Malays J Pathol 2020; 42(2): 245 – 252

ORIGINAL ARTICLE

Susceptibility patterns of anaerobes isolated from clinical specimens in tertiary Hospital, Malaysia

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Abstract

Introduction: The susceptibility patterns of anaerobes are becoming less predictable due to the emergence of anaerobic resistance trends to antibiotics; hence increasing the importance of the isolation and antimicrobial susceptibility testing of anaerobes. *Materials and Methods:* This study investigated the isolation of anaerobes from the clinical specimens of Hospital Sungai Buloh, Malaysia, from January 2015 to December 2015. All isolates were identified using the API 20A system (bioMérieux, France). Antimicrobial susceptibility testing was performed using the E-test (bioMérieux, France). *Results:* The proportion of obligate anaerobes isolated from the clinical specimens was 0.83%. The Gram-positive anaerobes were most susceptible to vancomycin and imipenem, showing 100% sensitivity to these antimicrobials, followed by clindamycin (86.3%), penicillin (76.7%), and metronidazole (48.9%). Meanwhile, Gram-negative anaerobes were most susceptible to metronidazole (96%) followed by imipenem (89%), clindamycin (79%), and ampicillin (32%). The present study also showed that 3 out of 12 *Bacteroides fragilis* isolates were resistant to imipenem. *Conclusion:* This study demonstrated the differences in the susceptibility patterns of anaerobes towards commonly used antimicrobials for the treatment of anaerobic infections. In summary, continuous monitoring of antimicrobial resistance trends among anaerobes is needed to ensure the appropriateness of treatment.

Keywords: anaerobes, antimicrobial resistance, susceptibility patterns

INTRODUCTION

Anaerobes are part of normal human flora and normally cause infections that are polymicrobial in nature. This type of infection is sometimes overlooked, as isolation is not routinely performed in many microbiology laboratories because of the special anaerobic culturing method required.[1] The isolation technique is difficult to perform, as it requires a strict anaerobic condition during collection, transportation, and isolation. A few anaerobic studies have identified varying incidence rates as well as anaerobic resistance trends, which differ according to anaerobe species and geographical region.[2] There is a growing incidence of antimicrobial resistance among anaerobes globally making the in vitro sensitivity of anaerobes towards antimicrobial agents no longer predictable. Therefore, continuous surveillance of the susceptibility patterns of anaerobes should be emphasized to ensure the appropriateness of therapy.[3] Limited data is available on the resistance trends of anaerobes in Malaysia. Therefore, this present study aims to determine the antimicrobial susceptibility patterns of anaerobes isolated from clinical specimens in a hospitalised patient at a tertiary hospital in Malaysia.

MATERIALS AND METHODS

This study was conducted from 1 January 2015 to 31 December 2015 using purposive sampling, in the Microbiology laboratory of Hospital Sungai Buloh, Malaysia.

Culture and identification

Specimens for the anaerobic blood culture

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were received in either BD BACTEC Plus Anaerobic vials or BacT/Alert FN Plus vials and incubated for 5 days. Positive anaerobic vials were inoculated onto blood agar, a Mac Conkey agar, and/or a Chocolate agar and incubated at 35°C in O_2 or CO_2 condition for 48 hours depending on the Gram stain findings. In addition, all positive anaerobic vials were also inoculated onto a Schaedler agar (Thermofisher Scientific®) regardless of the Gram stain findings and incubated at 35°C in anaerobic conditions (either in an anaerobic chamber or in an anaerobic jar) for 48 hours. For the pus aspirate, tissue and bone specimens were inoculated onto the blood agar and the MacConkey agar, which were incubated at 35°C in O_2 condition and also onto the Schaedler agar, which was incubated at 35°C in anaerobic condition (either in an anaerobic chamber or in an anaerobic jar) for 48 hours. The stool specimens requested for *C. difficile* isolation were tested for both toxin A and toxin B using a commercial immunochromatographic technique (ICT) kit (Remel Xpect®; Oxoid, UK) and immediately inoculated onto a *C. difficile* agar and incubated at 35°C in anaerobic condition (either in an anaerobic chamber or in an anaerobic jar) for 4 days. The primary plates were examined for all different colony types and an aerotolerance test was done to differentiate between facultative and obligate anaerobes. Presumptive identification was made using Gram stain and special potency antibiotic discs.[4] The potency antibiotic discs consisted of 10 µg colistin, 1000 µg kanamycin, 5 µg vancomycin, 2 units of penicillin, 15 µg rifampicin, and 60 µg erythromycin. These discs can be used as an aid for clarifying Gram stain reaction and in the presumptive identification of anaerobes, as shown in Table 1. A definitive identification was made with the API 20A system (bioMérieux, France). A percentage identification of more than 80% was set as the threshold for positive identification of the anaerobe isolates.

Antibiotic sensitivity testing

Antimicrobial susceptibility testing (AST) was performed using an E-test (bioMérieux, France) and the Schaedler agar was used as the media for AST. Gram-positive anaerobe isolates were tested against benzyl-penicillin, imipenem, clindamycin, metronidazole, and vancomycin while Gram-negative anaerobes were tested against ampicillin, imipenem, clindamycin, and metronidazole. *C. difficile* was only tested against vancomycin and metronidazole. Minimum inhibitory concentration (MIC) interpretation was carried out following the Clinical and Laboratory Standards Institute (CLSI) M100, 25th edition (January 2015), for all anaerobes except for vancomycin. For vancomycin, the interpretation was carried out following the European Committee Antimicrobial Susceptibility Testing (EUCAST) Version 3.1 (2013) for Gram-positive anaerobes.

RESULTS

A total of 116 anaerobes were isolated from 13,995 clinical specimens comprising blood culture, tissue, bone, pus aspirate, and *C. difficile* from the stool, which accounted for 0.83% of the anaerobes. Out of the 116 anaerobes, 70 were isolated from blood culture specimens (60.3%), 19 from tissue and bone specimens (16.4%), 12 from pus aspirate specimens (10.3%), and 15 *C. difficile* were isolated from stool specimens (13%). The most frequently encountered anaerobe was *Propionibacterium*

TABLE 1: Potency discs for the presumptive identification of anaerobes

Bacteria	Erythromycin 60 µg	Rifampicin 15 µg	Colistin 10 µg	Penicillin 2 units	Kanamycin 1000 µg	Vancomycin 5 µg
B. fragilis	S	S	R	R	R	R
P. melaninogenica	S	S	V	S	R	R
B. oralis	S	S	S	S	R	R
B. ureolyticus	S	S	S	S	S	R
Fusobacterium spp	R	R	S	S	S	R
Gram-positive cocci	S	S	R	S	S	S
Gram-negative cocci	S	S	S	S	S	R

S = sensitive, V = variable, R = resistant

TABLE 2: Distribution of anaerobes isolated from clinical specimens

Anaerobe	Specimen type				
	Blood n (%)	Pus aspirate n (%)	Tissue & bone n (%)	Stool n (%)	Total
***Propionibacterium* spp.**	29 (41.4)	1 (8.3)	2 (10.5)	0	32
***Clostridium* spp.**					
Clostridium difficile	0	0	0	15 (100)	15
Other Clostridium spp.	6 (8.6)	2 (16.7)	0	0	8
***Bacteroides* spp.**	15 (21.4)	1 (8.3)	2 (10.5)	0	18
Gram-positive anaerobic cocci (GPAC)					
Peptostreptococcus	5 (7.1)	3(25)	7(36.9)	0	15
Peptoniphilus asaccharolyticus	3(4.3)	0	4(21)	0	7
Other Gram-positive rods	8 (11.4)	3 (25)	0	0	11
***Prevotella* spp.**	2 (3)	2 (16.7)	2 (10.5)	0	6
***Prophyromonas* spp.**	1 (1.4)	0	1 (5.3)	0	2
Veilonella parvula	1 (1.4)	0	0	0	1
***Fusobacterium* spp.**	0	0	1 (5.3)	0	1
Total	70	12	19	15	**116**

spp. The distribution of anaerobes according to the type of clinical specimen is shown in Table 2.

Antimicrobial susceptibility patterns of anaerobes
The antimicrobial susceptibility patterns of both the Gram-negative and Gram-positive anaerobe isolates are shown in Fig. 1 and Fig. 2, respectively. Among the antibiotics tested, the Gram-negative anaerobe isolates were most susceptible to metronidazole (96%) followed by imipenem (89%), clindamycin (79%), and ampicillin (32%). Only Gram-negative (11%) anaerobe isolates were found resistant to imipenem. The Gram-positive anaerobe

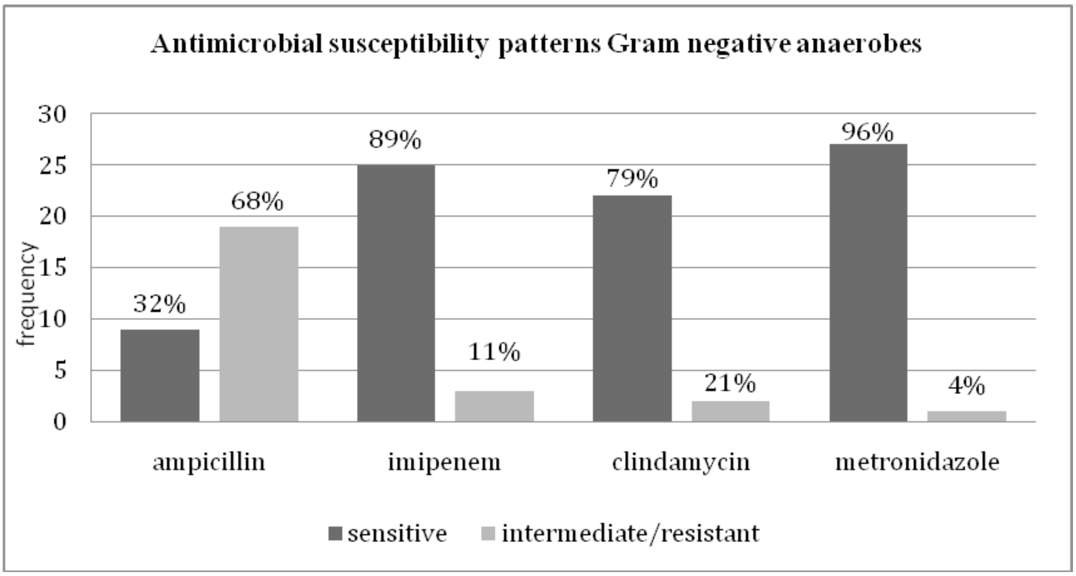

FIG. 1: Antimicrobial susceptibility patterns of the Gram-negative anaerobes

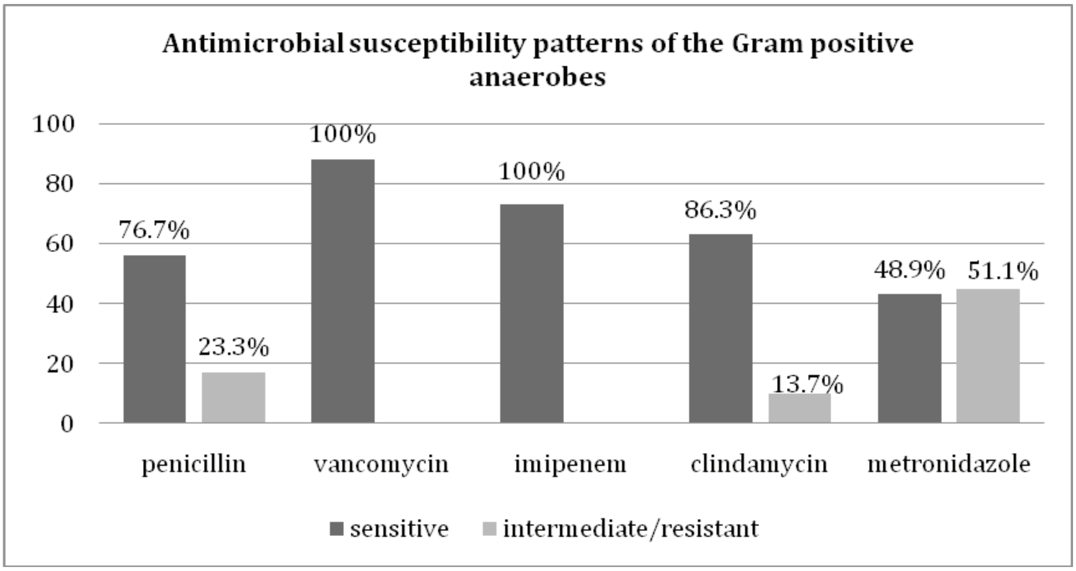

FIG. 2: Antimicrobial susceptibility patterns of the Gram-positive anaerobes

isolates were most susceptible to vancomycin and imipenem, and showed 100% sensitivity to the antibiotics. The susceptibility of the Gram-positive anaerobe isolates to clindamycin, penicillin, and metronidazole were 86.3%, 76.7%, and 48.9% respectively.

Further details of the antimicrobial susceptibility patterns of the Gram-negative anaerobes and the Gram-positive anaerobes are summarized in Table 3 and Table 4, respectively.

TABLE 3: Antimicrobial susceptibility patterns of the Gram-negative anaerobe isolates

Organism and antimicrobial agent	Number of isolates (%)with susceptibility	
	S	R
Gram-negative rods		
Bacteroides fragilis **(n = 12)**		
Ampicillin	1 (8.3)	11 (91.7)
Imipenem	9 (75)	3 (25)
Clindamycin	8 (66.7)	4 (33.3)
Metronidazole	12 (100)	0
Other *Bacteroides* spp. (n = 6)		
Ampicillin	3 (50)	3 (50)
Imipenem	6 (100)	0
Clindamycin	4 (66.7)	2 (33.3)
Metronidazole	5 (83.3)	1 (16.7)
Other Gram-negative rods (n = 9)		
Ampicillin	5 (55.6)	4 (44.4)
Imipenem	9 (100)	0
Clindamycin	9 (100)	0
Metronidazole	9 (100)	0
Gram-negative cocci		
Veillonella parvula **(n = 1)**		
Ampicillin	0	1 (100)
Imipenem	1 (100)	0
Clindamycin	1 (100)	0
Metronidazole	1 (100)	0

S = sensitive, R = resistant

TABLE 4: Antimicrobial susceptibility patterns of the Gram-positive anaerobe isolates

Organism and antimicrobial agent	Number of isolates (%) with susceptibility	
	S	R
Gram-positive Rods		
***Propionibacterium* spp. (n = 32)**		
Penicillin	26 (81.3)	6 (18.7)
Vancomycin	32(100)	0
Imipenem	32 (100)	0
Clindamycin	31 (96.9)	1 (3.1)
Metronidazole	0	32 (100)
***Clostridium difficile* (n = 15)**		
Vancomycin	15(100)	0
Metronidazole	15 (100)	0
***Clostridium* spp. (n = 8)**		
Penicillin	8 (100)	0
Vancomycin	8 (100)	0
Imipenem	8 (100)	0
Clindamycin	5 (62.5)	3 (37.5)
Metronidazole	6 (75)	2 (25)
Other Gram-positive rods (n = 11)		
Penicillin	8 (72.7)	3 (27.3)
Vancomycin	11 (100)	0
Imipenem	11 (100)	0
Clindamycin	7 (63.6)	4 (36.4)
Metronidazole	4 (36.4)	7 (63.6)
Gram-positive anaerobic cocci (GPAC) (n = 22)	14 (63.6)	8 (36.4)
Penicillin		
Vancomycin	22 (100)	0
Imipenem	22 (100)	0
Clindamycin	20 (90.9)	2 (9.1)
Metronidazole	18 (81.8)	4 (18.2)

S = sensitive, R = resistant

A reduced susceptibility towards ampicillin was observed for the majority of Gram-negative anaerobe isolates. Ampicillin resistance was found in *B. fragilis* (91.7%), other *Bacteroides* spp. (50%), other Gram-negative rods (44.4%), and *Veillonella parvula* (100%), as shown in Table 3. Imipenem showed good activity against most Gram-negative anaerobe isolates but the resistance to imipenem was observed in only 3 of the *B. fragilis* isolates (25%). Clindamycin and metronidazole had a good in vitro activity against most of the Gram-negative isolates. However, clindamycin resistance was found in both *B. fragilis* (33.3%) and other *Bacteroides* spp. isolates (33.3%). On the other hand, metronidazole resistance was only observed in other *Bacteroides* spp. isolates (16.7%).

Penicillin resistance was found in 18.7% *Propionibacterium* spp. isolates, 27.3% of other Gram-positive rods, and 36.4% of Gram-positive anaerobic cocci (GPAC). The resistance to clindamycin was observed in *Propionibacterium* spp. isolates (3.1%), *Clostridium* spp. (37.5%), other Gram-positive rods (36.4%), and GPAC (9.1%). Gram-positive anaerobe isolates showed varying sensitivity against metronidazole in which all *Propionibacterium* spp. isolates were intrinsically resistant to metronidazole and the same resistance was also observed among *Clostridium* spp. (25%), other Gram-positive rods (63.6%), and GPAC (18.2%). In this study, *C. difficile* isolates were only tested for MIC vancomycin and metronidazole. All fifteen *C. difficile* isolates were found sensitive to vancomycin (100%) and metronidazole (100%).

DISCUSSION

The proportion of anaerobes in this study is much lower (0.83%) compared to that recorded by the Japanese Society of Chemotherapy and the Japanese Association for Infectious Diseases, which reported 7% to 8% of anaerobes isolated from all clinical specimens, respectively.[5] The low proportion might be due to the fact that other studies may have used different types of clinical specimen and different methods of isolate identification. Besides that, there is a lack of request for anaerobic bacteria culture among clinicians. In addition, the improper collection and transportation of clinical specimens were identified as a major drawback in these studies. [6] *Propionibacterium* spp. was the most common anaerobe isolated in previous studies. Even *P. acnes* would usually be regarded as a contaminant, and it was found to be significant in 3.5% of bacteraemia cases, especially in those with hospital-acquired bacteraemia and that had undergone invasive procedures. [7] However, the clinical significance of *Propionibacterium* spp. in this study was not determined.

Little is known about the antimicrobial susceptibility profiles of anaerobes in Malaysia. Penicillin and ampicillin are often used to treat various types of infections including anaerobic infections. Generally, Gram-positive anaerobes such as *Peptostreptococcus* spp., *Clostridium* spp. and *Propionibacterium* spp. are sensitive to penicillin.[2,8] Unlike these previous findings, a few *Propionibacterium* spp. isolates, other Gram-positive rods, and GPAC were found resistant to penicillin. This is comparable to different surveys that reported the resistance of 7% of GPAC.[9] and 4% of *P. acnes* to penicillin.[10] In this present study, it was found that most *Bacteroides* spp. and other Gram-negative rod anaerobes were resistant to ampicillin, similar to the findings in various studies albeit with a difference in resistance rate.[8,11] The production of β-Lactamases by anaerobic bacteria has limited the use of penicillin, ampicillin, and amoxicillin.[2] Other mechanisms of resistance against β-lactam antibiotics include low-affinity penicillin-binding proteins (PBPs) or the decreased permeability through porins channel alterations.[12]

The carbapenem group of antibiotic is one of the choices for treating anaerobic infection. Imipenem showed good activity against most anaerobe isolates in the present study and the resistance towards this antibiotic was only found in 3 out of 12 *B. fragilis* isolates. However, several studies have shown an emerging resistance towards carbapenem among anaerobes, especially *B. fragilis*, *Fusobacterium* spp., and *Prevotella* spp.[13-14] A European study reported that carbapenem resistance was usually mediated by a chromosomal zinc metallo-β-lactamases enzyme encoded by the *cfi*A gene present in *B. fragile*.[14] The emergence of this resistance to carbapenem among anaerobes was also reported in other countries including Taiwan, Kuwait, and Spain.[15]

Clindamycin is considered a treatment of choice for anaerobic infections. However, a recent review reported a significant increase in clindamycin resistance among *B. fragilis* in many countries including Europe, France, Spain, the USA, Canada, Kuwait, and Korea.[15] Clindamycin resistance was also found in other anaerobes such as *Bacteroides* spp., *Veillonella* spp., *Peptostreptococcus* spp., *Fusobacterium* spp., *Prevotella* spp., *Propionibacterium* spp., *Clostridium* spp., and other Gram-positive rod anaerobic bacteria.[16-17] These past findings are consistent with the findings of the current study. A multivariate logistic regression analysis conducted in Japan reported the presence of clindamycin resistance in anaerobic isolates from blood culture, and that it was significantly associated with fatal outcomes.[18]

Metronidazole is one of the most commonly used drugs for the treatment and prophylaxis of anaerobic infections. Metronidazole resistance has been increasing among *C. difficile* in the UK, *Bacteroides* spp. and *Parabacteroides* spp. in France, and *Prevotella* spp. in the Netherlands.[15] Metronidazole resistance is linked to metronidazole (*nim*) genes.[19-20] In this study, GPAC, *Clostridium* spp., other Gram-positive rods, and *Bacteroides* spp. were also found resistant to metronidazole, which is more or less comparable to similar studies conducted in Croatia[8] and in Canada[16], albeit with different rates of resistance. Overusing metronidazole as an empirical treatment in diarrhoea or dysentery in general practice may lead to a high resistance rate among anaerobic isolates.[21] Metronidazole is universally used as the first-line therapy for treating *C. difficile* infection.[22] In line with this finding, the *C. difficile* isolates in this study remained susceptible to this antibiotic. However, a few articles have reported metronidazole resistance in several *C. difficile* isolates.[23-24]

In general, the differences in the rate of resistance in previous studies may be influenced by anaerobe species, geographical region, types of specimens, as well as the differences in the local usage of antimicrobial therapy. Multi-central studies on the antibiotic susceptibility of anaerobes should, therefore, be conducted to determine the susceptibility pattern of anaerobes in Malaysia. The resulting information would be helpful in formulating local treatment guidelines and to ensure the appropriateness of antimicrobial therapy.

CONCLUSION

The anaerobes investigated in this study exhibited different susceptibility patterns to commonly used antimicrobials for anaerobic infections. It is therefore important to monitor the resistance trends of anaerobes, as this will guide clinicians to select the appropriate antimicrobial agent as well as increase their awareness of the antibiotic stewardship programme.

Abbreviations: AST: Antimicrobial susceptibility testing; CLSI: Clinical and Laboratory Standards Institute; GPAC: Gram-positive anaerobic cocci; MIC: Minimum inhibitory concentration (MIC)

Acknowledgement: The authors would like to thank all the staff of Microbiology laboratory, Hospital Sungai Buloh, Malaysia, and the Department of Medical Microbiology and Parasitology, FMHS, UPM, Malaysia. This project was funded by UPM Postgraduate Supervision Grants (FPSK/14/PSG(3).

Conflict of interest: The authors declare they have no conflict of interest.

Ethics approval: This study has obtained ethical approval from the Ethics & Medical Research Committee of the Ministry of Health, Malaysia (Reference number: NMRR-14-1674-23834).

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