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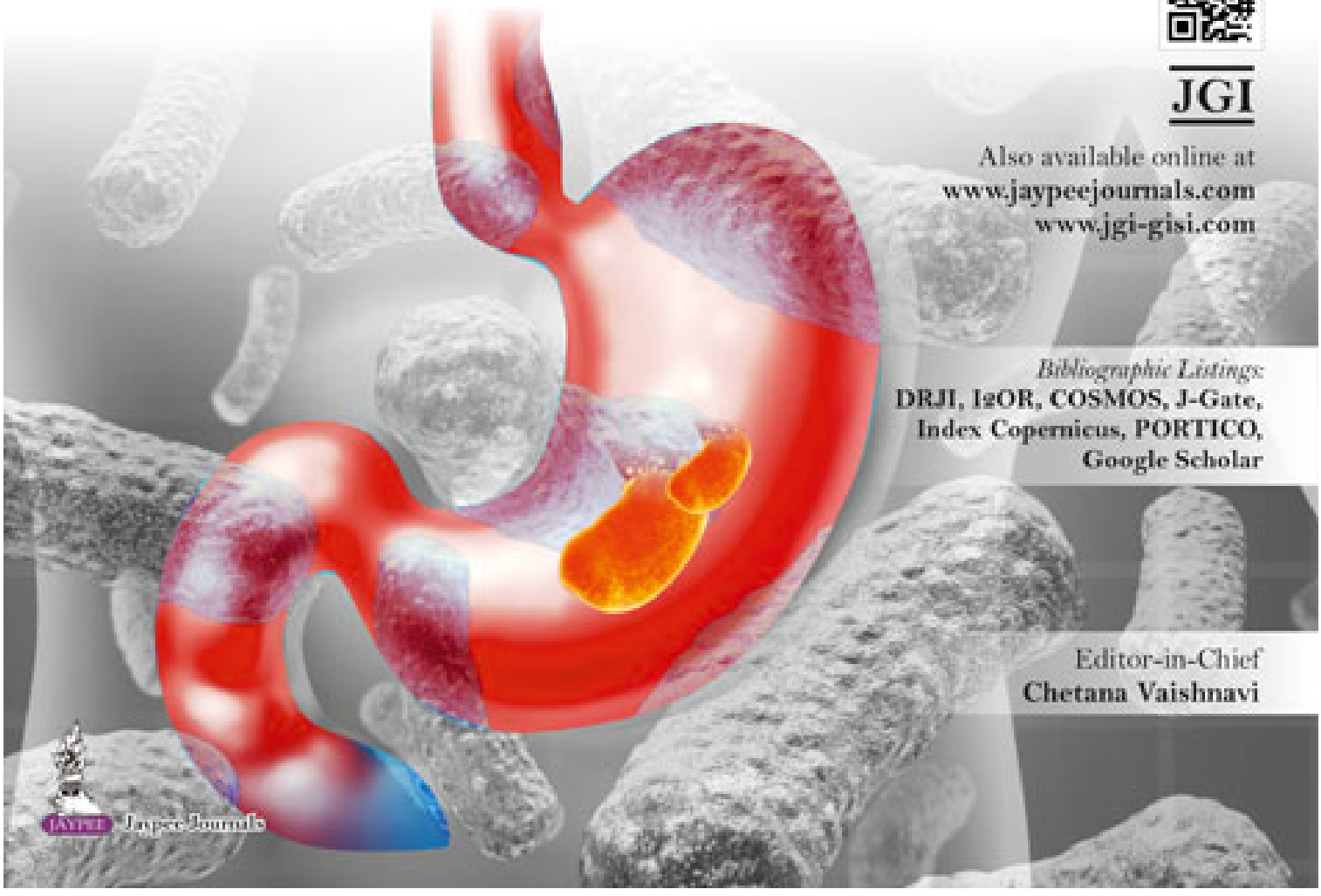

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Journal of Gastrointestinal Infections

1. Aims and Scope

The Gastrointestinal Infection Society of India (GISI) was born in 2007 as a result of earnest endeavors by the Founder Members. It is duly registered as 'Gastrointestinal Infection Society of India' under the Societies Act XXI of 1860. GISI began its operations with a view to work in line with the aspirations and objectives of some of the renowned gastroenterologists of the world. The society commenced its working with a membership of just 14 members. Down the line, GISI has been successful in developing its strength. The society currently maintains a membership of 300 members. The active members of GISI have been vigorously involved in creating awareness about gastrointestinal problems by holding conferences throughout the country. The efforts of the GISI members have filled the long-standing need for generating alertness in the minds of people as regards common gastrointestinal infections. The primary objective of GISI is to disseminate information related to GI infections. The society attempts to act as a forum of discussion for gastroenterologists, microbiologists and other healthcare professionals dealing with gastrointestinal infections.

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Contents

EDITORIAL

Role of 16S Ribosomal RNA in Etiological Diagnosis of Pyogenic Liver Abscess

Navneet Kaur[1], Ashwini Agarwal[2], Guduru Gopal Rao[3]

Keywords: 16S rRNA, Diagnosis, Liver abscess.
Journal of Gastrointestinal Infections (2021): 10.5005/jp-journals-10068-3045

Liver abscess is an uncommon but life-threatening condition seen in all parts of the world with mortality rates ranging from 2.5 to 26.4%.[1] Liver abscesses are most commonly caused by either bacteria (pyogenic) or *Entamoeba histolytica* (amebic) infection. The incidence of liver abscesses varies between different parts of the world as also in the relative proportion of pyogenic and amebic abscesses. In India, amebic abscesses are more frequent than pyogenic abscesses whereas in the Western countries, bacterial abscesses are by far commoner except in patients who give history for travel or past residence in countries where *E. histolytica* is endemic.[2]

Advances in imaging, less invasive interventive procedures for source control, and antibiotic treatment of pyogenic liver abscesses have improved the outcomes and reduced the mortality of this condition. However, in making an etiological diagnosis, limitations of traditional culture methods are being increasingly recognized. Many studies on the etiology of pyogenic liver abscesses have described that the traditional culture methods fail to detect the causal bacteria in up to 30% of cases or may not fully detect all the bacteria causing the abscesses.[1] The failure to culture organisms could be due to the inherent lack of sensitivity of culture methods, slow growth, fastidious growth requirements, or particular incubation needs. Furthermore, many patients with suspected liver abscess are treated with empirical antibiotics, which may significantly reduce the ability to successfully culture the causative organisms.

In the recent decades, molecular methods such as polymerase chain reaction (PCR) have transformed the etiological diagnosis of various infections and indeed have replaced traditional culture-based diagnosis. However, PCR-based methods are limited by the requirement of specific primers against specific targets. It follows that PCR-based methods cannot detect the etiology of infections where the range of organisms is diverse and the necessary primers are not included in the test. New molecular methods have emerged to overcome this important limitation of PCR.

One such method is the sequencing of 16S ribosomal RNA (16S rRNA) in the pus aspirated from the liver abscess. The bacterial ribosome (70S ribosome) structurally consists of 30S and 50S subunits. The 30S subunit is composed of mainly 16S rRNA whereas 50S subunit contains 5S and 23S rRNA. Importantly, 16S rRNA is evolutionarily conserved and found in all the bacteria. Detection and identification of bacteria based on the 16S rRNA sequence have become particularly useful because the gene encoding 16S rRNA has a "conserved" region that exists universally among bacteria and a hypervariable region that is specific for identifying bacteria at a species level.[3] Analysis of entire 16S gene sequences can even distinguish between strains of the same species of bacteria.

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Sequencing of 16S rRNA gene involves extraction of DNA from clinical specimens and using it as a template for PCR to amplify a segment of about 500 or 1500 bp of the 16S rRNA gene sequence. Bacteria can be identified by nucleotide sequence analysis of the PCR product followed by comparison of this sequence with known sequences stored in a database.[3]

Many recently published reports have employed 16S rRNA sequencing in the timely identification of unusual non-cultivable bacteria, which has helped in the early initiation of definitive antibiotic therapy in liver abscess. The identified bacteria using this technique comprise *Eggerthella lenta*, *Aggregatibacter aphrophilus*, *Pannonibacter phragmitetus*, *Parvimonas micra*, *Streptococcus oralis*, *Fusobacterium* spp, *Bacteroides* spp, *Prevotella* spp, *Peptostreptococcus*, Unassigned *Enterobacteriaceae*, etc.[4–6]

Clearly, 16S rRNA sequencing is proving to be useful in detection and identification of bacteria causing liver abscess, but it has important limitations. First, it does not provide antibiotic susceptibility data that have to be inferred from the identity of the organism.[7] Second, low taxonomical resolution of the sequencing reads has been observed for some bacterial genus. Thirdly, for superior species identification, where interspecies hypervariable regions are similar as observed between *Escherichia coli* and *Shigella* spp. and between *Streptococcus viridians*; additional sequencing of other genes are needed.[8] In addition, at a practical level, especially in resource-limited settings, establishing a laboratory for sequencing requires substantial funds and technically competent laboratory personnel.[9]

Provided resources and funding are available, 16S rRNA has the potential to change our understanding of etiology of liver abscess and make an important contribution in patient's management. PCR

based on 16S rRNA has also proven its diagnostic utility in etiological diagnosis of sepsis, infective endocarditis, and osteomyelitis particularly in culture-negative cases. Indeed, 16S rRNA promises to usher in a new era of diagnostic microbiology for a variety of infections.

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ORIGINAL RESEARCH

Does Over-colonization of *Klebsiella pneumoniae* in the Gut Cause Obesity?

Gopal Nath[1], Shweta Singh[2], Rajesh Kumar[3]

Abstract

Background and objectives: Gut microbes have been implicated in human weight gain and involve a few species of the Enterobacteriaceae family such as *Klebsiella pneumoniae*. We have tried to explore the effect of early colonization of the *K. pneumoniae* and subsequent eradication through bacteriophage therapy in rat pups on weight gain and loss.

Materials and methods: Three pairs of rats selected for mating were grouped separately. Group I having five pups were kept on a sterile diet. Five pups each belonging to group II and III were fed with *K. pneumoniae*. At the end of 10th week, the pups belonging to the group III were fed with *K. pneumoniae*-specific phages for 8 weeks. At the end of 30th week, group III were again fed with the bacterium, while group II received bacteriophage therapy for the next 8 weeks. The weight of each of the pups was noted every Monday of the week till the completion of the study.

Results: There was significantly higher weight gain (p <0.001) in the rats colonized by the bacterium (50% higher) than those without the colonization by *K. pneumoniae* by the end of the seventh week. When the bacterium was eradicated using a specific bacteriophage cocktail orally, the mean weight decreased and became almost similar to that of the control rats in about 12 weeks.

Conclusion: The bacterial species *K. pneumoniae*, which is a saprophyte with voracious metabolic activities, may lead to more harvesting of energy from the food and in turn lead to obesity.

Keywords: Bacteriophage therapy, Charles Foster rats, *Klebsiella pneumoniae*, Obesity.

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Introduction

There has been a substantial increase in the prevalence of obesity among rural residents and older Indians since 1975.[1] Consequently, obesity-related comorbidities, i.e., cardiovascular diseases, type-II diabetes, osteoarthritis, gallbladder diseases, backache, obesity-associated cancers, hypertension, breathlessness, including psychological disturbances, are on the increase.[2] The etiopathogenesis of obesity is multifactorial. Various factors like genetics, economic, psychological, physical exercise, diet, reproductive, and pharmacological etc., have been proposed to contribute to the genesis of obesity.[3–9] The human metagenome is considered a composite of genes of *Homo sapiens* and those trillions of microbes colonizing the body.[10] The introduction of antibiotics for the last 70 years may have induced obesity as it affects the gut microbiome.[11] Gut microbiota exerts many functions, such as stimulating effect on the intestinal epithelium, leading to the appearance of microvilli and mobility affecting the quantity of energy absorbed.[11–14]

It has been demonstrated that germ-free mice eat more but gain less weight than conventionally reared mice, indicating the importance of gut microbiota and weight gain.[15] The transplantation of gut microbiota from discordant human twins to the two groups of germ-free mice ensued into the expression of the donor's respective phenotypic character again shows the significance of the type of gut microbiota on weight gain.[16] Therefore, the new term has been coined as "Infectobesity."[17] Gut dysbiosis in terms of preponderance of either Firmicutes or Bacteroides or Actinobacteria has been proposed in the causation of obesity; however, phylum-level differences of gut microbiota between lean and obese individuals may not be universally real.[18] To establish the concept of "Infectobesity", we should try to determine etiological agent/s causing obesity.[19] *Chlamydiae trachomatis, Selenomonas noxia*, *Helicobacter pylori, Chlamydophila pneumoniae*, and viruses, e.g. certain adenovirus, canine distemper virus, Borna disease virus, enteroviruses, and Herpes simplex 1 and 2, etc., have been implicated in the genesis of obesity.[20,21]

In a pilot study, we cultured stool samples from five lean and obese subjects. Interestingly, all the five stool samples from obese subjects yielded *K. pneumoniae* as a predominant growth, while this bacterium was absent from the samples of leans subjects (unpublished data). A few Chinese studies claim that *K. pneumoniae* and *Enterobacter cloacae* have been associated with nonalcoholic fatty liver disease in human being[22] and enhanced subcutaneous fat accumulation in rats.[23]

With this background, we have planned to explore the effect of *K. pneumoniae* colonization and its eradication by using its specific bacteriophage cocktail on weight gain or loss in an animal model.

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Materials and Methods

The Institute Animal Ethics Committee of Banaras, Hindu University, approved the experimental protocol (Dean/2018/C.A.E.C./821 dated August 29, 2018). This study was carried out from July 2019 to May 2020.

Study Design

Three pairs of Charles Foster adult male and female rats were selected for mating. The selected rats did not have prior colonization of *K. pneumoniae* in their gut, as proved by culturing stool samples on MacConkey agar. All three pairs of rats were fed *ad libitum* with a standard chow diet and sterile drinking water. The animals were divided into three groups:

Group I: This group comprised the five pups delivered from the mother who was only on a standard chow diet and sterile drinking water.

Group II: In this group, the five pups delivered from the mother who was given *K. pneumoniae* in the drinking water at the final concentration of 10^9 CFU/mL since put for mating were included. The bacterial feeding was continued up to 10 weeks after birth. The bacteriophage cocktail was initiated at the concentration of 10^{12} PFU/mL at the end of 30th week and continued for further 2 weeks.

Group III: This group consisted of the four pups delivered from the mother who was given *K. pneumoniae* in the drinking water at a final concentration of 10^9 CFU/mL since put for mating. The bacterial feeding was continued up to 10 weeks after birth. The bacteriophage cocktail at a concentration of 10^{12} PFU/mL was initiated at the end of 10th week and continued for further 2 weeks. At the beginning of 31st week, the rats belonging to this group were again fed with *K. pneumoniae* at a final concentration of 10^9 CFU/mL for 8 weeks.

The weight of each of the experimental animal was recorded every Monday of the week. Blood samples were collected from the retro-orbital vein of the rats at the end of 30th week to estimate serum urea, creatinine, cholesterol, triglyceride, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), total bilirubin (TB), and direct bilirubin. These parameters were estimated by using the Johnson & Johnson-4600 Chemistry System Autoanalyzer (Mumbai, India) working on the principle of Dry Chemistry Technology.

Isolation of *K. pneumoniae* Strains

A total of 100 isolates of *K. pneumoniae* were isolated from clinical and environmental samples. These isolates were used to test specific bacteriophage activity in order to pick up the three most lytic phages for the strain used for feeding the rats. One isolate of *K. pneumoniae* (*Kpn*ob01) from an obese individual was isolated and identified. This isolate was suspended in normal saline at a concentration of 10^9 CFU/mL and was given to the animals in the study through drinking water.

Isolation of Bacteriophages

For isolation of respective bacteriophages, water specimens in the volume of 100 mL were collected from different sources like hospital sewage, river Ganga, ponds, sewer of the municipal corporation, etc. The water was centrifuged, and the supernatant was collected and treated with 1% chloroform for 10 minutes. The lawn culture of the different isolates of *K. pneumoniae* was brought into the log phase by incubating for 4 hours in a 90 mm Petri plate. The chloroform-treated water was poured on each plate in the volume of 2 mL and incubated overnight at 37°C for plaque formation. If plaques were not seen, the surface of the plate was washed with 5 mL Tris-Magnesium chloride buffer (pH 7.0). The washing obtained was centrifuged and treated with 1% chloroform to lyse the bacteria and to spare the protein-coated viruses. The supernatant was then dropped on fresh lawn culture of the host bacterium in the log phase. After overnight incubation the plaques seen with different morphology were cut and propagated on the host bacterium. The number of phages was increased by inoculating a larger surface area of the host bacterium lawn culture on Roux bottles. The sufficient volume of the harvest was subjected to membrane dialysis at 4°C with three changes of 25% polyethylene glycol buffer three times. The purified phages were suspended in normal saline to have ready to use phages at the concentration of 10^{12} PFU/mL.

Statistical Analysis

Statistical analysis was done using SPSS trial version 21.0 software. For comparing the mean values among the groups, ANOVA, and between the two groups, Student's *t*-test, have been used, if the data followed the Gaussian distribution. If the data did not follow the normality, the Wilcoxon signed-rank test and the Kruskal-Wallis test were applied. If ANOVA/Kruskal-Wallis tests resulted in significant differences, a post-hoc test (Student-Newman-Keuls) was used to determine pairwise differences. The critical value of *p* indicating the probability of significant difference was taken as <0.05 for comparisons at two-tailed tests.

Results

At the beginning of the experiment, none of the stool samples collected from six adult rats yielded *K. pneumoniae*. Table 1 and Figure 1 show that the weight of the pups in all the three study groups was similar at birth. At the end of 7 days, the mean percentage weight gain in groups II and III was 4.8 and 10.1, respectively, compared to group I. The highest percentage of weight gain was observed in the rats fed with *K. pneumoniae* (groups II and III) than the control (group I) during the sixth and seventh weeks (55.7–62.9%, respectively). At the end of the 10th week, the overall percentage of weight gain was 18.4 in group II and 13.3% in group III. The mean percentage weight gain (18.4) was significantly higher in rats fed with *K. pneumoniae* (group I vs group II; $p < 0.021$).

Interestingly when the bacteriophage cocktail therapy was started at the end of 10th week and continued for 15 days, the mean weight of the intervention group III decreased (220.7 g) and was comparable ($p = 0.772$) with the mean weight of the control group (229 g). *K. pneumoniae* could not be isolated from the stool samples after 1 week of the phage therapy. However, the phage therapy was continued for 8 weeks, and comparable weight could be seen in both groups I and III. Interestingly *K. pneumoniae* was observed continuously getting excreted by all the rats of group II. At the end of 30th week, the mean weight in the control group was 228 g, while those on *K. pneumoniae* were 288 g, which was 26.3% higher ($p < 0.029$). The mean weight of group III was 2.8% less at the end of 30th week. However, the mean weight difference between groups I and III was statistically comparable ($p > 0.5$).

Table 1: Percentage gain/loss in rats on oral *K. pneumoniae* and its bacteriophages as compared to the control group of rats

Weeks	*Group I (Control group)*	*Group II (K. pneumoniae + phage therapy at 30th week)*	*Group I vs group II (% weight gain/ loss)*	*Group III (K. pneumoniae + phage therapy at 10th week + repeat K. pneumoniae at 30th week)*	*Group I vs group III (% weight gain/loss)*
0	6	6	00	6	00
1	16.8	17.6	+4.8	18.5	+10.1
2	26.2	25	−4.5	25	−4.5
3	29.4	41.9	+42.5	42.25	+43.7
4	51.8	72.8	+40.5	73.25	+41.4
5	69.4	99	+42.6	108.75	+56.7
6	87.6	136.4	+55.7	115.75	+62.9
7	97.6	151.8	+55.7	142.75	+62.9
8	132.8	165.8	+24.8	156.5	+29.1
9	142.8	180.2	+15.2	171.5	+15.8
10	161.2	190.8	+18.4	182.75	+13.3
11	168.8	209	+23.8	174.25	+9.9
12	182.4	222.6	+22.03	180.75	−0.9
13	193.6	237.6	+22.4	181.5	−6.1
14	194.4	241	+23.9	187.5	−3.5
15	197.2	255.6	+29.6	192	−2.6
16	207.8	248	+19.3	196.75	−5.3
17	207.4	251.8	+21.1	200.25	−3.4
18	207.4	253.4	+22.2	205.75	−0.9
19	207.8	265.6	+27.8	219.25	+5.5
20	206.8	271.6	+31.3	213.25	+3.1
21	202	275.2	+36.2	217.75	+7.8
22	201.2	275.8	+37.1	223.5	+11.1
23	211.2	279.2	+32.2	220.25	+4.3
24	212.6	279	+32	216.5	+1.8
25	213.2	282	+32.3	221	+3.6
26	215.6	285.6	+32.4	215	−0.09
27	226	279	+23.4	223	−1.3
28	229	294	+22.1	226.75	−0.9
30	228	288	+26.3	221.5	−2.8
32	227.4	280	+23.1	219.75	−3.3
34	230.8	259.25	+8.0	219.75	−4.8
36	206	258.5	+25.5	201.5	−2.1
38	222.4	208.5	−6.2	154.4	−30.6
40	233	213	−8.5	280	+20
42	230	223	−3.0	283	+23
44	232.5	225.5	−3	268.5	+15.5
46	235.2	213.75	−9.1	280	+19.1

We did a reversal of therapy at the end of the 30th week. We administered bacteria to the rats on phage therapy (group III) and initiated phage therapy to those who were exclusively on bacteria (group II) up to 10 weeks; we observed a weight gain by 20% in group III in comparison to group I at the end of 38th week. However, group II, which was on phage therapy, lost weight and become comparable to the control group ($p = 0.168$) by the end of 8 weeks (Table 1 and Fig. 1). The percentage of weight gain in group III was observed to be 19.1. Contrary to this, group II had a weight loss of 9.1% compared to the control group I (Table 1). The other significant

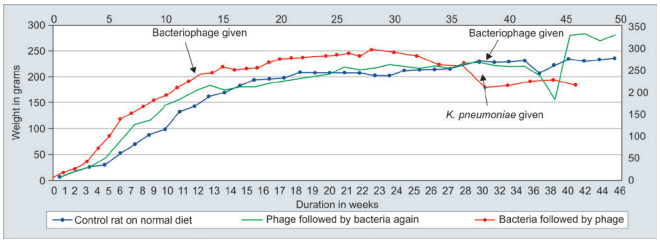

Fig. 1: Weight pattern of rats fed with *K. pneumoniae* and later treated with specific bacteriophage

observation was that one rat in group II died at the age of 32 weeks, weighing exceptionally high, i.e., 375 g, while the average weight of the rest of the rats in group II was 260 g at 30th week.

The levels of cholesterol, LDL, and SGOT were the highest in group I. In group II, the highest level of SGOT, total bilirubin, and direct bilirubin was observed. Further, group II also had the best ratio of HDL/CHOL and TG/HDL. The HDL level in group II was significantly higher than group III (p <0.029). The VLDL, however, was significantly higher in group III than group II (p <0.003). Further, the highest levels of TG and VLDL were observed in group III. Interestingly, the worst ratio between TG/CHOL was also observed in group II (Table 2).

Discussion

In this study, we observed that *K. pneumoniae* contributed to excess weight gain by rats made to colonize the bacterium in their gut. When the oral bacteriophage therapy eradicated the bacterial colonization of 10 and 30 weeks duration in rats, the weight loss could be observed on both occasions. The further surprising observation was that when the bacteriophage treated group of rats were again fed with fed *K. pneumoniae*, they gained a significantly higher weight (p <0.5) than the control group in the next 16 weeks.

Reports indicate that bacteria may produce several metabolites that may affect the composition of the gut microbiota. The metabolites may enter the blood circulation and act on distant organs like the liver, the adipose depot, and even the brain.[24] Pieces of evidence are available suggesting that certain bacteria do colonize the gut of obese persons, which may breakdown the food substrates (polysaccharides). These polysaccharides are usually undigestible by the flora available in lean subjects. These bacteria, if inhabited, may provide up to 40% more calories to the host for absorption and assimilation. Saprophytes can survive on simple carbon sources, e.g., citrate, nitrate, indigestible complex polysaccharides, etc. These saprophytes may produce various short-chain fatty acids (SCFA) like acetate, butyrate, and propionate as well as other metabolites (leptins, leptin receptor inhibitors, other neurotransmitters, trimethylamine, indole etc.),[25] which may help in lipogenesis. These metabolites and neurotransmitters may stimulate the hunger center. The SCFA like acetate may lead to fat deposition. Acetate is known for its potential for increased lipogenesis.[26]

Further, these bacteria may also cause auto-brewery syndrome because of their high fermentative abilities leading to the adverse effect of alcoholism, apart from obesity.[22] The authors have stated that alcohol drinkers following weekly low-risk drinking guidelines are not insulated from harm.[27]

We have to consider the present data with caution that *K. pneumoniae* may not be the only bacterium involved in excess weight gain. This is quite possible that other bacteria of saprophytic nature with good fermenting activity can cause more energy harvesting in the gut. The possible mechanism might be that these saprophytes can utilize even the citrate like simple carbon substrates. In support of this speculation, the plant *Garcinia* extract, hydroxy-citric acid, has been found to cause weight loss by competitively inhibiting the enzyme adenosine triphosphatase (ATP)-citrate-lyase.[28–31] Cytosolic acetyl-CoA synthesized by ATP citrate lyase is the primary enzyme responsible in many tissues. Cytosolic acetyl-CoA is used in several critical biosynthetic pathways, including lipogenesis and cholesterologenesis.[32] *K. pneumoniae* can utilize the citrate through a fermentative pathway involving carrier CitS, citrate lyase, oxaloacetate decarboxylase.[33] *K. pneumoniae* is also known for bioconversion of pentose sugars of hemicelluloses to ethanol.[34] Therefore, it is worth looking for the colonization of alcohol-producing bacteria in the human gut, which might cause all the adverse effect of alcohol consumption including weight gain despite being a teetotaler. The significance of the present work is that if a bacterial association is established with obesity; the culprit bacteria may be eradicated using specific bacteriophages.

Table 2: Showing lipid profile and liver function tests in different groups

	Group I (mean ± SEM)	*Group II (mean ± SEM)*	*Group III (mean ± SEM)*	*Significance*
Cholesterol (mg/dL)	109.40 ± 29.11	87.80 ± 32.51	91 ± 17.31	
TG (mg/dL)	216.40 ± 50.25	137.60 ± 74.40	307.25 ± 71.66	2 vs 3, p <0.003
HDL (mg/dL)	41.80 ± 4.87	37.00 ± 6.44	46.00 ± 4.24	
LDL (mg/dL)	24.32 ± 35.77	23.28 ± 31.13	16.20 ± 19.73	
VLDL (mg/dL)	43.28 ± 10.05	27.52 ± 14.88	61.45 ± 14.33	2 vs 3, p <0.003
SGOT	239.80 ± 73.67	162.20 ± .20.33	138.75 ± 33.95	
SGPT	101.00 ± 118.32	112.60 ± 98.50	35.25 ± 8.06	
Total bilirubin	0.34 ± 0.05	0.44 ± 0.22	0.38 ± 0.10	
Direct bilirubin	0.10 ± 00	0.12 ± 0.04	0.10 ± 0.00	
TG/HDL				1 vs 2, p <0.012
HDL/VLDL				2 vs 3, p <0.033
LDL/VLDL				2 vs 3, p <0.038

TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; SGOT, serum glutamic-oxaloacetic transaminase, SGPT, serum glutamic pyruvic transaminase

The implication of the detection of the highest HDL level, the highest HDL/CHOL, and the lowest TG/HDL ratios in the rats on bacterial therapy needs explanation. However, the highest SGPT, along with elevated total bilirubin and direct bilirubin (an indicator of liver damage), indicates that prolonged colonization with *K. pneumoniae* may cause injury to liver parenchyma. In support of this statement, *E. cloacae* have been reported in inducing hepatic damage and subcutaneous fat accumulation in mice on a high-fat diet.[35]

In conclusion, significant advances have been made, and our understanding concerning obesity is improving. The data presented in this study are based on a minimal number of rats. The high SGPT in group II might be indicating liver damage. Contrary to this, the excellent ratio between HDL/CHOL and HDL/TG in group II may indicate that *K. pneumoniae* may help maintain these healthy ratios. The highest levels of TG and VLDL in group III remain to be explained. Therefore the experiment may be repeated with many more animals to have robust data about the relationship between *K. pneumoniae* colonization and obesity and also its amelioration by specific bacteriophages can be verified. Exclusive dietary foods are usually associated with unpredictable outcomes. Therefore, the ultimate aim is to develop a personalized intervention if the causative agent/s or factors are known. Specific bacteriophage therapy may be a significant modality in this direction. There is an *in vitro* study where feces treated with specific bacteriophage before transplantation prevented the development of nonalcoholic fatty liver disease.[22] Our study is unique as we have fed the rats with the bacteria and eradicated it with a particular cocktail of phages, establishing the role of *K. pneumoniae* in obesity. However, a lot more is required to delineate the relationship between obesity and microbes and specific bacteriophage therapy.[36]

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ORIGINAL RESEARCH

Study of Prevalence of *Campylobacter* Gastroenteritis among Pediatric Population Using a Multiplex PCR in a Tertiary Care Hospital in Puducherry, South India

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Abstract

Background and objectives: *Campylobacter* is one of the four common causes of diarrheal illness worldwide. *Campylobacter* infection is more common in developing than in developed countries. As conventional methods pose a great difficulty for the isolation and identification of this organism, molecular methods are much preferred.

Materials and methods: A total of 133 stool samples were collected from children less than 13 years presenting to pediatric outpatient department and the emergency department in a tertiary care hospital in Puducherry. The stool samples were extracted, and the DNA was subjected to multiplex PCR to detect *Campylobacter* species, followed by sequencing.

Results: *Campylobacter* species was detected in 13 children (9.7% of the study population) (95% CI: 5.5–16.4), with *Campylobacter jejuni* (11 of 13) being the predominant species. The prevalence was higher in children less than 24 months (18.18%) with a higher predisposition to girls (14.29%). The most common clinical presentation was found to be acute watery diarrhea (10%). *Campylobacter* detection was higher from August to November (62%), with the highest incidence in October (22.3%). *Campylobacter* was detected in six (21.4%) children who had contact with pets.

Conclusion: The study reveals that the prevalence of *Campylobacter* infection was high in Puducherry. There is an increased need to implement molecular assays for the routine detection of *Campylobacter* in all clinical pediatric stool samples.

Keywords: *Campylobacter*, Children, Gastroenteritis, Multiplex PCR.

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Introduction

Campylobacter is one of the four common causes of diarrheal illness worldwide.[1] CDC estimates that about 1.5 million U.S. residents are infected by *Campylobacter* every year.[2] In India, diarrhea is the third common cause of mortality, with a mortality rate of 13%, and is responsible for the death of nearly 300,000 under 5 years children in India every year.[3] *C. jejuni* and *C. coli* are the primary agents of gastroenteritis in humans. *Campylobacter* is a slender, curved/spiral, "S"-shaped motile gram-negative, nonspore-forming microaerophilic organism with polar flagella.[4–6] Ingestion of contaminated food and water remains the primary mode of transmission.

The traditional method for the isolation of *Campylobacter* species from stool samples is by inoculating the sample onto the selective media such as blood or charcoal-based media and incubating the plates at 42°C in a microaerophilic (5% O_2, 10% CO_2, 85% N_2) environment.[7,8] Despite the public health problems posed by this organism, the routine isolation and identification of this organism are often missed due to the inappropriate diagnostic modalities. Therefore, this study was conducted to utilize multiplex PCR to identify *Campylobacter*, thereby determining the prevalence of *Campylobacter* infection among the pediatric population in a tertiary care hospital in Puducherry.

Materials and Methods

The study was a hospital-based cross-sectional descriptive study and was approved by the Institute Ethics committee (IEC No JIP/IEC/2018/0398). The duration of the study was from January 2019 to June 2020.

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Conflict of interest: None

Study Population

Stool samples from children less than 13 years of age with acute diarrhea and dysentery were included in the study after obtaining informed written consent from the parents/guardians. Stool samples from children with hospital-acquired (the onset of loose or watery stools at least 48 hours after hospital admission), persistent, and chronic diarrhea were excluded.

Sample Size Calculation

The approximate sample size calculated was 133 using sample size for sensitivity specificity studies by Naing.[9] This was calculated

considering the detection rate of *Campylobacter* using PCR to be 34% (0.34), the sensitivity of PCR to be 97% (i.e., 0.97) from a study conducted in New Delhi with a precision of 5% (0.05) and with a confidence level of 95% (0.95).[10]

Stool Processing

All the stool samples underwent routine processing as per our laboratory standard operating procedures upon reception at the laboratory. Briefly, each sample was examined macroscopically for the consistency, presence of obvious blood/mucus, and visible worms/proglottids. This was followed by microscopic examination of saline wet mount preparation to look for the presence of pus cells, red blood cells (RBCs), ova, cyst, and trophozoites. Subsequently, each sample was plated onto MacConkey agar (MAC), xylose lysine deoxycholate agar (XLD), and inoculated into selenite F enrichment broth. Watery samples were subjected to an additional plating on thiosulfate citrate bile salt sucrose agar (TCBS) and alkaline peptone water (APW). The plates and the broth were incubated aerobically at 37°C for 18 hours. After incubation the plates were read for the presence of any suspected colonies of Salmonella/*Shigella*/Vibrio and Aeromonas on XLD and MAC agar. Subculture from selenite F broth was done after 16–18 hours of incubation on MAC and XLD agar and incubated at 37°C for 18 hours. Subculture from APW was done within 6–8 hours of incubation on TCBS agar and incubated at 37°C for 18 hours. The remaining stool samples were preserved at 2–8°C till the DNA extraction is performed.

Stool DNA Extraction and PCR

Genomic DNA was extracted directly from the feces using QIAamp® Fast DNA Stool Mini Kit obtained from (Qiagen, Germany). The steps of extraction were done as per the manufacturer's protocol. Every extraction set was carried out along with the extraction of nuclease-free water, which served as the negative extraction control to rule out the carry over contamination during the extraction. The yielded DNA was stored at −80°C till further analysis.

Multiplex PCR was performed in a 25 µL reaction mixture containing a 2× master mix, 1.0 µL of each primer of the targets[11,12] and the internal control.[13] The target genes for the multiplex PCR were *16S rRNA* (genus *Campylobacter*), *mapA* (*C. jejuni*), *ceuA* (*C. coli*), and *actB* for internal control. β-*actin* was used as the internal control to rule out the presence of PCR inhibitors in the stool samples. Amplification reactions were as follows: one cycle of 10 minute at 95°C followed by 35 cycles each consisting of 30 seconds at 95°C, 90 seconds at 59°C, and 60 seconds at 72°C. The PCR was terminated by a final extension step of 10 minutes at 72°C. Amplification was expected to generate 857, 589, 462, and 619 base pair DNA fragments specific for the genus *Campylobacter* and for *C. jejuni*, *C. coli*, and β-*actin* genes, respectively. Post PCR, the PCR products were subjected to 1.5% gel electrophoresis and were visualized under ultraviolet light.[11] A 100 bp DNA ladder was used in gel electrophoresis that aided in determining the basepair size of the bands that are formed. Reference strain (ATCC *C. jejuni* 33291) was used as the positive control. Each set of multiplex PCR reactions was carried out along with a no template control, negative extraction control, and a positive control. The primers used in the study are mentioned in Table 1.

Sequencing of PCR Products

The products of PCR amplification were subjected to sequencing using an ABI 3730 XL sequencer (Applied Biosystem, Foster, California, USA) at Eurofins Genomics Private Limited, India. The sequences generated were assembled and compared using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information. Translation of DNA sequences into protein sequences was done using Expert Protein Analysis System translate. The translated protein sequences were assembled and compared using the protein BLAST of the NCBI.

Statistical Analysis

All the data were entered and analyzed using STATA 2.0 statistical software. Continuous variables, like age, were summarized as median depending on the distribution of the data. Categorical variables like gender, duration of symptoms, blood, or mucus in stools, duration of antibiotic use were expressed as a percentage. The Chi-square test was used to compare the two groups. A *p*-value of <0.05 was considered statistically significant. Outcome variable like detection of *Campylobacter* was expressed in percentage with 95% confidence interval.

Results

A total of 133 children were included in our study. Out of this, 77 (57.8%) were boys, and 56 (42.1%) were girls. The age distribution of these 133 children were 66 children (49.6%) less than 24 months, 22 (16.5%) between 25 and 60 months, 34 (25.5%) between 61 and 120 months and 11 children (8.2%) between 121 and 156 months. Of these 133, 111 (83.4%) presented with acute watery diarrhea and 22 (16.5%) with dysentery. 105 children (79%) had no contact with pets, and 28 (21%) had contact with pets. Of them, 96 (72.1%) presented in 3–5 days, 32 (24%) presented in 2 days, 5 (3.7%) presented after 5 days. Among the 133 children, 11 (8.3%) were already started on antibiotics, of which nearly nine were under antibiotic coverage for less than 48 hours, and two were between 48 hours to 120 hours. Out of the 133 stool samples, 55 (41.4%) were watery, 49 (36.8%) were semi-formed, 21 (15.8%) were blood-tinged, and 8 (6%) were formed. Microscopically, 100 (75.2%) samples had no pus cells, 26 (19.6%) had pus cells, 3 (2.3%) had RBC, 4 (3%) had both pus cells and RBC. Stool culture had grown enteric pathogens in 23 samples accounting for about 17%. The most common pathogen was found

Table 1: Details of multiplex PCR for the detection of members of the genus and of the thermophilic species of *Campylobacter*[*]

Target gene	*Sequence 5′–3′ of primers*[*]	*Amplicon length specificity*	*References*
16S rRNA	ATC TAA TGG CTT AAC CAT TAA AC GGA CGG TAA CTA GTT TAG TAT	857 bp *Campylobacter*	Denis et al., 1999[12*]
mapA	CTATTTTATTTTTGAGTGCTTGTG GCTTTATTTGCCATTTGTTTTATTA	589 bp *Campylobacter jejuni*	Denis et al., 1999[12*]
ceuA	TGATTTTATTATTTGTAGCAGCG AATTGAAAATTGCTCCAACTATG	462 bp *Campylobacter coli*	Denis et al., 1999[12*]
actb	GCACCACACCTTCTACAATG TGCTTGCTGATCCACATCTG	619 bp beta actin	Glare et al., 2002[13]

to be *Salmonella* species (10.3%), followed by *Shigella* species (6.7%) in the stool culture.

All these stool samples were subjected to multiplex PCR, of which 13 children were tested positive for campylobacteriosis, among which 8 (14.3%) were girls and 5 (6.5%) were boys ($p = 0.13$). The majority (18.9%) were less than 24 months which was found to be statistically significant. ($p \leq 0.05$). Acute watery diarrhea was found to be the common clinical presentation accounting for about 10%.

Distribution and association of campylobacteriosis with demographic and clinical variables is depicted in Table 2.

Table 2: Distribution and association of campylobacteriosis with demographic and clinical variables*

Variables	*Total no. of samples (n)%*	*No. of campylobacter positive cases, n (%)*	*95% CI*	*p value*
Gender				
Boys	77 (57.8%)	5 (6.5)	2.1–14.5	1
Girls	56 (42.1%)	8 (14.3)	6.4–26.2	0.13
Age				
<24 months	66 (49.6%)	11 (16.7)	8.6–27.9	0.12
25–60 months	22 (16.5%)	0	—	—
61–120 months	34 (25.5%)	2 (5.9)	0.7–19.7	1
121–156 months	11 (8.2%)	0	—	—
Clinical presentations				
Acute watery diarrhea	111 (83.4%)	11 (9.9)	5.1–17.0	0.9
Dysentery	22 (16.5%)	2 (9.1)	1.1–29.2	1
Fever				
Yes	37	6 (16.2)	6.2–32.0	0.12
No	96	7 (7.2)	3.0–14.4	1
Abdominal pain				
Yes	51	5 (9.8)	3.3–21.4	0.99
No	82	8 (9.8)	4.3–18.3	1
Nausea/vomiting				
Yes	27	5 (18.5)	6.3–38.1	0.08
No	106	8 (7.5)	3.3–14.3	1
Duration of symptoms				
<2 days	32 (24%)	3 (9.4)	2.0–25.0	0.7
3–5 days	96 (72.1%)	8 (8.3)	3.7–15.8	1
>5 days	5 (3.7%)	0	—	—
Contact with pets				
Yes	28 (21%)	6 (21.5)	8.3–40.1	0.9
No	105 (79%)	7 (6.7)	2.7–13.3	1
Under antibiotic coverage				
Yes	11 (8.3%)	0	—	—
<48 hours	9 (6.8%)	0	—	—
48–120 hours	2 (1.5%)	0	—	—
No	122 (91.7%)	13 (10.7)	5.8–17.5	—
Stool macroscopy				
Watery	55 (41.4%)	8 (14.5)	6.5–26.7	0.16
Semi-formed	49 (36.8%)	3 (6.1)	1.3–16.9	1
Formed	29 (21.8%)	2 (6.9)	8.5–22.8	0.89
Stool microscopy				
Pus cells+	26 (19.6%)	3 (11.5)	2.4–30.2	0.56
RBC+	3 (2.3%)	2 (66.7)	9.4–99.2	<0.001
Pus cells and RBC+	4 (3%)	0	—	—
Nil	100 (75.2%)	8 (8.0)	3.5–15.2	1
Stool culture				
Salmonella species	13 (10.3%)	0	—	—
Shigella species	10 (6.7%)	1 (10.0)	0.2–44.5	1
Nil	110	12 (10.9)	5.8–18.3	0.92

Of 133 samples, 13 (9.8%) were positive for *16S rRNA* gene, which corresponds to the genus *Campylobacter*, and 11 (84.6%) among the 13 samples were positive for the *mapA* gene confirming the species *jejuni*. The two remaining samples detected *Campylobacter* species other than *C. jejuni* and *C. coli*. The internal control, β *actin* gene, had flagged positively in all the stool samples ruling out the presence of PCR inhibitors.

Aligned sequences were searched in NCBI-BLAST (megablast) for the similarity of significant matches in the database. The sequences of *16S rRNA* showed 99.75% similarity with *C. jejuni* strain BfR-CA-12970 chromosome (Accession ID-CP054848.1) and *mapA* sequences showed 99.45% homology with *C. jejuni* strain 129108 chromosome (Accession ID-CP053854.1).

Discussion

Campylobacteriosis is common in developing countries. The overall burden was estimated to be 9.8% in the present study (95% CI: 5.5–16.4). This is in concordance with an earlier study from our institute in 2011, which showed a prevalence of 10%[14] and with the study in Assam and Nagaland in 2014–2016.[15] A report from Bhubaneswar from 2016 to 2017 found that 16.77% were positive for *Campylobacter* PCR.[16] A study conducted in Kolkata in 2008–2010 observed campylobacteriosis was 7% by culture[17] and 16.2% by real-time PCR. The prevalence of *campylobacter* was observed to be 8.5% in Meghalaya, Assam in 2010.[18] In 2012, the prevalence of campylobacter reported from Vellore[19] was 4.5%. In 2015, a low prevalence of campylobacteriosis (2.6%) from 1,145 diarrheal samples was reported from North India with *C. jejuni* as the most common species detected by culture and molecular investigation.[20] In a study from South India, the prevalence of *C. fetus* subsp. *jejuni* was found to be 14.8% isolated from the feces of healthy individuals.[21]

Asian studies showed an isolation rate of 14.9% from China,[22] 17.7% from Bangladesh, 8% from Tehran, 12% from Lahore, and 18% from Rawalpindi.[14] European studies revealed an isolation rate of 71.4% in the Netherlands, 31.9% in Portugal, 4.7% in Southern Ireland, 9.6% in North Poland.[23] Moreover, African studies stated a prevalence of 21% in Malawi, 8.9% in Madagascar, 5.8% in Kenya,[24] and 0.5% in Nigeria.[25]

The median age of presentation in our study was 36 months. Campylobacteriosis was higher (18.9%) in children less than 24 months which is in concordance with the study conducted earlier in our institute.[14] Similar findings were observed in Odisha, Israel,[15,26] and Bangladesh[27] and was also in line with the World Health Organization, indicating that the *Campylobacter* infection is common in children less than 2 years in the developing countries.[1] Another study from Denmark found a higher incidence in 1–4 years and young adults (15–24 years).[28] Kappareud et al.[29] found a higher incidence of *Campylobacter* in 0–4 years of age. This increased incidence in infants and toddlers could be because of the immature immune system, poor hand hygiene practices and contact with soil, water, and pets.[23]

Campylobacteriosis was found to be more in girls (14.3%) than boys (6.5%). This is in concordance with the study from Odisha, which showed a female preponderance (20%),[16] whereas in an earlier study conducted in our institute males had a higher prevalence.[14] This difference is not attributable to any host-specific or pathogen-specific factors related to virulence or manifestations of the disease.

The clinical presentation varies between developing countries and developed countries. In the former, it was watery diarrhea, whereas in the latter, it was bloody diarrhea. The most common presentation was found to be acute watery diarrhea (10%) in the present study. This is in concordance with the earlier study conducted in our institute.[14] The duration of symptoms was 3–5 days in 13.2% of the children. The mean duration of symptoms was 3 ± 1.1 days which is in line with the WHO data, suggesting that the symptoms typically last for 3–6 days.[1] In our study, the detection of *Campylobacter* was higher in watery stools (61.5%), followed by semi-formed stools (23%) and the least in formed stools (15%). Nearly 70% of the stools were devoid of pus cells and RBC.

The positivity rate was found to be higher (21.4%) in children who had contact with pets, similar to a Denmark study which found 52% isolation in children with pets. A study in North Poland found an isolation rate of 8.1% from pets.[23] Pintar et al.[30] found that the prevalence of *Campylobacter* in pet animals and petting zoo animals was 24.7 and 6.5%, respectively. Increased contact with pets has been associated with increased disease manifestations because the pets are known to serve as reservoirs.

Campylobacteriosis was highly detected during August–November (62%), with the highest in October (22.3%) as depicted in Figure 1. This is in concordance with a study from Denmark where the highest prevalence was from June to October.[28] In China, the isolation rate was the highest (6.29%) during June–August,[31] and in Beijing, it was in June.[22] This could be attributed to the monsoon changes as diarrheal episodes are relatively higher in the rainy season because of possible contamination of the ingested food and water with potential enteric pathogens.

None of the children with *Campylobacter* infection was started on antibiotics before sample collection. This is because fluid and electrolyte replacement is of prime importance for treating all forms of acute diarrhea. Antibiotics were not routinely recommended for all diarrhea cases in our hospital. This is in line with the existing guidelines for treating diarrheal diseases where antibiotics are indicated for dysentery and immunocompromised patients.[32]

Only 1 (7.6%) out of the 13 samples had grown *Shigella sonnei* in culture. There was no co-infection of other diarrheal disease-causing bacteria with *Campylobacter* observed in our study. Co-infection of *Campylobacter* with *Shigella* 0157 (2.2%) and

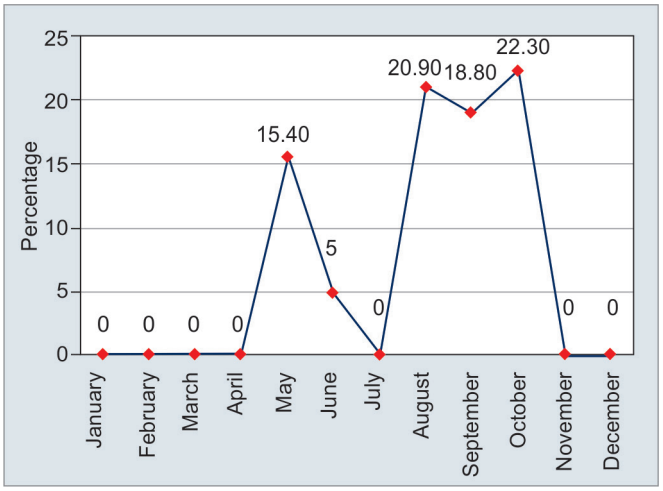

Fig. 1: Seasonal trends of campylobacteriosis from January to December 2019

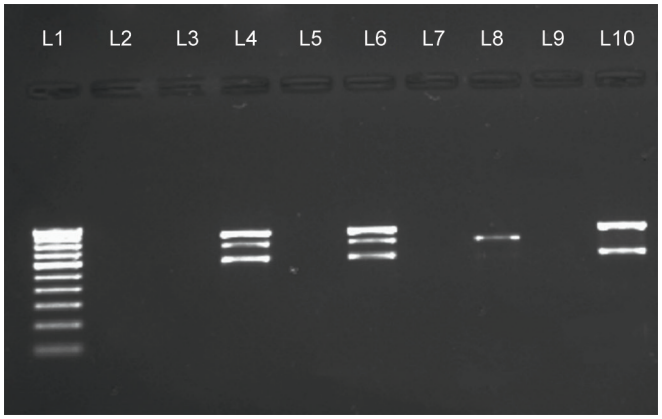

Fig. 2: Gel electrophoresis image showing bands for genus *Campylobacter* (857 bp) and species *jejuni* (589 bp) β actin gene (619 bp). Lanes: L1, 100 bp DNA ladder; L2, No template control (NTC); L3, Negative extraction control (NEC); L4, L6, Samples positive for *Campylobacter jejuni*; L8, Sample negative for *Campylobacter* species with detection of internal control; L5, L7, L9, Blank; L10, Positive control (ATCC *C. jejuni* 33291) without internal control

Rotavirus (2.2%) was observed in an earlier study.[16] Rotavirus was not investigated in the present study.

Multiplex PCR detected the genus *Campylobacter* in 13 (9.8%) samples, of which 11 (84%) were *C. jejuni*. Figure 2 depicts the Gel electrophoresis image showing bands for Genus *Campylobacter* (857 bp) and species *jejuni* (589 bp) β actin gene (619 bp). Species identification could not be made in the other two samples. The reason for this could be attributed to issues with multiplexing, where amplification of one target could have had a suppressive effect on the amplification of the other target. *C. coli* or other species were not detected in any of the samples. A similar picture was observed in an earlier study from Assam, where 80% ($n = 41$) of the positive samples had detectable *C. jejuni*.[15] Nadeem et al. in Kolkata and Chen et al. in China observed 70% ($n = 142$) and 89% ($n = 142$) of the positives were *C. jejuni*, respectively.[17,22] In a study from North India, 27 (90%) out of 30 positive samples were *C. jejuni* and 3 (10%) were *C. coli*.[20] This finding suggests that *C. jejuni* is the most predominant species in humans than the other species.

The strength of our study is that multiplex PCR was performed where genus and species level identification of *Campylobacter* was done in a single step. However, there were some limitations in the present study, i.e., (i) lesser sample size, (ii) inability to retrieve detailed history as the population comprised largely of infants and toddlers, and (iii) real-time PCR could have been a better choice for detecting pathogens in clinical samples because of its increased sensitivity and least contamination rate. We could not do that in the present study because of the limited financial resources.

Conclusion

As there are a subtle number of children with undiagnosed *Campylobacter* infection, there is a need for the implementation of molecular tests for diagnosis. The advantage of this multiplex PCR is that the detection and species differentiation can be done in a single step with a relatively less turnaround time. This aids in the early diagnosis of the infection and better patient management. This study highlights the prevalence of *Campylobacter* as a common agent of gastroenteritis and a need to employ sensitive and robust PCR-based tools for its detection.

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REVIEW ARTICLE

New Insights into Molecular Diagnostics for Common Gastrointestinal Infections

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Abstract

Gastrointestinal (GI) infections are a major health problem all over the world, causing an increase in hospitalization, morbidity, and mortality. The etiological agents of infectious gastroenteritis are viruses, bacteria, and parasites. A precise identification of GI pathogens is crucial for proper treatment and/or isolation, management, and further investigations like designing specific prevention modalities, vaccination strategies, and empiric treatment regimens to prevent the spread of the infectious agents. Routinely, the laboratory diagnosis of GI infections depends on microscopy, culture, and antigen detection. The drawbacks of conventional method are its low sensitivity and 3–5 days of turnaround time in the finalization of report. Quick turnaround time is of paramount value in diagnosis, clinical management, and infection control. From the last decade, molecular-based diagnostic tools have emerged for GI infections in the microbiological laboratory analyses. Culture-independent diagnostic tests typically involve nucleic acid amplification of the genetic material of several bacteria, viruses, and parasites simultaneously. Even whole-genome next-generation sequencing is important for symptomatic patients that remain negative by both routine and multiplex PCR-based diagnostic methods. Therefore, the use of proficient methods for pathogen detection is necessary to ensure prompt turnaround time. This review includes various conventional and molecular tools in identifying various enteropathogens and also analyzes the advantages and drawbacks of all methods.

Keywords: Culture, Diarrhea, Enteropathogens, Gastrointestinal infections, Real-time PCR.

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Introduction

Gastrointestinal (GI) infections remain an unmet challenge in global health. GI infections can be categorized as gastritis, enteritis, and gastroenteritis. An inflammation of the protective lining of the stomach is known as gastritis, and it is further classified into acute and chronic gastritis.[1–3] Enteritis is the inflammation of the small intestine part only. Gastroenteritis involves both inflammation of the stomach and the intestine and is also known as infectious diarrhea, which is the major illness related to GI infections. Diarrhea is defined as passage of three or more loose stools in a day.[4] Worldwide, diarrhea leads to a number of outpatient visits, inpatient load, and loss of quality of life, in both domestic settings and among people traveling abroad. It has been estimated that 4–6 million children die each year of diarrheal diseases, mainly in developing countries of Asia and Africa.[5] Developed countries like United States have reported infectious enteritis and foodborne illness in around 1.3 million patients diagnosed with enteritis or GI symptoms.[6]

Accurate detection of GI pathogens is crucial for appropriate management, treatment and/or isolation, as well as further investigations like designing specific prevention modalities, vaccination strategies, and empiric treatment regimens to prevent the spread of the infectious agents. Rapid turnaround time is also imperative for clinical management, diagnosis, and infection control, and consequently, the use of effective methods for pathogen detection is necessary, which decreases the turnaround time. The challenge in evaluating a patient is to decide what measures to follow that will lead to a most direct and efficient diagnosis. There are different diagnostic modalities required for evaluating patients with GI illness, which are laboratory studies, endoscopy, and diagnostic imaging. Advancement in these three areas has provided clinicians with a medley of testing modalities at their fingertips. This article reviews and compares both conventional and molecular-based laboratory tests, and advantages and limitations of diagnostics for common GI infections.

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Etiological Agents of Infectious Gastroenteritis

Diarrhea due to viral and bacterial infections is a crucial public health problem especially in developing countries. A wide range of enteric pathogens can cause gastroenteritis. The causative agents of infectious diarrhea may vary according to geographical locales, urban to rural areas, and depend on a few factors such as comorbidities and host immune status. However, the most common among these agents are viral pathogens, especially in children up to 9 years. Parasitic infections are also an important cause, particularly in a tropical country like India. Bacterial causes are more responsible for severe cases of infectious diarrhea compared

to other infections. Ironically, the routine processing tests available for bacterial pathogens are more than those for viral pathogens.

- **Viral gastroenteritis:** It has been estimated that 4–38% of deaths among children <5 years of age are caused by viral infections. Viruses, including Adenovirus (enteric types 40 and 41), Astrovirus, Coxsackie virus, Norovirus, Rotavirus, and Sapovirus, are substantiated to be the most common causative agents. In current years, numerous novel enteric viruses such as Aichivirus, Kobuvirus, Enteroviruses, Parechoviruses, Salivirus, Parechoviruses (family Picornaviridae), and human bocaviruses (family Parvoviridae) have also been found to be associated with acute gastroenteritis.[7]
- **Bacterial gastroenteritis:** Bacterial pathogens like non-typhoidal *Salmonellae* and *Campylobacter* spp. are the most common cause of severe bacterial infections in the United States. Other bacterial causes include *Clostridium perfringens* and enterotoxigenic *Escherichia coli* (ETEC), which cause watery diarrhea. The list of diarrheagenic pathogens is extensive and includes *Bacillus cereus*, *Campylobacter* spp., *Clostridium difficile* (toxigenic), *C. perfringens*, Shiga toxin-producing *E. coli* (STEC), *E. coli* O157:H7, ETEC, diarrheagenic *E. coli* other than STEC and ETEC, *Helicobacter pylori*, *Listeria monocytogenes*, *Plesiomonas shigelloides*, *Shigella* spp., *Salmonella enterica* non-typhi, *S. enterica* serotype Typhi, *Vibrio* and Vibrio-like spp., *Staphylococcus aureus*, *Yersinia enterocolitica*, and other species.[8]
- **Parasitic infections:** The infections caused by parasites can present with different clinical manifestations, depending on the agent and various host factors. They result in more severe infections in immunocompromised individuals. These infections can cause enteritis, diarrhea, dysentery (*Giardia lamblia*, *Cryptosporidium parvum* or *C. hominis*, and *Entamoeba histolytica*, etc.), invasive disease (*E. histolytica* and *Balantidium coli*), nutritional depletion (*Cryptosporidium*, *Cystoisospora*, *G. lamblia, Ancylostoma, Necator americanus,* etc.), and mechanical obstruction (*Ascaris*).[9]

Conventional Approaches for GI Pathogens

By tradition, the laboratory diagnosis of GI infections relies on microscopy, culture, and antigen detection. The stool culture still remains the gold standard for identifying bacterial enteropathogens, even though it has a relatively low sensitivity and is laborious.[10] Staining techniques also help in the detection of bacterial pathogens as well. For bacterial culture, a delay in transport or processing can lead to a decreased viability of certain pathogens, including *Campylobacter* and *Vibrio* spp., unless transported in a transport medium like Cary-Blair. The advantages of culture method include its specificity for the pathogenic organism isolated in patient and the availability of the isolate for antibiotic sensitivity testing.[11] Additionally, the strains isolated can be referred to state public health laboratories for further identification, epidemiological studies, or outbreak investigations. The drawbacks of this conventional approach are its low sensitivity and 3–5 days of turnaround time in the finalization of report. For virus cultures, the specimen should be refrigerated if not inoculated into cell cultures within 2 hours. Apart from microscopy and culture, antigen and antibody detection can be done by tests like ELISA. Enzyme immunoassays can be used to detect numerous microorganisms, which cause GI infections like *E. coli* O157:H7 and *Campylobacter* spp., the presence of the Shiga toxins produced by STEC, or the presence of *C. difficile* toxins A or A and B.[12] Furthermore, antigen tests are also available to detect some viruses causing gastroenteritis, such as rotavirus and adenovirus; limitations of the same are that these tests show a variable sensitivity and are not available for all enteropathogens.

For parasitic infections, concentration techniques, indirect wet mount, and permanently stained smear can be employed for the detection of ova and parasites. Additionally, antigen tests are also available to detect certain specific parasites such as *G. lamblia, Cryptosporidium* spp., or *E. histolytica*. Infectious inflammatory and secretory diarrhea can also be differentiated on the basis of the presence of leukocytes in case of inflammatory diarrhea.[13]

Emerging Molecular Approaches

From the last decade, molecular-based diagnostic tools have emerged for GI infections in the microbiological laboratory. Culture-independent diagnostic tests typically involve nucleic acid amplification of the genetic material of several bacteria, viruses, and parasites simultaneously. This not only allows for a rapid diagnosis of previously difficult-to-detect and culture pathogens, including several bacteria, viruses, and parasites, and is not limited to *E. coli* 0157, *Salmonella* spp., ETEC, Norovirus, and *Giardia*.

Culture-independent diagnostic tests include:

- Singleplex/monoplex techniques and multiplex molecular assays
- Microfluidics and array technologies
- Fully automatic platforms in which a single-step nucleic acid extraction, amplification, and analysis are done.

Of late, isothermal amplification has also gained popularity in which no expensive thermal cycling equipment pieces are required and isothermal helicase-dependent amplification can detect a single pathogen at a time.[14]

Real-time Polymerase Chain Reaction

Real-time PCR can detect various organisms such as *Shigella* spp., *Salmonella* spp., *Campylobacter* spp., and various diarrheagenic *E. coli* strains often with better sensitivity compared to bacterial culture.[15] The selection of detection method is the basis for efficient diagnosis since each method has different sensitivity and specificity. Real-time PCR assays have good performance, but are labor-intensive and are time-consuming. Molecular tests for various GI pathogens are commercially available in the form of real-time PCR, which can be singleplex or multiplex. These days, multiplex molecular assays based on PCR methodology are applied for the detection and identification of pathogens responsible for causing diarrhea and other infectious diseases.[16] These syndromic panels permit healthcare providers to achieve a timely diagnosis, which is crucial in certain patient populations, like immunocompromised hosts and the critically ill patients by allowing the diagnosis of a wide range of pathogens.[17]

Singleplex assays are used for a single pathogen like in the case of *C. difficile.* Being one of the most important pathogens causing antibiotic-associated diarrhea particularly in hospitalized patients, several molecular platforms are available for it.[18] The virulent genes of this pathogen are present on pathogenicity island covering toxigenic genes: toxin A, toxin B, and binary toxins.[19] In the last decade, several outbreaks have occurred due to the hypervirulent strain of *C. difficile*.[20] According to Infectious Diseases Society of

America/Society for Healthcare Epidemiology of America (IDSA/SHEA), *C. difficile* testing is suggested with onset of ≥3 unformed stools in 24 hours in high-risk adults and children of ≥2 years of age following antimicrobial treatment, in healthcare-associated diarrhea, and in patients with persistent chronic diarrhea without any etiology.[21] Several FDA-approved platforms are available for *C. difficile* toxin identification: Illumigene® *C. difficile* targeting *tcdA* and *tcdB* (Meridian Bioscience, Milan, Italy), PCRFast® *C. difficile* A/B targeting *tcdA a*nd *tcdB* (GmbH, Berlin, Germany), GeneXpert® *C. difficile/*Epi targeting *tcdA, tcdB, Δ*117*tcdC* and *cdt* (Cepheid, Sunnyvale, California, USA) etc are available. This not only helps in specific detection but also detects 027 and 066/078 *C. difficile*, which are hypervirulent ribotypes (GeneXpert® *C. difficile/*Epi and in-house PCR) leading to better patient care as well as timely epidemiological control measure application.[22]

Another important organism causing GI illness is norovirus. The most frequently used modality for its diagnosis is RT-PCR, like RIDAGENE Norovirus (R-Biopharm, Darmstadt, Germany) and AccuPower Norovirus Real-time RT-P CR Kit (Bioneer Co., Daejeon, South Korea). These PCR assays detect Noroviruses GI/GII.[23] Genogroup IV has been recently included in some PCR assays as it also causes acute gastroenteritis.[24]

However, there are a wide range of pathogens that have non-distinguishable clinical presentations caused by GI infections; therefore, the identification of multiple organisms is a more efficient approach for appropriate management. There are several FDA- or CE-IVD- approved open and closed multiplex commercial systems that identify the most common pathogens.[25] These assays are helpful in the detection of multiple enteropathogens especially in relation to local epidemiology and prevalence. Thus, before acquisition of these investigations, institutional need should be considered. Presently, 11 FDA-approved multiplex assays are available for enteric pathogens. These assays not only detect multiple microorganisms at one fell swoop but also select resistance genes that are important for therapy and management.

- **Verigene Enteric Pathogens Test:** It is manufactured by Luminex Corporation (Luminex, Austin, USA). This system received FDA approval/clearance in 2012. It is an integrated system that can detect five bacterial (*Campylobacter* spp., *Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Y. enterocolitica*, and Shiga toxins 1 and 2) and two viral (Norovirus and Rotavirus) pathogens. This panel cannot detect parasites. This platform uses a processor and a reader that can simultaneously perform nucleic acid extraction, amplification, and hybridization to probes on a glass slide in a microarray format. However, a comparative study done on three different platforms, viz., Verigene EP test, BioFire FilmArray GI panel, and Luminex xTAG GI panel, showed this technique to be less sensitive and specific as compared to BioFire Array GI panel.[26]
- **BioFire FilmArray:** It is manufactured by BioFire Diagnostics, LLC (BioFire, USA), which got FDA approval in 2013. Film array is a fully automatic multiplex PCR system, which simultaneously performs nucleic acid extraction, reverse transcription, amplification, and analysis within 1 hour. It has a long list of bacterial, viral as well as parasitic pathogens that can be identified by it, including *E. histolytica*, *Cyclospora cayetanensis*, *Y. enterocolitica*, etc. The advantages of this system are a comprehensive coverage of many of the major enteropathogens and rapid turnaround time.[17] In a multicenter study, a comparison of BioFire GI Panel, conventional stool culture, and molecular methods was done, which showed the FilmArray GI Panel sensitivity as 100% for 12 of the 22 and >94.5% for an additional 7 of the 22 target pathogens tested. For the rest of the three targets due to the low prevalence of the pathogens in the study, sensitivity could not be calculated.[27] In another retrospective cohort study done in immunocompromised patients, 124 patient samples were tested positive by BioFire GI Panel, compared with 45 patient specimens by conventional testing.[17] The automatic panel group as compared to conventional method was found to be highly advantageous as it demonstrated a higher co-infection rate (48.4 vs 13.3%) and quicker turnaround time (23.4 vs 71.4 hours). Moreover, this panel was also able to identify 29 potential viral infections that were undetectable by conventional stool tests, which warrants against unnecessary prescription of antibiotics.
- **xTAG GPP Panel:** This syndromic panel manufactured by Luminex Corporation (Luminex, Austin, USA) got FDA approval in 2014. This multiplex RT-PCR assay detects 15 enteropathogens, including 5 viruses (Adenovirus 40/41, Norovirus GI/II, Rotavirus A), 9 bacteria [*Campylobacter*, *C. difficile*, *E. coli* O157, ETEC (LT/ST), STEC (stx1/stx2), *Salmonella*, *Shigella*, *Vibrio cholera* and *Y. enterocolitica*, and three parasites (*Cryptosporidium* spp., *Giardia* spp., and *E. histolytica*)]. This assay can detect enteropathogens with sensitivity ranging from 90 to 100%, depending on pathogen present, and specificity between 91 and 99%.[28–30] This assay is helpful in identifying mixed infections requiring 5 hours for analysis and can process 96 samples at once. However, the disadvantages of this system is the requirement of separate nucleic acid extraction, and a high level of technical skill required to prevent cross-contamination as the operator must handle the PCR product before the hybridization step. In this system, the mean fluorescence intensity (MFI) is generated for each bead population and analyzed automatically by the xTAG Data Analysis Software GPP. Although Luminex is not designated as a quantitative assay, MFI values between consensus-positive and false-positive cases can be compared.[31]
- **BD Max™ Enteric and Extended Bacterial Panel (BD Max EBP):** As the name suggests, BD Max Enteric panel can detect bacterial pathogens only, which include *Salmonella*, *Shigella*, and *Campylobacter*, STEC (stx1/stx2). However, the other system of BD, i.e., BD Max EBP, can detect *Y. enterocolitica*, ETEC, *Vibrio*, and *P. shigelloides*. The system is a walkaway microfluidic RT-PCR instrument manufactured by Dickinson (Becton Dickinson, USA). It can process 24 samples at once in 3 hours. The advantage is that it requires 2 minutes of hands-on time per sample and thus has less chances of contamination. Stool specimen is placed into the BD MAX sample buffer tubes and vortexed. The tubes along with the BD MAX enteric bacterial panel reagent strip are then loaded into the instrument. This is then processed using the multiplex PCR assay after preparing the sample followed by lysis and extraction of the nucleic acid through an automated process. In various studies comparing conventional methods and the BD Max EBP assay for the detection of enteric bacterial pathogens in stool specimens, the BD Max EBP assay demonstrated a higher sensitivity and excellent specificity.[32,33]

Apart from bacterial identification, this company also offers separate systems for parasites and viruses detection known as the BD Max enteric parasite panel (Max EPP), which detects *G. lamblia*, *E. histolytica*, and *Cryptosporidium* spp. (*C. parvum* and *C. hominis*). It has shown good specificity and sensitivity for all targets, whereas sensitivity for *G. intestinalis* was equivalent to microscopic detection

with the BD Max enteric parasite panel.[34] The BD Max enteric viral panel (Max EVP), which detects rotavirus type A, norovirus genogroup I (GI) and GII, adenovirus type F 40/41, human astrovirus, and sapovirus (genogroups I, II, IV, and V), has also come up in the market and has shown to be valuable for the differential diagnosis of enteric disease caused by these viruses.[35]

- **Seegene Allplex Gastrointestinal Full Panel Assay (AGPA):** This is a one-step reverse transcription real-time multiplex PCR assay manufactured by Seegene (Seegene, Seoul, South Korea), which is CE-IVD-approved. This system recognizes 13 bacteria, 6 parasites, and 5 viruses in four multiplex PCR panels (bacteria I, bacteria II, virus, and parasite). The nucleic acid is extracted separately after which the Microlab Nimbus IVD or CFX96™ real-time PCR system (Bio-Rad Laboratories, Richmond, California, USA) automatically performs the nucleic acid processing and PCR setup. The principle used in this assay involves using novel analytical multiple detection temperature (MuDT) technique, thus perceiving multiple targets using a single fluorescence channel without the use of melting curve analysis. After the setup, fluorescence is sensed at two temperatures (60°C and 72°C). A distinct exponential fluorescence curve is observed beyond the crossing threshold at a value of less than 42 for the individual targets, if the result is positive. The four aforementioned panels can be selected according to the patient condition: virus and bacteria I panels could be used for hospitalized patients with a suspected nosocomial infection and bacteria I and II panels for patients having occult blood in stool.

Overall, this system had a >2-fold higher detection rate compared to conventional methods (44.4 vs 17.8%) in a study done at Children's Hospital of Eastern Ontario, Canada. The study showed that norovirus genogroup II detection by the AGPA was higher in number, as it also detected the same in specimens which were negative by electron microscopy. Similarly, the bacterial pathogens, i.e., non-O157 STEC, enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC), which were overlooked by conventional culture methods, were also evident by AGPA.[36]

In one study comparing Seegene, Luminex, and BD MAX for detecting GI pathogens, Seegene Allplex GI had the highest overall positive percent agreement (94%; 258 of 275) and negative percent agreement (98%; 571 of 583), respectively, while the BD MAX Enteric assay occupied second place with overall percentage agreement of 96% (362 of 379) in bacterial pathogen detection except *C. difficile*. Additionally, Seegene or Luminex was more sensitive for the detection of *Campylobacter* spp. as compared to BD MAX Enteric assay.

The company also offers an alternative multiplex PCR-based CE-IVD-approved kit named as Seeplex Diarrhea ACE Detection kits (Seegene, Seoul, Korea) with three assays (bacteria 1, bacterial 2, and virus) with the ability to simultaneously detect common bacterial and viral multipathogens including nine bacteria, four viruses, and a *C. difficile* toxin-producing gene. In this assay, independent nucleic acid extraction is needed, and then reverse transcription PCR is done and products formed are separated by capillary electrophoresis. The disadvantages include separate nucleic acid extraction and no detection of parasitic pathogens. There is a variation in sensitivity (40–100%) and specificity (96–100%) of these assays according to the pathogen in the sample.[37–39]

- **ProGastro SSCS Assay:** This is another commercially available FDA-approved kit (Hologic, San Diego, California, USA) used for the simultaneous qualitative detection of four bacterial pathogens [*Campylobacter*, *Salmonella*, *Shigella*, and STEC (*stx1* and *stx2* genes)]. As it is not an integrated system, it requires a separate nucleic acid extraction step, followed by PCR amplification in SmartCycler (Cepheid, Sunnyvale, California, USA) and data analysis. The overall sensitivity of this assay is 98.5% and specificity is in the range of 98.9–99.4%, depending on the target pathogen.[40,15]
- **RIDA Gene Real-time PCR Kits:** These RT-PCR kits are CE-IVD-approved and manufactured by R-BioPharma (R-Biopharm AG, Darmstadt, Germany) that can detect various bacteria, viruses, and parasites simultaneously. A comparative study of seven different RT-PCR kits showed a less diagnostic capacity (PPA-81.7%) of this kit, which could be due to failure in the detection of *Campylobacter* species other than *C. jejuni* and *C. coli* (*C. upsaliensis*, *C. hyointestinalis*, *C. helveticus*, or *C. rectus*) rather than in the sensitivity of the test, since most of the samples were in high concentration.[41] However, in a different study comparing this kit with traditional methods for the detection of *Campylobacter* and *Shigella* species, the results of the kit were found to be more sensitive.[42]
- **FTD Bacterial Gastrointestinal Panel:** This FTD panel (Fast Track Diagnostics, Junglinster, Luxembourg) is a CE-IVD-cleared two-step multiplex RT-PCR test for the detection of pathogen genes by TaqMan technology. Being not integrated system, it requires separate nucleic acid extraction step. In this technique, the first tube performs multiplex detection of three species of *C. coli*/*C. jejuni*/*C. lari* and enterohemorrhagic *E. coli* (EHEC), while the second tube is used for detecting *Salmonella* spp., *Shigella*/enteroinvasive *E. coli*, *Y. enterocolitica*, and *C. difficile*. A study by Biswas et al.,[42] evaluated and compared the diagnostic accuracy and the turnaround time of three multiplex molecular panels: the RIDA®GENE Bacterial Stool and EHEC/EPEC Panels, the FTD® Bacterial Gastroenteritis, and the BD MAX™ Enteric Bacterial Panel, suggesting all of the three as more sensitive as compared to conventional culture method by detecting additional 13 targets that were negative by culture. On comparing the turnaround time, all the three multiplex panels were much faster as compared to conventional technique (<3 vs 66.5 hours).
- **CLART EnteroBac Panel:** There is one another two-tube PCR array-based molecular technique manufactured by Genomica (Genomica, Madrid, Spain) that simultaneously allows the detection of eight bacterial pathogens. The assay follows the steps for nucleic acid extraction and amplification, and detection is carried out on a low-density microarray analyzed by the company's CAR reader. Apart from the company itself, no validation has been done outside.
- **QIAstat-Dx Gastrointestinal Panel:** The QIAstat GIP (Qiagen, Hilden, Germany), an integrated multiplex PCR system, having a closed system for nucleic acid extraction, real-time PCR amplification, and fluorescent amplicon detection, uses cartridge and QIAstat-Dxanalyzer. It can detect and identify 24 gastroenteritis pathogens directly from stool samples in Cary-Blair transport medium concurrently. A multicenter comparative study of QIAstat GIP with BioFire FilmArray GIP and Seegene Allplex GIP shows positive percent agreement of 98.2% and good correlation of QIAstat GIP with other assays.[43]
- **GastroFinder 2SMART:** The GastroFinder 2SMART assay (PathoFinder, the Netherlands), a CE-IVD-approved multiplex real-time PCR-based assay, can detect nine bacterial, five viral, and four parasitic enteropathogens but needs a separate nucleic acid extraction process, which is then followed by real-time

PCR amplification and melting curve analysis based on the identification of organisms. Validation of this kit's performance is assessed by the manufacturer itself.

- **EasyScreen Enteric Assay:** This is non-FDA and non-CE-IVD kit (Genetic Signature's, Sydney, Australia), based on company's 3base technology that converts all cytosine bases (C) in the starting nucleic acid samples to thymidine (T) and results in a reduction in sequence variation. This allows for a greater number of multiplex targets to be run under similar conditions. There are separate panels for the detection of common bacterial, parasitic, viral pathogens, as well as *C. difficile* including hypervirulent 027 and 078 strains. In one of the studies, done by Stark et al., sensitivity was shown to be 92–100%, specificity was 100%, and the assay detected all commonly found subtypes and genotypes of clinically important human parasites.[44]
- **Fecal Pathogens M Detection Assay:** Aus Diagnostics Fecal Pathogens M detection assay (AusDiagnostics, Mascot, Australia) (non-FDA and non-CE-IVD) can detect 14 common bacterial, viral, and parasitic enteropathogens. This assay uses multiplexed tandem PCR technique comprising two amplification steps. In the first step, extracted nucleic acid is pre-amplified as a single-well multiplex reaction. The amplified product in the first step is then diluted, followed by the second step multiplex real-time PCR using SYBR green dye, and finally a melting curve analysis-based identification of organisms is done. Table 1 shows multiplex commercial tools with the list of pathogens, reported sensitivities, amount of automation, and pros and cons.

Benefits and Drawbacks of Multiplex GI Panels

The overall advantages of multiplex panels include increased diagnostic yield, improved workflow, and reduced hands-on time as well as the important impact on infection control. One important benefit it provides is shorter hospital stay and reduction of antibiotic therapy. Also, these tests are more user-friendly, not needing highly trained personnel. Besides, several enteropathogens that have the capability to cause rapidly spreading outbreaks and epidemics can also be readily identified by these panels.

However, some microorganisms may not be clinically relevant such as those that can be shed in feces for several weeks. Similarly, some people may be asymptomatic carriers for certain organisms. Also, these methods cannot distinguish between viable and nonviable microorganisms. One major disadvantage of the molecular diagnostics is the unavailability of the isolates for further prospective studies. Another limitation of molecular-based testing is that antimicrobial testing cannot be performed by this method. Sometimes, the specimen collected for culture-independent testing might be incompatible with culture-based testing due to the use of inappropriate collection media used or inadequate method of collection.

The main current concern is cost as these tests cost much more than the conventional methods and at the same time the pathogens detected might not be clinically significant. Thus, the judicious use of multiplex panel is of paramount importance to diagnose the causative organism taking care not to overuse the drugs meant to treat these organisms, which create the problem of drug resistance in them.

Next-generation Sequencing

Next-generation sequencing (NGS) helps to sequence mixed populations of DNA or RNA genomes rapidly. This technique has found its application predominantly in areas where conventional diagnostic approaches present limitations, such as in understanding the epidemiology of many diarrhea-associated bacterial pathogens, identifying novel pathogens, and also identifying acknowledged pathogens.[45] The etiology of suspected GI infections in acutely ill hospitalized or immunosuppressed patients usually remains undiagnosed, resulting in increased mortality and morbidity due to delayed or inadequate treatment, prolonged stays, or readmissions. In these types of patients, the identification of known or unknown pathogen is of utmost importance so as to start the correct therapy. NGS is also an effective approach for the detection of novel pathogens as well as to identify several putative diarrheal pathogens. In time, it is likely that probe-based detection will be taken over by sequencing, for the detection of unknown pathogens too.[46]

Whole-genome next-generation sequencing (WG-NGS) is important for samples that are negative by both routine and multiplex PCR-based diagnostic methods while the patient remains symptomatic. It helps us to allow the identification of non-predefined targeted microorganisms, and it also allows enteric disease surveillance, thus helping to detect and investigate outbreaks and to monitor disease trends. The implementation depends on the ratio between costs and clinical benefits. Recently, origins of the Haitian cholera outbreak were analyzed using WG-NGS and phylogenetic analysis.[47] However, there are few limitations with this technology: the cost is high, it takes time for diagnosis, and more importantly, it is unable to identify the causative agent in a large part of the samples.[48]

CRISPR-Cas9 System

C. difficile infection has become a grave health problem, which results in thousands of deaths all over the world annually. The dearth of genome engineering tools for *C. difficile* has delayed the machine-like understanding of the interaction between this pathogen and its hosts, as well as its pathology. Plasmids that carry the CRISPR-Cas9 system were created and conjugated into *C. difficile*. Colony PCR having primers that anneal to regions flanking the target gene deletion/integration locus was then used to identify the mutants, while heat-survival assay was done and comparison of the sporulation frequency between the mutant with spo0A deletion and the wild-type strain was observed. The resulting fluorescence in the mutant which has insertion of the green fluorescent protein (GFP) gene was then seen under a fluorescent microscope. This tool enabled the mutation efficiency of 100% for spo0A deletion. Conversely, required genes can be inserted into the *C. difficile* chromosome: an anaerobic GFP gene was inserted with a mutation efficiency of 80%. This tool has the potential for the advancement of novel strategies for CDI diagnostics as well as therapies.[49]

After rotavirus, the second most important diarrheal pathogen is *Cryptosporidium*. It is also an important opportunistic pathogen in AIDS and organ transplant patients. Drug and vaccine development for cryptosporidiosis is restricted because of the poor traceability of this pathogen as there is deficiency of culture tools, inappropriate animal models, and molecular genetic tools.[50] In such a scenario, CRISPR/Cas9 technology provides us with a valiant new era to help study this pathogen. The application of *Cryptosporidium* genetic modification will not only help us to increase our understanding of the basic biology of the parasite and its virulence, but will also help us in the development of upgraded vaccines and therapeutics.[51]

Table 1: Multiplex commercial tools with the list of pathogens, reported sensitivities, amount of automation, and pros and cons

Name of the multiplex tool	*List of pathogens*	*Reported sensitivities*	*Amount of automation*	*Pros and Cons*	
Verigene Entericpathogen Test	5 bacterial (*Campylobacter spp., Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Y. enterocolitica*, Shiga toxin 1 and 2) and 2 viral (norovirus, rotavirus) pathogens	Less sensitive and specific as compared to BioFire Array GI panel	Utilize multiplex PCR followed by either hybridization to microarray, hybridization probes or melting curve analysis.	Cannot detect parasites	
BioFire FilmArray	Bacterial, viral as well as parasitic pathogens that can be identified by it, including *E. histolytica, C. cayatenensis, Y. enterocolitica*	100% for 12 >94.5% for an additional 7 of the 22 target pathogens	Fully integrated system simultaneously performs nucleic acid extraction, reverse transcription, amplification, and analysis within 1 hour.	Comprehensive coverage of most of the pathogen and low hands-on and turnaround time.	Low throughput and inability to separate bacterial, viral, or parasitic testing if needed from patients' need or billing point of view.
xTAG GPP Panel	Detects 15 enteropathogens, including 5 viruses (adenovirus 40/41, norovirus GI/II, rotavirus A), 9 bacteria [*Campylobacter, C. difficile, E. coli* O157,enterotoxigenic *E. coli* (ETEC LT/ST), STEC (stx1/stx2)], *Salmonella, Shigella, V. cholera* and *Y. enterocolitica*, and 3 parasites (*Cryptosporidium* spp., *Giardia* spp., and *E. histolytica*).	Sensitivity ranging from 90 to 100%. Specificity between 91 and 99%	Not an integrated system	High sample throughput and the ability to detect multiple pathogens.	Requires separate nucleic acid extraction and post-PCR handling, which increases the potential of cross-contamination and false-positive results.
BD Max™ Enteric and Extended Bacterial Panel (BD Max EBP)	Bacterial pathogens, i.e., *Campylobacter, Salmonella, Shigella,* EIEC, and STEC (stx1 and stx2 genes), *Y. enterocolitica, V. cholera, V. parahaemolyticus, and V. vulnificus,* viral panel (adenovirus, astrovirus, norovirus, and sapovirus), and parasite panel (*C. parvum, C. hominis, E. histolytica,* and *G. lamblia*)	Increased sensitivity and specificity in the detection of *Campylobacter*, *Salmonella*, *Shigella* and STEC	An integrated system that incorporates simultaneous sample preparation, nucleic acid extraction, amplification, and detection.	Batches up to 24 samples within 3 hours and require 2 minutes of hands-on time per sample.	
Seegene Allplex Gastrointestinal Full Panel Assay (AGPA)	Detects 13 bacteria, 5 viruses, and 6 parasites in 4 multiplex PCRs. the bacterial pathogens, i.e., *E. coli* non-O157, STEC, EPEC, and EAEC, which were overlooked by conventional culture methods that were also evident by AGPA	New CEIVD marked multiplex real-time PCR assay [uses the novel analytical multiple detection temperature (MuDT)] technique, which is able to detect multiple targets in a single fluorescence channel without melting curve analysis.	More sensitive and specific as compared to traditional methods	Overall 94% positive percent agreement for the detection of GI pathogens when compared with the conventional procedure and two other NAT methods.	Separate nucleic acid extraction from stool samples.
ProGastro SSCS Assay	4 bacterial pathogens [*Campylobacter, Salmonella*, *Shigella*, and STEC (*stx1* and *stx2* genes)]	ProGastro SSCS is not an integrated system that requires a separate nucleic acid extraction step, followed by PCR amplification in Smart-Cycler (Cepheid, USA) and data analysis.	Sensitivity is 98.5% and specificity 98.9–99.4%		Requires a separate nucleic acid extraction step, followed by PCR amplification in Smart-Cycler (Cepheid, USA) and data analysis.

RIDA Gene Real Time PCR Kits	Can detect various bacteria, virus, and parasites simultaneously	Not an integrated system	More sensitive than culture methods for the detection of *Campylobacter* and *Shigella* species, the sensitivity of RIDA GENE GI Kit for the detection of *Salmonella* spp. was found to be low at 25% as compared to the culture method	Can be performed on most commonly available real-time PCR equipment	Require separate nucleic acid isolation procedure
FTD Bacterial Gastrointestinal Panel	First tube performs multiplex detection of *C. coli, C. jejuni, C. lari*, and EHEC. Second tube performs multiplex PCR detection of *Salmonella* spp., *Shigella*, EHEC, Enteroinvasive *E. coli, Y. enterocolitica*, and *C. difficile*.	Two-tube multiplex real-time PCR test for the detection of pathogen genes	More sensitive than culture methods for the detection of *Campylobacter* and *Shigella* species. However, for *Salmonella* spp., FTD Bacterial GI panel showed a low sensitivity of 50% as compared to the culture method		Requires separate nucleic acid extraction step.
CLART EnteroBac Panel	Simultaneously allows the detection and identification of 8 bacterial pathogens	Test procedure includes nucleic acid extraction, multiplex PCR amplification, microarray hybridization, and automated data analysis		High throughput	Does not detect any viral and parasitic pathogens.
QIAstat-Dx Gastrointestinal Panel	Can simultaneously detect and identify 24 gastroenteritis pathogens from stool samples	An integrated system	Overall assay sensitivity and specificity to be 97.9 and 97.8%, respectively.	A good correlation and positive percent agreement of 98.2% with other assays.	
GastroFinder 2SMART	Is able to detect 9 bacterial, 5 viral, and 4 parasitic pathogens causing GI infections	Not an integrated assay, requires separate nucleic acid extraction followed by real-time PCR amplification and identification of organisms on the basis of melting curve analysis.			Limited independent studies.
EasyScreen Enteric Assay	Detect common bacterial, viral, parasitic pathogens, and *C. difficile*, including hypervirulent 027 and 078 strains.	92–100% sensitivity and 100% specificity	Reduction in sequence variation allows for a higher number of multiplex targets to be run under similar conditions.		
Fecal Pathogens M detection Assay	Detect 14 common bacterial, viral, and parasitic pathogens		Multiplex real-time PCR is performed using SYBR green dye, and identification of organisms is performed by melting curve analysis.		

Usage of Newer Techniques

Gastroenteritis is not always severe, often resolving spontaneously and rapidly, thus raising the question of which patients presenting with GI symptoms should undergo these tests. Considering the patient factors and cost-effectiveness is important in making this decision. Patient factors such as severity of symptoms and immune status have to be kept in mind. Also, rapid diagnosis is important in public health contexts as several enteropathogens can cause outbreaks and epidemics. With the shift of population dynamics toward older population, an advancement in treatment for patients with various life-threatening conditions of the past, such as hematological, rheumatologic, and oncological conditions, has led to an increase in people with immunocompromised status whose conditions can quickly deteriorate. These tests present us with the opportunity to identify the causative organism rapidly and adequately. Rapid molecular tests can also help us to differentiate the diarrhea due to host vs graft disease and that due to infectious etiology.

According to IDSA,[52] the best approach to use these tests includes the following: The specimen with positive result on a culture-independent testing method should be made available for future testing purposes and culture to the laboratory.

- Future testing can then be done for the identification of species and determination of the serotype and further subtype by the molecular methods, like pulsed-field gel electrophoresis or whole-genome sequencing.
- Determination of the subtype further allows the detection of increased infections occurring due to a specific strain and hence facilitates in outbreak investigations by helping in finding a common exposure source for suspected case patients.
- Antimicrobial susceptibility can be done, which not only provides information about the drugs effective against that particular organism but also helps in the outbreak settings and in ongoing surveillance, which provides the local trends in resistance patterns as well as their mechanisms.

To conclude, we can surely say that these panel-based GI testing techniques are here to stay. Even though not all patients are likely to need these tests, in certain patients they could make the difference of life and death. Their use and interpretation of the result now depends on the use by the clinicians and their wisdom in making these panels a useful tool in diagnosis, without blindly treating for everything that comes positive.

Future Aspects

Judicious use of these culture-independent methods is beneficial for the patients as well as for clinicians. However, there are some lacunae yet to be filled by proper studies and improvement in technologies. One such issue is the lack of significant number of studies on the cost-effectiveness for the use of these assays that are needed to guide us. Another important issue is regarding the importance of mixed infections and their implication in patient presentation as well as treatment required. More studies are also required to study the role of quantitative diagnostics in regard to these assays. These assays can be used to study the epidemiology of diarrheal disease during vaccine efficacy trials to determine the relation to particular benefit of the vaccine. Another area where these assays might help us to understand is regarding mucosal immunology, of which our knowledge is limited.

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REVIEW ARTICLE

Virulence Factors Associated with *Clostridiodes difficile*: An Overview

Chetana Vaishnavi

Abstract

Clostridiodes difficile is a health threat mainly acquired *via* the feco-oral route and colonizes the human gut. There is a wide range of clinical presentation of *C. difficile* infection (CDI). *C. difficile* can be accountable for 15–25% of antibiotic-related diarrhea and up to 100% of pseudo-membranous colitis. Clinically important *C. difficile* are evolving and increasingly being reported globally. The pathogenesis of *C. difficile* is associated with many established and potential virulence factors. They include toxins, surface layer proteins, cell wall proteins, flagella, fimbriae, spores, etc. The main virulent factors of CDI are toxin A and toxin B, both of which share a high structural and functional resemblance between them. Both these toxins are responsible for neutrophil infiltration marked by mucosal insult and colitis which is a significant feature of CDI. These toxins also influence the cytoskeletal features, despite the difference in activity potency. A third toxin, produced by some *C. difficile* strains, contains components of both toxin A and toxin B and is referred to as the binary toxin. The role of this toxin in CDI virulence is not clear. Besides the above described virulence features there are other probable factors that could be involved in *C. difficile* colonization. They are flagella, surface layer protein, production of tissue degradative exoenzymes, and sporulation. In this overview, the virulence factors associated with *C. difficile* shall be discussed to highlight their potential role in the disease.

Keywords: Binary toxin, *Clostridiodes difficile* infection, Flagella, Spores, Surface layer proteins, Toxin A, Toxin B, Virulence factors.

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Introduction

Clostridiodes difficile is an anaerobic, heat-resistant-endospore-producing gram-positive bacillus with peritrichous flagella. It belongs to the Phylum Firmicutes and Family *Clostridiaceae*. Toxigenic and epidemic *C. difficile* is a well-established health threat and a leading cause of infectious diarrhea in patients exposed to the hospital environment[1,2] as well as in persons in the community.[3]

The organism is mainly acquired *via* the feco-oral route and colonizes the intestinal tract of humans. The clinical presentation of *C. difficile* infection (CDI) ranges from asymptomatic carriage, diarrhea, simple colitis, pseudo-membranous colitis, acute severe colitis, and recurring CDI. *C. difficile* could be accountable for 15–25% of antibiotic-related diarrhea and up to 100% of pseudo-membranous colitis.[4,5] The severity of infection includes high rates of leukemoid reactions, severe hypoalbuminemia, toxic megacolon, need for colectomy, and ultimately shock and death.[6] Exacerbation of ulcerative colitis due to CDI[7] and a higher risk for CDI in pancreatic disease patients[8] have also been reported.

Heightened awareness of CDI outbreaks has led to an increase in the surveillance for the disease. Epidemic and clinically important *C. difficile* with several PCR (polymerase chain reaction) ribotypes are evolving and is increasingly being reported from all over the world. The hypervirulent NAP1/BI/027 (North American Pulse Field type I/Restriction Endonuclease Assay type BI/Ribotype 027) strain of *C. difficile* is linked with a higher incidence of the disease and an increased rate of morbidity and mortality. In this overview, the virulence factors associated with *C. difficile* shall be discussed to highlight their potential role in the disease.

Virulence Factors of *C. difficile*

The pathogenesis of *C. difficile* is associated with many established and potential virulence factors. They include toxins, surface layer proteins, cell wall proteins, flagella, fimbriae, spores, etc. The main virulent factors of CDI are the two exotoxins A and B, the genes for which are positioned closely to each other within a pathogenicity locus (PaLoc) in the pathogenicity island.[9] Both these toxins are responsible for neutrophil infiltration marked by mucosal insult and colitis which is a significant feature of CDI.[10] These toxins also influence the cytoskeletal features, despite the difference in activity potency.

Both the *C. difficile* exotoxins have a high molecular weight making them the largest bacterial protein toxins, along with some other clostridial proteins like those of *C. sordellii* and *C. novyi*. These large clostridial toxins and glycosylate small guanine triose phosphate (GTP)-binding proteins[11] are solely present in the Rho and Ras GTPases[12] which are a family of hydrolase enzymes. Zhu et al.[13] reported identification and characterization of a new cell wall hydrolase Cwl0971 from a *C. difficile* strain. The 0971 gene deletion mutant showed delayed cell autolysis and increased cell viability which impaired the release of toxin A and B and affected sporulation.

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Toxin A

Toxin A (TcdA) is a 308 kDa proteinaceous enterotoxin encoded by gene *tcd*A. It is a lethal enterotoxin causing hemorrhage and fluid secretion in the rodent gut. Toxin A induces extensive damage to the intestinal epithelial cells and therefore accounts for almost all of the gastrointestinal symptoms. It is considered as the main virulence factor of *C. difficile* as it causes severe damage to the gut.[14] Toxin A has also been reported to disrupt the tight junctions of the intestinal epithelial lining by acting as a cytotoxin and this might be a significant mechanism involved in toxin enterotoxicity.[15] Various cytokines and neurokinins, playing a significant role in CDI pathogenesis, are induced by toxin A.[16] Toxin A causes cell rounding and cell detachment from the basement membrane, leading to apoptosis. Rapid loss of macrophages, T cells, and eosinophils also occur. Massive inflammation due to neutrophil infiltration results in denuding of the gut mucosa and damage to the intestinal epithelium. Katyal et al.[17] observed disturbances in the intestinal brush border membrane enzymes of CDI patients.

Toxin B

Toxin B (TcdB) is a 269 kDa potent toxin encoded by *tcdB* gene. It is largely a cytotoxin identified by its cytopathic effect on tissue culture cells[18] and is 1,000 fold more potent than toxin A as a cytotoxin. Toxin B does not by itself damage the gut possibly because of its lack of ability to attach to particular receptors on the brush border membrane of the gut.[19] Toxin A bind to the specific receptors on the intestinal wall to bring about the damage. Next, toxin B connects to gain access to the underlying tissue.[19] Partial detachment of cells occurs due to development of neurite-like retraction. Later on the cell-spanning stress fibers wane and actin filaments gather in the perinuclear space.[20] It disorganizes the actin filaments, brings about a loss of intracellular potassium, and a reduced protein and nucleic acid synthesis.[21] Toxin B has been found to suppress interleukin-2 expression, disrupt tight junctions, and stimulate nitric oxide production.[22,23]

Structure and Function of Major *C. difficile* Toxins

Toxin A and toxin B share a high structural and functional resemblance between them with a 63% sequence of amino acids.[24] The structure of *C. difficile* toxins was earlier described as having three parts, namely, a binding domain of C-terminal, a catalytic domain of N-terminal,[24] and a central hydrophobic region.[25] However, later on this toxin structure representation was substituted with a structural model of four-domains comprising the glucosyltransferase, the cysteine protease, the translocation, and the receptor-binding domains.[26]

A number of messenger RNAs are transcribed from the toxinogenic element, including a 17.5 kb polycistronic transcript.[27] Owing to the sequence similarity and the position on PaLoc, both *tcd*A and *tcd*B genes are supposed to have a common ancestor and are the result of gene duplication.[24] Together with three additional genes, namely, *tcd*C, *tcd*D, and *tcd*E, the *tcd*A and *tcd*B genes form the 19.6 kb PaLoc found only in the toxinogenic isolates.[28] Gene *tcd*E is a cell wall hydrolase gene, and *tcd*R gene—an alternative sigma factor—helps in the positive transcriptional regulation while *tcdC* serves as a presumed negative regulator.[29] The *tcd*A gene with 8,133 nucleotides is found between *tcd*E gene and the divergently transcribed *tcd*C gene.[30]

Sequencing and transcription analysis has shown that *tcd*D encoding a 22 kDa protein necessary for transcription of the toxin genes[31] when interacts with *tcd*C works as a positive regulator for TcdA and TcdB expression.[27,30] This has therefore been renamed as *Tcd*R.[32] The *tcd*B gene having 7,098 nucleotides is located between *tcd*R and *tcd*E genes. Tan et al.[33] demonstrated the bactericidal effect of *tcd*E when expressed in *Escherichia coli*. *Tcd*E is structurally and functionally similar to holins. It may facilitate the release of toxins to the extracellular environment.[33] Olling et al.[34] reported that a *tcd*E mutant neither delays nor inhibits the release of toxins A and B.

Due to the lack of negative regulation there is an increased production of toxins A and B as a consequence of the deletions of 18 and 39 bp found in *tcd*C gene forming truncated TcdC proteins.[35] There is a marked increase in the virulence of the NAP1/BI/027 strains due to 18 bp deletion in the *tcd*C gene thus producing both toxins in higher quantities and at higher rates.[36] NAP1/BI/027 strains are reported to generate about 16 times more toxin A and 23 times more toxin B.[36]

A second deletion at position 117 of a single-base-pair in the *tcd*C gene was found in all Canadian NAP1/BI/027 strains, and in a United Kingdom reference strain.[37] This strain has enhanced toxin production, a faster sporulation rate, and increased antimicrobial resistance, particularly to fluoroquinolones.[36,38,39] Surprisingly hyper-production of toxins has also been reported in a *C. difficile* strain with no *tcd*C mutations and normal levels of toxin production in a strain with *tcd*C mutation.[40] Thus, it appears that mutation in the *tcd*C gene is not definitively related to increased clinical virulence[41,42] and there could be other regulators of toxin expression involved in the hyper-production of toxin in some isolates of *C. difficile*.

The superfamily Ras comprising Rho, Rac, and Cdc42 GTPases within the intestinal cells gets targeted for alteration *via* glycosylation by the toxins. When this alteration occurs, it leads to activation of the guanosine triphosphate (GTP) binding Rho proteins after the toxins gain entry into the cytoplasm, resulting in interruption of critical signaling pathways in the cell.[29] In addition to the intracellular inactivation of GTPases, toxins A and B also bring about other morphological and physiological changes to the intestinal epithelial cells. The Rho proteins are engaged in the creation of focal adhesion complexes and stress fibers. They polymerize the actin, maintain the cytoskeletal structural design, as well as the cell movement.[43] The actin cytoskeleton gets regulated by these GTPases. The changes in the epithelial cell wall *via* Rho protein glycolysation involves at least two pathways including disaggregation of actin microfilaments leading to increased permeability of tight junctions and untimely discharge of proinflammatory cytokines from the intestinal epithelium resulting in stimulation of mast cells, vascular endothelium, and immune cells.[44] F-actin cytoskeleton forms aggregates after the spherical cells become thin and rope-like.[45]

The Rac proteins are responsible for membrane ruffling as well as lamellipodia formation. In some cell types, this is also induced by Rho proteins. Cdc42 brings about the formation of filopodia or microspikes. This change activates the tiny regulatory proteins and causes interruption in the fundamental cell signaling pathways[29] and tight junctions, causing excessive fluid accumulation and destruction of the intestinal epithelial lining.[46]

After colonization of the gut, toxin A along with toxin B comes into play. Both these clostridial toxins bind to the surface

receptors present on the intestinal epithelial cells, damage them to undergo apoptosis, modify the actin cytoskeleton, and increase the permeability of the tight junctions.[9] In the beginning, TcdA forms homodimers to bind the carbohydrate groups. Then, the toxin appears in coated pits which are then internalized. Once the toxin B accesses the underlying tissue, it brings about widespread damage with the disease getting progressed further. Thus it appears that both the toxins work synergistically.[19] *C. difficile* enters the intestinal cells and thereby inactivates the important intracellular signals. Concurrently there is a release of pro-inflammatory interleukins and tumor necrosis factor-α with an increase in vascular permeability. Toxin A has been found to stimulate substance P—an inflammatory mediator—thus triggering inflammation.[47] Neutrophils and monocytes get recruited to the site of injury and tissue degradation starts due to the production of hydrolytic enzymes leading to the formation of pseudomembranous colitis. A severe inflammatory reaction occurs in the lamina propria, because of the activity of the toxins. This is followed by the development of tiny ulcerations in the mucosa of the colon enclosed by a pseudomembrane.[48]

Apart from their role in precipitating CDI, toxin A and toxin B together are the principal markers for the disease diagnosis and can be detected in the fecal samples of patients by laboratory assays. Strains of *C. difficile* that are nontoxigenic do not cause disease.

Typing of *C. difficile* isolates can be done by restriction endonuclease analysis, pulse field gel electrophoresis, or PCR ribotyping. *C. difficile* strains can be distributed into 34 currently known toxinotypes (I to XXXIV) depending on the changes in both toxin genes.[49] Singh et al.[50] reported toxigenic culture of 95 (54.6%) toxigenic and 79 (45.4%) nontoxigenic *C. difficile* isolates from stool samples of CDI patients. Toxinotyping revealed that 121 (69.5%) of these isolates were toxigenic with 76 (62.8%) belonging to toxinotype 0 and 45 (37.2%) to toxinotype VIII. PCR ribotyping revealed that 36.8% of these belonged to ribotype 001, 33.9% to ribotype 017, and 13.2% to ribotype 106.[51] Partial sequencing of genes from 10 isolates showed changes in toxin A sequences of 5 (50%) isolates with translated nucleotide substitution in just 3 (30%) of them.[51]

Binary Toxin

Since 1987, another iota-like toxin produced by some *C. difficile* strains was identified. This toxin known as the binary toxin (CDT) contains components of both toxin A and toxin B. The role of this toxin in CDI virulence is not clear. This toxin was not cytotoxic to tissue epithelial cells, nor it was found to be lethal to animals upon intraperitonial inoculation.[52] Despite this, cytotoxicity brought about by CDT appears to be analogous to that of both toxin A and toxin B.[53] It has the potential to act in conjunction with toxins A and B or to act alone in so-called "nontoxinogenic" strains.

Up to 2% of *C. difficile* produce only binary toxin and 4–12% of isolates are positive for this toxin.[40,54,55] Binary toxin could be a significant virulence factor of *C. difficile* as it is present in the epidemic NAP1 strain. It is envisaged that CDT alone is not enough to commence the disease, but might play a role in the later stages of infection. Cytotoxic activity to Vero cells[56] and significant morphological changes to Caco-2 cells *in vitro* by purified binary toxin have been demonstrated.[57] CDI patients infected with a CDT positive isolate compared to those with a CDT negative isolate have a higher case-fatality rate infection.[58] All upcoming *C. difficile* hypervirulent strains possess this toxin, suggesting that the binary toxin could be a marker for increased virulence or that it might contribute to increased virulence, by acting in synergy with toxin A and B, exacerbating the toxicity of the strain.[58] As a matter of fact, the binary toxin is linked with the majority of severe outbreaks of drug-resistant CDI in the present century.[59]

Structure and Function of Binary Toxin

Binary toxin has been encoded in a different region called CdtLoc outside the PaLoc. This toxin comprises two unlinked molecules—one, the 48 kDa enzymatic component encoded by the 1,392 nucleotides (*cdt*A gene) and the other is a binding component of 94 kDa encoded by the 2,631 nucleotide (*cdt*B gene). Both these genes act synergistically.[53] Upstream of the *cdt*AB genes, *cdt*R a regulator gene, belonging to the LytTR family of response regulators, is located. It has no detectable sensor kinase common to other members of the family and therefore is considered as an orphan response regulator.[60] The lack of a functional *Cdt*R results in a 15-fold decrease in binary toxin production.[61]

Due to proteolytic cleavage, the binding component of the binary toxin gets activated and binds to the exposed cell surface receptor forming heptamers and prepore. Next the enzymatic component of the toxin binds to prepore-receptor complex.[62,63] Subsequently the toxin-receptor complex gets endocytosed. A conformational change in the heptamers occurs due to the low pH of the endosome, leading to membrane insertion and pore formation. The enzymatic component then gets translocated into the cytosol with the help of the host chaperones.[64] Once entry is gained, the enzymatic component ribosylates adenosine diphosphate monomeric G-actin at Arg177 and thereby inhibits the polymerization of G-actin to F-actin.[65] This toxin induces the production of a new kind of microtubule structures, which consist of long microtubule-dependent protrusions on the epithelial cell surface which promote bacterial adherence and colonization.[57]

Additional Virulence Factors

Besides the above-described virulence features, there are other probable factors that could be involved in *C. difficile* colonization. They are flagella, surface layer protein (SLP), production of tissue degradative exoenzymes,[66] and sporulation. The surface proteins and the flagella of *C. difficile* adhere to the colonic wall, particularly in individuals with depleted normal gut flora. Fimbriae may also act as potential mediators of attachment to intestinal mucosa thus enhancing the pathogenesis.[67] However, these factors are not clearly understood and their roles in *C. difficile* virulence are greatly speculative.

Surface Layer Proteins

The SLP is an adhesion factor, represented as one of several potential surface associated genes present in a group of 17 open reading frames along with cwp66, a cell wall protein. SLP is paracrystalline, proteinaceous arrays that envelop the cell wall of all *C. difficile* strains. The unique *slp*A gene comprises 2,160 bp codes for the SlpA precursor protein of 73.4 kDa.[68]

The *C. difficile* S-layer is composed of the precursor protein, SlpA. The S-layer proteins are composed of a surface protein with a low molecular weight (32–38 kDa LMW-SLP) and a cell wall-associated protein with a high molecular weight (42–48 kDa HMW-SLP).[69] The two subunits of the protein self-assemble to form a lattice and are structurally placed over one another showing square symmetry of the external LMW-SLP layer and hexagonal symmetry of the inner HMW-SLP layer.[69–71]

Surface layer protein can cause binding of *C. difficile* to host intestinal brush border membrane and thereby permit targeted delivery of toxins to enterocytes. Next, after toxin-induced epithelial damage occurs, SLP binding to extracellular matrix components is also liable to add considerably to further the tissue damage. Calabi et al.[72] reported the existence of a high degree of variability in the molecular masses of the two proteins of the S-layer of *C. difficile*.

Cell-associated Protein

Cell-associated proteins help in *C. difficile* adherence to the intestinal epithelial cells and are also considered as virulent factors. Cell wall protein, CwpV, is a large SlpA homolog, expressed in a phase variable manner. Antibodies against cell wall proteins have been observed in sera of CDI patients signifying their immunogenicity and *in vivo* expression.[73] Emerson et al.[74] suggested that it may be associated with immune evasion.

Flagella

Before establishing infection, the bacteria need to adhere to the tissue to start colonization or else shall be immediately removed by nonspecific host defense means.[75] Flagellum is required for movement, adherence, and invasion of mucosal surfaces as well as direct interaction with the host immune system.[76] Bacterial flagella consist of three parts: basal body, hook, and helicoidal filament. Even though nonflagellated strains occur, flagella are found on the surface of most *C. difficile* strains.[77] The presence of amplified flagellum genes in nonmotile strains propose that the flagella expression could be phase-variable.[78] Environmental signals regulate the translation of flagellum proteins; those strains that appear nonmotile *in vitro* may actually be motile *in vivo*.[79] Tasteyre et al.[80] reported that flagellated *C. difficile* led to a ten times higher adherence to mouse cecum tissue compared to unflagellated strains.

The virulence factor associated with adherence is the flagellar filament, protein C (FliC), along with the flagellar cap protein D (FliD).[81] *fliC* gene comprises 870 bp and its corresponding protein of 290 amino acid.[75] *C. difficile* genome has only one copy of *fliC*. Quite a lot of conserved alanine residues accountable for the α-helical conformation of the filament are present in FliC. Its N-terminal responsible for secretion and C-terminal for polymerization are also conserved. High conservation of FliC has been found between clinical strains isolated over a short stretch of time.[78] Between different *C. difficile* strains the central region is divergent, as it is surface-exposed; antigenic drift causes selection of variants and is therefore a useful genetic marker for epidemiological studies.[82]

The 39 kDa protein of *C. difficile* flagella shows similarity in all flagellated strains and is therefore responsible for the cross-agglutination observed in serogrouping reactions.[77] The genetic differences for the analysis of *fliC* can be seen using a typing method involving restriction fragment length polymorphism (RFLP).[75,79] The *fli*D is a 1524 bp gene coding for the 56 kDa FliD cap protein and composed of 507 amino acids.[80] FliD is highly conserved, surface-exposed, and does not have variable domains. It has a very precise purpose of attaching to cell or mucus receptors. Two main RFLP patterns have been observed by treatment of *fli*D with a variety of restriction endonucleases highlighting the conservation of its genetic sequence.

Tissue Degrading Exoenzymes

Other virulence factors found in some *C. difficile* strains are protease, collagenase, hyaluronidase, and other hydrolytic enzymes. They also add to the adhesion and dissemination of organism *in vivo*.[83]

Fimbriae

Infrequently, the presence of fimbriae has also been implicated for their role in infection.[83] However, their absence does not suggest affecting colonization or infection.[84,85]

Capsule-like Material

Some strains of *C. difficile* also possess a capsule-like material which might be implicated in adhesion and evasion of the immune system through its antiphagocytic properties.[86]

Spores

Spores are also factors for *C. difficile* pathogenesis because of its hard coat which helps the organism to survive disinfectants, heat as well as drying conditions. The spores shield *C. difficile* from unfavorable situations like antibiotics, nutrient deficiency, and bactericidal immune response, thereby increasing the virulence of the organism. Spore formation is regulated by gene *spo0*A, a master regulator of the sporulation pathway. This is related with high spore production as also with formation of biofilms, which is a possible reservoir for the restitution of CDI after initial therapy of the patients.[87]

Conclusion

Even though *C. difficile* has several virulence factors associated with its pathogenesis, the most important ones are toxin A and toxin B. The role of binary toxin is also being delineated as a virulence factor as all emerging hypervirulent strains of *C. difficile* possess this toxin, suggesting that it might contribute to increased pathogenesis, by acting in synergy with toxin A and B. Other virulence factors like flagella, surface layer protein, sporulation, etc., add to the virulence of the organism.

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REVIEW ARTICLE

Gastrointestinal Mucormycosis: A Challenge during COVID-19 Pandemic

Jagdish Chander

Abstract

In India, the second wave of COVID-19 pandemic came with unprecedented number of patients in the months of April, May, and June, 2021. This time correspondingly there was a record number (~50,000) of mucormycosis, which was popularized by the mainstream media as "black fungus." The most common presentation was rhino-orbital mucormycosis followed by pulmonary and gastrointestinal mucormycosis. Scientifically this disease should be termed as COVID-19-associated mucormycosis (CAM). The Government of India had to declare it as an emergency situation under the Epidemic Diseases Act, 1897, on May 20, 2021. A large number of professional bodies in medical sciences, particularly in the field of microbiology, came out with various guidelines to tackle the challenging issue. The most common species involved is *Rhizopus arrhizus*, which is found in our surroundings, food material, and different places. Sometimes it is taken as a contaminant or "bread-mold" in houses or "lid-lifter" in the laboratory. But when there is an underlying background of any disease like diabetic mellitus, the same very fungus also becomes life-threatening. During this intervening period of second and looming third wave, patients are presenting with recurrence of the disease. The diagnosis is not very difficult when the index of suspicion is high. The direct finding of nonseptate hyphae with right-angle branching followed by cultural confirmation on Sabouraud Dextrose Agar clinch the final diagnosis. As far as the treatment is concerned, it is mainly surgical intervention of the necrotic tissue followed by antifungals like amphotericin B (conventional/liposomal), posaconazole, isavuconazole, apart from taking care simultaneously of the underlying risk factors like diabetes mellitus. With timely management the patient can be saved easily otherwise it can prove fatal.

Keywords: Amphotericin B, CAM, COVID-19, Gastrointestinal mucormycosis, Pandemic.

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Introduction

During the last four decades, fungal infections are increasingly being reported among the population at large. Some of them, i.e., candidiasis, cryptococcosis, pneumocystosis, talaromycosis, aspergillosis, etc., were frequently encountered after the onset of AIDS pandemic in 1980s to the extent that they were designated as AIDS-defining illnesses. However, lately, another infection was also added to this opportunistic fungal list, not going parallel to earlier ones, that is, mucormycosis. Although rare but one of its clinical components is gastrointestinal (GI) manifestation. Majority of the presenting cases are rhino-orbital, a few belong to pulmonary type followed by GI mucormycosis. The main disease itself, along with GI tract manifestations, has posed a very serious challenge for the medical fraternity during this on-going COVID-19 pandemic.

The media has branded the disease as "black fungus," which is essentially a misnomer as there is designated category of "black fungi" already existing in the medical literature causing tinea nigra, chromoblastomycosis, pheohyphomycosis, etc. The deciding factor for any "black fungus" is the presence of melanin in the fungal cell wall otherwise they are considered as colorless, i.e., hyaline and the Mucorales are definitely hyaline fungi. There is no end to such unfounded and fancy nomenclatures. Today it is a "white fungus" or "yellow fungus" and tomorrow there will be red, blue, or green fungi, which should be discouraged. Preferably, it should be called as COVID-19-associated mucormycosis.

The understanding about Mucormycetes has evolved as these are now emerging as highly pathogenic organisms invariably entailing fatal consequences, especially when an obvious underlying predisposing factor like diabetes mellitus already exists in a particular clinical setting. The cases are increasingly reported among absolutely healthy individuals also where there is no obvious underlying risk factor. Unlike other fungal diseases, even the AIDS pandemic could not significantly affect the incidence as well as prevalence of mucormycosis during the last four decades; however, an upsurge in the number of diabetic patients has really changed the entire scenario more or less like an epidemic in the Southeast Asian Subcontinent by posing a very serious health threat. The present COVID-19 or the future pandemics will aggravate the situation in the times to come.

The so-called "black fungus" disease, i.e., mucormycosis is in general an acute infection caused by several agents belonging to phylum Glomeromycota. These saprotrophic fungi are found ubiquitously in the atmosphere, i.e., soil and environment, in our surroundings. Earlier these mucoralean fungi used to be taken as nonpathogenic to man and animals. Rather they were considered as merely fungal contaminants in the diagnostic microbiology laboratory. During the first wave of COVID-19, these presentations were not observed; however, in the second

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wave about 50,000 cases were reported, half of them being life-threatening. Now, the third wave is also looming, which may lead to alarming consequences. The upsurge in cases is because of the COVID-19 pandemic, contributed by various risk factors. In the present review, description of the causative agent, upsurge of mucormycosis during COVID-19 pandemic, virulence, clinical manifestations, diagnosis, and treatment of the disease have been discussed in detail.

What is Mucormycosis?

The nomenclature, mucormycosis, is an umbrella term used for diseases caused by many nonseptate filamentous fungi. Classified under orders Mucorales and Entomophthorales, they were previously considered members of phylum Zygomycota, which are elevated to the rank of subphylum Mucoromycotina and Entomophthoromycotina, respectively, under phylum Glomeromycota. The phylum Zygomycota is now abandoned, hence, consequently the term zygomycosis also no longer exists. Some investigators prefer to call the disease caused by members belonging to subphylum Mucoromycotina as mucormycosis and to subphylum Entomophthoromycotina as entomophthoromycosis or even subcutaneous mucormycosis, which is further subdivided into two clinical types, i.e., conidiobolomycosis and basidiobolomycosis.[1]

The Mucormycete is a group of lower fungi and their hyphae are generally nonseptate. However, when these septa occur they are solid cross-walls with no pores and there is no flow of cytoplasmic material between adjoining cells. These reproduce asexually by way of sporangiospores formed within a sac called as sporangium and/or by means of conidial development. These fungi also reproduce sexually by formation of a single, dark, thick-walled spore called zygospore.

These fungi are seen as broad, nonseptate hyphae in tissue with a strong predilection to invade blood vessels due to their angioinvasive nature thereby leading to extensive necrosis of the surrounding area eventually formation of embolism. Since the morphology of these organisms is indistinguishable in histopathological sections, culture is essential for the exact identification of the causative Mucorales species. The course and outcome of disease differ according to anatomical site involved as well as the nature of fungal species isolated in a particular patient.

The fungi are found in food items, soil, air, and may be frequently encountered as laboratory contaminant. The spores are widely distributed, growing on leaf litter and other decaying carbohydrate substrates. The spores are found in large number in damp interiors and around composting vegetation and even present as "bread molds" appearing as grayish, fluffy, and rapidly spreading growth. Because of their rapid growth and prolific spore-forming capacity, inhalation of conidia is a routine occurrence. It is now the third most common invasive mycoses after candidiasis and aspergillosis.

Upsurge of Mucormycosis Cases

The fundamental question is why there is an upsurge of cases of mucormycosis during this pandemic of COVID-19 including the GI tract manifestations. This situation was not so grim during its first wave in 2020 but all of a sudden, like a sort of tsunami, within a short span of time, many cases were reported all over the country. This began with a few case reports from Ganga Ram Hospital, New Delhi, and almost simultaneously from Gujarat. Subsequently, cases started pouring in from almost every nook and corner of the country. Most of the patients had already recovered from COVID-19 episode and therefore discharged; however, some of them came back to the hospital with a fulminant disease. The second wave of COVID-19 has now declined but recurrence is common among the already treated cases. The various risk factors are contemplated and evidence-based studies need to be carried out before reaching a definitive conclusion.

First and foremost, there is injudicious use of steroids during the course of the management of COVID-19 and that too in high doses than the prescribed ones for a prolonged duration. The other risk factors may be like prolonged use of contaminated masks, without periodically changing as per the prescribed guidelines. There is a strong possibility of use of contaminated accessory while inhaling oxygen among patients. During the second wave of COVID-19 pandemic, there was an acute shortage of medical oxygen in the country hence most of the hospitals had to shift on to the industrial oxygen. Although it is said that there is no fundamental difference between both the grades of oxygen except for a minor one of dispensing but the public at large still suspect it to be one of the causes. The humidifiers used while delivering the oxygen, i.e., distilled water vs sterile or tap water is also suspected to be the source. Moreover, frequent and excessive steam inhalation is also considered as one of the risk factors.

Among other suspected risk factors, there is a higher level of iron in the form of ferritin among the COVID-19 patients, which is also favorable for copious growth of Mucormycetes. In addition almost all patients of COVID-19 are taking zinc as a preventive and/or therapeutic agent, which is also conducive to the fungal growth. This fungal infection also affects people who are already on some medication like prolonged use of antibiotics. Some of the reports state that the delta variant of the SARS-CoV-2 (B.1.617.2) is targeting the islets of Langerhans in the pancreas through ACE2 receptors, jeopardizing insulin production thereby leading to diabetic state among patients during their recovery phase.

Mucormycosisis declared as a notifiable disease by various Indian states beginning with Rajasthan. Eventually Indian Council of Medical Research (ICMR)/Government of India (GOI) also announced this disease under Epidemic Diseases Act, 1897, on May 20, 2021, wherein all government and private health facilities and medical colleges are to follow guidelines for its screening, diagnosis, and management. The advisory goes on to say that mucormycosis, if remain uncared, may turn out to be fatal. Hence to prevent the disease, blood glucose level should be regularly monitored during the post-Covid period. Consequently, all cases reported are to be conveyed to the World Bank-funded Integrated Disease Surveillance Project of the respective States or Union Territories.

In addition to the NCDC/ICMR/GOI, Fungal Infection Study Forum, European Confederation of Medical Mycology (ECMM), and International Society for Human and Animal Mycology (ISHAM) have also released their respective guidelines on this acutely emerging disease during the COVID-19 pandemic.

Virulence of Mucormycosis

The virulence of these fungi is simply of low order and usually sporadic infections occur throughout the world particularly in severely debilitated patients. Being an opportunistic infection, mucormycosis is produced by contaminant fungi in a host whose immunological defense mechanisms are weakened by endogenous causes like uncontrolled diabetes mellitus in Southeast Asia whereas malignancy, leukemia, or exogenous causes like immunosuppressive therapy in the Western world.

The breakthrough mucormycosis cases are also reported among patients with hematologic malignancies receiving hematopoietic stem cell transplants or intensive chemotherapy, given voriconazole for preventing aspergillosis or have undergone COVID-19 vaccination.

The neutrophils appear to play a major role in the defense of host from infection caused by Mucormycetes. In normal hosts, macrophages prevent initiation of infection by phagocytosis and oxidative killing of spores. On the other hand, among hosts with uncontrolled diabetes mellitus and other underlying factor, monocytes/macrophages are dysfunctional and fail to suppress spore germination process. The hosts with neutrophil defects, either qualitative or quantitative, are predisposed to infection by fungal agents of this class.

Similar to aspergillosis, phagocytes, polymorphonuclear neutrophils, and macrophages play a significant role among patients of mucormycosis. Infection can occur by inhalation, percutaneous inoculation, or ingestion. The spores are inhaled into lungs where they are ingested by alveolar macrophages. These cells are known to inhibit the germination of ingested spores to some extent but their activity to kill them is limited. Further, when they evade antifungal activity of macrophages and germinate into mycelial form, polymorphonuclear neutrophils and peripheral monocytes are expected to work against fungi. The former, which possess property of fungicidal activities against Mucormycetes, are known to play an important role in defense against mucormycosis; therefore, leukocytopenic patients are extremely susceptible. The increased risk of mucormycosis in patients with ketoacidosis may also be due to the release of iron bound to proteins. Due to ketoacidosis, low serum pH diminishes the phagocytic effect of macrophages, chemotactic, and oxidative burst of neutrophils.

The most common encountered mucormycete, *Rhizopus arrhizus*, has several virulence factors like angioinvasive nature, growth at or above body temperature, production of destructive enzymes, dormant spores which are resistant to destruction at extremes of temperature along with active ketone reductase system and hydroxamate siderophores. The diabetic patients with ketoacidosis are usually more affected by mucormycosis. *Rhizopus* species have an active ketone reductase system hence thrive in high glucose and acidotic conditions. These patients also have decreased phagocytic activity because of an impaired glutathione pathway. The exact mechanism of increased susceptibility among these patients remains somewhat unknown, probably a combination of metabolic abnormalities present in the patients with diabetes. Hyperglycemia or acidosis alone does not permit fungal growth *in vivo* although acidosis without hyperglycemia is reported with invasive mucormycosis. The normal serum inhibits *Rhizopus* species whereas serum from patients of diabetic ketoacidosis stimulates its growth.

This has been observed that patients, on dialysis and iron overload, who are being treated with deferoxamine, an iron chelator, are more susceptible to mucormycosis. It is probably because Mucorales use this chelator as a siderophore to obtain more iron. The other risk factors include neutropenia, high-dose systemic steroids, protein-calorie malnutrition, solid organ and bone marrow transplants, immunodeficiency, leukemia and intravenous drug abusers who may inject spores of Mucorales with the drugs and then present with space-occupying lesions of the central nervous system.

Clinical Manifestations of Mucormycosis

As such the nomenclature as mucormycosis comes from Mucorales and not from its one of its genera, i.e., *Mucor*, which is confused. Irrespective of the genus or species, based on the sites involved, the disease is called mucormycosis, which presents as the following six clinical categories.[2]

The clinical manifestations are (i) rhino-orbito-cerebral, (ii) pulmonary, (iii) cutaneous, (iv) gastrointestinal, (v) isolated renal, and (vi) disseminated mucormycosis.

Each category is associated with certain underlying disorders, relationship, and existence of variable factors. The commonest type is rhino-orbito-cerebral with almost 50% mortality and *R. arrhizus* is the usual causative agent.[3,4]

The signs and symptoms of GI mucormycosis are nonspecific and vary considerably depending on the exact site as well as the extent of involvement. These include abdominal pain, distension, vomiting, diarrhea, hematemesis, and melena. The patients on dialysis are reported to develop the disease as most of them are treated with desferrioxamine (deferoxamine and desferal) used as chelator for either iron or aluminum and may be for both.[5]

The necrotic and gangrenous lesions of intestine are rarely reported among the COVID-19 patients due to GI manifestations of mucormycosis. This may be a primary disease where there is no involvement of other systems. However, mostly it is found to be presenting with rhino-orbital or disseminated infections. Simultaneous involvement of renal and GI tract is also reported. As such GI type occurs in general accounting for about 7% of all cases of mucormycosis, most often involving the stomach. It is primarily found among patients suffering from extreme malnutrition and is acquired by ingesting food contaminated with fungal spores. Moreover, ingestion of fermented milk, porridge, and alcohol made from corn and herbal products have been implicated in GI mucormycosis. The lesions in stomach are followed by colon, ileum, and esophagus. This may follow surgery from filthy trauma to abdomen or contaminated ileostomy. Ulceration of gastric mucosa with thrombosis of associated vessels has been observed (Figs 1 and 2). The disease is usually found in adult patients but has also been reported in neonates, low-birth weight infants, and young children.[6]

The digestive system in mucormycosis is primarily involved in patients suffering from malnutrition. The manifestations range

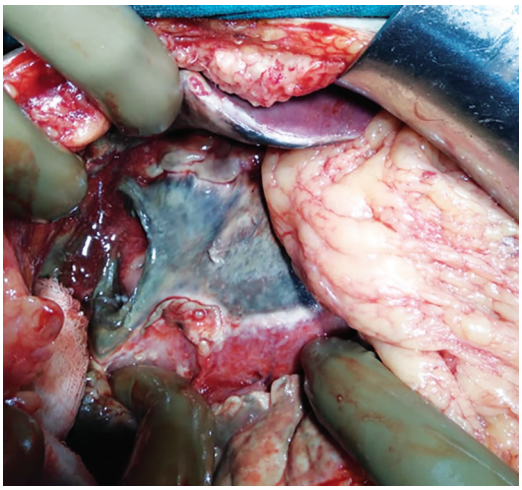

Fig. 1: Extensive necrotizing gastric lesions due to mucormycosis over the pyloric end of the stomach of a patient

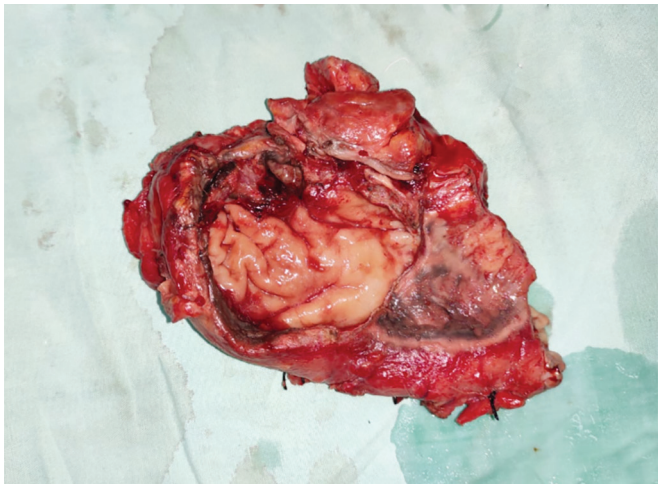

Fig. 2: Extensive necrotizing gastric lesions surgically removed from the pyloric end of the stomach of a patient

from mere colonization of peptic ulcers to infiltrative disease with vascular invasion and dissemination. It can be categorized into three forms: colonization, infiltration, and vascular invasion. Colonization usually occurs in preexisting gastric ulcers and is not fatal. Invasive form of gastric mucormycosis has variable presentation and is usually fatal. This form can either invade preexisting peptic ulcer or invade the stomach *de novo*. The disease usually presents as epigastric discomfort, GI bleeding, or viscous perforation in patients with established predisposing factors. In the invasive variant, there is involvement of vessel walls producing thrombosis, hemorrhage, necrosis, and ulceration of local tissue, and usually has a fatal outcome. Intestinal tract involvement is relatively rare with terminal ileum, cecum, and colon being primarily affected sites.[7]

Diagnosis of GI Mucormycosis

Earlier, diagnosis of gastric mucormycosis used to be made on the basis of postmortem examination and was rarely possible during life. More recently, radiological imaging and endoscopic biopsies have established diagnosis at an earlier stage during life, allowing attempt to a successful treatment. Gastric brushing is less invasive than biopsy and allows early identification of organisms as compared to histopathological processing. A very similar type of disease, GI basidiobolomycosis, is caused by *Basidiobolus ranarum*, members of order Entomophthorales, which is a rare clinical entity and about 80 cases have been reported in the literature. It is usually found in Middle East countries like Iran, Saudi Arabia, Kuwait, Oman, etc., but occasionally reported from India also.[8]

The patient with GI mucormycosis is often misdiagnosed as having an intra-abdominal abscess. It is usually confused with GI basidiobolomycosis, other inflammatory bowel diseases, malignancies, appendicitis, and diverticulitis. GI basidiobolomycosis involves the stomach, small intestine, and colon; however, it can disseminate to the liver, pancreas, and renal system. This may also present with complications like bowel perforation, obstructive uropathy, esophageal varices, duodenobiliary fistula, or even death. The histopathological examination is useful in establishing the accurate diagnosis, which reveals Splendore-Hoeppli phenomenon; however, culture remains the gold standard to diagnose GI basidiobolomycosis.[9]

The clinician should keep high index of suspicion about mucormycosis, which is necessary in post-COVID-19 patients presenting with mesenteric ischemia or bowel perforation especially if they were diabetic or have used high-dose steroids. It is established that computed tomography scan and particularly magnetic resonance imaging are most helpful in enabling an early detection of orbital, sinus, meningeal, intraparenchymal, cerebral lesions, intracranial vascular occlusion as well as GI involvement even before full-fledged clinical signs develop. These imaging techniques are helpful in defining the extent of soft tissue involved and are more useful in planning surgical intervention in addition to establishing the diagnosis. The COVID-19 case may present with markedly increased inflammatory cytokines like IL-2, IL-6, IL-10, and tumor necrosis factor-alpha, impaired cell-mediated immunity, both CD4+ and CD8+ T cells.

In the laboratory, a thorough understanding of GI mucormycosis is of paramount importance to establish an early diagnosis thereby appropriate treatment. The diagnosis is challenging and that too in COVID-19 times because of rapid fulminating course of disease and doubtful significance of isolates, which are commonly encountered as laboratory contaminants. One has to compete with the race of time. Therefore, detection of fungus in tissues is supplemented to establish significance of cultural isolate. The necrotic clinical materials from infected site may contain fungal elements; however, deeper tissue section may also be required. Detection of galactomannan or β-glucans in patients' serum, which is often helpful in diagnosis of other systemic fungal infection like aspergillosis, is of little value because Mucormycetes do not produce substantial amount of both these biomarkers.

The microscopic examination of biopsy material in potassium hydroxide/calcofluor-white (KOH/CFW) wet mount shows characteristic broad, nonseptate ribbon-like hyphae with right-angle branching at irregular intervals (Figs 3 and 4). These hyphae usually are very sparsely distributed in infected tissue, and therefore, histopathological stains are mandatory like hematoxylin and eosin (H&E) (Fig. 5), Periodic acid-Schiff (PAS) (Fig. 6), and Gomori's methenamine silver. The inflammatory response is of neutrophilic nature and is associated with infarct, vascular, and perineural invasion. Sometimes, distorted hyphae are also seen in the tissues. The disease may also be established on the basis of fine-needle aspiration cytology from the affected site. Frozen

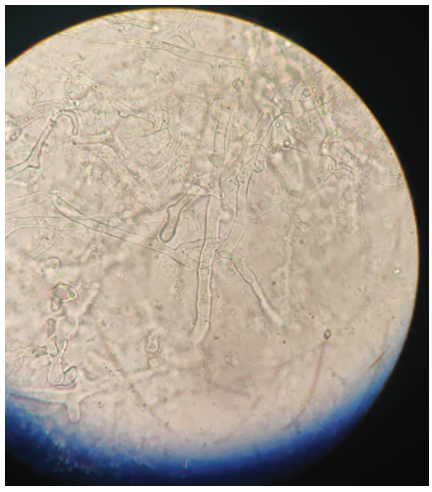

Fig. 3: Broad, ribbon-like, nonseptate hyphae with right-angle branching seen in wet mount (KOH × 400)

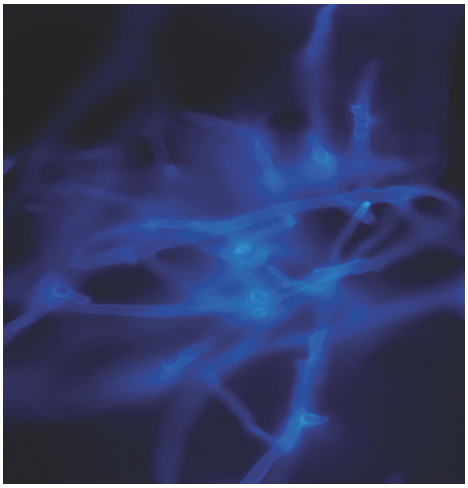

Fig. 4: Broad, ribbon-like, nonseptate hyphae with right-angle branching seen in wet mount (CFW × 400)

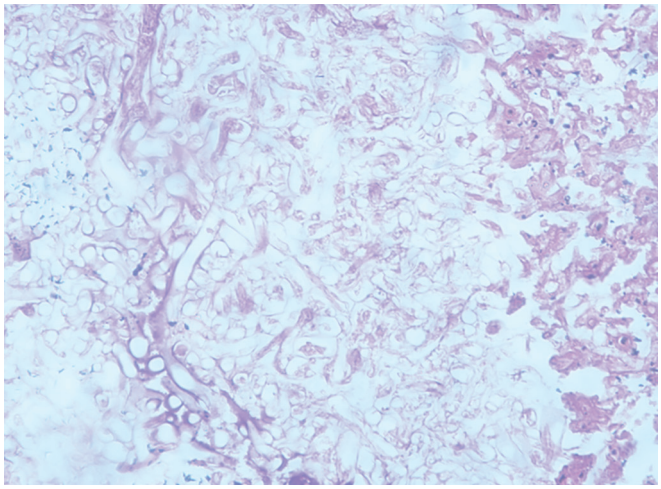

Fig. 5: Nonseptate hyphae of Mucormycetes with right-angle branching in a tissue sections (H&E × 400)

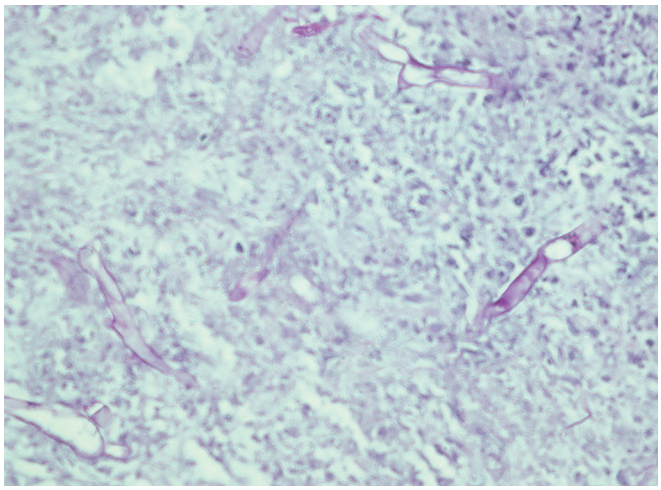

Fig. 6: Nonseptate hyphae of Mucormycetes with right-angle branching in a tissue sections (PAS × 400)

section may be useful when operative procedure was contemplated without suspicion of GI mucormycosis.

The hyphal elements of Mucormycetes are recognized as nonseptate thick-walled (10–20 µm) hyphae, with right-angle branching. Due to the absence of cross-walls, fluids from hyphae are free to escape during handling of tissues, hyphae collapse, and crinkle giving characteristic ribbon-like appearance. They do not radiate from a single point in tissue. These features distinguish them from slender hyphae of *Aspergillus* species, which have regular dichotomous branching at acute angle with frequent septation. Therefore, it is recommended not to homogenize tissue material and specimens must be as such directly inoculated on to culture media to keep texture intact thereby viability of fungal cells.

The Mucormycetes can be easily grown on conventional media like Sabouraud dextrose agar with antibiotics at both temperatures, i.e., 25 and 37°C but without cycloheximide, as it is inhibitory to most of them. Although the Mucormycetes are not fastidious fungi, still sometimes they fail to grow during primary isolation due to careless handling of specimen. Therefore, to avoid growth failure, some portion of the tissue may be kept in water added with a few drops of Yeast Malt Broth. In about 50% of cases there is no growth despite direct demonstration of the fungi. In such circumstance, molecular technique directly from the sample should be done to establish the diagnosis.

As mentioned the hyphal elements of Mucormycetes are prone to physical damage; therefore, specimens must be directly inoculated onto fungal culture media avoiding undue grinding. The relevance of isolates in clinical material may be difficult to establish if coenocytic hyphal elements are not seen in direct examination of KOH/CFW wet mount or histopathological section. Hence repeated attempt to isolate organism from consecutive specimens provides strong evidence that isolate is clinically significant.

The rapidly growing mycelial colonies are floccose, dense, and hairy in appearance (Fig. 7). The mycelia are described as fibrous or with cotton-candy growth, which is very vigorous hence some of Mucormycetes of order Mucorales are called as "lid-lifters" as they press upon lid of petri dish from below. The isolate is identified on the basis of morphological features observed in lactophenol cotton blue (LCB) mount (Fig. 8) followed by molecular sequencing for confirmation. The antifungal susceptibility testing is also done to

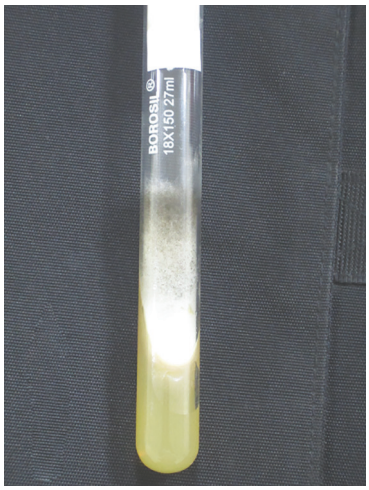

Fig. 7: Grayish mycelial growth of *Rhizopus arrhizus* in fungal culture tube after 3 days of incubation on Sabouraud dextrose agar at room temperature

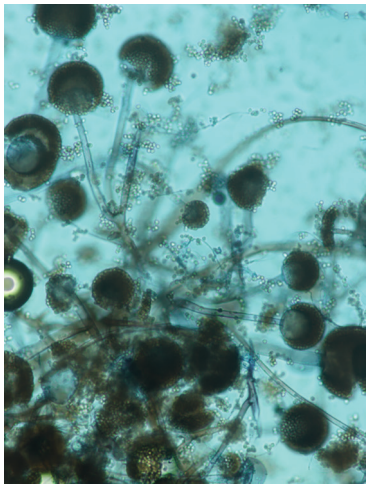

Fig. 8: Nonseptate hyphae of *Rhizopus arrhizus* with rhizoids, sporangia, and sporangiospores (LCB × 400)

establish whether the isolate is sensitive to a particular antifungal drug or not using CLSI or EUCAST methods. There is no reliable serological test for mucormycosis thereby cannot be recommended for the routine use in a diagnostic microbiology laboratory.

Treatment

During this pandemic of COVID-19, most of the national and international organizations like WHO, ISHAM, ECMM, FISF, and NCDC/ICMR/GOI have issued clinical guidelines for the diagnosis and treatment of mucormycosis including the GI manifestations. The consensus is resultant from the experience of clinicians treating such cases. As GI mucormycosis is a life-threatening condition hence invariably it proves to be fatal within a very short span, particularly when specific diagnosis is not established well in time thereby leading to substantial delay in surgery as well as institution of proper antifungal regimen.

For treating the disease, first of all proper management of COVID-19 infection has to be instituted depending on its stage and severity, ensuring all the prescribed precautions. Try to avoid steroids therapy as far as possible; however, if at all indicated then regulated dose to be given for a minimum time period. Immediately remove all the risk factors like diabetes mellitus by starting insulin or other appropriate antidiabetic regimens.[10]

The therapeutic modalities can be divided into four concurrent approaches. These are (a) rapid correction of underlying predisposing condition of the host like diabetic ketoacidosis using insulin; (b) surgical debridement of necrotizing tissue for better circulation and penetration of drugs; (c) antifungal therapy; and (d) consideration of adjunctive treatment such as hyperbaric oxygen, if available. A combination of surgical debridement and antifungal drugs is required for an ideal treatment of mucormycosis. The temptation for small and frequent piecemeal debridement should be avoided, which further aggravates morbidity and mortality among the patients. It should be preferably done taking adequate surrounding healthy tissue for debridement.[11]

There are only three drugs available for treating GI mucormycosis, i.e., intravenous amphotericin B (conventional/liposomal), posaconazole, and isavuconazole. It is observed to start with higher doses of antifungal drugs instead of stepwise increment. Drug resistance may be another hurdle but in case of majority of mucormycetes strains, these are found to be sensitive. Topical amphotericin B gel is a useful adjunct, which is applied locally after surgery.

The azole derivatives are not useful in treating GI mucormycosis due to lack of both *in vivo* and *in vitro* antifungal activity. However, exceptionally posaconazole and isavuconazole are found to be useful, which are available as oral and intravenous preparations. Some of the azole like voriconazole is otherwise counterproductive which enhances the disease process among mucormycosis patients. Similarly, echinocandins have no role in the treatment and there is no oral chemoprophylactic agent available for this fungal disease. Cytokines such as gamma interferon and granulocyte-macrophage colony-stimulating factors have also been used to treat GI mucormycosis.

The advisory guidelines, most of the times, prove to be powerless because there is nothing mandatory. Hence keeping in view the rapid and devastating course of mucormycosis, every institution should resort to its local mandatory protocol, wherein KOH/CFW wet mount should be performed within half an hour of the arrival of suspected patient followed by surgical debridement within 2 hours in cases found to be positive. If this promptness is adopted, the patients may not require antifungal drugs also, which were in inordinate scarcity due to increased demand. However, comprehensive antifungal regimen is to be instituted as per the clinical and financial condition of the patient. If proper measures are not followed in letter and spirit one is bound to lose the patient of a treatable disease. All the COVID-19 patients should be made cautious at the time of discharge that they should check and regulate their sugar level at least for a period of few weeks.

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REVIEW ARTICLE

Biofilm and Chronic Typhoid Carriers with Special Reference to Bacteriophage Therapy

Virendra Bahadur Yadav[1], Gopal Nath[2], Sudhir Kumar Singh[3]

Abstract

Salmonella enterica serovar Typhi is a human-restricted pathogen and the primary etiologic agent of typhoid fever with an incidence of 21 million cases each year, resulting in 200,000 deaths annually. About 3–5% of the individuals with an acute clinical or subclinical infection ultimately develop a chronic asymptomatic carrier state. These new chronic carriers are being added to the existing pool every year. This chronic carriage state not only serves as a reservoir for further spread of the disease *via* bacterial shedding in feces but is also being reported to be associated with malignant transformations in the biliary system. The acute and chronic carrier states are also becoming challenging to resolve with antibiotics due to the emergence of multiple drug-resistant strains. Moreover, biofilm formation is another hindrance in eliminating the infection. It is crucial to understand the development of each of these states to design and test targeted approaches to resolve the more recalcitrant chronic carriage. Bacteriophage therapy is emerging as one of the potential alternatives to deal with acute and chronic infection associated with biofilm formation. In this review, we have discussed the natural process of biofilm formation along with the intelligent role of bacteriophages to resolve such complicated infections, particularly in relation to typhoid.

Keywords: Antibiotics, Bacteriophage therapy, Biofilm, Chronic carrier, Typhoid fever.

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Introduction

Bacteria are simple entities when one compares them with eukaryotic organisms. However, they have better adaptive capabilities to survive in various environmental conditions. The gene expression is modulated according to the availability of nutrients.[1] Where most eukaryotes already possess a multicellular system, bacteria can also shift from a free-floating single-cell (planktonic) state to a biofilm-like community against a harsh environment for millions of years.[2] Biofilm consists of a three-dimensional (3D) microbial structure (either aggregation of mixed or single species) enclosed within a self-produced extracellular matrix.[3] It appears that biofilms are a beneficial trait in pathogenesis, as it exhibits distinct metabolism and gene expression than their planktonic forms. The altered phenotype has increased tolerance to host immune response and exogenously administered antiseptics and antibiotics.[4]

Biofilms have evolved on earth for 3.4 billion years. They perform several biochemical cycling processes. Biofilms may be present in a free-floating form or can form on various biotic and abiotic surfaces.[5] The bacteria have acclimatized to live at 37°C; they find the human body a perfect biotic microenvironment for bacterial colonization and biofilm formation. The human body surfaces have a reserve of nutrients, humidity, pH apart from appropriate temperature. Interestingly in 1985, Costerton introduced biofilm in medical microbiology.[6] It has been stated that approximately 65% of microbial infections have a biofilm-related etiology. The microbial infections based on biofilm can be classified as (i) intrinsic to host tissue and (ii) associated with indwelling medical devices.[7] The intrinsic biofilm to host tissues leads to chronic infections such as cystic fibrosis, osteomyelitis, conjunctivitis, vaginitis, urethritis, nonhealing wound, bacterial endocarditis, dental caries, sinusitis, otitis media, periodontitis, etc.[8] The other type of biofilm-associated infections is usually associated with medical devices, e.g., catheters, pacemakers, heart valves, breast implants, contact lenses, endotracheal tubes, and orthopedic implants.[9]

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Conflict of interest: None

The evolution of biofilm on any surface primarily results in diseases of chronic nature. The inherent high resistance to antimicrobial substances and antibiotics is the added economic burden for the global healthcare system. Chronic typhoid carrier state is primarily a biofilm etiology in the gallbladder. These carriers are the sole source of typhoid and paratyphoid infection as the bacteria causing these conditions are human restricted. Further, it has been very strongly proposed that chronic bacterial infections often culminate into carcinogenesis.[10] The association between chronic typhoid carrier state and gallbladder cancer has been already reported with evidence.[11] In this review, we have highlighted the process of biofilm formation, mechanism of chronicity, and different modalities to tackle the issues of treating biofilm-associated diseases with special reference to chronic typhoid carriers.

Basis of Biofilm Development

Understanding the fundamental concept of biofilm development will enable us to develop effective antimicrobial modalities for their eradication. Microbes colonizing and surviving in the human

body face several stresses, including cellular and humoral immune mechanisms. In addition, variations in physical conditions (pH, oxygen concentration, nutrients, other competing microbes, osmolarity) occur at different body sites.

Both prokaryotic (bacteria) and eukaryotic (fungi) cells tend to form a biofilm,[12] but the composition may vary. Intriguingly, irrespective of the type of microbes involved and physical conditions, the complete series of events during the biofilm development are almost identical (Fig. 1). However, the actual processes under native conditions are pretty complex, varied, and dynamic.

Composition of Biofilm

Production of an extracellular polymeric matrix is a biofilm's hallmark; however, the biofilms formed by most organisms commonly comprise of DNA, lipids, exopolysaccharides (EPS) and extracellular proteins. Furthermore, many of these proteins exhibit amyloid-like properties.[13] Thus, the biofilm matrix's production is primarily the critical key point for the success of biofilm communities in terms of propagation and survival of the cells.

Antibiotics penetrance for biofilm slows down by a factor of 2–3 due to the excretion of highly charged membrane-bound glycocalyx, which also plays a vital role in cohesion and adhesion with solid surfaces.[14] Another factor responsible for antibiotic resistance is the altered microenvironment and slow growth of the bacteria. It is known that within the biofilm, microgradients occur in the concentration of critical nutrients and oxygen, which results in heterogeneity in growth states extending from rapidly growing to metabolically inactive. As a result, the dormant bacteria in a biofilm can survive the antibacterial challenges.[15]

There is persistence in the biofilms, which evade killing by antibiotics and become resistant to chemical disinfectants. Although the proportion of this persistence is tiny, they evolve as a spore-like state. These mechanisms altogether increase the resistance of resident bacteria against conventional antibiotics by around 1000 folds.[16] In addition, the biofilms protect the resident bacteria against the immune system of host-mediated by impaired phagocytosis and complement system.[17]

Strategies to Combat Biofilm Formation and Biofilms

The biofilm formation on abiotic and biotic surfaces can be minimized by removing the indwelling devices and coating the abiotic surfaces with antibiofilm substances.[18] Antifungal or antimicrobial surfaces have also been proposed to prevent biofilm formation.[19] Impregnation of antibiotics or disinfectants such as polyurethane polymers, loaded with the safest antibiotics, and photodynamic therapy (to kill photosensitized microbes) can also be used.[20]

The EPS protect the microorganisms from various antimicrobial agents. So, the substances with EPS degrading ability would expose the biofilm cells to antibiotic agents. Here, it is worth mentioning that bacteriophages encode a unique enzyme class called endolysins (peptidoglycan hydrolases).[21] Endolysins are primarily species-specific. Moreover, it is vital to know the bacteria present in the biofilm for bacteriophage-derived endolysin. Specific extracellular proteases (sarA, Sigma B, ESP) have also been reported for biofilm disassembly.[22] The addition of extraneous DNase and restriction enzymes for certain species has been reported to disrupt the biofilm matrix as eDNA is a significant cementing matter for EPS.[22] Neutralization of lipopolysaccharides may also result in disassembling of the biofilm. Change in membrane permeability due to alteration in membrane potential may also disrupt the biofilm. This alteration may be pursued by harnessing bacteriocins as antimicrobials alone or existing antimicrobials to target biofilms. Lantibiotics have already been reported to be effective at permeating biofilms.[23]

Any molecule damaging the plasma membrane will stop the cell division and affects the microorganism's viability. Some antimicrobial peptides (AMPs) (e.g., pyrrhocoricin, apidaecin, drosocin) use the exact mechanism to inhibit the cell division and act as antibiofilm. In addition, certain AMPs are known to inhibit adhesion as they stop the synthesis of adhesion molecules.[24] While EPS is an essential component of biofilm, few EPS have been found to inhibit the synthesis of polysaccharides in biofilm. Instead, they have been reported to induce dispersion of the performed biofilm.[25] Inhibition of the cyclic di-GMP signaling system leads to

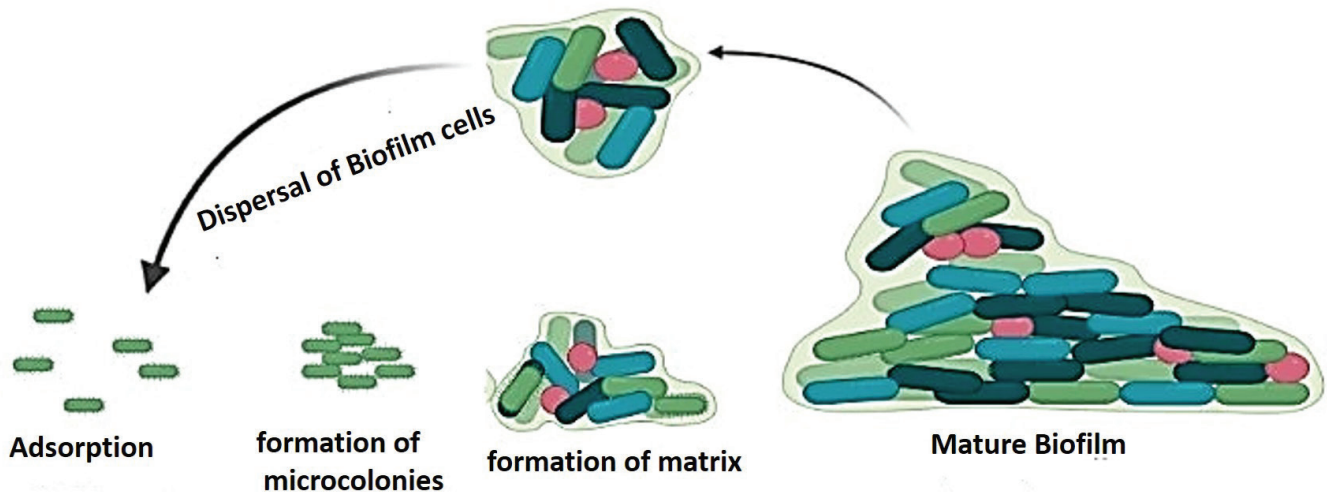

Fig. 1: Stages of biofilm formation

biofilm dispersal, and this phenomenon occurs under stress such as starvation, nitrosative conditions, etc.

Some of the secondary metabolites such as fisetin and esculetin affect biofilm maturation. As a result, they reduce the thickness of biofilm. Other agents, namely bispyridinamineoctenidine hydrochloride, have also been found to have antibiofilm activity, but the exact mode of action is unknown.[26]

Chronic Carriage of *Salmonella* Infection

It is now established that asymptomatic *Salmonella* Typhi carriage may develop following symptomatic typhoid fever or even subclinical infection. Intriguingly, very famous German microbiologist, Robert Koch, very well predicted in 1902 that the main reservoir of *S. Typhi* is humans who are usually symptom-free but excreting the pathogen. At about the same time, two persons known as Typhoid Mary in the USA and Mr N in the United Kingdom were traced as the source of infection. The studies carried out later on, demonstrated that about 1–4% of individuals infected with *S. Typhi* become chronic carriers and keep shedding 10^4–10^5 CFU/g of stool beyond 12 months of initial infection.[27] It is important to note that about a quarter of the chronic carriers never had a symptomatic infection due to *S. Typhi*, and possibly these individuals have a subclinical infection.

Since typhoid- and paratyphoid-carrying serotypes of *Salmonella* are human restricted, the persistence is of particular concern as a source of infection. The other serious concern is the recent report of a strong association between chronic typhoid carriage and cancer gallbladder.[11,28] Therefore, finding out the modalities to eradicate the bacterium from the entire human population seems significant.

As we have seen the grave public health concern of both acute infection and chronic carriage of typhoid-causing bacteria, it is imperative to understand the evolution of both states to design the strategies to combat the recalcitrant chronic carriage.

The key feature of chronic carriage of *S. Typhi* is successful colonization of the biliary system, especially biofilm formation on the surface of the gallbladder and gallstones.[29] As mentioned earlier regarding the development, maturation and dispersal of biofilm in general, the same phenomenon also occurs with this serotype of *Salmonella*. The biofilm protects the bacteria from the gallbladder's harsh environment, e.g., bile, host immune responses, and antibiotics.[30] However, in chronic carriage, the immune response of TH1 shifts from acute infection to TH2 when chronic carriage develops. Therefore, there is apprehension that chronic carriage causing *S. Typhi* may be genotypically different from acute infection. However, this speculation has been nullified by Ong et al.[31] report where complete genome sequencing of an isolate from chronic carrier revealed no difference from an isolate of acute typhoid fever.

Strategies to Combat Persistent *Salmonella* Infection

Most of the time, the administration of antibiotics has been observed to be infective against persistent typhoidal infection. The gallbladder removal has been reported with some success but not in absolute terms as persisters have been reported in other body parts, e.g., liver, lymph nodes, bone marrow[32] Devraj et al.[33] have demonstrated that despite being genetically indistinct, two isolates from chronic carriers tended to form thicker biofilms with a higher level of eDNA and DNABII proteins than those formed by acute infection isolate. In addition, the authors have demonstrated that antibodies against DNABII proteins disrupted biofilm *in vitro.* The extracellular DNABII proteins consist of integration host factor and histone-like protein. These proteins are critical to the structural integrity of bacterial biofilms.

Phages for Biofilm Removal

Biofilm formation is a form of cooperative group behaviors and probably the initial evolutionary structure of multicellular organisms. Different phages and bacteria evolve together in antagonistic, coevolutionary cycles, enhancing the speed of evolution of several traits, e.g., virulence and biofilm formation. Interestingly, biofilm gives shelter and protection to the bacteriophages either in the EPS or inside as prophage form. However, bacteria have several mechanisms to limit the access of phages.[34] As our understanding of the underlying mechanisms of coevolutionary interactions between biofilm and phages is getting bettered, biofilm's phage-based treatment can be designed.[35] Phage-based treatment modalities may include phages combined with antibiotics using a single phage or phage cocktail or phage-derived molecules such as enzymes and genetically modified phages.[36]

Phage Therapy

The lytic phages are the key point to disrupt biofilms. The phages must penetrate, diffuse, and propagate through the biofilm. The phages harboring EPS-degrading enzymes can degrade the EPS matrix components and thus facilitate the penetration.[37]

It has also been reported that bacteria infected with phage undergo stress, leading to EPS-degrading enzymes release.[38] Often the bacterial biofilm may be multispecies. The cocktail of phages takes care of such conditions and the emergence of phage resistance in bacteria.[39]

Genetically Modified Phages

Transduction is the major issue raised by opponents of phage therapy. Further, many phages may not have the mandatory genes/gene products for penetration and degradation of biofilms. Therefore, the phage may be genetically modified to express EPS-degrading enzymes extracellular and hydrolases intracellular and without virulence and antibiotic resistance genes.[40] Specific temperate phages with phenotypic characteristics disrupting the biofilm may also be genetically modified to lytic phages.[41] The phage can be made with broad spectrum activity, programmable DNA nucleases associated with CRISPR in temperate phages can be used to reverse the antibiotic resistance.[42] Further combination of gold nanorods which, after infrared light, induces photothermal lysis of both target cells and phages will take care of transduction-mediated problem of gene transfer.

Phages in Combination with Antibiotics

The phenomenon of phage-antibiotic synergy can be utilized for better results in biofilm disposal. Often bacteriophages revert the antibiotic-resistant phenotypes.[43] However, phage–antibiotic combination has certain drawbacks also, i.e., the emergence of resistant bacteria may promote antibiotic resistance if resistant variants escape killing and decreased metabolic activity of cells may lead to decreased replication of the virus.[44]

Phage-derived Enzymes

The enzymes encoded by phages called enzybiotics may help treat bacterial infection and biofilms. The lysins and depolymerases are the two powerful enzymes performing biofilm disposal and killing the bacteria.

- **Lysins:** Lysins are of two types; one is present in the phage tail as phage-associated lysins, acting on receptors after identification to degrade the cell was locally to facilitate the injection of the viral genome inside the host cell.[45] The other lysins are found inside the phage, which lyses the bacterial cell wall after the replication cycle. The unique feature of lysins as a therapeutic agent is that the lytic activity depends on the bacterial metabolic state. It means the phage lysins may also lyse the persisters.[46]
- **Depolymerases:** The polymerases produced by the phages are capable of degrading the extracellular substances of the bacteria, e.g., capsular polysaccharides, EPS, O-polysaccharides, and peptidoglycan. As these substances are abundantly present in the biofilm, they help the phages enter the biofilm's EPS structure.[47] Like lysins, depolymerase is also found in the bound form attached to the tail of phages.

Depolymerases may be divided into different groups. They are hydrolases, lyases, and triglycerol lipases. These enzymes are host-specific and highly diversified because phages and bacteria are pretty diverse and have intense horizontal gene transfer.[45,47] Depolymerases can be used to treat human and animal infections due to biofilms. These enzymes can enhance the penetration of the immune system by degrading the EPS matrix.[48] Interestingly, lysin and depolymerases have shown synergistic behavior in reducing the viable cells in the biofilm.[49]

Problematic Biofilm in Humans

All the mucosal surfaces with commensal flora of the human body are prone to bacterial infections. Most such infections (>65%) are associated with bacterial biofilms. The oro-gastro-intestinal tract, periodontitis, gingivitis, dental caries, peptic ulcer, cholecystitis, ulcerative colitis, etc., are associated with biofilm. Pyelonephritis, chronic prostate cystitis, urethritis, etc., are also usually associated with biofilm formation. Ironically, bacteriophage therapy has not been tried on most of the infections mentioned earlier.[50] Therefore, it will be advisable to shift to bacteriophage therapy when conventional antibiotic therapy fails after 6 weeks of duration.

One such infection is cholecystitis, and cholelithiasis affects the gallbladder for the long term. If *S. Typhi* causes it, it may be an inducing factor for the biliary tract cancer apart from being a constant source of infection. The aforementioned mechanisms of bacteriophages dealing with biofilm suggest that they may be deployed to treat acute typhoid fever and eradicate its chronic carriage when all the available therapeutic drugs are ineffective.

Many case reports mention that the treatment of typhoid fever using bacteriophage therapy during the pre-antibiotic era was done in many parts of the world. However, due to the incidences of the severe reaction after therapy, most likely due to constituent endotoxins in the bacteriophage preparations, this modality could not continue. Further, the decline in the application of phage therapy could be observed because of the introduction of the then magic drug, antibiotics. However, we may go for bacteriophage therapy in the present scenario of antimicrobial resistance and antibiotics often failing to eradicate the chronic carrier state. Many types of research have been carried out in recent times about bacteriophage biology, including the therapeutic aspects. The endotoxin in the phage composition and a release after lysis of the infecting bacteria has been worked out. In animal models, safe doses in different clinical conditions have already been determined with septicaemia.[51] It will facilitate clinical trials on humans. The question of killing intracellular bacteria by bacteriophages has been addressed by Broxmeyer et al.[52] They have demonstrated the killing of *Mycobacterium avium* and *M. tuberculosis* by a mycobacteriophage delivered by nonvirulent mycobacterium. We have carried out the killing of intracellular *S. Typhi* using a bacteriophage cocktail (Unpublished data).

Further, an acute and chronic model of mimicking typhoid may be created in a susceptible mouse model using *S. Typhimurium* as a surrogate model. The efficacy of bacteriophages may be evaluated. Moreover, phage therapy may also be assessed by putting the mice on a chalcolithic diet with *S. Typhimurium* infection leading to biofilm formation. If encouraging results are seen, we may proceed with human cases as well, not only to treat the chronic typhoid carriers but also the acute infections. This therapy may ultimately result in the complete eradication of *S. Typhi* from the human population.

Limitation of Phage Therapy and Preferable Approach

Although isolating bacteriophages is not a tough job for common target bacteria,[53] identifying therapeutic grade phages is complicated. Before starting phage therapy, knowledge of phage specificity toward other nontarget bacteria is also equally important. Importantly, bacteriophage genome sequencing is needed before their therapeutic application to confirm the absence of integrase genes (found in lysogenic type), antibiotic-resistant genes,[54] and genes for phage-encoded toxins. Furthermore, the formulation and stabilization of phages for use is bacteriophage dependent and optimized for each phage separately. This optimization is time-consuming and costly affairs of phage therapy clinical trials, which discourages the research and production of phage preparations.

The rapid development of resistance in host bacteria against phages in bacteria has been reported.[55] However, using bacteriophage cocktails to target different bacterial receptors and bacteriophage–antibiotics combined treatment prevents resistant development.[56] Furthermore, bacteriophages host range can also be expanded by genetic modification in phage tail ligand proteins.

Another approach of synthetic biology techniques generates various chimeric phages belonging to family T2, T4, and T7, targeting different bacterial receptors for synergistic therapeutic effects and delayed phage resistance development.[57]

Phage stability in the bloodstream is another obstacle in the path of phage therapy. Viruses used in therapy lose their potency soon due to the effect of humoral- and cell-mediated immunity. However, phage stability can be improved in circulation by altering the viral capsid proteins or through PEGlation (conjugation of PEG onto bacteriophages).[58]

Although bacteriophages are safe for humans,[59] phage purity is another severe concern before its therapeutic use. Phage lysates used in therapy may contain several harmful components, especially endotoxins (in the case of gram-negative bacteria) and protein toxins (produced by many pathogenic bacterial species). Due to their highly immunogenic properties, the endotoxins produced after the lysis of the bacteria aggravate the septic shock

via cytokine storm. The maximum permitted value of endotoxin in therapy is 5.0 Endotoxin Units /kg/h for intravenous injection. Therefore, removing these endotoxins is necessary before therapy, and today, many techniques are available to remove these harmful components. Some are PEG precipitation, membrane dialysis, ultracentrifugation, ion exchange chromatography, and extraction with 1-Octanol. Bacteriophage production in a cell-free system (synthetic bacteriophages) is another advanced synthetic approach to overcome these endotoxin-mediated side effects.[60]

Conclusion and Future Perspective

Asymptomatic, chronic typhoid carriers have been recognized for over a century. Unfortunately, despite our increased understanding regarding the persistence of *S. Typhi* in the gallbladder, we still do not have an effective method to cure it. As we are convinced now that antibiotics cannot do the miracle in any case of biofilm formation if given alone, bacteriophage therapy is now (*) being seen as one of the potential modalities to deal with such infections. However, acquiring several genes by bacteriophages coding for several enzymes during the evolutionary process might help deal with persistent infection and long-term carriage of typhoidal and nontyphoidal *Salmonellae*.

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CASE REPORT

Forme Fruste of Septic Arthritis in a Patient with Ulcerative Colitis

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Abstract

Musculoskeletal involvement is the most common extraintestinal manifestation of inflammatory bowel disease (IBD). Typically, peripheral joint involvement in IBD is classified into two types depending upon involvement of joint pattern. Apart from IBD associated arthritis, patient of IBD is also at risk of developing non-IBD related arthritis. Patients with IBD are on immunosuppressive medication and are at risk of developing septic arthritis, which is a medical emergency and needs emergent drainage and antibiotic therapy. Here we report a case of ulcerative colitis in a woman who was on azathioprine, presenting with unilateral pain and swelling of knee joint where a diagnosis of septic arthritis was established. The case highlights difficulties in diagnosis especially in patients on immunosuppression.

Keywords: Arthritis, Inflammatory bowel disease, Septic arthritis, Ulcerative colitis.

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Introduction

Inflammatory bowel disease (IBD) could be associated with many extraintestinal manifestations of which musculoskeletal involvement is a common one.[1,2] Joint involvement could be either peripheral arthritis or axial spondyloarthropathy. However, given the need for immunosuppression to achieve and maintain disease remission, these patients could be at a heightened risk of infections.[3,4] It is unclear if the manifestations and synovial fluid analysis are impacted by underlying drug treatment including immunosuppression. We report about a lady in remission of ulcerative colitis and receiving mesalamine and azathioprine, who presented with pain and swelling of right knee with equivocal findings on evaluation.

Case Description

A 48-year-old female presented with two-day history of fever and painful swelling of right knee. She was suffering from ulcerative colitis since the last 21 years without any extraintestinal manifestation. According to the Montreal classification, the extent of ulcerative colitis was E3, i.e., extensive disease (beyond the splenic flexure). Her disease had relapsing and remitting course that necessitated multiple steroid courses with the last one given 3 years ago. She had done well on maintenance therapy with oral mesalamine 4.8 g and azathioprine 75 mg/day since then. On examination, her right knee was swollen, tender with decreased active and passive movements of the right knee joint. Examination of rest of joints was normal. Right knee X-ray did not reveal any joint erosion (Fig. 1A) and ultrasound of right knee showed mild synovial thickening and effusion of the joint (Fig. 1B). Routine blood investigation showed hemoglobin of 10.8 g/L with normal leukocyte count (7400/mm^3). Serum uric acid level was 2.5 mg/dL and C-reactive protein was 135 mg/L. Blood culture was sterile. Magnetic resonance imaging of knee showed joint effusion and synovial thickening and enhancement without any joint erosion (Fig. 1C). Routine stool works up was normal with fecal calprotectin of 75 µg/g of stool.

Ultrasound guided therapeutic and diagnostic aspiration of synovial fluid showed straw colored fluid with total count 11275/mm^3, 89% polymorphonuclear cells, sugar 142 mg/dL, and protein 3.8 g/dL. Gram stain of the synovial fluid showed gram-negative bacilli but the culture did not grow any organism. Patient also underwent therapeutic aspiration of synovial fluid, of which 80 mL fluid was aspirated. In the wake of suspicion of septic arthritis, piperacillin and tazobactam were started and azathioprine was withheld which resulted in improvement in fever and resolution of pain, redness and swelling of the right knee joint. She was discharged after 4 days with oral antibiotic for 2 weeks, and after 4 weeks of follow-up, she did not have recurrence of joint symptoms and her stool frequency was two times without any blood in stool.

Discussion

This case highlights the clinical dilemma in diagnosing musculoskelteal system involvement in the patients with IBD. IBD related arthritis is typically divided into axial and peripheral arthritis. Arthritis in patients with IBD can be IBD associated or non IBD related. As per Oxford criteria, IBD-associated peripheral arthritis

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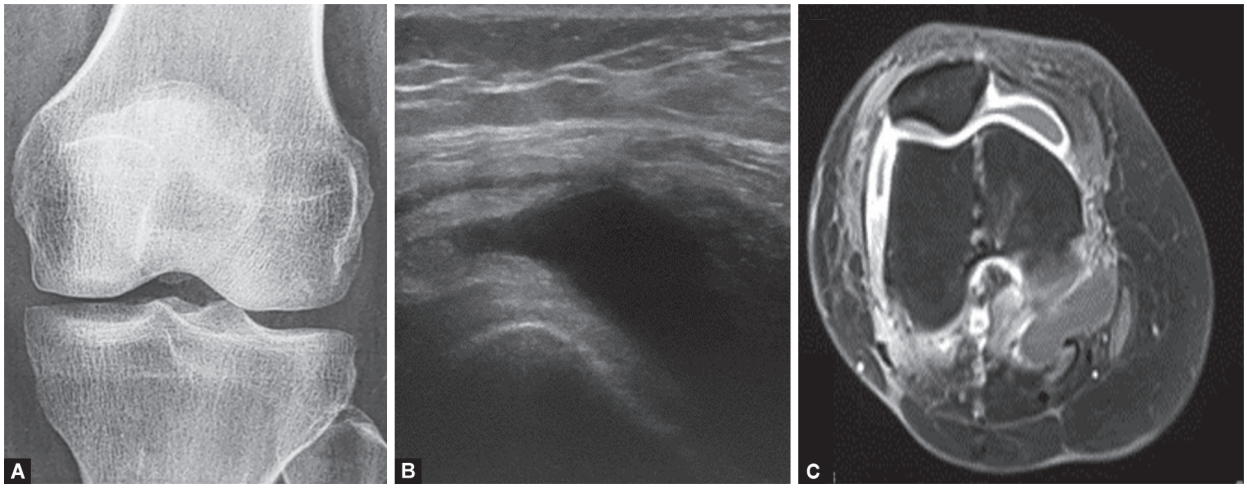

Figs 1A to C: (A) Anteroposterior radiograph of right knee was unremarkable; (B) Longitudinal ultrasound image of the right knee shows joint effusion and mild synovial thickening; (C) Contrast-enhanced axial MR image of right knee shows joint effusion with synovial thickening and enhancement

are classified in to type I, which is mono or oligo arthritis (<5 joints) asymmetrical predominantly involving the weight bearing large joints of lower limb, and type II, which is symmetrical polyarthritis predominantly involving the small joints of the hand. Type I arthritis, associated with activity of bowel disease, runs a course parallel to bowel disease but type II arthritis is not related to bowel disease and runs an independent course.[2] While type I arthritis was a consideration in our patient, the lack of significant IBD activity and presence of monoarthritis with joint tenderness and redness argue against this possibility.

Differential diagnosis of monoarthritis include septic arthritis, crystal induced arthropathy or trauma. Patients on immunosuppressive therapy have an increased risk of developing septic arthritis.[3,4] Septic arthritis is a medical emergency and delay in medical therapy can cause permanent joint damge.

High leucocyte counts in synovial fluid (typically >50,000/mm^3) with >90% polymorphs favor the diagnosis of septic arthritis.[5] There are multiple reports which suggest that cell count could be lower in certain setting. However, the most definitive test to diagnose septic arthritis is synovial fluid culture. It is unclear if the patients who are on immune suppressant therapy mount an effective immune response and whether the leukocyte counts in synovial fluid may be lower than the typically described counts. These counts could also be lower in early disease course, partially treated septic arthritis, peripheral leukopenia, prosthetic joints, etc. In such a clinical situation a lower threshold of synovial fluid total leukocyte count should be used to avoid delay in treatment initiation.[6–8] Our patients were on azathioprine and did not demonstrate peripheral leukocytosis, and this could be one reason for a lower cell count in the synovial fluid.

Conclusion

Bacterial infection of joint can progress rapidly causing cartilage damage, septicemia, and even death. So, a high index of clinical suspicion, early diagnosis, and treatment are essential to preserve the joint function and to have a good clinical outcome.

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CASE REPORT

Shigella flexneri Sepsis in a Well-nourished Immunocompetent Child: A Rare Case Report

Rama Gupta[1], Veenu Gupta[2], Deepak Bhat[3], Reetu Goyal[4], Jyoti Chaudhary[5], Menal Gupta[6]

Abstract

Shigella infections are usually restricted to the intestine. There are a few reports of *Shigella* isolated from the blood and most of these are from children, usually the neonates and the malnourished. In the small number of adult cases of *Shigella* bacteremia which have been reported, there appears to be an association with underlying disease and immunosuppression including acquired immunodeficiency syndrome. We report a rare case of septicemia with *Shigella flexneri* in a well-nourished, obese child, with no other predisposing underlying condition. With the prompt and aggressive symptomatic treatment along with administration of appropriate antibiotics based upon the antimicrobial susceptibility pattern of the isolate, the patient's recovery was uneventful.

Keywords: Bacteremia, Intravenous antibiotics, *Shigella flexneri*, Stool culture, Well-nourished child.

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Introduction

Shigellosis remains an important public health problem in developing and underdeveloped countries, including India. The disease is transmitted through feco-oral route due to consumption of contaminated potable water. There are four important species of *Shigella*, i.e., *Shigella dysenteriae, S. flexneri, S. boydii,* and *S. sonnei*, also known as groups A, B, C, and D, respectively.[1] The spectrum of the disease varies from mild gastrointestinal (GI) symptoms to severe and fatal septicemia/septic shock, though rare.[2,3] The primary GI symptoms include acute bloody diarrhea and fever, which are enterotoxin mediated.[4] Other extraintestinal organ system which may rarely get involved, include urinary tract, joints, liver, and central nervous system especially in young malnourished children. In most of the cases disease is self-limited or requires only supportive care, and symptoms resolve within 1 week.[5,6]

Shigella bacteremia seems to be a rare complication of GI shigellosis and is usually associated with underlying disease like malnutrition. Other predisposing conditions which have been associated include age, diabetes, leukemia, sickle cell anemia, malignancy, cirrhosis, immunosuppression, and HIV infection.[7–9]

This is a rare case report of shigellosis leading to bacteremia in a well-nourished, obese child, who did not have any other underlying condition, except for her young age.

Case Description

A 5-year-old female child from Ludhiana admitted to a referral hospital during August, 2021, presented with multiple episodes of watery diarrhea and high-grade fever since the past 4 days. In addition, the patient complained of abdominal pain for the last 2 days. On physical examination, the liver and the spleen were not palpable; abdomen was soft, tender, nondistended, with the presence of tenesmus and bowel sounds. The heart rate was 124/minute, respiratory rate 23/minute, blood pressure was 100/70 mm Hg, and body temperature was 98.6°F. Anthropometric details of the child were weight 27 kg (expected weight—16 kg), length 108 cm (expected—109 cm) and BMI 23 (expected—13). On auscultation, bilateral air entry was observed with no crepitations/ronchi. CVS-S1S2 was normal without any murmur. CNS-E4V5M6, bilateral pupil ESRL, tone/power normal, no signs of cranial nerve palsy/meningeal irritation.

The child was allergic to ceftriaxone and metronidazole. Provisional diagnosis of acute dysentery with obesity was made and the child was managed as a case of acute dysentery and treated with intravenous antibiotics (amikacin and ciprofloxacin) along with intravenous fluids and other supportive care. The stool routine examination showed white blood cells 10–15/HPF with 2–3 red blood cells/HPF. Ultrasound abdomen was suggestive of mild splenomegaly, and thickened large gut bowel in left hemi-abdomen, suggestive of infective/inflammatory etiopathogenesis.

Complete blood count reported hemoglobin 10.8 mg/dL, total leukocyte count: 8700/cu mm (neutophils 40.7%; lymphocytes 45.8%; monocytes 11.3%; eosinophils 02%, and basophils 0%) with a platelet count of 320×10^3/µL. No parasites were observed in the blood film. Liver function tests revealed total bilirubin of 0.38 mg/dL with all other tests being normal. Renal functions parameters were normal and C-reactive protein was 34.1.

Considering septic shock as a possibility, blood culture and other relevant investigations were requested. Blood culture was performed using BACTEC 9120 and identification and susceptibility testing was done by Vitek 2. Blood culture was positive for

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S. flexneri. The isolate was sensitive to cotrimoxazole (MIC ≤20 µg/mL), cefipime (MIC ≤0.12 µg/mL), ceftriaxone (MIC ≤0.12 µg/mL), azithromycin, and resistant to amikacin (MIC 4 µg/mL), gentamicin (MIC 4 µg/mL), ciprofloxacin (MIC >4 µg/mL), levofloxacin (MIC >8 µg/mL). Susceptibility to azithromycin was done by Kirby Bauer disk diffusion test. Based upon the antibiotic susceptibility pattern, azithromycin was added to the treatment regimen. Additionally, a request for stool culture was also sent after the blood culture turned out to be positive for *S. flexneri*. Culture of stool was done on MacConkey agar and deoxycholate citrate agar which grew nonlactose fermenting colonies after incubation at 37°C for 24 hours and was identified using automated identification system (Vitek 2 compact, BioMerieux, place) as *S. flexneri* and further confirmed by serotyping.

The general condition of the child improved gradually and was discharged in a stable condition after 8 days of hospital stay.

Discussion

Shigellosis is primarily caused by *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*. *S. dysenteriae* type I, and *S. flexneri* are among the most virulent serotypes, associated with invasive shigellosis leading to septicemia.[10,11] Though an invasive disease, it usually does not invade the tissue beyond the lamina propria and hence infrequently cause bacteremia in some patients with predisposing conditions. These predisposing conditions include young age, malnutrition, and immune-suppression.[12] A case report of *S. sonnei* septicemia with enterocolitis in a newborn infant had been documented.[13] Authors had emphasized that septicemia with enterocolitis was responsible for exudative loss of immunoglobulins, complement, and various plasma proteins which resulted in compromised immune response in the form of impaired lysis and opsonization of invading bacilli leading to devastating sepsis.

A study from Argentina has described two cases of *S. flexneri* bacteremia in infants.[14] Both the patients presented with fever and diarrhea. One of them had primary immune deficiency, whereas the other patient had no other underlying predisposing condition. Another study from Mumbai has reported four cases of *Shigella* septicemia in children admitted to Lokmanya Tilak Municipal General Hospital. Out of these four shigellosis cases, three were caused by *S. dysenteriae* serotype I and one by *S. flexneri*; a mortality rate of 75% was reported. Probable reason for high mortality with *Shigella* septicemia in these patients could be multidrug resistant *Shigella* species and a majority of them had *S. dysenteriae* type I infection, which is most virulent of all the serotypes of *Shigella*. All these isolates were sensitive to gentamicin, amikacin, norfloxacin, and nalidixic acid but resistant to amoxicillin, chloramphenicol, tetracyclin, and cotrimoxazole.[11] However, another meta-analysis of *Shigella* isolates had reported 20% resistance to nalidixic acid increasing levels of resistance to third-generation cephalosporins and azithromycin.[15] On the other hand, the present isolate was susceptible to azithromycin. Another rare case of fatal septicemia due to multidrug-resistant *S. flexneri* foregoing an episode of GI infection has also been reported in a 6-month-old infant from New Delhi.[5] It was an exceptional presentation of *Shigella* septicemia with severe shock, disseminated intravenous coagulation, and convulsions.

Although shigellemia is primarily reported from malnourished infants and children, there are a few reports where adult population was also reported to manifest bacteremia due to different species of *Shigella*. Sharma and Arora reported an uncommon case of *S. flexneri* bacteremia in a 65-year-old nondiabetic, hypertensive male with a history of dysentery. The patient from New Delhi was a known case of chronic kidney disease, dilated cardiomyopathy, and permanent pacemaker device implanted since the past 7 years.[12] Another series of case reports have documented three adult cases in which *Shigella* was isolated from the blood. Two of these patients made an uneventful recovery while the third died. An underlying cause of immunosuppression was suspected in this patient but unproven.[9]

Though shigellosis results in considerable morbidity in endemic areas, mortality is rare in developed countries. However, a study from Israel has reviewed all pediatric (age 5 months to 11 years) deaths ($n = 15$) following shigellosis during a span of 10 years. Predominantly, *S. flexneri* was the concerned *Shigella* species in these patients ($n = 8$) followed by *S. sonnei* ($n = 4$) and *S. dysenteriae* ($n = 1$). However, two of the *Shigella* species could not be identified. The cause of death in all these patients was unswerving with noxious encephalopathy, except for one case where "Reye-like" syndrome was found to be associated with the death of the patient. Case-control study of these patients with the surviving shigellosis hospitalized patients was found to have similar incidence of fever, diarrhea, vomiting, dehydration and convulsions, and toxic encephalopathy due to childhood shigellosis in a developed country.[16]

In the present case report, septicemia was caused by *S. flexneri*, as isolated from both the blood and the stool samples. Additionally, the isolate was susceptible to many antibiotics and the patient was treated symptomatically along with azithromycin. The patient was discharged in a stable condition and recovery was uneventful after a stay of 8 days in the hospital.

Conclusion

The present study emphasizes the importance of considering *Shigella* infection as a differential diagnosis when a patient with severe sepsis associated with diarrhea and vomiting is encountered. The possibility of shigellemia should be considered not only in malnourished/immunocompromised children but also in well-nourished/immunocompetent patients presenting with acute febrile gastroenteritis. The patient should be aggressively treated as the disease may progress very rapidly. Further, antibiotic susceptibility profile of the isolate should be kept in mind while treating such patients especially when the patient does not respond even after 48 hours of empirical antibiotic treatment, which could be life-saving.

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CASE REPORT

Isolation of *Comamonas* Species from the Stool Samples of Patients with Underlying Gastrointestinal Pathology

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Abstract

Comamonas species are saprophytes, thought to be rarely associated with clinical infections. We report five cases of *Comamonas* species associated with gastrointestinal (GI) manifestations isolated over a period of 2 years. All these patients had underlying GI pathologies like malignancy or inflammatory bowel disease. This report tends to introspect on the clinical significance of such rare pathogens in stool samples and emphasize its possible role in causing GI infections.

Keywords: *Comamonas aquatica*, *Comamonas testosteroni*, Gastrointestinal infection.

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Introduction

Comamonas are aerobic, oxidase-positive gram-negative bacilli, which belongs to the family *Comamonadaceae* in the *Pseudomonas* rRNA homology group III.[1] They emerged as a separate genus in 1985 with a single species *C. terrigena*. Later in 1987, two more species, *P. testosteroni* and *P. acidovorans* were reclassified into this genus. *C. acidovorans* were later reclassified as *Delftia acidovorans*.[1] *C. terrigena* initially comprised of three DNA hybridization groups which was later described as three distinct species, namely *C. terrigena*, *C. aquatica*, and *C. kerstersii*.[2] *Comamonas* spp. are generally present in natural habitats such as soil, water, and plants. They were considered non-pathogenic saprophytes until 1987, after which a few case reports emerged, where *Comamonas* spp. were isolated from various clinical samples. Most commonly, they were associated with intra-abdominal infections. The most common species reported are *C. testosteroni* followed by *C. kerstersii*.[3] Here, we describe five cases of isolation of *Comamonas* spp. (four of *C. aquatica* and one of *C. testosteroni*) from stool samples of patients with gastrointestinal (GI) manifestations over a period of 2 years (January 2019–December 2020).

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Source of support: Nil

Conflict of interest: None

Case Descriptions

Case 1

A 60-year-old man presented with complaints of passage of watery stool along with hematochezia. Stool frequency was 5–7 times per day and associated with crampy abdominal pain. There was a history of significant weight loss over the past 4 months. On per abdominal examination, a mass of approximately 5 × 4 cm was felt in the left iliac fossa. On examination, vitals and other systems were within normal limits. His routine blood investigation showed mild anemia and a slight elevation of total leukocyte count. A stool sample was sent for microscopy and culture to rule out any GI infection. There were no pus cells, red blood cells (RBCs), or parasitic elements on microscopy. Stool culture was done on two selective media, MacConkey agar, and Xylose Lysine Deoxycholate agar (XLD) and an enrichment media-Selenite F, which was further subcultured onto the above mentioned selective media after 16–18 hours. On direct plating, pale non-lactose-fermenting colonies were noted on the MacConkey agar (Fig. 1), which were oxidase-positive and red-colored colonies on XLD. The colonies were identified using conventional biochemical tests and automated method MALDI TOF MS (VITEK MS V3/KB V3.2.0, bioMerieux, Marcy L'Etoile, France). The biochemical reactions were as follows. Indole was not produced, citrate was not utilized, urea was not hydrolyzed, Kligler iron agar showed alkaline slant and alkaline butt without gas or hydrogen

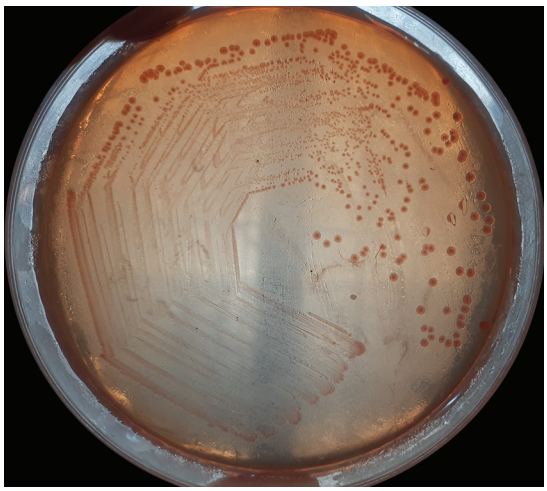

Fig. 1: Pale non-lactose-fermenting colonies of *Comamonas* spp. on MacConkey agar

sulfide. MALDI TOF MS identified the colonies as *C. aquatica* with a 99.9% confidence level.

Antimicrobial susceptibility testing was performed as per the CLSI 2020 using *Pseudomonas aeruginosa* as the reference standard. It was found susceptible to ampicillin, cotrimoxazole, ceftriaxone, piperacillin-tazobactam, cefoperazone sulbactam, and meropenem and resistant to ciprofloxacin. The patient was treated with ampicillin for 5 days. Ultrasound of abdomen and pelvis revealed cholelithiasis and bilateral medical renal disease. Sigmoidoscopy of the patient showed ulceroproliferative growth at the rectosigmoid junction. CECT of the abdomen revealed a circumferential wall thickening in the mid sigmoid colon with a serosal breach involving the posterior wall. Biopsy from the growth was suggestive of villous adenoma with low-grade dysplasia. The patient underwent robotic-assisted anterior resection, intraoperative, and postoperative period was uneventful. The patient was discharged in stable condition, tolerating a normal diet and advised for follow-up.

Case 2

A 62-year-old man with 10 years history of diabetes being managed on oral hypoglycemic agents presented with complaints of vomiting, loose stool, abdominal distension, and intolerance for solid and semisolid food for the past 3 weeks. He also noted the passage of dark colored stool for the past 3 weeks. On examination, the abdomen was soft, nontender, with no palpable mass or organomegaly and shifting dullness was present. Vitals and examination of other systems were within normal limits. His blood investigations revealed a low hemoglobin count of 6.8 g/dL, with a slightly elevated total leukocyte count of 16,080 mm^3 and decreased albumin level of 2.4 g/dL. Ultrasound of abdomen revealed massive ascites with septations and omental thickening with a few nodules. In upper GI endoscopy, circumferential growth in the gastroesophageal junction extending to the body of the stomach was noted. Multiple biopsies were taken, which revealed the evidence of moderately differentiated adenocarcinoma of the gastroesophageal junction. Ascitic fluid cytology was also positive for malignant cells. A stool sample was sent for microscopy and culture. There were no pus cells, RBCs, or parasitic elements on microscopy. Stool culture and identification was performed as described in case 1, and the primary culture yielded *C.aquatica*. The isolate was susceptible to ampicillin, ceftriaxone, ciprofloxacin, cotrimoxazole, cefoperazone sulbactam, and meropenem. The patient was given injection ciprofloxacin 200 mg IV 12 hourly and injection metronidazole 500 mg IV 8 hourly. He was planned for best supportive care and chemotherapy with oral capecitabine and follow-up in a regional cancer center. At the time of discharge, the patient was stable, tolerating oral feeds, and vomiting subsided.

Case 3

A 43-year-old woman, known case of ulcerative colitis on irregular medications, came with complaints of increased stool frequency, 8–10 episodes per day, associated with blood in stool, urgency, tenesmus, and pain abdomen for the past 1 month. On examination, vitals and all systems were within normal limits. Routine blood investigations showed mild anemia (Hb: 9.8 g/dL, MCH: 24.9 pg, MCHC: 30 g/dL) and elevated ESR (110 mm/hour). Stool culture was sent to rule out any associated infection. Pus cells and RBCs were present in microscopy. Culture yielded *C. aquatica* susceptible to ampicillin, ceftriaxone, cotrimoxazole, cefoperazone sulbactam, meropenem, and resistant to ciprofloxacin. The patient was treated with oral cotrimoxazole for 3 days. Colonoscopy findings were suggestive of severe flare of ulcerative colitis. Therefore, the patient was initially started on tablet mesalamine and mesalamine enema, which was subsequently changed to sulfasalazine due to evidence of acute sacroiliitis. The patient improved symptomatically and was discharged in stable condition.

Case 4

A 65-year-old man presented with complaints of fecal incontinence and pain during defecation for the past 8 months. On general examination, bilateral inguinal lymph nodes were palpable and vitals were stable. A circumferential growth of 4 × 4 cm, firm in consistency was felt on per rectal examination, which did not bleed on touch. Other systems examinations were within normal limits. A stool sample was sent for microscopy and culture to rule out any infection. Pus cells and RBCs were present on microscopy, and *C. testosteroni* was isolated from the primary stool culture, which was susceptible to ampicillin, ceftriaxone, cotrimoxazole, cefoperazone, sulbactam, and meropenem but resistant to ciprofloxacin. The patient was treated with injection ampicillin for 5 days. MRI scan of pelvis and screening of upper abdomen revealed a mass involving the proximal two-third portion of the rectum, rectosigmoid junction and distal sigmoid colon along with florid regional lymph nodes and multiple discrete regional lymph nodes on either side. A solitary gallstone was also detected. Biopsy findings were suggestive of well-differentiated adenocarcinoma of the rectum. A whole-body FDG-PET scan revealed mesorectal lymph node metastasis. The patient was managed with neoadjuvant chemotherapy and radiotherapy.

Case 5

A 62-year-old man presented with complaints of passing loose stool for the past 2 months, 6–7 episodes per day, occasionally associated with blood in the stool. On examination, vitals, all systems, and baseline laboratory parameters were within normal limits. A provisional diagnosis of inflammatory bowel disease was considered. On chest X-ray, there was bilateral upper lobe consolidation, CECT of the thorax revealed bilateral apical lobe cavities. The patient was worked up to diagnose pulmonary tuberculosis/ileocecal tuberculosis. The sputum acid-fast smear and the cartridge-based nucleic acid amplification test for *Mycobacterium tuberculosis* turned out to be negative. A stool sample was sent for microscopy and culture. Pus cells were present on microscopy, and *C. aquatica* was isolated from the primary stool culture susceptible to ampicillin, ceftriaxone, cefoperazone-sulbactam, and meropenem but resistant to ciprofloxacin and cotrimoxazole. The patient was treated with ampicillin for 5 days, following which diarrhea subsided. The patient is under further evaluation for his respiratory pathology.

The clinical details and laboratory findings of all the patients have been summarized in Table 1.

Discussion

Comamonas spp. are nonfermenting bacteria, rarely associated with clinical infections. The majority of the cases reported till now includes intra-abdominal infections and sepsis.[4] Most of these had been associated with predisposing appendicular perforation.[3] There are a few reports which describe the isolation of *Comamonas* spp. from the stool sample. *C. testosteroni* was isolated from the stool sample of an elderly with a history of colostomy and presented with watery diarrhea.[5] Biswas et al. reported the isolation of *C. kerstersii*

Table 1: Clinical and laboratory findings of the cases

Cases	*Age/gender*	*Clinical features*	*Coexisting conditions*	*Stool microscopy*	*Organism isolated in culture*	*Antimicrobial susceptibility*						
						A	*Ci*	*Cfs*	*Pt*	*M*	*Cot*	*Cf*
1	60/Male	Loose stool, hematochezia	Villous adenoma of rectum, gallstones	No pus cells, RBC's or parasitic elements	*C. aquatica*	S	S	S	S	S	S	R
2	65/Male	Vomiting, loose stool, abdominal distension	Adenocarcinoma stomach, Diabetes mellitus	No pus cells, RBC's or parasitic elements	*C. aquatica*	S	S	S	S	S	S	S
3	43/Female	Increased frequency of stool, blood in stool, tenesmus and pain abdomen	Ulcerative colitis	Pus cells and RBC's present, no parasiticelements	*C. aquatica*	S	S	S	S	S	S	R
4	66/Male	Fecal incontinence, pain while defecation	Adenocarcinoma rectum, gallstones	Pus cells and RBC's present, no parasitic elements	*C. testosteroni*	S	S	S	S	S	S	R
5	62/Male	Loose stool, occasionally associated with blood	Ileocaecal tuberculosis	Pus cells present, no parasitic elements	*C. aquatica*	S	S	S	S	S	R	R

A, ampicillin; Ci, ceftriaxone; Cfs, cefoperazone-sulbactam; Pt, piperacillin tazobactam; M, meropenem; Cot, cotrimoxazole; Cf, ciprofloxacin; S, susceptible; R, resistant

from 27 stool samples from patients who presented with diarrhea over a period of 2 years. There are several reports of isolation of *C. testosteroni* from cases of bacteremia, septic shock, appendicitis, catheter-related infections, infective endocarditis, etc.[6,7]

The reports of isolation of *C. aqautica* from clinical samples are very scanty. After a thorough literature search, no other account of the isolation of *C. aquatica* from the stool sample was found. Interestingly, four out of five of our isolates were identified as *C. aquatica*. There are a few reports of sepsis caused by *Comamonas* spp. where GI colonization due to environmental exposure to contaminated water was thought to be the probable source.[1,8,9] *C. testosteroni* was isolated from the blood of a 12-month-old child who presented with acute gastroenteritis, which later progressed into sepsis.[10] *C. kerstersii* was isolated along with *Bacteroides fragilis* from the blood sample of an elderly patient with sudden onset of fever, diarrhea, and vomiting, which later progressed to sepsis. An abdominal computed tomography scan revealed diverticulosis, and the patient also had a history of drinking water from the river. All these factors prompted the authors to think of gut as the source of infectiont.[8] *C. aquatica* has been reported from the blood sample of a patient with septic shock, whose initial presentation was with diarrhea and fever. The patient was a known diabetic and had a history of ischemic heart disease and removal of the sigmoid polyp.[4] Although the organism was not isolated from the stool, the potential of this organism to translocate from the gut to cause systemic infection has to be considered, owing to the initial presentation with diarrhea and the coexisting conditions of the patient.[4] They are also known to be colonizing hospital devices such as intravenous lines, respiratory equipment, and humidifiers. But most commonly, they are associated with community-acquired than nosocomial infections.[1]

Although they are considered to be low-virulent organisms, pan-genome and core genome analysis of various species of *Comamonas* have revealed that they possess diverse virulence mechanisms such as factors for adherence, antiphagocytosis factors, motility systems, and metabolic enzymes for adaptation.[11] They possess several advanced environmental sensors and signaling systems, which enables them to persist in the environment and form biofilms.[11] All the five patients in our cases were from rural areas, and no specific history of environmental exposure to contaminated water could be elicited. Most of the previous cases reported were associated with some other comorbidity or immunosuppression such as malignancy, chronic liver disease, HIV, diabetes mellitus, etc. Three of the patients in our case were associated with GI malignancy in which one of them also had diabetes, and one had inflammatory bowel disease. Since all had underlying GI pathology, it is difficult to determine whether *Comamonas* spp. were responsible for the clinical symptoms or were primarily due to the underlying pathology. Nevertheless, *Comamonas* spp. could be contributing to any of these clinical features, which needs further exploration. But as it was isolated as the predominant pathogen compared to the normal gut microbial flora in all the five cases, and considering the previous reports of association with disease, one cannot ignore its presence. Two of our patients had ultrasonographic evidence of gallstones. A similar observation was noted in another study, where they considered gallbladder to be a potential source of harboring this organism since *Comamonas* spp. have the genes for the utilization of aromatic and short-chain fatty acids as the source of carbon.[5]

In many of the previous reports, *Comamonas* spp. were found to be susceptible to aminoglycosides, cephalosporins, cotrimoxazole, ciprofloxacin, etc., and the majority recovered with appropriate antibiotic therapy.[6,7] In our case, except for one isolate of *C. aquatica*, all others were found to be resistant to ciprofloxacin. Some workers have reported similar observations.[4,5,9] But whether antimicrobial agents are required for the treatment is obscure.

Conclusion

Inadequate phenotypic methods for the identification of *Comamonas* spp. could be a reason for its underreporting. There is a chance that *Comamonas* spp. could be misidentified as *Pseudomonas* spp. using the routine, conventional biochemical reactions. Although the advent of automated identification systems has increased the detection rate, species-level identification is still a challenge as many of the automated systems had a limited

number of *Comamonas* spp. in their databases.[12] There is a high chance of ignoring such pathogens as commensals, especially from stool samples.

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