter is a fastidious organism and it requires the microaero philic environment for growth and optimum temperature for growth is 30-370C. Most of the isolates causing human gastroenteritis are said to be of thermotolerant variety (can grow at incubation temperatures of 42-43oC. These include Campylobacter jejuni, C.coli, C.lari [(3-6)] Microaerophilic atmosphere refers to the presence of around 2-10% of oxygen which can be created manually (e.g candle jar) or using chemical substances (e.g gas generating packs) or by the automated system. The use of candle jar was demonostrated as early as the late 1990's for the isolation of Campylobacter spp[(7)].

The present study was performed to isolate this pathogen from the stool specimen on routinely available laboratory media using candle jar for creating the microaerophiic atmosphere.

MATERIALS AND METHODS

This study was approved by Institute Ethics Committee and informed consent was obtained from the respective guardians accompanying the children before collecting their stool samples for the study.

Sample collection details:-

The study included consecutive 100 stool samples from children suffering from diarrhoea, dysentery,or an acute gastroenteritis who had reported to the Department of Pediatrics, King George Hospital, Visakhapatnam between June2015 and June 2016.

Samples were processed as soon as received: in cases of delay we had stored the samples in Carry-Blair medium at 4oC for 1 day. Filtrates of each stool sample were kept at -20oC for Polymerase Chain Reaction (PCR)

Isolation by Culture:-

Three media were tested simultaneously namely, the modified charcoal-cefoperazone deoxycholate agar (mCCDA) (HIMEDIA, Mumbai, India) with supplement, 5% sheep blood agar, chocolate agar. Samples were inoculated after filtration through a 0.45 um

membrane filter . The inoculated media were simultaneously incubated in microaerophilic atmosphere in candle jar 37OC and 42oC. The candle jar was in-house devised made of stainless steel with a tight fitting lid. In each jar of 1L Capacity 10 plates of 90 mm diameter could be kept at a time with 7-10 wax candles lighted to create the microaerophilic atmosphere. While closing the jar each time, petroleum jelly (Vaseline) was put on the side of the rim of the jar and then the jar was closed tightly, the jar was kept at 37OC and 42OC for 2 days. ATCC Pseudomonas aeruginosa 27853 was inoculated simultaneously in a plate as control to check for the maintenance of the microaerophilic status while the ATCC C. jejuni strain 33291 was inoculated every time as a positive control. The same was also used for all the phenotypic tests as a positive control. Suspected colonies from the plates were checked by Gram stain, (slender curved "gull wing" shaped Gram negative rod), motility,0xidase and catalase tests. Hippurate test was used to differentiate C. jejuni and C.coli. The same stool samples were screened simultaneously for other pathogens like Salmonella, Shigella, Vibrio, Aeromonas and Enterohemorrhagic E. coli as described earlier[13]

Subsequently, a simplex PCR was performed, using primers targeting the 16S ribosomal deoxyribonucleicacid ,[14] for the confirmation of the culture isolates as well as Campylobacter directly from the sample. Oligonucleotides were obtained from Merck Bangalore genei (Bengaluru, India). For this purpose, two sets of deoxyribonucleic acid (DNA) were extracted, one set from the culture isolates by inoculating one colony in 100 ul of sterile milli Q water. This was kept in a water bath at 1000C for 5 min, then immediately placed into ice and centrifuged at 10,000 rpm for 5 min. The supernatant was taken as DNA as mentioned earlier . The second set of DNA was extracted from the filtrates of the stool samples using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. ATCC C.jejuni strain 33291 was used as a positive control. The reaction mixture for the PCR was prepared by using 9.5 ul of sterile milli Q water, forward and reverse primers 1 ul (0.4umol each primer) each, DNA 2.5 ul and ready to use master mix (Merck Bangalore genei, Bangalore,India) 11 ul. The forward primer was 5' AAT CTA ATG GCT TAA CCA TTA-3' and the reverse primer was 5'GTA ACT AGT TTA GTA TTC CGG 3', the expected product size being 1706 bp. The total reaction volume was 25ul. Amplification was carried out using 25 cycles of the following PCR conditions. Denaturation at 94oC for 1 min. annealing at 58oC for 1 min and extension at 72oC for 1 min. The amplicons (5 ul) were electrophoresed in 1% agarose gel stained with ethidium bromide and visualized under ultraviolet light.

Data was expressed as a proportion.

RESULTS

Out of 100 stool samples 66 were from males and 34 were from females aged from 2 days to 14 years. Of these 100 stool samples, 10 (10% demonstrated the presence of C. jejuni, 8(12.5%) of whom were males.

On all the three media tested growth of Campylobacter spp. was observed. Amongst all the media tested , the best colony morphology was observed on the blood agar and mCCDA medium followed by the chocolate agar. Also, the morphology on gram stain was well preserved, all the forms were observed , when the smears were prepared from the blood agar than when made from the colonies on the other media tested for the same. ATCC C.jejuni 33291 grew on all the, though the most luxuriant growth were seen on blood agar followed by mCCDA medium and chocolate agar. However in media like blood agar, there are chances of contamination with other facultative anaerobes which may not be noted in the selective media.

Campylobacter colonies were translucent and moist on blood agar (water droplet like colonies) while on the selective media i.e the mCCDA medium the colonies were creamy/ grey, moist with a tendency to spread with a metallic silver sheen. These typical colony morphologies were obtained after 48 h of incubation under microaerophilic conditions at 37Oc as well as at 42oC.

In the present study we used primers which could detect both C. jejeuni and C. coli the product size of the amplicon 1706 bp. The PCR offered no additional advantage in detecting this organism as the findings were corroborative with that of the culture methods.

TABLE I:

Camparison of culture and PCR for the detection of Campylobacter in stool samples

Number of samples Processed (N=100)	Isolation of Campylo bacter byculture (%) mCCDA Blood agar/chocolate agar		Detection of Campylo bacter by PCR(%)
No. of positives	10(10%)	10(10%) 90	10(10%)
No. of negatives	90		90

mCCDA:Modified charcoal-cefoperazone deoxycholate agar,PCR: Polymerase Chain Reaction

DISCUSSION

C.jejuni has been recognized as a major food borne pathogen resulting in diarrhoeal illness. The frequency of isolation of C. jejuni in various parts of the world varies due to the varying standards of living standards , water supply and feeding habits. Among the studies carried out in Europe,9.5% isolation rate was reported from France [15] and 6.7% from UK [14] while among studies from Africa indicates 9% isolation rate from Central Africa [16] and 44% from South Africa [17] studies from Asia show 17.7% isolation rate from Bangladesh [18]8% from Tehran [12] 12% from Lahore,[19] 18% from Rawalpindi, and varying reports from India, viz 16% from the rural population in Mumbai [21] 8.6% from Ranchi[22]and 14.8% from Vellore [23] . The isolation rate was 10% (10/100) in the present study.

Campylobacter mostly affects children. Majority of children suffering from Campylobacter associated diarrhoea in this study were less than 48 months of age,with the average age ranging between 2 days of life and 24 months of age. The maximum age at which Campylobacter could be isolated in our study was 5 years of age while minimum age for isolation was 2 days of life. We did not isolate any Campylobacter beyond 5 years of age. This is possibly the lowest age recorded in the available literature. In another study conducted at Rawalpindi,[20] the maximum and minimum age of isolation was 48 months and 3 months respectively. In a study carried out in China showed the peak incidence to be between 12 and 24 months of age,[24]. In Bangladesh, the maximum rate of isolation was obtained from children between 12 and 24 months,[18] . Study from Ranchi showed that maximum isolation was from below the age of 6 years of age , [22], while another study from vellore showed that the maximum rate of isolation was from preschool children.[23]. Our findings are very similar to the earlier studies and may add to the existing pattern. This emphasises that we should suspect this agent as a cause of diarrhoea in children above 1 day of life.

The total number of pathogens isolated in this study was 12(12%) which comprised of 10(10%) isolates of C. jejuni and 2(2%) of Escherichia coli. According to Allos, Campylobacter causes diarrhoea 2-7 times as frequently as infection with Salmonella, Shigella or E. coli,[4]. Amongst the patients infected with C.jejuni, 80% (8 out of 10 culture positive has diarrhoea and only 20%(2 0ut of 10 culture positive had dysentery . Out of 100 cases studied 32 (32%) had diarrhoea, 42 (42%) had dysentery and 26 (26%) had acute gastroenteritis. None from acute gastroenteritis cases yields Campylobacter on culture.Adenkule et al. found out that clinical spectrum of Campylobacter enteritis in their study ranged from a watery, mucoid non-bloody diarrhoea to that of abdominal pain and fever. [6]

There was no difference in the isolation of Campylobacter on mCCDA with that of blood agar. The advantage of using blood agar is that some of the drug susceptible strains of Campylobacter may be able to grow which might be inhibited on the mCCDA medium,hence the use of non-selective blood agar may prove better. The use of different temperatures for incubation also did not show any difference in the culture yields.

Out of 100 stool samples 10 (10%) yields Campylobacter species on culture.Al l these isolates hydrolyzed hippurate based on which we phenotypically identified our isolates to be C. jejuni, and were further confirmed by the PCR assay. In addition, the findings of the PCR assay when applied directly on the stool filtrates were similar to that of the culture methods.

We found this method of isolation of C. jejuni to be cost-effective,

simple in operation reliable and possessing good culture efficiency. Limitations of the study include cost effective. So we need more studies to determine the prevalence of campylobacteriosis. So, by early diagnosing the infection, we can prevent other diseases like endocarditis, meningitis, Gullian-Barre syndrome etc';

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