Microbiological Diagnosis of Severe Diarrhea in Kidney Transplant Recipients by Use of Multiplex PCR Assays

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Diarrhea is a frequent complication after kidney transplantation, ascribed to adverse effects of the immunosuppressive therapy in case of negative microbiological examination of the stools. The aim of this study was to improve the microbiological diagnosis by implementing molecular tests. Fifty-four severe diarrhea events that occurred in 49 adult kidney transplant recipients from September 2010 to November 2011 were investigated. One or several enteric pathogens were detected in 13 (23%) stool samples using classical microbiological methods versus 39 (72%) for the seven commercially available multiplex PCR assays used retrospectively ($P = 0.006$). Interestingly, molecular diagnosis identified 15 multiple infections compared to none using classical techniques. The primary pathogens detected were enteropathogenic *Escherichia coli* (EPEC) ($n = 15$; 38%), *Campylobacter* spp. ($n = 15$; 38%), and *Norovirus* ($n = 14$; 36%). Specificities for *Campylobacter* and *Norovirus* infection diagnosis were 75 and 100%, respectively, by comparison to reference methods. Based on molecular findings, a cyclosporine-mycophenolate mofetil combination was identified as a risk factor for developing *Norovirus*-induced diarrhea. *Norovirus* infections were also responsible for higher weight loss than all the other causes of diarrhea. In samples from asymptomatic immunocompromised and immunocompetent patients, EPEC but not *Norovirus* and *Campylobacter* infections were detected at a frequency similar to that observed in symptomatic kidney transplant recipients. In conclusion, molecular tools significantly improved the detection of single and multiple enteric infections by comparison to classical techniques and could quickly become the key element in the management of severe acute diarrhea in transplant recipients.

Diarrhea is a frequent complication in kidney transplant recipients (1–6). Although diarrhea is often considered to be benign, severe episodes can cause dehydration with weight loss, high creatinine levels, and a fluctuation in serum immunosuppressive drug concentrations, resulting in a significant increase in mortality and morbidity and a poorer functional prognosis for the graft (1, 2, 6). Etiological diagnosis is difficult given the numerous causes of diarrhea in transplant recipients. All immunosuppressive treatments administered posttransplantation, especially mycophenolate mofetil (MMF), were associated with the onset of gastrointestinal complications (7, 8). Diarrhea may also be due to drug toxicity not related to immunosuppressive therapy, such as oral hypoglycemic agents, inflammatory bowel disease exacerbation, broad-spectrum antibiotics, or, lastly, infections (6). In practice, negative microbiological diagnosis leads nephrologists to perform invasive complementary tests like colonoscopy and often to modify immunosuppressive treatment considered to be the cause of diarrhea, with a risk of acute or chronic transplant rejection (9–11). Yet in various studies, it has been reported that, in 38 to 64% of cases, diarrhea was of infectious origin (1, 12–14). The implicated microorganisms corresponded mainly to enteric bacteria (*Campylobacter* spp., enterovirulent *Escherichia coli*, *Salmonella* spp., *Clostridium difficile*), parasites (*Giardia intestinalis*), and viruses (*Norovirus* and *Rotavirus*) that are already responsible for gastroenteritis in the general population (1, 2, 6, 12–16). However, some pathogens, like human cytomegalovirus (CMV), mi-

crosporidia, and cryptosporidia, were specifically found in immunocompromised patients (1, 12, 13, 17–20, 34).

Microbiological diagnosis of diarrhea episodes in transplant recipients is currently based on using classical techniques involving stool culture for bacteriology, parasitological examination of stool, and virus detection using rapid antigen detection tests (RADT) (1, 6, 12, 13). These methods have in general a poor ability to detect fragile microorganisms that are present at low levels in the stool of immunocompromised patients or microorganisms that demonstrate significant antigenic variations. The recent development of faster and more-sensitive molecular tests that can detect simultaneously a wide range of bacterial, viral, and parasitic pathogenic agents might improve the etiological diagnosis of infectious diarrhea (16, 21, 22). Their contribution to the management of severe diarrhea episodes in renal transplant recipients still needs to be evaluated. In this context, the objective of this study was to compare molecular methods to classical methods for

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microbiological diagnosis of severe diarrhea events in kidney transplant recipients during a 15-month study.

MATERIALS AND METHODS

Patients. (i) **Cases.** Between September 2010 and November 2011, every adult kidney transplant recipient, whatever the posttransplantation term, admitted in the nephrology department of the Reims university hospital for severe diarrhea was enrolled in the study. Severe diarrhea was defined as 3 stools/day for ≥ 7 consecutive days or ≥ 3 stools/day accompanied by weight loss ≥ 2 kg and/or fever and/or mucous and/or blood in the stool and/or hospitalization. Patients with inflammatory bowel diseases were excluded from the study. Demographic profiles (age, sex, posttransplantation term), clinical data (hospitalization, hospitalization duration, number of stools per day, weight loss compared to the last three preinclusion weigh-ins), biological data (hematocrit, variations in creatinine levels compared to the average value observed during the last three consultations, protein C reactive level, leukocyte counts), and immunosuppressive therapy of all investigated patients were recorded at inclusion. The same patient could be included several times if a period of 1 month had elapsed after the resolution of the preceding episode.

(ii) **Controls.** Three control groups, consisting of (i) the same patients ($n = 30$) sampled again 6 months after resolution of the episode of severe diarrhea, (ii) renal transplant recipients ($n = 30$) with no history of diarrhea and matched for age, sex, and immunosuppressive therapy, and (iii) asymptomatic immunocompetent patients ($n = 30$) hospitalized in the cardiology department for defibrillator implantation, were made up and investigated.

Informed consent was obtained from each patient included. The present study was conducted by the university hospital of Reims (Champagne Ardenne, France) and was approved by the hospital's ethics committee.

Clinical specimens. A stool sample was taken from each study participant at the time of the severe diarrhea episode [see "Patients (i) Cases"] or during consultation or hospitalization for another reason [see "Patients (ii) Controls"]. For kidney transplant recipients with severe diarrhea, parasitological tests were performed again on a second sample taken 72 h after inclusion to deal with the shedding of intestinal protozoa in the stool. Fibroscopy with colonic biopsy specimens was performed if symptoms persisted more than 21 days with negative microbiological analyses and/or worsening symptoms (rectal hemorrhage, fever).

Conventional microbiology diagnosis. Stool samples for the cases were routinely addressed at the time that they were sent to the bacteriology, parasitology, and virology laboratories for analysis. Bacteriology diagnosis prospectively performed consisted of conventional culture on Hektoen agar plates (bioMérieux, Marcy l'Etoile, France) after 24 h of selenite broth enrichment to detect *Salmonella* and *Shigella*, on *Yersinia* agar plates (bioMérieux, Marcy l'Etoile, France), on *Campylosel* agar plates (bioMérieux, Marcy l'Etoile, France) for the isolation of *Campylobacter* spp. (*C. jejuni*, *C. coli*, *C. fetus*, and *C. lari*) under a microaerobic atmosphere, on nonselective chromogenic medium UriSelect 4 (Bio-Rad, Marnes-la-Coquette, France), and sorbitol-MacConkey agar plates (Oxoid, Basingstoke, United Kingdom). These tests were combined with the detection of *eae*, *stx*₁, and *stx*₂ genes by an in-house PCR to detect enteropathogenic *E. coli* (EPEC) and Shiga toxin-producing *E. coli* (STEC). *Enterobacteriaceae* isolates were identified by Vitek 2 ID-GN (bioMérieux, Marcy l'Etoile, France). *Salmonella enterica* and *Shigella* spp. isolated from stool samples were serotyped by slide agglutination with commercially produced antisera (Bio-Rad, Marnes-la-Coquette, France). Diagnosis of *Clostridium difficile* infection was performed by direct toxin testing on fecal specimen using an enzyme immunoassay (EIA) (ImmunoCard toxin A/B; Meridian Bioscience, Cincinnati, OH) and by toxigenic culture on *Clostridium* agar plates (bioMérieux, Marcy l'Etoile, France) combined with EIA for toxin on isolates. Parasite diagnosis consisted of routine microscopic ovum and parasite examination directly from fresh stool and from specimens concentrated by physicochemical methods using sedimentation associated with the acid-fast Ziehl-Neelsen staining technique

for cryptosporidium detection. For virology, clinical samples were analyzed with RADT for *Rotavirus*, *Adenovirus* (Biorapid Rota-Adeno; Biokit, Barcelona, Spain), and *Norovirus* genogroups I (GI) and II (GII) (Ridaquick Norovirus; R-Biopharm, Darmstadt, Germany). Stool specimens were then stored at -80°C until processing with the multiplex PCR tests.

In case of intestinal endoscopy, colonic tissue was subjected to real-time PCR assay for CMV detection (23). Results were compared to those of histopathological examination, including immunohistochemical staining of CMV antigens.

Multiplex PCR assays for enteric pathogen detection. At the end of the patient inclusion phase, the collected stool samples from the cases were retrospectively tested with seven commercially available multiplex PCR assays (xTAG GPP [Luminex molecular diagnostics, Toronto, Canada], Seeplex Diarrhea ACE Detection method [Seegene, Seoul, South Korea], gastroenteritis virus and gastroenteritis parasite panels [Diagenode, Liege, Belgium], Ridagene Norovirus I & II and Ridagene EHEC/EPEC [R-Biopharm, Darmstadt, Germany], and Clart Enterobac [Genomica, Madrid, Spain]). This allowed the detection of enteric bacteria (*Aeromonas* spp., *Campylobacter* spp. [*C. lari*, *C. laridis*, *C. upsaliensis*, *C. coli*, *C. jejuni*], enteropathogenic *Escherichia coli* [EPEC; *eae* gene], Shiga toxin-producing *Escherichia coli* [STEC; *stx*₁ and *stx*₂ genes], enterotoxinogenic *Escherichia coli* [ETEC], *Shigella* spp. [*S. dysenteriae*, *S. sonnei*, *S. boydii*, and *S. flexneri*], *Salmonella* spp., *Vibrio cholerae*, *Yersinia enterocolitica*, and *Clostridium difficile* A/B), parasites (*Cryptosporidium parvum*, *Entamoeba histolytica*, and *Giardia intestinalis*), and viruses (*Rotavirus* A, *Adenovirus* serotypes 40 and 41, *Astrovirus*, and *Norovirus* GI and GII). Stool samples from the three control groups were tested with the xTAG GPP, Ridagene, and Clart Enterobac kits.

Total nucleic acid extraction (DNA and RNA) was performed from approximately 150 mg of stool using the NucliSens EasyMAG instrument (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Briefly, 200 μl of preextracted stool obtained by glass bead grinding and clarification by centrifugation was added to 2 ml of lysis buffer in a plastic vessel and incubated for 10 min at room temperature. Fifty microliters of silica was then added to the mixture. This was followed by an automatic magnetic separation phase. Nucleic acids were recovered in 70 μl elution buffer and divided into 7 aliquots of 10 μl , which were stored at -80°C until use. Each technique was then performed using a different aliquot according to the manufacturer's instructions.

Confirmation of the molecular findings by reference methods. Stool samples positive for one of the enteric viruses and for *Campylobacter* spp. were sent to the National Reference Centers (NRC) for Enteric Viruses (P. Pothier, Dijon, France) or the NRC for *Campylobacter* and *Helicobacter* (F. Megraud, Bordeaux, France) for confirmation and typing. Enteric virus detection confirmation was obtained by amplification and sequencing of part of the polymerase and capsid protein-coding genes (24–26). *Campylobacter* detection confirmation was obtained by the combination of an in-house PCR assay specific for *Campylobacter jejuni* and *Campylobacter coli*, targeting the *gyrA* gene, and two different immunoenzymatic methods (Premier Campy [Meridian Bioscience, Inc., Cincinnati, OH] and Ridascreeen *Campylobacter* test [R-Biopharm, Darmstadt, Germany]) (27, 28).

Statistical analysis. Quantitative values for variables were given as medians $\pm$ extreme values or means $\pm$ standard deviations, and qualitative data were given as numbers and percentages. Pearson's chi-square test or the Fisher exact test was used to compare qualitative variables; the Student *t* test or the Wilcoxon *W* test was used to compare quantitative variables. The accuracy of the commercially available kits for diagnosing *Norovirus* and *Campylobacter* sp. infections was determined by calculating sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) by comparison to the methods used by the NRCs as the "gold standard." Demographic profiles, immunosuppressive treatments (for each drug independently or in association), and clinical and biological data were compared between patients suffering from infectious

FIG 1 Monthly distribution of samples collected during the 15-month study and seasonal pattern of circulation of enteric pathogens detected by molecular tools from September 2010 to November 2011. Bacterial, parasitic, and viral etiologies are shown. The black line indicates the number of samples collected each month.

and noninfectious diarrhea. Results were considered to be statistically significant for two-sided P values <0.05 . All statistical analyses were performed using the SAS system, release 9.0 (SAS Institute Inc.).

Nucleotide sequence accession numbers. The sequences obtained in this study were deposited in GenBank under accession numbers [KC223582](#) to [KC223598](#).

RESULTS

Demographic and clinical parameters of patients with severe diarrhea. During the 15 months of the study, 54 severe diarrhea events occurred in 49 patients. The median age was 51 years (range, 18 to 78 years), with a male-to-female ratio of 1.6. The median posttransplantation term at the time of assessment in the study was 6.3 years (range, 3 days to 24.2 years). The three main immunosuppressive therapies reported among patients at inclusion were cyclosporine-MMF (43%), tacrolimus-MMF (22%), and MMF alone (12%). Forty-four patients (89.8%) presented a single event of diarrhea, while five (10.2%) had two. At inclusion, patients presented an average of 5 ± 2.5 stools per day, an average weight loss of 1.8 ± 2.3 kg, and an average increase in serum creatinine of 39 ± 81 $\mu\text{mol/liter}$. Intestinal endoscopy was performed in 9 patients (18%). Immunosuppressive therapy switching or dose reduction was observed in 13 of the 54 episodes (24%). The monthly distribution of samples collected during the 15-month study is presented in Fig. 1.

Microbiological findings. Of the 54 severe acute diarrhea episodes investigated, one or several enteric pathogens were detected in 13 stool samples using classical microbiological techniques versus 39 for at least one of the seven multiplex PCR assays used retrospectively (23% versus 72%; $P = 0.006$) (Fig. 2). Every infectious etiology found through classical methods was confirmed by retrospective molecular diagnosis. The summary of the results obtained for each molecular biology kit tested is presented in Table 1, while the details of the results of each sample with each molecular biology kit are shown in Table 2. Among the 39 positive samples, molecular diagnosis identified 15 multiple infections (38%) compared to none using classical techniques (Fig. 2). Bac-

teria ranked first among the infectious causes of diarrhea in kidney transplant recipients since they were detected in 10/13 (77%) and 32/39 (82%) of the positive samples tested by classical techniques and molecular tools, respectively, viruses were second, with 2/13 (15%) and 16/39 (41%), and parasites were third, with 1/13 (8%) and 2/39 (5%). The primary pathogens detected by classical techniques were EPEC ($n = 6$; 46%), *Campylobacter* spp. ($n = 2$; 15%), and *Rotavirus* ($n = 2$; 15%), while multiplex PCR assays mainly detected EPEC ($n = 15$; 38%), *Campylobacter* spp. ($n = 15$; 38%), and *Norovirus* ($n = 14$; 36%) from the 39 positive samples (Fig. 2). The seasonal pattern of circulation of enteric pathogens during the 15-month study period is depicted in Fig. 1.

Of the nine colonic biopsy specimens analyzed, one was positive for CMV using the real-time PCR assay but did not present histological evidence of CMV infection or positive CMV immunohistochemical staining.

Confirmation by reference methods. Samples positive for enteric viruses and *Campylobacter* spp. were sent to the French NRCs for confirmation and typing as described in Materials and Methods. The 17 enteric viruses detected by the commercially available multiplex PCR assays were confirmed by the NRC. Typing was obtained for 12 of the 14 *Norovirus* isolates and revealed two GII.4 isolates, six GII.4 isolates, two GII.12 isolates, one GII.3 isolate, and one GII.14 isolate. The two *Rotavirus* isolates detected were G1P[8] and G8P[14], while the *Astrovirus* strain belonged to serotype 5 (GenBank accession numbers [KC223582](#) to [KC223598](#)). Twenty-three negative samples from molecular kits were also tested for *Norovirus* by the NRC. Three were positive, whereas 20 were confirmed to be negative. The multiplex PCR assays demonstrated, by comparison to the techniques used by the NRC, considered the gold standard, a sensitivity of 82%, a specificity of 100%, a PPV of 100%, and a NPV of 87% for *Norovirus* detection in the stool samples of adult kidney transplant recipients.

Nine of the 15 *Campylobacter* infections were confirmed by the NRC. The species identified were *Campylobacter jejuni* in eight cases and *Campylobacter coli* in one. Eighteen negative samples

FIG 2 Comparison of enteric pathogens detection in adult kidney transplant recipients by classical microbiological techniques and the multiplex PCR assays.

from molecular kits were also analyzed and confirmed to be negative by the NRC. The multiplex PCR assays demonstrated a sensitivity of 100%, a specificity of 75%, a PPV of 60%, and a NPV of 100% for *Campylobacter* sp. detection in the stool samples of adult kidney transplant recipients.

Biological and clinical characteristics of infectious diarrhea. The analysis of the data obtained from patients tested positive for enteric pathogens with the multiplex PCR assays showed that the posttransplantation term of diarrhea onset caused by *Norovirus* was significantly prolonged compared to that of other causes of

TABLE 1 Summary of results of 7 commercially available multiplex PCR kits for the detection of enteric pathogens in the stool samples collected from kidney transplant recipients with severe diarrhea in Reims, France, September 2010 to November 2011

Infectious etiology	No. of stool samples positive (out of 54) by:					Total no. of enteric pathogens detected
	xTAG GPP (Luminex)	Ridagene Norovirus I & II EHEC/EPEC (R-Biopharm)	Clart Enterobac (Genomica)	Seeplex Diarrhea ACE Detection (Seegene)	Gastroenteritis virus and parasite panels (Diagenode)	
Bacteria	18	17	29			35
<i>Campylobacter</i>	14		14			15
EPEC (<i>eae</i>)		15	14 ^a			15
STEC (<i>stx₁/stx₂</i>)	2	2				3
<i>Clostridium difficile</i>	1		1			1
<i>Salmonella</i>	1					1
Viruses	16	8		11	11	17
Norovirus GI/GII	14	8		9	9	14
Rotavirus	2			1	1	2
Astrovirus				1	1	1
Parasites	2				2	2
<i>Cryptosporidium parvum</i>	1				1	1
<i>Giardia intestinalis</i>	1				1	1
Total no. of enteric pathogens detected	36	25	29	11	13	54

^a No distinction between enterovirulent *E. coli* spp.

TABLE 2 Details of the results obtained with the 7 commercially available multiplex PCR kits for each of the 54 stool samples collected from 49 kidney transplant patients with severe diarrhea in Reims, France, September 2010 to November 2011

Sample	Classical methods	Result for detection by:				
		Molecular diagnosis				
		xTAG GPP (Luminex)	Ridagene Norovirus I & II EHEC/EPEC (R-Biopharm)	Clart Enterobac (Genomica)	Seeplex Diarrhea ACE Detection (Seegene)	Gastroenteritis virus and parasite panels (Diagenode)
001-1	Negative	Negative	EPEC	Pathogenic <i>E. coli</i>	Negative	Negative
002-1	<i>Giardia</i>	<i>Giardia</i> + <i>Campylobacter</i>	Negative	<i>Campylobacter</i>	Negative	<i>Giardia</i>
003-1	Negative	<i>Salmonella</i>	Negative	Negative	Negative	Negative
004-1	EPEC	Negative	EPEC	Pathogenic <i>E. coli</i>	Negative	Negative
005-1	<i>C. difficile</i>	<i>C. difficile</i>	Negative	<i>C. difficile</i>	Negative	Negative
006-1	Negative	Norovirus	Norovirus + EPEC	Pathogenic <i>E. coli</i>	Norovirus	Norovirus
006-2	EPEC	Norovirus	EPEC	Pathogenic <i>E. coli</i>	Norovirus	Norovirus
007-1	EPEC	Negative	EPEC	Negative	Negative	Negative
008-1	Negative	<i>Campylobacter</i> + Norovirus	Negative	<i>Campylobacter</i>	Negative	Negative
009-1	Negative	<i>Campylobacter</i> + Norovirus	Norovirus	<i>Campylobacter</i>	Norovirus	Norovirus
010-1	Negative	Norovirus	Norovirus	Negative	Norovirus	Norovirus
011-1	Negative	Norovirus	Norovirus	Negative	Negative	Negative
012-1	Negative	Norovirus	Norovirus	Negative	Norovirus	Norovirus
013-1	Negative	Negative	Negative	Negative	Negative	Negative
014-1	Negative	<i>Campylobacter</i> + Norovirus	Negative	<i>Campylobacter</i>	Negative	Negative
015-1	Negative	Negative	Negative	Negative	Negative	Negative
016-1	Negative	<i>Campylobacter</i>	Negative	Negative	Negative	Negative
017-1	Negative	Norovirus	Norovirus	Negative	Norovirus	Norovirus
018-1	Negative	Norovirus	EPEC	Pathogenic <i>E. coli</i>	Norovirus	Norovirus
019-1	Negative	Negative	Negative	Negative	Negative	Negative
020-1	Rotavirus	Rotavirus	EPEC	Pathogenic <i>E. coli</i>	Negative	Negative
020-2	Negative	Negative	Negative	<i>Campylobacter</i>	Negative	Negative
021-1	EPEC	<i>Campylobacter</i> + STEC	STEC	<i>Campylobacter</i> + pathogenic <i>E. coli</i>	Negative	Negative
022-1	Negative	Negative	Negative	Negative	Negative	Negative
023-1	Negative	Negative	Negative	Negative	Negative	Negative
024-1	Negative	Negative	Negative	Negative	Negative	Negative
025-1	Negative	Norovirus	Norovirus + EPEC	Negative	Norovirus	Norovirus
026-1	Negative	Negative	Negative	Negative	Negative	Negative
027-1	Negative	Negative	Negative	Negative	Negative	Negative
027-2	EPEC	<i>Cryptosporidium</i>	EPEC	Pathogenic <i>E. coli</i>	Negative	<i>Cryptosporidium</i>
028-1	STEC	Negative	STEC	Pathogenic <i>E. coli</i>	Negative	Negative
029-1	Negative	Negative	Negative	Negative	Negative	Negative
030-1	Negative	STEC	Negative	Negative	Negative	Negative
031-1	Negative	Negative	Negative	Negative	Negative	Negative
032-1	<i>Campylobacter</i>	<i>Campylobacter</i>	Negative	<i>Campylobacter</i>	Negative	Negative
033-1	<i>Campylobacter</i>	<i>Campylobacter</i>	Negative	<i>Campylobacter</i>	Negative	Negative
034-1	Negative	<i>Campylobacter</i>	Negative	<i>Campylobacter</i>	Negative	Negative
035-1	Negative	Negative	Negative	Negative	Negative	Negative
036-1	Negative	<i>Campylobacter</i>	Negative	<i>Campylobacter</i>	Negative	Negative
037-1	Negative	Negative	Negative	Negative	Negative	Negative
038-1	Negative	<i>Campylobacter</i>	EPEC	<i>Campylobacter</i> + pathogenic <i>E. coli</i>	Negative	Negative
039-1	EPEC	<i>Campylobacter</i>	EPEC	<i>Campylobacter</i> + pathogenic <i>E. coli</i>	Negative	Negative
040-1	Rotavirus	Norovirus + Rotavirus	Norovirus	Negative	Norovirus + Rotavirus	Norovirus + Rotavirus
041-1	Negative	Negative	EPEC	Pathogenic <i>E. coli</i>	Negative	Negative
042-1	Negative	Negative	Negative	Negative	Negative	Negative
043-1	Negative	Negative	Negative	Negative	Astrovirus	Astrovirus
044-1	Negative	<i>Campylobacter</i>	Negative	<i>Campylobacter</i>	Negative	Negative
044-2	Negative	Norovirus	Negative	Negative	Negative	Negative
045-1	Negative	<i>Campylobacter</i> + Norovirus	Negative	<i>Campylobacter</i>	Negative	Negative
045-2	Negative	Negative	EPEC	Pathogenic <i>E. coli</i>	Negative	Negative
046-1	Negative	Negative	EPEC	Pathogenic <i>E. coli</i>	Negative	Negative
047-1	Negative	Negative	EPEC	Negative	Negative	Negative
048-1	Negative	Negative	Negative	Negative	Negative	Negative
049-1	Negative	Negative	Negative	Negative	Negative	Negative

infectious and noninfectious diarrhea ($P = 0.04$). Furthermore, the immunosuppressive protocol combining cyclosporine and MMF was associated with a significantly higher risk of developing viral gastroenteritis ($P = 0.003$; odds ratio, 5.8) and particularly

diarrhea caused by *Norovirus* ($P = 0.002$; odds ratio, 6.7) than of developing any other cause of infectious or noninfectious diarrhea. The cyclosporine-MMF combination was also associated with a higher risk of onset of multiple enteric infections ($P = 0.01$) (Table 3).

TABLE 3 Risk factors for developing infectious diarrhea in adult kidney transplant recipients^b

Patient status (n ^c)	Age (yr)		Sex		Posttransplantation term (yr)		Cyclosporine-MMF ^a treatment		Tacrolimus-MMF treatment		Antibiotic therapy	
	Mean ± SD	P	No. male	P	Mean ± SD	P	No. treated	P	No. treated	P	No. treated	P
Infected (34)	51 ± 14	0.9	19	0.5	6.5 ± 6.3	0.9	16	0.4	8	0.7	8	0.7
Not infected (15)	51 ± 14		11		5.6 ± 6.3		5		3		5	
Single infections (21)	51 ± 14	0.5	14	0.7	5.3 ± 6.1	0.7	9	0.01	7	0.6	7	0.6
Mixed infections (13)	49 ± 16		9		5.9 ± 5.3		13		2		2	
Norovirus positive (12)	51 ± 15	0.8	6	0.5	9.5 ± 7.7	0.04	10	0.002	1	0.3	3	0.9
Norovirus negative (37)	51 ± 14		24		5.2 ± 5.4		11		10		10	
EPEC positive (12)	50 ± 13	0.7	7	0.7	6.1 ± 5.1	0.4	7	0.5	2	0.7	2	0.7
EPEC negative (37)	51 ± 14		24		6.3 ± 6.6		15		9		11	
<i>Campylobacter</i> positive (14)	46 ± 15	0.7	9	0.7	5.8 ± 5.3	0.6	6	0.9	5	0.4	5	0.7
<i>Campylobacter</i> negative (35)	52 ± 14		21		6.3 ± 6.5		15		7		9	

^a MMF, mycophenolate mofetil.^b Significant P values are in boldface.^c n, number of patients.

Norovirus infections were responsible for significantly higher weight loss than all the other infectious and noninfectious causes of diarrhea ($P = 0.04$). In regard to biological parameters, patients with diarrhea caused by *Norovirus* had an average protein C reactive level at inclusion statistically lower than those observed for all of the other causes of diarrhea ($P = 0.01$). A tendency to have a higher number of stools per day was observed in patients coinfecting with several enteric pathogens compared to patients with a single enteric infection ($P = 0.06$) (Table 4).

Molecular microbiological analyses of the three control groups. Of the stool samples taken from 30 of the initially investigated patients sampled again 6 months after resolution of the episode of severe diarrhea, one enteric pathogen was detected in 14 (45%). Thirteen (43%) of the 30 renal transplant recipients with no history of diarrhea were positive for at least one enteric pathogen, while seven enteric infections were detected in the 30 asymptomatic immunocompetent patients (23%) tested (Fig. 3).

DISCUSSION

Retrospective implementation of molecular techniques helped detect one or several enteric pathogens in 39 of 54 analyzed samples (72%). This number was higher than that found using classical methods, whether in our study (23%) or in other studies on kidney transplant recipients, where the detection rates using classical techniques varied from 20.4 to 63.5% (1, 6, 12). The difference observed compared to classical techniques was also larger

than what has been described in a study on immunocompetent subjects, showing a progression in the detection rate from 53% to 75% using molecular techniques (16). Pathogen by pathogen, it is interesting to note that, in our study, *Norovirus* was the only pathogen that could be detected by molecular methods only since none of the positive samples were positive by RADT. The fact that *Norovirus* strains belonged to different genotypes of genogroups I and II would seem to indicate a lack of sensitivity of the RADT usually used rather than a problem detecting strains belonging to a given genotype. Furthermore, the genetic diversity of the *Norovirus* strains detected suggested sporadic cases rather than cross-contaminations between kidney transplant recipients. Another enteric pathogen underestimated by classical diagnosis was *Campylobacter*. Molecular biology kits detected *Campylobacter* spp. in 15 (28%) of 54 diarrhea episodes investigated, whereas only 2 were detected by culture. The lack of culture sensitivity has already been observed in immunocompetent subjects, where culture could detect only around 60% of the positive results by combining a molecular technique with two immunoenzymatic techniques (27). The fragility of these microaerophile bacteria in room air along with a lower infectious dose in immunocompromised patients than in immunocompetent ones could explain the observed gap between culture and detection of *Campylobacter* with molecular biology techniques (27).

The increased sensitivity of the molecular methods was demonstrated by the percentage of samples in which coinfections

TABLE 4 Clinical and biological characteristics of adult kidney transplant recipients^b

Patient status (n ^a)	Hospitalization		Hospitalization duration (days)		No. of stools per day		Wt loss (kg)		Hematocrit		Creatinine variation (μmol/liter)		Protein C reactive (mg/liter)		Leukocytes (10 ⁹ /liter)	
	No.	P	Mean ± SD	P	Mean ± SD	P	Mean ± SD	P	Mean ± SD	P	Mean ± SD	P	Mean ± SD	P	Mean ± SD	P
Infected (34)	21	0.8	8 ± 7	0.2	5.2 ± 2.7	0.7	-2.1 ± 2.5	0.2	34 ± 6	0.5	+38 ± 80	0.8	63 ± 99	0.2	7.3 ± 3.6	0.5
Not infected (15)	9		17 ± 24		4.8 ± 2.3		-1.0 ± 1.8		35 ± 5		+41 ± 84		43 ± 70		6.9 ± 3.0	
Single infections (21)	12	0.7	9 ± 8	0.3	4.7 ± 2.3	0.06	-2.3 ± 2.6	0.5	35 ± 5	0.7	+32 ± 82	0.5	72 ± 109	0.2	7.0 ± 3.7	0.6
Mixed infections (13)	9		5 ± 5		7.0 ± 3		-1.6 ± 2.4		34 ± 8		+56 ± 77		28 ± 33		7.9 ± 3.5	
Norovirus positive (12)	6	0.7	8 ± 10	0.3	6.1 ± 2.7	0.6	-3.0 ± 2.7	0.04	35 ± 6	0.5	+22 ± 75	0.8	23 ± 29	0.01	7.5 ± 3.1	0.7
Norovirus negative (37)	21		12 ± 16		4.6 ± 2.4		-1.4 ± 2.1		35 ± 5		+43 ± 83		63 ± 96		7.0 ± 3.5	
EPEC positive (12)	7	0.7	10 ± 5	0.8	5.4 ± 2.5	0.7	-2.3 ± 2.5	0.6	33 ± 6	0.4	+67 ± 110	0.3	58 ± 120	0.9	7.4 ± 3.0	0.8
EPEC negative (37)	21		11 ± 17		5.0 ± 2.6		-1.6 ± 2.3		35 ± 5		+31 ± 69		55 ± 81		7.1 ± 3.5	
<i>Campylobacter</i> positive (14)	8	0.9	7 ± 9	0.09	6.1 ± 3	0.5	-2.2 ± 1.3	0.5	36 ± 5	0.9	+51 ± 63	0.4	49 ± 28	0.8	6.4 ± 3.7	0.6
<i>Campylobacter</i> negative (35)	22		13 ± 16		4.9 ± 2.5		-1.7 ± 2.5		35 ± 5		+36 ± 85		57 ± 96		7.3 ± 3.3	

^a n, number of patients.^b Significant P values are in boldface.

FIG 3 Microbiological findings obtained from the molecular analyses of the three control groups. (A) Transplant patients ($n = 30$) sampled 6 months after resolution of severe diarrhea. (B) Renal transplant recipients ($n = 30$) with no history of diarrhea. (C) Immunocompetent patients without diarrheal symptoms ($n = 30$).

could be detected compared to that for classical detection methods (38% versus none). These results corroborated the data found in immunocompetent subjects, in whom 41% of diarrhea episodes were due to multiple enteric infections (16). The identification of coinfections could constitute one of the major advantages of multiplex PCR kits since our study has shown a tendency to increased severity of multiple infections in kidney transplant recipients, with a higher number of stools per day.

The more sensitive detection of gastrointestinal pathogens by using molecular tests retrospectively identified an infectious cause in the stool of five of the nine patients who underwent colonoscopy and during 6 of the 13 diarrhea episodes initially attributed to immunosuppressive therapy (data not shown). In all these cases, testing of samples from kidney transplant recipients with severe acute diarrhea by molecular methods could have potentially avoided invasive procedures or modifications in the immunosuppressive therapy.

However, the detection of 54 enteric pathogens from 39 positive samples by multiplex PCR assays raises the question of the analytical reliability of the results obtained. The detection of viruses was well correlated with the results obtained by reference techniques since all of the viruses detected (*Norovirus*, *Rotavirus*, and *Astrovirus*) were confirmed, thus establishing the positive predictive value of molecular techniques at 100%. However, a lack of specificity when diagnosing bacterial infections was observed. Only 9 of 15 *Campylobacter* infections were confirmed by the reference method. This gap highlights the difficulty in evaluating a new technique more sensitive than the reference method used for comparison. For EPEC, using molecular biology led to a nearly 50% increase in detection rates compared to standard stool culture. Given the absence of a reference technique and epidemiological data available for kidney transplant recipients, we could make no conclusions as to the validity of EPEC infections detected with multiplex PCR assays. Nevertheless, the 6 strains of *E. coli* detected prospectively by stool culture were retrospectively detected by molecular tools, indicating at least partial accuracy of the molecular results obtained.

The significant number of pathogens detected with molecular techniques during this study also presents the question of the clinical relevance of the results obtained. Molecular techniques are able to detect a microorganism present at extremely low levels in a clinical sample that can either be the cause of the symptoms ob-

served or the mark of a subclinical and long-term asymptomatic carriage, as reported for *Norovirus* in kidney transplant recipients (29). The absence of a prior study using the same methodology in kidney transplant recipients makes any comparisons impossible. Nevertheless, from our study, it can be noticed that several specific characteristics of infected patients diagnosed by molecular techniques have been identified. In particular, significantly higher weight loss was observed in patients infected by *Norovirus* than in patients with other infectious and noninfectious causes of diarrhea. This confirms the previously reported specific severity of *Norovirus*-induced diarrhea in transplant recipients and supports an active role of the enteric pathogen detected (20, 30, 31).

Furthermore, among the 5 patients who had several diarrheal episodes, an identical pathogen was detected only in one patient in the multiple samples. In the other 4 patients, multiple pathogens were detected in follow-up samples, contradicting the hypothesis of a long-term carriage of the pathogens following the infection detected by molecular tools.

Finally, stool samples from three control populations of asymptomatic immunocompromised and immunocompetent patients were analyzed using three of the multiplex PCR kits covering the complete panel of enteric pathogens initially detected in kidney transplant recipients with severe diarrhea. The results obtained showed an unexpected high frequency of enteric pathogen detection, ranging from 23% in asymptomatic immunocompetent patients to 45% in renal transplant recipients sampled again 6 months after resolution of the episode of severe diarrhea. Enteropathogenic *Escherichia coli* (EPEC) expressing the *eae* gene was the main pathogen diagnosed in both asymptomatic kidney transplant recipients (37%) and asymptomatic immunocompetent patients (23%). This bacterium represented 58 to 100% of the enteric pathogens detected in the three control groups. The clinical relevance of its detection by both classical and molecular techniques during diarrhea episodes in kidney transplant recipients is thus unlikely, leading to the question of the interest of its inclusion in the multiplex PCR kit detection panels. Molecular tools also revealed asymptomatic carriage of *Norovirus* and *Campylobacter* spp. in kidney transplant recipients with or without a history of diarrhea. However, the frequency of their detection, as well as the detection of other enteric pathogens, was clearly lower than that observed in kidney transplant recipients at the time of the diarrhea episode. Interestingly, the mean cycle threshold values of *Norovi-*

rus detection by Ridagene Norovirus I & II were 27.1 and 23.6 (not shown) in kidney transplant recipients at the time of the diarrhea episode and in the control groups, respectively, contradicting the hypothesis of a link between high viral load and the disease, which is still debated (29, 32, 33).

In conclusion, this study has demonstrated the power of molecular techniques for the microbiological diagnosis of enteric pathogens in kidney transplant recipients. The application of molecular diagnostics increased the detection rate of enteric pathogens significantly by comparison to classical techniques, and this can help to better define the characteristics of infectious diarrhea as well as to avoid unnecessary invasive procedures and modifications of the immunosuppressive therapy in kidney transplant recipients. Their analytical performances and clinical relevance, validated for viruses during this study, remain, however, to be specified for bacteria and parasites. Rapid and simultaneous molecular detection of a wide range of bacterial, parasitic, and viral enteric pathogens could quickly become the key element of the management of severe acute diarrhea in transplant recipients.

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