JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1991, p. 2503–2506
0095-1137/91/112503-04$02.00/0

Vol. 29, No. 11

Recovery of Vancomycin-Resistant Gram-Positive Cocci from Pediatric Liver Transplant Recipients

MICHAEL GREEN,[1,2]* KAREN BARBADORA,[1] AND MARIAN MICHAELS[1]

Departments of Pediatrics[1] and Surgery,[2] Children's Hospital of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213

Received 3 June 1991/Accepted 16 August 1991

Between November 1988 and October 1989, 49 first-time pediatric liver transplant recipients at the Children's Hospital of Pittsburgh were prospectively monitored for the presence of stool colonization and the development of disease caused by vancomycin-resistant gram-positive cocci (VRGPC). Quantitative stool culturing was done on a weekly basis, and cultures were planted onto a selective medium for VRGPC. Isolates for which the MIC was ≥8 were considered resistant to vancomycin. Patients were monitored clinically for the development of infection, and their charts were systematically reviewed for the use of antibiotics. Eighty-six isolates were recovered from 36 of the 49 patients. Enterococcal species were isolated from 31 patients and included *Enterococcus gallinarum* ($n = 28$), *E. casseliflavus* ($n = 14$), *E. faecium* ($n = 9$), *E. faecalis* ($n = 2$), *E. mundtii* ($n = 2$), and *E. durans* ($n = 1$). Stool colonization with vancomycin-resistant enterococci was noted to increase steadily during the first month after transplantation. Only 9 of 31 patients demonstrated clearance of these organisms in serial repeat cultures. Additional isolates of VRGPC included *Lactobacillus confusus* ($n = 13$), *Lactobacillus* spp. ($n = 12$), and *Pediococcus pentosaceus* ($n = 4$). Infection due to VRGPC developed in three patients: a urinary tract infection in two and peritonitis in one. *E. faecium* was the pathogen in each of these cases. The ranges of MICs of vancomycin were 8 to 32 μg/ml for all enterococcal isolates and >128 μg/ml for *Lactobacillus* and *Pediococcus* isolates. All *Lactobacillus* and *Pediococcus* isolates were resistant to teicoplanin, although they were susceptible to daptomycin. All other isolates were susceptible to both teicoplanin and daptomycin. This study demonstrates that stool colonization with VRGPC may be a common and early finding among pediatric liver transplant recipients. However, infection appears to be uncommon.

Vancomycin resistance among gram-positive cocci has recently been recognized (1, 8, 11, 16, 18, 23). Great interest has focused on the mechanisms of resistance; however, few data regarding the frequency of disease due to these organisms exist. A previous report from our hospital suggested that low densities of vancomycin-resistant gram-positive cocci (VRGPC) are common in the gastrointestinal tracts of children (6). Recently, several episodes of bacteremia due to VRGPC were observed among pediatric liver transplant recipients at our hospital. This observation prompted us to undertake a prospective surveillance study of fecal colonization and infection with VRGPC among children who have undergone liver transplantation to better define the frequency of colonization by and disease due to these organisms.

(This research was presented in part at the 30th Interscience Conference on Antimicrobial Agents and Chemotherapy, Atlanta, Ga., 21 to 24 October 1990.)

MATERIALS AND METHODS

Study subjects. Between November 1988 and October 1989, first-time liver transplant recipients at the Children's Hospital of Pittsburgh were prospectively monitored for the presence of stool colonization and the development of disease caused by VRGPC. Stool specimens were obtained weekly in sterile containers from the time of transplant until discharge from the hospital.

Isolation of VRGPC from stool specimens. Quantitative counts were determined by culturing serial 10-fold dilutions of fecal suspensions (initial fecal specimens, 0.5 to 4.5 g) on a selective medium containing 5 μg of vancomycin per ml (6). The plates were incubated at 35°C in the presence of 5% carbon dioxide and were inspected for the presence of colonies at 24, 48, and 72 h. The number of CFU per gram of feces was determined. Cellular morphology was determined from Gram stains of these cultures. No further tests were performed on isolates identified as gram-negative rods or fungi.

Bacteriological studies. Isolates of VRGPC were identified in accordance with the recent guidelines proposed by Facklam and colleagues at the Centers for Disease Control (3, 5). Morphology was confirmed by Gram staining of wet-mount smears of overnight cultures grown in thioglycolate broth. Gram-positive isolates were subjected to a battery of identification tests, including catalase, PRYase, gas production in MRS broth with an overlay of 2% agar (8), and growth in 6.5% NaCl, on bile-esculin agar, and at 10 and 45°C. Additionally, all isolates were tested with the API Rapid Strep system (Analytab Products, Plainview, N.Y.). This commercial system has been shown to be useful in the identification of gram-positive isolates, although some discrepancies occur in comparison with conventional tests (3).

Isolates that produced gas in MRS broth were initially classified as either *Lactobacillus* group 3 or *Leuconostoc* species. For determination of their identities, these isolates were additionally evaluated for the production of slime on 5% sucrose agar. Isolates were identified as *Lactobacillus confusus* if they produced slime, grew at 10 and 45°C, and had positive reactions for arginine, leucine aminopeptidase, the Voges-Proskauer test, hydrolysis of esculin, xylose, salicin, maltose, D-galactose, and cellobiose. Isolates were identified as *Leuconostoc* spp. if they did not produce slime, grew at 10 but not 45°C, and had negative reactions for

* Corresponding author.

leucine aminopeptidase and arginine. Isolates which had the morphology of gram-positive rods, did not produce gas, and had positive reactions for leucine aminopeptidase and arginine were identified as *Lactobacillus* spp.

Isolates presumptively identified as streptococci by colony morphology and Gram stains were tested for the presence of group D antigen by latex agglutination (Burroughs Wellcome Co., Research Triangle Park, N.C.) after extraction by the autoclave method (4). Isolates positive for group D antigen, PRYase, hydrolysis of esculin, and growth in 6.5% NaCl were identified as enterococci. Positive results for mannitol and arginine identified isolates as *Enterococcus* group 2 (5). Isolates were examined for the production of pigment and motility (19). Final identification of these isolates was based on a review of reactions with arabinose, sorbitol, and lactose, as well as the presence of pigment and motility (5). Additionally, isolates identified as *Enterococcus casseliflavus* or *E. gallinarum* had their identifications confirmed by DNA hybridization as part of a collaborative study (22).

Isolates were identified as *Pediococcus* species if gram-positive cocci in tetrads were observed (3), growth was noted at 45 but not 10°C, and esculin was hydrolyzed. The identifications of these isolates and of all vancomycin-resistant streptococci other than enterococci were confirmed by Richard Facklam, Centers for Disease Control.

Susceptibility testing. Initial susceptibility testing was performed by broth microdilution (15) in cation-supplemented Mueller-Hinton broth with the Sensititre system (Radiometer, Copenhagen, Denmark) by use of visual endpoints. Low-level resistance was defined as MICs of 8 to 32 μg/ml. High-level resistance was defined as MICs of at least 64 μg/ml. MICs of vancomycin were confirmed for isolates identified as *E. casseliflavus* and *E. gallinarum* by agar dilution as part of a collaborative project (22) and by macrotube dilution (15) for all other enterococci and pediococci. Additional susceptibility testing was performed with daptomycin by both Kirby-Bauer disk and macrotube dilution techniques and with teicoplanin only by the macrotube dilution technique. MBCs of daptomycin and teicoplanin were also determined for these isolates (20).

Inducibility of vancomycin resistance. Induction from low-level to high-level resistance was attempted for 10 of the *Enterococcus* isolates (12). Isolates were cultured overnight in brain heart infusion broth containing 5 μg of vancomycin per ml. After serial dilutions of each isolate were made, a 100-μl aliquot of each dilution was subcultured onto a Columbia CNA agar plate supplemented with 16 or 150 μg of vancomycin per ml. After overnight incubation, the agar plates were observed for growth. Viable colonies were subcultured onto 5% sheep blood agar containing 150 or 80 μg of vancomycin per ml for 10 generations, and the MICs of vancomycin were determined.

Curing procedures. Several isolates of each of the enterococcal species were subjected to novobiocin curing in an effort to eliminate plasmid-mediated resistance as previously described (13). Curing experiments for each vancomycin-resistant enterococcal isolate were performed at least twice.

Mating conditions. Mating was attempted between enterococcal isolates with low-level vancomycin resistance and *E. faecalis* ATCC 29212 (21). Donor and recipient strains were grown in brain heart infusion broth with gentle shaking for 3 to 5 h at 37°C. At 20 min prior to mating, the recipient strains were placed in a 45°C incubator to enhance plasmid transfer between species. Donor and recipient strains were mated by mixing 0.1-ml aliquots of each strain on a sterile filter (type HAEP, 0.45-μm-pore size, 47 mm; Millipore Corp., Bedford, Mass.) and then placing the filter on a 5% horse serum–brain heart infusion agar plate. After overnight incubation at 37°C, the filters were removed from the plates and washed several times with brain heart infusion broth. Dilutions of the broth washes were plated on both blood agar and VRGPC agar and incubated at 37°C overnight. Donors were differentiated from possible transconjugants by colony morphology.

TABLE 1. Ranges of MICs of ampicillin, vancomycin, daptomycin, and teicoplanin

Organism (no. of isolates)	Range of MICs (μg/ml) of:			
	Ampicillin	Vancomycin	Daptomycin	Teicoplanin
E. gallinarum (29)	0.5–8.0	8.0–32	0.5–8.0	<2
E. casseliflavus (15)	0.5–1.0	8.0–16	0.5–2.0	<2
E. faecium (9)	0.5–>16	8.0	1.0–4.0	<2
L. confusus (13)	0.2–2.0	>128	<0.5	<2
Lactobacillus spp. (12)	0.2–2.0	>128	<0.5	>32
P. pentosaceus (4)	2.0–4.0	>128	<0.5	>32

Clinical data collection and analysis. Clinical progress and antibiotic use were systematically monitored during the study period. The presence of infection was determined by use of preestablished definitions (10). Rates of acquisition and clearance of colonization with vancomycin-resistant enterococci were determined by use of the Kaplan-Meier life table analysis for the first 12 weeks after liver transplantation. Colonization with enterococci was considered to be acquired if any culture was positive. Colonization was considered to be cleared if two or more consecutive stool specimens were negative or, alternatively, if the last stool specimen obtained was negative. Colonization was considered to be persistent if it was not cleared.

Relationships between density and persistence of colonization with VRGPC were determined with the chi-square test.

RESULTS

Forty-nine first-time liver transplant recipients were prospectively monitored during the study. The age range of these children was from 4 months to 17 years, with a mean of 4.2 years. The median number of stool specimens collected per patient was three (range, one to nine).

Eighty-six isolates of VRGPC were recovered from 36 of the 49 children (80%). Enterococcal species were recovered from 31 patients (63%) and included *E. gallinarum* (n = 28), *E. casseliflavus* (n = 14), *E. faecium* (n = 9), *E. faecalis* (n = 2), *E. mundtii* (n = 2), and *E. durans* (n = 1). Nonenterococcal isolates of VRGPC identified during the study included *L. confusus* (n = 13), *Lactobacillus* spp. (n = 12), and *Pediococcus pentosaceus* (n = 4). One additional isolate presumptively identified as *Streptococcus salivarius* appeared to be an unidentified *Pediococcus* species (2). Vancomycin-resistant staphylococci were not recovered during the study period.

The ranges of MICs of ampicillin, vancomycin, daptomycin, and teicoplanin are shown in Table 1. Only one of the stool isolates identified in this study was resistant to ampicillin. All identified enterococcal isolates had low-level resistance to vancomycin. The highest MIC for the enterococcal isolates was 32 μg/ml. No difference was noted between MICs determined by microtube dilution and either agar

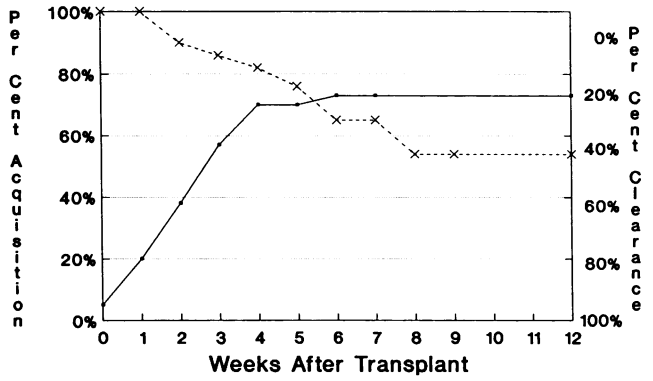

FIG. 1. Acquisition (■) and clearance (×) of colonization with vancomycin-resistant enterococci.

dilution or macrotube dilution. All enterococcal isolates were susceptible to both daptomycin and teicoplanin.

High-level vancomycin resistance was demonstrated for isolates of *Lactobacillus* spp. and *P. pentosaceus*. All four isolates of *P. pentosaceus* as well as the isolates of *Lactobacillus* spp. were resistant to teicoplanin, although they were susceptible to daptomycin. All other isolates were susceptible to both daptomycin and teicoplanin. MBCs were also determined and revealed that both daptomycin and teicoplanin were bacteriostatic against all susceptible isolates tested.

Curing experiments were performed with 17 isolates of enterococci (7 *E. casseliflavus*, 6 *E. gallinarum*, 2 *E. faecium*, 1 *E. faecalis*, and 1 *E. mundtii*). MICs for two isolates of *E. casseliflavus* were reproducibly decreased after curing: 16 to 4 μg/ml and 8 to 2 μg/ml. No change was seen in the MICs for the other isolates. None of 10 tested isolates, including the 2 curable *E. casseliflavus* isolates, were inducible to higher levels of resistance.

Preoperative stool cultures obtained within 1 month of transplantation were available for six patients. Three patients were colonized pretransplant; one of the three did not have resistant enterococci isolated from any of three stool specimens submitted posttransplant. Of the three remaining patients, who were not colonized pretransplant, one acquired fecal colonization with vancomycin-resistant enterococci.

The acquisition and clearance of fecal colonization with vancomycin-resistant enterococci posttransplantation are shown in Fig. 1. The percentage of children having at least one isolate of resistant enterococci recovered from stool specimens increased steadily during the first postoperative month. Only one child acquired colonization after this time. Twenty-two of the 31 colonized children had persist colonization. Fecal colonization was cleared at a relatively constant rate between 2 and 8 weeks posttransplantation. Stool colonization was cleared in a single patient 14 weeks posttransplantation; this patient was included in the 12-week actuarial analysis as having persistent colonization.

The relationship between quantitative culture results and persist colonization is shown in Table 2. An increased risk of persistent colonization was associated with the presence of at least 10^6 CFU/ml in any single stool specimen ($P = 0.008$).

The relationship between the use of vancomycin and both the incidence and the density of colonization with vancomycin-resistant enterococci was examined. Sixteen of 30 colonized patients and 8 of 19 noncolonized patients were treated with parenteral vancomycin. However, 12 of the 16 colonized patients received their vancomycin after colonization with resistant enterococci was already present. Only three patients received oral vancomycin. We did not observe a relationship between the use of vancomycin and either the presence or the density of colonization with these organisms in this small series of patients.

TABLE 2. Relationship between quantitative culture results and persistence of colonization with vancomycin-resistant enterococci

Maximum log CFU/ml	No. (%) with persistent colonization
≤3	0/4 (0)
4	0/1 (0)
5	3/6 (50)
6	3/4 (75)
>7	17/21 (81)[a]

[a] Increased risk of persistent colonization with ≥6 log CFU/ml in any culture ($P = 0.008$).

Three infectious episodes due to VRGPC occurred during the study period. *E. faecium* was the pathogen in each case. Two of the three episodes were urinary tract infections, and the third was a case of peritonitis. Each episode occurred within the first 2 weeks after the liver transplant. In only one of the three cases was the organism ever isolated from stool, and in that case stool isolation occurred 2 days after the urine was cultured and found to be positive. All three children were treated with ampicillin successfully; they included two children with ampicillin-resistant *E. faecium* in their urine, probably attributable to the concentration of ampicillin by the kidneys.

DISCUSSION

This study attempted to identify the incidence and significance of colonization by and disease due to VRGPC in pediatric liver transplant recipients. Colonization with VRGPC was found in 80% of these children; resistant enterococci were identified in 63%. This rate is higher than the 22% rate of colonization seen in both our previous study (6) and that of Rouff et al. (14). However, some patients in both of these series were not hospitalized, raising the possibility that the increased rate of stool colonization in the present series may be explained by nosocomial spread in an area in which colonization was endemic.

Because preoperative stool cultures were available from only six of our patients, the exact timing of acquisition of fecal colonization with VRGPC and resistant enterococci in particular is unclear. Three of the six patients were colonized with resistant enterococci, but all of these patients had been in the hospital for days to weeks prior to transplantation. The nearly linear increase in the percentage of colonized patients during the first month following transplantation suggests nosocomial acquisition of colonization. Liver transplant recipients are predominantly cared for by the transplant surgical service both in the intensive-care setting and on the ward. It is possible that nosocomial spread of VRGPC by one or more colonized individuals, either from patient to patient or from the hands of personnel to patient, explains the high rate of colonization in these children. Alternatively, these organisms may have been present in concentrations which were initially below the sensitivities of our detection methods (600 CFU/g of feces) and which increased to detectable levels following transplantation. A third possible explanation for the increased colonization rate observed in this study is the frequent exposure to vancomy-

cin of pediatric liver transplant recipients during the course of their clinical care. However, an increased risk of colonization in patients exposed to vancomycin was not demonstrated in this study. We are currently conducting ongoing surveillance of this and additional control populations at our hospital in an effort to better understand the epidemiology of this colonization in the pediatric population as a whole.

Illness due to VRGPC was uncommon during the study period. *E. faecium* was the pathogen in each of the three cases, despite the fact that it represented only 16% of the resistant enterococcal isolates recovered in our study. This result may be explained by the fact that the other, more frequently recovered vancomycin-resistant species of enterococci are less virulent than *E. faecium*. However, the true incidence of infection due to the 12 enterococcal species is unclear, as reliable identification procedures for these species have only recently been described (3, 5) and may not be used in many clinical laboratories. Reidentification of clinical isolates may alter current concepts of pathogenicity (9).

Vancomycin-resistant enterococci were identified in the stool of only one of the three children who developed an infection in this study. It is possible that a low density of fecal colonization prevented detection (<600 CFU/g of feces). Alternatively, the vancomycin-resistant enterococci may have colonized another site which was not sampled. Surveillance cultures for other sites (e.g., throat or skin) may help to delineate the relationship between colonization and disease.

If infections due to VRGPC increase in frequency, the development of additional antimicrobial agents to treat these infections may be necessary. Two of three isolates associated with disease in this study were resistant to both ampicillin and vancomycin. The increasing recognition of ampicillin resistance among enterococci (7) is a concern. Newer agents, including teicoplanin and daptomycin, appear to offer promise in the treatment of VRGPC infections. In vitro susceptibility testing performed in this study suggests that daptomycin may be an alternative treatment, as all strains were susceptible to this drug. Teicoplanin also appeared promising but does not appear to have activity against *Lactobacillus* or *Pediococcus* spp.

The enterococcal isolates in this study, as well as those in our previous study (6), showed moderate levels of resistance to vancomycin (MIC range, 8 to 32 μg/ml). None of the tested isolates were inducible, suggesting that they would fall into the VanC class of resistance (17). Seventy-five percent of our stool isolates were either *E. gallinarum* or *E. casseliflavus*. These two species constitute the motile enterococci. Previous reports have suggested that vancomycin resistance among *E. gallinarum* isolates may be a property of the species (6, 17, 19). *E. casseliflavus* isolates are biochemically similar to *E. gallinarum* isolates, with the production of yellow pigment being the major difference between the two species. It now appears that low-level vancomycin resistance is a property of all motile enterococci (22).

ACKNOWLEDGMENTS

We thank Ellen R. Wald and Robert M. Wadowsky for review of the manuscript. We also thank the nursing staff of Children's Hospital of Pittsburgh for help in obtaining stool specimens.

This work was supported by grants from NIH General Clinical Research Center (grant RR00084) and from Eli Lilly & Co.

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