SUPPLEMENT ARTICLE

Gastrointestinal Microflora Studies in Late-Onset Autism

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Some cases of late-onset (regressive) autism may involve abnormal flora because oral vancomycin, which is poorly absorbed, may lead to significant improvement in these children. Fecal flora of children with regressive autism was compared with that of control children, and clostridial counts were higher. The number of clostridial species found in the stools of children with autism was greater than in the stools of control children. Children with autism had 9 species of *Clostridium* not found in controls, whereas controls yielded only 3 species not found in children with autism. In all, there were 25 different clostridial species found. In gastric and duodenal specimens, the most striking finding was total absence of non–spore-forming anaerobes and microaerophilic bacteria from control children and significant numbers of such bacteria from children with autism. These studies demonstrate significant alterations in the upper and lower intestinal flora of children with late-onset autism and may provide insights into the nature of this disorder.

Autism is characterized by delays in understanding and use of language, unusual response to sensory stimuli, insistence on routines and resistance to change, and difficulties with typical social interactions. Some children are aggressive, and some have even been described as animalistic. It is a devastating disease for the children and their families. The disease usually manifests itself in early infancy [1], but in at least one-third of patients, the onset is delayed until age 18–24 months [2]. Autism occurs in 1 of every 500 births [3], but some recent studies have indicated an increasing incidence. Some 10% of cases have a genetic background, but no underlying etiology can be determined in the majority of patients [4]. Therapy of autism has centered around intensive speech and language therapy, intensive psychological intervention, and behavior modification. This treatment helps, but it is labor-intensive and expensive. Anecdotal reports have indicated that patients on a gluten- and casein-free diet may show improvement. It has been hypothesized that antimicrobial use might disrupt the indigenous intestinal flora and allow colonization by $\geq$1 organisms that produce a neurotoxin [5], because a number of parents date the onset of the regressive form of the disease to antimicrobial administration that was followed by chronic diarrhea and gradual evolution of autistic symptoms. On the basis of this hypothesis, an open-label trial of oral van-

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comycin was done [6] that led to improvement in a number of parameters in 8 of 10 children studied. The purpose of the present study was to do microbiological studies of the intestinal contents of autistic and control children in hopes of detecting ⩾1 organisms unique to the flora of the children with autism.

PATIENTS, MATERIALS, AND METHODS

Study patients. Stool specimens were obtained at Rush Children's Hospital, Chicago, under the jurisdiction of its Institutional Review Board (IRB) and with written informed consent by a parent or guardian. Specimens of gastric juice and duodenal/jejunal fluid were collected at the Children's Hospital Medical Center, Cincinnati, by upper gastrointestinal endoscopy (via the mouth) under the jurisdiction of its IRB and with written informed consent of a parent or guardian. The patients with autism had late-onset disease, and all had gastrointestinal symptoms, primarily diarrhea and/or constipation. Many of these patients were on a gluten-free, casein-free diet; we are not aware of any studies that have indicated whether such a diet influences the makeup of the bowel flora. All patients had received no antibacterial agents for at least 1 month before the study. A number of the patients were on oral nystatin or fluconazole.

Fecal flora studies. Stool specimens were shipped to the VA Medical Center, West Los Angeles, by overnight air express. The entire stool specimen was collected in a grocery store–type Ziploc bag; all the air was squeezed out manually and closure effected. Specimens were then frozen at −70°C until they could be shipped, packed in dry ice. All microbiological manipulations were done in an anaerobic chamber, and all media were prereduced. The entire stool specimen was homogenized by use of a sterile stainless steel blender with 1–3 volumes of peptone (0.05%) added as diluent, if needed. An aliquot of the specimen of ~1 g weight was used, and serial 10-fold dilutions were made in prereduced, anaerobically sterilized (PRAS) dilution blanks (Anaerobe Systems). Another aliquot was weighed before and after thorough drying in a vacuum oven to permit the calculation of counts on a dry-weight basis. Initially, culturing was done on 27 different media or incubation setups; this proved to be so formidable that we subsequently elected to culture primarily for clostridia. Various dilutions were plated (100 μL/plate) onto brucella blood agar (BAP; Anaerobe Systems), CDC ANA blood agar (BBL Microbiology Systems), egg yolk agar (EYA; Anaerobe Systems), and brain-heart infusion blood agar (SBA; Becton Dickinson) with trimethoprim (Sigma Chemical; final concentration, 4 mg/L) and sulfamethoxazole (Sigma; final concentration, 1 mg/L). Additional sets of dilution tubes, 1 heated at 70°C and the other at 80°C for 10 min, were also inoculated onto sets of the above media. Plates were incubated in the anaerobic chamber at 37°C for 120 h. Single colonies were selected, described, and identified according to standard methods [7, 8]. Analysis of metabolic end products, analysis of cellular fatty acids, PCR of the 16S–23S spacer region, and 16S rRNA sequencing were performed on all gram-positive anaerobic rods and selected other organisms.

Gastric and small-bowel specimens. These specimens were collected in 6-mL crimped-top anaerobic vials with silicone-coated polytetrafluoroethylene septa, maintained at room temperature, and shipped to the VA Medical Center, West Los Angeles, by overnight air express. All work was performed in anaerobic chambers with prereduced media. Specimens were serially diluted in PRAS dilution blanks and plated to BAP, CDC, EYA, SBA, and chocolate agar with 10 mg/L pyridoxal (CAP; Becton Dickinson). Dilution blanks were then heated at 70°C for 10 min and plated onto another set of the above plates. CAP plates were incubated in a CO_2-enriched atmosphere for 48 h. The other plates were incubated anaerobically at 37°C for 120 h. Isolates were selected and identified as described above, except that aerobic isolates were identified by other standard methods [9]. The pHs of gastric specimens were determined with a pH meter probe unless the volume was too small, in which case litmus paper was used.

16S rRNA gene sequencing. Genomic DNA was extracted and purified from cells in the midlogarithmic growth phase with a QIAamp DNA Mini kit (Qiagen). The PCR products of the 16S rRNA gene fragments were generated using universal primers to the 16S rRNA gene. The almost complete 16S rRNA gene was amplified between positions 8 and 1485 (*Escherichia coli* numbering) with 2 pairs of primers (8UA, 907B and 774A, 1485B). The amplification was performed for 35 cycles at 95°C for 30 s for denaturation and at 50°C for 2 min for extension. The PCR product was excised from a 1% agarose gel after electrophoresis and purified, using a QIAquick Gel Extraction kit (Qiagen). It was then sequenced directly with an ABI 377 sequencer (Applied Biosystems). Sequences were compared with sequences in the Ribosomal Database Project (RDP) 16S rDNA database (release 7.0), using the SIMILARITY_RANK and CHECK_CHIMERA software, and with GenBank sequences, using BLAST software, and the percentage similarity to other known sequences was determined.

Statistical analysis. The difference between the mean counts of the clostridia and ruminococci, taken together, for the stools of the children with autism and the control children's stools was analyzed by Student's *t* test, using the logs of the numbers and assuming both equal and unequal variance.

Institutional review. The protocols and informed consent forms were approved by the IRBs on human experimentation and the research committees of all institutions involved, and all research was done in accordance with the ethical standards of these committees and with the Helsinki Declaration of 1975, as revised in 1983.

RESULTS

Stool data. The clostridia and ruminococci (the latter organisms often survive the procedures designed to select out clostridia from mixtures) recovered from the fecal samples of 13 children with autism and of 8 control children are listed in table 1. In all, we encountered 25 different species of *Clostridium* and 6 of *Ruminococcus*. There were 23 species of *Clostridium* and 5 of *Ruminococcus* found in the autistic group and 15 clostridial species and 5 ruminococcal species in the control group. The number of these strains per specimen ranged from 2 to 10 in the children with autism and from 3 to 12 in the controls; the average number of strains per specimen was 6 in both groups. The peak counts of these organisms ranged from 10^2 to 10^9 in the children with autism and from 10^4 to 10^8 in the controls; the geometric mean count was 1 log higher in the stools of children with autism (2.1×10^6 vs. 1.6×10^5). The statistical analysis of the counts showed that they were significantly different ($P = .0393$ under the assumption of equal variance and $P = .0289$ under the assumption of unequal variance). A value of ⩾98% similarity to the closest RDP/GenBank organism indicates identification to the species level or to a genomically closely related species, and this figure applied to 21 (67.8%) of 31 species isolated. A value of 99% similarity is essentially diagnostic of identification to the species level; this applied to 17 (54.8%) of 31 isolates. Organisms that displayed >2% sequence divergence with described species were considered novel species. Figure 1 is a phylogenetic tree that shows the relationships of the clostridia and ruminococci isolated from both the autistic and the control children.

Gastric and small-bowel data. Data on 7 children with autism and 4 controls are presented in table 2 and table 3, respectively. The gastric pH was elevated in 2 of 4 children with autism who had this measurement (these children had not been receiving H_2 blockers or proton pump inhibitors). Two children with autism (patients 1 and 2) had only duodenal/jejunal fluid studied, one (patient 1) of whom also had jejunal biopsy material studied. These were the first specimens we received, and they were not collected or transported in optimum fashion. One of the control children did not have gastric juice sampled. Two patients with autism had no organisms recovered from either the gastric or the duodenal specimens, and a third had only 3 species of aerobes recovered. All 3 of these subjects and all control subjects had normal gastric pH. One of these 3 patients had never had either diarrhea or constipation, and a second had had a number of courses of antimicrobial agents for recurrent sinusitis, although he had not had any antimicrobials during the month prior to his endoscopy. The most striking finding was that non–spore-forming anaerobic and microaerophilic bacteria were totally absent from the control children's specimens. In contrast, 4 of 5 children with autism whose specimens yielded any growth at all had such organisms present.

Table 1. *Clostridium* and *Ruminococcus* species isolated from stool specimens of children with autism and control children.

Identified or nearest known species	Similarity,[a] %	Highest counts (cfu/g) in stool specimens	
		From autistic children	From control children
Clostridium spp.			
C. aminobutyricum	92.7[b]	2.0×10^5	—
C. bifermentans	95.0	2.0×10^9	—
C. butyricum	100.0	1.6×10^3	3.2×10^4
C. clostridioforme	95.0	4.0×10^5	—
C. cocleatum[c]	93.5	3.0×10^4	—
C. difficile[d]	100.0	8.0×10^2	—
C. disporicum 1	98.3	—	1.8×10^4
C. disporicum 2	98.1	3.0×10^6	4.2×10^6
C. glycolicum	97.5	4.0×10^5	9.0×10^5
C. innocuum	98.7	2.0×10^9	3.0×10^4
C. lactifermentum	99.7	8.0×10^5	3.0×10^5
C. nexile	96.0	3.0×10^7	—
C. orbiscindens 1	99.8	8.0×10^7	3.0×10^6
C. orbiscindens 2	97.9	4.0×10^4	9.0×10^2
C. orbiscindens 3	97.0	4.6×10^4	—
C. paraputrificum	99.9	2.1×10^6	3.0×10^6
C. perfringens	99.9	1.8×10^4	2.1×10^5
C. ramosum	100.0	6.0×10^7	—
C. roseum	99.6	3.2×10^3	—
C. scindens	99.2	9.0×10^7	—
C. sordellii	98.6	4.8×10^4	1.2×10^4
C. spiroforme	99.5	4.0×10^4	1.8×10^6
C. subterminale	99.4	1.0×10^8	3.0×10^3
C. symbiosum	99.0	4.0×10^9	6.0×10^3
C. tertium	99.9	—	2.7×10^6
Ruminococcus spp.			
R. albus	94.9	—	1.3×10^6
R. flavefaciens	92.0	1.2×10^7	1.2×10^3
R. gnavus	99.8	4.0×10^9	1.7×10^4
R. lactaris	99.3	6.0×10^5	9.0×10^5
"R. luti"	100.0	3.0×10^9	1.2×10^8
R. torques[c,d]	99.1	4.0×10^5	—

[a] Similarity to the closest Ribosomal Database Project/GenBank organism, as determined by use of nearly full-length 16S rDNA sequences.
[b] Isolates with <98% sequence similarity with described species can be regarded as novel species.
[c] Not shown in figure 1: the sequences are not available.
[d] Isolated from a child with autism after mebendazole treatment.

Two of those 4 children with autism had 7–9 different species of non–spore-forming anaerobes or microaerophiles in each of their gastric and duodenal fluids.

In terms of clostridia, both the gastric and small-bowel specimens from children with autism were more likely to have clostridia and were more likely to have a higher number of

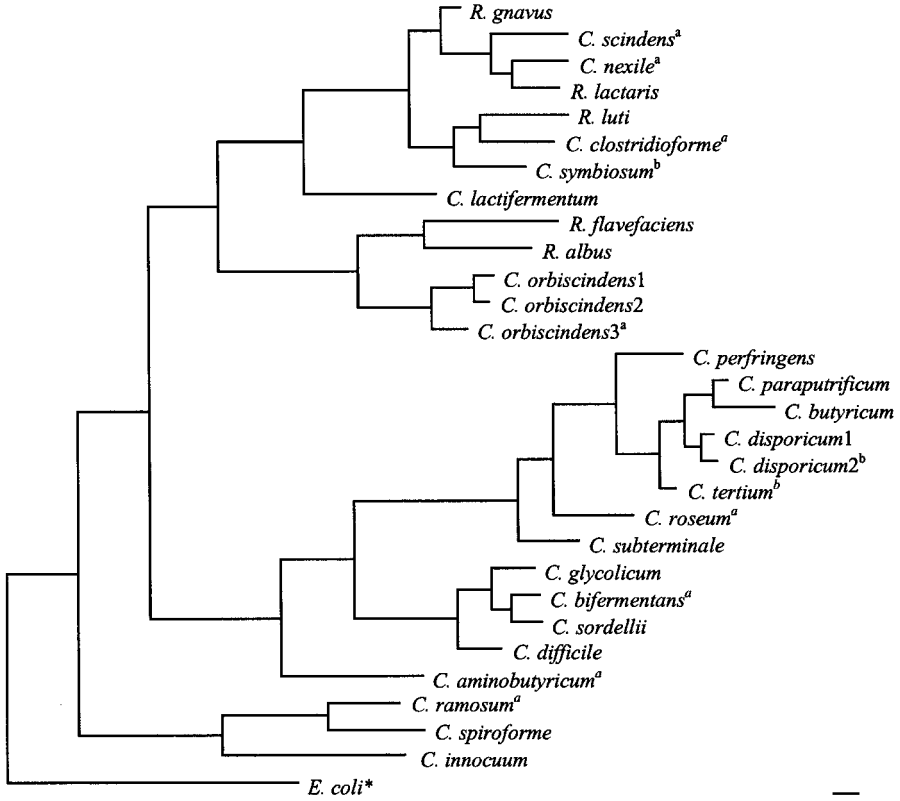

Figure 1. Dendrogram showing the interrelationships within the *Clostridium* and *Ruminococcus* species isolated from autistic and control children's stool specimens. The bar denotes 1% sequence divergence. The asterisk (*) denotes the outgroup standard. [a]*Clostridium* species isolated only from the stool specimens of children with autism. *Clostridium cocleatum* is not shown in this figure because the sequence data are not available. [b]*Clostridium* species isolated only from the stool specimens of control children.

species of clostridia than was true for control specimens of these types. Geometric mean counts, however, were somewhat higher in the control specimens. All the clostridial species isolated from the gastric and/or small-bowel specimens were also recovered from fecal samples (different patients) except for *Clostridium acetobutylicum/beijerinckii, Clostridium ghonii/Eubacterium tenue,* and *Clostridium cochlearium. Candida albicans* was found in 1 gastric fluid and 2 duodenal fluid samples from control patients; non-*albicans Candida* was found in the gastric fluid of a child with autism.

DISCUSSION

Reasons to consider that microorganisms may be involved in late-onset autism include the following: (1) onset of the disease often follows antimicrobial therapy, (2) gastrointestinal symptoms are common at onset and often persist, (3) other antimicrobials (e.g., oral vancomycin) may lead to a clear-cut response and relapse may occur when the vancomycin is discontinued, and (4) some patients have responded to several courses of vancomycin and relapsed each time it was discontinued. The issue can be raised as to whether the effectiveness of vancomycin might be related to some unknown property of the drug aside from its antimicrobial activity (e.g., an effect on the CNS). Because vancomycin is only minimally absorbed when given orally, it is much more likely that the effect is mediated through its activity on intestinal bacteria. The relapse after discontinuation of therapy may be related to the persistence of spores from a spore-forming organism such as *Clostridium* during therapy and then germination of the spores after the drug is stopped [10]. Although waxing and waning of autistic symptomatology is well known [11], the repeated responses and relapses in the same patient treated on several occasions and the degree of improvement on vancomycin therapy argue against a coincidental improvement. However, a double-blind, placebo-controlled trial should be performed with an agent effective in patients with autism in an open-label trial. The reasons that we suspected clostridia initially were (1) anecdotally, the antimicrobial that most commonly predisposes to late-onset autism is trimethoprim/sulfamethoxazole, a drug that is notably poorly active against clostridia, (2) the patients had responded to oral vancomycin and, again anecdotally, to oral metronidazole, which suggests the possibility that a gram-positive anaerobic organism is involved (although the very high

Table 2. Microbiological data from examination of gastric and small-bowel specimens obtained from children with autism.

Specimen pH level and organism isolated	Microbiological data, by patient number and specimen number (type)											
	Patient 1, 1324 (J/D)	Patient 2, 1327 (D)	Patient 3		Patient 4		Patient 5		Patient 6		Patient 7	
			1333 (G)	1334 (D)	1337 (G)	1338 (D)	1339 (G)	1340 (D)	1346 (G)	1347 (D)	1350 (G)	1351 (D)
Specimen pH level	no pH	no pH	6.3	6.8	1.25	4.5–5	4	5.7	1.8	7.2	1.25	2.6
Clostridium spp.												
C. acetobutylicum/beijerinckii			2.0×10^2	20			1.0×10^4					
C. bifermentans		1.0×10^3										
C. cochlearium	10^a											
C. ghonii/Eubacterium tenue				4.5×10^6								
C. glycolicum			10									
C. orbiscindens	10^a											
C. perfringens	10											
C. ramosum		1.0×10^4										
C. subterminale			1.0×10^4									
Nonclostridial anaerobes												
Bacteroides ovatus			1.0×10^5	5.0×10^5								
Prevotella sp.								1.1×10^2				
Selenomonas sp.			1.0×10^4									
Actinomyces D01							2.1×10^4					
Actinomyces odontolyticus				1.0×10^5			2.0×10^4					
Actinomyces sp.		1.0×10^3		1.0×10^5								
Probable *Eubacterium* sp.	2.4×10^3											
Propionibacterium acnes			1.0×10^5	1.0×10^6								
Anaerobic GPR							1.1×10^4					
Anaerobic rod								40				
Veillonella sp.			2.0×10^3				1.37×10^5	1.0×10^3				
Anaerobic GPC							9.7×10^4					
Anaerobic coccus							7.0×10^3					
Anaerobic GNCB								30				
Microaerophilic isolates												
Campylobacter concisus			5.0×10^2									
Campylobacter gracilis			2.0×10^3									

Lactobacillus sp.			1.0×10^3	3.5×10^6		9.0×10^3	50	
Sporolactobacillus racemicus			3.0×10^5	1.0×10^5				
Streptococcus intermedius				3.0×10^5				
Large gram-positive cocci						1.0×10^3		
Aerobes								
Enterococcus faecium						4.9×10^3	10	
Enterococcus sp.	20[a]		2.0×10^6	6.0×10^6				
α-Hemolytic *Streptococcus*	30,[a] 2000					3.0×10^3	3.0×10^1	
Streptococcus parasanguinus				1.0×10^6				
Streptococcus salivarius				7.0×10^5				
Streptococcus sp.	10,[a] 1400					7.0×10^2	10	
Aerobic GPC		1.7×10^5						
Micrococcus sp.						2.0×10^2		
Staphylococccus aureus				8.0×10^4				3.6×10^5
Coagulase-negative *Staphylococcus*	2.0×10^2			1.0×10^5		1.0×10^5	10	1.0×10^3
Neisseria perflava			1.0×10^4					
Neisseria sp.			1.43×10^4					
Haemophilus sp.								1.45×10^6
Pasteurella sp.			1.0×10^4					
Bacillus sp.				1.0×10^6				
Cellulomonas sp.				2.0×10^3				
Corynebacterium singulare								
Coryneform sp.				2.0×10^6		1.0×10^4		
Oerskovia sp.				2.5×10^7				
Aerobic gram-positive rod	2.0×10^3							
Yeast								
Candida albicans								
Non-*albicans Candida*			2.0×10^5					

NOTE. D, duodenal fluid; G, gastric juice; GNCB, gram-negative coccobacillus; GPC, gram-positive coccus; GPR, gram-positive rod; J/D, jejunal/duodenal fluid.

[a] Jejunal biopsy.

Table 3. Microbiological data from gastric and small-bowel specimens obtained from control children.

Specimen pH level and organism isolated	Microbiological data, by patient number and specimen number (type)						
	Subject 8		Subject 9		Subject 10	Subject 11	
	01-1341 (G)	01-1342 (D)	1343 (G)	1344 (D)	1345 (D)	1348 (G)	1349 (D)
Specimen pH level	1	6.4	1.5	6.6	6.8	1.5	6.3
Clostridium spp.							
C. acetobutylicum/beijerinckii							2.0×10^2
C. bifermentans				4.0×10^2			
C. cochlearium							
C. ghonii/Eubacterium tenue							
C. glycolicum							
C. orbiscindens							
C. perfringens							
C. ramosum							
C. subterminale			1.0×10^3	2.0×10^4			
Nonclostridial anaerobes							
Bacteroides ovatus							
Prevotella sp.							
Selenomonas sp.							
Actinomyces D01							
Actinomyces odontolyticus							
Actinomyces sp.							
Probable *Eubacterium* sp.							
Propionibacterium acnes							
Anaerobic GPR							
Anaerobic rod							
Veillonella sp.							
Anaerobic GPC							
Anaerobic coccus							
Anaerobic GNCB							
Microaerophilic isolates							
Campylobacter concisus							
Campylobacter gracilis							
Lactobacillus sp.							
Sporolactobacillus racemicus							
Streptococcus intermedius							
Large gram-positive cocci, slightly microaerophilic							
Aerobes							
Enterococcus faecium							
Enterococcus sp.							
α-Hemolytic *Streptococcus*		10			1.96×10^6		
Streptococcus parasanguinus							
Streptococcus salivarius							
Streptococcus sp.					1.0×10^3		
Aerobic GPC							
Micrococcus sp.							
Staphylococccus aureus							1.7×10^4
Coagulase-negative *Staphylococcus*		7.0×10^4	3.0×10^2	6.0×10^4	4.0×10^6		
Neisseria perflava							
Neisseria sp.							
Haemophilus sp.					1.5×10^6		
Pasteurella sp.							
Bacillus sp.				6.0×10^4			
Cellulomonas sp.							
Corynebacterium singulare							7.8×10^3
Coryneform sp.							
Oerskovia sp.							
Aerobic gram-positive rod							
Yeast							
Candida albicans			20	3.0×10^7			2.14×10^7
Non-*albicans Candida*							

NOTE. D, duodenal fluid; G, gastric juice; GNCB, gram-negative coccobacillus; GPC, gram-positive coccus; GPR, gram-positive rod; J/D, jejunal/duodenal fluid.

levels of vancomycin achieved in the bowel when it is given orally are sufficient to eliminate the *Bacteroides fragilis* group and most other gram-negative anaerobic bacteria), (3) unusually high tetanus antitoxin titers have been noted in several patients with late-onset autism [12], and (4) clostridia are the principal bacteria that produce both an enterotoxin and a neurotoxin and are generally very active metabolically (e.g., may produce potentially toxic metabolites such as phenols, p-cresol, or certain indole derivatives). Other antimicrobial agents may also lead to overgrowth of clostridia; for example, *Clostridium perfringens* and *Clostridium innocuum* were found in the salivary flora of patients treated with clindamycin [13]. For the above reasons, then, early during the course of our studies we focused primarily on clostridia in stool specimens that we studied. In our initial article [6], however, we noted a relative scarcity of peptostreptococci in stools of children with autism compared with control children. This suggested the possibility that peptostreptococci might be protective. We have elected not to follow up on this until we develop a good selective medium for these anaerobic cocci.

The studies reported herein should be interpreted in light of the following considerations: (1) there may be $\geqslant$1 key organisms other than clostridia and ruminococci, (2) uncultivatable clostridia or other organisms may be present, (3) the key organism(s) may be present in small numbers (considering that autism is a low-grade, chronic process) and cannot be detected without the use of a selective medium or the availability of some special marker, (4) the organisms involved in autism may be mucosa-associated and therefore not cultivatable by the methods we used, (5) the key organisms may be farther down in the small bowel than we are able to sample, (6) the organisms of concern may be cell wall–deficient and therefore unable to grow on conventional media (although the response to vancomycin, a cell wall–active drug, indicates otherwise), and (7) different types of autism and/or different degrees of severity of the process may be different in terms of whether there is an abnormal flora either in the colon (reflected in the feces) or in the small bowel, and perhaps these factors may even be associated with different specific flora and/or sites or degrees of abnormal colonization.

The stool flora studies showed higher counts of *Clostridium* and *Ruminococcus* spp. in the stools of the children with autism than in those of the control children. There were also a number of species of these genera present only in the stools of children with autism. Additional studies are needed to further analyze the differences in the flora recovered or differences in toxin or toxic metabolite production.

We were interested in the possibility of there being an abnormal microbial flora in the small bowel in autism and, of course, recognized that if that should prove to be the case, our task would be much easier than analyzing stool flora because the flora of the small bowel, even under abnormal circumstances, would likely be considerably less diverse and profuse than would be true of the large bowel and feces. To the extent that toxins were produced by an abnormal flora and gained access to the CNS via the vagus nerve (as hypothesized by Bolte [5]), the small bowel would be a logical site for the abnormal flora because the vagus nerve innervates primarily the small bowel. Also, Wakefield et al. [14, 15] have described pathology in the terminal ileum and proximal colon in autism. Some studies [16] have reported increased small-bowel permeability in autism, and, finally, some children with autism have watery diarrhea, which is characteristic of small-bowel disease.

Older studies from the literature have shown a relatively sparse flora, with relatively few anaerobes and no clostridia in the proximal jejunum [17–20], and even less flora in the duodenum [17, 18] in healthy adult and pediatric subjects. In most cases, the specimens for these studies were obtained by oral intubation. Our laboratory's previous studies of material obtained from the proximal jejunum [21] yielded similar results; these specimens were obtained by aspiration with needle and syringe at the time of surgery. All patients in the latter study were adults. Studies of the gastric flora [17, 18, 22] revealed many sterile specimens in 1 study [17] and counts that were usually in the range of up to 10^4–10^6, when positive. A positive correlation between elevated pH and higher counts in gastric specimens was noted in all these studies, as it was in our patients. Studies of the bacterial content of saliva from 132 subjects were done in one of the above investigations [17]; a wide variety of aerobes and anaerobes were found, with counts as high as 10^7/mL. No clostridia were recovered.

Our current study of upper gastrointestinal specimens has revealed definite abnormality. Nevertheless, there were some children with autism whose specimens yielded no growth or only a few aerobes. Therefore, the possibilities listed above with regard to other types of studies that might be indicated (see above) deserve exploration in this setting as well. Clostridia, as well as non–spore-forming anaerobes, were recovered from 4 upper gastrointestinal tract specimens of children with autism. After the first 2 patients yielded clostridia from duodenal/jejunal contents, we obtained gastric specimens as well, when possible, with the thought that if we found clostridia in the duodenum but not the stomach, this would likely rule out an oral source of these organisms. Clostridia have been reported in the oral cavity on occasion, in conjunction with gingivitis or periodontal disease [23] and in relation to antimicrobial therapy [13]. We were surprised to find elevated gastric pH and bacterial overgrowth in the stomach in 2 of the patients subsequently studied. In 1 of these cases, cholecystokinin had been administered during the endoscopy, and there was likely some reflux of duodenal contents into the stomach; however, the flora at these 2 sites were not at all identical. Cholecystokinin

was not used with any of the other endoscopies. We were also surprised to find clostridia in 2 of 4 control children, despite normal gastric pH; however, none of the 4 controls had non–spore-forming anaerobes or microaerophiles present. Thus, a normal gastric pH may provide a reliable negative screening test for significant bacterial overgrowth. Non–spore-forming anaerobes and microaerophilic bacteria were found in 4 of 5 children with autism whose specimens yielded any growth at all but not in any of the control children. The relatively greater frequency of recovery of yeasts from the control children may reflect the fact that children with autism are not uncommonly given antifungal agents, with the feeling that yeast may contribute to the clinical picture of autism.

The reasons for abnormal colonization of the stomach and upper small bowel in some patients with autism remain to be determined. One factor, already documented in our studies, is hypochlorhydria, but it may be that other factors are responsible in addition to or instead of that factor in some patients. The 2 most reasonable possibilities are impaired gastrointestinal motility and IgA deficiency. Either a genetic predisposition (or defect) or an acquired defect might be involved in any of the above scenarios. There is an excellent recent review of the immune abnormalities noted in patients with autism, including the association of major histocompatibility complex genes with autism [24].

Finally, the question as to how the bacteria in the gut effect the damage that results in the picture of autism is important to consider (see table 4). As noted, one possibility is the production of a toxin or toxins. One organism might produce both an enterotoxin and a neurotoxin, or different organisms might produce these or other toxins separately. One or more of the species of *Clostridium* or of *Ruminococcus* that were found in the fecal flora of the children with autism but not the controls could be a toxin producer. Additional studies of stool samples may narrow the possibilities (i.e., there may be fewer organisms of these types found only in the children with autism). The same points made with regard to fecal flora may also pertain to the clostridia found in the gastric or small-bowel flora of children with autism but not in the control children.

A second possibility, mentioned in our previous study [6], is that autoantibodies or some other bacterial-antibody interaction might lead to autism. Autoantibodies to neuron-axon filament protein [25], glial fibrillary acidic protein [25], and myelin basic protein [26] have been found in patients with autism and might contribute to the pathology and clinical picture of the disease. Such autoantibodies related to *Proteus mirabilis* have been postulated to be involved in rheumatoid arthritis [27], and autoantibodies to *Campylobacter jejuni* are felt to be important in associated Guillain-Barré syndrome [28].

Finally, it may be that the pathogenesis of autism related to abnormal microbiology in the gut relates to microbial production of toxic metabolites. An elegant article by Elsden et al. in 1976 [29] discussed the end products of metabolism of the aromatic amino acids phenylalanine, tyrosine, and tryptophane by clostridia. Twenty-one species of clostridia plus 2 toxin types of *Clostridium botulinum* were studied, and the amounts of phenylacetic, phenylpropionic, phenyllactic, hydroxyphenylacetic, hydroxyphenylpropionic, indole acetic, and indole propionic acids as well as the amount of phenol, p-cresol, and indole produced were given. Another excellent article, by Smith and Macfarlane in 1997 [30], reported similar studies that investigated the effects of pH and carbohydrate availability and compared the effects of free amino acids and peptides as substrates on aromatic amino acid metabolism and the production of toxic metabolites. The latter investigators also showed interconversion of some of these products. They worked with batch cultures of colonic anaerobes rather than with pure cultures. It is interesting to note that urinary myelin basic protein-like material is increased in progressive multiple sclerosis, and p-cresol sulfate is an immunologic mimic of myelin basic protein [31]. Elevated whole blood levels of serotonin (5-hydroxytryptamine), the precursor of which is tryptophan, have been noted in 30%–40% of children with autism, although there are no clear-cut studies to indicate that such elevation plays a role in the disease [32]. Serotonin is not formed by bacterial action, but the extensive activities of bacteria related to other indole derivatives is interesting, particularly in the light of serotonin-function effects on social interaction, mood, obsessive-compulsive activity, motor stereotypies (spinning activity, etc.), and little reaction to painful stimuli [33, 34].

A curious phenomenon reported by parents of some children with autism is a mothball-like odor to the stools of their children. A study by Moore et al. [35] suggested that this odor is not uncommon in healthy adult subjects and is due to the presence in such stools of indole, skatole (3-methyl-indole), or both. These compounds are formed in the human gut from

Table 4. Possible pathways for bacteria-related autism.

Immune-mediated
Toxin
Toxic metabolic product(s)
p-cresol and phenols
Casomorphin, gliadomorphin, and endorphins
Products required for sulfation (e.g., glycosaminoglycans and sulfotransferases) or interfering with sulfation (sulfatase and sulfate-reductase)
Indole derivatives, including serotonin and indolylacryloyl glycine
Naphthalene derivatives
Naphtha derivatives
Mercury (especially methyl mercury) or other metallic ions
Interferons and cytokines

tryptophan by microbial action. Skatole and indole are rapidly absorbed in the jejunum, ileum, and colon of humans [36]. It should be noted, however, that other compounds (e.g., naphthalene and naphtha and their derivatives) also give the odor of mothballs. After the infusion of tryptophan or casein into the colon of normal subjects, indican and indole-3-acetic acid are excreted in large amounts in the urine [36]. Indolylacryloylglycine excretion in urine has been noted in several situations, including autism and other behavioral disorders [37].

Sulfate metabolism is very important in the normal physiology of the body. Specifically, large macromolecules called proteoglycans bear chains of carbohydrate called glycosaminoglycans (GAGs) that are modified and patterned posttranslationally by sulfate. The body makes sulfate from the amino acid cysteine and indirectly (via cysteine) from methionine. The regulation of sulfate may play a significant role in autism. There is depressed phenolsulfotransferase activity in autism, and patients with autism excrete significant amounts of sulfate in the urine [38], despite abnormally low plasma sulfate levels [39]. Colonic bacteria may produce sulfide, which is a neurotoxin [40]. Quite a few bacteria are known to desulfate mucins, including *B. fragilis, Bacteroides thetaiotaomicron, Prevotella, Bifidobacterium, Helicobacter pylori, Clostridium, Ruminococcus torques,* and various oral streptococci [41]. Twenty-three isolates of human fecal bacteria were studied, and some or all produced sialidase, sialate O-acetylesterase, N-acetylneuraminate lyase, arylesterase, and glycosulfatase, all of which are implicated in mucin degradation in the human colon [42]. A *Bacteroides* strain from the human intestinal tract produced polysaccharide lyases (including heparin lyase and chondroitin sulfate lyase) that could degrade GAGs [43]. The effect of gastrointestinal surgery and bacterial overgrowth on the urinary excretion of sulfur amino acids and their degradation products has been studied [44].

Caseinomorphin, gliadomorphin, and various endorphins may play a role in autism. The benefits of a casein-free and gluten-free diet may derive from the elimination of some of these compounds.

There has been much speculation about the role of mercury in autism, particularly in relation to the use of mercury-containing compounds in vaccines administered to children. In vitro studies have shown that the bowel microflora can transform mercury, sometimes to more toxic compounds such as methylmercury (by methylation of mercuric salts), and, in other cases, to less toxic compounds (by demethylation of methylmercury), but such metabolism seems to be of significance to the host only in the case of demethylation. Elimination of the bowel flora by antimicrobial administration in the rat leads to increased mercury content in tissues and a greater proportion of the total mercury as methylmercury [45]. Thus, an intact bowel flora is protective. On the other hand, methylation of mercury has been demonstrated in rats with an experimental jejunal blind loop [46]. Studies with individual components of the human intestinal microflora have indicated that facultative bacteria were more often able to methylate mercury than were lactobacilli, *Bacteroides,* and *Bifidobacterium* [47]. More information is needed on the relative importance of methylation and demethylation by specific elements of the bowel flora and, therefore, the impact of abnormal flora in autism on the toxicity that might be encountered.

The present study provides up-to-date information on the gastric, small-bowel, and fecal flora of young children, using current techniques for identification of the various taxa. In all, 28 species of *Clostridium* were encountered. The geometric mean count of clostridia in stools of children with autism was 1 log greater than that in control children ($P = .0393$). Nine clostridial species were found only in children with autism compared with only 3 in control children. Nonclostridial anaerobes and microaerophilic bacteria were common in upper gastrointestinal specimens of children with autism but were absent from controls. Two children with autism had hypochlorhydria with attendant bacterial overgrowth in gastric and small-bowel specimens. These significant alterations in bowel flora may provide insight into the nature of some types of autism.

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Real-Time PCR Quantitation of Clostridia in Feces of Autistic Children

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Based on the hypothesis that intestinal clostridia play a role in late-onset autism, we have been characterizing clostridia from stools of autistic and control children. We applied the TaqMan real-time PCR procedure to detect and quantitate three *Clostridium* clusters and one *Clostridium* species, *C. bolteae*, in stool specimens. Group- and species-specific primers targeting the 16S rRNA genes were designed, and specificity of the primers was confirmed with DNA from related bacterial strains. In this procedure, a linear relationship exists between the threshold cycle (C_T) fluorescence value and the number of bacterial cells (CFU). The assay showed high sensitivity: as few as 2 cells of members of cluster I, 6 cells of cluster XI, 4 cells of cluster XIVab, and 0.6 cell of *C. bolteae* could be detected per PCR. Analysis of the real-time PCR data indicated that the cell count differences between autistic and control children for *C. bolteae* and the following *Clostridium* groups were statistically significant: mean counts of *C. bolteae* and clusters I and XI in autistic children were 46-fold ($P = 0.01$), 9.0-fold ($P = 0.014$), and 3.5-fold ($P = 0.004$) greater than those in control children, respectively, but not for cluster XIVab (2.6×10^8 CFU/g in autistic children and 4.8×10^8 CFU/g in controls; respectively). More subjects need to be studied. The assay is a rapid and reliable method, and it should have great potential for quantitation of other bacteria in the intestinal tract.

Autism is a complex disease with unclear causes. Many autistic subjects exhibit a range of gut disorders, which include constipation, diarrhea, retention of gas, and abdominal pain and discomfort. Abnormal gut microflora may play a role in these problems. Research into the characteristics of the gut flora in autism has been limited. In our initial studies that characterized the fecal bacterial composition by culturing, we noted abnormalities in the fecal bacterial composition of children with autism compared to age- and sex-matched controls. We found higher counts of clostridia overall and more species of clostridia in stools of autistic children than in healthy children (11). In particular, *Clostridium bolteae*, a novel species that we described previously (29; called *Clostridium clostridioforme* in reference 11), caught our attention because it was cultured from 5 of 15 autistic children, but none of 8 controls. However, it is well known that traditional culture-based methods, while very important, result in a significant underestimation of bacteria present in fecal samples (14, 19, 30).

Molecular techniques introduced in microbial ecology have made it possible to study the composition of intestinal flora in a culture-independent way based on the detection of rRNA genes. Although these methods, such as fluorescent in situ hybridization (12, 13, 19), denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis (8, 10, 28), and the 16S rRNA gene clone library method (15, 27, 30), have been applied successfully for studying the ecology of intestinal flora, PCR analysis using specific primers achieves the most sensitive results as well as providing ease of use and speed (23, 24, 32). Most recently, real-time quantitative PCR has been used for the specific detection and quantitation of selected bacteria from fecal DNA (1, 2, 4, 9, 16, 18, 21, 22, 25, 33).

Few studies have reported on using real-time PCR for quantitation of clostridia in different environments. Belanger et al. (2) and Kimura et al. (17) reported on the successful quantitation of *Clostridium difficile* in feces and *Clostridium botulinum* type E in fish samples using specific primers and probes targeted to toxin genes, respectively. A very recent study investigated the feasibility of using 16S rRNA gene-targeted specific primers and probes for quantitation of major intestinal bacteria, including certain *Clostridum* species by real-time PCR (26). In this study, we evaluated the suitability of a real-time PCR (5′ nuclease PCR assay) to detect and quantitate *C. bolteae* and some *Clostridium* groups (clusters) in fecal specimens of autistic and control children.

MATERIALS AND METHODS

Bacterial strains and fecal specimens. All reference strains used in this study are listed in Table 1. The strains were obtained from different sources, as indicated in Table 1: ATCC is the American Type Culture Collection, CCUG is the Culture Collection, University of Göteborg, DSM is the Deutsche Sammlung von Mikroorganismen und Zellkulturen, and WAL is the Wadsworth Anaerobe Laboratory. All the strains were cultivated on brucella agar (Anaerobe Systems, Morgan Hill, Calif.) supplemented with 5% sheep blood and incubated anaerobically at 37°C under N_2 (86%), H_2 (7%), and CO_2 (7%) gas phase in an anaerobic incubator. The WAL strains are human fecal isolates; they were identified by phenotypic testing and 16S rRNA sequencing (11).

Fecal specimens were obtained at Rush Children's Hospital, Chicago, Ill., under the jurisdiction of its Institutional Review Board (IRB) and with written informed consent by a parent or guardian. Our IRB approved receipt of these specimens by our laboratory. Stool specimens were packed in dry ice and shipped to our laboratory by overnight air express. The entire fecal specimen was ho-

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TABLE 1. Strains used in this study

Strain	Source[a]
Anaerococcus spp.	
A. lactolyticus	CCUG 31351^T
A. octavius	CCUG 38493^T
A. prevotii	CCUG 41932^T
A. tetradius	CCUG 46590^T
A. vaginalis	CCUG 31349^T
Clostridium spp.	
C. beijerinckii	ATCC 25752^T
C. bifermentans	ATCC 638^T
C. bifermentans	WAL 16469
C. bolteae	ATCC BAA-613^T
C. bolteae	WAL 14578
C. bolteae	WAL 14510
C. bolteae	WAL 16099
C. bolteae	WAL 12258
C. bolteae	WAL 7642
C. butyricum	ATCC 13732^T
C. butyricum	WAL 16108
C. cadaveris	ATCC 25783^T
C. carnis	ATCC 25777^T
C. celerecrescens	DSMZ 5628^T
C. fallax	ATCC 19400^T
C. glycolicum	ATCC 14880^T
C. glycolicum	WAL 14424
C. indolis	ATCC 25771^T
C. irregulare	ATCC 25756^T
C. limosum	ATCC 25620^T
C. paraputrificum	ATCC 25780^T
C. paraputrificum	WAL 14502
C. pasteurianum	ATCC 6013^T
C. perfringens	ATCC 13124^T
C. perfringens	WAL 14572
C. putrificum	ATCC 25784^T
C. septicum	ATCC 12464^T
C. sordellii	ATCC 9714^T
C. subterminale	ATCC 25774^T
C. subterminale	WAL 16483
C. symbiosum	ATCC 14940^T
C. symbiosum	WAL 14673
C. tertium	ATCC 14573^T
C. tertium	WAL 16200
C. tyrobutyricum	ATCC 25755^T
C. xylanolyticum	DSMZ 6555^T
C. acetobutylicum	ATCC 824^T
C. baratii	ATCC 27638^T
C. bolteae	WAL 16351
C. celatum	ATCC 27791^T
C. clostridioforme	ATCC 25537^T
C. clostridioforme	WAL 12260
C. clostridioforme	WAL 7855
C. coccoides	ATCC 29236^T
C. cochlearium	ATCC 17787^T
C. difficile	ATCC 9689^T
C. difficile	WAL 14186
C. ghonii	ATCC 25757^T
C. malenominatum	ATCC 25776^T
C. methoxybenzovorans	DSMZ 12182^T
C. novyi	ATCC 27606
C. oroticum	ATCC 13619^T
C. sphenoides	ATCC 19403^T
C. sporogenes	ATCC 19404
Eubacterium spp.	
E. tenue	ATCC 25553^T
Finegoldia spp.	
F. magna	CCUG 17636^T
Peptoniphilus spp.	
P. anaerobius	CCUG 7835^T
P. asaccharolyticus	CCUG 9988^T
P. harei	CCUG 38491^T
P. indolicus	CCUG 17639^T
P. ivorii	CCUG 38492^T
P. lacrimalis	CCUG 31350^T
Peptostreptococcus spp.	
P. micros	CCUG 46357^T
Ruminococcus spp.	
R. flavefaciens	WAL 16464
R. gnavus	CCUG 43222^T
R. gnavus	WAL 16098
R. lactaris	WAL 14694
R. productus	CCUG 10976^T
R. torques	WAL 14161
Sarcina spp.	
S. maxima	CCUG 45682^T
S. ventriculi	WAL 16376

[a] All WAL strains were identified by 16S rDNA sequencing.

mogenized by use of a sterile stainless steel Waring blender, and aliquots of each specimen were frozen at −80°C until DNA was extracted.

Extraction of DNA from pure cultures and feces. Genomic DNA from all bacterial strains was purified from cultures with a QIAamp DNA extraction kit, (QIAGEN, Valencia, Calif.), and the concentration was determined by spectrophotometer (A_{260}). Duplicate samples of genomic DNA (20 ng) from each bacterium were amplified for 40 cycles with each of four real-time PCR primer/probe sets (Table 2) to assess specificity.

One-milliliter aliquots of stool (previously diluted 1:3 in sterile bi-distilled water and thoroughly homogenized under anaerobic conditions in an anaerobic chamber) were centrifuged at 14,000 × *g* for 3 min to pellet fecal bacterial cells. The supernatant was carefully removed and discarded. Two hundred milligrams of cell pellet was transferred to a fresh tube and subjected to DNA extraction by using a commercial extraction system (QIAamp DNA stool mini kit; QIAGEN) according to the instructions of the manufacturer. Our pilot studies have shown that this QIAamp product produces high-quality DNA free of PCR-inhibiting substances. DNA extraction was performed in duplicate.

Design of oligonucleotide primers and probe. Sequences of the 16S rRNA genes of the organisms of interest and of closely related bacteria were aligned with CLUSTAL-W (http://genome.kribb.re.kr) and inspected for regions of conserved and variable sequences. Based on the multialignment analysis data, four pairs of 16S rRNA gene-targeted cluster- and species-specific primers were designed, one each for *Clostridium* clusters I, XI, and XIVab (7) and *C. bolteae*, respectively. They were designed from a collection of 128 *Clostridium* 16S rRNA sequences obtained from the GenBank database. Potential candidates for PCR primers were compared to the aligned SSU_rRNA database of the Ribosomal Database Project (RDP) using the CHECK_PROBE utility (20) and were compared to all available 16S ribosomal DNA (rDNA) sequences by using the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST) (3). The primers were used in combination with internal nonspecific probes (Table 2). The primers' target regions were located as near as possible to the probe in order to enable an efficient hydrolysis of the probe during primer elongation by the polymerase. The primer and probe sequences were analyzed for T_m (melting temperature), secondary structure formation, G+C content, and primer-dimer formation with NetPrimer analysis software (http://www.premierbiosoft.com/netprimer). The probe was 5′ labeled with carboxyfluorescein (FAM) as the reporter dye and Black Hole quencher (BHQ) as the 3′ quencher dye. The BHQ dye was used as the quencher dye because of its broad quenching spectrum and a lower signal/noise ratio than that of other quenching dyes.

SmartCycler real-time PCR assay. The amplification reactions were carried out in a total volume of 25 μl, which consisted of 2 μl of DNA samples and 23 μl of the master mixture. The latter contained 1× puReTaq Ready-To Go PCR bead (2.5 U of puReTaq DNA polymerase, 10 mM Tris-HCl [pH 9.0 at room temperature], 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate [dNTP], and stabilizer bovine serum albumin [Amersham Biosciences, Piscataway, N.J.]), 300 nM each primer, 200 nM fluorescence-labeled probe, and

TABLE 2. Sequences of oligonucleotide primers and probe

Target bacteria and primer or probe[a]	Oligonucleotide sequence (5′→3′)	T_m (°C)[b]
Probe-I	GTGCCAGCAGCCGCGGTAATACG	72.4
Group I (*Clostridium* cluster I)		
Forward primer, CI-F1	TACCHRAGGAGGAAGCCAC	54.6
Reverse primer, CI-R2	GTTCTTCCTAATCTCTACGCAT	53.0
Group II (*Clostridium* cluster XI)		
Forward primer, CXI-F1	ACGCTACTTGAGGAGGA	46.5
Reverse primer, CXI-R2	GAGCCGTAGCCTTTCACT	52.3
Group III (*Clostridium* cluster XIVab)		
Forward primer, CXIV-F1	GAWGAAGTATYTCGGTATGT	46.2
Reverse primer, CXIV-R2	CTACGCWCCCTTTACAC	44.8
Probe-II (*C. bolteae*)	CAGGTGGTGCATGGTTGTCGTCAG	69.2
Forward primer	CCTCTTGACCGGCGTGT	56.7
Reverse primer	CAGGTAGAGCTGGGCACTCTAGG	62.1

[a] Probe-I is an internal universal probe that corresponds to a region of the 16S rRNA gene that is conserved in all eubacteria (5); it was used with three sets of cluster-specific primers. Probe-II was used with *C. bolteae*-specific primer set. *Clostridium* clusters are as described in reference 7.
[b] The T_m of DNA was determined with NetPrimer software (PREMIER Biosoft International).

2.5 mM $MgCl_2$, made up to 23 μl with distilled water. The assay was performed with the Cepheid (Sunnyvale, Calif.) SmartCycler instrument in a protocol comprising 1 cycle of 2 min at 95°C (hot start) followed by 45 cycles of 95°C for 20 s for denaturation. Annealing was performed for 30 s at 58°C for *C. bolteae*, 63°C for *Clostridium* cluster I, 58°C for *Clostridium* cluster XI, and 52°C for *Clostridium* cluster XIVab. Extension was performed at 72°C for 45 s for all. Data analysis was performed with Cepheid software. The C_T values (i.e., the threshold cycles in which exponential amplification of PCR products was first detected) were determined on the basis of the mean baseline signals during the early cycles of amplification.

Standard curve. The standard curve for *C. bolteae* was constructed by using DNA recovered from fecal samples spiked with different numbers of *C. bolteae* type strain ATCC BAA-613 (30 to 3.0×10^7 CFU ml^{-1}). Briefly, *C. bolteae* ATCC BAA-613 was collected from thioglycolate broth cultures while in logarithmic growth phase. Ten-fold dilutions were made in dilution blanks (Anaerobe Systems), and the number of CFU of bacteria in each dilution was determined by plating on brucella blood agar plates. All determinations were performed in triplicate. The average titer (CFU per milliliter) of three replicates was determined. One milliliter of each dilution was added to tubes containing 200-mg aliquots of a stool sample that was verified to be *C. bolteae* negative by both bacteriological and PCR techniques and pelleted by centrifugation. The pellets with different numbers of *C. bolteae* cells were subjected to DNA extraction by using a QIAamp DNA Stool Mini kit. The DNA was used to establish a standard curve.

To generate standard curves for each of the three *Clostridium* clusters, the C_T values were plotted relative to the corresponding serial 10-fold dilutions of template DNA extracted from cultures of representative clostridial stool isolates of different *Clostridium* clusters: a mixed culture of *C. subterminale* WAL 16483, *C. perfringens* WAL 14572, and *C. paraputrificum* WAL 14502 for cluster I; a mixed culture of *C. bifermentans* WAL 16496, *C. difficile* WAL 14186, and *C. glycolicum* WAL 14424 for cluster XI; and a mixed culture of *C. symbiosum* WAL 14673, *C. bolteae* WAL 16351 and *Ruminococcus gnavus* WAL 16098 for cluster XIVab. Briefly, 1 ml of the overnight thioglycolate broth cultures of these bacterial strains was subjected to DNA extraction by using the QIAamp DNA stool mini kit. Ten-fold serial dilutions of these bacterial DNAs were prepared. The corresponding CFU per PCR were calculated based on plate counts, as described above.

For comparison of PCR amplification efficiencies and detection sensitivities among different experiments, slopes of the standard curves were calculated by performing a linear regression analysis with the SmartCycler software. A mixture of all PCR reagents without any DNA was used as a negative control. Amplification efficiency (E) was estimated by using the slope of the standard curve and the formula $E = (10^{-1\text{slope}})^{-1}$. A reaction with 100% efficiency will generate a slope of −3.32. The standard curves were used for determining the detection limits of the assays and enumeration of *C. bolteae* and members of each of the three *Clostridium* clusters in stool samples.

Enumeration of *Clostridium* in stool samples by real-time PCR. Purified DNA from stool samples was used with optimized PCR conditions and an appropriate standard curve to enumerate the load of *C. bolteae* and members of different *Clostridium* clusters in the stool samples. Each stool sample was subjected to four PCR runs (2 PCR repetitions × 2 DNA extractions; $n = 4$). The amount of DNA measured by real-time PCR was converted to cell numbers to allow comparison with the CFU data. This was accomplished by using the standard curve that was generated by plotting the C_T against CFU. This approach was used because, for stool samples, it is easier to understand results in actual CFU numbers than in DNA concentrations or copy numbers.

The C_T standard deviations were calculated as shown in Table 3. The CFU of *C. bolteae* and the members of each cluster were determined from C_T values by using the standard curves (Fig. 1 and 2).

Statistical analysis. Parametric t tests and multivariate logistic regression and/or classification tree (CART) method (6) was used to compare mean CFU values between autistic and control samples.

RESULTS

Specificity of oligonucleotide primers. The results of comprehensive cross-specificity checks on the RDP database enabled the final selection of four 16S rDNA-targeted PCR primer pairs theoretically specific for *C. bolteae* or each of three *Clostridium* clusters (Table 2). Degenerate PCR primers were used in order to broaden the specificity within each cluster. The primer pair CI-F1 and CI-R2 is specific for cluster I, which includes most species of the *Clostridium* cluster I and three species of cluster II. Primer pair CXI-F1 and CXI-R2 is specific for cluster XI, which includes part of *Clostridium* cluster XI. Finally, primer pair CXIV-F1 and CXIV-R2 is specific for cluster XIVab, which includes most species belonging to *Clostridium* cluster XIVab. The specificity of species- and cluster-specific primers was further confirmed by amplifying genomic DNA from target and phylogenetically related non-target bacterial strains (Table 1) with each of the four sets of primers by conventional PCR and real-time PCR. In conventional PCR, the primer pairs were specific for their target species or cluster at the appropriate annealing temperatures and yielded PCR products of the expected size only from their targets. The optimized conventional PCR conditions were applied to real-time PCR. In real-time PCR, no fluorescent signal was detected from non-target bacterial DNA (data not shown). Although our TaqMan probe and reverse primer for *C. bolteae* also showed 100% homology with 16S rDNA of its most closely related species, *C. clostridioforme*, the forward primer did not

TABLE 3. *Clostridium* detection in stool samples of autistic and control children by real-time PCR[a]

Sample[b]	*C. bolteae*		*Clostridium* cluster I		*Clostridium* cluster XI		*Clostridium* cluster XIVab	
	Mean C_T (SD)	CFU/g of fecal specimen	Mean C_T (SD)	CFU/g of fecal specimen	Mean C_T (SD)	CFU/g of fecal specimen	Mean C_T (SD)	CFU/g of fecal specimen
Control								
98-1306C	33.6 (0.22)	$(4.0 \pm 0.3) \times 10^2$	28.6 (0.31)	$(3.1 \pm 0.4) \times 10^5$	25.4 (0.21)	$(3.5 \pm 0.3) \times 10^6$	21.7 (0.33)	$(5.9 \pm 0.3) \times 10^7$
98-1332C	35.2 (0.21)	$(2.8 \pm 0.2) \times 10^2$	31.6 (0.46)	$(4.9 \pm 0.4) \times 10^4$	26.3 (0.27)	$(1.7 \pm 0.3) \times 10^6$	18.5 (0.22)	$(2.5 \pm 0.4) \times 10^8$
98-1333C	36.9 (0.29)	$(9.2 \pm 0.4) \times 10^1$	27.6 (0.45)	$(6.6 \pm 0.4) \times 10^5$	24.6 (0.25)	$(6.2 \pm 0.5) \times 10^6$	21.0 (0.23)	$(7.5 \pm 0.2) \times 10^7$
98-1334C	31.4 (0.18)	$(2.7 \pm 0.2) \times 10^3$	28.4 (0.25)	$(3.9 \pm 0.3) \times 10^5$	26.4 (0.31)	$(1.7 \pm 0.2) \times 10^6$	18.0 (0.35)	$(3.5 \pm 0.3) \times 10^8$
98-1335C	31.3 (0.24)	$(3.3 \pm 0.4) \times 10^3$	27.6 (0.46)	$(6.6 \pm 0.3) \times 10^5$	26.6 (0.21)	$(1.5 \pm 0.2) \times 10^6$	20.5 (0.26)	$(9.6 \pm 0.2) \times 10^7$
98-1342C	30.8 (0.22)	$(5.6 \pm 0.4) \times 10^3$	31.9 (0.39)	$(4.3 \pm 0.3) \times 10^4$	25.7 (0.13)	$(2.6 \pm 0.4) \times 10^6$	18.1 (0.31)	$(3.4 \pm 0.3) \times 10^8$
00-1318C	<DL	<DL	28.5 (0.23)	$(3.5 \pm 0.4) \times 10^5$	25.5 (0.12)	$(2.9 \pm 0.4) \times 10^6$	22.0 (0.25)	$(4.6 \pm 0.3) \times 10^7$
00-1319C	28.6 (0.15)	$(1.9 \pm 0.1) \times 10^4$	27.1 (0.21)	$(8.5 \pm 0.5) \times 10^5$	23.6 (0.11)	$(1.2 \pm 0.1) \times 10^7$	16.5 (0.38)	$(8.8 \pm 0.2) \times 10^8$
Avg		$(3.9 \pm 0.3) \times 10^3$		$(4.1 \pm 0.3) \times 10^5$		$(4.0 \pm 0.4) \times 10^6$		$(2.6 \pm 0.2) \times 10^8$
Autistic								
97-1301A	31.5 (0.17)	$(2.7 \pm 0.2) \times 10^3$	24.6 (0.22)	$(5.5 \pm 0.2) \times 10^6$	21.4 (0.08)	$(6.7 \pm 0.2) \times 10^7$	18.5 (0.29)	$(2.5 \pm 0.3) \times 10^8$
97-1316A	30.0 (0.16)	$(7.6 \pm 0.2) \times 10^3$	24.4 (0.24)	$(6.6 \pm 0.2) \times 10^6$	22.9 (0.17)	$(2.2 \pm 0.3) \times 10^7$	17.4 (0.41)	$(4.9 \pm 0.3) \times 10^8$
97-1321A	24.1 (0.10)	$(3.9 \pm 0.1) \times 10^5$	25.1 (0.32)	$(3.3 \pm 0.3) \times 10^6$	22.5 (0.13)	$(3.0 \pm 0.3) \times 10^7$	18.6 (0.25)	$(2.4 \pm 0.4) \times 10^8$
98-1301A	28.4 (0.18)	$(2.0 \pm 0.3) \times 10^4$	30.9 (0.44)	$(8.2 \pm 0.6) \times 10^4$	25.3 (0.23)	$(3.8 \pm 0.5) \times 10^6$	22.4 (0.09)	$(3.9 \pm 0.1) \times 10^7$
98-1343A	27.7 (0.22)	$(3.2 \pm 0.4) \times 10^4$	25.7 (0.34)	$(2.6 \pm 0.3) \times 10^6$	26.6 (0.22)	$(1.6 \pm 0.3) \times 10^6$	18.9 (0.17)	$(1.5 \pm 0.2) \times 10^8$
98-1344A	29.3 (0.17)	$(1.2 \pm 0.2) \times 10^4$	28.2 (0.21)	$(4.7 \pm 0.4) \times 10^5$	24.3 (0.20)	$(8.6 \pm 0.4) \times 10^6$	15.5 (0.30)	$(1.6 \pm 0.3) \times 10^9$
00-1311A	22.9 (0.10)	$(7.3 \pm 0.1) \times 10^5$	27.8 (0.44)	$(5.9 \pm 0.3) \times 10^5$	21.4 (0.14)	$(1.6 \pm 0.3) \times 10^6$	18.9 (0.17)	$(1.4 \pm 0.2) \times 10^8$
00-1312A	33.0 (0.13)	$(1.2 \pm 0.1) \times 10^3$	26.1 (0.38)	$(2.4 \pm 0.3) \times 10^6$	25.5 (0.15)	$(3.2 \pm 0.6) \times 10^6$	18.7 (0.24)	$(2.2 \pm 0.4) \times 10^8$
00-1313A	26.0 (0.08)	$(9.5 \pm 0.1) \times 10^4$	26.6 (0.37)	$(1.4 \pm 0.3) \times 10^6$	24.6 (0.22)	$(6.1 \pm 0.6) \times 10^6$	17.7 (0.52)	$(4.2 \pm 0.5) \times 10^8$
00-1314A	26.3 (0.21)	$(5.0 \pm 0.4) \times 10^4$	23.3 (0.24)	$(1.3 \pm 0.2) \times 10^7$	22.5 (0.18)	$(2.8 \pm 0.2) \times 10^7$	18.5 (0.22)	$(2.7 \pm 0.2) \times 10^8$
00-1315A	28.0 (0.15)	$(2.7 \pm 0.2) \times 10^4$	29.1 (0.29)	$(2.2 \pm 0.5) \times 10^5$	23.8 (0.15)	$(1.1 \pm 0.1) \times 10^7$	20.6 (0.25)	$(8.4 \pm 0.1) \times 10^7$
00-1316A	31.4 (0.17)	$(3.3 \pm 0.2) \times 10^3$	24.8 (0.30)	$(4.8 \pm 0.3) \times 10^6$	23.3 (0.18)	$(1.5 \pm 0.1) \times 10^7$	15.1 (0.34)	$(2.4 \pm 0.4) \times 10^9$
00-1317A	30.4 (0.19)	$(7.1 \pm 0.3) \times 10^3$	23.6 (0.24)	$(1.1 \pm 0.2) \times 10^7$	23.8 (0.13)	$(1.1 \pm 0.1) \times 10^7$	22.1 (0.27)	$(4.4 \pm 0.3) \times 10^7$
02-1409A	22.0 (0.10)	$(1.3 \pm 0.1) \times 10^6$	29.9 (0.31)	$(1.7 \pm 0.3) \times 10^5$	24.6 (0.25)	$(6.8 \pm 0.3) \times 10^6$	17.3 (0.42)	$(5.1 \pm 0.1) \times 10^8$
03-1404A	26.6 (0.13)	$(5.0 \pm 0.2) \times 10^4$	25.4 (0.33)	$(2.9 \pm 0.2) \times 10^6$	22.4 (0.07)	$(3.0 \pm 0.2) \times 10^7$	18.5 (0.17)	$(2.7 \pm 0.2) \times 10^8$
Avg		$(1.8 \pm 0.1) \times 10^5$		$(3.7 \pm 0.4) \times 10^6$		$(1.4 \pm 0.1) \times 10^7$		$(4.8 \pm 0.6) \times 10^8$

[a] Mean C_T represents two PCR replicates of two replicate DNA extractions ($n = 4$). CFU were calculated by using the standard curve. <DL, below the detection limit of 0.6 CFU/reaction; SD, standard deviation.
[b] C, control children; A, autistic children.

show any major sequence homology (70.5% only); there was no signal detected from DNA from *C. clostridioforme* strains.

Standard curve and detection limits. In real-time PCR assay, the C_T at which the fluorescent signal is statistically significant above background is measured. The C_T value is proportional to the amount of target DNA and hence the number of bacteria in the samples. The detection sensitivity for *C. bolteae* was determined by two sets of genomic DNA prepared from 10-fold serial dilutions (30 to 3.0×10^7 CFU/ml) of *C. bolteae* type strain ATCC BAA-613 spiked into a *C. bolteae*-negative stool sample. The detection sensitivities for three *Clostridium* clusters were determined by two sets of 10-fold serial dilutions of genomic DNA prepared from representatives of each cluster. Representative data are shown in Fig. 1 and 2. The results are reported as the C_T numbers versus CFU numbers.

As shown in Fig. 1 and 2, plotting the obtained C_T values relative to the CFU of bacteria resulted in a linear correlation with square regression coefficient of ≥0.99, demonstrating that quantification of the target DNA was possible. To evaluate the robustness of the assays, the same experiment, which included an unknown stool sample, was repeated six times on different days with two different sets of DNA. The result for *C. bolteae* is given in the inset in Fig. 1. The standard deviation of C_T values for the unknown sample based on the six PCR runs (3 PCR repetitions × 2 DNA extractions; $n = 6$) were 0.08, 0.37, 0.22, and 0.52 (Table 3); determined by the *C. bolteae* and cluster I, XI, and XIVab standard curves, respectively. This showed that both DNA extraction by the QIAamp stool DNA extraction kit and the real-time PCR assay were highly reproducible.

The function describing the relationship between C_T values and CFU for the assay has been calculated based on the mean values obtained from the six PCR runs (Fig. 1and 2). Average slopes of −3.52, −3.52, −3.28, and −3.14 were generated for *C. bolteae* and clusters I, XI, and XIVab, respectively. According to the formula $\log E = \text{slope}^{-1}$, the standard curves generated from the PCR data resulted in a reaction efficiency of above 96.5% for all four PCR assays.

The estimated detection limits for the primer and probe sets specific for *C. bolteae* and clusters I, XI, and XIVab were 0.6, 2, 6, and 4 bacteria per PCR, respectively. All nontemplate controls and all dilutions with DNA corresponding to less than these numbers of cells per reaction gave negative results.

Enumeration of *Clostridium* in stool samples by real-time PCR. The bacterial load of *C. bolteae* and each *Clostridium* cluster in stools was estimated by real-time PCR with DNA extracted from the 23 samples (Table 3). The reproducibility of the assay was high, with the standard deviation of C_T values for stool samples ranging from 0.08 to 0.29 for *C. bolteae*, 0.21 to

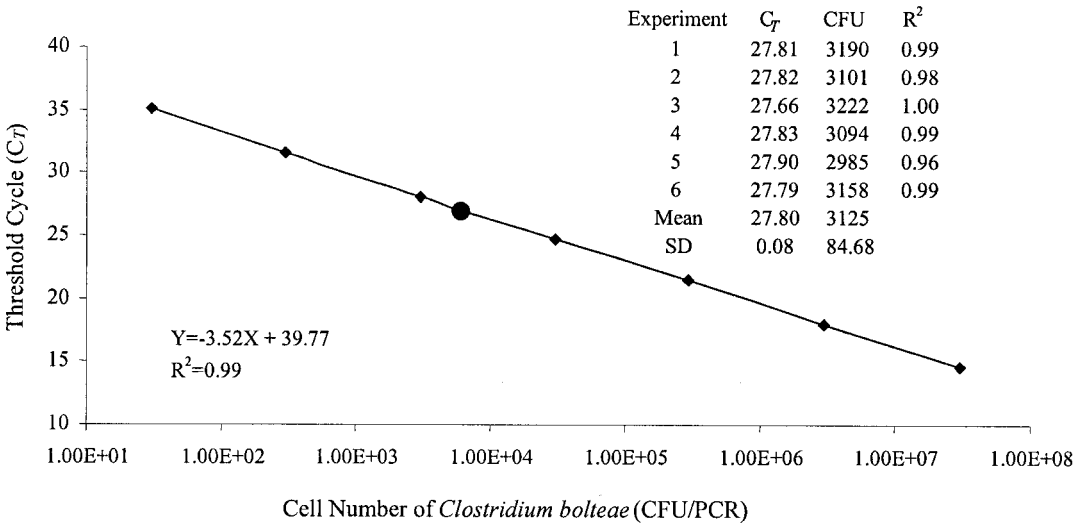

FIG. 1. Standard curve generated by analysis of a dilution series of *C. bolteae* cells spiked in stools by real-time PCR. Quantitation was performed by determining the C_T. The same experiment, which included a known *C. bolteae*-positive stool with an unknown count (●), was repeated six times. The C_T values, the CFU/PCR determined, and R^2 values of each experiment are listed in the inset. Means and the standard deviation were calculated from these six experiments. ◆, *C. bolteae* standards.

0.46 for cluster I, 0.07 to 0.31 for cluster XI, and 0.09 to 0.52 for cluster XIVab. The mean counts (CFU per gram [wet weight of centrifuged specimens] of feces) of *C. bolteae* and members of clusters I, XI, and XIVab were $1.8 \times 10^5 \pm 0.1 \times 10^5$, $3.7 \times 10^6 \pm 0.4 \times 10^6$, $1.4 \times 10^7 \pm 0.1 \times 10^7$, and $4.8 \times 10^8 \pm 0.6 \times 10^8$ in autistic children, respectively, and were $3.9 \times 10^3 \pm 0.3 \times 10^3$, $4.1 \times 10^5 \pm 0.3 \times 10^5$, $4.0 \times 10^6 \pm 0.4 \times 10^6$, and $2.6 \times 10^8 \pm 0.2 \times 10^8$ in controls, respectively. Analysis of the real-time PCR data indicated that the cell count differences between autistic and control children in *C. bolteae* and the following *Clostridium* groups were statistically significant: mean counts of *C. bolteae* and clusters I and XI in autistic children were 46-fold ($P = 0.01$), 9.0-fold ($P = 0.014$), and 3.5-fold ($P = 0.004$) greater than those in control children, respectively, but the differences in cluster XIVab between autistic and control children were not statistically significant ($P = 0.3$). More subjects need to be studied for all groups.

DISCUSSION

Real-time PCR has several advantages over conventional PCR. It focuses on the logarithmic phase of product accumulation rather than on the end product abundance. Therefore, it is more accurate since it is less affected by amplification efficiency or depletion of a reagent. In addition, it has an increased dynamic range for quantification of target sequence (at least 5 orders of magnitude). Furthermore, without any post-PCR manipulation of the samples, cross-contamination between samples is greatly reduced. Finally, PCR results can be

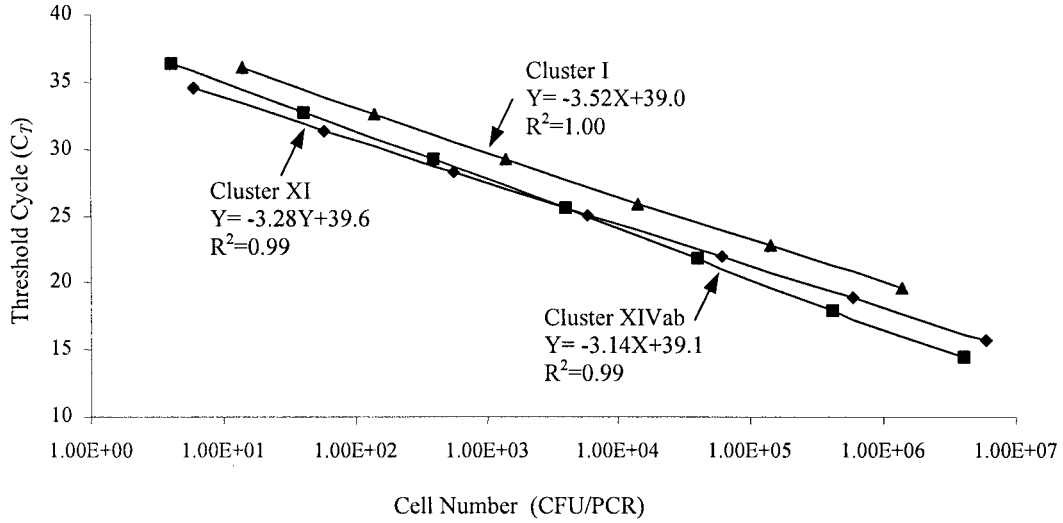

FIG. 2. Standard curves generated by analysis of a dilution series of DNA extracted from mixed cultures of representative strains for each *Clostridium* cluster by real-time PCR. The C_T values are plotted against the corresponding cell numbers in the PCR. ▲, *Clostridium* cluster I; ◆, *Clostridium* cluster XI; ■, *Clostridium* cluster XIVab.

obtained within 1 h. In the present study, we developed quantitative real-time TaqMan PCR assays targeting the 16S rRNA for detection and quantitation *of C. bolteae* and members of three *Clostridium* clusters (I, XI, and XIVab) in stool samples. The methods were successful in detecting and quantitating *Clostridium* in pure culture and in stool samples from autistic children and control children.

Good detection methods share the following features: specificity, sensitivity, and reproducibility. Real-time PCR appears to possess all these features. In the present study, specificity, a key factor for accurate quantitation of the bacteria of interest, was determined both by homology search in nucleotide databases and by testing bacterial strains with different degrees of relatedness to the target species based on 16S rRNA comparative analysis. Although theoretical cross-specificity analysis of the primers indicated that the cluster XIVab-specific primer pair may cross-react with some nontarget bacteria (only one side primer contained 2-bp mismatches), such as *Anaeroplasma bactoclasticum*, *Anaeroplasma varium*, and *Mycoplasma feliminutum*, these bacteria have never been reported as being isolated from human gut. Since the primers were designed on the basis of currently available 16S rRNA sequences, the specificity is not guaranteed for unknown gut bacteria. It is an advantage, then, that these probes are designed on a phylogenetic basis and that related species are more likely to have the same target sequences.

The reason we used 16S rDNA as a target to design the probe and primers is that the large 16S rDNA database available allows the identification of sequences exclusive to our target species. As we know, the complex microflora of the human gut is difficult to study with probes or primers on a species level due to the diversity of the ecosystem. Therefore, it is more convenient to have probes or primers specific for genera or groups present in the gut. For intertwined genera such as *Clostridium*, genus-specific probes or primers cannot be designed and primers and/or probes for phylogenetic clusters have to be considered. Analysis of *Clostridium* 16S rRNA sequences has enabled the phylogeny of the genus *Clostridium* to be described and major *Clostridium* clusters to be identified (7). This in turn has enabled the design and development of cluster-specific 16S rDNA-targeted oligonucleotide probes and PCR primers (12, 13, 23, 31). The major disadvantage of this approach to design species-specific primers is that the variable regions within the 16S rDNA can be almost identical for closely related bacteria. To overcome this problem, stringent conditions such as the hot-start PCR technique and high annealing temperature, were used to exclude amplification of organisms closely related to *C. bolteae*. Thus, even though the TaqMan probe and the reverse primer show 100% homology with the corresponding regions of 16S rRNA of *C. clostridioforme*, no signal was generated when the real-time PCR was performed with this set of oligonucleotides. It is also unlikely that other non-target closely related DNA in stool specimens would be amplified.

The TaqMan assays we developed proved very sensitive and reproducible. The standard curves showed that the estimated detection limit for primer and probe sets specific for *C. bolteae* and *Clostridium* clusters I, XI, and XIVab were 0.6, 2, 6, and 4 CFU per PCR, respectively. We found that the reproducibility of the quantitative experiments was over 99%, based on multiple replicate PCR runs. When quantifying targets using real-time PCR, both the variability attributable to DNA yields between extractions and the PCR assay itself need to be considered. Commercially available kits for the isolation of genomic DNA from stool samples are desirable due to the high throughput and elimination of phenol, which requires special disposal protocols and can affect PCR results. In this study, the QIAamp stool DNA extraction kit was used for stool DNA extraction. It proved to be good in providing DNA suitable for the assay. The small standard deviation of mean C_T values (Table 3) confirmed the reproducibility of the DNA extraction method and was associated with only a small amount of error during the PCR analysis.

A challenge in using the real-time PCR method is to convert measurements of fluorescent signal into target cell densities. The approach used in this study is to directly relate the signal to cell numbers by a plate counting method. It is therefore necessary to exercise some caution in selecting which *Clostridium* strains are used to establish standard curves. It is known that the final determination of bacterial load by real-time PCR in a multispecies population will be influenced by the variation in the number of rRNA operons in a given species. Unfortunately, information on the number of 16S rRNA genes per genome in *Clostridium* species is very limited (rRNA Operon Copy Number Database; http://rrndb.cme.msu.edu/rrndb/servlet/controller/controller?page = home), so only presumptions can be made about the size of the different clostridial cluster populations. However, in a complex ecosystem like a stool sample, other methodologies are likely to be far less sensitive or precise. In this study, several *Clostridium* stool isolates in each cluster (*C. butyricum*, *C. paraputrificum*, *C. perfringens*, *C. subterminale*, and *Clostridium tertium* in cluster I; *C. bifermentans*, *C. difficile*, and *C. glycolicum* in cluster XI; and *C. symbiosum*, *C. bolteae*, *R. gnavus*, *R. torques*, and *R. lactaris* in cluster XIVab) were tested for constructing a standard curve. The standard curves obtained from the different strains within the same cluster were in good agreement (data not shown). This suggests that the sensitivity of measurement by these assays may not vary greatly depending on the species or strain. We used three *Clostridium* strains, which were isolated from stool specimens of autistic or control children in another study (11), for each cluster to construct the standard curves.

Since stool samples contain diverse bacterial communities, the amplification of *C. bolteae* DNA to construct a standard curve was performed with the background of stool community DNA. We spiked the dilution series of *C. bolteae* cells into one *C. bolteae*-negative stool sample to determine the PCR efficiency and reliability under the usual stool community DNA conditions. However, there was no stool sample that was *Clostridium* negative; therefore, we used DNA extracted from pure cultures of representative strains from each cluster to construct their standard curves.

Our previous study compared culture methodologies with PCR for the detection of *C. bolteae* from stool samples, with the conclusion that *C. bolteae* detection by PCR is more sensitive and more reliable than culture (29). The findings in this study by real-time PCR are in agreement with the previous observations and provide reliable quantitative information. Analysis of the real-time PCR data indicated that the cell count differences between autistic and control children in *C.*

bolteae and the *Clostridium* clusters I and XI were statistically significant; however, we feel that to be confident about it, we need to study large numbers of samples.

In summary, we developed a new method based on real-time PCR that allowed for fast, reliable quantitation of *C. bolteae* and three *Clostridium* clusters in stool samples. *C. bolteae* is also a pathogen encountered in clinically significant infections in humans. Therefore, this procedure may also be useful in recovering *C. bolteae* from clinical specimens with complicated mixed floras. To our knowledge, this study demonstrates for the first time the potential of the real-time PCR technique for quantifying *Clostridium* clusters in human stool. Investigation of the intestinal microflora is crucial for obtaining an understanding of the role of the gut microflora in health and also an understanding of the role of the microflora in disease (e.g., inflammatory bowel disease—Crohn's disease and ulcerative colitis). The assay is rapid and reliable, and we assume that it has great potential for quantitation of other bacteria in the intestinal tract. A major advantage of this method is that it lends itself to high throughput.

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Therapy and epidemiology of autism—clostridial spores as key elements

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Summary This manuscript reviews evidence indicating that intestinal bacteria, specifically clostridia, may play a role in certain cases of autism and hypothesizes that the clostridial spores (which are notably resistant to antimicrobial agents and commonly used germicides) are involved in: (1) relapse in the autistic subject after a response to an agent such as oral vancomycin, after the drug is discontinued, (2) the unexplained increased incidence of autism in recent years, and (3) the unexplained increase in numbers of multiple cases in the same family. Hypothesis (1), if established as valid, would spur research to find well-tolerated and safe agents that could be given together with vancomycin (or other appropriate antimicrobial agent) to eliminate spores; this would revolutionize the therapeutic approach. Hypotheses (2) and (3) relate to widespread use of antimicrobial agents, poor hygiene in young autistic children, and difficulty in removing spores from the home environment. These latter two hypotheses have major implications with regard to the epidemiology of this important and distressing disease and would encourage research into methods to eliminate clostridial spores from the home and other environments.

Introduction

Autism is a syndrome involving language deficits, lack of social skills, reduced behavioral flexibility and often hyperfocus. There is a lot of variability as to degree of involvement and there may be unusual talents as well. There are often gastrointestinal abnormalities and there may be immune defects and involvement of other systems.

Recent studies have indicated a striking increase in the incidence of autism; the first national survey of incidence of autism conducted by the CDC indicates the disease has become widespread, afflicting an estimated 300,000 US schoolchildren, about one in every 175 [1].

A recent critical review of the literature by Herbert [2] indicates that while genetic factors are often involved in autism, it is likely that

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environmental factors play an important role as well.

Studies on a possible role of intestinal bacteria in autism [3—8]

In 1998, Bolte (a non-professional mother of an autistic child) published an intriguing hypothesis that *Clostridium tetani* (or other bacteria in the gut) might play a role in late-onset autism. On the basis of this paper, studies were carried out that suggest that intestinal bacteria are a factor in autism in that autistic children showed improved behavior, communication, and social skills and in the gastrointestinal manifestations of their disease after oral vancomycin, an antibiotic that is essentially not absorbed from the gut. Anecdotally, individual patients have responded to antimicrobials such as oral vancomycin and metronidazole on several occasions, relapsing each time after discontinuation and then responding again to re-institution of these antimicrobials.

Using cultural methods, we found the geometric mean count of clostridial species in stools of children with autism was 1 log higher than in stools of control children ($P = 0.039$) and more clostridial species were found in autistic children (23 species in stools of 13 autistic children vs. 15 in eight control children). In upper gastrointestinal studies, we found nonsporeforming anaerobes and microaerophilic bacteria were absent from control children whereas they were plentiful in autistic children's specimens. We also tested fecal specimens from both autistic and control children using real-time PCR and found that cell counts of *Clostridium bolteae* and of clostridial clusters I and XI, but not cluster XIVab, were statistically higher in autistic children.

A fluorescense in situ hybridization study by Parracho et al. compared fecal flora of autistic children with that of two control groups. They noted a higher incidence of *Clostridium* clusters I and II in autistic children than in healthy controls, and an intermediate level in siblings of the autistic children. MacFabe et al. injected propionic acid intraventricularly in rats; this led to dystonic behavior, hyperactivity, turning behavior and other findings that demonstrated some aspects of human autism spectrum disorders. Propionic acid is produced as a metabolic product by a number of intestinal bacteria, including some clostridia.

Hypotheses

Hypothesis 1. Relapse in autistic subjects after discontinuation of an appropriate agent such as oral vancomycin which produces a response is due to the presence of spores (which are not killed by the action of antibiotics) and which then germinate to reproduce the disease. It might represent a new infection with appropriate clostridia, but the inevitable relapse after discontinuation suggests it is reinfection with the original offending organism(s). Relapse during therapy would suggest that the offending organisms have acquired resistance to the antibiotic (not common with clostridia) or that other clostridia already resistant have taken over. Typing of implicated clostridial strains from the original ''infection'' and after relapse, by PFGE or other typing methods, would help establish this mechanism. Determination of the specific clostridia involved in autism and development of an appropriate animal model would permit definitive documentation (or refuting) of this hypothesis.

Hypothesis 2. The increased incidence of autism is related to the relatively widespread dissemination of clostridial spores in the environment leading to infection of other children who may be immunosuppressed by virtue of contact with certain chemicals or other materials or who are immunosuppressed on a genetic basis. Consider first of all that many of the clostridial species in the human gut are prolific spore producers , secondly that children at the age of two years (or even children of five or six years) or less are not very hygienic in their habits, and thirdly that autistic children are likely to be less hygienic than other children of the same age. There is ample opportunity for contamination of floors, or other surfaces on which children play or various fomites, with bacteria from the bowel, perhaps particularly in children wearing diapers. Other children playing on these surfaces or contacting the fomites may acquire bacteria by fecal-oral contamination. Although many clostridia, being anaerobic in nature, may not survive for long periods in the environment, the spores will stay alive for prolonged periods [9] and then germinate into vegetative bacterial forms in the intestinal tract of the ''victim''. Spores, of course, resist gastric acid and other host defense mechanisms. It should be noted, also, that spores are resistant to the usual household germicides and, for that matter, to virtually all the routine germicides used in hospitals, and parents would be reluctant to use germicides or other harsh chemicals on the surfaces where infants play. Mechanical removal of spores by thorough and frequent cleansing is probably the best bet at present, but simple and safe methods of destroying spores will likely become available. A form of ''isolation'', such as keeping the potential index case in a playpen with a raised

floor may be useful. To the extent it is feasible, periodic handwashing with conventional soap (not germicidal soaps which may irritate the skin or induce local allergic reactions) and water would reduce the number of spores [10,11]. The ''wipes'' and lotions, etc. which are used in homes and hospitals also only reduce the number of spores [10,11].

Hypothesis 3. The increase in families with multiple cases of autism is also due to contact with spores, as outlined in Hypothesis 2. Young children in the family who are predisposed would typically have much greater contact with disseminated spores than playmates or casual contacts.

Testing of Hypotheses 2 and 3 also depends on determination of the specific clostridial species that are involved in autism (and documentation of involvement of intestinal bacteria by means of a double-blind, placebo-controlled treatment study of autistic children with an antibiotic such as oral vancomycin that is not absorbed from the bowel). Microbiologic studies of surfaces and fomites in homes of autistic children and control children (age- and sex-matched) should provide data to support or refute these hypotheses.

Discussion

Reasons to consider that intestinal bacteria may be involved in autism are: (1) onset of the disease often follows antimicrobial therapy, (2) gastrointestinal symptoms are common at the onset and often persist, (3) other antimicrobials (e.g., oral vancomycin) may lead to a clear-cut response, and relapse occurs when the antimicrobial is discontinued, and (4) anecdotally, a number of patients have responded to several courses of vancomycin and relapsed each time when it was discontinued. The reasons that we have suspected clostridia specifically are: (1) anecdotally, one of the antimicrobials that most commonly predisposes to late-onset autism is trimethoprim/sulfamethoxazole, a drug that is notably poorly active vs. clostridia [12], (2) patients respond to vancomycin and again anecdotally, to oral metronidazole, suggesting that a gram-positive anaerobic organism is involved, (3) unusually high tetanus antitoxin titers have been noted in several patients with late-onset autism [13] even though *C. tetani* has never been recovered from autistic subjects in any of our studies (other clostridia may produce toxins similar to tetanus toxin), and (4) clostridia are the principal bacteria that produce both an enterotoxin and neurotoxins as well as a number of other metabolic products that are potentially toxic (e.g., phenols, *p*-cresol, certain indole derivatives).

Vancomycin, given orally, is essentially not absorbed from the gut so that any effect that is seen with its use is likely due to its action on intestinal bacteria. As discussed above, relapse after discontinuation of oral vancomycin is likely due to germination of spores that persisted through the vancomycin therapy.

Implicit in the hypotheses proposed herein is the relatively widespread use of antimicrobials. The points made in this paper regarding clostridial spores being important in: (1) persistence of the clostridia despite oral vancomycin and other therapy and accounting for relapse after discontinuation of therapy, and (2) spread of the clostridial infection to other susceptible individuals are also important in certain other clostridial diseases involving the gastrointestinal tract, notably *Clostridium difficile*-caused antimicrobial-associated colitis or diarrhea.

If Hypothesis 1 is validated, it will dictate the need for benign agents that might be used together with or following antimicrobial therapy to eliminate spores. If such agents became available, their use could establish the validity of the hypothesis. Hypotheses 2 and 3 have profound implications regarding the epidemiology of this important and distressing disease. If these hypotheses are validated, research on removing spores from the home environment and other pertinent environments will be spurred.

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Original Article

Short-Term Benefit From Oral Vancomycin Treatment of Regressive-Onset Autism

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ABSTRACT

In most cases symptoms of autism begin in early infancy. However, a subset of children appears to develop normally until a clear deterioration is observed. Many parents of children with "regressive"-onset autism have noted antecedent antibiotic exposure followed by chronic diarrhea. We speculated that, in a subgroup of children, disruption of indigenous gut flora might promote colonization by one or more neurotoxin-producing bacteria, contributing, at least in part, to their autistic symptomatology. To help test this hypothesis, 11 children with regressive-onset autism were recruited for an intervention trial using a minimally absorbed oral antibiotic. Entry criteria included antecedent broad-spectrum antimicrobial exposure followed by chronic persistent diarrhea, deterioration of previously acquired skills, and then autistic features. Short-term improvement was noted using multiple pre- and post-therapy evaluations. These included coded, paired videotapes scored by a clinical psychologist blinded to treatment status; these noted improvement in 8 of 10 children studied. Unfortunately, these gains had largely waned at follow-up. Although the protocol used is not suggested as useful therapy, these results indicate that a possible gut flora–brain connection warrants further investigation, as it might lead to greater pathophysiologic insight and meaningful prevention or treatment in a subset of children with autism. (*J Child Neurol* 2000;15:429–435).

Autism is a devastating and largely untreatable disorder first described by Kanner in 1943.[1] Currently classified as a pervasive developmental disorder in the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition (*DSM-IV*),[2] it usually manifests in early infancy, with impairment typically persisting into adulthood.[3] Incidence estimates vary from 10 to 20 per 10,000 children, with boys four times more likely to be affected.[4] Although some children are later found to have chromosomal aberrations or metabolic disorders that might explain their autistic features, no underlying etiology can be identified in the vast majority of cases.[5] "Autistic regression" occurs in approximately one third of cases, with regression typically occurring before 2 years of age, and involving loss of language, social, and play skills.[6]

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HYPOTHESIS

Several parents of children with regressive-onset autism reported to us their observation of the following sequence: repeated broad-spectrum antimicrobial use (usually for chronic otitis media), followed by chronic diarrhea, then loss of language, play, and social skills, and subsequent onset of autistic symptoms. Although these observations could be unrelated, they led to speculation regarding a possible etiologic link in this sequence. We developed the hypothesis that repeated antimicrobial use might have disrupted a protective effect of indigenous intestinal organisms and allowed colonization by one or more neurotoxin-producing species.[7] If this were true, then appropriately targeted antimicrobial therapy might reduce autistic symptoms in these individuals.

We therefore performed an exhaustive literature review to search for candidate organisms. The details of this review

are beyond the scope of this article, but it appeared most plausible that the agent(s), if present, might be a clostridial species. Several members of this genus have been implicated in diarrheal diseases of humans and animals. *Clostridium tetani* and *C botulinum* produce potent, nonnecrotizing neurotoxins that severely disrupt neurotransmitter release.[8] Also, at least one clostridial species (*C difficile*) has demonstrated its ability to proliferate enterically during use of certain antimicrobials.[9]

TREATMENT RATIONALE

If, in fact, this conjecture were correct, therapeutic options would include oral metronidazole, bacitracin, or vancomycin. The latter was chosen for its efficacy, minimal absorption (ie, the antibiotic remains in the intestinal tract and is excreted in the stool), and benign taste (the unpleasant-tasting metronidazole or bacitracin would have required a nasogastric tube for drug delivery). The decision to use vancomycin was not made lightly, however, since this drug is of paramount importance in treating life-threatening antibiotic-resistant bacterial infections, and significant public health concerns exist should its use become widespread in the community.[10]

INDEX CASE

The index case was a 4½-year-old Caucasian boy with chronic diarrhea and autism whose motor, cognitive, and social development was normal until 18 months of age. Diarrhea began at approximately 17 months of age after three 10-day courses of broad-spectrum antimicrobials prescribed over a 6-week period for chronic otitis media. There was no blood or pus in the stool nor associated constitutional symptoms. At 19 months of age there was profound behavioral and developmental deterioration, along with emergence of severe autistic features.

Extensive genetic, neurologic, gastrointestinal, and immunologic evaluations were all unrevealing. Neither conventional (eg, full-day special education program, speech, and play therapy) nor unconventional interventions (eg, special diets, megavitamin loading) had a significant effect on his autistic symptoms.

A 12-week therapeutic trial of oral vancomycin (125 mg four times per day) was begun with expanded observations by a pediatric neuropsychologist pre- and post-treatment. At baseline, the child was not on a special diet nor was he taking any vitamin supplements. Three days after initiation of the vancomycin therapy, a hyperactivity pattern emerged that lasted 4 days. This was followed by 2 days of lethargy and subsequently by a rapid and dramatic clinical improvement. He became affectionate and relatively calm. He promptly achieved toilet training and increased vocabulary. Follow-up behavioral observations after 8 weeks of therapy noted an increase in on-task performance, compliance with parental requests, awareness of environmental surroundings, and persistence when engaging in positive activities. A significant reduction in repetitive and self-stimulatory behaviors was also noted. The child's educational therapies remained unchanged for both 6 months before and during the vancomycin trial. Shortly after vancomycin discontinuation, behavioral deterioration was observed. Though still improved over baseline, he eventually lost most of the initial gains.

METHODS

Subjects and Study Design

To explore whether our index case's improvement represented a true therapeutic effect, institutional human investigation committee approval was obtained for an open-label trial in a narrowly defined subgroup of autistic children. Eleven children (10 boys, 1 girl; age range, 43 to 84 months) were enrolled. Inclusion criteria for the study are listed in Table 1 and were derived from our central hypothesis and index case characteristics. All children had diarrhea and regressive onset of autistic features (occurring at a mean of 17.7 ± 3.4 months) as previously defined in the literature.[11]

The Developmental Profile II[12] provided descriptive developmental levels to contrast with developmental age. While mean chronologic age of the children was 59.4 ± 12.7 months, the mean developmental age for the domains of communication (23.0 months ± 13.0), socialization (25.6 months ± 12.9), and self-help (34.4 months ± 12.4) is evidence of their significant developmental delay. The Childhood Autism Rating Scale was also administered. This is a 15-item behavioral rating scale developed to identify children with autism, and to distinguish them from developmentally handicapped children without autism syndrome. Based on Childhood Autism Rating Scale diagnostic categories, six children met the criteria for severe autism, two for moderate autism, and three for mild autism.[13] The vancomycin dose was 500 mg/day given orally as a liquid (500 mg/6 mL), divided 2 mL three times per day for 8 weeks. This was followed by 4 weeks of oral treatment with a probiotic mixture of *Lactobacillus acidophilus, L bulgaricus,* and *Bifidobacterium bifidum* (40×10^9 colony-forming units/mL).

Psychologic Evaluations

Two measures of potential improvement were examined: (1) Children were videotaped for 30 minutes at baseline and once during therapy in a playroom environment. At each session, the child was directed to play with a series of puzzles, books, blocks, and dolls by the mother and then by the evaluator. At the end of the trial,

Table 1. Study Entry Criteria

1. Meets diagnostic criteria for autistic disorder (*DSM-IV* 299.00)
2. Other genetic and medical diagnoses have been adequately evaluated and ruled out
3. Definable, rapid onset after 12 months of age
4. Antecedent antimicrobial use (2 months or less before autism symptom onset)
5. Persistent loose stool history, with diarrhea onset before autism symptoms
6. Symptoms for 4 years or less
7. Child is between 2 and 8 years of age
8. No evidence of any significant medical problem that might complicate treatment, such as renal, cardiac, or pulmonary disease; severe enterocolitis (visible blood or pus in the stool), or chronic infection (eg, tuberculosis)
9. Clinically static for 3 months or more (no new neuroleptic, seizure, or other medications), with no elective changes during the study
10. No antimicrobial use for at least 2 months prior to entry into the study

Table 2. Scoring System for Videotapes

Observer Rating Analog Assessment Scales for Behavior, Communication, and Social Skills

Child's Name: _____

Does the child appear "better" overall in one tape over the other? Yes No

If yes, in which tape does the child appear better? _____

Place mark on line where 10 = normal behavior and 0 = horribly abnormal. Note "NR" on scale if not ratable.

Tape number:_____	Tape number:_____
GLOBAL IMPRESSION	GLOBAL IMPRESSION
0 5 10	0 5 10
BEHAVIOR SUBDOMAIN RATINGS	
0 5 10	0 5 10
Global Behavior Rating	**Global Behavior Rating**
0 5 10	0 5 10
Perseveration	Perseveration
0 5 10	0 5 10
Noncompliance	Noncompliance
0 5 10	0 5 10
Oppositional Behavior	Oppositional Behavior
0 5 10	0 5 10
Self-Stimulation Behaviors	Self-Stimulation Behaviors
COMMUNICATION SUBDOMAIN RATINGS	
0 5 10	0 5 10
Global Communication Rating	**Global Communication Rating**
0 5 10	0 5 10
Expressive Language	Expressive Language
0 5 10	0 5 10
Receptive Language	Receptive Language
SOCIAL SKILLS SUBDOMAIN RATINGS	
0 5 10	0 5 10
Global Social Scale Rating	**Global Social Scale Rating**
0 5 10	0 5 10
Eye Contact	Eye Contact
0 5 10	0 5 10
Approach Behavior	Approach Behavior
0 5 10	0 5 10
Play Skills	Play Skills

a clinical child psychologist (who was provided with a brief explanation of our working hypothesis) compared coded, paired videotapes of 10 of the 11 children studied (video was not available for one child). The psychologist viewed each pair of tapes and scored them using the rating scale shown in Table 2. To diminish the possibility of investigator bias, the tapes were randomly numbered and the psychologist did not have any personal contact with the children. (2) Behavior and communication analog rating scales (Table 3) were completed by the study physician at baseline, during therapy, and at follow-up in a manner similar to previously validated methods for other disease states.[14] Results are presented as median scores to account for potential nonlinear score increment.

Table 3. Physician Analog Rating Scales

Analog Assessment Scales for Behavior and Communication

Physician Rating

Date: _____

Child's Name: _____

Behavior: 1 5 10

Communication: 1 5 10

1 = Severely impaired, can't be any worse; 10 = Age appropriate

Behavior: Compliance to requests. Mood (temper, outbursts, irritability), eye contact, attention, and alertness. Activity level. Interaction with others present in room. Stereotyped behaviors (degree of severity).

Communication: Pointing or gesturing. Babble and quality of babble. Receptive language. Use of sign language and gesture (prompted/spontaneous). Verbal language (prompted/spontaneous, understandable). Appropriate use of language (single words, two words together, sentences). Verbal perseveration.

Laboratory Evaluations

Extensive medical evaluations were conducted in parallel with the detailed psychologic assessments. Stools were examined for occult blood, inflammatory cells, *Aeromonas hydrophila*, *Cryptosporidium*, *C difficile* toxin, routine bacterial pathogens, and ova and parasites. Blood tests included complete blood cell counts, chemistry panels, and erythrocyte sedimentation rates. Urinalyses were also obtained. Detailed quantitative aerobic and anaerobic fecal microbiologic studies were conducted at the Wadsworth Anaerobic Bacteriology Laboratory on specimens from four children. Each stool was cultured with a total of 27 different media and atmospheric conditions, modified from the procedure described in Summanen et al.[15]

RESULTS

Analog Rating Scales, Videotapes, Treatment Observations, and Laboratory Evaluations

As shown in Table 4, unblinded assessment using an analog rating scale noted improvement for the group as a whole in communication (Wilcoxon Signed Rank Z-score = –2.9, $P = .003$) and behavior (Wilcoxon Signed Rank Z-score = –2.9, $P = .003$). To ensure that changes attributed to intervention were not a reflection of differences at baseline, Spearman correlations were conducted. There were no significant correlations between the baseline measure and scores

Table 4. Results of Two Measures of Short-Term Improvement

Demographics			Measures of Improvement				
				Subjective Visual Analog Rating Scales			
				Behavior*		Communication*	
Subject	Age at Onset of Autism, months	Age at Initiation of Treatment, months	Paired Videotapes	Baseline	Post-treatment	Baseline	Post-treatment
1	14	78	+	1.5	4.7	1.7	2.2
2†	16	61	+	5.2	5.6	5.1	5.6
3	16	43	–	1.9	2.1	1.8	2.1
4	15	57	Not available	1.5	3.3	1.4	1.7
5	19	47	+	2.3	4.6	1.2	4.3
6	18	70	+	2.2	6.0	1.7	5.1
7	27	84	+	2.4	5.0	2.0	4.6
8	18	63	+	2.3	7.7	1.7	5.3
9	18	44	±‡	1.4	6.7	1.0	5.2
10	16	56	+	2.2	3.3	2.2	2.8
11	18	56	+	2.5	5.5	2.8	5.6
Mean	17.7 (±3.4)	59.9 (±13.3)	Median§	2.2/1.5, 2.4§	5.0/3.3, 6.0§	1.7/1.4, 2.2§	4.6/2.2, 5.3§

*Behavior scale rated compliance to requests, mood, eye contact, attentiveness, activity level, and severity level of stereotyped behaviors. Communication scale rated receptive and expressive language (10 = "normal," 1 = "worst" autistic features).
†Testing instruments were relatively insensitive at measuring improvement observed in this subject, who was "high functioning" at baseline (eg, communication skills shifted from "need-based" language to "conversational" while on therapy).
‡Although blinded rater did not observe clear difference between paired tapes, study physician, psychologist, coordinator, and parents unanimously agreed that child clearly improved while on therapy.
§Results are presented as median, 25th and 75th percentiles. Subjective analog rating scale post-treatment scores were significantly improved over baseline rating ($P = .003$ for both behavior and communication scales).
+ = on-therapy videotape rated as clearly better than baseline; – = baseline videotape rated as clearly better than on-therapy;
± = no clear difference between the two tapes.

during intervention for either communication ($\rho = .35$, $P = .28$) or behavior ($\rho = .22$, $P = .51$). Blinded assessment of the coded, paired videotapes noted an improvement during therapy in 8 of 10 children studied, no change in one, and a possible deterioration in one.

As previously observed in the index case, a brief (1 to 4 days) period of hyperactivity was noted in six children within 3 days of initiating antibiotic treatment. One subject then experienced a day of marked lethargy. Otherwise, aside from obvious autistic features, all children had normal physical examinations at baseline and throughout the study, as well as unremarkable basic blood, stool, and urine tests as outlined in Methods.

Long-Term Follow-Up

Although improvement was clear by several measures, unfortunately these gains did not endure. One child who had responded significantly to treatment deteriorated toward the end of the study while still on vancomycin therapy. During telephone follow-up (conducted weekly during the probiotic therapy), most parents reported substantial behavioral deterioration within 2 weeks of discontinuance of vancomycin treatment. Because of difficulty in disguising the taste, probiotic treatment compliance was very poor in several children. Behavioral deterioration appeared to occur whether or not the child was compliant with the probiotic therapy regimen. Therefore, it would appear that the probiotic therapy used as an adjunct after vancomycin treatment had no discernible beneficial or adverse effect. All children were observed in follow-up ranging from 2 to 8 months after discontinuance of vancomycin. In all but one child, the analog ratings returned toward baseline (Figure 1).

Quantitative Fecal Flora

Given the extreme labor intensiveness of such studies, it will be some time before detailed microbiologic analysis of all pre- and post-therapy stool specimens is completed. Stool specimen data from four autistic children prior to vancomycin therapy were compared to those of 104 normal adult subjects from previously published studies (performed under the supervision of the same principal investigator).[16] Anaerobic cocci, chiefly peptostreptococcal species, were present in 93% of the adults' specimens, comprising some 10% of the stool microorganisms. In stark contrast, these

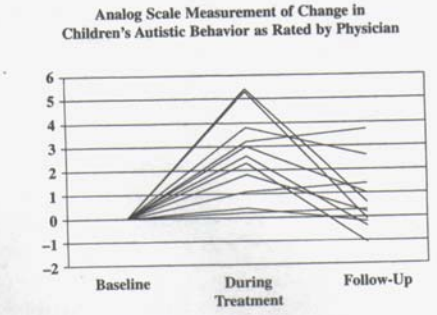

Figure 1. Analog Behavioral Rating Scale. Shown is a comparison of analog behavioral ratings for each of the 11 study children at three points: baseline, during vancomycin treatment, and after longer-term follow-up (2 to 8 months). Scores for each child are adjusted for differences in baseline. Positive scores indicate improvement; negative scores indicate worsening relative to baseline. Ratings for communication skills showed a similar pattern and are not shown separately.

Table 5. Fecal Flora Data

Organism	*Autistic Patient A*	*Autistic Patient B*	*Autistic Patient C*	*Autistic Patient D*	*Adults (N = 104*)*
Enterobacteriaceae	6	7	7	7	9
Streptococcus	3	5	0	4	9
Enterococcus	0	6	0	0	8
Bacteroides fragilis group	8	8	9	8	11
Bacteroides, other	8	0	9	8	11
Anaerobic gram negative rod, other	6	4	7	5	8
Peptostreptococcus species	0	0	0	0	10†
Anaerobic cocci, other	0‡	0	0‡	0	11†
Lactobacillus species	9	9	10	8	10
Bifidobacterium species	7	9	9	8	10
Eubacterium species	8	0	9	8	11
Clostridium species	9	7	8	8	10

Units are $\log_e$ colony-forming units per gram dry weight.
*Mean of positive specimens. Subjects were normal adults on various diets (vegetarian, traditional Japanese, or standard Western); there were no statistically significant differences in results between these various groups.
†93% of the 104 subjects had *Peptostreptococcus* species or other anaerobic cocci.
‡Ethanol and heat-resistant coccoid forms were present (probably clostridia).
§Heat-resistant coccoid forms were present (probably clostridia).

species were absent from the stools of each of the four autistic children tested (Table 5).

DISCUSSION

The apparent, though short-term, improvement during treatment with this minimally absorbed antibiotic is not explainable using current conventional genetic hypotheses[17] alone for autism. Results of this preliminary study, along with previous reports of increased intestinal permeability[18] and a "nonspecific colitis" in children with autism,[19] suggests a possible "gut-brain" etiologic connection in a subset of these children.

Although the hypothesis that autism (in a defined subset of children) might be a sequela to the colonization of the intestinal tract by one or more neurotoxin-producing bacteria is novel, published data along several paths could lend credence to the notion that an alteration in colonic flora contributes to autism symptoms. The first line of evidence is from the infant botulism literature. This condition was first recognized as a distinct clinical entity in 1976.[20] It differs from classic (foodborne) botulism in that the intestinal tract becomes colonized by *Clostridium botulinum* and elaboration of the neurotoxin occurs in vivo.[21] Age is a primary risk factor for the development of infant botulism; diagnosis of the disease is rare after 1 year of age.[22] Studies in animals have demonstrated a similar age-dependent susceptibility.[23] However, the colonization resistance observed in mature animals is greatly diminished when they are treated with broad-spectrum antimicrobials.[23] Similarly, antimicrobial use has been identified as a risk factor for the development of botulism related to intestinal colonization with *C botulinum* in older children and adults.[24]

The second line of evidence is from human and animal studies that have demonstrated repeatedly that intestinal colonization by opportunistic pathogens (eg, *Escherichia coli*,[25,26] *Klebsiella pneumoniae*,[25] *Pseudomonas aeruginosa*,[26,27] *Salmonella enteritidis*,[28] *Shigella flexneri*,[29] and *Vibrio cholerae*[29]) is greatly enhanced when protective intestinal microbiota are disrupted by broad-spectrum antimicrobials. In humans, the best-documented example of opportunistic colonization of the intestinal tract following antimicrobial use is that by *C difficile*, the causative agent of pseudomembranous colitis.[9]

Another potentially relevant condition is D-lactic acidosis, in which associated psychiatric symptoms are well documented. D-lactic acidosis, a complication of short bowel syndrome or intestinal bypass surgery for obesity, is a condition caused by a change in bacterial flora to an acid-tolerant, aciduric (*Lactobacillus*, *Bifidobacterium*, *Eubacterium*, and *Streptococcus*) flora.[30] Patients present with a range of behavioral changes such as hostility, slurred speech, stupor, altered mental status, dizziness, asterixis, and ataxia.[31] Treatment is with oral antimicrobials, resulting in rapid cessation of neurologic signs.

No validated instrument is currently available for quantitative measurement of improvement in autistic symptomatology and there is an urgent need to correct this deficit for use in future autism intervention trials. In the absence of a preexisting standardized method, the current study used two independent assessment tools. Although the analog rating scales were completed by the study physician, who was aware of the children's treatment status, the formal videotape ratings were performed in a blinded manner. The improvement observed after vancomycin intervention appeared to be significantly greater than could normally be attributable to the characteristic waxing and waning of autistic symptomatology.[32]

A substantial deterioration of the behavioral improvements made while on therapy was reported by most parents within 2 weeks of ending the vancomycin trial. While the cause is not known for either the apparent improvement or the later decline, it is possible that the deterioration is due to the offending organism being spore forming, and hence surviving therapy to germinate after vancomycin discontinuation, as has been documented with *C difficile* infection.[33] An additional possibility is that the therapy was sublethal because of antimicrobial choice, dosage regimen,

or both, permitting emergence of an antimicrobial-resistant bacterium.

Since vancomycin is not absorbed, it appears likely that the behavioral improvement was related, in some way, to the drug's effect on the intestinal-tract flora (and not a "drug effect" per se on the central nervous system). Although we theorize that the short-term benefit from vancomycin treatment might be due to the temporary elimination of a neurotoxin-producing pathogen, there are other possible mechanisms. For example, autoantibodies to neuron-axon filament protein,[34] glial fibrillary acidic protein,[34] and myelin basic protein[35] have been reported in autism and it has been postulated that these autoantibodies might contribute to autistic symptomotology.[34,35] It is, at least, theoretically possible that the production of these autoantibodies is related to the presence of an infectious pathogen, as has been postulated for rheumatoid arthritis.[36]

The significance of the possible fecal flora changes in these autistic children is unknown. It is unlikely that specimen collection or shipping contributed to the absence of *Peptostreptococcus* and other anaerobic cocci as other equally oxygen-sensitive organisms were recovered. Although all of the children previously had received broad-spectrum antimicrobials (capable of severely disrupting intestinal flora), fecal bacterial counts typically return to their pretreatment composition within 2 weeks of discontinuance of the antimicrobial agent.[37] Therefore, since none of the children, at baseline, had a history of antimicrobial treatment for at least 2 months prior to entering our study, it is unlikely that the absence of these species reflects a transient alteration in the children's fecal flora. An uncharacterized *Peptostreptococcus* species has been documented to inhibit certain organisms, including clostridia, in vitro and in animals,[38] and it is intriguing to speculate that the absence of such organisms in certain autistic children might permit growth of clostridial or other toxin-producing bacteria through loss of competitive inhibition.[39]

The fecal flora of pediatric subjects has been studied extensively.[40–42] Use of normal adult control fecal specimens in the present study, though not ideal, is justifiable given documented similarity to pediatric stool flora. For example, one recent review of bacterial colonization patterns states that "by 12 months (of age) the anaerobic fecal populations begin to resemble that of adults in number and composition as the facultative anaerobes decrease. By two years of age, the profile resembles that of the adult."[43]

CONCLUSIONS

In this study, an open-label vancomycin trial indicates the possibility of a gut flora–brain connection in a subset of children with autism and diarrhea. If such a connection is later verified, it might be possible to identify an offending organism, and from this, effective prophylactic (eg, vaccine) or therapeutic measures.

Given the devastating nature of autism and the current lack of effective medical treatments, parents are understandably anxious to try newly reported therapies. However, we must emphasize that the benefit of oral vancomycin appears to be only short-term, and the potential threat of vancomycin-resistant organisms must be seriously considered. Therefore, we urge that vancomycin not be used to treat autistic symptomatology outside of a study protocol, and that further research of a possible gut–brain connection be vigorously pursued.

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