Difficulties in the serodiagnosis of infection with the fragilis group of *Bacteroides*

K. M. ELHAG, A. M. BAHAR* and K. K. MAHAJAN†

Departments of Microbiology, *Obstetrics and Gynaecology and †Surgery, Faculty of Medicine, University of Kuwait, P.O. Box 24923, Kuwait

Summary. *Bacteroides* antibodies were studied in sera from 74 patients infected with the fragilis group of *Bacteroides* and 74 healthy control persons, by immunofluorescence of 26 different serotypes of the fragilis group. Antibodies were present at titres of 10–320 in 65 (88%) patients and 50 (68%) controls ($p < 0.01$). Titres of ≥ 80 were demonstrated in sera of 38 (51%) patients and 5 (7%) controls ($p < 0.01$). Specific IgM antibodies were detected in sera of 42 (57%) patients at a geometric mean titre (GMT) of 30, and 8 (11%) controls at a GMT of 11 ($p < 0.01$). High antibody titres as well as specific IgM were found in 32 (43%) patients, while none of the controls showed such a combination ($p < 0.01$). The majority of positive patients' sera (57%) reacted with five or more serotypes, whereas most positive control sera (51%) reacted against only one or two serotypes ($p < 0.01$). A selected combination of serotypes not reacting with the control sera showed positive reactions with 52 (70%) patients' sera. These findings may be useful in devising schemes for the serodiagnosis of infection caused by the fragilis group of *Bacteroides*. However, there are indications of geographic variation in prevalence of serotypes, which may prevent the development of a single universal scheme.

Introduction

The fragilis group of *Bacteroides* are the anaerobic bacteria most frequently associated with infections (Moore *et al.*, 1969; Gorbach and Bartlett, 1974). Among these, *B. fragilis* is the most significant, accounting for over 70% of the strains isolated from clinical specimens (Polk and Kasper, 1977). The diagnosis of deep-seated bacteroides infections may require invasive procedures, not always without risk. Furthermore, factors such as poor technique of obtaining specimens, inappropriate transport and prolonged culture procedures tend to reduce the diagnostic accuracy and to delay the commencement of appropriate chemotherapy. These factors prompted a search for serological means of diagnosing such infections (Ross and Gilmore, 1978; Rissing *et al.*, 1979; Paavonen *et al.*, 1981). Although it was possible to detect specific antibodies in patients' sera, serological diagnosis of these infections has remained far from perfect. The poor immunogenicity of the fragilis group (Elhag and Tabaqchali, 1978a; Elhag *et al.*, 1986) and the presence of antibodies in the sera of healthy

individuals (Danielsson *et al.*, 1974; Hofstad, 1974; Elhag *et al.*, 1986) may cause difficulties in interpretation of the findings. Furthermore, the antigenic heterogeneity of the group (Elhag *et al.*, 1977) and the geographic variation of their serotypes (Elhag and Tabaqchali, 1978b) may hamper the selection of appropriate test antigens.

In this study, 26 serotypes were selected to evaluate the immune responses of patients infected by this group of bacteria compared with normal levels of these antibodies in healthy subjects.

Materials and methods

Patients

This study included patients admitted to the Maternity Hospital and the surgical wards at Mubarak Al-Kabeer Hospital, Kuwait, in 1983–85. Patients were selected randomly, with a variety of conditions: wound infections following hysterectomy or surgery of the bowel or biliary tract, septicaemia, intra-abdominal sepsis, deep-seated abscess, acute pelvic inflammatory disease, osteomyelitis, otitis, skin infections.

Infection with the fragilis group of *Bacteroides* was found in 74 patients (39 male, 35 female) aged 13–90 years (mean 39 years). They were grouped according to

the type and site of infection into: intra-abdominal sepsis (20), post-operative wound infection (29), skin abscess or gangrene (16), septicaemia (4) and miscellaneous infections (5). The latter group consisted of osteomyelitis (2), cholecystitis (1), lung abscess (1) and otitis media (1).

Controls. A control group consisted of 74 blood donors and other healthy persons coming to Mubarak Hospital for routine medical examination; they were similar to the patients in sex (39 male, 35 female) and age (14–88 years; mean 39 years).

Clinical specimens. Specimens collected from patients were blood, exudate from deep-seated abscesses, wound swabs, bile and transtracheal aspirates. Samples of venous blood (10 ml) were taken from all patients as soon as a diagnosis of bacteroides infection was established. The duration of infection to the time of specimen collection was 4–25 days (mean 9 days). Blood samples (10 ml) were collected from the controls.

Microbiological methods

Blood cultures were performed as described by Isenberg *et al.* (1980), using commercially prepared Tryptic Soy Broth and Thioglycollate medium (6440 and 6436; Gibco Europe Ltd, Paisley, Scotland). The remaining specimens were sent to the laboratory in anaerobic transport media (Portagerm 41994 and 41995; bioMérieux, Charbonnières-Bains, France) within 24 h of collection. Specimens were inoculated on to Blood Agar (CM 55; Oxoid, Basingstoke), Schaedler Blood Agar (CM 437; Oxoid) with Kanamycin-Vancomycin mixture (55681; bioMérieux), and Schaedler Agar with laked horse blood 5% v/v and gentamicin 80 µg/ml, and into Brain Heart Infusion (BHI) Broth (CM 225; Oxoid). Cultures were incubated for 48 h in an anaerobic system (1028; Forma Scientific, Marietta, OH, USA) with an atmosphere of N₂ 90%, H₂ 5%, CO₂ 5%. After incubation, BHI-enrichment cultures were subcultured on to the above media. Blood Agar and MacConkey Agar (CM 7; Oxoid) were also inoculated and incubated aerobically for 24 h.

Identification of bacterial strains. Aerobic bacteria were identified by standard techniques (Isenberg *et al.*, 1980). Anaerobic bacteria were identified to the genus level by their colonial appearance, cell morphology, and the end products of glucose metabolism as determined by gas-liquid chromatography (Shimatzu Gas Chromatograph GC90, Shimatzu Corporation, Kyoto, Japan). They were further identified biochemically (Holdeman *et al.*, 1977) by the Anaerobe Tek System (Flow Laboratories Inc., McLean, VA, USA). Gram-negative anaerobic bacilli were tested also for tolerance to bile and brilliant green and resistance to disks containing kanamycin 1000 µg, penicillin 2 U, colistin 10 µg, erythromycin 60 µg, rifampicin 15 µg (Anaerodiscs 57311–57371; bioMérieux) and metronidazole 5 µg (x195B; Oxoid).

Fragilis group. The fragilis group of *Bacteroides* were identified as gram-negative anaerobic bacilli that formed non-pigmented non-pitting colonies on blood agar and that were resistant to penicillin, kanamycin and colistin, sensitive to metronidazole and rifampicin, and tolerant

to bile but inhibited by brilliant green. They fermented glucose and lactose and most strains hydrolysed aesculin. The species of the isolates of the fragilis group were differentiated by indole production and fermentation of mannitol and trehalose (Duerden *et al.*, 1980).

Bacteroides serotypes

By a process of successive screening, 26 distinct serotypes of the fragilis group were obtained. Serotypes 1–20 have been described previously (Elhag *et al.*, 1977; Elhag and Tabaqchali, 1978a), while serotypes 21–26 were selected in Kuwait (table I). Antisera were raised in rabbits; each agglutinated the homologous strain, but cross-reactions occurred also between serotypes within each of four groups (2, 11 and 22; 5, 16 and 23; 1 and 18; 17 and 24). All cross-reacting antibodies, however, were successfully removed by absorbing the sera with heterologous strains.

Preparation of bacterial suspensions for immunofluorescence tests. The 26 serotypes were grown anaerobically

Table I. Strains of the fragilis group of *Bacteroides* used for serological differentiation

Serotype	Species of <i>Bacteroides</i>	Source of isolate
1	<i>B. fragilis</i>	NCTC 9343
2	<i>B. fragilis</i>	NCTC 10584
3	<i>B. thetaiotaomicron</i>	NCTC 10582
4	<i>B. vulgatus</i>	NCTC 10583
5	<i>B. fragilis</i>	NCTC 9344
6	<i>B. fragilis</i>	NCTC 8560
7	<i>B. fragilis</i>	NCTC 10581
8	<i>B. ovatus</i>	ATCC 8483
9	<i>B. distasonis</i>	ATCC 8503
10	<i>B. fragilis</i>	Cholecystectomy wound
11	<i>B. fragilis</i>	Appendicectomy wound
12	<i>B. fragilis</i>	Peritonitis exudate
13	<i>B. distasonis</i>	Faeces
14	<i>B. distasonis</i>	Faeces
15	<i>B. distasonis</i>	Small intestinal tissue
16	<i>B. fragilis</i>	Appendicectomy wound
17	<i>B. fragilis</i>	Cholecystectomy wound
18	<i>B. fragilis</i>	Blood
19	<i>B. fragilis</i>	Blood
20	<i>B. fragilis</i>	Blood
21	<i>B. fragilis</i>	Peritonitis exudate
22	<i>B. fragilis</i>	Appendicectomy wound
23	<i>B. distasonis</i>	Appendicectomy wound
24	<i>B. fragilis</i>	Appendicectomy wound
25	<i>B. fragilis</i>	Otitis media exudate
26	<i>B. fragilis</i>	Appendicectomy wound

NCTC = National Collection of Type Cultures, London; ATCC = American Type Culture Collection.

BH = St Bartholomew's Hospital, London; TH = St Thomas's Hospital, London; MH = Mubarak Al-Kabeer Hospital, Kuwait.

for 48 h in 30 ml of peptone yeast broth, giving c. 5×10^8 bacteria/ml. Formalin was added (final concentration 0.5% v/v), and after 1 h at room temperature the bacteria were deposited by centrifugation, washed three times, and resuspended in physiological buffered saline (PBS) pH 7.2 to give an optical density of 0.05 at 405 nm (Titer Tek Multiscan; Flow Laboratories).

Testing of sera. Sera from the patients and controls were tested against the 26 bacterial suspensions by the immunofluorescence technique of Wulff and Lange (1975). The sera were first tested at a dilution of 1 in 10, with fluorescein isothiocyanate (FITC) conjugated with goat antihuman immunoglobulin (75602 bioMérieux), and those sera that gave a positive reaction were then tested in two-fold dilutions from 1 in 10 to 1 in 1280, with FITC goat antihuman IgG (75692 bioMérieux) and FITC goat antihuman IgM (75671 bioMérieux) conjugates. To suppress nonspecific fluorescence, slides were counterstained with Evans blue (22274; George T. Gurr Ltd, London) 0.2% w/v in PBS, and examined by a fluorescence microscope (Leitz Ortholux II, Germany) with a mercury lamp HBO 50 as light source.

Reproducibility and expression of titration results. All sera were tested against all 26 bacterial suspensions on three separate occasions. Many of the sera reacted with more than one suspension; in such a case, the results with the suspension that had given the highest titre were used. Frequently, the triplicate results were identical but, not surprisingly, there was often a two-fold variation. The recording of such titres has been simplified (table III) by using the two-fold dilution closest to the geometric mean: e.g., observed titres of 40, 20 and 40 in the triplicate tests have been recorded as 40.

Results

Bacteria isolated from patients

Bacterial species isolated from the various groups of patients are listed in table II. *B. fragilis* was the most commonly isolated anaerobe, followed by other species of *Bacteroides*; and *Escherichia coli* was the most common aerobe.

Fragilis antibodies in patients and controls

The titres obtained from the triplicate titrations of patients' and control sera are shown in table III. Antibodies against the fragilis group were detected at titres of 10–320 in the sera of 65 (88%) of the 74 patients and 50 (68%) of the 74 controls ($p < 0.01$). No sera had titres higher than 320. Among the different groups of patients, antibody was detected in 79–100%, the highest occurrence being in the small numbers of patients with septicaemia and miscellaneous infections and the lowest in those with post-operative wound infections. The sera of 38 patients (51%) showed high titres of specific IgG (80–320), while only five controls (7%) showed such titres ($p < 0.01$). Specific IgM antibodies were detected in the sera of 42 patients (57%) at titres of 10–320, geometric mean titre (GMT) 36, and in eight controls (11%) at titres of 10–20, GMT 11 ($p < 0.01$). There were 32 patients (43%) with high

Table II. Bacterial species isolated from patients in five clinical groups

Bacterial species	Number of isolates from patients (n) in					
	group 1* (20)	grp 2 (29)	grp 3 (16)	grp 4 (4)	grp 5 (5)	all groups (74)
<i>Bacteroides</i> spp.						
<i>B. fragilis</i>	16	21	11	3	3	54
<i>B. ovatus</i>	3	6	5	0	1	15
<i>B. distasonis</i>	3	3	4	0	1	11
<i>B. thetaiotaomicron</i>	1	3	1	1	0	6
Other anaerobes						
<i>Peptococcus</i> spp.	1	0	1	0	0	2
Aerobes						
<i>Escherichia coli</i>	10	13	4	0	1	28
<i>Streptococcus</i> spp.	3	5	2	0	0	10
<i>Proteus</i> spp.	1	3	3	0	1	8
<i>Pseudomonas</i> spp.	4	1	0	0	2	7
<i>Klebsiella pneumoniae</i>	0	1	2	0	0	3
<i>Staphylococcus</i> spp.	0	1	1	0	0	2
All species	42	57	34	4	9	146

* Patient groups: 1, intra-abdominal sepsis; 2, post-operative wound infection; 3, skin abscess or gangrene; 4, septicaemia; 5, miscellaneous infections (see text).

Table III. Antibodies against the fragilis group of *Bacteroides* in sera of patients and of healthy controls

Patient group	Number of sera tested	Ig class†	Number of sera with bacteroides antibodies at a titre of*						
			10	20	40	80	160	320	Total (%)
Intra-abdominal sepsis	20	IgG	3	5	1	2	5	3	19 (95)
		IgM	3	5	1	2	2	0	13 (65)
Post-operative wound infection	29	IgG	2	3	4	8	1	5	23 (79)
		IgM	2	3	4	5	0	1	15 (52)
Skin abscess or gangrene	16	IgG	3	2	1	5	1	2	14 (88)
		IgM	3	2	1	1	0	0	7 (44)
Septicaemia	4	IgG	1	0	0	1	0	2	4 (100)
		IgM	1	0	0	1	0	1	3 (75)
Miscellaneous infections	5	IgG	0	0	2	2	0	1	5 (100)
		IgM	0	1	1	2	0	0	4 (80)
All patients	74	IgG	9	10	8	18	7	13	65 (88)
		IgM	9	11	7	11	2	2	42 (57)
Healthy controls	74	IgG	14	13	18	5	0	0	50 (68)
		IgM	7	1	0	0	0	0	8‡ (11)

*For each serum, the recorded titre represents triplicate immunofluorescence tests with a bacteroides suspension, the chosen serotype being that which gave the highest titre in a preliminary test of the serum with 26 different serotypes (see text).

†In none of the patients, and in only one of the controls, was the specific IgM titre higher than the specific IgG titre.

‡In these eight controls with specific IgM, the specific IgG titre was <80.

specific IgG titres as well as specific IgM, while none of the controls showed such reactions.

There was no correlation between antibody titre and the age of the subjects. However, the antibody response to infection was rapid and subsequently showed an early decline, especially of IgM. Of the 74 patients, 23 had been infected for 4–7 days, 36 for 8–10 days and 15 for more than 10 days. Antibodies were detected in 96% of patients in the first group (GMT 80), 86% in the second group (GMT 57), and 80% in the third (GMT 53). Specific IgM antibodies were found in 83% of patients in the first group, 53% in the second, but in only 27% in the third.

Range of fragilis serotypes reacting with sera of patients and controls

Each of 42 patients' sera (57%) reacted with five or more different serotypes, and 10 (14%) reacted with only one or two serotypes. On the other hand, none of the control sera reacted with five or more serotypes but 38 (51%) did so with only one or two serotypes (fig. 1).

The reactions of patients' and control sera with the various serotypes are illustrated in fig. 2. Several patients' sera reacted with each of the 26 serotypes

while the control sera reacted with only 17, mostly with one of the new local strains (type 21). Amongst the nine serotypes reacting only with patients' sera,

Fig. 1. Percentage of 74 patients' (●—●) and 74 control (●---●) sera reacting in immunofluorescence with one or more serotypes of the fragilis group of *Bacteroides*.

Fig. 2. Percentage of patients' (□) and control (■) sera reacting in immunofluorescence with each of 26 serotypes of the fragilis group of *Bacteroides*.

type 10 showed the highest frequency of reactions (42%), followed by types 3 (38%) and 4 (37%).

The proportions of patients' and control sera reacting with various combinations of serotypes are shown in fig. 3. These combinations are A (2 and 10), B (2, 3 and 10), C (2, 3, 4 and 10), D (2, 3, 4, 5 and 10), E (2, 3, 4, 5, 7 and 10), F (2, 3, 4, 5, 7, 8 and 10), G (2, 3, 4, 5, 7, 8, 10 and 11), H (2, 3, 4, 5, 7, 8, 10, 11 and 22), I (2, 3, 4, 5, 7, 8, 10, 11, 12 and 22), J (2, 3, 4, 5, 7, 8, 10, 11, 12, 22 and 24), and K (2, 3, 4, 5, 7, 8, 10, 11, 12, 21, 22 and 24). There was a gradual increase in the number of positive sera as the results with more serotypes were included. The combinations of A to F reacted only with patients' sera and F showed the highest proportion of reactions (70%). The twelve serotypes in combination K reacted with all positive patients' and control sera.

Discussion

Antibodies to the fragilis group of *Bacteroides* were studied in 74 patients with various infections caused by these bacteria and in 74 normal subjects, using suspensions of 26 different serotypes. Antibodies were detected in all nine patients with septicaemia, lung abscess, osteomyelitis, cholecys-

titis or otitis media, and in 95% of patients with intra-abdominal sepsis. However, fewer patients with wound or skin infections (79–88%) showed antibody responses. The proportion of patients with antibodies was higher than that reported by other workers, who used only one to three different strains of *B. fragilis* (Rissing *et al.*, 1979, Paavonen *et al.*, 1981). Because the fragilis group are antigenically heterogeneous (Elhag *et al.*, 1977), a wide variety of antigenic types is needed; and our findings suggest that the rate of detection of antibodies in patients' sera increases with the number of serotypes used for their detection. However, antibodies may also be detected in a large number of healthy persons, if a wide range of serotypes is used. Such antibodies were not detected in normal sera when antigens of only one or three strains of *B. fragilis* were used (Ross and Gilmore, 1978; Rissing *et al.*, 1979); but other workers have demonstrated them in normal sera (Quick *et al.*, 1972; Danielsson *et al.*, 1974; Hofstad, 1974).

We found that the titres of many patients were ≥ 80 , while those of controls were predominantly low. These findings are consistent with those of Beckmann *et al.* (1979), who demonstrated high titres in patients' sera. Similarly, we have found IgM antibodies more frequently in patients' sera and at significantly higher titres (GMT 36) than the

Fig. 3. Percentage of patients' (▨) and control (■) sera reacting in immunofluorescence with serotypes of the fragilis group of *Bacteroides*. Combinations A to K represent increasing numbers of serotypes, from two to twelve (see text), that gave positive results with a single serum. Up to F (seven selected serotypes), only patients' sera reacted; but an increasing proportion of control sera also were positive when the results with eight or more serotypes were included.

controls (GMT 11). It seems, therefore, that the presence of only IgG may not be diagnostic; but the combined presence of high antibody titres and specific IgM appears to be indicative of a current infection, as we found this combination in 43% of patients but in no controls.

Most positive patients' sera reacted with several serotypes of the fragilis group, while most of the controls reacted with only one or two serotypes. This response of patients was probably due to the wide variety of intestinal and other bacteria found in such infections. For example, Leigh *et al.* (1974) and Lari *et al.* (1976) isolated several types of micro-organisms from the peritoneal cavities of patients with acute appendicitis. The high reactivity of our control sera with serotype 21, isolated in Kuwait, confirms an earlier report suggesting geographical variation in distribution (Elhag and Tabaqchali, 1978*b*). Moreover, nine serotypes reacted only with patients' sera while the other 17 reacted with patients' and control sera. Similar findings were reported by Beckmann *et al.* (1979); also, Elhag and Tabaqchali (1978*b*) found that some serotypes

were associated with infections more frequently than others. Further studies may show whether there are virulent and avirulent serotypes, and perhaps reveal certain antigenic determinants associated with virulence.

Our study showed that all positive sera, of patients and controls, could be detected by the use of twelve serotypes. An optimum combination (F, fig. 3) of seven serotypes reacted with 52 patients' sera (70%) but did not react with any of the control sera. The initial use of a wide range of serotypes was therefore necessary to select appropriate strains for use in serodiagnosis. In view of the antigenic diversity of the fragilis group and their geographic variations (Elhag and Tabaqchali, 1978*a* and *b*), however, this set of strains may not be suitable for use in other areas, where a similar preliminary screening may be necessary. Nevertheless, it may be possible to devise a system that could be used widely to differentiate antibodies in a high proportion of patients from those in normal people. Sera would need to be tested against at least ten serologically distinct strains of the fragilis group,

including some local strains, and only titres of ≥ 80 would be considered significant. Reactivity with several strains, and the detection of specific IgM, may be suggestive of a current infection.

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