The *Bacteroides fragilis* Group: The Next Superbugs in Waiting?

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ABSTRACT

Treatment of infections caused by anaerobic bacteria remains largely empiric, despite recent knowledge and reduced antimicrobial susceptibility to commonly used antibiotics. The objective of this study was to obtain current surveillance data for the *Bacteroides fragilis* group in Australia. We obtained 255 faecal isolates, 100 derived from the community, 98 derived from hospital high-dependency unit samples, and 57 derived from blood cultures and other sterile sites. Susceptibility testing was performed using both the BSAC disc diffusion method and the CLSI agar dilution method. Molecular markers of resistance (*erm* genes, *nim* genes, *gyrA* mutations) were tested to determine the presence of resistance to clindamycin, metronidazole and fluoroquinolones respectively, and to assess their potential diagnostic use. By comparison with previous Australian studies last conducted in 1992, overall resistance of the *Bacteroides fragilis* group in Australia has increased from 11% to 44% for clindamycin, 1% to 3% for the carbapenems, and from 1% to 9% for ticarcillin/clavulanic acid. No resistance to metronidazole was observed. At species level, *B. fragilis* isolates (n=82) showed low rates of resistance, whilst *B. distasonis* (n=47) displayed high resistance to clindamycin (83%) and ticarcillin/clavulanic acid (21%), *B. uniformis* (n=32) showed high resistance to moxifloxacin (53%) and meropenem (9%), and *B. thetaiotaomicron* (n=31) presented high resistance to clindamycin (58%) and ticarcillin/clavulanic acid (19%). Comparison of the two susceptibility methods used demonstrated a lack of correlation. Examples of all molecular markers of resistance tested were found within the study population, with 66/255 strains harbouring at least one *erm* gene, 2/255 strains harbouring *nim* genes, and 2/6 selected strains carrying a *gyrA* mutation.

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INTRODUCTION

The culture, isolation and identification of anaerobic bacteria has long been considered more time-consuming, labour-intensive and expensive than that of aerobic bacteria, and is therefore considered to be too demanding for many diagnostic microbiology laboratories (7). As a result, the culture and isolation of anaerobic bacteria is often either minimal or omitted, lending a bias to the identification of aerobes or facultative anaerobes as the infective agent. However, when all the appropriate investigations are undertaken, anaerobes have been found to comprise a significant proportion of most infections. Overall, anaerobic bacteria are implicated in up to 20% of bacteraemias, 70% of bite wounds, 90% of community-acquired aspiration pneumonia, and 73% of appendicitis cases (18). Anaerobes are also known to be associated with many diverse infections including intra-abdominal infection, brain abscess, otogenic meningitis, chronic sinusitis, breast abscess, vaginitis, post-partum sepsis, endometritis, gas gangrene, clostridial myonecrosis, perirectal abscess and diabetic foot infections. The members of the *Bacteroides fragilis* group have been widely recognised as the most common anaerobic bacteria encountered in clinical specimens (13, 22).

With the increasing recognition of the importance of anaerobic bacteria as pathogens, as well as growing suspicion and interest regarding changing susceptibility profiles, a large number of laboratories world-wide have undertaken investigations into their local anaerobic population (3, 4, 5, 6, 15, 25, 28, 34, 35, 36, 38, 39, 41, 42, 47, 50, 60, 63). In Europe, North America and Japan, this has included the formation of large study groups with a view to conducting regular surveillance studies (22, 30, 31, 32, 33, 40, 52, 53, 54, 56, 57, 58). In contrast, there is little recent Australian data on the antibiotic susceptibility patterns of the local anaerobic population, with the last such study having been conducted in 1992 (9). This has meant that the selection of antibiotic treatment is empiric, based on identification of the suspected pathogen and the anticipated susceptibility pattern. The antibiotics most commonly used to treat infections caused by the members of the *Bacteroides fragilis* group are currently metronidazole, clindamycin, the carbapenems and the β-lactam/β-lactamase-inhibitor combinations.

Recent anaerobe research has also focused on investigation of molecular markers of anaerobic resistance as potential screening methods. The implicated molecular marker has been identified for the main antimicrobials used in the treatment of infections caused by members of the *Bacteroides fragilis* group. Research to date has been primarily focused on the *erm* genes associated with clindamycin resistance (21, 25, 41, 55), the *nim* genes associated with resistance to metronidazole (14, 16, 20, 24, 29, 50, 62), and mutations in the quinolone-resistance-determining region (QRDR) of the *gyrA* gene that has been related to fluoroquinolone resistance (37, 43, 45).

This study presents the first Australian epidemiological data into the susceptibility patterns of the *Bacteroides fragilis* group for over 14 years. In addition, two of the most commonly used methods of susceptibility testing were compared for reproducibility and correlation of results. Finally, we also present the first Australian data looking at the prevalence of some of the more common molecular markers of resistance found in *Bacteroides* species: *erm* genes (*ermF, ermG and ermB*), *nim* genes and *gyrA* mutations.

MATERIALS AND METHODS

Bacterial Strains. Faecal samples were collected from 125 community and 256 hospital high dependency unit (HDU) patients during the six-month period between February and July 2004. An HDU patient was considered to be one currently admitted to an intensive care unit (ICU), high dependency unit (HDU) or burns unit who had been hospitalized for no less than 48 hours. Following collection, each sample was plated directly onto selective Bacteroides Bile Esculin (BBE) agar (26). Isolates were identified to genus level by anaerobic growth, presentation of aesculin hydrolysis and Gram morphology. This resulted in 100 community and 98 HDU isolates. Identification to species level was conducted using RapID™ ANA II test panels (Remel INC, USA). An additional clinical group of 57 previously identified isolates from blood culture and other sterile site infections was also included. Control strains used in susceptibility testing were *Bacteroides fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29741. The positive control strain used in screening for *nim* genes was strain BF8 (19). The *erm* gene screening positive controls were strain BFV479 containing the pBF4 plasmid known to harbour the *ermF* gene (64) and a wild-type *Bacteroides fragilis* determined during the course of this study to harbour both the *ermB* and *ermG* genes.

Antimicrobial Agents. The following antimicrobial agents were investigated in this study: penicillin G and metronidazole (Sigma-Aldrich); moxifloxacin (Bayer Pharmaceuticals); meropenem (Astra Zeneca); ticarcillin and clavulanic acid (Glaxo-Smith Kline); and clindamycin (Pfizer). All powders were stored at –20°C prior to use.

Susceptibility Testing. MIC determination was conducted on all 259 isolates using the CLSI agar dilution method. The antimicrobial agents were suspended, diluted and incorporated into brucella agar supplemented with 5% laked sheep blood according to the CLSI published standard (10). The inoculum of each isolate was prepared by suspending colonies from a 48-hour-old anaerobic blood agar plate culture directly into 3mL of brucella broth to a density equivalent to that of a 0.5 McFarland turbidity standard. A Steers replicator was used to inoculate the plates, delivering approximately 10^5 CFU per spot. Plates were incubated anaerobically for 48 hours at 35°C in a Modular Atmosphere Controlled System anaerobic chamber (Don Whitley Scientific, UK). The MIC was defined as the lowest concentration of antibiotic that caused a marked reduction in the appearance of growth compared to the control plate.

Susceptibility testing was also performed on the community and HDU isolates using the disc diffusion method outlined by the British Society for Antimicrobial Chemotherapy (BSAC) (8). The inoculum of each isolate was prepared by suspending colonies from a 48-hour-old anaerobic blood agar plate culture directly into 5mL of sterile distilled water to a density equivalent to that of a 0.5 McFarland turbidity standard. Each inoculum was swabbed evenly over the surface of a brucella agar plate supplemented with 5% horse blood and 1mg/L vitamin K. The following antimicrobial susceptibility testing discs (Oxoid, Australia) were placed evenly over the surface of the inoculated plate: clindamycin (2µg), meropenem (5µg), metronidazole (5µg), moxifloxacin (5µg), penicillin G (2units), and ticarcillin/calvulanic acid (7:5.1; 85µg). Plates were incubated anaerobically for 48 hours at 35°C.

DNA Extraction. The target DNA was extracted using a previously described rapid extraction procedure (61). Bacterial colonies were picked from agar and suspended in 100µL of 1x TE buffer, pH 7.5. The suspension was heated for 10 minutes at 95°C and centrifuged at 13,000rpm for 5 minutes. 90µL of the DNA-containing supernatant was collected and stored at –20°C until use.

***ermF* gene amplification.** PCR amplification was performed using the previously described primers ERMF1 (5'-CGG GTC AGC ACT TTA CTA TTG-3') and ERMF2 (5'-GGA CCT ACC TCA TAG ACA AG-3') (46) synthesised by Invitrogen, Australia. Each assay was carried out in a final volume of 25µL containing 12.5µL BioMix ready-to-go reaction mixture (Bioline, London, UK), each primer at a concentration of 25pM, 5µL of template DNA and sterile distilled water to volume. An initial denaturation step of 94°C for 10 minutes was followed by 32 cycles of amplification consisting of denaturation at 94°C for 30 seconds, annealing at 62°C for 1 minute and extension at 72°C for 1 minute, and a final extension step of 72°C for 10 minutes. 10µL of each PCR product was analysed by electrophoresis in TBE buffer on a 1.5% (w/v) agarose gel containing ethidium bromide. Template DNA of strain BFV479 was included in each PCR run as a positive control and DNA fragments of approximately 466-bp were considered indicative of the presence of an *ermF* gene.

***ermB* and *ermG* gene amplification.** Screening of isolates for the *ermB* and *ermG* genes was conducted as a multiplex PCR reaction. PCR amplification of the *ermB* gene was carried out using primers ERMB1 (5'-GAA AAG GTA CTC AAC CAA ATA-3') and ERMB2 (5'-AGT AAC GGT ACT TAA ATT GTT TAC-3'). Amplification of the *ermG* gene was carried out using primers ERMGfor1 (5'-ACA TTT CCT AGC CAC AAT C-3') and ERMGrev1 (5'-CGC TAT GTT TAA CAA GC-3'). Both primer pairs were previously described (55) and were synthesised by Invitrogen, Australia. Each assay was carried out in a final volume of 25µL containing 12.5µL BioMix ready-to-go reaction mixture (Bioline, London, UK), each primer at a concentration of 25pM, 5µL of template DNA and sterile distilled water to volume. PCR conditions were as previously described (55). An initial denaturation step of 95°C for 5 minutes was followed by 30 cycles of amplification consisting of denaturation at 95°C for 1 minute, annealing at 52°C for 1 minute and extension at 72°C for 2 minutes, and a final extension step of 72°C for 5 minutes. 10µL of each PCR product was analysed by electrophoresis in TBE buffer on a 1.5% (w/v) agarose gel containing ethidium bromide. Template DNA from a wild type *B. fragilis* strain found to possess both the *ermB* and *ermG* genes was included in each run as a positive control. DNA fragments of approximately 639-bp were considered to be indicative of the *ermB* gene, whilst DNA fragments of approximately 442-bp were considered to be indicative of the *ermG* gene.

***nim* gene amplification.** Previously described primers NIM-3 (5'-ATG TTC AGA GAA ATG CGG CGT AAG CG-3') and NIM-5 (5'-GCT TCC TTG CCT GTC ATG TGC TC-3') were used for the universal PCR amplification of the six members of the *nim* gene family (61). Primers were synthesised by Invitrogen, Australia. Each PCR assay was carried out in a final volume of 25µL containing 12.5µL of BioMix ready-to-go reaction mixture (Bioline, London, UK), each primer at a concentration of 25pM, 5µL of template DNA and sterile distilled water to volume. PCR conditions were as previously described (61). An initial denaturation step of 94°C for 10 minutes

was followed by 32 cycles of amplification consisting of denaturation at 94°C for 30 seconds, annealing at 62°C for 1 minute and extension at 72°C for 1 minute, and a final extension step of 72°C for 10 minutes. 10µL of each PCR product was analysed by electrophoresis in TBE buffer on a 1.3% (w/v) agarose gel containing ethidium bromide. Template DNA from strain BF8 was included in each run as a positive control and DNA fragments of approximately 458-bp were considered to be indicative of a *nim* gene.

***gyrA* amplification.** PCR amplification of the *gyrA* QRDR of selected strains was carried out with previously described primers Pr-BFGBA03 (5'-ATG CTT GAA CAA GAC AGA ATT ATA AAG-3') and Pr-BFGA02 (5'-GAC TGT CGC CGT CTA CAG AAC CG-3') (37) synthesised by Invitrogen, Australia. Each assay was carried out in a final volume of 100µL containing 50µL of BioMix ready-to-go reaction mixture (Bioline, London, UK), each primer to a concentration of 25pM, 5µL of template DNA and sterile distilled water to volume. PCR conditions were as previously described (37): 25 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 5 minutes, and extension at 72°C for 1 minute. An initial denaturation step of 94°C for 5 minutes and a final extension step of 72°C for 5 minutes were added to the protocol. Free primers and nucleotides were removed from the PCR products by the JETQUICK PCR Purification Spin Kit (Genomed, Germany). 10µL of purified PCR product was analysed by electrophoresis in TBE buffer on a 1% agarose gel containing ethidium bromide. DNA fragments of approximately 282 to 296-bp were obtained as expected. PCR products were sent to the Sydney University Prince Alfred Macromolecular Analysis Centre (Sydney, Australia; http://www.supamac.com) for genetic sequencing using ABI PRISM® dye terminator cycle sequencing.

RESULTS

Agar Dilution MIC Results. All isolates were individually tested by the CLSI agar dilution method (10) to provide a MIC value for each antibiotic. The percentage of strains inhibited at each concentration, range of MIC values, MIC_{50}, MIC_{90}, and overall percentage of isolates resistant for each antibiotic and each study group are presented in Table 1.

The MIC results for penicillin revealed 100% resistance across all three study groups. Moderately high levels of resistance were found to both clindamycin and moxifloxacin, with percentages ranging between 35-53% and 18-29% respectively. Results for these antibiotics displayed a spread of isolates across the entire range of MIC values tested. Overall resistance to meropenem, metronidazole and ticarcillin/clavulanic acid were each below 10% (see Table 1).

There was significantly elevated resistance to ticarcillin/clavulanic acid seen in strains isolated from HDU patients as compared to the community and sterile site groups ($p<0.01$ in both cases). The HDU group also showed a significant increase in clindamycin resistance compared to the sterile site sample group ($p<0.05$). Levels of resistance presented by the three groups for penicillin, metronidazole, moxifloxacin and meropenem showed no significant difference ($p>0.1$ in all cases).

Disk Diffusion Susceptibility Results. The diameter of the zone of inhibition of each isolate was measured for each antibiotic. BSAC (8) has determined a definite breakpoint for penicillin,

clindamycin and metronidazole, with zone sizes of ≤17mm, ≤9mm, and ≤17mm respectively, considered indicative of resistance. There are currently no inhibition zone sizes established for moxifloxacin, ticarcillin/clavulanic acid, or the 5μg meropenem antibiotic disc as used in this study. The results are presented in Table 2.

The isolates taken from HDU patients presented higher rates of resistance than the community isolates. A statistically significant difference in resistance rates between the two groups occurred with metronidazole ($p<0.05$).

Methodology Comparison. The results obtained by both the disc diffusion and agar dilution were compared to determine the level of correlation between the methods. Whilst the two methods were comparable for penicillin and clindamycin, a statistically significant difference was determined for metronidazole ($p<0.05$).

A designation of errors was also used to determine compatibility between disc diffusion and MIC breakpoint methods (11): a very major error (VME) indicates that the isolates were susceptible by disk diffusion and resistant by the reference method; a major error (ME) indicates that the isolates were resistant by disk diffusion and susceptible by the reference method; and a minor error (MiE) indicates that the isolates were intermediate by MIC method and resistant or susceptible by disc diffusion. Applying this, the present study was found to have 20 very major errors (2.6%), 9 major errors (1.2%) and 33 minor errors (4.3%).

Resistance By Individual Species. The resistance profiles obtained by the different species of the *Bacteroides fragilis* group were analysed. The results obtained by the agar dilution were used to classify isolates as resistant to allow for literature comparison. The rate of resistance for each *Bacteroides* species in each group for each antimicrobial agent is presented in Table 3.

Bacteroides fragilis was observed to have lower levels of resistance to all antibiotics than other species. *Bacteroides distasonis* isolates showed a higher level of resistance to clindamycin across all three sample groups compared to other species. *Bacteroides uniformis* isolates displayed a high level of resistance to most of the antibiotics, in particular to moxifloxacin. A similar profile of resistance was shown by *Bacteroides thetaiotaomicron*, with resistance levels comparatively high for all antibiotics except metronidazole.

Differing resistance rates were also observed within species depending on the source of the isolates. Of the *Bacteroides fragilis* isolates, those from the community group showed a lower rate of resistance to clindamycin than those isolated from the hospital group ($p<0.05$), while the hospital group isolates demonstrated a lower level of resistance to moxifloxacin than was found in the clinical group ($p<0.05$). Within the *Bacteroides distasonis* species, the level of resistance to ticarcillin/clavulanic acid displayed by the hospital group was higher than the community group isolates ($p<0.02$).

Molecular Markers of Resistance

***erm* Gene Analysis**. All isolates were tested for the presence of the three *erm* genes known to be associated with *Bacteroides* species. Results were compared to the MIC value obtained by the agar dilution method to establish any correlation between genotype and phenotype. This is presented in Table 4. Isolates with a resistant MIC value showed a higher prevalence of *erm* genes (54/115 = 47%) than those isolates with a sensitive MIC value (12/140 = 9%). In isolates determined resistant by agar dilution MIC value (n=115), the *ermF* gene was the most common gene present, either alone (38/54) or in combination (10/54). In isolates determined sensitive by agar dilution MIC testing (n=140), *ermB* was the most commonly found *erm* gene (7/12). The *ermB*/*ermG* combination was the only combination not associated with a resistant phenotype.

***nim* Gene Analysis.** Isolates were tested for the presence of *nim* genes using a universal primer enabling detection of all six known genes in the family. Products of approximately 458-bp were considered to be indicative of the presence of a *nim* gene. Based on this classification, two isolates were found to be *nim* gene positive. On comparison with the results obtained by agar dilution, it is noted that these isolates demonstrated an elevated metronidazole MIC value of 4 and 8µg/mL respectively.

***gyrA* Gene Analysis.** The quinolone-resistance-determining region of the *gyrA* gene of six isolates was sequenced to determine the presence of mutations known to convey resistance to fluoroquinolones (37). These six isolates were selected to include two with high level resistance (MIC >32µg/mL), two isolates considered intermediate range (MIC = 2µg/mL), and two susceptible strains (MIC <1µg/mL). Genetic sequences obtained were compared to their respective ATCC strain GenBank sequence to determine mutations. Both highly resistant strains showed a C→T mutation in amino acid 82, which would result in a Ser→Phe amino acid change. All other isolates showed complete homology with the reference sequence.

DISCUSSION

One of the main issues of contention in the susceptibility testing of anaerobes has long been the lack of standardisation of testing methods. Whilst individual organisations (CLSI, BSAC, CDS) have attempted to overcome this through the development of their own standard methods of testing, there have been very few studies into the correlation of results obtained by methods outlined by the different organisations. The results obtained by this study demonstrate the potential for discrepancies to arise between the levels of resistance determined by each of the two methods employed. In this study, the disc diffusion method presented a 5% resistance level to metronidazole where no resistance was detected by the agar dilution method. This suggests that the levels of resistance obtained in a sample study are highly dependent upon the method used. As further evidence, over 10% of results obtained by the two methods used in this study differed in their classification of the isolate with 20 very major errors (2.6%), 9 major errors (1.2%) and 33 minor errors (4.3%). Given that the CLSI recommends major and minor error rates of <1.5% and <3% respectively (10), and the BSAC recommends major and minor error rates of <1% and <5% respectively (8), the comparison of these methodologies resulted in too many errors for them to be considered equivalent. This poor correlation between disc diffusion and

MIC results has also been observed in other comparative studies (11), and illustrates why the standardisation of susceptibility testing methodologies for anaerobes remains a highly contentious issue.

Regardless of any discrepancies between the different methodologies, the results of this study clearly demonstrate there has been a significant increase in antimicrobial resistance in *Bacteroides fragilis* group isolates from Australia over the past 14 years. The levels of resistance to each antimicrobial under investigation parallel, and in some cases exceed, those reported in various regions worldwide.

The results obtained for penicillin show essentially 100% of isolates lie within the highly resistant range. This can be accounted for by the fact that up to 95% of *Bacteroides fragilis* group isolates have been reported to express a β-lactamase (1).

Since its production in 1966, clindamycin has been considered a mainstay in the treatment of anaerobic infections. However, international studies report the levels of resistance to this antibiotic have increased alarmingly in recent times. The results obtained by this study indicate this also appears to be the case in Australia, with an increase in clindamycin resistance to over 30% - an increase which parallels and in some cases exceeds that reported by world-wide studies. The European Society for Clinical Microbiology and Infectious Diseases (ESCMID) found resistance to clindamycin to have almost doubled from 9% in 1992 to 15% in 2003 (22). Results found by other investigators include 32% resistance shown in a French multicentre survey (3) and 49% resistance reported from Spain (38). Similar results have been reported throughout North America, where the regular national multicentre survey (56, 57, 58) has shown clindamycin resistance increasing from 15% in 1994 to 23% in 2000. As a consequence of these results, many areas no longer consider clindamycin as a first-line choice for therapy of anaerobic infections, particularly those caused by the *Bacteroides fragilis* group.

Historically, the fluoroquinolones were not considered to be active agents against anaerobic bacteria. This changed with the advent of some of the newer fluoroquinolones such as trovafloxacin and moxifloxacin, which showed increased anaerobic activity. However, a major issue of concern in the use of fluoroquinolones is that of increasing resistance in the face of increased prescription. As a more recent addition, moxifloxacin has only recently been included in many of the antimicrobial susceptibility surveys. The resistance rate of greater than 15% as determined by this study is well supported by global literature. The 2003 ESCMID study (22) demonstrated resistance to moxifloxacin in 9% of isolates, whilst Spanish data (39) documented a resistance increase from 0% in 1998 to 12% in 2001, and data from the North American national survey (56, 57, 58) reported continuing increases in resistance from 7% in 1996 to 17% in 2000. Similarly, a surveillance study of moxifloxacin resistance in the United States between 1998 and 2001 (17) showed a resistance rate rise from 30% in 1998 to 42% in 2001.

Recently a number of sporadic cases of true metronidazole resistance in the *Bacteroides fragilis* group have been reported in both Europe and North America, raising concerns of an overall decrease in susceptibility. Whilst no true resistance was found to metronidazole in this study

(MIC ≥32µg/mL), two isolates were identified as having reduced susceptibility (4 and 8µg/mL), with both isolates testing positive for the presence of a *nim* gene. These results correlate well with the findings of other global studies. The ESCMID study group (22) reported 18 isolates with reduced susceptibility to metronidazole, whilst another European multicentre survey reported up to 4% of isolates demonstrating reduced susceptibility (3). This world-wide trend is also supported by recent figures from South America, where a Brazilian study found 16% of their isolates with a reduced susceptibility to metronidazole (41). In addition to these increased numbers of isolates demonstrating reduced susceptibility to metronidazole, resistant isolates are also emerging in other parts of the world, including Canada (23), Sweden (14), United Kingdom (16) and the United States (50).

From an Australian clinical viewpoint, a major point of concern surrounds the results obtained for meropenem and ticarcillin/clavulanic acid, as these are two common empiric choices for anaerobic infections in hospital units. In this study, it was shown that MIC values indicating resistance to meropenem could be found in between 1 and 5% of isolates, whilst resistance to ticarcillin/clavulanic acid varied between 5 to 17%, depending on the particular source population of isolates. Although the majority of international studies still place these antimicrobials at >98% and >95% effective against the *Bacteroides fragilis* group respectively (22, 58), there are growing concerns regarding potential for resistance rates to increase. In terms of carbapenem resistance, the metallo-β-lactamase-producing *cfiA* gene has been reported to be present in up to 9% of the *Bacteroides fragilis* group (2, 12, 59). As the use of carbapenem antibiotics increases due to raised levels of resistance to most other antibiotics, it is possible that *Bacteroides fragilis* is subjected to sufficient selective pressure to convert a larger proportion to gene expression - an event which would have significant clinical ramifications. With respect to ticarcillin/clavulanic acid, increasing resistance poses the question of extended-spectrum β-lactamase production, particularly given that there have been recent reports of *Bacteroides* strains testing positive for the production of ESBL-like enzymes (48, 49).

In addition to the disturbing increase in resistance, this study also highlights the importance of accurate identification to species level in order to formulate the most effective therapeutic regimens. Similar to what has been demonstrated in previous studies (1, 22, 58), *Bacteroides fragilis* and *Bacteroides distasonis* isolates were relatively susceptible to the antibiotics tested, whilst *Bacteroides uniformis* and *Bacteroides thetaiotaomicron* isolates showed higher rates of resistance to the antimicrobials investigated, particularly against moxifloxacin, meropenem and ticarcillin/clavulanic acid. Investigation of this study into the resistance profiles obtained by the individual species of the group has emphasised the importance of accurate species identification in the diagnostic and clinical settings. Given that the different members of the *Bacteroides fragilis* group show significantly different profiles of resistance to the antimicrobials tested, the results of this study would suggest the increasing importance of species identification in order to optimise the treatment choice given to patients. The importance of species identification in selecting therapies has long been recognised in the case of enterococci, where *Enterococcus faecium* is known to be much more resistant to many antibiotics than *Enterococcus faecalis*. The list may well be expanded soon to include the *Bacteroides fragilis* group.

A final objective of this research was to assess the viability of molecular testing as a diagnostic tool for the resistance screening of anaerobic bacteria. The findings of this study show it to be limited at this point in time, as in the genetic markers investigated, some lack of correlation with MIC resistance was shown.

Approximately 25% of isolates in this study were found to harbour an *erm* gene. This result is almost identical to that achieved by a 2001 study (55), where *erm* genes were detected in 23% of isolates. However, the most interesting result to note is that a number of isolates determined to be sensitive to clindamycin by MIC value were found to carry one or more *erm* genes. This suggests that *erm* gene activity may be susceptible to selective pressure. Indeed, it has been shown that resistance to the macrolide-lincosamide family of antibiotics is inducible (44) and this may be caused by the presence of previously silent *erm* genes. Consequently, the presence of *erm* genes in MIC-sensitive strains largely invalidates the use of e*rm* gene screening for the accurate detection of clindamycin resistant *Bacteroides* isolates.

The investigation into the presence of *nim* genes revealed two isolates possessing one of the family of six *nim* genes, although the use of a universal primer pair prevented the identification of which specific *nim* gene(s) without the use of sequencing. The result of note is the correlation between the presence of a *nim* gene and the MIC value obtained. In this study, it was shown that a *nim* gene correlated 100% to an MIC value of $\geq$4µg/mL, which although still susceptible by established MIC breakpoint, is considered to exhibit reduced susceptibility. Whilst some studies such as support this finding of *nim* genes in isolates considered to have an increased MIC value (24, 62), there are also a number of studies that have found results contradictory to this. One such study tested 16 isolates with MIC values of 2-4µg/mL and found none possessing a *nim* gene (41), whilst other studies have found *nim* genes to be present in isolates with MIC values as low as 1.5µg/mL (14, 16, 20, 29). The presence of these seemingly unexpressed genes in isolates considered to be susceptible by most published breakpoints is of interest. There is a concern that selective pressures brought about by overuse of this antibiotic could lead to the irreversible induction and expression of this gene. Indeed, as with the *erm* genes conferring clindamycin resistance, it has recently been shown that resistance to metronidazole can be induced (16, 27, 51).

The third genetic marker of resistance assessed in this study was the quinolone resistance-determining region (QRDR) of the *gyrA* gene. Mutations in this region have previously been proven to be associated with resistance to the fluoroquinolones, most commonly a serine-to-phenylalanine amino acid change at position 82 (37). Two highly resistant, two intermediate and two highly sensitive isolates that showed no resistance to any other antibiotics tested except penicillin were examined for the presence of this and other mutations that may have occurred. The two sensitive and the two intermediate strains showed 100% homology to the reference sequences. In contrast, the resistant strains displayed the expected mutation in amino acid 82. Although this study gave a 100% correlation between fluoroquinolone resistance and the *gyrA* mutation, the small sample number prevents this from being a definitive confirmation of this occurrence. Other studies have shown that resistant strains can have alternate or second-step

mutations in the *gyrB* or *parC* genes, and that resistant strains can be observed to possess no mutations at all (43, 45). Further characterisation of these genes, as well as known efflux pumps, would be required before any molecular screening method could be considered conclusive.

In conclusion, there has been a clear increase in antimicrobial resistance of the *Bacteroides* family in Australia when compared with data collected in 1992. The results of this study also demonstrate varying resistance profiles for individual species within the *Bacteroides* family, thus emphasising the importance of accurate identification to species level in order to determine the most appropriate course of treatment. Furthermore, evidence of both silent and expressed molecular markers of anaerobe resistance was found within all three isolate populations indicating that this group of organisms should at the very least have periodic surveillance to determine institution resistance trends.

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TABLE 1. Minimum inhibitory concentration data as determined by CLSI agar dilution

Antibiotic Agent	Group[a]	No. of isolates	MIC Value (µg/mL)[b]								Range	MIC_{50}	MIC_{90}	%R[c]
CLN	Comm	100	19 (19)	10 (29)	11 (40)	17 (57)	15 (72)	2 (74)	1 (75)	25 (100)	<1 - >32	4	>32	43
	Hosp	98	6 (6)	12 (18)	12 (31)	16 (47)	11 (58)	6 (64)	5 (69)	30 (100)	<1 - >32	8	>32	53
	Clin	57	5 (9)	13 (32)	12 (53)	7 (65)	5 (74)	1 (75)	0 (75)	14 (100)	<1 - >32	2	>32	35
	All	255	30 (12)	35 (25)	35 (39)	40 (55)	31 (67)	9 (71)	6 (73)	69 (100)	<1 - >32	4	>32	44
			<1	1	2	4	8	16	32	>32				
MER	Comm	100	88 (88)	6 (94)	1 (95)	3 (98)	1 (99)	1 (100)	0 (100)	0 (100)	<1 – 16	<1	1	1
	Hosp	98	66 (67)	17 (85)	7 (92)	2 (94)	2 (96)	1 (97)	0 (97)	3 (100)	<1 - >32	<1	2	4
	Clin	57	35 (61)	9 (77)	0 (77)	6 (88)	4 (95)	2 (98)	1 (100)	0 (100)	<1 – 32	<1	8	5
	All	255	189 (74)	32 (87)	8 (90)	11 (94)	7 (97)	4 (98)	1 (99)	3 (100)	<1 - >32	<1	2	3
			<1	1	2	4	8	16	32	>32				
MTZ	Comm	100	55 (55)	37 (92)	7 (99)	1 (100)	0 (100)	0 (100)	0 (100)	0 (100)	<1 – 4	<1	1	0
	Hosp	98	57 (58)	36 (95)	5 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	<1 – 2	<1	1	0
	Clin	57	25 (44)	21 (81)	10 (98)	0 (98)	1 (100)	0 (100)	0 (100)	0 (100)	<1 – 8	1	2	0
	All	255	137 (54)	94 (91)	22 (99)	1 (99)	1 (100	0 (100)	0 (100)	0 (100)	<1 – 8	<1	1	0
			<1	1	2	4	8	16	32	>32				
MOX	Comm	100	52 (52)	7 (59)	12 (71)	12 (83)	8 (91)	5 (96)	4 (100)	0 (100)	<1 - >32	<1	8	29
	Hosp	98	39 (40)	18 (58)	23 (82)	4 (86)	4 (90)	4 (94)	3 (97)	3 (100)	<1 - >32	1	8	18
	Clin	57	25 (44)	7 (56)	11 (74)	7 (88)	1 (89)	0 (89)	5 (98)	1 (100)	<1 - >32	1	16	25
	All	255	116 (45)	32 (58)	46 (76)	23 (85)	13 (90)	9 (94)	12 (98)	4 (100)	<1 - >32	1	8	24
			<0.5	0.5	1	2	4	8	16	>16				
PEN	Comm	100	0 (0)	0 (0)	0 (0)	2 (2)	4 (6)	18 (24)	25 (49)	51 (100)	2 - >16	16	>16	100
	Hosp	98	0 (0)	0 (0)	1 (1)	0 (1)	5 (6)	9 (15)	12 (28)	71 (100)	1 - >16	>16	>16	99
	Clin	57	0 (0)	0 (0)	0 (0)	3 (5)	7 (18)	3 (23)	11 (42)	33 (100)	2 - >16	>16	>16	100
	All	255	0 (0)	0 (0)	1 (1)	5 (2)	16 (9)	30 (20)	48 (39)	155 (100)	1 - >16	>16	>16	100
			<8/2	8/2	16/2	32/2	64/2	128/2	256/2	>256/2				
TIM	Comm	100	62 (62)	14 (76)	10 (86)	6 (92)	2 (94)	1 (95)	3 (98)	2 (100)	<8/2 - >256/2	<8/2	32/2	6
	Hosp	98	42 (43)	17 (60)	11 (71)	3 (74)	7 (82)	4 (86)	6 (92)	8 (100)	<8/2 - >256/2	8/2	256/2	18
	Clin	57	32 (56)	13 (79)	2 (82)	3 (88)	5 (96)	2 (100)	0 (100)	0 (100)	<8/2 – 128/2	<8/2	64/2	4
	All	255	136 (53)	44 (71)	23 (80)	12 (84)	14 (90)	7 (93)	9 (96)	10 (100)	<8/2 - >256/2	<8/2	64/2	9

[a] Denotes isolate study groups: Comm = community isolate group; Hosp = hospital high-dependency unit isolate group; Clin = clinically significant isolate group; All = all isolates, irrespective of source

[b] MIC values determined by CLSI agar dilution method (10)

[c] The following MIC values (ug/mL) were used as resistance breakpoints as recommended by the CLSI (10): penicillin G, ≥2; clindamycin, ≥8; metronidazole, ≥32; moxifloxacin, ≥4; meropenem, ≥16; and ticarcillin/clavulanic acid, ≥128/2.

TABLE 2. Comparison of resistance obtained for the three study groups by methodology, and errors determined

Antimicrobial Agent	Group[a]	No. of isolates	BSAC[b]		CLSI[c]		Errors[d]		
			%R	%S	%R	%S	VME	ME	MiE
Penicillin G	Comm	100	100	0	100	0	0	0	0
	Hosp	98	100	0	99	1	0	1	0
	Clin[e]	57	-	-	100	0	-	-	-
Clindamycin	Comm	100	39	61	43	57	10	2	17
	Hosp	98	50	50	53	47	10	1	16
	Clin[e]	57	-	-	35	65	-	-	-
Metronidazole	Comm	100	0	100	0	100	0	0	0
	Hosp	98	5	95	0	100	0	5	0
	Clin[e]	57	-	-	0	100	-	-	-
Moxifloxacin	Comm	100	-	-	29	71	-	-	-
	Hosp	98	-	-	18	82	-	-	-
	Clin[e]	57	-	-	25	75	-	-	-
Meropenem	Comm	100	-	-	1	99	-	-	-
	Hosp	98	-	-	4	96	-	-	-
	Clin[e]	57	-	-	5	95	-	-	-
Ticarcillin/Clavulanic Acid	Comm	100	-	-	6	94	-	-	-
	Hosp	98	-	-	18	82	-	-	-
	Clin[e]	57	-	-	4	96	-	-	-

[a] Denotes isolate study groups: Comm = community isolate group; Hosp = hospital high-dependency unit isolate group; Clin = clinically significant isolate group

[b] BSAC (8) has no designated zone diameter breakpoint for the moxifloxacin, meropenem or ticarcillin/clavulanic acid antibiotic discs used. Thus resistance and susceptibility rates could not be determined, nor could errors.

[c] The following MIC values (ug/mL) were used as resistance breakpoints as recommended by the CLSI (10): penicillin G, $\geq$2; clindamycin, $\geq$8; metronidazole, $\geq$32; moxifloxacin, $\geq$4; meropenem, $\geq$16; and ticarcillin/clavulanic acid, $\geq$128/2.

[d] Errors found when comparing the results obtained by the two methods for individual isolates. Designations as previously described (11): VME = very major error = susceptible by disk diffusion and resistant by the reference (agar dilution) method; ME = major error = resistant by disk diffusion and susceptible by the reference method; MiE = minor error = intermediate by reference method and resistant or susceptible by disc diffusion.

[e] Clinical group isolates were not subjected to disc diffusion testing. Accordingly, error rates could not be established.

TABLE 3. Comparison of resistance rates of individual species of the *B. fragilis* group

Species	Group[a]	No. of isolates	%R[b]					
			CLN	MER	MTZ	MOX	PEN	TIM
B. caccae	Comm	11	36	0	0	45	100	0
	Hosp	6	17	0	0	33	100	0
	Clin	1	100	0	0	0	100	0
	All	18	33	0	0	39	100	0
B. distasonis	Comm	24	75	0	0	4	100	8
	Hosp	20	90	0	0	5	100	40
	Clin	3	67	0	0	0	100	0
	All	47	83	0	0	4	100	21
B. eggerthii	Comm	6	83	0	0	0	100	17
	Hosp	8	25	0	0	0	88	13
	Clin	0	-	-	-	-	-	-
	All	14	50	0	0	0	93	14
B. fragilis	Comm	22	9	0	0	18	100	5
	Hosp	24	38	4	0	4	100	4
	Clin	36	28	6	0	25	100	3
	All	82	26	4	0	17	100	4
B. merdae	Comm	1	100	0	0	0	100	0
	Hosp	0	-	-	-	-	-	-
	Clin	0	-	-	-	-	-	-
	All	1	100	0	0	0	100	0
B. ovatus	Comm	6	33	0	0	67	100	0
	Hosp	2	100	0	0	50	100	0
	Clin	5	60	0	0	0	100	0
	All	13	54	0	0	38	100	0
B. stercoris	Comm	1	0	0	0	0	100	0
	Hosp	0	-	-	-	-	-	-
	Clin	0	-	-	-	-	-	-
	All	1	0	0	0	0	100	0
B. thetaiotaomicron	Comm	8	50	0	0	38	100	13
	Hosp	19	53	5	0	11	100	26
	Clin	4	100	25	0	100	100	0
	All	31	58	6	0	29	100	19
B. uniformis	Comm	16	44	6	0	56	100	6
	Hosp	13	54	15	0	62	100	23
	Clin	3	100	0	0	0	100	0
	All	32	53	9	0	53	100	13
B. vulgatus	Comm	5	0	0	0	40	100	0
	Hosp	6	50	0	0	33	100	0
	Clin	5	40	0	0	0	100	20
	All	16	31	0	0	25	100	6

[a] Denotes isolate study groups: Comm = community isolate group; Hosp = hospital high-dependency unit isolate group; Clin = clinically significant isolate group; All = total of species, irrespective of isolate source

[b] Resistance as determined by CLIS agar dilution method (10)

TABLE 4. Genetic markers of resistance

MIC Value[a]	Group[b]	No. of isolates	Gene/s present (%)								
			ermF	*ermB*	*ermG*	*ermF + ermB*	*ermF + ermG*	*ermB + ermG*	All 3	No *erm* genes	*nim* gene[e]
Resistant[c]	Comm	43	13 (30)	3 (7)	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)	26 (60)	-
	Hosp	52	18 (35)	2 (4)	0 (0)	4 (8)	0 (0)	1 (2)	1 (2)	26 (50)	-
	Clin	20	7 (35)	0 (0)	0 (0)	3 (15)	0 (0)	0 (0)	1 (5)	9 (45)	-
	All	115	38 (33)	5 (4)	0 (0)	8 (7)	0 (0)	1 (1)	2 (2)	61 (53)	-
Susceptible[d]	Comm	57	4 (7)	1 (2)	0 (0)	0 (0)	0 (0)	3 (5)	0 (0)	49 (86)	1 (1)
	Hosp	46	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)	2 (4)	0 (0)	43 (93)	0 (0)
	Clin	37	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	36 (97)	1 (2)
	All	140	5 (4)	2 (1)	0 (0)	0 (0)	0 (0)	5 (4)	0 (0)	128 (91)	2 (1)

[a] MIC value as determined by CLSI agar dilution method (10)

[b] Denotes the three isolate study groups: Comm = community isolate group; Hosp = hospital high-dependency unit isolate group; Clin = clinically significant isolate group, All = total isolates, irrespective of source

[c] Resistant isolate classified by CLSI (10) as having an MIC value of ≥8µg/mL for clindamycin or ≥32µg/mL for metronidazole

[d] Susceptible isolate classified by CLSI (10) as having an MIC value of <8µg/mL for clindamycin or <32µg/mL for metronidazole

[e] No isolates were found to be resistant to metronidazole. Susceptible isolate group numbers for metronidazole are: community = 100, hospital = 98, clinical = 57, all = 255