Prevalence and characteristics of bacteria and host factors in an outbreak situation of antibiotic-associated diarrhoea

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Antibiotic-associated diarrhoea (AAD) represents a clinical entity leading to prolonged hospital stays and diagnostic and therapeutic procedures, and results in additional costs. The aim of the present study was to assess the prevalence and characteristics of different bacteria in stools of patients with AAD. The reliability of diagnostic procedures under routine conditions was evaluated. Host factors were also analysed. From June 2002 to April 2003 89 cases of diarrhoea were reported at a hospital unit for internal medicine. Clostridium difficile and Clostridium perfringens toxin enzyme-immunoassays (EIAs), and culture for C. difficile, C. perfringens and Staphylococcus aureus were performed on stool samples from all patients. Toxin production was determined in isolated S. aureus strains. In vitro susceptibility of S. aureus for oxacillin and of C. difficile for vancomycin, metronidazole, linezolid, fusidic acid and tetracycline was tested. Host factors, such as age, comorbidities, antibiotic exposure and contact with other patients, were evaluated. Twenty-six stools were positive for C. difficile toxins by an EIA technique, while C. difficile was cultured from 39. C. difficile was isolated from 21 stools that were EIA negative. Additionally, from 28 stools S. aureus and/or C. perfringens could be isolated. Nine samples contained only S. aureus and/or C. perfringens. Thirty-one stools were negative in all tests. All C. difficile isolates were susceptible to vancomycin and metronidazole. Age >60 years, and diseases of the vascular system, the heart, the kidneys and the lungs were identified as risk factors for acquiring C. difficile in this setting (P values < 0.05). Stool culture for C. difficile was shown to be more sensitive than toxin EIA in this study. Risk factors for the acquisition of C. difficile in outbreak situations seem to differ from risk factors in the normal hospital setting. The role of toxin-producing S. aureus in cases of AAD needs further investigation.

INTRODUCTION

Antibiotic-associated diarrhoea (AAD) is a major health problem. Clostridium difficile, Clostridium perfringens and Staphylococcus aureus are the most frequent causes of AAD; however, many cases remain undiagnosed. C. difficile is the most commonly identified and accepted pathogen in nosocomial diarrhoea. For patients whose hospital stay is complicated by C. difficile-associated diarrhoea (CDAD) a prolonged hospital stay, and additional diagnostic and therapeutic procedures, leading to increasing health care costs, were reported (Kyne et al., 2002). Increased hospitalization and death possibly due to C. difficile diarrhoeal disease were found in a study that included data from 1980 to 1994 from the USA (Frost et al., 1998).

Evidence for antibiotic-induced C. perfringens diarrhoea was shown in several studies (Abrahao et al., 2001; Modi & Wilcox, 2001; Borriello et al., 1984). The prevalence of C. perfringens enterotoxin (CPE) in stool samples in these studies was reported to vary between 1-6 and 6-4 %. S. aureus was first suspected to cause AAD in 1954 (Oeding & Austarheim, 1954). Since then a couple of studies reporting the occurrence of MRSA in stools of patients with AAD and data on specific toxin production have been published (McDonald et al., 1982; Adesiyun et al., 1992; Gravet et al., 1999). No recommendation or guidelines for routine testing for CPE or S. aureus in faecal samples from patients with AAD have been presented until now.

Abbreviations: AAD, antibiotic-associated diarrhoea; CDAD, Clostridium difficile-associated diarrhoea; CPE, Clostridium perfringens enterotoxin; EIA, enzyme-immunoassay.
Diagnostic procedures for AAD mostly include *C. difficile* toxin detection using EIA only. However, the sensitivities of toxin ELAs vary and the impact of toxin variants on the test sensitivity is not known. Stool culture, susceptibility testing and typing of *C. difficile* isolates are only performed in special laboratories.

The lack of clear guidelines for *C. difficile* diagnosis was also evident in a recent European survey of diagnostic methods and testing of *C. difficile*. This study showed major discrepancies between laboratories and between countries (Barbut et al., 2003).

First-line antimicrobials for the treatment of CDAD are metronidazole and vancomycin. Impaired susceptibility was observed for both substances; however, this was not directly linked to treatment failure (Brazier et al., 2001; Jang et al., 1997; Peláez et al., 2002; Wong et al., 1999). On the other hand, as many as 20 % of patients had at least one recurrence of CDAD after the initial therapy was discontinued (Fekety et al., 1997; Pérez et al., 2002). Thus, the search for alternative treatments includes substances for the reconstitution and balance of colonization resistance as well as new antibacterial compounds.

This study analysed the prevalence of *C. difficile*, *C. perfringens* and *S. aureus* in stool samples of hospitalized patients in an AAD outbreak situation. Isolated strains were characterized regarding antimicrobial susceptibility, toxin production and clonality.

**METHODS**

**Patient population.** Patients included in this study were patients at the hospital unit for internal medicine of a tertiary care teaching hospital (St Georg Hospital, Leipzig). Stools from 89 patients with diarrhoea were collected from June 2002 to April 2003. All samples were investigated for *C. difficile* toxin and *C. perfringens* enterotoxin by EIA, and cultured for *C. difficile*, *C. perfringens* and *S. aureus*.

Physicians were asked to complete a questionnaire on patients’ data, which was submitted, together with the stool sample, to the laboratory. Additionally, the medical records for 81 patients were available and analysed for special conditions associated with AAD.

**Culture and identification of bacteria.** All stools were cultured anaerobically on a selective medium (cycloserine-cefoxitin-fructose agar, CCFA) and on Columbia agar supplemented with sheep erythrocytes, haemin and vitamin K at 37 °C for 48 h, and aerobically on mannitol-salt-agar (both Oxoid) at 37 °C for 24 h (George et al., 1979). The bacterial strains were identified with the RapID ANA II System, the PRO KIT (both Remel) and the plasma-coagulase test.

**Antimicrobial-susceptibility testing.** Minimal inhibitory concentrations (MICs) of five antibiotics (metronidazole, vancomycin, fusidic acid, linezolid and tetracycline) were evaluated for all isolated strains of *C. difficile* using Etest (AB Biodisk). For antimicrobial-susceptibility testing, strains were grown on supplemented Columbia agar. Etest was performed by inoculating the surface of pre-reduced Columbia agar plates containing vitamin K1, haemin and 5 % defibrinated sheep red blood cells with a 1 McFarland standard-matched inoculum. The inoculation was applied with cotton-tipped swabs that were streaked three times, rotating the plate approximately 90° each time to ensure an even distribution of inoculum. Etest strips were used according to the manufacturer’s instructions.

Susceptibility of *S. aureus* isolates to oxacillin was tested using Etest. Strains were grown on Columbia agar. Using a McFarland 1 standard-matched inoculum, Mueller–Hinton agar plates containing 2 % sodium chloride were inoculated.

Reference strains used in this study were *C. difficile* VPI 10463, *Bacteroides fragilis* ATCC 25285, and *S. aureus* ATCC 29213 and ATCC 43300.

**Toxin assays.** *C. difficile* toxin A/B EIA (r-biopharm) and *C. perfringens* enterotoxin EIA (r-biopharm) were performed for all stool samples according to the instructions of the manufacturer. Stools were stored at 4 °C until testing. Isolated *S. aureus* strains were tested for toxin production using the SET-RLPA staphylococcal enterotoxin test kit (Oxoid).

All *C. difficile* strains grown from EIA-negative stools were analysed by PCR for tcdA/tcdB gene sequences as described elsewhere (Cohen et al., 2000).

**Genotyping.** Epidemiological analysis of *C. difficile* isolates was performed using PCR ribotyping. For PCR ribotyping, template nucleic acid was prepared by resuspension of cells in a 5 % solution of Chelex100 (Bio-Rad) and boiling for 12 min. Cellular debris was removed by centrifugation (15 000 g for 10 min) and the supernatant was used for PCR reaction. PCR ribotyping was performed using two primers (16S: 5’ CTGGGGTGAAGTCGTAACAAGG 3’ and 23S: 5’ CGGCCCTTTTG TAGCTTGGACC 3’). Amplification products were concentrated to a final volume of 50 μl using a speedvac. Electrophoresis (400 mA, 80 V) was run in 1:5 % Metaphor agarose (BMA) for about 4 h. Products were visualized by staining the gel in ethidium bromide (Stubbs et al., 1999).

**Statistical analysis.** Chi-square analysis was performed with SPSS 11.0 for Windows (SPSS).

**RESULTS**

**Prevalence of bacteria**

*C. difficile* was cultured from 39 stools. *S. aureus* could be cultured from 25 samples, and *C. perfringens* from five (Table 1). *C. difficile* was isolated from 21 of the EIA-negative stools. Nine EIA-positive samples were negative using culture (Table 2). Additionally, in 28 stools *S. aureus* and/or *C. perfringens* could be isolated. From three samples *C. difficile* as well as *C. perfringens* and *S. aureus* grew. Nine samples contained only *S. aureus* and/or *C. perfringens*. *S. aureus* only was found in seven samples. Forty-one stools were culture-negative for specific bacteria in this study.

**Table 1. Results of bacterial culture**

<table>
<thead>
<tr>
<th>Culture</th>
<th>CD</th>
<th>SA</th>
<th>CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>39</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>Negative</td>
<td>50</td>
<td>64</td>
<td>84</td>
</tr>
</tbody>
</table>
Susceptibility of C. difficile and S. aureus

All C. difficile isolates were susceptible to vancomycin, metronidazole and fusidic acid. Ten (26%) strains were resistant to tetracycline (MIC $> 8$ mg l$^{-1}$) and one strain showed an enhanced MIC for linezolid (MIC $= 8$ mg l$^{-1}$).

Since the Deutsches Institut fuer Normung (DIN) has not yet established acceptable ranges for testing reference strains and linezolid, published data from the NCCLS (National Committee for Clinical Laboratory Standards) were used. For fusidic acid no ranges for control strains exist in either organization. For the reference strains, the MICs of metronidazole, vancomycin and linezolid were all within the acceptable ranges described by the DIN and the NCCLS (Deutsches Institut fuer Normung e.V., 2000; National Committee for Clinical Laboratory Standards, 2004). According to DIN recommendations, resistance was defined as follows: vancomycin, $> 16$ mg l$^{-1}$; metronidazole, $> 8$ mg l$^{-1}$; fusidic acid, $> 4$ mg l$^{-1}$; linezolid, $> 8$ mg l$^{-1}$; tetracycline, $> 8$ mg l$^{-1}$ (Deutsches Institut fuer Normung e.V., 2000).

All S. aureus isolates were susceptible to oxacillin. Resistance to oxacillin was defined as MIC $> 2$ mg l$^{-1}$ (Deutsches Institut fuer Normung e.V., 2000).

Toxigenicity

Out of 38 culture-positive stools 17 were positive for C. difficile toxin A/B using EIA. All 21 strains cultured from EIA-negative stools contained toxin A and B sequences as shown by PCR. Nine samples were toxin positive and did not grow C. difficile. One sample was positive using CPE EIA.

Seventy-two percent ($n = 18$) of the S. aureus isolates exhibited enterotoxin production: 15 strains produced enterotoxin D, two enterotoxin A and one enterotoxin B and D.

Altogether C. difficile was detected in 52% of cases of AAD using culture and/or toxin EIA. One or more of the pathogens (C. difficile and/or C. perfringens and/or S. aureus) was found in 58 (65%) stool samples. Thirty-one stools were negative in all tests.

Genotyping

Using PCR ribotyping, about 10 ribotypes were identified (Fig. 1). A PCR ribotype was defined as the existence of clearly discernible, reproducible differences in the PCR ribotype pattern from those of the other existing types (Stubbs et al., 1999). The 10 tetracycline-resistant strains showed the same ribotyping pattern and had similar MIC values for the other antimicrobials tested here. Almost half of the 39 C. difficile isolates were grouped in two ribotyping groups (I: strains 3, 4, 6, 9, 10, 11, 12, 14, 16, 21; II: strains 57, 58, 64, 65, 66, 67).

Table 2. Results of C. difficile culture and toxin EIA

<table>
<thead>
<tr>
<th>C. difficile culture</th>
<th>C. difficile toxin A/B EIA*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>17</td>
<td>38</td>
</tr>
<tr>
<td>Negative</td>
<td>9</td>
<td>50</td>
</tr>
</tbody>
</table>

*One specimen was not determined for the detection of toxin A/B of C. difficile.

Fig. 1. PCR ribotyping of 39 C. difficile strains. L, ladder.

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Host factors

Twenty-eight out of the 39 stools from which C. difficile was cultured were from patients on a geriatric ward. Ninety-seven percent of the C. difficile-positive patients and 80% of the C. difficile-negative patients were >60 years ($P$ value = 0.013). Medical records for 81 patients were searched for the following data: transfer from another hospital or within the hospital; vascular, heart, pulmonary, urogenital, infectious, neurological, skeletal, gastrointestinal and endocrine diseases; cancer; and surgery or wound infection. There were no significant associations between the acquisition of C. difficile and the transfer from another hospital or within the hospital, or underlying diseases such as infections, cancer, gastrointestinal disorders, neurological diseases and disor-
ders of the skeletal or endocrine system. However, vascular and heart diseases, and disorders of the kidneys and the lungs were strongly associated with C. difficile infection (P < 0.05).

DISCUSSION

C. difficile was detected in an outbreak situation in more than half of the cases (52%) of AAD. Nosocomial transmission was shown for most of the cases by genotyping and is suggested to have started on the geriatric ward.

Risk factors for C. difficile colonization and toxin production have been described previously. Antibiotics such as third generation cephalosporins are accepted as the main risk factors, and antibiotic restriction is the most effective control measure (Starr et al., 2003; Schwaber et al., 2000; McFarland et al., 1999; Al-Eidan et al., 2000). Older age, female gender and a prolonged hospital stay were identified risk factors in hospitalized CDAD patients (Al-Eidan et al., 2000). More recent studies reported the association of the use of proton pump inhibitors within the preceding 8 weeks, the use of nasal feeding tubes and exposure to antineoplastic agents with an increased risk of C. difficile diarrhoea (Cunningham et al., 2003; Komatsu et al., 2003). Some of the risk factors identified in this study differ from those reported in other investigations. In the outbreak situation described here vascular and heart diseases, disorders of the kidneys and the lungs, and age ≥60 years were associated with CDAD.

A European survey of diagnostic methods for C. difficile showed that culture of the organism is performed in only a few countries. Mostly C. difficile toxin EIAs are used for the diagnosis of CDAD (Barbut et al., 2003). Stool culture is used in 20% (Great Britain) to 100% (Denmark) of laboratories.

The sensitivity of C. difficile stool culture is superior to that of toxin EIA and the cytotoxicity assay. In addition, culturing the organism offers the possibility of further analyses, such as antimicrobial-susceptibility testing and molecular typing. In this study using both toxin A/B EIA and culture, the detection of C. difficile in patient stools could be enhanced. Using either method would have led to false-negative results in 13% (toxin EIA) and 9% (culture) of cases. Negative culture and a positive toxin assay might reflect the presence of the toxin or specific bacteria only in certain portions of the stool sample. The high specificity of the C. difficile toxin EIA excludes false-positive results. The investigation of a repeated specimen or of homogenized stool samples could correct this constellation. Possible explanations for a positive culture and negative toxin assay from one stool sample are the presence of toxin A- and toxin B-negative C. difficile strains or low toxin levels that would be missed by the toxin EIA. The detection limit of the EIA used in this study is 1.2 ng ml⁻¹ purified toxin (data from the manufacturer). Toxin B of C. difficile is stable at 4°C for several weeks, but stool samples might have been exposed to room temperature for hours before arrival in the microbiological laboratory (Freeman & Wilcox, 2003).

Susceptibility testing of the isolated strains in this study gave no surprising results. Fusidic acid showed good activity and deserves further consideration. Linezolid was shown to be active against C. difficile in an earlier study (Ackermann et al., 2003).

To look only for C. difficile in patients hospitalized for more than 72 h and who are suffering from diarrhoea is accepted and applied in most hospitals. However, S. aureus was first suggested to be associated with AAD 50 years ago (Oeding & Austarheim, 1954). Since then a few studies reported the prevalence of enterotoxin-producing S. aureus in AAD patients; some studies focused on the resistance to methicillin in the isolated strains and found controversial results (McDonald et al., 1982; Adesiyan et al., 1992; Gravet et al., 1999). In our patient population, enterotoxin-producing S. aureus could be detected in 20% of the patients stools, most of them (15 out of 18) were isolated from stools from which C. difficile could also be cultured. Six S. aureus strains were recovered from C. difficile toxin EIA-negative specimens. None of the S. aureus isolates were identified as MRSA. Toxin D was produced by most of the isolated S. aureus strains (64%). Other studies have reported the predominance of staphylococcal enterotoxins C and A in diarrhoeal strains (Adesiyan et al., 1992; Gravet et al., 1999).

C. perfringens has been reported and discussed as a cause of AAD since 1984 (Borriello et al., 1984; Boone & Carman, 1997; Abrahao et al., 2001; Modi & Wilcox, 2001). However, in our study CPE was detected in one stool (1%) and the organism was cultured from only five samples (6%). Considering the high frequency of C. difficile and S. aureus detected in stools in this study, C. perfringens seems not to be an important cause of AAD.

Since causes of AAD other than C. difficile have only been searched for in studies, the number of investigations we can look at is small and may not reflect the real situation. Whether stools of patients with nosocomial diarrhoea should be cultured for other pathogens, i.e. S. aureus, was questioned many years ago. Patients suffering from AAD and the frustrating course of repeated relapses motivate physicians to search for causes of AAD other than C. difficile. To further analyse the role of S. aureus in AAD another study was started at our institute.

REFERENCES


