RESEARCH ARTICLE
INTERNATIONAL MICROBIOLOGY (2005) 8:43-48
www.im.microbios.org

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Received 14 December 2004
Accepted 23 February 2005

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Colonization by *Clostridium difficile* of neonates in a hospital, and infants and children in three day-care facilities of Kanazawa, Japan

Summary. The intestinal-carriage rates of *Clostridium difficile* in neonates hospitalized in the University Hospital's Center for Perinatal and Reproductive Health and in infants and children enrolled in two day-nurseries and a kindergarten were examined. Swab samples from the floors of these facilities were also analyzed to determine the extent of environmental contamination by this organism. *C. difficile* was found in the stool of only one of 40 neonates during the normal 1-week stay in the hospital after delivery. The isolate from the neonate was identical to that of her mother, as determined by PCR ribotyping, pulsed-field gel electrophoresis analysis, and toxin gene type, suggesting that the *C. difficile*-positive neonate acquired the organism from her mother rather than from the environment. By contrast, 47 (48.0%) of the 98 infants and children, comprising 50 enrolled in two day-nurseries who were ≤ 3 years old and 48 enrolled in a kindergarten who were 2–5 years old, carried *C. difficile*. The carriage rate in infants under 2 years of age was much higher (84.4%) than in children 2 years old and older (30.3%). When analyzed according to age group, the carriage rates were 100, 75.0, 45.5, 24.0, 38.5, and 23.5% in infants and children 0, 1, 2, 3, 4, and 5 years old, respectively. The observation that several children were colonized with the same type of *C. difficile* strain in each day-care facility, and that the floors of day-nursery A and kindergarten C were contaminated with *C. difficile* strains identical to those colonizing the intestines of children enrolled in those facilities suggests that cross-infection of *C. difficile* among children occurs through *C. difficile*-carrying children or their contaminated environments. [**Int Microbiol** 2005; 8(1):43-48]

Key words: *Clostridium difficile* · bacterial colonization · carriage rate · molecular typing· neonates · infants · children

Introduction

Clostridium difficile is a strictly anaerobic, gram-positive, spore-forming rod and is well-established as the main causative agent of pseudomembranous colitis associated with antimicrobial or cancer chemotherapy. Pathogenic strains produce two kinds of virulence factors, toxin A and toxin B, which are enterotoxigenic and cytotoxic, respectively [10,28]. In a study by Brazier [5], neonates and infants in European countries and the USA showed high frequencies of carriage of *C. difficile*, and neonates were found to have been infected with the organism during their short stays in the hospital after birth. Other studies have reported that neonates were infected through contaminated hospital environments or via infection of their mothers [1,6,8,19]. In Japan, the *C. difficile* carriage rate of adults ranges from 4.2% to 17.5% [13,16,22], but there have been few investigations of the carriage rates of

neonates, infants, and children. In this study, we investigated both the carriage of *C. difficile* in neonates and their mothers during the short hospitalization following normal delivery and contamination by *C. difficile* of the floors of the hospital. In addition, the carriage rates of *C. difficile* in children and infants enrolled in two day-nurseries and a kindergarten as well as contamination by the organism of the floors of those facilities were examined. The origin of infection was determined by PCR ribotyping, pulsed-field gel electrophoresis (PFGE) analysis, and toxin gene typing of the isolates.

Materials and methods

Subjects. The sources of the human samples examined for intestinal carriage of *C. difficile* were: (i) stool specimens of 40 neonates hospitalized in the Center for Perinatal and Reproductive Health, Kanazawa University Hospital (Kodatsuno, Kanazawa, Japan), collected on day 5 or day 6 after birth during their normal stay in the hospital; (ii) stool specimens of 14 of the studied neonates' mothers, collected 2 or 3 days after admission; and (iii) stool specimens of 98 infants and children in two day-nurseries (A and B), and a kindergarten (C). None of the subjects had been exposed to antimicrobial agents for at least the 4 weeks previous to the beginning of the study.

Isolation of *C. difficile* from stool specimens. Stool specimens were collected and frozen at –80°C within a few hours until use. *C. difficile* spores were isolated by homogenizing the stool specimens with an equal volume of ethyl alcohol and cultured on cycloserine-cefoxitin-mannitol agar (CCMA): proteose peptone no. 2 (Becton Dickinson, Sparks, MD, USA) 40 g; agar 20 g; Na_2HPO_4 5 g; KH_2PO_4 1 g; NaCl 2 g; $MgSO_4$ 0.1 g; mannitol 6 g; neutral red (1% in ethanol) 3 ml; sodium taurocholate (Nacalai Tesque, Kyoto, Japan) 1 g; cycloserine (Nacalai Tesque) 300 mg; cefoxitin (Sigma) 10 mg/l [13]. To investigate the possible colonization of individuals by multiple strains, colonies (a maximum of five) were isolated at random from the primary culture plate for each specimen and subcultured on CCMA [23]. *C. difficile* was identified as described previously [15].

Isolation of *C. difficile* from floors. The floors of the hospital ward where the *C. difficile*-positive neonate was born and stayed were examined for environmental contamination by *C. difficile*, as were the floors of day-nursery A and kindergarten C. A total of 95 samples were examined. Using sterile plastic gloves, an area of 30 × 30 cm of the floor of each institution was swabbed with a piece of sterilized gauze, measuring 30 × 30 cm, soaked in 30 ml of sterilized distilled water. The swabs were immersed and shaken in 100 ml of sterilized distilled water, followed by addition of 99.5% ethyl alcohol to a final concentration of 70%. Samples were allowed to stand for 30–60 min at room temperature before passage through a Millipore filter (type HA, 0.45-μm pore size, 47-mm diameter, Millipore, Bedford, MA, USA), which was then placed on CCMA medium and incubated anoxically at 37°C for 72 h. If no colonies grew on the medium, the incubation period was extended to 5 days. All of the colonies grown on CCMA medium were subcultured and kept in cooked-meat medium.

Typing of *C. difficile*. The toxin gene type of the isolates was determined using a previously described PCR assay system [13]. *C. difficile* strains were classified as toxin-A-positive, toxin-B-positive (A+B+); toxin-A-negative, toxin-B-positive (A–B+); or toxin-A-negative, toxin B-negative (A–B–). Typing analyses using PCR ribotyping and PFGE were performed as described previously [14]. From each *C. difficile*-positive stool specimen, a maximum of five colonies were selected and examined by PCR ribotyping and toxin gene typing. Isolates with patterns that differed by one or more major bands were assigned to different PCR ribotypes; differences in faint bands were ignored. Major PFGE types were defined by differences in more than three fragments; these major types were subtyped further according to the criteria described by Tenover et al. [30].

Results

Colonization by *C. difficile* of hospitalized neonates and their mothers and of infants and children in day-care facilities. The frequency of colonization by *C. difficile* in neonates was quite low: only one of the samples obtained from the 40 neonates examined yielded a positive culture. Two of the mothers, including the mother of the *C. difficile*-positive neonate, tested positive. The carriage rates in infants and children in two day-nurseries and a kindergarten are summarized in Table 1. When the carriage rates were analyzed in relation to age group, the rate in infants < 2 years of age was higher (84.4%), than in children 2 years old and older (30.3%).

Detection of *C. difficile* in the floors of the hospital and the day-care facilities. Table 2 and 3 show the number of *C. difficile* strains isolated from the floors of the hospital and from the two day-care facilities (day-nursery A and kindergarten C), respectively.

Genetic analysis of *C. difficile* isolates. The *C. difficile*-positive specimens consisted of three specimens, from a neonate and two mothers, in the hospital; 23 specimens from infants and children in day-nursery A; 11 specimens from infants and children in day-nursery B; and 13 specimens from children in kindergarten C. Of these 50 specimens, 48 yielded colonies with the same PCR ribotype and toxin gene type. The specimens yielding more than one type were those of a mother whose baby was colonized with *C. difficile* and infant k11 in day-nursery B. From the mother, three colonies of tyg2/A+B+ and two colonies of tyg1/A–B–, which were the same types as the isolates from her baby, were obtained. From the infant in day-nursery B, two colonies of mnm/A–B+ and three colonies of hrt/A–B– were obtained. Therefore, a total of 52 isolates, one from each of the 48 specimens, two isolates from the mother, and two isolates from infant k11 were analyzed further by PFGE. The isolates were then classified according to PCR ribotype, PFGE type, and toxin gene type.

The types isolated from the day-care facilities were: fkd/C37-a/A–B–, sgu/C44/A–B–, hrt/S18-a/A–B–, and kmt/C9/A+B+. With regards to the toxin gene type, 41 (87.2%), five (10.6%), and one (k11) (2.1%) of the 47 infants and children were colonized by types A–B–, A+B+, and a mixture of A–B– and A–B+, respectively. The isolate from

Table 1. Asymptomatic intestinal colonization by *Clostridium difficile* in healthy infants and children in three day-care facilities

	Carriage rate (%) of *C. difficile* in infants and children according to age group (years)						
Nursery facility	Under 1	1	2	3	4	5	Total
Day-nursery A	5/5[a] (100.0)	12/15 (80.0)	4/6 (66. 7)	2/8 (25.0)	NS[b]	NS	23/34 (67.6)
Day-nursery B	7/7 (100.0)	3/5[c] (60.0)	1/3 (33.3)	0/1 (0)	NS	NS	11/16 (68.8)
Kindergarten C	NS	NS	0/2 (0)	4/16 (25.0)	5/13 (38.5)	4/17 (23.5)	13/48 (27.1)
Total	12/12 (100.0)	15/20 (75.0)	5/11 (45.5)	6/25 (24.0)	5/13 (38.5)	4/17 (23.5)	47/98 (48.0)

[a]Number of *C. difficile*-positive subjects/number of subjects tested.
[b]There were no subjects in this age group.
[c]Two *C. difficile* colonies differing in toxin gene type and PCR ribotype were isolated from subject k11.

Table 2. Recovery of *Clostridium difficile* from the floors of the hospital (Center for Perinatal and Reproductive Health)

Location of test strains	No. of sites examined	No. of *C. difficile*-positive sites	No. of *C. difficile* strains isolated
Delivery room	3	0	0
Nursery room for neonates	8	0	0
In-patient room	10	1	4
Restroom for patients	2	2	15[a]
Restroom for staff	1	0	0
Pre-, and post-delivery room	4	1	3
Operating room	1	0	0
Total	29	4	22

[a]Five and 10 strains were isolated from each of the two sites, respectively.

Table 3. Detection of *Clostridium difficile* from the floors of day-nursery A and kindergarten C

Location of test strains	No. of sites examined	No. of *C.difficile*-positive sites	No. of *C. difficile* strains isolated
Day-nursery A			
Nursery-room 1	3	0	0
Nursery-room 3	8	4	17
Restroom 1	4	0	0
Restroom 2[a]	4	0	0
Restroom 3[b]	3	1[c]	1
Staff restroom	1	1	1
Entrance	2	1	3
Kitchen	3	0	0
Kindergarten C			
Nursery-room 4	3	0	0
Nursery-room 5	6	0	0
Nursery-room 6	4	0	0
Restroom 4	7	1	16
Restroom 5	5	0	0
Staff restroom	2	1	1
Play room	7	0	0
Entrance	2	0	0
Total	66	9	39

[a]Two fixed and two portable toilets.
[b]One fixed and two portable toilets.
[c]A portable toilet.

the single hospitalized neonate who was positive for *C. difficile* had a type (tyg1/N005/A–B–) identical to one of the two types (tyg1/N005/A–B– and tyg2/N006/A+B+) of isolates from her mother, but different from those of the floors of the hospital (Fig. 1), suggesting that *C. difficile* had been transmitted to the neonate from her mother.

Also, all of the 61 environmental isolates (i.e. 22 isolates from the floor of the hospital and 39 from the floors of the two day-care facilities) were examined by PCR ribotyping, PFGE type, and toxin gene typing. The PCR ribotype/PFGE types/toxin gene type of the 22 isolates from the hospital were not identical to those from the neonate or the mothers. The PCR ribotype/PFGE type/toxin gene type of 21 of the 22 isolates from the floors of day-nursery A were identical to those of one of the isolates from infants in that facility (Fig. 2). Among the 21 isolates, 15 were fkd/C37-a/A–B– (13 from nursery-room 3, one from restroom 3, and one from the staff restroom); 5 were sgu/C44/A–B– (4 from nursery-room 3, and one from the entrance); and one (from the entrance) was ymm/C16/A–B–; 18, 2, and 2 isolates from infants and children in the facility were of these respective PCR ribotypes/PFGE type/toxin gene type. Among the 17 isolates from the floors of restrooms of kindergarten C, only two had a PCR ribotype/PFGE type/toxin gene type (hrt/S18-a/A–B–) identical to that of an isolate obtained from one of the children in the facility; one out of 13 isolates from children belonged to this type.

Discussion

Carriage rates of *C. difficile* in neonates in European countries and in the USA have been reported to range from 13% to 70%, and it has been suggested that longer stays in the hospital are associated with higher rates of colonization [1,3,8]. By contrast, the carriage rate in neonates in this study was very low (2.5%), a finding that needs to be confirmed by further epidemiological studies in neonates.

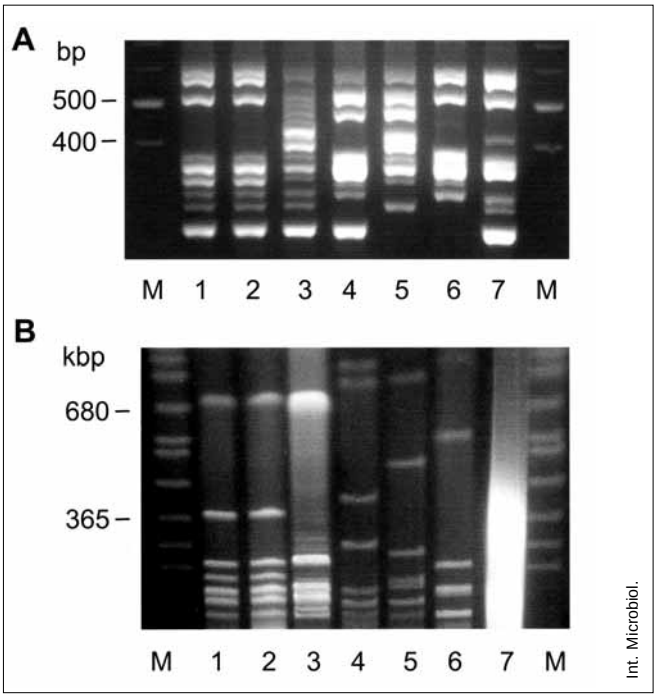

Fig. 1. Typing of *Clostridium difficile* isolates from a neonate, her mother, and the floors of the hospital (Center for Perinatal and Reproductive Health). (**A**) Results of PCR ribotyping and (**B**) results of PFGE typing. *Lanes M*: markers (standard 100-bp DNA ladder and *Saccharomyces cerevisiae* DNA ladder, respectively); *lane 1* (PCR ribotype tyg1/PFGE type N005/toxin gene type A–B–): isolate from the neonate; *lanes 2* (tyg1/N005/A–B–) and *3* (tyg2/N006/A+B+): isolates from the mother of the neonate; *lanes 4* (ptb/OTB1/A+B+), *5* (ctb/OTB2/A–B–), *6* (ptw/OTB3/A–B–), and *7* (phr/NT/A+B+): isolates from the floors of the hospital.

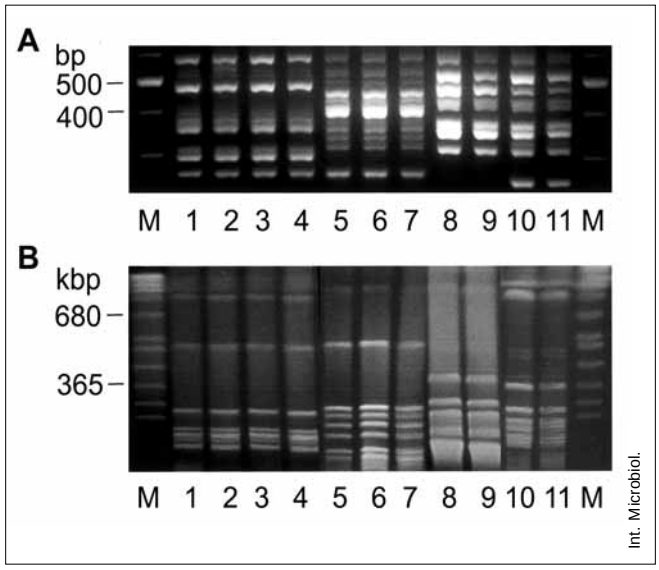

Fig. 2. Typing of *C. difficile* isolates from infants and children as well as the floors of the day-care facilities. (**A**) Results of PCR ribotyping and (**B**) results of PFGE typing. *Lanes M*: markers (standard 100-bp DNA ladder and *S. cerevisiae* DNA ladder, respectively). *Lanes 1*, *5*, and *8*: isolates from infants and children of day-nursery A; *lanes 2* and *6*: isolates from the floor of nursery-room 3 of day-nursery A; *lane 3*: isolate from the floor of the restroom with a portable toilet for infants of day-nursery A; *lane 4*: isolate from the floor of a staff restroom of day-nursery A; *lanes 7* and 9: isolates from the entrance of day-nursery A; *lane 10*: isolate from a child of kindergarten C; *lane 11*: isolate from the restroom 4 for children of kindergarten C. PCR ribotype/PFGE type/toxin gene type: lanes 1, 2, 3, and 4, fkd/C37-a/A–B–; lanes 5, 6, and 7, sgu/C44/A–B–; lanes 8 and 9, ymm/C16/A–B–; lanes 10 and 11, hrt/S18-a/A–B–.

Yamamoto-Osaki et al. [32] reported that, of the 67 infants and children under 3 years of age with no medical disorders who were examined in a day-nursery, 25 (37%) carried *C. difficile*, while the carriage rate in infants < 1 year of age was much higher (65%). The carriage rates in the present study (see Table 1) were higher than those in the study by Yamamoto-Osaki et al. [32].

In the present study, the carriage rate of *C. difficile* in infants under 2 years of age was 84.4%, whereas in children 2 years of age and over the average was 30.3%, ranging from 23.5% to 45.5% depending on the exact age. These results are consistent with those of previous studies in European countries and the USA, where the carriage rates in infants under 2 years of age were found to be high, although they varied from 26.6% to 43.5% [5,6,9,11,26,31], and the rates in children 2 years of age and over were as low as those in adults, whose carriage rates were generally reported to be less than 4% [7,9,11,26,27]. The carriage rates of infants under 1 year of age were extremely high in this study. The reason why the rates decrease in older infants remains unclear, but it has been suggested that other microorganisms in the microbiota of the developed intestine inhibit colonization or growth by *C. difficile* [20,32]. Several investigators reported that high densities of enterococci were related to colonization of *C. difficile* [12,21,23,27]. The decrease in *C. difficile* densities and those of other components of the intestinal microbiota in the intestine should be investigated in future studies.

Acquisition of *C. difficile* by neonates is believed to occur mainly via the hospital environment, although some studies have suggested that maternal transmission is also a frequent route [1,3,5,6,8,19,29]. A previous study indicated that neonates born to mothers carrying *C. difficile* in the rectum or vagina also carried *C. difficile*; however, the types of isolates were not analyzed [29]. In the present study, the floors of wards of the hospital were also contaminated with, in agreement with numerous earlier reports [1–5,17,18,25]. However, it was highly likely that one neonate acquired *C. difficile* from her mother, because the type of organism isolated from both mother and neonate was the same (Fig. 1) but different from those isolated from the floors of the hospital.

The results of the present study also suggest that cross-infection occurs in day-care facilities either through *C. difficile*-carrying infants and children or through environmental contamination, such as that of the nursery-room or restroom floors of day-nursery A and kindergarten C, since in each day-care facility the same types of *C. difficile* were isolated from the infants and children, and some of the isolates were of the same types as those present in the floors of the facilities, especially in day-nursery A. Thus, cross-infection may play an important role in the carriage of *C. difficile* in infants and children in day-care facilities. The *C. difficile* isolates of fke/C37-a/A–B–, sgu/C44/A–B–, and hrt/S18-a/A–B– were distributed among both infants and children as well as environments in the different facilities. Since several reports have shown that specific types of *C. difficile* can induce environmental contamination in hospitals [5,13,24], these three types of organism might easily infect neonates, infants, and children or spread readily in some areas.

Acknowledgements. We thank Satoshi Shimura for preparing the manuscript. This work was supported in part by the Yakult Bio-Science Foundation, Japan.

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Colonización por *Clostridium difficile* en recién nacidos en un hospital y en niños menores de cinco años en tres guarderías de Kanazawa, Japón

Resumen. Se examinaron las frecuencias de portadores intestinales de *Clostridium difficile* en recién nacidos y en niños menores de cinco años en las salas del centro de obstetricia y neonatología de un hospital universitario, en dos guarderías y en un parvulario. También se examinaron muestras de los suelos de estas instalaciones tomadas con torundas, para determinar la contaminación ambiental por el mencionado organismo. *C. difficile* se encontró solamente en las defecaciones de uno de los 40 recién nacidos durante la semana de estancia en el hospital después del parto. El aislado del recién nacido era idéntico al aislado de su madre en el ribotipado de PCR, en el análisis mediante electroforesis en gel de campo pulsado, y en el tipo de gen de la toxina. Esto sugiere que el recién nacido positivo para *C. difficile* adquirió el organismo de su madre y no del ambiente. Del total de 98 niños menores de cinco años estudiados (50 alumnos de una guardería, que tenían tres años o menos, y 48 alumnos de un parvulario, con edades entre dos y cinco años), 47 (48%) eran portadores de *C. difficile*. Cuando se analizó la frecuencia de portadores en niños menores de dos años de edad era más elevada (84,4%), mientras que en niños de dos o más años era menor (30,3%). Las frecuencias en los grupos de edades de 0, 1, 2, 3, 4, y 5 años fueron de 100, 75,0, 45,5, 24,0, 38,5, y 23,5% respectivamente. La observación de que en cada guardería hubiese varios niños colonizados con la misma cepa de *C. difficile*, y de que el suelo de la guardería A y el del parvulario C estuvieran contaminados con varios tipos de *C. difficile* idénticos a los que colonizaban el intestino de los niños menores de dos años, sugiere que existe una infección cruzada de *C. difficile* entre los niños. Esta infección se transmitiría en las instalaciones de la guardería a partir de los niños portadores de *C. difficile* o por contaminación del ambiente. [**Int Microbiol** 2005; 8(1):43-48]

Palabras clave: *Clostridium difficile* · colonización bacteriana · frecuencia de portadores · tipado molecular · recién nacidos · niños menores de cinco años

Colonização pelo *Clostridium difficile* de neonatos no hospital e de crianças menores de cinco anos em três escolinhas de Kanazawa, Japón

Resumo. Foram examinadas as freqüências de portadores intestinais de *Clostridium difficile* em neonatos e crianças menores de cinco anos nas salas do centro de obstetrícia e neonatologia de um hospital universitário, assim como em duas creches e uma pré-escola. Também foram examinadas amostras dos solos destas instalações coletadas com “swabs”, para determinar a contaminação ambiental pelo citado organismo. *C. difficile* só foi encontrado nas fezes de um dos 40 neonatos durante a semana de estadia no hospital depois do parto. O isolado foi idêntico ao isolado da mãe quando foi feita a ribotipagem por PCR, a análise mediante eletroforese em gel de campo pulsado, e a análise do tipo de gene da toxina. Isto sugere que o neonato positivo para *C. difficile* adquiriu o microorganismo da mãe e não do ambiente. Do total das 98 crianças menores de cinco anos estudadas (50 alunos de uma creche com três anos ou menos, e 48 alunos de uma pré-escola, com idade entre dois e cinco anos), 47 (48%) foram portadores de *C. difficile*. Quando foi analisada a freqüência de portadores em crianças menores de dois anos foi mais elevada (84,4%), enquanto que em crianças de dois ou mais anos foi menor (30,3%). As freqüências nos grupos de idades 0, 1, 2, 3, 4, e 5 anos foram de 100, 75,0, 45,5, 24,0, 38,5, e 23,5% respectivamente. A observação de que cada creche apresentava varias crianças colonizadas pela mesma cepa do *C. difficile*, e de que o piso da creche A e da pré-escola C estiveram contaminados com vários tipos de *C. difficile* idênticos aos que colonizaram o intestino das crianças menores de dois anos, sugere que existe uma infecção cruzada do *C. difficile* entre as crianças. Esta infecção seria transmitida nas instalações da creche a partir das crianças portadoras do *C. difficile* ou pela contaminação do ambiente. [**Int Microbiol** 2005; 8(1):43-48]

Palavras chave: *Clostridium difficile* · colonização bacteriana · freqüência de portadores · tipagem molecular · recém nascido · crianças menores de cinco anos