CHANGES IN BACTERIAL PROFILE DURING AMEBIASIS: DEMONSTRATION OF ANAEROBIC BACTERIA IN ALA PUS SAMPLES

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Abstract: Little is known about the changes in gut resident flora during amebic colitis and amebic liver abscess (ALA) caused by *Entamoeba histolytica* infection. Fecal samples from ALA patients, from healthy *E. histolytica* negative and positive (asymptomatic) individuals, and from pre- and post-metronidazole–treated healthy volunteers and pus samples from ALA patients were tested for the presence of various bacterial genera using 16S rRNA–based primers. Statistically significant reduction in *Lactobacillus* due to *E. histolytica* infection was observed in asymptomatic individuals and ALA patients. On the other hand, reduction in *Bacteroides*, *Bifidobacterium*, and *Clostridium* in the same samples was due to metronidazole treatment. Two anaerobic genera, viz. *Bacteroides* and *Peptostreptococcus*, were detected in ALA pus samples, and this observation is unprecedented. In addition, PCR revealed metronidazole resistance genes in fecal and pus samples of metronidazole-treated individuals. Re-examination of the ameba-bacterium relationship in amebiasis is suggested.

INTRODUCTION

Amebiasis is a common worldwide disease in developing countries, caused by infection with the protozoan parasite *Entamoeba histolytica*. About 40,000 people are estimated to die each year from amebic colitis and amebic liver abscess (ALA).[1] In most infected individuals, trophozoites in the intestine live as commensals without causing any noticeable damage to the host. A small fraction of *E. histolytica*–infected people present with clinical symptoms of colitis or extraintestinal invasion. The total number of infected individuals is very high (up to 20% of the Indian population). In India ALA is endemic.[2] Most patients with an amebic liver abscess do not have co-existent amebic colitis.[3]

In their natural environment, trophozoites of *E. histolytica* live in the colonic region of the human intestine together with resident microbial flora, which under normal conditions is composed of a complex mixture of mostly anaerobic or microaerophilic bacteria.[4] The predominant anaerobic species in this flora belong to *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Peptococcus*, *Peptostreptococcus*, and *Ruminococcus*, whereas facultative anaerobes, such as *Escherichia coli*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus*, and *Proteus*, are among the subdominant genera.[5] The composition of the individual's flora can fluctuate in conditions like acute diarrhea[6] and antibiotic treatment. Although the microbiota in healthy adults have been studied extensively, little is known about the changes in flora that occur in protozoan-associated gastrointestinal infection.

It has been suggested that the bacterial flora provides anaerobic conditions or low redox potential beneficial for amebic growth.[7] Trophozoites of *E. histolytica* grown in association with bacteria are active feeders that phagocytose numerous bacteria.[8] *E. histolytica* appears to be selective with respect to association with different bacterial species, and only those bacteria that possess the appropriate recognition signals will become attached and ingested by the ameba. It

has been speculated that certain bacterial species of the gut may trigger the virulent potential of the trophozoites while others may have no effect or may even cause avirulence.[9] Metronidazole is known to be clinically more effective against ameba in tissue than lumenal ameba.[10] This may be, in part, due to anaerobic bacteria that co-exist with the amebic trophozoites *in vivo* may bring about the reduction and elimination of metronidazole molecules needed for the killing of amebic trophozoites.[10] The incidence of metronidazole-resistant anaerobic bacteria, especially some species of *Bacteroides*, appears to be on the rise.[11] A molecular study demonstrated that the moderate resistance phenotype of these clinical strains was in all cases associated with the presence of the 5-NI (nitroimidazole) resistance gene (*nim*).[12]

Systematic studies are lacking to provide a clinical correlation between resident bacterial flora of the gut and the severity and spectrum of amebic disease. In the present study, we have looked into the profile of predominant gut flora of healthy individuals, asymptomatic *E. histolytica* carriers, and amebic liver abscess patients. We have also addressed the issue whether the changes observed in the gut flora may be attributed to the administration of metronidazole or the presence of the parasite. We have also looked for the presence of gut bacteria in the liver abscess aspirates. The prevalence of the metronidazole-resistance gene (*nim*) was also scored in these bacterial species. Our results indicate a significant loss of some predominant gut bacteria in amebiasis patients and the presence of some anaerobic species in amebic liver abscess aspirates.

MATERIALS AND METHODS

Subjects. The present study included 35 patients with amebic liver abscess admitted to the Gastroenterolgy Department (All India Institute of Medical Sciences, New Delhi, India) and 30 healthy individuals who were residing in a slum area of New Delhi. Healthy individuals tested ranged in age from age 6 to 40 years and had not been on antibiotics for 3 months prior to sample collection. They had no complaints of diarrhea, abdominal pain, or fever. Nineteen of 30 individuals were negative for *E. histolytica* and were therefore considered as "healthy, *E. histolytica* negative." The remaining 11 (out of

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30) individuals were positive for *E. histolytica*, therefore considered as "healthy *E. histolytica* carriers" (asymptomatic).

Samples were also collected before and after metronidazole treatment (300 mg twice per day; 3-day course) from a group of 11 individuals: 8 healthy volunteers (residents of urban area, Jawaharlal Nehru University Campus, India) and 3 patients suffering from irritable bowel syndrome (IBS) reporting to AIIMS.

Informed consent was obtained from patients and healthy individuals prior to sample collection. Amebic liver abscess patients were from different economic backgrounds and came from different local areas in Delhi. Diagnosis of amebic liver abscess was based on four or more of the following criteria: (i) A space, occupying lesion in the liver diagnosed by ultrasonography and suggestive of abscess; (ii) clinical symptoms (fever, abdominal pain), (iii) enlarged and/or tender liver, usually without jaundice, (iv) in some cases diarrhea, and bloody diarrhea in a few cases, and (v) positive amebic serology. Of 35 patients, 33 were positive for amebic serology with ELISA Kit containing IgG antibodies against *E. histolytica* (R-Biopharm AG, Darmstadt, Germany). To avoid any secondary infection, amebic liver aspirates were collected only from those patients who were being aspirated for the first time. Out of 35 ALA patients, fecal samples were collected from 19 ALA patients.

Most of the ALA patients were males (33/35). Most of them had consumed metronidazole (mean duration of drug intake, 6 days).

Fecal and ALA pus specimens. Fecal and pus samples were collected from various categories of individuals, as listed in the Tables 1 and 2. Aliquots of all fecal samples as well as pus samples were preserved at –20°C till DNA isolation was done. The aspiration procedure for ALA was carried out in a sterile environment in the Intensive Care Unit by a clinician with the required precautions.

Extraction of DNA from fecal or ALA pus specimens. Fecal (0.2 g) and ALA pus samples (500 μL) were used for DNA extraction. DNA was extracted using the QIAamp DNA stool mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions except that the suspension was incubated in the kit's stool lysis buffer at 95°C followed by a 3-minute incubation with the InhibitEx tablets. The DNA was eluted in 0.2 mL of AE buffer (supplied with the QIAGEN kit).

TABLE 1
Prevalence of selected bacteria in fecal samples from healthy individuals and ALA patients

Bacteria	1 (n = 19)	2 (n = 11)	3 (n = 19)	1 vs 2	1 vs 3	2 vs 3
Bacto	12	9	3	–	0.0069	0.0013
Bif	16	8	4	–	0.0002	0.0086
PSP	12	10	14	–	–	–
Clos	14	8	4	–	0.0029	0.0086
Lacb	18	4	3	0.0011	0.0001	–
Rum	17	7	12	–	–	–
Camp	7	7	10	–	–	–
Pep	12	9	8	–	–	–
E. coli	10	2	12	–	–	0.0259
Pseudo	6	10	7	0.0024	–	0.0067

P value header spans columns 1 vs 2, 1 vs 3, 2 vs 3.

* Only the significant *P* values are indicated; 1, healthy individuals, *E. histolytica* negative; 2, healthy individuals, *E. histolytica* positive; 3, stool samples from ALA patients.

TABLE 2
Effect of metronidazole* on the prevalence of selected bacteria in fecal samples

Bacteria	4 (n = 11)	5 (n = 11)	*P* value 4 vs 5
Bacto	11	2	0.0002
Bif	11	4	0.0039
PSP	11	8	–
Clos	11	5	0.0124
Lacb	11	11	–
Rum	10	5	–
Camp	9	1	0.0019
Pep	11	6	0.0351
E. coli	2	8	0.0300
Pseudo	7	4	–

* In individuals not suffering from amebiasis (healthy volunteers and IBS patients).
† Only the significant *P* values are indicated; 4, before metronidazole treatment; 5, after metronidazole treatment.

PCR using genus-specific primers. Universal primer set; *S*-D-Bact-0008-a-S-20 (5′ AGA GTT TGA TCC TGG CTC AG 3′,[13] which targets the domain *Bacteria*, and S-*-Univ-1492-b-A-21 (5′ ACG GCT ACC TTG TTA CGA CTT 3′,[14] which targets all living organisms, were used to amplify bacterial 16S rDNAs from the collected samples to ensure the DNA quality. Later genus-specific primers were used to amplify the selected bacteria DNA from fecal and pus samples.

For all primer sets, 30-cycle PCR at different annealing temperatures was performed in a Tech gene thermal cycler (NuGEN Scientific, San Carlos, CA).[15] Genus-specific primer sets were designed from 16S rRNA sequences of the following bacteria: *Bifidobacterium* (Bif), *Clostridium* (Clos), *Ruminococcus* (Rum), *Campylobacter* (Camp), *Pseudomonas aeruginosa* (Pseudo), *Lactobacillus* (Lacb), and *Peptococcus* (Pep). Specificity of these primers was checked by using target and non-targeted bacterial genomic DNA as described previously.[15] Primers for *Bacteroides* (Bacto) and *Peptostreptococcus productus* (PSP) were obtained from the literature.[16,17] All primers were commercially synthesized by Microsynth GmbH (Balgach, Switzerland).

Primer set used for the detection of *Escherichia coli* was based on the *mal* B gene[17] while for *Staphylococcus aureus*, the primers mecA-1 and mecA-2 used were synthesized from methicillin-resistant gene.[18]

Genomic DNA isolated from human blood (Genomic DNA isolation kit, MBI Fermentas, St. Leon Rot, Germany) was used as a template DNA for ensuring primers' specificity. PCR products derived from healthy individuals' fecal DNA were cloned and sequenced. These were used as controls in subsequent PCR experiments.

Detection of *E. histolytica*-positive samples. For the PCR-based detection of *E. histolytica* among all fecal and pus samples, *E. histolytica*-specific UEE primers were used.[19]

Detection of *nim* genes. PCR assays were carried out using the universal set of primers, Nim-3 and Nim-5, for all known *nim* genes.[20] These primers amplify a PCR product of 458 bp. Amplification was done under conditions as previously described[21] using an annealing temperature of 52°C. Wild-type strain *Bacteroides fragilis* (MTCC 1045) was used as a negative control.

Selected amplicons of *nim* genes (size = 458 bp) obtained for fecal and pus samples, were purified using a Qiagen Gel Extraction kit and cloned into a pGEMT-Easy vector

(Promega Co., Madison, WI) as per the manufacturer's instructions. Clones were further sequenced.

Analysis of sequenced data. Sequences obtained for representative cloned amplicons from each category were analyzed using the BLASTN program for a homology search within the existing database for each genus.

Statistical analysis. One-way ANOVA and Fisher's exact tests were performed to check the statistical significance of the data. Statistical significance was accepted at the level of 0.05, and probability values were calculated for two-tailed possibilities.

RESULTS AND DISCUSSION

Changes in gastrointestinal flora. In their natural habitat, the human large intestine, *E. histolytica* trophozoites co-exist with hundreds of different bacterial species. The contribution of these bacteria to the viability and virulence potential of *E. histolytica* has been difficult to establish. Previous studies have shown that trophozoites administered intracecally in germ-free animals failed to establish themselves and produce disease, whereas the introduction of single bacterial species prior to administration of *E. histolytica* trophozoites in the same animal resulted in active infection.[22] *E. histolytica* is known to efficiently phagocytose bacteria that may be a source of nutrients for the growing trophozoites.[9] In addition, bacteria may provide the optimum conditions of pH and low redox potential needed for *E. histolytica* survival. For a better understanding of ameba-bacteria relationship, it is important to determine what perturbations occur, if any, in the human gut flora as a result of *E. histolytica* infection.

In the present study, two major aspects have been dealt with: (i) changes in gastrointestinal flora as a result of *E. histolytica* infections and (ii) bacterial co-infection in amebic liver abscess.

Stool samples were analyzed from the following categories of individuals: (1) healthy *E. histolytica* negative ($N = 19$); (2) healthy, asymptomatic, *E. histolytica* positive ($N = 11$); and (3) ALA patients ($N = 19$). Pus samples were also analyzed from ALA patients ($N = 35$) for the presence of 11 prominent, mostly anaerobic bacteria found in the human gut. Bacterial detection was done by PCR amplification of total DNA from samples, using genus specific primers for 16S rRNA gene. The specificity of detection was ascertained by sequencing the PCR-amplified bands and confirming their identity by comparing with genus specific sequences available in the database.

Figure 1 represents typical pattern for the PCR amplification of various genera.

Percent incidence values were calculated from the PCR results (Table 1). The significance of the PCR-based percent incidence results was evaluated by statistical analysis. One-way ANOVA test and two-tailed Fisher's exact test indicated a significant variation in fecal flora in the various categories tested (Table 3). To confirm the results of PCR analysis from stool and pus samples, randomly selected amplicons from each primer set were cloned and sequenced. BLASTN results revealed 94–100% identity with 16S rRNA sequences of the corresponding bacterial species (from which the primer was designed; data not shown). The sequences were submitted to the database, and accession numbers were obtained (Table 4). The high level of sequence identity showed that the assign-
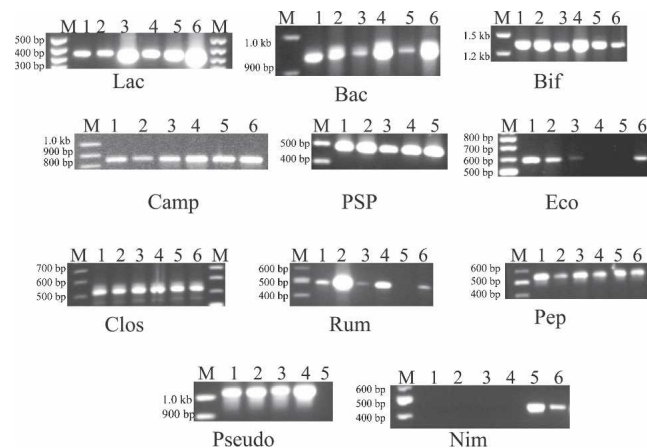

FIGURE 1. Typical PCR profile from fecal samples, using 16S rRNA sequence–based primers from the indicated bacteria. Abbreviations are as in Materials and Methods. Expected amplicon sizes (in bp) for each genus are as follows: Bacto, 948; Clos, 619; Nim, 458; Camp, 969; Rum, 489; Lacb, 318; Pep, 539; Eco, 585; PSP, 267; Pseudo, 1078; and Bif, 1300 (M = 100-bp marker). Profiles shown correspond to the healthy volunteers of Table 2.

ment of bacterial genera based on 16S rRNA was reliable. Species names mentioned in Table 4 are based on the highest score values in BLAST N results for the query sequence.

There was a statistically significant drop in the frequency of four of the 11 bacterial genera, namely, *Bacteroides* ($P = 0.0069$), *Bifidobacterium* ($P = 0.0002$), *Lactobacillus* ($P = 0.0001$), and *Clostridium* ($P = 0.0029$) in fecal samples of ALA patients (percent incidence = 16–22%) when compared with healthy, *E. histolytica*–negative controls. Of these, three genera (*Bacteroides*, *Bifidobacterium*, and *Clostridium*) were not reduced in healthy asymptomatic carriers (*E. histolytica*–positive) compared with healthy *E. histolytica*–negative individuals (Table 1). However, *Lactobacillus* was reduced in *E. histolytica*–asymptomatic carriers also (percent incidence = 36.3%; $P = 0.0011$). Asymptomatic carriers also showed a statistically significant decrease in the incidence of *E. coli* and increase in *Pseudomonas aeruginosa* compared with the other two categories of individuals. No significant changes were observed for *Peptostreptococcus productus*, *Ruminococcus*, *Campylobacter*, and *Peptococcus* among healthy controls and ALA patients (Table 1).

Because ALA patients were on metronidazole treatment for ethical reasons, and *E. histolytica*–asymptomatic carriers had not taken metronidazole treatment, it was possible that, the observed reduction in *Bacteroides*, *Bifidobacterium*, and *Clostridium* in ALA patients alone was due to metronidazole treatment while reduction in *Lactobacillus* seen in both ALA patients and asymptomatic carriers was due to *E. histolytica* infection.

TABLE 3
One-ANOVA test results*

Source of variation	Sum of squares	Degrees of freedom	Mean square ($= S$ of S/df)	F ratio = Mean square due to individual/ residual mean square
Due to individual	158.17	2	79	5.95
Residual	358	27	13.259	
Total	516.17	29		

H_0, no variation in bacterial flora among different categories; H_1, variation in bacterial flora among different categories; Tab $F_{(2,27)(0.05)} = 3.4$; Cal $F_{(2,27)(0.05)} = 5.95$. Because Cal $F >$ Tab F, H_0 is therefore rejected and H_1 is accepted.

TABLE 4
NCBI database accession numbers obtained for sequences of cloned products

Bacteria	Source	Accession no.
Bacteroides species	Healthy Eh −ve	AM117604
B. vulgatus	Healthy Eh −ve	AM042696
B. species	Healthy Eh +ve	AM117577
B. species	ALA stool sample	AM117578
B. species	ALA plus sample	AM117579
Uncultured *Clostridium* species	Healthy Eh −ve	AM042697
C. species	Healthy Eh +ve	AM117582
C. species	ALA stool sample	AM117583
C. species	ALA stool sample II	AM117584
Uncultured *Bifidobacterium* species	Healthy Eh −ve	AM042698
B. species	Healthy Eh +ve	AM117580
B. species	ALA stool sample	AM117581
Campylobacter coli	Healthy Eh −ve	AM042699
C. species	Healthy Eh +ve	AM117593
C. species	ALA stool sample	AM117594
Peptococcus species RR-2005	Healthy Eh −ve	AM042700
Peptococcus species	Healthy Eh +ve	AM117585
P. species	ALA stool sample	AM117586
L. acidophilus	Healthy Eh −ve	AM042701
L. species	Healthy Eh +ve	AM117595
L. species	ALA stool sample	AM117596
Peptostreptococcus productus	Healthy Eh −ve	AM117587
P. productus	Healthy Eh +ve	AM117588
P. productus	ALA stool sample	AM117589
P. productus	ALA pus sample 10	AM117590
P. productus	ALA pus sample 17	AM117591
P. productus	ALA pus sample 21	AM117592
Ruminococcus species	Healthy Eh −ve	AM117597
R. species	Healthy Eh +ve	AM117598
R. species	ALA stool sample	AM117599
E. coli	Healthy Eh −ve	AM117600
E. coli	ALA stool sample	AM117601
nim gene	ALA stool sample	AM117602
nim gene	ALA pus sample	AM117603

* Species were assigned on the basis of the highest score values in the BLAST results.

This was confirmed by directly testing the effect of metronidazole on the gut flora of healthy volunteers (residents of urban area) and IBS patients who were not suffering from amebiasis. Bacterial flora in fecal samples of urban area resident healthy volunteers (taken before metronidazole treatment) differed from the slum area resident healthy volunteers. This could be due to differences in their living conditions and dietary habits.[23] These individuals were given metronidazole for a period of 3 days. None of these individuals was *E. histolytica*–positive, as tested by *E. histolytica*–specific UEE primers.[21]

There was no change in the incidence of *Lactobacillus* in these individuals as a result of metronidazole treatment, although there was a marked drop in the incidence of *Bifidobacterium*, *Bacteroides*, and *Clostridium*. Metronidazole is known to be very effective against obligate anaerobes, which explains the drop in *Bifidobacterium*, *Bacteroides*, and *Clostridium*.[24] However, *Lactobacillus*, being facultative anaerobe, is not significantly affected by this drug. Thus, our results show that, of the 11 bacterial genera tested, at least *Lactobacillus* is significantly reduced in individuals infected with *E. histolytica*. Such a loss of anaerobic flora has been reported under conditions of severe diarrhea[25] and *Clostridium difficile*–associated diarrhea.[26]

In keeping with the facts that metronidazole is effective only against obligate anaerobic bacteria and that *P. aeruginosa* is a strictly aerobic bacterium, no change was observed in the incidence of *P. aeruginosa* in healthy volunteers and IBS patients after metronidazole administration (Table 2). Our observations regarding the low prevalence of *E. coli* in the fecal samples of healthy adult individuals could be attributed to the fact that the level of this population may have been below detectable limits before the drug treatment. This could be due to the lower sensitivity of *mal* B gene–based PCR primers used for the detection of *E. coli*. The 16S rRNA gene could not be used for *E. coli* due to the lack of specificity.[27] As a result of drug-treatment overgrowth of aerobic bacteria including *E. coli* may have occurred (Table 2), concomitant with the reduction in anaerobic bacteria as reported earlier.[28] This was also supported by a study carried out with human fecal samples in which bifidobacteria were present in higher number than *E. coli*.[29]

No significant change was observed in *P. productus* or *Ruminococcus* titers among the various categories of individuals tested. Members of these genera are likely to be resistant both to drug action as well as *E. histolytica* infection.

To our knowledge, an extensive investigation of bacterial prevalence using sensitive DNA probes has not been carried out with *E. histolytica* infected individuals. This needs to be substantiated with more patients from a variety of geographical locations. If a general pattern emerges, then a probiotic therapy to replace the depleted bacterial species could be envisaged.

Bacteria in pus samples. DNA was extracted from ALA pus samples and tested for the presence of various bacterial genera as described for stool samples. No amplification was observed with primers specific for aerobic bacteria (*E. coli*, *S. aureus*, and *P. aeruginosa*).

A remarkable observation reported in this study is the highly significant occurrence of *Peptostreptococcus* (25/35 ALA cases, 71.4% occurrence) and, less frequently, *Bacteroides* (5/35 ALA cases, 14.2% occurrence) in the pus samples of ALA patients (Figure 2). Pus from these patients is generally considered to be sterile on the basis of culturing and microscopy.[9] Because our detection method was based on PCR amplification, there could be a chance of primer cross-reaction or bacterial contamination.

This was unlikely for the following reasons: (1) The primers for *Peptostreptococcus* and *Bacteroides* did not have any match with human DNA sequences and did not give any amplification with human blood DNA. (2) All precautions were taken to collect the aspirated sample under sterile conditions; these patients had never been aspirated before, hence there was no possibility of prior infection for this reason. (3) Bacteria amplified by PCR (*Peptostreptococcus* and *Bacteroides*) were both anaerobes, while primers from aerobic species (*E. coli*, *Pseudomonas aeruginosa*, and methicillin-resistant *S. aureus*) did not give any amplification. Aerial contamination of pus samples during aspiration would be more likely due to

FIGURE 2. Amplicons for Bacto and PSP in selected ALA pus samples (Table 4). Abbreviations are as in Materials and Methods. Expected amplicon sizes (in bp) are as follows: Bacto, 948; and PSP, 267 (M = 100-bp marker).

aerobic species than anaerobes. (4) The PCR-amplified products obtained from *Peptostreptococcus*- and *Bacteroides*-specific primers were sequenced. These showed 98% and 99% identity, respectively, with the known 16S rRNA sequences of these bacteria. Thus, it is not likely that the PCR primers cross-reacted with contaminating aerobic bacteria in the pus samples. From this we can conclude that ALA pus samples do, indeed, contain DNA from *Peptostreptococcus* and *Bacteroides*. Direct demonstration of bacteria by culturing needs to be made.

Several species of *Peptostreptococcus* and *Bacteroides* including *P. productus* and *B. fragilis* are known to be involved in causing abscesses and other septicemia-related infections in humans. They are frequently associated with pyogenic liver abscess.[30] However, their involvement with ALA as suggested here has not, so far, been reported. In a previous study, superinfection of an amebic abscess with *Salmonella enteridis* has been reported.[31] However, in this case there was hemorrhage into the abscess due to physical trauma, which was followed by secondary bacterial superinfection. Reports are available about possible mechanisms of bacterial translocation to extraintestinal sites. These mechanisms, including intestinal bacterial overgrowth, are increased permeability of mucosal barrier (leaky gut syndrome) and deficiencies in host immune response.[32] Whether these mechanisms continue to operate in mixed infection of these anaerobic bacteria along with the *E. histolytica* or whether bacteria passively gain entry into the liver abscess caused by *E. histolytica* trophozoites needs to be studied. It is important to know the extent of involvement of bacteria in initiating or enhancing the severity of ALA to effectively treat these patients.

Whether the bacteria detected by us were viable cannot be assessed because PCR amplification alone was used. In an attempt to cultivate bacteria from ALA pus samples from a population of Bangladesh, only 19% samples were found to be positive for a few aerobic gram- negative bacteria like *E. coli*, *Proteus*, and *Pseudomonas*. However, presence of any anaerobic bacteria was not reported.[33] This could be due to the difficulty in cultivating anaerobes. The very high incidence of *Peptostreptococcus* reported here in pus samples (25 out of 35 ALA cases) is unprecedented. *Bacteroides* although found in 5 out of 35 ALA cases, is unreported so far from pus samples. Further confirmation of this data will require anaerobic cultivation of viable bacteria from ALA pus samples.

Presence of *nim* genes in ALA patients. In the last few years, the occurrence of *nim* genes associated with metronidazole resistance has been found to be widespread among anaerobes, including *Bacteroides*[34] and *Peptostreptococcus*.[35] Many of these *nim* genes are plasmid encoded, while some are also chromosomally located.[36] Five *nim* genes, *nimA* to -*E*, have been identified that confer reduced susceptibility to 5-nitroimidazole antibiotics (e.g., metronidazole) on species of the *B. fragilis* group.[34] The proposed resistance mechanism conferred by the *nim* genes is that they encode a 5-nitroimidazole reductase.[37] Four of the *nim* genes have been shown to be associated with different mobile insertion sequence (IS) elements flanked by inverted repeats. There is strong evidence that these IS elements carry regulatory signals for expression of certain resistance genes, including the *nim* genes.[38]

The presence of *nim* genes was determined in all fecal and

TABLE 5
Nitroimidazole-resistant gene (*nim*) in fecal samples from healthy and ALA patients

Category*	1 (*n* = 19)	2 (*n* = 11)	3 (*n* = 19)	4 (*n* = 11)	5 (*n* = 11)
nim	0	0	11	2	8

* For explanation of category, see Tables 1 and 2.

pus samples by PCR amplification with *nim*-specific primers.[20] No amplicons were observed in healthy *E. histolytica*–negative individuals and asymptomatic carriers, while amplicons of the sizes expected for *nim* gene were present in both pus and fecal samples of ALA patients (Table 5). Selected clones of representative PCR products from both fecal and pus samples of ALA patients were sequenced. Sequence analysis using BLASTN revealed 99–100% identity with *nim* E gene from *B. fragilis* (database entry) for amplicons from both fecal and pus samples. Because ALA patients were receiving metronidazole, this result shows that *nim* genes may be rapidly amplified in bacterial populations after antibiotic challenge.

Among the healthy volunteers and IBS patients who were given metronidazole treatment for a short duration (600 mg/day, for 3 days), the *nim* gene amplicon was present in two of the healthy volunteers before metronidazole treatment (percent incidence, 18.8%); this frequency increased to 72.7% after metronidazole treatment. Thus, metronidazole intake is likely to result in the rapid appearance of metronidazole-resistant bacteria in the Indian population.

The study presented here initiates discussion of the need to rethink and reconsider the ameba-bacterium relationship inside the intestine and in extraintestinal tissues. The mechanism by which anaerobic bacteria reach the amebic liver abscess and their role, if any, in amebic pathogenesis need to be explored using more experimental evidence.

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