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Hepatitis B virus-related liver diseases: Identity and evidence-based control strategy

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Hepatitis B virus (HBV), a member of Hepadnaviridae family, represents a small virus of about 3200 bp and infects human and higher primates. Although the virus is hepatotropic in nature, it has been detected in many organs and tissues including blood of infected human. HBV infection is universal in nature and about 2 billion people of the world have been infected with the HBV at some point of their life. Among them, considerable numbers of HBV-infected subjects have developed protective antibody to HBV (antibody to hepatitis B surface antigen [anti-HBs]) and are immune from future HBV challenge. On the other hand, several million HBV-infected subjects express antibody to hepatitis B core antigen (anti-HBc) without expressing replication product of HBV (hepatitis B surface antigen [HBsAg]). However, these inactive HBV carriers may enter to active HBV replication cycle due to alteration of life style or several other causes. Finally, about 240 to 370 million people allow active HBV replication and express HBsAg and HBV DNA in the sera. Among these chronic HBV carriers, several millions develop chronic hepatitis B (CHB), liver cirrhosis (LC) and hepatocellular carcinoma (HCC). If these epidemiological evidences are compiled, it is evident that primary, mid-point and ultimately fate of HBV infection is not only diverse but also poorly understood. More importantly, the kinetics of HBV infection and liver-damaging properties and potentials of HBV could not be explained by HBV-related factors. Neither the viral load (HBV concentrations) nor the expression levels of HBV-related antigens (HBsAg, HBeAg) or antibodies (anti-HBe, anti-HBc), nor HBV genotype or viral mutation could be accounted for genesis of protection, in active HBV-carrier state, chronic HBV-carriers, and pathogenesis of patients with CHB, LC and HCC (natural course of HBV infection).[1,2]

The liver damages induced in some HBV-infected patients seem to be inflicted by host immune system, the nature and property of which is yet to be completely clarified by future investigations. It seems that when an appropriate cellular and humoral immunity is induced after HBV infection that leads to development of protective immunity from future HBV challenge. However, if the host immunity is impaired or distorted in HBV-infected patients, the patients start to allow HBV replication and subsequently immune-mediated damages and destruction of hepatocytes and progression of hepatic fibrosis leading to pathogenesis of CHB, LC and possibly HCC.[3] These observations have been supported by the fact that HBV is a non-cytopathic virus and it is unable to induce direct damage and death of any hepatocyte or other tissues. This scenario is more complicated by the fact that there are various forms of HBV in infected human, such as replicating HBV, covalently closed circular DNA (cccDNA) and extra-hepatic HBV DNA.

Under these realities, some new insights are required to manage patients with CHB, LC, and HCC because commercially-available antiviral drugs are capable of controlling only replicating HBV DNA, but not cccDNA. Their role on extra-hepatic HBV DNA is yet to be analyzed. At the same time, these antiviral drugs should be used for prolonged period or even for life and their long-term usage may be related to emergence of mutation and some of these may be life-threatening as well. Although a new and novel therapeutic approach for CHB may be restoration of host immunity in

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appropriate manner, the design and nature of therapeutic immunity in CHB patients is under investigation. It has been found that non-antigen specific immune modulators may not be a proper therapeutic choice for these patients.\(^4\) Rather, HBV antigen-specific immune therapy may be worthy and different designs of HBV antigen-specific immune therapies are on trial at clinical levels.\(^5,6\)

HBV, a terrorist virus of our time, needs to be properly dissected so that millions of HBV-infected patients may be rescued from more time-consuming complications. The good information about HBV is the presence of a prophylactic vaccine, but its limitation should also be borne in mind. Thus, simultaneous development of more potent prophylactic vaccine and innovative therapies remain the challenge of our time to tackle HBV.

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Non-antibiotic management for Clostridium difficile infection

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ABSTRACT

Clostridium difficile infection (CDI) is a key cause of diarrheal illness due to outbreaks by the hyper-virulent C. difficile NAP1,027 strain. The mainstay and time-honored antibiotic therapies for the management of CDI apart from killing C. difficile, also disturbs the standard healthy gut flora leading to dysbiosis. Mismanagement of antibiotics has led to a widespread increase in antibiotic resistance which has jeopardized the efficacy of antibiotics. This article has shed some light on the present-day vista of non-antibiotic approaches to combat CDI which include bacteriotherapy (fecal microbiota transplant, probiotics, non-toxigenic C. difficile spores), immunoglobulin therapy (monoclonal antibodies, polyclonal antibodies, bovine antibodies, whey protein concentrate, colostrum, C. difficile vaccine), photodynamic therapy and other miscellaneous therapies like the use of adsorbents, prebiotics and corticosteroids.

Keywords: Bacteriotherapy, immunoglobulin therapy, photodynamic therapy

INTRODUCTION

Clostridium difficile, generally acquired by the feco-oral route, is an important cause of gastrointestinal (GI) illness occurring as a complication of therapy by antibiotics, chemotherapeutics or other drugs that alter the gut microbiota in hospitalized patients. The spectrum of C. difficile infection (CDI) ranges from mild diarrhea, infectious colitis or pseudomembranous colitis (PMC) and toxic megacolon. This illness is responsible for a high burden on the health-care management.

The two first line antibiotics – metronidazole and vancomycin – are most often used as antibiotics of choice for the treatment of CDI. Metronidazole is generally used for patients with initial episodes of mild to moderate CDI whereas vancomycin is reserved for patients with serious illness as it hardly has any side effect and is not absorbed by the intestine. However because of its high cost as also the risk of development of vancomycin-resistant enterococci, its routine use is discouraged. Both the drugs have unacceptably high rates (15-35%) of recurrence of infection. In May 2011, fidaxomicin, a new antibiotic, was approved for CDI by the US Food and Drug Administration (FDA). All these three antibiotics are generally used to treat most patients with CDI. The leading risk factor linked with CDI development is prior antibiotic use. Antibiotics disturb the normal healthy intestinal microflora causing dysbiosis and may thus provide an opportunity to C. difficile to cause CDI. Furthermore, the overuse and misuse of antibiotics has led to a widespread increase in antibiotic resistance which jeopardizes the efficacy of the antibiotics.

Due to the increase in CDI incidence, rise in the rate of recurrence, greater mortality and morbidity, appearance of hypervirulent C. difficile strains, and a widespread increase in antibiotic resistance, there is a strong need to prospect novel treatment paradigms and non-antibiotic ways to manage CDI. During the past few years, numerous non-antibiotic approaches have
been developed for the management of CDI with each having its own pros and cons. This review is an attempt to streamline all the non-antibiotic strategies involved in the management of CDI.

**Non-antibiotic management for CDI**

Several non-antibiotic strategies are available and some have been put into practice to manage CDI. The important ones are listed below:

1. **Probiotics, Prebiotics and Bacteriocins:** Several different probiotics have been used oft and again to treat CDI. They are Lactobacillus rhamnosus GG, L. acidophilus, Bacillus clausii, Enterococcus faecium SF68, Bifidobacterium longum, Clostridium butyricum, Saccharomyces boulardii and even nontoxigenic C. difficile. CDI in mice was well resolved by the cocktail of six species including Porphyromonadaceae, Lachnospiraceae, Lactobacillus, Coriobacteriaceae, Staphylococcus and Enterococcus isolates.\(^8\) C. difficile colonization resistance is an attribute of multiple microbial assemblies interacting in a context-dependent manner and not conferred by a single microorganism. Increased C. difficile colonization were reported with Escherichia and Streptococcus isolates whereas Porphyromonadaceae, Lachnospiraceae, Lactobacillus, and Alistipes isolates were protective.\(^9\) Colonization resistance in recurrent CDI was successfully restored by a more diverse consortium of bacterial species (n=33) including Lactobacillus, Porphyromonadaceae, Ruminococcaceae, Lachnospiraceae and Eubacteriaceae isolates with diarrheal resolution by six months of treatment.\(^10\) Enache et al\(^11\) examined in four studies the combination of probiotic with either vancomycin or metronidazole for treatment of initial episode or recurrence of CDI in adults. It is believed that probiotics merely aid in restoring the microorganisms milieu within the intestine. Again it should be used with caution as in a case-series, use of S. cerevisae led to invasive fungemia.\(^11\) Moreover the use of probiotics is not recommended in elderly, immunocompromised patients with increased colonic permeability due to CDI. Ollech et al\(^12\) recently discussed the recommendations of probiotics, relying on evidence gathered from in-vitro laboratory and pre-clinical studies, and observed that probiotics are efficacious at preventing initial cases of CDI and also for its recurrences. However, because of inadequate substantiation, more research and large controlled clinical trials are required to use probiotic therapy by itself or in combination with antibiotic therapy for C. difficile colitis.

Oligofructose and inulin are prebiotics used as adjunct therapy to CDI which help to promote the growth of beneficial gut flora.\(^13\) With combination of oligofructose with either vancomycin or metronidazole, fewer CDI relapse was reported by Lewis et al.\(^14\)

Some gut bacteria can also produce bacteriocins that can act as bactericidal or bacteriostatic agent. Rea et al\(^15\) reported one such C. difficile inhibiting bacteriocin “Thuricin CD” produced by Bacillus thuringiensis DPC 6431. This bacteriocin was shown to be effective against a large number of clinically significant C. difficile isolates, together with Bl/NAP1,027-type strains while also having the least impact on the indigenous microbiota.\(^15\) The same group of researchers later on in another experiment demonstrated that rectal administration of thuricin CD was more effective against C. difficile than oral gavage of the same due to lower bioavailability.\(^16\) Another contractile R-type bacteriocin from C. difficile strain CD4 was genetically modified to diffocins (a.k.a. Avidocin-CDs) which was found to be effective against all 16 tested Bl/NAP1,027 strains of C. difficile and also harmless to the host microbiota.\(^17\)

2. **Fecal microbiota transplantation:** As CDI is an antibiotic-associated disease, treatment with antibiotic is best avoided, and replacement of the lost normal microflora with bacteria obtained from a healthy donor is required. Fecal microbiota transplantation (FMT) is another such method apart from probiotic therapy that serves the purpose. It involves the transplantation of stool from healthy individual to the patients’ large intestine via enemas.\(^18\) It has been efficaciously used to treat diarrhea for over 50 years even before C. difficile was documented as the chief cause of PMC. Eiseman et al\(^19\) in their study reported four patients with PMC and after successful treatment with fecal enemas the authors showed vivid resolution of...
diarrhea over 24-48 h post-treatment. Many methods have been used so far for stool delivery to patients which includes endoscopy/gastroscopy or via nasogastric tube for upper GI tract passage into the duodenum and via colonoscopy, rectal tube or enema for infusion into the lower GI tract. Fresh stool samples are used for FMT preferably within six hours but not later than 24 h. The volume of stool taken should be 50 g suspended in about 500 ml of fluid. Ideally 5–300 g of stool in volume ranging from 25 to 960 ml has been transplanted by investigators. Lower volume was used when delivered via the upper GI tract, but increased when delivered by colonoscopies to the lower GI tract.

Castro et al recently observed that antibiotics were not any longer needed after the transplantation of fecal microbiota. This was in accordance with the findings of Ganc et al where a 60-year-old woman suffering with PMC received sequentially oral vancomycin and metronidazole followed by a course of intravenous (IV) meropenem and oral metronidazole. Despite this her diarrhea still continued, till she received FMT from two different donors. Recently, Asonuma et al also reported successful FMT in a 49-year-old woman diagnosed with PMC and not responding to antibiotics. Diarrhea disappeared over three days of post-treatment and resolution of pseudomembranes was revealed after four days by colonoscopy and within the first year after discharge, recurrences were not reported.

FMT was used as a rational and relatively simple alternative approach to combat high recurrence rates of CDI. Nood et al reported 81% success rate for recurrent CDI treatment by duodenal infusion of donor stools compared to only 31% by vancomycin therapy. A cure rate of 93% was achieved when stool was taken from two healthy donors and used for 27 patients in a study performed at McMaster University, Canada. In the remaining 7% the lack of retention of enema led to unsuccessful FMT.

Human feces had been regulated as drug by the US FDA in May 2013. In February 2014, a gastroenterologist and co-founder of the stool bank OpenBiome, a biological engineering Professor and a PhD candidate from Massachusetts Institute of Technology recommended that human stool should be regarded as a tissue and not as a drug for medical use. Though very large unfavorable effects have not been reported with FMT so far, some risks and limitations do definitely exist viz. screening of suitable donor, threat of introducing opportunistic pathogens, patient preparation and various discomforts like short-lived abdominal uneasiness and bloating. By and large risks can be reduced by obtaining the stools from a donor of close physical association to the recipient, be it a spouse or another family member. FMT is still considered successful for CDI and recurrent CDI on the basis of recent clinical trials. Growing evidences show that FMT has hit the mark and is considered as a suitable method for restoration of microflora and competent non-antibiotic management of CDI. More investigations and clinical trials will help to establish the most efficient methods of gut microbiota restoration.

3. Non-toxigenic Clostridium difficile spores: C. difficile strains like M3 (VP20621) do not have genes for toxin production and thus are non-toxigenic C. difficile (NTCD). These strains are widespread in the hospital environment without evidence of CDI, thereby suggesting its asymptomatic carriage among patients. Oral administration of spores of a single NTCD-M3 has been found as an unconventional, alternative biotherapeutic approach to FMT. NTCD strains were selected for their high frequency of isolation from colonized patients and were identified using restriction endonuclease analysis typing. A phase II, randomized, double-blind, placebo-controlled study was performed among 173 CDI patients with a successful treatment of oral vancomycin, metronidazole or both. Patients were given one of four random treatments comprising of oral liquid formulation of NTCD-M3, 10⁶ spores/d for 7 days, 10⁷ spores/d for 7 days or 10⁸ spores/d for 14 days or placebo for 14 days. Among 168 patients, 157 completed the treatment. In 69% of patients receiving NTCD-M3, 63% fecal colonization was reported with 10⁷ spores/d and 71% with 10⁸ spores/d. Amongst patients receiving placebo 30% showed recurrence of CDI compared to only 11% of
patients receiving NTCD-M3; lowest recurrence was seen in 5% of patients receiving $10^7$ spores/d for 7 days. CDI recurrence was seen in only 2% of patients who were colonized, but in 31% who were not colonized, but received NTCD-M3. The study shows that NTCD-M3 colonization in the GI tract considerably reduces CDI recurrence. In another study, successful colonization of the same strains, in volunteers aged 60 years or older was reported when doses ranging from $10^4$ to $10^5$ spores per day were given for 14 days. To disturb the normal microbiota and stimulate CDI treatment, vancomycin was given for 5 days. In an earlier study it was shown that colonization with NTCD was found to be effective against toxigenic CDI in hamsters also. The mechanism by which NTCD colonization prevents CDI and its recurrence is unknown, though it is assumed that NTCD strains exploits the same colonization niche as that of toxigenic strains to damage their inhabitation.

4. Adsorbents: Cholestyramine and colestipol – the ion exchange resins – have been used time and again as an adjunct therapy to CDI as they attach to C. difficile toxins in the gut lumen before they bind to the intestinal epithelial cells to produce illness. Sequences of oligosaccharide bound to inert silica based support (Synsorb 90) work as a bait toxin receptor. Many successful cases have been reported with cholestyramine in pediatric and adult patients with numerous relapses and did not respond to conventional treatments. Another high molecular weight styrene sulfonate polymer (tolevamer) binds non-covalently to C. difficile toxins to block their activity has also been used as CDI therapy. The active ingredients of tolevamer are poly 4-styrenesulfonate containing either 100% sodium (tolevamer sodium) or a combination of 63% sodium and 37% potassium (tolevamer potassium-sodium) as counter ions. In a multicenter phase II trial Louie et al examined three and six gram doses of tolevamer for 14 days with vancomycin (500 mg x 10 days) in CDI patients. They observed that the six gram tolevamer dose was non-inferior to vancomycin for treatment of mild to moderate CDI. But hypokalemia was one of the side effects of tolevamer therapy. A phase III trial was conducted comparing vancomycin and metronidazole with a reformulated higher dose of tolevamer which included potassium. This trial showed that tolevamer did not appear to be non-inferior to vancomycin, but recurrent CDI was not common with tolevamer indicating that flora sparing drugs could possibly reduce recurrences.

DAV132 is a novel medicinal product which is adsorbent-based and has been reported recently for the treatment of CDI. This enteric-coated formulated activated-charcoal based product delivers the adsorbent to the ileum and neutralizes the drug compounds and antibiotic residues in the proximal part of the intestine before the latter can make a significant change in the microbiota. Three clinical trials of DAV132 have been performed successfully.

5. Immunoglobulin therapy

(i) Monoclonal antibodies: Lowy et al in a phase II randomized, double-blind, placebo-controlled trial investigated the effects of monoclonal antibodies on the extent and severity of the initial occurrence of CDI as well as on the length of hospital stay. A single infusion (10 mg/kg body weight) of the human monoclonal antibodies comprising of CDA1 and CDB1 against both the C. difficile toxins was administered to 101 patients getting one of the two antibiotics viz. metronidazole or vancomycin and to 99 volunteers receiving a placebo. The collective infusion of these two monoclonal antibodies along with antibiotics drastically reduced the CDI recurrence. Humanized monoclonal antibodies (HuMAbs) were found effective against both toxin A (HuMAb CDA1) and toxin B (MDX-1388). HuMAb CDA1 was found safe and well-tolerated in doses between 0.3 and 20 mg/kg. HuMAb CDA1 protected hamsters from mortality when used alone.
and more effective results were seen when synergized with a combination therapy.\cite{53}

Another set of HuMAbs – actoxumab and bezlotoxumab – that target toxin A and toxin B respectively, were found effective against several clinically relevant C. difficile strains and many BI/NAP1.027 and BK/NAP7.078 strain isolates, at antibody concentrations below the plasma levels as seen in humans.\cite{51} A 73% reduction in the rates of CDI recurrence has been reported in phase II clinical trials when these monoclonal antibodies were given with either vancomycin or metronidazole.\cite{48} These antibodies were found effective against C. difficile associated inflammatory response as well as damage to the gut wall, in murine CDI models together with mice challenged with a hypervirulent ribotype 027 strain.\cite{52} Two phase III randomized controlled trials (MODIFY I and MODIFY II; MODIFY: Monoclonal Antibodies for C. difficile Therapy) demonstrated the effectiveness of standard care antibiotics in addition to either actoxumab, bezlotoxumab, both monoclonal antibodies together or placebo. Monoclonal antibody against toxin B was found as an effective adjunct against recurrent CDI in the study.\cite{53} Bezlotoxumab alone was shown to work better than the combination of actoxumab and bezlotoxumab for prevention of CDI.\cite{54}

(ii) Polydonal antibodies: The presence of low serum antibody to C. difficile toxin A is a major risk factor for CDI.\cite{55,56} Polyclonal ovine antibodies have been found effective against C. difficile. They react with an epitope each of N-terminal (1-957), mid-region (958-1831) or C-terminal (1832-2710) domain of toxin. Antibodies can be raised in sheep by introducing a C. difficile toxin and the toxicity of immunogen is reduced by recombinant or chemical method. Recombinant method discriminately turns off the active site of C. difficile toxin by deletion or mutation, like alteration of aspartates and/or other residues to alanine, or by modifying the DXD motif in the N-terminal domain of the toxin and chemically modifying by treatment with UDP-dialdehyde, glutaraldehyde, formaldehyde etc.\cite{57}

In recent years IV immunoglobulins (IVIg) have been found beneficial in treating CDI patients.\cite{58} Initially Leung et al\cite{59} reported the use of IVIg (400 mg/kg once every three weeks) in five pediatric patients having recurrent CDI leading to complete resolution of symptoms. Abougergi et al\cite{60} reported treatment with IVIg in severe CDI patients (n=21) having pancolitis or ileus. The total IVIg dose administered over a period of 1-3 days ranged from 200 mg/kg to 1250 mg/kg. Complete clinical resolution was observed in nine patients within 2-20 days. The remaining 12 patients did not survive during the hospital stay, showing that the advantage of the use of IVIg was based on the level of systemic involvement. It has been suggested that IVIg therapy should only be administered when the albumin status deteriorates. However additional studies are required to standardize the timing for administration of IVIg, the right dosage and the right selection of patients, before this kind of treatment can be fully accepted as one of the CDI management strategies. Oral immunoglobulin obtained from eggs of immunized leghorn hens were found to have neutralizing effects against toxins A and B in a hamster model.\cite{60} Colonization factor-specific egg yolk antibodies (IgY) derived from chickens immunized with recombinant C. difficile colonization factors were found effective against C. difficile strain in hamsters and can be used as a treatment measure against acute and recurrent CDI in humans.\cite{61}

(iii) Bovine antibodies and whey protein concentrate:

Whey protein concentrate against C. difficile (anti-CD-WPC) is prepared from the milk of cows immunized against C. difficile to produce immunoglobulins (predominantly slgA and smaller amounts of IgG and IgM). The immunoglobulin slgA neutralizes in vitro the cytotoxicity of toxins and also protects hamsters against the otherwise lethal cecitis due to C. difficile.\cite{62} Many studies have reported the efficacy of anti-CD-WPC against CDI.\cite{63,64} In a double-blind randomized study, anti-CD-WPC was observed to be non-inferior to metronidazole in the prevention of CDI recurrences with a sustained recovery of 56% and 55% respectively.\cite{65} Van et al\cite{66} found that immune whey protein concentrate-40 when administered after a course of standard antibiotics helps in the prevention of relapse of CDI. In a prospective study
on 77 CDI patients, anti-CD WPC was found safe and effective against the illness.\textsuperscript{[64]}

(iv) Colostrum: Human colostrum is efficiently active against \textit{C. difficile}. It nullifies the \textit{C. difficile} toxin activity and therefore protects the newborns from these toxins\textsuperscript{[65,66]}. “Immune milk” has also been developed from bovine colostrums.\textsuperscript{[67]} Specific antigens and pathogens are used for the immunization of animals like cows to produce colostrum which has a large concentration of antibodies against specific pathogens.\textsuperscript{[68]} Some animal models like cows, when vaccinated during gestation, produce hyperimmune bovine colostrum (HBC) that is rich in IgG. It has been shown that cows immunized against \textit{C. difficile} resisted digestion and inactivation in the human intestine.\textsuperscript{[69]} Patients were also able to inactivate the toxins A and B of \textit{C. difficile} after oral consumption of the same bovine immunoglobulin concentrate.\textsuperscript{[70]} Artiushin \textit{et al.}\textsuperscript{[70]} also had shown that the colostrum blocks the cytopathic activity of \textit{C. difficile} toxins. In their experiment, pregnant mares were immunized with recombinant binding domains of toxin A and toxin B of \textit{C. difficile} and toxin neutralizing antibodies were passed to the newborn in colostrums. Anti-\textit{C. difficile} HBC was found to prevent in \textit{vitro} binding of \textit{C. difficile} to enterocyte-like Caco-2 cells.\textsuperscript{[71]} Sponseller \textit{et al.}\textsuperscript{[71]} demonstrated that HBC does not alter the gut microbiota and mild or no diarrhea was developed in piglets that were treated with HBC either in liquid or lyophilized forms.

(v) \textit{C. difficile} vaccine: A 50 times increase in serum antitoxin A production was reported by Aboudala \textit{et al.}\textsuperscript{[72]} when a vaccine prepared from culture filtrate toxoids A and B were administered intramuscularly to 30 volunteers. Thus \textit{C. difficile} toxoid vaccine was found to be safe and immunogenic in healthy volunteers. Souigiotzis \textit{et al.}\textsuperscript{[73]} showed an increase in serum IgG to toxin A in 2/3 patients with recurrent CDI. No additional recurrence was observed when vancomycin treatment was discontinued. Suggesting that inoculation of \textit{C. difficile} vaccine could help in the prevention and treatment of CDI. Another vaccine containing DNA which encodes the receptor binding domain (RBD) of \textit{C. difficile} toxin A has been developed and found to be immunogenic in \textit{vivo} and well-expressed in \textit{vitro}.\textsuperscript{[74]} Passive immunization with alpaca-derived polyclonal sera or immunization with toxoid was found effective against CDI in mouse.\textsuperscript{[75]} Recombinant lipoprotein-based vaccine candidates eliciting antibodies by C-terminal receptor binding domain of TcdA (A-rRBD) were found to neutralize TcdA toxicity in Vero cell cytotoxicity assays in mice, rabbits and hamsters.\textsuperscript{[76]} Insignificant protection (10-20\%) against \textit{C. difficile} spores was seen in hamsters but on formulation of rlipoA-RBD with B-rRBD protection was enhanced up to almost 100\%. The authors believe that this could be an excellent vaccine candidate for future clinical trials and preclinical studies.\textsuperscript{[76]} CDI vaccines currently in clinical development are “Sanofi Pasteur \textit{C. difficile} toxoid vaccine” with antigen formalin-inactivated toxins A and B from VPI 10463 in phase III clinical trial, “Valneva Austria GmbH VLA84 \textit{C. difficile} vaccine” with two other candidates in phase II clinical trial – recombinant fusion protein of toxin A and B binding regions and a genetically modified and chemically treated recombinant vaccine “\textit{Pfizer} 3-dose \textit{C. difficile} vaccine.”\textsuperscript{[77]}

6. Photodynamic therapy: The successful use of Photodynamic Antimicrobial Chemotherapy (PACT), a novel approach for CDI treatment has been reported in literature.\textsuperscript{[77,78]} PACT comprises of locally applied visible light and photosensitiser which is a light sensitive dye\textsuperscript{[77,78]} that targets microbial cells which produce reactive oxygen species containing free radicals and/or singlet oxygen. These dyes when combined with visible light in the presence of oxygen, eradicate the microorganisms. Target cells are inactivated by two oxidative mechanisms. Comprising electron/hydrogen transfer reactions from the photosensitiser excited state and then the production of singlet oxygen from molecular oxygen by energy transfer from the long-lived triplet state.\textsuperscript{[79,80]} Numerous photosensitizers have been studied for their use in photodynamic therapy. Nile blue derivative with a benzophenothiazinium dye structure known as EtNBS, a photosensitiser, was found to eliminate \textit{C. difficile} via oxygen-independent photodynamic therapy without damaging the host intestine and methylene blue active in the presence of oxygen.\textsuperscript{[18]} PACT was found to be an efficient method for killing most
hypervirulent C. difficile strains. Sordi et al. reported 3/13 photosensitizers capable of killing 99.9% of C. difficile in both planktonic and biofilm state after exposure to red laser light (0.2 J/cm²) with no harmful effects on model colon cells. C. difficile spore germination was induced by bile salt taurocholate, followed by PACT to demonstrate the applicability of PACT to eradicate C. difficile germinative spores. The efficacy of these photosensitizers is not restricted to certain genotypes since they were found effective against five extra recent C. difficile clinical isolates of different ribotypes. They found that non-cytotoxic photosensitizers were significantly more bactericidal against C. difficile both in aerobic and anaerobic conditions in vitro thereby making them as good candidates for in vivo investigations as well.

7. **Corticosteroid treatment:** The role of corticosteroid as therapy for CDI has been contentious. In a case report by Cavagnaro et al. a 5-year-old child with C. difficile-associated bloody diarrhea and PMC, was unresponsive to standard C. difficile antibiotics despite two weeks of therapy. Upon treatment with IV methylprednisolone (1 mg/kg twice daily) resolution of diarrhea occurred within 24 hours. The exact mechanism of action is not known. Corticosteroids obstruct phospholipase activity, which prevents arachidonic acid release from membranes, thereby decreasing eicosanoid production. The enhancement of colonic water and sodium absorption help in decreasing diarrhea.[84] Chang et al. observed that binding of the C. difficile toxin to human erythrocyte lysate was inhibited by number of sterols. Therefore it might have an effect on the binding of C. difficile to human colonic epithelial cells even though they are usually not used as therapy for C. difficile colitis. These observations suggested corticosteroids as a useful therapy for C. difficile induced colitis which does not respond to standard treatment. Wojciechowski et al. in a retrospective study found that corticosteroids tend to reduce the incidence of CDI. However more studies are required to authenticate the efficacy of corticosteroids and to establish a clear association between the therapy and CDI.

**CONCLUSION**

Even though antibiotics are the key options for CDI, due to their several disadvantages, alternative strategies are required for CDI management. This review provides an overview and evidence of non-antibiotic therapies for the treatment of CDI. Since researchers are exploring all avenues to find better and more effective efficacies to combat C. difficile infections and various clinical trials are hitherto under way so modifications to the discussed treatment stratagem are likely.

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**ABSTRACT**

*Helicobacter pylori* is an organism that is a worldwide cause of significant morbidity and mortality. There has been a sea change in our understanding and hence diagnosis and treatment of this ubiquitous bacterium over the last few years and more is in the offing. Though it still affects over half the world’s population, there has been an identification of genes and epigenetic motifs which can modify disease expression and cancer occurrence with *Helicobacter* infection. Newer diagnostic modalities like urine antibody analysis, immune-chromatographic culture methods, pepsinogen assays and micro-RNA detection promise earlier identification of more virulent forms. Advances in endoscopy have also incorporated Chromo-endoscopy, Narrow Band Imaging, Confocal endomicroscopy and Raman spectroscopy for diagnosis of *H.* pylori infections with greater accuracy. Advent of genotype drug resistance assays and newer therapeutic regimens have afforded greater efficacy in eradicating this infection. An interesting area of research is novel drug delivery systems, like the gastro-retentive systems, which have increased efficacy of existing drugs against *Helicobacter*. Vaccine development is also underway with ongoing animal trials on EPIVAC vaccine among others, showing some benefits. Though there is still a long way to go, all these newer modalities hold out hope for the possibility of a reduction in the burden associated with this widespread infection.

**Keywords:** Diagnostic modalities, Endoscopy, *Helicobacter pylori*, Treatment strategies

*Helicobacter pylori* is a bacterium that has transcended centuries, unchanged and thriving. From its first discovery in 1982 by Warren and Marshall, there has been a slow but steady change in the management and diagnosis of the infection. It still continues to colonize more than half of the world's populace and more than 60% of India's population.[1]

Most of the people with this infection develop some form of the disease. The degree of the manifestation differs from person-to-person depending on the host susceptibility genes, virulence factors and environmental factors. The disease manifests as chronic gastritis after infection via the feco-oral route. The bacterium is an obligate pathogen of the human stomach causing a form of superficial gastritis. The cytotoxin associated gene A (cagA) is an important inductor of inflammation and is an important marker for complications. There is marked variability in the manifestation of *H. pylori*, mainly due to the variations in the epigenetic EPIYA C motifs.[2] The presence of two or more of these motifs predisposes to a greater risk of atrophic gastritis and gastric carcinoma. The disease caused by the organism ranging from atrophic gastritis to gastric cancer is a continuous spectrum of the manifestations. The disease merits such global importance, because of its association with gastric cancer. Seventy five percent of all gastric lymphomas and all gastric MALTomas are attributable to *H. pylori* infection.[3]
An important recent discovery in the pathogenesis of \textit{H. pylori} is the FOXD3 mRNA and gene. The mRNA of the gene acts as a promoter for a tumor suppressor gene. It has been found that patients with intestinal metaplasia and, more so, those with gastric cancer have a lower FOXD3 mRNA level in their blood.\[^{[4]}\] This may be a future diagnostic or therapeutic target. One important point to note in the pathogenesis of \textit{H. pylori} gastric cancer is that the extent, severity and the atrophy correlates with the presence of gastric cancer and is almost always preceded by a period of chronic gastritis where diagnosis and intervention can be yielding and preventive.

There is a cohort of patients in whom \textit{H. pylori} detection and eradication should be actively pursued. According to the Maastricht IV guidelines,\[^{[5]}\] this population includes – first degree relatives of gastric cancer patients, past carcinoma of stomach, severe pangastritis or atrophy, chronic acid inhibition therapy (>1 year), environmental risk factors like smoking and if the patient fears carcinoma of the stomach. There have been reports of association of the infection with many extraintestinal manifestations. To name a few, they are coronary artery disease, iron deficiency anemia, Idiopathic Thrombocytopenic Purpura, B12 deficiency, Alzheimer's disease, Parkinsonism, migraine, alopecia, urticaria, Raynaud's phenomenon, Sjogren's autoimmune thyroiditis and hyperemesis gravidarum.\[^{[6]}\]

There have been a few diagnostic modalities in use for \textit{H. pylori} over the last few decades, each with its own set of advantages and disadvantages. All, except IgG serology, require stopping of acid suppressants for at least 2 weeks prior to application of the method. The current strategy advocated is to “Test and Treat” which means, to treat only those patients who test positive for \textit{H. pylori}.\[^{[3]}\] This precludes any empirical therapy. After \textit{H. pylori} eradication, current consensus is to test a select cohort for eradication. That cohort includes patients with persistent symptoms after four weeks, associated ulcers, MALTomas and early gastric cancer.\[^{[7]}\] The investigative techniques in use traditionally include rapid urease test, urea breath test, stool and urine antigen tests, histopathology and culture.

With rapidly changing technology, newer tests are now either being used, or are in the pipeline. Immunohistochemistry based techniques of staining improve sensitivity and specificity of biopsy specimens. Urine antibody tests are a new method which can be done either by the immunoassay or the immuno-chromatographic method.\[^{[8]}\] Serum Cag A antibodies have been found to correlate with gastric cancer in a meta analysis, though its significance in India has not been found to be as much.\[^{[9]}\] Serum pepsinogen assays have been called “serological biopsy” and the ratio of Pepsinogen I/II is a new marker for presence of gastric atrophy.\[^{[10]}\] The ratio is lower in the presence of \textit{H. pylori}. The Japanese classify their patients into four groups on the basis of \textit{H. pylori} and Pepsinogen status. The risk of gastric cancer varies in each group, with group D (\textit{H. pylori} negative but Pepsinogen positive) denoting the highest risk of gastric cancer.\[^{[11]}\] This screening tool cannot be used in India as the Pepsinogen levels are lower and, hence, fallacious.\[^{[12]}\] Serum miRNA levels can also be used for detecting early gastric cancer in \textit{H. pylori} infection, namely the miR–187, miR–371-5p and miR–378. There is also interest in the CYP2C19 polymorphism, which though does not aid in diagnosis, has important therapeutic implications as the patients with the said polymorphism are resistant to eradication therapies which are based on Omeprazole, Lansoprazole or Levofloxacain.

With the focus on newer endoscopic techniques in all areas of gastroenterology, really, \textit{Helicobacter} detection cannot be left behind. Endoscopy is basically a surrogate for definitive tissue diagnosis of \textit{Helicobacter}, but with newer techniques, that line has become blurred. Presence of a non bleeding duodenal ulcer itself has more than 90% positive predictive value for \textit{H. pylori} presence. Presence of antral nodularity(chicken skin appearance) has a specificity of 96% for \textit{Helicobacter} but is only 32% sensitive. Conversely, presence of star fish like appearance of the gastric mucosa, also known as RAC (regular arrangement of collecting venules) has a high negative predictive value for \textit{H. pylori} infection.\[^{[13]}\] Extent of chromoendoscopic staining with 0.1% phenol red solution also correlates with urea breath test and \textit{H. pylori} density on histology.\[^{[14]}\] The use of magnifying endoscopy for \textit{H. pylori} has also been recently validated. Yagi et al\[^{[10]}\] classified the appearance of \textit{H. pylori} infected gastric mucosa visible on magnifying endoscopy into 4 patterns, - Z0, Z1, Z2 and Z3. The Z0 pattern has 93.8%
sensitivity and a 96.2% specificity for predicting normal gastric mucosa. Z1, Z2 and Z3 correspond with \textit{H. pylori} infection.\cite{15} Narrow band imaging endoscopy has also classified \textit{H. pylori} infections into three types depending on pit pattern and vessel architecture with around 95% sensitivity and 82% specificity.\cite{16} Infrared Raman Spectroscopy has a near 100% specificity at 1542 cm$^{-1}$ frequency for detecting \textit{H. pylori} infection as a result of differential Porphyrin concentration.\cite{17} Confocal Laser Endomicroscopy can also be used to detect \textit{H. pylori} infection by visualizing organisms, neutrophils and microabscesses.

Not just diagnostic protocols but treatment strategies are also showing a slow but steady change. Various therapeutic regimes are now in use including the Sequential, Concomitant, probiotic based therapies and Hybrid regimen in addition to the standard Quadruple and Bismuth based therapies. The standard triple regimen uses 10 to 14 days of proton pump inhibitor along with clarithromycin and amoxicillin. The sequential therapy employs the use of 5 to 7 days of simultaneous PPI and amoxicillin followed by 5 to 7 days of PPI with clarithromycin and metronidazole. The concomitant therapy has treatment with 10 to 14 days of simultaneous PPI, clarithromycin, amoxicillin and metronidazole. The probiotic based regimen adds a probiotic to the standard triple regimen. The hybrid regimen is similar to the sequential therapy with the difference of continuing amoxicillin with clarithromycin and metronidazole even in the second half of the course of therapy.

When evaluated for side effects and overall efficacy, it was found in a recent meta-analysis, that the 14 day sequential regimen has overall good tolerance and efficacy, and is a good compromise on both while neither being the most efficacious, nor the best tolerated.\cite{18} With rising antimicrobial drug resistance, there is a need to evaluate the bacterium for the presence of the same. Conventional anti microbial susceptibility testing techniques depend on culture and is cumbersome as well as time consuming. An alternative to culture techniques are molecular assays which, though expensive and research based at present, may be useful adjuncts to therapy in the near future. The GenoType HelicoDR assay determines resistance patterns to Clarithromycin and quinolones.\cite{19} The sensitivity for Clarithromycin resistance is nearly 100% while being around 85% for quinolones.

With the development of advances in imaging, diagnostic and treatment methods, drug delivery systems are an interesting area of development. Gastroretentive drug delivery systems increase the time of contact of drugs against \textit{H. pylori} as the drugs are site specific.\cite{20} Floating systems cause the drug to float on the stomach contents till they are passed out. A disadvantage of this system is that the drug is not available specifically at the intended site.\cite{21} Bio-adhesive systems adhere to the mucosa and offer promising results in increasing drug contact and efficacy with a disadvantage of being dependent on gastric emptying and turnover. Dual systems, in development, combine the advantages of both systems and may be the drug delivery system of the future, improving the efficacy of antibacterial therapy.

While all these measures aim to diagnose and treat \textit{H. pylori} infection, there is a need for thrust in the development of a vaccine to prevent the disease all together. The three parts of a vaccine are the antigen, the adjuvant and the vector system. Various adjuvants have been tried but animal tests show the highest efficacy for alkyl hydroperoxide reductase and alum based vaccines.\cite{22} Attenuated \textit{Salmonella} and Poliovirus have been evaluated as vector delivery systems. The antigen for immunogenicity showing best outcomes is EPIVAC, a fusion protein of CD4+ T cell epitopes from HpA, UreB amd Cag A antigens. All these have shown some degree of effectiveness in animal studies and human studies are pending. A recombinant urease antigen vaccine is currently undergoing human studies. The problem with developing a vaccine for \textit{H. pylori} is the fact that it is an excellent parasite because it behaves more like a commensal. All human vaccination trials have never shown vaccine induced clearance. The question facing researchers at the moment is the right choice of antigen and adjuvant for the development of the vaccine.

\textit{H. pylori} is an infection that has spanned centuries, unchanged in virulence yet adapting for survival. With all the new armaments in our arsenal, it seems the time may have come to finally banish, if not eradicate this scourge of humanity.
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Antibiotic resistance has been an emerging concern for common bacterial infections worldwide. *Helicobacter pylori*, commonly associated with chronic bacterial infections, is also included in bacteria with drug resistant problems. Its infection, once considered curable, is now becoming a matter of grave concern with rising antibiotic resistant patterns reported worldwide. Resistance is mainly reported to the key antibiotics in the treatment of infection i.e. metronidazole and clarithromycin, and to a lesser extent to amoxicillin and tetracyclin, thereby decreasing the cure rates of the combination therapies used. Recently resistance to quinolones has also been reported.

**INTRODUCTION**

*Helicobacter pylori* is a fastidious Gram negative bacillus, found under the mucus layer on the epithelium of the gastric antrum of patients with chronic disease. Chronic infections caused by the organism include chronic gastritis, gastric and duodenal ulcers, mucosa associated lymphoid tumors (MALT) and adenocarcinoma of stomach. The bacterium was incidentally discovered by Marshall and Warren in 1983, and later in 2005 they were awarded the Nobel Prize for the discovery of *H. pylori.*[1] The organism is microaerophilic in nature and requires gaseous mixture of CO₂/O₂/N₂ in a ratio of 10:5:85% for growth, with high level of humidity. Culture media also require supplementation with 10% debrinated horse blood and selective antibiotic supplement containing vancomycin (10 mg/L), cefsulodin (5 mg/L), trimethoprim (5 mg/L) and amphotericin B (5 mg/L). The culture plates need incubation for 3-5 days at 37°C before considering the culture as negative. Biopsy is the most common specimen used for the culture of organisms. As proton pump inhibitors (PPIs) or antimicrobials inhibit the growth of *H. pylori*, their use reduces the chances of successful culture. Therefore patients should avoid taking PPIs for at least two weeks and antimicrobials for at least four weeks prior to endoscopy.[2] Biopsy specimens should be transported and processed for culture as soon as possible, ideally within six hours. *H. pylori* is further identified based on colony characteristics identified (circular, convex and translucent) in media, cellular morphology (Gram-negative and helix-shaped under the microscope) and positive biochemical tests (urease, catalase and oxidase).

This article is compiled by reviewing various studies to sum up the prevalence of *H. pylori* and the resistant pattern of antibiotics used as combination therapies in order to formulate an opinion regarding treatment of infection in areas with low and high prevalence of *H. pylori*.

**Prevalence and pathogenesis**

Prevalence of *H. pylori* infection varies in different geographical regions and ranges from 40% in developed countries to 90% in developing nations.[3] Prevalence is reported the least in children, increasing with age and is seen the maximum in the elderly.[3] The prevalence of *H. pylori* infection in India is extremely high. Up to 70–90% of patients with duodenal ulcer and 50–80% of patients with dyspepsia or healthy asymptomatic adults harbor the bacteria.[44] The infection is acquired feco-orally; the bacterium resides under the layer of mucus of the gastric antrum epithelium and its habitat provides protection from the lethal action of gastric acid, thereby contributing in its pathogenesis. Most of the patients in high prevalence...
area with H. pylori are asymptomatic, whereas others are diagnosed with the infection during evaluation of dyspeptic symptoms. H. pylori infection may manifest as chronic gastritis, peptic ulcer disease, gastric neoplasms including adenocarcinoma and MALT. Besides its carcinogenic potential, the organism also has the potential to cause recurrent infections. H. pylori is also associated with iron deficiency anemia due to alterations in iron absorption and occult blood loss through the development of erosive esophagitis or peptic ulcer disease. It has also been suggested that H. pylori may utilize iron itself, further contributing for iron deficiency anemia.\(^7\)

Eradication of H. pylori is associated with healing of ulcers and prevention of recurrence of infection. It may also cure low grade MALT lymphoma and incidence of gastric carcinoma is also reduced as reported by a meta-analysis study.\(^8\) Chey et al\(^9\) reported 25% reduction in the incidence of gastric cancer and a 67.4% reduction in the development of peptic ulcer disease after treatment.

**Methods used for H. pylori detection**

Methods used for the detection of H. pylori are histology, rapid urease test (RUT), culture, polymerase chain reaction (PCR) and various serology tests.\(^9\) Histology was the first method used for the detection of H. pylori. Presence of typical bacteria along with inflammatory reaction was suggestive of H. pylori infection. Detection of urease production has been used as a surrogate marker of H. pylori infection, as this organism is a strong and rapid urease enzyme producer.\(^9\) The organism can be cultured but it is microaerophilic and requires complex media for growth. Culture of the organism is a tedious, time-consuming procedure, and unnecessary for routine diagnosis of H. pylori infection because other non-invasive tests can detect evidence of the organism in a majority of the patients.\(^9\) Advantage of the culture method is that it allows the testing of the antibiotic sensitivity of H. pylori.

Molecular methods are widely used for the detection of bacterium as well as the characterization of pathogenic genes and specific mutations associated with antimicrobial resistance. The conserved genes used for detection of H. pylori are urease operon: ureA\(^10\) and glmM, also known as ureC,\(^11\) or the 16S rRNA,\(^12,13\) 23S rRNA,\(^14,15\) and hsp60\(^16\) gene. There are many modifications of the PCR for increasing the sensitivity of detection e.g. nested or semi-nested PCR using internal primer targeting conserved gene (heat shock protein; Hsp60) which increases the specificity and sensitivity up to 100%\(^14,17\) (approximately 3 organisms) after the nested cycles of amplification.\(^14\) New methods like liquid phase DNA-enzyme immunoassay\(^18,19\) and the reverse dot blot line probe assay (LIPA)\(^20\) have also been proposed to increase its specificity and sensitivity. There are reports suggesting use of reverse transcription PCR (RT-PCR) which successfully shows the viability of the bacterium.\(^21,22\) The PCR technology may also be used to target pathogenic and resistant genes of H. pylori.

**Antibiotics and Resistance Patterns**

Several options are available to test the antimicrobial susceptibility testing of H. pylori. The disc diffusion method though cost-effective and widely used for antimicrobial susceptibility testing for most of the bacterial isolates, have not yet been defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for H. pylori.\(^23\) Both EUCAST and the British Society for Antimicrobial Chemotherapy recommend E-test strips, a culture-based antimicrobial susceptibility testing for H. pylori as it enables the quantitative determination of the minimum inhibitory concentration of an antimicrobial agent required to inhibit bacterial growth.\(^24\) Besides these phenotypic methods, molecular methods are reliable alternatives to culture as genetic identification of mutations in H. pylori directly from biopsy samples and culture material is rapid, thereby enabling to report the diagnosis the same-day. Molecular testing has been recommended to detect H. pylori genes and resistant genes in clarithromycin and quinolone. These mutations can be detected by a number of PCR based molecular methods. Several molecular testing kits are commercially available for the detection of clarithromycin resistance, including the MutaREAL H. pylori kit\(^25\) (Immunodagnostik; Bensheim, Germany), the ClariRes real-time PCR assay\(^26\) (Ingentix; Vienna Austria) and the Seeplex ClaR-H. pylori ACE detection system\(^27\) (Seogene; Eschborn, Germany). Accumulating evidence has demonstrated that the presence of mutations detected by molecular tests correlates well with culture-based susceptibility testing. A recently developed method i.e. GenoType HelicoDR assay (Hain Lifescience; Nehren, Germany) enables determination of resistance. This is highly accurate with a sensitivity and specificity of 94-100% and 88-99% respectively for clarithromycin resistance and 83-87% and 95-98.5% respectively for quinolone resistance.\(^28,29\) In spite of good sensitivity and specificity, the majority of molecular tests available do not detect resistance based on rare genetic mechanisms. Therefore phenotypic methods should be paired along to ensure...
Various treatment regimes including dual, triple and quadruple therapies are used for the eradication of H. pylori. Eight antibiotic drugs are available to treat H. pylori infection i.e. amoxicillin, tetracycline, clarithromycin, metronidazole, levofloxacin, tinidazole, furazolidine and rifabutin. Adjunct drugs i.e. PPI, H2-blockers and bismuth containing agents enhance the performance of antimicrobials.

One week triple therapies combining a PPI such as omeprazole and a low dose of two antibiotics (metronidazole or amoxicillin plus clarithromycin) are the overall most effective eradication regimens with eradication rates of about 90%. Though cure rate after treatment is up to 90%, a matter of concern is the rising antimicrobial resistance for mainly three antibiotics i.e. Clarithromycin, metronidazole and levofloxacin. These being the key antibiotics for treatment of H. pylori, they have reduced the success rate of treatment from 90 to 70% in the past decade. Antibiotic resistance is associated with both genetic alteration and biofilm formation.

Reports of H. pylori resistance have been published from different geographic regions worldwide with different ranges. Prevalence of clarithromycin, metronidazole and levofloxacin resistance is increasing over time with higher reports from developing countries than developed countries.

Clarithromycin is a second generation macrolide. Resistance to clarithromycin is the major reason for decrease in susceptibility as it is the most effective antibiotic of combination therapy against H. pylori. Studies from European countries have reported resistance in clarithromycin ranging from 31 to 36.7% (2012-13) which is much higher than that reported in 2008-09 (7% to 21%). Clarithromycin resistance has almost doubled during the past 10 years. Studies from India have reported varying results. Clarithromycin was reported as sensitive in all the strains tested from Kolkata and Delhi, although other studies have reported resistant strains. The resistant strains reported were 96% from Hyderabad, 91% from Mumbai, 58.8% from Gujarat and 10% from Chandigarh. Clarithromycin resistance is due to point mutations in 23S rRNA genes, different nucleotide positions involved are 2143 (A2143G) and 2144 (A2144G). About 90% of the cases of primary clarithromycin resistance in western countries are due to A2143G, A2142G and A2142C mutations.

Clarithromycin resistance is the major reason for decrease in susceptibility as it is the most effective antibiotic of combination therapy against H. pylori. Studies from European countries have reported resistance in clarithromycin ranging from 31 to 36.7% (2012-13) which is much higher than that reported in 2008-09 (7% to 21%). Clarithromycin resistance has almost doubled during the past 10 years. Studies from India have reported varying results. Clarithromycin was reported as sensitive in all the strains tested from Kolkata and Delhi, although other studies have reported resistant strains. The resistant strains reported were 96% from Hyderabad, 91% from Mumbai, 58.8% from Gujarat and 10% from Chandigarh.

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Mutation varies with geographical area. Resistance is most commonly due to the use of macrolides in treatment of respiratory tract infections. Mcmohan et al in his study demonstrated a statistical significant relationship between patients with previous use of antibiotics and subsequent isolation of resistant H. pylori strains. This study also includes metronidazole. Resistance to metronidazole has remained high in the past decade ranging from 35.5% to 45.5%. Resistance reported in Arctic countries ranges from 42 — 66%. Studies from India show comparatively higher resistance rate. A study from Kolkata has reported 85% resistant strains. High metronidazole resistance has also been reported from other states like Delhi, Lucknow and Mumbai. Pandya et al has reported 83.8% resistance in Gujarat, 37.5% in Delhi and 38.2% resistant strains from Chandigarh. A multicentric study from South India has reported 100% resistant strains from Hyderabad, 88.2% from Chennai and 68% from Lucknow.

Metronidazole resistance of H. pylori is closely related to the indiscriminate use of this antibiotic as it is easily available and cheap. It is used as an anti-parasitic, in the treatment of genital and dental infections, and also in anaerobic infections. Resistance varies geographically, corresponding to its use. A high rate of resistance up to 90%, is seen in African countries while it is around 95% to 100% in India and China, 45% in Spain, 22.5% in Norway and 3.3% to 4.9% in Japan. Varied resistance pattern for amoxicillin has also been reported from India and abroad. Mainly low prevalence is reported, except for a few studies from India were 72.5%, 73% and 80% resistance is reported from Gujarat, Mumbai and Hyderabad. All sensitive strains have been reported from Kolkata, North India (Lucknow, Delhi and Chandigarh) and south India (Chennai). Low amoxicillin resistance (0.7%) has been reported from Europe. Other studies have also indicated that the rates of resistance to amoxicillin are <1% in China, Bahrain, Malaysia, Bhutan and Vietnam but are >10% in Japan. Low prevalence was also reported in Southeast Asia, Latin America and Brazil. Thus the inclusion of this antibiotic in empirical eradication regimens can still be considered.

Quinolones generally are not effective antibiotics against H. pylori, but levofloxacin or ciprofloxacin could be given in combination with amoxicillin as the second line antibiotic in strains resistant to clarithromycin and metronidazole. Initially the eradication success using
new regimen containing levofloxacin was as high as 90%, but same studies have reported increasing quinolone resistance. [30-34] In Europe, levofloxacin resistance rates have reached 18% and 18.8% rates have been reported from Germany in 2013. [35,36] Resistance rates are low (8.8%) in Taiwan, but higher in Brazil (23%), Iran (72.5%) and India (50%). [37,38] Ciprofloxacin resistance was 1-8% in Western countries. [39,40] Reports from India also show low level resistance. Hyderabad and Mumbai reported zero level ciprofloxacin resistance but 20% resistance rate has been reported from Lucknow. [41,42] A multicentric study from north India reported 2.7% ciprofloxacin resistance. [43] Resistance again is due to the use of quinolones both in respiratory tract infections and urinary tract infections.

Tetracyclin resistance ranging from 1.9 to 6.0% has been reported in various studies. [44,45,46] In a multicentric study from India, tetracyclin resistance was 4% from Hyderabad strains whereas Mumbai and Lucknow reported 27% and 10% tetracyclin resistance respectively. [47,48]

Rapidly increasing prevalence of antibiotic resistance has called for an urgent need to think about newer antibiotics such as furazolidone. Triple therapy with furazolidone-based regimens cannot achieve ideal eradication rates therefore it cannot be considered as a suitable option for first-line treatment. Quadruple furazolidone-containing regimens including probiotic is recommended, which could achieve more than 90% eradication rates. [49] A study from Iran has reported resistance rate to furazolidone between 0 to 4.5%. [50] Datta et al. [51] has reported no resistant strain to this antibiotic from Kolkata, India. Till date no furazolidone resistance has been reported from any part of the world except 13.8% resistance from Gujarat and Mumbai, India. [52] This antibiotic is not used in developed countries due to its purported carcinogenic effect which is not proven yet. [53,54]

Multi drug resistant (MDR) strains have also been reported in various studies; Pandya et al. [55] have reported 85.1% MDR H. pylori strains resistant to metronidazole, clarithromycin, and tetracyclin. A multicentric study from north India reported 31.6% resistant strains for metronidazole, amoxyccillin and clarithromycin. [56] No MDR H. pylori strain has been isolated from Delhi. [57] In addition, 18.1% of the strains in a study from Turkey were found to harbor clarithromycin, metronidazole and levofloxacin resistance. [58]

Various attempts have been made to improve the efficacy of standard treatments in use, e.g adding bismuth, increasing the dose of PPI, prolonging treatment duration and introducing new antibiotics, but none proved to be fruitful. An attractive option was to introduce probiotics in standard regimes. Studies have reported variable results, but currently there is evidence to support the use of probiotic supplementation to standard triple therapy. [59,60] This benefit is particularly reported in patients with recurrent infection and those with history of antibiotic side effects. Further studies are ongoing to establish the evidence of probiotics for their significant benefit in the H. pylori infection.

Guidelines have been developed in the United States and Europe (areas with low prevalence) for the management of H. pylori infection. 'Test and treat' strategy is applied to those with dyspepsia, but these guidelines will not be appropriate in high prevalence areas like India. A group of international experts performed a targeted literature review and formulated an expert opinion for evidence-based benefits and harms for screening and treatment of H. pylori in high-prevalence countries. They concluded that in Arctic and Asian countries where H. pylori prevalence exceeds 60%, treatment of persons with H. pylori infection should be limited only to instances where there is strong evidence of direct benefit in reduction of morbidity and mortality, associated peptic ulcer disease and MALT lymphoma and that the test-and-treat strategy may not be beneficial for those with dyspepsia in high prevalent areas. [61]

To provide effective treatment, susceptibility testing of antibiotics is very important, but H. pylori being microaerophilic, its culture and susceptibility testing needs different laboratory set up which is difficult and costly in developing countries like India. Therefore surveillance studies on H. pylori resistance patterns at regional and national levels are recommended. Periodic review of reports with antimicrobial susceptibility pattern can at least provide general guidelines for effective and successful eradication of H. pylori.

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Opportunistic agents causing diarrhea in HIV

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ABSTRACT

Opportunistic infections (OIs) in Human Immunodeficiency Virus (HIV) infection continue to cause morbidity and mortality in HIV infected patients. Virtually all OIs occur when the CD4+T cell count is less than 200/mm$^3$. The gastrointestinal (GI) tract is a common site for clinical expression of HIV. Diarrhea is the most common GI symptom in HIV/AIDS. Prevalence ranges from 0.9% to 14%. Diarrhea may be the presenting symptom of lymphoma and Kaposi's sarcoma. It may affect up to 40–50% of those taking anti-retroviral therapy (ART) and can be induced by other medications. It may also be the result of HIV-associated enteropathy. The agents can be classified as bacterial, parasitic, viral and fungal agents. Almost all bacterial diarrheic agents can cause diarrhea in HIV infected patients, including Clostridium difficile. Intracellular parasites like Isospora belli, Cryptosporidium parvum and Cyclospora are the main causative agents of diarrhea in HIV positive patients. Cytomegalovirus is an AIDS defining opportunistic infection. Candida is the main fungal agent of diarrhea in AIDS. Despite the availability of ART, OIs are common in India as HIV-infected persons in India present with an OI as the initial indicator of their disease. Some of them are aware of their HIV infection but do not take ART and some patients are enrolled in HIV care and prescribed ART but do not attain an adequate virologic and immunologic cure. Thus awareness about optimal strategies for diagnosis, prevention and treatment of OIs in GI tract is essential to provide comprehensive, high quality care for these patients.

Keywords: Diarrhea, gastrointestinal tract, HIV/AIDS, opportunistic infections

INTRODUCTION

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS). HIV related opportunistic infections (OIs) continue to cause morbidity and mortality in HIV infected patients. Virtually all OIs happen when the CD4+T cell count is less than 200/mm$^3$ and subsequently respond to immune reconstitution facilitated by highly active antiretroviral therapy (HAART). The gastrointestinal (GI) tract is a common site for clinical expression of HIV. Progressive immunosuppression is linked to an increase in the prevalence of GI features. Abdominal pain and diarrhea are common in HIV disease.$^{[5]}$ Diarrhea is, however, the most common GI symptom in HIV/AIDS. Prevalence ranges from 0.9% to 14%. Significantly, prevalence is highest in homosexual men and individuals with lower CD4+ T counts. Diarrhea occurs in 30-60% of patients with AIDS in developed countries and in about 90% in developing countries.$^{[8]}$ Chronic diarrhea in tropical countries is concomitant with weight loss (slim disease) which according to World Health Organization together with a positive HIV-1 serology test is an AIDS defining norm.$^{[6]}$ Diarrhea in AIDS patient is multifactorial. The various agents causing diarrhea are listed in Table 1. Diarrhea is a common problem for people with HIV in both resource-poor and resource-rich settings, regardless of antiretroviral exposure. In the pre-HAART era, 30–70% of HIV-seropositive individuals experienced diarrhea.
and among European patients with CD4+ T counts <50 cells/µl, 49% would expect to develop diarrhea within 1 year and 96% within 3 years. In resource-poor areas, incidence and severity continue to be higher. Early clinical observations confirmed that diarrheal illness was linked to reduced quality of life and poorer survival. Diarrhea may be the presenting symptom of lymphoma and Kaposi’s sarcoma. It may affect up to 40–50% of those taking antiretroviral therapy (ART), it can be induced by other medications and may be the result of an incompletely defined direct effect of HIV on the gut mucosa termed HIV-associated enteropathy. AIDS enteropathy was described in 1984 in patients having diarrhea with no detectable pathogen.

(i) **Bacterial causes**

Various strains of the diarrheagenic E. coli are E. coli enterotoxigenic (ETEC), E. coli enterohemorrhagic (EHEC), E. coli enteroinvasive (EIEC), E. coli enteropathogenic (EPEC). In a study conducted in Nasik, E. coli was the most common bacterial pathogen isolated in HIV seropositive patients. Among the diarrheagenic E. coli isolated, enteropathogenic E. coli was the most common isolate. In HIV seropositive diarrheic patients, E. coli was the most common bacterial isolate (37; 53.6%) followed by Proteus species (14; 20.2%) and Pseudomonas species (10; 14.5%). Diarrhea in HIV patients with Shigella are at higher risk of developing gastroenteritis. The prevalence of Shigella species was reported 10.5% for HIV seropositive diarrheic individuals from Southern India. Salmonella was isolated 20 to 100 times more frequently in HIV positive patients. Nontyphoidal Salmonellae (NTS) and Campylobacter jejuni cause prolonged and invasive infection. Recurrent NTS is well thought of as an AIDS defining illness since 1985. Chances of diarrhea in AIDS patients is 39 times higher with C. jejuni. Diarrhea may be associated with disseminated infection with Mycobacterium avium

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Table 1

The infectious causes of diarrhea in HIV/AIDS

and enteropathogenic E. coli (EPEC). In a study conducted in Nasik, E. coli was the most common bacterial pathogen isolated in HIV seropositive patients. Among the diarrheagenic E. coli isolated, enteropathogenic E. coli was the most common isolate. In HIV seropositive diarrheic patients, E. coli was the most common bacterial isolate (37; 53.6%) followed by Proteus species (14; 20.2%) and Pseudomonas species (10; 14.5%). Diarrhea in HIV patients with Shigella are at higher risk of developing gastroenteritis. The prevalence of Shigella species was reported 10.5% for HIV seropositive diarrheic individuals from Southern India. Salmonella was isolated 20 to 100 times more frequently in HIV positive patients. Nontyphoidal Salmonellae (NTS) and Campylobacter jejuni cause prolonged and invasive infection. Recurrent NTS is well thought of as an AIDS defining illness since 1985. Chances of diarrhea in AIDS patients is 39 times higher with C. jejuni. Diarrhea may be associated with disseminated infection with Mycobacterium avium

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complex, especially in patients with CD4+ count less than 50 cells/mm³. Patients having diarrhea and taking antibiotics should be investigated for Clostridium difficile which is more common in patients having CD4+ count less than 50 cells/mm³. Invasive NTS was recognized early in the HIV epidemic to be strongly associated with immunosuppression in Western tropical settings, but there is no association between HIV and typhoid or paratyphoid. Patients with HIV and NTS infections present with febrile illness or sepsis syndromes and diarrhea may be absent or present as a less prominent feature. As in HIV negative individuals, other bacterial pathogens include C. difficile, Campylobacter species and Shigella species may cause diarrhea. C. difficile was the most common cause of diarrhea in a US cohort study and has been described in British and resource-poor settings. It has been implicated in over 50% of cases of acute diarrhea in studies spanning both the pre- and post-HAART eras. Bacteria causing gastroenteritis may cause bloody diarrhea and abdominal pain. Bacteremia is more common, but still unusual, in HIV-related campylobacter and Shigella infections. Presenting symptoms of C. difficile infection are similar to HIV-seronegative individuals. Case series show that C. difficile infection is no more severe in HIV-seropositive individuals though case reports of complications such as toxic megacolon and leukemoid reactions exist as in other populations. Sexually transmitted diarrheal pathogens are encountered in patients with multiple sexual partners or receptive anal sex.

Initial evaluation should include stool smears and cultures for enteric bacteria. The sample is also sent for C. difficile toxin in the setting of antibiotic usage. It is encouraged to send at least three stool specimens for ova and parasites and acid fast bacilli smear and tuberculosis cultures. However, it has been shown from previous studies that isolation of pathogens is more likely from watery than from formed stools.

Acute bacterial diarrhea in HIV-seropositive individuals with CD4+ counts >200 cells/µl usually does not require treatment, but should be treated when the CD4+ count is <200 cells/µl. Acute bacterial diarrhea should be treated as per susceptibility tests and local guidance. C. difficile infection should be treated with metronidazole and vancomycin reserved for severe, relapsing or metronidazole nonresponsive infection. Trimethoprim-sulfamethoxazole reduced the incidence of infectious diarrhea in the pre-HAART era. Retrospective studies suggest that introduction of antiretroviral therapy, including zidovudine monotherapy, has been more effective than targeted antimicrobial prophylaxis in preventing recurrence of NTS, and that duration of antimicrobial prophylaxis, with agents such as fluoroquinolones need not exceed 30 days in patients established on HAART. The incidence of bacterial diarrhea declined steadily after the introduction of HAART, therefore HAART is the mainstay of preventing bacterial diarrhea. Specific therapy is then directed to the enteric pathogen detected. Recurrent infections with Salmonella, Shigella, Campylobacter species.
Campylobacter and Isospora will require administration of alternating antibiotics.

Various algorithms have been proposed for the investigation and/or empirical management of chronic HIV-related diarrhea (three or more loose stools for 28 or more days) in Western and tropical settings. Parasitic causes are more likely in those with prolonged diarrhea, considerable weight loss and CD4+ count <100 cells/µl, and may coexist with cytomegalovirus (CMV), mycobacterial or other infections.

(ii) Parasitic causes

Parasitic OIs are divided into protozoa and helminths. Isospora belli, Cryptosporidium parvum, Cyclospora which are intracellular parasitic infections are main causative agents of diarrhea in HIV positive patients. In intestinal infections caused by parasites the main defence mechanism is cellular immunity. Thus in HIV positive patients with reduced CD4+ T lymphocytes, chances of intracellular parasitic infections are more. However, the extracellular parasitic infections caused by Entamoeba histolytica, Giardia intestinalis and Strongyloides stercoralis have also been found as causative agent of diarrhea in HIV positive patients.

C. parvum was first reported as human pathogen in 1975 by Nime. C. parvum, C. hominis and C. meleagridis are the important species of cryptosporidiosis causing life threatening gastroenteritis in immunocompromised host. As the CD4+ cell count falls below 200 cells/µl the chances of cryptosporidiosis increases. Mode of transmission of Cryptosporidium is feco-oral route. Infection is spread by drinking water contaminated with oocysts which are resistant to chlorine and being small in size difficult to filter. Ingestion of even 30 oocysts can cause infection. There are reports of infection having occurred with even one oocyst. Cryptosporidium usually resides in small intestine and is minimally invasive in immunocompetent host. The patient present with diarrhea, abdominal cramps, nausea, vomiting, weight loss and fever; these symptoms are self limiting. Whereas in immunocompromised host it can involve epithelial cells of biliary tract, pancreatic duct, stomach, esophagus and respiratory tract. The infection in this group of patient is severe and presents with malabsorption leading to wasting syndrome in HIV positive patients along with jaundice and pancreatitis. For diagnosis, three consecutive stool samples are examined. Highly refractile, spherical/oval oocyst measuring 4-5mm in diameter are seen in wet mount. Staining is done by modified acid-fast staining, auramine-rhodamine, acridine orange and immunofluorescent antibody (IFA). Other methods are detection of cryptosporidial antigen in the fecal sample by the enzyme-linked immunosorbent assay (ELISA). Polymerase chain reaction (PCR) can also be done and is more sensitive but requires training. The treatment options are limited. Food and Drug Administration has approved nitazoxanide for its treatment. Other pharmacological interventions include treatment with paromomycin and immunotherapy. Passive immunotherapy is done by oral administration of colostrum-derived bovine immunoglobulin. To decrease the incidence of this life threatening diarrheal disease it is very important to reduce its transmission. The chemical method effective for decontamination of water is ozone. Newer approaches are reverse osmosis, membrane filtration, electronic or radiation methods.

There is no specific treatment targeting cryptosporidium directly. Early HAART is imperative and is associated with complete resolution of infection following restoration of immune function. In individuals with profuse diarrhea, therapeutic drug monitoring may be required to confirm adequate absorption of antiretroviral agents.

A study combining paromomycin with azithromycin reported substantial reduction in stool frequency and volume, together with diminished oocyst shedding. Paromomycin was given orally as 500 mg four times daily or 1 g twice daily for up to 12 weeks. The dose of azithromycin was 500 mg daily. However the small numbers in this study and the limited experience of this combination preclude its choice as a front line therapy. Nitazoxanide has been approved for use in immunocompetent individuals but has not been shown to be superior to placebo in the severely immunocompromised. When an anti-cryptosporidial agent is chosen nitazoxanide is the preferred agent but its efficacy is limited in more immunocompromised patients.

Supportive therapy with IV fluid replacement/ antimotility agents is essential.
• First-line treatment for cryptosporidiosis is with effective ART (category III recommendation).

• Nitazoxanide is effective in adults and children who are not severely immunosuppressed (category II b recommendation).

The use of optimized HAART should be continued to prevent relapse. Standard drinking water chlorination techniques are not sufficient to eradicate the parasite. Specific filtration employing an ‘absolute’ 1-micron filter is required.[46] Bottled water is not necessarily a safer option. Boiling of water should be advocated.

Microsporidia are unicellular obligate intracellular organisms which were once thought to be parasites but now by molecular phylogeny are placed among fungus. Infective stage is spore form which is highly resistant due to thick double layered spore wall and its mode of infection is ingestion, inhalation, inoculation, rarely transplacental route and is linked to homosexual intercourse. Two most important species found in immunocompromised host are Encephalitozoon species and the species Enterocytozoon bieneusi. It is associated with advanced stage of immunosuppression where CD4+ count is less than 100/mm³.[47] Most common symptom is diarrhea leading to wasting. The infection spreads from intestinal tract to gall bladder, pancreatic and bile duct, respiratory system as well as the eye. For diagnosis, repetitive examination is advised because of its intermittent shedding. Various staining procedures done are Gram staining, acid fast staining, periodic acid Schiff (PAS) and Giemsa staining. These intracytoplasmic spores are Gram positive, acid fast and PAS positive. Electron microscopy is taken as gold standard. Other methods are carbon immunoassay, indirect IFA test, ELISA, counter immune electrophoresis and Western blotting.[34,39]

Some species cause GI disturbance, such as diarrhea and cholangitis, and other genera are associated with upper respiratory and ophthalmic infections. The microsporidia most commonly linked to GI illness are Enterocytozoon bieneusi and Encephalitozoon (formerly Septata) intestinalis. Gut infection is acquired by swallowing cysts, usually in water.[48] Pre-HAART studies showed variability in the prevalence of microsporidiosis (2–70%) in the immunosuppressed HIV population with diarrhea.[49] The incidence has decreased with the introduction of HAART.

Watery, non-bloody diarrhea, with associated malabsorption, is the commonest presentation of GI infection. Sclerosing cholangitis may occur. Examination of three stools with chromotrope and chemofluorescent stains is often sufficient for diagnosis. If stool samples are consistently negative, a small bowel biopsy should be performed.[46] Stains such as Giemsa, acid-fast or hematoxylin and eosin can be used to visualize microsporidia in biopsy specimens. In disseminated infections due to Encephalitozoon species, organisms may also be found in the deposit of spun urine samples. Electron microscopy remains the gold standard for confirmation and speciation.[50] PCR may be used to identify up to species level.

There is no specific treatment for microsporidial infection. Early HAART is imperative and associated with complete resolution of GI symptoms following restoration of immune function. Therapeutic drug monitoring may be required to confirm adequate absorption of antiretroviral agents. This agent is not currently widely available. Nitazoxanide, albendazole and itraconazole have also been studied. Of these agents, albendazole (400 mg twice daily for 21 days) is recommended for initial therapy, particularly for E.intestinalis (category III recommendation).[51] Optimized HAART should be used to maintain CD4+ cell counts and prevent relapse.

I. belli now known as Cystoisospora belli was discovered by Rudolf Virchow in 1860. In India the prevalence of C. belli ranges from 2.5% to 14% in HIV positive patients.[52] In immunocompetent hosts the infection is limited to diarrhea, headache, fever, abdominal pain, vomiting, dehydration and weight loss, whereas in immunocompromised hosts diarrhea is more persistent, watery, chronic and associated with extra intestinal manifestations. Mode of infection is by ingestion of food or water contaminated with oocyst.

To diagnose isospora in stool sample, cyst is demonstrated in wet mount or iodine mount. Staining methods which can be used are acid fast staining in which oocyst appear red in color or auramine-rhodamine staining. A highly sensitive and specific method for diagnosis is PCR. I. belli has no known animal host but is widespread geographically, causing self-limiting small bowel diarrhea in HIV-seronegative individuals. It is implicated in 10–20% of cases of chronic
HIV-related diarrhea in the tropics and is an occasional cause of biliary disease. Treatment traditionally has been with TMP-SMX 960 mg qid PO for 10 days though 960 mg bd appears also to be effective (category III recommendation) and secondary prophylaxis with the same antibiotic (960 mg three times a week) is essential as relapse is common and there is indirect and direct evidence for this.[53,54]

Cyclospora cayetanensis, a parasitic infection was first discovered in late 1880s. It is an important cause of traveller’s diarrhea and diarrhea in immunocompromised hosts. In immunocompetent hosts, diarrhea is self limiting. But in HIV positive patients and other immunocompromised conditions it can lead to severe intestinal injury, prolonged diarrhea and associated with sequel such as Guillain-Barré syndrome, reactive arthritis syndrome and acalculous cholecystitis.[55] Laboratory diagnosis is done by examination of wet smear, staining with modified acid fast staining and safranine.

The oocysts of _C. belli_ are large (20–33 μm x 10–19 μm) and ellipsoidal, whereas that of _C. parvum_ and _Cyclospora cayetanensis_ are spherical or oval, measuring 4–5 μm and 8–10 μm respectively. The oocysts of _I. belli_ require 3-4 days and that of _C. cayetanensis_ require 5-13 days to mature in environment. On the other hand oocyst of _C. parvum_ are infective as they are passed in feces.[56]

_C. cayetanensis_, a coccidian parasite of the small bowel is widespread throughout the tropics and has caused large outbreaks of food-borne illness in the USA in imported foods. It causes prolonged watery diarrhea that may last for months in patients with HIV, in whom biliary involvement has also been reported.[57]

The diagnosis involves the microscopic detection of oocysts but fluorescence microscopy and real-time PCR may be used where available. The clinical and parasitological response to standard doses of TMP-SMX (960 mg twice daily) is rapid and 7 days is usually sufficient.[58]

The other two parasitic causes of diarrhea are _E. histolytica_ and _G. intestinalis_. First discovered in 1875 by Fedor A. Lösch, infection with _E. histolytica_ can lead to amebic liver abscess and colitis. The complications can be ulcerative colitis with toxic dilatation.[59] For diagnosis liver abscess aspiration is useful as it is typical ‘anchovy sauce’ appearance. Trophozoites can be seen in wet mount preparation of stool sample. Entamoeba infection is most commonly seen in men who have sex with men.[60] Fever, abdominal pain and either watery or bloody diarrhea are the most frequent symptoms and amoebic colitis occurs at a range of CD4+ counts and is not limited to individuals with CD4+ T-cell counts <200 cells/μl. Hepatic abscesses are the commonest extra-intestinal manifestation. Diagnosis involves microscopy of at least three stool samples for the detection of trophozoites or cysts. Antigen detection or PCR of stool may also be performed and endoscopy with biopsy can aid diagnosis if stool analysis fails to confirm the diagnosis or diagnostic uncertainty remains. Serology can be employed but remains positive for years after exposure and therefore direct identification of Entamoeba is desirable. Extra-intestinal lesions are diagnosed in the appropriate clinical setting by imaging combined with serology. Treatment is most often with metronidazole although tinidazole may be used as an alternative. These agents are followed by diloxanidefuroate or paromomycin both administered for 10 days to eradicate luminal infection.

Giardiasis was first discovered by Van Leeuwenhoek in his own stool in 1600s. Mode of infection is ingestion of contaminated food and water with cyst which is resistant to many disinfectants. The infection is usually asymptomatic; sometimes associated with abdominal cramps, diarrhea, weight loss. Diagnosis is made by microscopic examination of stool for trophozoite and cyst. Giardiasis usually presents with chronic diarrhea with constitutional symptoms. GI symptoms include nausea, bloating, crampy abdominal pain, indigestion and belching. Prolonged diarrhea may result in a malabsorptive state. Giardia species, including _G. lamblia_, have no increased prevalence in HIV-infected patients, and the clinical presentation and diagnostic methods are also similar to HIV-seronegative patients.[61] It is well recognized that multiple stool tests obtained on different days may be required for diagnosis as intestinal shedding is sporadic. Giardiasis is treated with metronidazole. Alternatives include albendazole, paromomycin or nitazoxanide.[41]

(iii) Helminthic causes

Intestinal helminth infections are usually found
in HIV positive patients; though the prevalence in HIV negative patients is more or less the same. The diagnosis and treatment hence remains same in both the groups. Strongyloides stercoralis was first found in 1876 in GI tract of five soldiers on post mortem examination who were returning from Indochina borders and is called Cochin China diarrhea. It is one of the neglected tropical diseases (NTDs). In India the prevalence is found to be 6.6% in community based surveys and 11.2% in hospital based surveys. Laboratory diagnosis of strongyloidiasis involves demonstration of larvae in stool in the wet mount method. Sensitivity of three fecal specimens if screened is 70%. Other methods for diagnosing infection are ELISA, radioallergosorbent test for detection of specific IgE antibodies, indirect immunofluorescence test, complement fixation test, gelatin particle agglutination test and western blot assay. Despite anecdotal reports, there is no conclusive evidence that infection or hyperinfection is more common in patients with HIV, although it may be implicated in immune reconstitution syndromes.

Hook worm causes hypochromic microcytic anemia in many developing countries. It has been found that hookworm infestation was not common in HIV positive anemic adults. Another helminth causing intense pruritus ani; Enterobius vermicularis has been studied in HIV positive patients and significant correlation has been found. Trichuris trichiura is mostly accompanied by protein energy malnutrition and anemia which is also common in HIV positive patients, thus increasing the chances of infestations.

(iv) Viral causes

Viruses are the common cause of diarrhea in HIV positive patients. CMV is an AIDS defining opportunistic infection presenting with diarrhea, weight loss, fever and abdominal pain. Most common site of CMV enteritis is colon. CMV is a member of the herpes family of viruses, usually acquired during childhood. CMV infection remains dormant unless an individual becomes immunosuppressed, when reactivation of latent infection may occur. In the pre-HAART era, retinitis was the most common presentation of CMV, followed by GI disease. Since the advent of HAART, CMV infection may occasionally occur as part of immune reconstitution syndromes, but the overall incidence of CMV in individuals living with HIV has dramatically reduced. CMV may affect all sections of the gut. Endoscopy may reveal classical CMV ulceration of the gut mucosa and biopsy with histopathological review may identify characteristic intranuclear and intracytoplasmic ‘owl’s eye’ inclusions. The absence of ulceration makes a diagnosis of CMV colitis very unlikely.

The culture of CMV from biopsy material is not sufficient for the diagnosis of gut infection as immunosuppressed individuals may shed the virus without intestinal disease. First line treatment for CMV colitis is intravenous ganciclovir (5 mg/kg twice daily) for 14–28 days. Immediate optimization of HAART should be considered. CMV colitis has traditionally been treated with ganciclovir 5 mg/kg bd iv for 14–28 days. Caution should be used in initiating treatment with the oral medication valganciclovir as there is a theoretical concern of decreased absorption, but HIV and non-HIV-related cases of CMV colitis have been successfully treated. Intravenous foscarnet (90 mg/kg twice daily) for 14–28 days is used as an alternative. Therapeutic drug monitoring may be required to ensure adequate HAART absorption. Continuous use of effective HAART is required to prevent relapse.

Few studies of HIV-related diarrhea include investigation for viruses other than CMV and there is only anecdotal evidence of increased severity or frequency of most viruses associated with gastroenteritis in HIV, including noroviruses and rotavirus. There have been reports implicating coronavirus, which may coexist with bacterial pathogens in acute diarrhea, and adenovirus, which may coexist with CMV in patients with chronic diarrhea. Adenovirus is associated with persistent diarrhea and disseminated infection in AIDS involving brain, lung and liver.

Herpes simplex infections (HSV-2 and HSV-1) cause relapsing and severe proctocolitis and should be treated with aciclovir 400 mg five times daily PO or valaciclovir 1 g bd PO for 7–14 days, while severe infection may necessitate aciclovir iv 5 mg/kg tid for the initial part of therapy. Prophylaxis should be considered for recurrent disease. CMV colitis can present with acute diarrhea and is a major opportunistic infection of the GI tract.
(v) Fungal causes

Most common fungal cause of diarrhea in HIV positive patients is Candida species. The diarrhea is mostly antibiotic associated. Systemic dimorphic fungi can cause disseminated infections including diarrhea in immunocompromised individuals. Thus before the widespread use of potent combination antiretroviral therapy, OIs, which have been defined as infections that are more frequent or more severe because of immunosuppression in HIV-infected persons were the principal cause of morbidity and mortality in this population. In the early 1990s, the use of chemophrophylaxis, immunization, and better strategies for managing acute OIs contributed to improved quality of life and improved survival. Subsequently, the widespread use of potent ART has had the most profound influence on reducing OI-related mortality in HIV infected persons. Despite the availability of ART, OIs continue to cause considerable morbidity and mortality in India due to the following reasons: HIV-infected persons in India are unaware of their HIV infection status and many present with an OI as the initial indicator of their disease. Some individuals are aware of their HIV infection, but do not take ART due to psychosocial or economic factors and some patients are enrolled in HIV care and prescribed ART, but do not attain an adequate virologic and immunologic response due to inconsistent retention in care, poor adherence, unfavorable pharmacokinetics, or unexplained biologic factors. Thus OIs continue to cause substantial morbidity and mortality in HIV-infected persons. Clinicians must be aware of optimal strategies for diagnosis, prevention, and treatment of OIs to provide comprehensive, high quality care for these patients.

REFERENCES

1986;91:651-9


Human parvovirus B19 co-infection aggravates liver dysfunction in patients with chronic Hepatitis B infection

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¹Department of Virology, ²Department of Transfusion Medicine and ³Department of Research Institute of Liver & Biliary Sciences Vasant Kunj, New Delhi, India

ABSTRACT

Background and objectives: Human parvovirus B19 (B19) has been reported to be detected in the sera of patients with acute or chronic hepatitis. The aim of this study was to investigate the prevalence of parvovirus B19 in patients with chronic hepatitis B virus (CHB) and hepatitis C virus (CHC) infection and understand its clinical significance. Materials and methods: Plasma samples from 40 adult chronic hepatitis patients (20 CHB and 20 CHC) and 20 healthy blood donors were investigated for antibodies to B19 (IgM and IgG both). Active viremia was confirmed in IgM positive patients by real time PCR for parvovirus B19. Results: IgG antibodies to B19 were seen in 5 (25%) CHB, 10 (50%) CHC and 10 (50%) healthy controls. IgM antibodies to B19 were seen in 3 (15%) CHB, 5 (25%) CHC and 1 (5%) controls. The liver dysfunction was significantly higher in B19 co-infected CHB patients. The serum ALT levels (Median 416.0, IQR 64.0-496.6 IU/L) and AST levels (Median 325, IQR 61.00-380.00 IU/L) among B19 co-infected CHB patients were significantly higher than serum ALT levels (Median 43.0, IQR 33.0-61.5 IU/L) (p=0.023) and serum AST levels (Median 31 (26.50-53.00) IU/L) (p=0.013) in CHB mono-infected patients. However the difference in serum bilirubin levels was not significant amongst the two groups (p=0.25). No aggravation of liver dysfunction was seen in B19 co-infected CHC patients. Interpretation and conclusions: Parvovirus B19 is prevalent equally amongst HBV, HCV infected and healthy population. Co-infection of B19 with HCV did not increase the frequency of liver dysfunction but it definitely aggravates the liver dysfunction in CHB co-infected patients.

Keywords: Chronic hepatitis B infection, chronic hepatitis C infection, parvovirus B19 infection

INTRODUCTION

Human parvovirus B19 is a DNA virus and is the type member of the erythrovirus genus of family Paroviridae. Parvovirus B19 propagates mainly in the erythroid progenitor cells and is a known etiologic agent of erythema infectiosum (fifth disease) and fever with rash illness in pediatric age group.¹² The sero-prevalence is reported to be nearly 50% in adult population.¹³ Blood transfusion and close contact are the modalities of effective transmission of the virus. Following exposure to the virus, viremia appears early and lasts approximately 6–8 days. IgM antibodies appear first with 1-2 weeks time and last for 1-2 months. IgG antibodies appear after 2-3 weeks and may persist for several years; therefore their presence indicates a past exposure.¹⁴ The spectrum of liver involvement caused by parvovirus B19 includes transient
elevation of transaminases, acute hepatitis, fulminant liver failure and chronic hepatitis. Parvovirus B19 DNA and antibodies against it have been detected in patients with chronic hepatitis B (CHB) or chronic hepatitis C (CHC) virus infection. However the exact association of parvovirus B19 co-infection in CHB or CHC infected patients and its effect on liver functions is still controversial. A few studies have concluded that persistent parvovirus infection in chronic hepatitis directly correlates with the extent of liver involvement and the probability of progression to more severe hepatitis is significantly higher in such patients. On the other hand other studies have maintained that persistent parvovirus B19 infection does not cause worsening of liver functions in such patients.

Hepatitis B virus (HBV) infection is a serious global health problem, with two billion people infected worldwide, and 350 million suffering from CHB infection. Globally, HBV and hepatitis C virus (HCV) together are estimated to have led to 500 million chronically infected persons and one million deaths annually. In India, nearly 3-4% of the population is infected by HBV, and CHB constitutes more than 50% of the chronic hepatitis cases in the country. The population prevalence of HCV infection in India is 1% and constitutes nearly 40% cases of chronic hepatitis. Sero-prevalence of parvovirus B19 in healthy blood donors has been reported to be 27% to 40% in various studies in Indian population. However, there is no published data from India regarding the role parvovirus B19 co-infection with HBV/HCV in aggravating chronic hepatitis.

The aim of this study was to investigate the prevalence of parvovirus B19 in patients with CHB and CHC infection and understand its clinical significance

MATERIALS AND METHODS

Research question: The research question for this study was to document the prevalence of parvovirus B19 virus among HBV and HCV infected patients and to find out whether the presence of this virus affects the clinical course of chronic hepatitis.

Study design: It was a cross-sectional study design. It was a pilot study with 20 cases in each group.

Duration: The duration of this study was January 2014-January 2015.

Study subjects: Blood samples were collected from symptomatic hepatitis patients attending to the outpatient and inpatient services of our Institute and healthy blood donors. Diagnosis of CHB or CHC infection was based on the presence of hepatitis B surface antigen (HBsAg) or anti-HCV antibodies (anti-HCV) for > 6 months. All the patients were also biopsy confirmed CHB or CHC cases. Patients with chronic hepatitis other than CHB or CHC (e.g. alcoholic liver disease, autoimmune hepatitis, others) were excluded from the study.

A total of 60 subjects were included in the study: 20 CHB and 20 CHC and 20 healthy age matched blood donors.

Sample collection: Twenty milliliters of blood was collected in EDTA (Ethylene diamine tetra acetic acid) vials from each patient. Plasma was separated and kept at -80°C till further testing.

Parvovirus B19 antibody detection: IgM and IgG antibodies against parvovirus B19 were detected in the plasma samples using ELISA classic parvovirus B19 IgG/IgM kit (Serion Immunodiagnostica GmbH, Germany) according to the manufacturer’s instructions. The test results were interpreted according to the kit literature and samples were reported as reactive or non-reactive.

DNA extraction and polymerase chain reaction (PCR): Viral DNA was extracted using High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Roche Applied Science, Germany) as per the manufacturer's instructions. Real time PCR was done to detect parvovirus DNA in the plasma samples using AmpliSens parvovirus B19-FRT PCR kit (Federal Budget Institute of Science “Central Research Institute for Epidemiology”, Russia). The reaction mixture containing PCR-mix-1-FRT parvovirus B19, PCR-mix-2-FRT, polymerase (TaqF) was added to 10 µl volume of the extracted DNA sample to make the total reaction volume of 25 µl. The cycling conditions used were: Holding at 95°C for 15 minutes followed by 5 cycles (95°C for 5s, 60°C for 30s and 72°C for 15s) and 40 cycles (95°C for 5s, 60°C for 30s and 72°C for 15s). Linear range of the assay is 10³ – 10⁷ IU/ml.

For the detection of HBV DNA/HCV RNA in CHB/CHC groups respectively, viral DNA/RNA was extracted using High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Roche Applied Science, Germany) as per the manufacturer's instructions and Real time PCR was performed to detect HBV DNA/ HCV RNA in the plasma samples using COBAS TaqMan 48 Analyser (Roche Diagnostics, North America).

Liver function tests: Alanine transaminase (ALT) and aspartate aminotransferase (AST) levels were determined in the patient sera using UniCel® DxC 600 (Beckman Coulter, Inc, Ireland)

Statistical analysis: Statistical analysis was done using the SPSS software version 22.0. Data was described in terms of mean (±2SD), median (interquartile range) and percentage. Non-parametric tests namely Mann Whitney U-test and Fisher’s exact were used to compare the sets of data.

Ethical considerations: A written and informed consent was
obtained from all the patients participating in the study. An approval from the institutional ethics committee was obtained.

RESULTS

Baseline characteristics of the study population

The baseline characteristics of the study population are depicted in Table 1. The study population consisted of 20 CHB, 20 CHC patients and 20 healthy controls. Overall mean age of the study population comprising of 37 males and 23 females was 41(±11.8) years.

In CHB group, median HBV DNA levels were 2.06 \times 10^5 (2.03 \times 10^4, 7.35 \times 10^4) IU/ml and all were genotype D isolates. 40% were HBe antigen positive. In the CHC group, median HCV RNA levels were 9.35 \times 10^5 (4.77 \times 10^5, 1.57 \times 10^6). Majority (80%) of the CHC patients were genotype 3 while rests were genotype 1.

Seroprevalence of parvovirus B19 in different groups

In order to determine the seroprevalence of parvovirus B19 in all the groups, parvovirus B19 specific IgG antibodies were tested. No difference in the seroprevalence amongst various groups was seen, 25% (5/20) in CHB, 50% (10/20) in CHC and 50% (10/20) in healthy controls.

IgM antibody positivity, was more in CHC patients 25% (5/20) than in CHB patients : 15% (3/20), or controls 5% (1/20) (Table 1). Parvovirus B19 DNA could not be detected in any of the IgM positive samples. Hence further comparisons were made between parvovirus B19 IgM positive (co-infected) and IgM negative (mono-infected) CHB and CHC patients.

Comparison of liver function abnormality between parvovirus B19 co-infected and mono-infected CHB patients

In the CHB patients, the difference in median values of ALT and AST (signifying the liver dysfunction) amongst the patients with parvovirus co-infection (IgM +ve) and mono-infected (IgM negative) was found to be significant (Fig.1). However the total bilirubin levels in the two groups was insignificant. Similarly, the difference in Hepatitis B viral load (signifying viral replication) was insignificant in the two groups (Table 2). The number of HBe Ag positive patients was 2/3 (66.7%) amongst the parvovirus B19 co-infected patients.

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CHB(n=20)</th>
<th>CHC(n=20)</th>
<th>Control(n=20)</th>
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<tr>
<td>Number</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Male: female</td>
<td>14:6</td>
<td>11:9</td>
<td>12:8</td>
</tr>
<tr>
<td>Mean age in years (± 2SD)</td>
<td>38(±11.09)</td>
<td>44(±12.4)</td>
<td>40(±11.3)</td>
</tr>
<tr>
<td>B19 IgM +ve, n (%)</td>
<td>3(15%)</td>
<td>5 (25%)</td>
<td>1(5%)</td>
</tr>
<tr>
<td>B19 IgG +ve, n(%)</td>
<td>5 (25%)</td>
<td>10 (50%)</td>
<td>10 (50%)</td>
</tr>
<tr>
<td>Median AST levels (IU/L)(Range)</td>
<td>36.00(27.25-60.00)</td>
<td>54.00(37.00-87.50)</td>
<td>20 (18.25-25.00)</td>
</tr>
<tr>
<td>Median ALT levels (IU/L)(Range)</td>
<td>46.30(33.25-92.5)</td>
<td>50.00(35.00-95.5)</td>
<td>22 (17.25-26.00)</td>
</tr>
<tr>
<td>Median bilirubin levels (mg/dl)</td>
<td>0.80 (0.70-1.47)</td>
<td>0.90 (0.70-1.57)</td>
<td>0.7 (0.60-0.87)</td>
</tr>
<tr>
<td>Median viral load (DNA/RNA)(IU/ml)</td>
<td>2.06 ? 10^2.03 ? 10^4-7.35 ? 10^6</td>
<td>9.35 ? 10^4.77 ? 10^2-1.57 ? 10^6</td>
<td>-</td>
</tr>
</tbody>
</table>

CHB= Chronic Hepatitis B, CHC= Chronic Hepatitis C, SD= Standard deviation, AST= aspartate transaminase, ALT= alanine transaminase, IU= International Units

Fig. 1a: Comparison of serum AST levels between parvovirus B19 co-infected and HBV mono-infected patients amongst the CHB patients.
Table 2  
Comparison of various parameters in parvovirus B19 co-infected and HBV/HCV mono-infected patients

<table>
<thead>
<tr>
<th>S. no</th>
<th>Parameter</th>
<th>CHB patients</th>
<th>P value*</th>
<th>CHC patients</th>
<th>P value*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Parvovirus B19 IgM +ve</td>
<td>Parvovirus B19 IgM-ve</td>
<td>Parvovirus B19 IgM +ve</td>
<td>Parvovirus B19 IgM-ve</td>
</tr>
<tr>
<td>1</td>
<td>Median AST levels (IU/L)</td>
<td>325 (61.00-380.00)</td>
<td>31 (26.50-53.00)</td>
<td>0.013</td>
<td>66 (33.50-240)</td>
</tr>
<tr>
<td>2</td>
<td>Median ALT levels (IU/L)</td>
<td>416 (64.00-96.50)</td>
<td>43 (33.00-61.50)</td>
<td>0.023</td>
<td>97 (31.00-188.50)</td>
</tr>
<tr>
<td>3</td>
<td>Median bilirubin levels (mg/dl)</td>
<td>3.4 (0.50-4.3)</td>
<td>0.80 (0.70-1.15)</td>
<td>0.25</td>
<td>0.9 (0.65-2.75)</td>
</tr>
<tr>
<td>4</td>
<td>Median viral load (DNA/RNA) (IU/ml)</td>
<td>4.83 x 10^6 (2.80 x 10^5 - 5.89 x 10^5)</td>
<td>1.18 x 10^6 (1.82 x 10^5 - 9.52 x 10^5)</td>
<td>0.26</td>
<td>1.09 x 10^6 (4.2 x 10^5 - 2.65 x 10^6)</td>
</tr>
<tr>
<td>5</td>
<td>Median HBsAg levels (IU/ml)</td>
<td>58688 (2860-68790)</td>
<td>15345 (5731-23779)</td>
<td>0.49</td>
<td>—</td>
</tr>
</tbody>
</table>

*Mann Whitney U-test  
CHB= Chronic Hepatitis B, CHC= Chronic Hepatitis C, AST= aspartate transaminase, ALT= alanine transaminase, IU= International Units

Fig. 1b: Comparison of serum ALT levels between parvovirus B19 co-infected and HBV mono-infected CHB patients.  
Fig. 1c: Comparison of serum Bilirubin levels between parvovirus B19 co-infected and HBV mono-infected CHB patients.
and 6/17 (35.3%) amongst the HBV mono-infected. The difference was not statistically significant (P = 0.53).

Amongst the CHC patients, the difference in median values of ALT, AST, bilirubin and Hepatitis C viral load in patients with co-infection with parvovirus and HCV mono-infected was found to be insignificant (Fig. 2).

**DISCUSSION**

Human parvovirus B19 has been reported to be detected in the sera of patients with acute or chronic hepatitis. The seroprevalence of B19 in CHB/CHC patients ranges from 21% to 47%. Acute infection with parvovirus has been reportedly higher in CHB and CHC patients. It has been established that it might be difficult to eradicate parvovirus B19 infection from chronic hepatitis patients. The role of parvovirus B19 infection in chronic hepatitis has been debated in various studies. There are published reports concluding that parvovirus B19 persistence in CHB/CHC patients positively correlates with liver involvement and increases the probability of worsening of hepatitis and others stating that persistent
Parvovirus B19 infection in such patients doesn’t worsen liver functions. No association of HBV genotype has been found with parvovirus prevalence in coinfected subjects. In the present study, we found the seroprevalence of parvovirus B19 infection to be similar in healthy controls (50%) and chronic hepatitis patients (25% in CHB and 50 % in CHC). The seroprevalence of parvovirus B19 found in this study is lesser as compared to the population from Vietnam or Taiwan; although similar to the Nigerian population as reported by Opalaye et al in 2011. However there is no published data from India highlighting the seroprevalence of parvovirus B19 in chronic hepatitis patients.

Acute infection with parvovirus IgM +ve was found to be higher in CHB and CHC patients as compared to controls. This significant difference might be due to the shared routes of infection like blood transfusion and intravenous drug abuse between hepatitis B virus, hepatitis C virus and parvovirus B19. Similar association has been reported earlier in the published literature.

Yu et al in a recently published study, reported that parvovirus infection was positively correlated to serum ALT production in CHB coinfected patients. Toan et al concluded in a study conducted on 463 hepatitis patients, that in HBV/B19 co-infection the probability of progression to more severe hepatitis is significantly higher. Pongratz et al reported that parvovirus B19 persistence in chronic hepatitis patients directly correlates with the extent of liver involvement. However, Wang et al in a study conducted on European patients, reported persistent parvovirus B19 infection in the CHB and CHC patients and concluded that the persistence of B19 virus infection does not cause any significant worsening of liver functions in the patients with chronic hepatitis related to HBV/HCV. In the existing study, the median values of liver enzymes (AST, ALT) were significantly higher in CHB patients co-infected with parvovirus B19 but not in the case of CHC patients. The difference in DNA/RNA levels in the co-infected and mono-infected groups was insignificant in both CHB and CHC patients. This suggests that co-infection with parvovirus B19 in CHB patients might have a role in aggravating the liver dysfunction but a larger sample size and a more detailed study might be needed to establish the same.

The results reflect that co-infection with parvovirus B19 in CHC patients neither aggravates the ongoing insult to the hepatocytes nor enhances replication rates of HCV. Therefore on the basis of these results the role of parvovirus B19 in aggravating the disease in CHC seems unlikely.

**Limitations of the study**

This is the first study on Indian population which explores the role of parvovirus B19 in chronic hepatitis. However due to small sample size and lack of follow up of the parvovirus B19 co-infected patients to monitor further worsening of liver function we cannot confirm the role of parvovirus B19 in aggravating liver disease. More structured studies should be planned that could provide a better insight into the same.

**CONCLUSION**

Parvovirus B19 is prevalent equally amongst HBV, HCV infected and healthy population. Co-infection of parvovirus B19 with HCV did not increase the frequency of liver dysfunction but it definitely aggravates the liver dysfunction in CHB co-infected patients.

**CONFLICTS OF INTEREST:** None

**REFERENCES**


Prevalence of Cryptosporidiosis in children with diarrhea and its correlation with mucosal immunity

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¹Department of Microbiology, ²Department of Pediatrics,
University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi-110 095

ABSTRACT

Background and objectives: Cryptosporidium is a recognized cause of diarrhea, particularly among children, in developing countries. Cryptosporidium diarrheal episode impinges heavily on the quantitative effector mucosal responses of subsets of T cell population, especially within the gut cytokines. The current study aims to estimate the prevalence of cryptosporidiosis in children of age ≤ 5 years old and also compared IL-10 and IFN-γ levels in immunocompetent children responding to gut infection with Cryptosporidium. Materials and methods: Diarrheal stool specimens from 175 young children (≤ 5 years) were collected. Kinyoun’s acid fast staining was performed for identification of Cryptosporidium. ELISA was performed for antigen detection of Cryptosporidium and cytokine (IFN-γ and IL-10) analysis. For comparison a total of 30 stool samples from age and sex matched healthy children were also tested for IFN-γ and IL-10. Results: Cryptosporidium oocysts were found in 7 (4.0%) out of 175 children whereas 48 (27.4%) children suffering from diarrhea had Cryptosporidium antigen. A marker of a proinflammatory immune response, IFN-γ and the counter-regulatory cytokine IL-10 was also exclusively elevated in the patient population (p <001). Interpretations and conclusion: Cryptosporidium is present in a significant portion of children suffering from diarrhea in our setting. Antigen detection has much higher isolation rate than Kinyoun’s acid fast staining. Results suggest that the Th response adapted at controlling cryptosporidial infection may be a dynamic one in which there is a strong early Th-1 response which later shifts to Th-2 response to facilitate parasite removal and to limit the infection.

Keywords: Cryptosporidium, immunocompetent children, mucosal immunity

INTRODUCTION

Our knowledge of the protozoan parasite Cryptosporidium parvum has increased considerably since 1976 when it was first discovered as a cause of diarrhea in humans and animals, and in present times the organism is recognized as one of the important opportunistic parasites. Cryptosporidium is an obligate intracellular protozoan parasite and is a major cause of diarrheal illness worldwide. Cryptosporidium primarily infects the distal small intestine (distal jejunum and ileum). Immunocompetent hosts control and eliminate the infection, which typically manifest as acute, self-limited watery diarrhea lasting 5 to10 days. However, in patients with defects in cellular immune responses, Cryptosporidium frequently causes persistent or chronic diarrhea and may also involve the biliary tract. In malnourished children, recurrent diarrheal episodes, can lead to death or chronic nutritional and cognitive sequelae. Thus, the host immune response plays a critical role in the control of human cryptosporidiosis. The mechanism by which C. parvum causes a spectrum of clinical illnesses with different outcomes are poorly understood. To understand the pathophysiology of cryptosporidiosis, it is important to define the intestinal mucosal immune responses to C. parvum.
infection and their potential impact on intestinal secretory responses. Although extensive studies with various animal models have provided important insight into the host immune response to *C. parvum*, the ability of these models to explain the human immune response is limited. [1] *C. hominis*, infects only humans and gnotobiotic pigs, thus limiting the data from animal models. Healthy human volunteers can be studied, but they typically experience a milder illness than malnourished children and AIDS patients. Human intestinal tissue samples can be obtained only by invasive procedures, limiting the number of subjects and samples available. Some data can be obtained from *in vitro* infections, but most of the target cells are immortalized cell-lines and may not be ideal for studying mechanisms involving apoptosis. Furthermore, the immune cells in the peripheral blood may exhibit properties different from the properties of cells found in the intestinal compartment. Thus, knowledge about the human immune response towards *Cryptosporidium* infection is far from complete. In the face of these limitations, important recent advances have been made.

In our study, an attempt was made to estimate the prevalence of *Cryptosporidium* in immuno-competent children, under the age of 5 years, presenting with acute or persistent diarrhea. This will help us to measure not only the parasitic burden, but will also correlate the demographic, nutritional and immunological status of the child with the disease burden. Additionally since the immune response to *Cryptosporidium* is not clearly defined till now, an attempt was made to investigate the indicators of an inflammatory component at mucosal surface, in naturally acquired cryptosporidial diarrhea, by stool assay of interferon-γ (IFN-γ) and interleukin-10 (IL-10) which may help to understand the intestinal immune response of young children with cryptosporidiosis.

**MATERIALS AND METHODS**

**i) Patient population**

In the present prospective study from 2012 to 2013, a total of 175 stool specimens were collected from children under 05 years of age and suffering from diarrhea. The samples were collected only once from the patient. Diarrhea episodes were defined as acute (three or more loose stools per day over 72 hours) or persistent (persisted more than 14 days). Ethical clearance was obtained from Institutional Ethics Committee. The parents/guardians of the children were interviewed using a designed proforma for demographic, epidemiological data and clinical history. Children with known immune suppression, history of receiving antibiotics or antiparasitic drug for current episode of diarrhea, known allergy to lactose, gluten or any other food, history of recurrent hospitalization due to infections, history of prolonged steroids intake in last three months or history of infection with unusual organisms were excluded from our study.

**ii) Routine microscopy and culture**

Fecal samples meeting the inclusion criteria were collected in a clean, dry, leak-proof plastic container. Routine microscopic examination inclusive of Kinyoun’s acid-fast staining of the stool samples were performed as per standard laboratory protocols. Bacterial culture was done using MacConkey’s media, xylose lysine deoxycholate agar and bile salt agar for primary plating of stool sample and selenite F broth and alkaline peptone water for enrichment of stool sample.

**iii) Antigen detection**

A portion of each sample was preserved in 10% formalin for antigen detection till the time of assay by commercially available *Cryptosporidium* antigen detection ELISA kit (DRG international Inc, USA).

**iv) Cytokine analysis**

A portion of stool sample was preserved at -70°C for cytokine analysis till the time of assay. Stool samples which were positive for *Cryptosporidium* on microscopy or ELISA test for antigen detection were further tested for IFN-γ and IL-10 analysis in stool supernatant. Thirty samples from healthy age, sex matched children were also subjected to cytokine analysis and served as controls.

Before the assay the stool samples were removed from -70°C freezer and thawed. Small aliquots were diluted in 1:2 (w/v) in PBS (phosphate buffered saline pH-7.2-7.4) containing protease inhibitor (AEBSF, E-64, pepstatin A.1, and 10-phenanthroline) (Sigma, St. Louis, Missouri). Stool samples were thoroughly homogenized and centrifuged for 10 minutes at 12,000 rpm. The supernatants were collected in fresh sterile eppendorf tubes. IFN-γ and IL-10 determinations were performed in stool supernatants using commercially available human IFN-γ and IL-10 ELISA kit (Gen-Probe Diaclon, France). Each test was performed according to the manufacturer’s specification. Briefly, standards and samples were pipetted into wells pre-coated with highly specific antibodies to IFN-γ and IL-10. After binding of IFN-γ and IL-10 in samples and known standards to the capture antibodies and subsequent binding of the biotinylated anti IFN-γ and anti IL-10 secondary antibody to the analyte was completed during the same incubation period; excess unbound analyte and secondary antibody was removed. The HRP conjugate solution was then added to every well including the zero controls.

A chromogen substrate...
was added to the wells resulting in the progressive development of colored complex with the conjugate. The absorbance of the color complex was then measured and the generated OD values for each standard were plotted against expected concentration forming a standard curve. Standard curve was used to accurately determine the concentration of IFN-γ and IL-10 in any sample tested.

RESULTS

Among the 175 patients of diarrhea, seven were positive by microscopy and 48 were positive by antigen detection kit. All microscopically positive samples were also positive for antigen by ELISA. According to the onset of illness (diarrhea) only one (2.08%) Cryptosporidium positive patient presented with persistent diarrhea whereas the remaining 47 (97.91%) presented with acute diarrhea. The demographic profile of these patients is represented in Table 1. Fever was present in nine (18.7%), vomiting in 19 (39.58%), abdominal pain in six (12.5%) and abdominal distention in two (4.16%) children with Cryptosporidium species in diarrheal stool. The epidemiological characteristic is presented in Table 2.

Hydration status was defined according to WHO classification as (No, some or severe dehydration). Twenty three (47.91%) children did not have dehydration; some dehydration was present in 21 (43.75%) whereas severe dehydration was present in four (8.33%) children positive for Cryptosporidium species. Breast-fed children with cryptosporidiosis were 64.58%.

Forty eight Cryptosporidium positive stool samples and 30 stool specimens from healthy controls were evaluated for IFN-γ and IL-10 assay. IFN-γ was measurable in 26 (54.16%) of 48 patients to a maximum of 159.2 pg/ml and in control groups it was measurable in four (13.3%) samples. IL-10 was measurable in 30 (62.5%) of 48 patients to a maximum of 320 pg/mL, while in the control groups it was detected in four (13.3%) samples.

DISCUSSION

Cryptosporidiosis is a common cause of gastrointestinal disease, and it has been recognized worldwide as a cause of diarrhea in otherwise healthy children. The disease is widespread in many developed and developing countries. The present prospective case control study of cryptosporidiosis in urban North Indian children with diarrhea has revealed similarities as well as differences in clinical and epidemiological features when compared with previous studies from this country, and from other developing

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cryptosporidium positive cases (n=48)</th>
<th>Cryptosporidium negative cases (n=127)</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diarrheal type</td>
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</tr>
<tr>
<td>Acute</td>
<td>47</td>
<td>121</td>
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<td>6</td>
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<tr>
<td>Hydration status</td>
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</tr>
<tr>
<td>No</td>
<td>23</td>
<td>76</td>
<td></td>
<td></td>
<td>0.304</td>
</tr>
<tr>
<td>Some</td>
<td>21</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>4</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Still breast feeding</td>
<td>31</td>
<td>67</td>
<td>1.633</td>
<td>0.822 - 3.244</td>
<td>0.160</td>
</tr>
<tr>
<td>Fever</td>
<td>9</td>
<td>25</td>
<td>0.942</td>
<td>0.404 - 2.195</td>
<td>0.899</td>
</tr>
<tr>
<td>Vomiting</td>
<td>19</td>
<td>40</td>
<td>1.425</td>
<td>0.715 - 2.838</td>
<td>0.313</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>6</td>
<td>9</td>
<td>1.873</td>
<td>0.629 - 5.579</td>
<td>0.254</td>
</tr>
<tr>
<td>Abdominal distention</td>
<td>2</td>
<td>10</td>
<td>0.509</td>
<td>0.107 - 2.411</td>
<td>0.387</td>
</tr>
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<td>WHZ Mean(SD)</td>
<td>0.80 (1.70)</td>
<td>-1.17 (1.90)</td>
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<td></td>
<td>0.244</td>
</tr>
<tr>
<td>WAZ Mean(SD)</td>
<td>-1.80 (1.54)</td>
<td>-2.14 (1.48)</td>
<td></td>
<td></td>
<td>0.179</td>
</tr>
<tr>
<td>HAZ Mean(SD)</td>
<td>-1.88 (1.93)</td>
<td>2.32 (1.54)</td>
<td></td>
<td></td>
<td>0.117</td>
</tr>
</tbody>
</table>
countries. In addition, to our knowledge the present study describes for the first time mucosal immune responses to Cryptosporidium species in Indian immunocompetent children.

Our study demonstrated clearly a high prevalence rate (27.4%) of Cryptosporidium infection in children less than five years. Similar high prevalence of cryptosporidiosis has been reported from India; (18.9 %),<sup>[5]</sup> Bolivia (31.4%)<sup>[6]</sup> and Mexico (29.6%).<sup>[7]</sup> High prevalence of Cryptosporidium infection found among 1-2 years (32%) followed by 4-5 years age group (31.8%) is similar to studies conducted in the tropical countries<sup>[8,9]</sup> showing highest C. parvum infection in less than two years of age group children.

Regarding gender variation male:female ratio of 29% versus 24% was not statistically significant. However the contact with domestic animals may act as a risk factor in zoonotic infection and previous studies have reported an association of this disease with the domestic animals in the household.<sup>[10]</sup> In our study, however there was no statistically significant association between Cryptosporidium infection and animal contact similar to studies in Mexico and Brazil.<sup>[11,12]</sup>

It is well known that, water borne transmission is a major route of infection with Cryptosporidium species and because of small size of Cryptosporidium oocyst and its ability to survive chlorination it was of interest to determine whether there was any difference in the type of water supply associated with the disease. In our study, consumption of untreated overhead tank water supply from municipality could have been a major source of contamination leading to infection and disease. Cryptosporidium species infected patients presenting with watery diarrhea (100%), vomiting (39.50%) and abdominal

<table>
<thead>
<tr>
<th>Cytokine level pg/ml</th>
<th>Cryptosporidium positive diarrhea (cases) (n=48)</th>
<th>Non-diarrheal Cryptosporidium negative (control) (n=30)</th>
<th>P value (Mann - Whitney Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No of detectable level</td>
<td>26</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>25.50 (38.19)</td>
<td>4.75 (14.63)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median (range)</td>
<td>5.68 (0-159.20)</td>
<td>0 (0-57.85)</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No of detectable level</td>
<td>30</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>47.34 (65.29)</td>
<td>12.88 (33.49)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median (range)</td>
<td>21.95 (0-320)</td>
<td>0 (0-103)</td>
<td></td>
</tr>
</tbody>
</table>
pain (12.5%) is consistent with the previous studies from developing countries. Cryptosporidial infection usually leads to chronic watery diarrhea with varying degree of dehydration as observed in our study.

Lack of breast-feeding has been reported to be associated with cryptosporidiosis in many studies in developing countries. However, some prospective studies have not shown a protective effect of breast-feeding, and one study found that Cryptosporidium species infection was actually more common in breast-fed children. Although in the present study too, infection was seen more common among breast-fed (64%) as compared to non-breastfed (35%) patients, there was no statistically significant association of disease with breast-feeding.

This study demonstrated that 54% of Cryptosporidium patients had detectable levels of IFN-γ compared to control group and this difference was statistically significant. The mechanism by which the increased expression of IFN-γ controls intestinal cryptosporidiosis is unknown. Studies in a variety of systems have implicated IFN-γ in several aspects of the cellular immune system. Intestinal epithelial cells express IFN-γ receptors and IFN-γ directly activate intestinal epithelial cells to kill intracellular parasites. IFN-γ could potentially activate the epithelial cells directly or activate cytotoxic lymphocytes, which in turn might destroy infected cells to limit infection. Most importantly, comparison of animal and human data has shown that the immune response towards Cryptosporidium in humans differ significantly from that in animals; for example, in mice gamma interferon (IFN-γ) production seems to be associated with the innate and primary immune responses, whereas in humans it is most probably associated with the memory response towards the parasite. Designing studies to elucidate human mucosal immune responses is difficult.

In a study in Haitan children, IFN-γ was not detected in the stool samples with cryptosporidiosis, possibly due to degradation of the cytokine in the luminal contents of the gut, or the insensitivity of the ELISA method used, and/or collection of the samples at too frequent time-points such that secretion of this cytokine was missed.

In addition to IFN-γ, our study population also revealed evidence of a counter regulatory (anti-inflammatory) mucosal response, as demonstrated by IL-10 elevation in stool samples of 62.5% of positive subjects. IL-10 is produced by a broad range of cells in the gastrointestinal tract (including intestinal epithelial cells, B cells, and macrophages) and plays a central role in the immune balance between pathology and protection through its broad immune regulatory and immunosuppressive functions. The production of IL-10 can be considered as an attempt to “restrain” the immune system, shown to have an important role in regulation of mucosal immunity. Thus IL-10 may be key factor for repairing the intestinal epithelium after cryptosporidiosis mediating parasite clearance.

Our study showed parallel increase in both IFN-γ (inflammatory cytokine) and IL-10, (anti-inflammatory cytokine). It suggests that there exists a balance between these two cytokines determining the shift of Th1 to Th2 response, when there is a deficiency in IFN-γ production or a condition involving high levels of IL-10. Alternatively it can be said that the Th responses to cryptosporidiosis is a dynamic one in which there is an early Th1 response but later shifts to Th2 response to facilitate parasite removal and to limit the infection. The important observation of our study is that there is a predominant Th2 (IL-10) response and alternative activation of IL-10 may minimize tissue injury, or help in the escape of the parasite from the host. The quick appearance of IL-10 in the present study favors acute episode of diarrhea in Indian immunocompetent children which is in contrast with previous studies in Pakistan, Haiti and Uganda, where most of the Cryptosporidium affected patients presented with persistent diarrhea.

Cryptosporidium species is prevalent in a significant proportion (27%) in immunocompetent children presenting with acute diarrhea in North Indian tertiary care hospital. Outcome of a diarrheal episode impinges heavily on the quantitative effector responses of subsets of T cell population, especially within the gut. The presence of gut microbiota and proteases in the stool specimens can destroy secreted cytokines and can result in false low levels. Influence of other enteric pathogens in modulating the cytokine regulation is a probability. The inflammatory process observed in cryptosporidiosis requires additional investigation to comprehend the complex interrelationship between the pathogens, host-factors, malabsorption and malnutrition, especially in developing countries. Therefore such a study should be followed up sequentially to understand the role of cytokines in either remission or persistence of diarrhea with this agent.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST: None

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Probiotic Lactobacillus rhamnosus GG inhibits the adhesion of Giardia intestinalis to murine enterocytes: An in vitro study

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Department of Microbiology, Panjab University, Chandigarh 160014

ABSTRACT

Background and Objectives: Giardiasis mainly affects young children causing diarrhea, malnutrition and growth retardation. Due to adverse effects of antiprotozoal treatment such as low compliance with drug therapy, reinfection, occurrence of resistant strains, headache and metallic taste, the use of natural live bacteriotherapy has been studied. The study was designed to investigate in vitro the colonizing ability of probiotic Lactobacillus rhamnosus GG (LGG) viz-a-viz its ability to inhibit the adherence of Giardia trophozoites to murine enterocytes under conditions simulating the intestinal environment. Materials and methods: Murine enterocytes were harvested and incubated with Giardia trophozoites either prior or simultaneously with probiotic LGG to assess the adhesion using scanning electron microscopy. Results: It was observed that 15% of Giardia trophozoites adhered to enterocytes at 37°C in Hank’s Balanced Salt Solution, after 1h of incubation. However, coinoculation of murine enterocytes with probiotic LGG either 30 min prior or simultaneously with Giardia trophozoites led to 23-27% reduction in the adherence of Giardia trophozoites compared with 46% adherence in the absence of LGG. Further, scanning electron microscopy also showed in vitro inhibition of Giardia trophozoites to murine enterocytes due to probiotic supplementation. Interpretations and conclusion: The data suggest the colonizing ability of probiotic LGG to murine enterocytes that modulates murine giardiasis mainly by displacing the Giardia trophozoites.

Keywords: Giardiasis, mouse enterocytes, probiotic, scanning electron microscopy

INTRODUCTION

Giardia intestinalis is a flagellated protist causing diarrheal disease in humans worldwide.\cite{1,2} Pathophysiology of giardiasis is due to the adherence of Giardia trophozoites to epithelial surface of the small intestine resulting in a series of events that initiate the onset of diarrhea.\cite{3,4} Besides the routine anti-Giardial drugs, live lactic acid bacteria have been employed in vivo as an alternative bio-intervention to control the duration and severity of Giardia infection by their ability to antagonize the adherence of trophozoite to enterocytes.\cite{5,6}

Probiotic lactobacilli are the part of complex bacterial communities of gastro-intestinal tract (GIT) and are able to survive extreme intestinal conditions. More specifically, adhesive properties of probiotics and their ability to produce antimicrobial substances active against a variety of bacteria, including Escherichia coli, Salmonella species, Clostridium species, Streptococcus species, and Bacteroides species, have made them a part of our daily life management.\cite{7,8} To this end, we have established a mouse model to assess the modulatory potentials of Lactobacillus rhamnosus GG (LGG) in ameliorating murine giardiasis and have reported that

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among the various probiotics, LGG was the most effective one in reducing both the duration and severity of murine giardiasis. Further, it was observed that the probiotic LGG modulated murine giardiasis due to its antioxidative and immunomodulatory properties. Thus, the aim of the present study was to assess in vitro the adherence and colonizing ability of LGG vis-à-vis its inhibitory effect on the adherence of Giardia trophozoites to murine enterocytes.

MATERIALS AND METHODS

Parasite

G. intestinalis trophozoites (Portland strain 1) were grown axenically in TYI-S-33 medium supplemented with antibiotic solution (streptomycin 4 ug/100ml, penicillin 2000 units/100ml and gentamycin 1000 units/100ml) and horse serum, pH 6.9, adjusted prior to filter sterilization using 0.22 µm seitz filter. Actively growing trophozoites (48-72 h old culture) were centrifuged at 200 g for 10min, after chilling the tubes in ice for 15 min, washed thrice with phosphate buffer saline (PBS) pH-7.2 and finally re-suspended in PBS.

Probiotic

Lactobacillus rhamnosus GG (MTCC (1408) procured from Institute of Microbial Technology, Chandigarh was grown in de Man Rogosa Sharpe (MRS) broth and maintained on MRS agar slants by regular sub-culturing at 15 days interval. For experimental use, LGG grown in MRS broth for 18 h was sedimented by cold centrifugation at 1200 g for 10 min, washed thrice and finally re-suspended in PBS.

Animals

BALB/c mice aged 5-6 weeks old (18-20g) of either sex were obtained from Central Animal House, Panjab University, Chandigarh, India. The mice were housed under standard conditions of light and dark cycle, fed with standard pellet diet (Hindustan Liver Products Limited, Kolkata, India) and were given water ad libitum. Only Giardia - free mice were employed. Care and use of animals was in accordance with the guidelines of the Institutional Animal Ethical Committee.

Isolation and characterization of murine enterocytes

After sacrificing the animals either by retro-orbital bleeding or by cervical dislocation, intestinal epithelial cells were isolated as per the Weiser method with minor modifications. A segment of small intestine (3 cm) was everted on glass rod, tied and rinsed with 0.9% cold NaCl. The rod was kept in a conical tube containing 10 ml solution A (1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH₃PO₄, 5.6 mM Na₂HPO₄) for 30min at 37°C and then transferred to another tube with 10ml solution B (277 mM trisodium citrate, 0.5 mM dithiothreitol, 55 mM sorbitol, 44 mM sucrose) followed by gentle shaking for 30 sec, centrifuged at 500g for 10 min at 4°C. Enterocytes were washed, suspended in Hanks Balanced Salt Solution (HBSS) and cell viability was monitored with trypan blue exclusion staining method.

The morphology of isolated murine enterocytes was observed under both light and phase contrast microscopes and purity was monitored by alkaline phosphatase assay as per Bergmeyer. Cells were disrupted by sonication for 5 sec at 8Kc/seconds (Soniprep-150MSE) and centrifuged at 12000g for 5min at 4°C. 100µl of cell extract was added to 500 µl buffered substrate (0.1M Glycine-NaOH buffer with 5.5 mM β-nitrophenyl phosphate, pH 10.5) and kept at 37°C for 5 min for equilibration. Reaction was stopped by adding 5 ml of 0.1N NaOH. Absorbance was read at 420 nm and results were expressed as µmoles of DNP released per mg of protein.

Giardia-murine enterocytes adherence assay

The adhesion study was performed as per Céu Sousa et al. Equal amount (1x10⁶ cells/ml in HSCB Hepes saline buffer comprising of 10 mM Hepes saline buffer (pH 7.2) containing 2 mmol/l CaCl₂) of Giardia trophozoites and epithelial cell suspension were mixed in tissue culture plate (Laxbro), incubated for 1h at 37°C in CO₂ incubator. Unadhered trophozoites were removed by gently pipetting followed by washing with PBS. Adhered trophozoites were sedimented by centrifugation at 200 g for 5 min and were counted. Percentage of adhered trophozoites to enterocytes was calculated by determining the ratio of adhered trophozoites/total Giardia trophozoites seeded.

Effect of various physicochemical factors on adherence

Effect of time

To investigate the ability of Giardia trophozoites to
adhere to the mouse enterocytes, adherence assay was performed at time intervals ranging from 30 min to 3h of incubation as per Céu Sousa et al. Briefly, equal amount (10^6 cells/ml in HSCB) of Giardia trophozoites and murine enterocytes were mixed in tissue culture plate and incubated at 37°C in CO₂ incubator for different time intervals.

**Effect of bile salt and pH**

The adhesion study was performed as per Céu Sousa et al both in the presence and absence of bile salt (0.03% sodium taurocholate) as well as by varying the pH of HSCB to 5.5, 7.8 and 9.2 either with 1N HCl or 1N NaOH.

**LGG-Giardia-Murine enterocytes adherence assay**

The inhibitory effect of LGG on the adherence of Giardia trophozoites to murine enterocytes was studied by co-incubating the trophozoites, enterocytes and probiotic LGG as per Céu Sousa et al. In one set of experiments, enterocytes were pretreated for 30 min with LGG before adding Giardia trophozoites to the assay mixture. In the second set of experiments LGG, Giardia trophozoites and enterocytes were added together to the assay mixture.

**Scanning Electron Microscopy**

Interactions of Giardia trophozoites, LGG and murine enterocytes were monitored by Scanning Electron Microscope (JEOL, JEM 1600 Model, Japan). Respective cells were incubated on coverslips for 1 h in HSCB. Specimens were fixed with 2.5% glutaraldehyde for 2 h and dehydrated in 50, 70, 80, 90 and 100% ethanol for 10, 15, 15, 20, 30 min respectively. Finally, the samples were desiccated, mounted on aluminium stubs, coated with gold-palladium at a thickness of 200Å and were observed by Scanning Electron Microscope.

**Statistics**

All experiments were carried out in triplicates. Results were expressed as mean ± SD of three independent experiments. Differences between the observations were evaluated by a two tailed Student's t-test.

**RESULTS**

**Effect of time on adherence**: It was observed that the adherence increased significantly from 0.5 h to 1 h and was maximum (56±1.3%) after 1h of incubation, compared with 20.8±1.9% of adhered trophozoites after 0.5 h and thereafter reached a plateau (Fig.1). Thus, for further in vitro experiments, 1 h was set as the standard incubation time.

**Effect of bile salt and pH**: It was observed 50 ±1.3% of Giardia trophozoites adhered to murine enterocyte in the presence of bile salt (0.3% sodium taurocholate) compared with 53.8±1.1% in its absence. The percentage of adhered Giardia trophozoites was 38.4±1.1, 51.9±1.0 and 42.1±1.1 at pH 5.5, 7.8 and 9.2 respectively. However, the adherence of Giardia trophozoites to murine enterocytes was most significant (p<0.05) at pH 7.8 as compared to pH 5.5 and 9.2 respectively (Fig.2).
The treatment of mouse enterocytes either 30min prior or simultaneously with trophozoites significantly (p<0.05) decreased the adherence of Giardia trophozoites (23±1.0 and 27±1.0% respectively) compared with 46±1.3% adherence without LGG treatment (Fig.3).

Scanning Electron Microscopy

In vitro interactions of Giardia trophozoites and LGG with murine enterocytes were also confirmed by SEM. The Giardia trophozoites and lactobacilli were found adhered to the murine enterocytes as observed under SEM (Fig. 4a, b and c). More specifically, treatment of enterocytes with the LGG either 30 min prior or simultaneously with Giardia trophozoites led to displacement of the Giardia trophozoites (Fig. 4d).

DISCUSSION

In giardiasis, there is a close association of Giardia trophozoites with the brush border membrane of small intestine resulting in intestinal lesions, malabsorption and diarrhea. Probiotics have the ability to adhere to intestinal mucosa due to which these are generally regarded as safe “GRAS” and are much in consideration for intestinal infections.

In the present study it was found that both the Giardia trophozoites and probiotic LGG adhered to the isolated mouse enterocytes and the adherence of Giardia trophozoites was maximum at pH 7.8 after 1 h of incubation in HBSS at 37°C in the presence of 0.3% bile salt. This observation is in accordance with earlier studies where rat intestinal epithelial cells and human intestinal cell lines were also employed as models to assess the adherence of Giardia trophozoites in vitro. Further, it was observed that co-incubation of mouse enterocytes with LGG either 30 min prior or simultaneously with Giardia trophozoites led to significant decrease in the number of adhered trophozoites. However, there could also be the display of various specific adhesions and other surface determinants by LGG that may be involved in the interaction with intestinal epithelial cells as suggested by earlier studies.

Moreover, the present observation is in concordance with Collodo who has shown that treatment of pig intestinal mucus with Bifidobacterium lactis Bb12 and LGG, alone or in combination, significantly reduced the adhesion of pathogens viz. Salmonella, Clostridium and E.coli. Based on these findings, it appears that the probiotic LGG has the potential to displace the Giardia trophozoites due to its ability to adhere and colonize with murine enterocytes.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

SSU rRNA target amplification of intestinal microsporidia: A sensitive diagnostic tool for accurate estimate of its prevalence
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ABSTRACT
The diagnosis of intestinal microsporidiosis has traditionally relied on light microscopy. Western literature shows PCR to be more sensitive and specific. The present study was conducted to assess the prevalence of enteric microsporidiosis in HIV seropositive patients using PCR. Five percent stool samples were found to be positive for microsporidia by pan microsporidia primers and found to be Enterocytozoon bieneusi on amplification using species specific primers. Microsporidia is grossly under-reported in our country and there is a dire need to institute measures to detect this organism particularly in HIV infected individuals to abate morbidity and mortality due to this organism.

Keywords: Diarrhea, Encephalitozoon, Enterocytozoon bieneusi, HIV, microsporidia

INTRODUCTION
Microsporidia are obligate, intracellular, spore forming protozoan parasites which infect human beings. Humans are vulnerable to infection with thirteen different species belonging to seven genera including Encephalitozoon and Enterocytozoon. The first case of human microsporidiosis was documented in 1959 in Japan and subsequently after the dawn of HIV-AIDS era in 1980s, these organisms have emerged as important etiological agents of opportunistic infections in hosts who are immunocompromised.

The spectrum of disease in infected individuals includes chronic diarrhea and a wasting syndrome as the most common manifestation, and even as hepatitis, peritonitis, keratoconjunctivitis, sinusitis, bronchitis, pneumonia, cystitis, nephritis, myositis, encephalitis and other cerebral infections also occur. This protozoan has emerged as an important enteric pathogen in patients with AIDS, with Enterocytozoon bieneusi and Encephalitozoon intestinalis being responsible for most cases. Enteric infections in AIDS patients due to Encephalitozoon intestinalis are likely to become disseminated. Enterocytozoon bieneusi is more commonly associated with chronic diarrhea in patients with AIDS in certain studies of the western world. It is imperative to identify the causative organism at the species level since Enterocytozoon bieneusi is known to be inherently resistant to albendazole, which is conventionally used in the treatment of intestinal microsporidiosis.

In the recent past, molecular techniques such as PCR that focus on small subunit (SSU) rRNA genes of microsporidia have been explored for detection as well as speciation of this organism. This has been possible due to limited homology of the SSU rRNA genes of microsporidia with other eukaryotic organisms and allows utility of these genes as probes in hybridization and PCR assays.

In the developing world, microsporidia is a neglected etiological agent of diarrhea in patients, especially in the HIV infected. Studies in the developing world including India on the HIV-microsporidia nexus and its amelioration arising out of pharmacological interventions are scarce. The paucity of proper
diagnostic facilities has led to gross under-reporting of this important parasite. Only a handful of studies have been conducted in India to ascertain the predominant species of microsporidia associated with enteric disease.\cite{5,6} To the best of our knowledge no studies on intestinal microsporidiosis have been undertaken in Delhi.

**MATERIALS AND METHODS**

The present cross-sectional observational study was conducted in our tertiary care hospital in east Delhi between November 2014 and April 2016 after obtaining Institutional ethics clearance and written consent from the patients.

A total of 60 HIV seropositive patients, all 12 years, who were ART naïve were included in the study and were further divided into two groups: (i) 30 HIV seropositive patients with diarrhea and (ii) 30 HIV seropositive patients without diarrhea. Subjects with history of intake of antiparasitic drugs, antibiotics or antimotility drugs in preceding two weeks were excluded.

Three consecutive fresh stool samples were collected and transported immediately to the Microbiology laboratory. Samples were subjected to routine processing and part of the sample was stored at -20°C for molecular work.

DNA from three stool samples of each subject was extracted using Qiagen 'QIAamp DNA Stool Mini Kit' (Germany) according to the manufacturer's protocol and quantified by nanodrop and stored at -20°C for further use. PCR was done using primers amplifying a conserved region of SSU rRNA gene of four common intestinal microsporidia: Enterocytozoon bieneusi, Encephalitozoon intestinalis, E. cuniculi and E. hellem. Speciation for Enterocytozoon bieneusi and Encephalitozoon intestinalis were done using species specific primers. Sequences of primers are shown in Table 1.

Uniplex PCR amplification assay was performed with the DNA template for the four microsporidia mentioned above in thermo cycler (Eppendorf). The amplification protocol included an initial denaturation of DNA at 94°C for 5 mins followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, elongation at 72°C for 1 min and 5 mins of extension at 72°C after 35 cycles.

**RESULTS**

Of a total of 60 HIV seropositive treatment naïve patients (30 with diarrhea and 30 without diarrhea), 5% (3/60) stool samples were found to be positive for microsporidia by pan microsporidia specific primers. Similar results were observed in all three consecutive stool samples. The three positives were found to be Enterocytozoon bieneusi on amplification using species specific primers (Fig 1). The patients infected with intestinal microsporidiosis were aged between 18 and 33 years with a male predominance.

Two of the three patients of intestinal microsporidiosis did not present with diarrhea and acute diarrhea was the presenting illness associated with nausea and vomiting in only one. The stool was semi-formed with frequency 10/day. This patient had an episode of diarrheal illness about 5 months back. The patient was in Stage 2 of clinical AIDS and the CD4 counts were below 100/mm³.

The other two patients did not have any enteric complaints. However, one of them affirmed to having a history of intermittent diarrhea three months before presentation. A history of significant weight loss was present in both of them. Clinical stage of AIDS at the time of presentation was Stage 3 and Stage 4 respectively. The CD4 counts were below 100/mm³ in both cases.

**DISCUSSION**

Aiming to detect the burden of intestinal microsporidiosis in HIV seropositive individuals in Delhi, the present study identified an overall prevalence of intestinal microsporidiosis in HIV seropositive individuals in Delhi to be 5% (3/60), which is comparable to studies from Gujarat and Chennai, where prevalence rates of 2% and 6.5% have been reported respectively.\cite{5,7} A higher prevalence of 15.9% in HIV seropositive individuals has been reported from Chandigarh.\cite{6}
Microsporidia detection in HIV seropositive individuals presenting with diarrhea in the present study was 3.33% (1/30) which is lower in comparison to studies from Mumbai (17.8%) and Pune (12.9%). A study from Chandigarh has documented even higher rates (30.4%). The prevalence of intestinal microsporidiosis in HIV seropositive patients without diarrhea in the present study was 6.66% (2/30), akin to a report from Chandigarh (7.6%). The varying rates of prevalence in different regions of the country maybe due to different techniques used for detection. Whether or not, geographical and ethnic differences play any role needs to be further investigated.
In this study it was noted that diarrhea was present only in one of the three patients of enteric microsporidiosis indicating that diarrhea need not necessarily be present in such patients. The symptomatology was varied including nausea, vomiting, weight loss and generalized body ache. It was also observed that patients with enteric microsporidiosis can present at any clinical stage of AIDS. Anemia was noted in all 3 cases. The CD4 counts were below 100 mm³ in all patients with enteric microsporidiosis.

All (100%) patients of intestinal microsporidiosis in present study were due to *Enterocytozoon bieneusi*, perhaps the predominant species in Delhi, reiterating its preponderance in HIV seropositive individuals. *Encephalitozoon intestinalis* was not detected in any sample. Our findings are comparable to studies from Chennai (100%) and Lucknow (100%), but unlike that from Chandigarh where *Encephalitozoon intestinalis* was found to predominate.  

*Enterocytozoon bieneusi* also predominates in Vietnam.  

*Enterocytozoon bieneusi* exhibits intrinsic resistance to albendazole, which is conventionally used in the treatment of intestinal microsporidiosis and speciation if done well in time can prevent its dissemination in advanced stages of HIV disease.

The clinical diagnosis of intestinal microsporidiosis traditionally depends upon direct visualization of the parasite by light and electron microscopy, and sensitivity and specificity of these techniques are dependent on expertise of the microscopist. Transmission Electron Microscopy, the conventional gold standard for diagnosis of intestinal microsporidiosis has limitations of cost and expertise. The sampling error in electron microscopy may be overcome by immune electron microscopy but these were beyond the scope of the present study. PCR involves an initial DNA extraction step from stool samples where inhibitors present may decrease the effectiveness. However PCR still has the advantage of speciation when used with species specific primers, thus augmenting therapeutic and epidemiological importance.

Two asymptomatic patients who had no enteric complaints are of special interest. One of them had intermittent diarrhea months before the most recent therapeutic intervention raising the possibility of persistence of this parasite in the host and becoming active at an opportune time. The patients under discussion had significant weight loss along with low CD4 counts. Microsporidia is grossly under-reported in our country and considering the varied manifestations, especially the potential to disseminate if undetected at early stages, may lead to avoidable complications. There is a dire need to institute measures to detect this organism particularly in HIV infected individuals to abate morbidity and mortality due to this organism.

**ACKNOWLEDGEMENT**

The authors would like to express their sincere gratitude towards Dr. Ujjala Ghoshal, Additional Professor, Department of Microbiology, SGPGI, Lucknow for providing the positive control sample of *Enterocytozoon bieneusi*.

**SOURCE OF FUNDING**: Institutional fund

**CONFLICTS OF INTEREST**: Nil

**REFERENCES**


Esophageal candidiasis in an immunocompetent host: case report and review of literature

Prerna Goyal1, Omesh Goyal2, Deepinder Chhina3, Pavneet Kaur4, Aminder Singh4, Rajoo Singh Chhina2

1Department of Medicine, 2Department of Gastroenterology, 3Department of Microbiology and 4Department of Pathology, Dayanand Medical College and Hospital, Ludhiana, Punjab

ABSTRACT

Infection by Candida species is the most common cause of infectious esophagitis in adults. Esophageal candidiasis (EC) occurs most commonly in immunocompromised hosts, such as those with human immunodeficiency virus infection. However, EC may also occur in immunocompetent individuals with or without apparent predisposing risk factors and symptoms. We report a case of EC incidentally discovered in an immunocompetent middle age male who underwent esophago-gastro-duodenoscopy for dyspeptic symptoms. The only potential risk factor was long-term use of alcohol. Treatment with fluconazole led to complete resolution of EC.

Keywords: Esophageal candidiasis, fluconazole, immunocompetent

INTRODUCTION

Esophageal candidiasis (EC) is one of the most common opportunistic infections in patients with impaired cellular immunity, such as human immunodeficiency virus (HIV) infection. However, EC has also been reported to occur in immunocompetent individuals with or without apparent predisposing risk factors and symptoms. Various risk factors implicated to cause EC in immunocompetent individuals include broad-spectrum antibiotics, proton-pump inhibitors (PPI), H2-receptor antagonists, esophageal diseases such as noninfectious esophagitis or achalasia, and use of corticosteroid/cytotoxic drugs. Other conditions associated with an increased incidence of EC include alcoholism, malnutrition, and advanced age. Most of the patients detected to have EC on esophago-gastro-duodenoscopy (EGD) are asymptomatic, and typical esophageal symptoms are uncommon. We report a case of EC incidentally discovered in an immunocompetent middle age male who underwent EGD for dyspeptic symptoms.

CASE REPORT

A 49 year old male patient presented to gastroenterology OPD with complaints of epigastric discomfort since one month. There was no history of nausea, vomiting, hematemesis, melena, diarrhea, constipation, weight loss, jaundice or fever. He used to consume about 100 gram of alcohol daily since last 20 years. There was no history of smoking, or drug abuse. He was taking PPI (Tab esomeprazole 40 mg, once daily) since 3 weeks. He denied any use of analgesics or antibiotics in the last one year. Physical examination revealed a well nourished, moderately built person. He had a blood pressure of 124/80 mm Hg and heart rate of 74 beats per minute. His oral cavity was normal with no signs of infection or thrush. He had mild epigastric discomfort with no rebound tenderness or guarding. Rest of physical examination was unremarkable. Laboratory investigations revealed 62 IU/ml alanine transaminase and 85 IU/ml aspartate transaminase. Other investigations including serum bilirubin, albumin, complete blood counts, renal function tests, thyroid function test and glycosylated hemoglobin were normal. Ultrasonography of abdomen and pelvis revealed hepatomegaly with fatty liver. Further, EGD was performed, which revealed multiple whitish plaques throughout esophagus with underlying ulceration (Fig.1). Stomach and duodenum were normal. Brushings from the lesions revealed spores and pseudohyphae of Candida infiltrating the mature squamous cells at places (Fig. 2). He was further investigated to rule out immunocompromised state. HBsAg, anti-HCV,
anti-HIV, syphilis screen, anti-nuclear antibodies and T-lymphocyte counts were normal. He was treated with Tab fluconazole 200 mg once daily for two weeks. Repeat endoscopy performed three weeks later showed complete resolution of esophageal candidiasis.

DISCUSSION

Esophageal candidiasis has been commonly reported to occur in immunocompromised patients, especially those infected with HIV infection. However, the regular use of screening EGD has led to more frequent diagnosis of EC in healthy individuals, The prevalence of EC among patients undergoing EGD has varied from 0.32% (281/88125) by Choi et al and 1.17% (41/3501) by Naito et al to 8.7% by Kakati et al from India. The development of EC is a two-step process consisting of colonization of the esophagus and subsequent invasion of the epithelial layer. It is already well accepted that Candida are known to colonize the esophagus of 20% of healthy adults. Once colonization has been established, impaired cellular immunity, such as due to HIV infection, permits invasion of the epithelial layer.

The usual causative agent is Candida albicans, but other Candida species have also been isolated from cases of esophagitis. In a recent report from India, C. albicans was recovered from the majority of patients (52.1%), followed by C. tropicalis (24%), C. parapsilosis (13.4%), C. glabrata (6.9%) and C. krusei (3.6%). Alarmingly, among the C. albicans isolates 8.6% were resistant to fluconazole.

In immunocompetent persons, various risk factors for EC have been identified. PPI, H2-receptor antagonists, and prior vagotomy may increase the risk of EC by elevation of gastric pH and altering the colonization of the esophagus by oral cavity bacteria and yeast. Broad-spectrum antibiotics may eliminate certain bacteria that inhibit fungal growth, thereby allowing overgrowth and colonization of the Candida species. Corticosteroids and various cytotoxic drugs predispose to candidiasis by suppressing both lymphocyte and granulocyte function. EC has also been seen in cases of functional or mechanical obstruction of the esophagus, where stasis leads to excessive growth of the fungus. Previously, diabetes was considered as risk factor for EC because of impaired immunity and stasis of esophageal contents. However, most of the cases of diabetes-related EC reported have been associated with chronic poor glycemic control (hyperglycemia more than 2 years) and combined with secondary factors, such as those causing an immunocompromised state. Our case had two potential risk factors for EC i.e. long standing alcohol intake and use of acid suppressing medications.

As in our case, esophageal symptoms are more frequently absent in individuals with EC. In studies by Choi et al and Naito et al about two-thirds of the patients had no symptoms. In those who were symptomatic, variable gastrointestinal symptoms included epigastric discomfort, dyspepsia, nausea etc. Classical symptoms of infectious...
esophagitis, such as dysphagia, odynophagia and chest discomfort have been reported in only about 10% patients.\textsuperscript{[2,7]} It is questionable whether the gastrointestinal symptoms are associated with EC and whether this has a clinical significance. On follow-up, about three-fourth of the cases of EC have been shown to disappear spontaneously.\textsuperscript{[2]}

The endoscopic appearance of Candida esophagitis ranges from a few superficial white plaques on the mucosal surface to a dense, thick pseudomembrane composed of desquamated squamous epithelial cells, fungus, and fibrin. Histologically, in the presence of invasive candidal esophagitis, Candida species are seen along with squamous cells and invading hyphae on smears.

Although most cases of EC may remain silent and invasion of esophageal wall is usually limited to the superficial epithelium, extensive tissue necrosis and ulceration resulting in esophageal perforation is possible.\textsuperscript{[10]} There have been a few reported cases of esophageal perforation associated with Candida infection, and most of these predominantly occurred in immunocompromised hosts, such as transplanted or leukemia patients. It is possible that neutropenia, irradiation and chemotherapy such as methotrexate might have led to mucosal disruption, thereby facilitating deeper invasion of the esophagus by Candida.\textsuperscript{[10]} In immunocompetent hosts, chronic alcohol consumption and long-standing gastroesophageal reflux may increase the risk of transmural invasive Candida infection and esophageal perforation.\textsuperscript{[10]}

Our case highlights the fact that EC can occur in immunocompetent persons without esophageal symptoms. Confirmation by histopathology and adequate treatment is warranted to prevent complications, especially in persons with underlying risk factors.

**CONFLICT OF INTEREST:** There is no potential conflict of interest

**REFERENCES**

HuMiX - The artificial gut

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Department of Gastroenterology, PGIMER, 160012, India

Human gut is an open system that encounters wide-range of diet-derived compounds and numerous microorganisms making it a complex system to explore. Alterations in intestinal morphology and dysbiosis lead to several gastrointestinal (GI) ailments and other disorders. In the present scenario metagenomics is considered as an important discipline to study the correlation of human microbiome with the health and disease. Although it provides association between microbial species and maladies, it does not help to infer the cause of disease. A thorough knowledge of fundamental molecular mechanism behind the host-microbe interaction is required to study the disease causality which necessitates experimental validation. Animal models have always been the subject of choice but they cannot recapitulate the human systems completely. Moreover the cross-talk between the host and its gut microbiota is host-specific and therefore this requires the model system i.e. human gut. However all experiments cannot be performed on human volunteers as it is relatively challenging to manipulate different variables on them. Therefore a system is needed that mimics the intestinal conditions and can represent GI human-microbe interface.

A microfluidics-based in-vitro model of the GI human-microbe interface named HuMiX (human-microbial crosstalk) has been reported in the journal, “Nature Communications” by researchers from the University of Luxembourg in collaboration with colleagues at the the University of Arizona. This model of human gut consists of three co-laminar microchannels viz. a perfusion chamber, which contains media, a human microchamber, which is a human epithelial cell culture microchamber and a microbial microchamber that supports the microbial growth. These microchambers are equipped with an inlet and outlet for the inoculation of cells and management of physicochemical parameters. The microchambers are separated by a porous membrane analogous to healthy epithelial cell layer. Optodes which are the oxygen sensors in the device monitors the dissolved oxygen concentration. Also the specially designed form of device accommodates an electrode to measure transepithelial electrical resistance which depicts the growth and differentiation of cell inside the device.

The researchers demonstrated the recapitulating ability of HuMiX in vivo. They co-cultured human epithelial cells (Caco-2) with GI commensals like Lactobacillus rhamnosus GG (LGG) a facultative anaerobe under anaerobic conditions as well as obligate anaerobe Bacteroides caccae along with LGG. Transcriptional response resulted were different amongst both the cultures. Results were in accordance to the published in vitro and in vivo data sets which makes HuMiX an efficient representative model of the GI human-microbe interface. Co-culture of Caco-2 cells with LGG and some strains of other bacterial species like Lactobacillus rhamnosus, were found to produce gamma-aminobutyric acic (GABA) a neurotransmitter. This makes HuMiX a suitable tool to study gut-brain axis and communication in future.

Thus HuMiX can be used as a key device to facilitate the exploration of host-microbe interactions and molecular processes behind these interactions. It could possibly be used in various other areas like probiotics, pharmacokinetics, drug discovery and development. It helps to comprehend microbiome impact on the actual gut milieu and in human health and disease onset. According to the authors, HuMiX is considered as a substitute to animal models for first-pass experiments which might help replace the animal testing in future. Similarly, the modular architecture of the regions apart from GI tract can also be designed to explore and study the different human organs. A wider and deeper understanding of the gut and the other regions could open the doors to new research avenues that can help us device personalized treatments and right therapeutic strategy in terms of patient treatment and better patient management.

REFERENCES

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The Editorial Board gratefully acknowledges the painstaking effort of the following reviewers of this issue who have contributed to our journal in building a strong peer reviewer system.

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INSTRUCTIONS FOR AUTHORS

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SCOPE

The journal will cover studies related to gastrointestinal (GI) infections. The Journal of Gastrointestinal Infections is devoted to the dissemination of new knowledge concerning the various aspects of human infections and infestations associated with GI tract. The journal will publish peer-reviewed original research papers, case reports, systematic reviews and meta-analysis. Current reports can be submitted as brief communications which must include review of current literature, clinical details, outcome and follow up. Letters to the editor must be a comment on or pertain to a manuscript already published in the journal or in relation to preliminary communication of a larger study. The journal will also regularly publish Review articles, Special Articles or Guest Editorials on various topics in GI infections, accepted on invitation. But those who are desirous of submitting a review article, special article or guest editorial are required to send a one page outline to the Editor-in-Chief.

EDITORIAL PROCESS

The manuscripts will be reviewed for possible publication with the understanding that they are being submitted solely to JGI and have not been published, simultaneously submitted or already accepted for publication elsewhere. The registered articles will be initially reviewed by the Editorial Board to judge their suitability for publication and then sent for peer review. Manuscripts with insufficient originality, serious scientific and technical flaws or lack of a significant message will be rejected. All accepted manuscripts will be suitably edited before publication and print proof will be sent to the corresponding author which has to be returned within 2 days. Corrections received after that period may not be included. The contributors will be informed about the acceptance/rejection of manuscript.

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Authorship criteria

All authors of a manuscript must have agreed to its submission and are responsible for its content (initial submission and any subsequent versions), including appropriate citations and acknowledgments, and must also have agreed that the corresponding author has the authority to act on their behalf in all matters pertaining to publication of the manuscript. For a study from a single institute the number of contributors should not exceed six. For a case-report, brief communication, letter to the editor and review article the number of contributors should not exceed four. A justification should be included, if the number of contributors exceeds these limits. Once submitted the order of authorship cannot be changed without written consent of all the contributors. All the authors should submit an undertaking in the format specified by the journal indicating their consent to agree upon the authorship in the sequence indicated on the title page as well as the undertaking.

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TYPES OF MANUSCRIPTS AND LIMITS

Submission Articles in the following categories are published:

**Original Articles:** Cover topics relevant to clinical and basic studies in the areas of gastrointestinal infections. Both adult and pediatric problems are included and clinical studies relevant to the care of critically ill patients may be submitted for publication. (Maximum 3000 words excluding about 30 references and structured abstract).

**Review articles:** Current topics which are usually those specially requested by the editorial board from persons who have done substantial work on the given subject. (Up to 4000 words excluding about 90 references and abstract)

**Case Reports:** New / interesting / rare cases can be reported. (Up to 1000 words excluding about 10 references and abstract)

**Brief communication:** Study with clinical interest or unusual presentation of a disease can be sent. (Up to 1500 words and 10 references). Illustrations and tables, when included, should be limited to one each. It should have an abstract limited to 100 words.

**Correspondence:** Correspondence offers opinions on papers published in Journal of Gastrointestinal Infections. It should be limited in length to 300 words and should be continuous without headings. It should not include more than 5 references and one table or figure. Letters commenting on papers are sent to the authors of those papers for a response.
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3) **Keywords:** 3 to 8 keywords, arranged alphabetically.

4) **Introduction:** Brief, most recent essential background only.

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Diagnostic algorithm in patients with suspected inflammatory bowel disease

Patients with suspected inflammatory bowel disease (IBD)

Measurement of faecal calprotectin

>50 µg/g

Urgent endoscopy

positive

Appropriate treatment

<50 µg/g

IBD unlikely

negative

Plan other investigations


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