Antimicrobial susceptibility patterns of anaerobic bacteria in Victoria, Australia.

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Abstract:

Mortality associated with anaerobic infections approximates 20%. Resistance of anaerobic bacteria to commonly used antimicrobials has been increasingly reported. The aim of this study was to describe antimicrobial susceptibility patterns of anaerobic bacteria isolated from clinical samples using a gradient diffusion method, E test (bioMérieux), in Victoria, Australia. Metronidazole, meropenem and amoxycillin-clavulanate were found to be active against almost all isolates tested. Most Gram positive anaerobic cocci (GPAC), except *Peptostreptococcus anaerobius* (64.6% penicillin-susceptible), remained susceptible to penicillin. All *Clostridium perfringens* isolates tested were penicillin, metronidazole and meropenem susceptible. Of *B. fragilis* isolates tested, 5% and 0.83% were meropenem and metronidazole non-susceptible, respectively. Clindamycin susceptibility in anaerobes other than the GPAC is approximately 75% and therefore should not be used as empirical treatment in the absence of susceptibility testing. Considering the global trend of antibiotic resistance among anaerobic bacteria, routine susceptibility testing of anaerobic bacteria, particularly when isolated from critical sites, as well as surveillance of local resistance trends is strongly encouraged. Gradient diffusion MIC determination of anaerobic bacteria is feasible in a clinical diagnostic laboratory and should be more widely utilised.

Introduction

Anaerobic infections cause significant morbidity and mortality and various clinical studies have demonstrated adverse survival outcomes in patients due to inappropriate therapy. Furthermore, anaerobic resistance to commonly used antimicrobial agents has increasingly been reported.(1, 2)

Despite this, routine antimicrobial susceptibility testing of clinical anaerobic isolates remains a contentious issue.(3) This is in part due to difficulties associated with identification, purification and manipulation of anaerobes. In the past decade, the introduction of matrix-assisted light desorption ionization- time of flight (Maldi-TOF) mass spectrometry in most diagnostic microbiology laboratories has greatly enhanced the ability for microbial identification in a time and cost-efficient manner. Multiple studies have corroborated the accuracy and reliability of anaerobic bacteria identification by MALDI-TOF.(4-7) At present, there is no ISO standard reference method for susceptibility testing of anaerobic bacteria. Procedural guidelines have been published by the Clinical and Laboratory Standards Institute (CLSI), (8) European Committee on Antimicrobial Susceptibility Testing (EUCAST),(9) and Calibration, Dichotomous Susceptibility (CDS).(10) CLSI recommends minimum inhibitory concentration (MIC) determination by broth microdilution for Bacteroides fragilis group and agar dilution for all anaerobes,(8) whereas the EUCAST recommends testing with an MIC method, and reference to the manufacturer's instructions of a commercial product.(9) Clinical MIC breakpoints for main classes of anaerobic antimicrobials are provided by each committee respectively; these differ and should be interpreted with care. The CDS recommends disc susceptibility testing method for anaerobes with interpretive annular radius cutoffs.(10)

Both broth microdilution and agar dilution methods for anaerobes are time-consuming, require expertise and are not practical to be implemented in a routine diagnostic laboratory. In the past, susceptibility testing by disc diffusion has not been recommended due to suboptimal correlation and reproducibility.(11) A recent correlation study by Nagy et al, demonstrated good agreement between zone diameter and MIC for Bacteroides fragilis group of bacteria using EUCAST rules and breakpoints,(12) although further validation is needed. MIC determination by gradient diffusion has shown reasonable correlation with broth microdilution and agar dilution methods.(13-17) Gradient diffusion MIC is easy to perform and readily implemented using commercially available products, E test (bioMérieux) and MIC Evaluator (M.I.C.E., Thermo Fisher Scientific) strips.

In Australia, antimicrobial susceptibility testing of anaerobes is done sporadically and treatment of anaerobic infections is largely empirical. As such, antimicrobial susceptibility trends of anaerobic bacteria over time is largely unknown.

The aim of this study was to describe the antimicrobial susceptibility patterns of anaerobic bacteria from clinical samples in a private clinical microbiology laboratory in Victoria, Australia.

Materials and methods:

Isolates:

Anaerobic bacteria isolated from clinical specimens were collected from January 2015 to January 2018 from both community and hospital samples from Victoria. Clinical sources included blood culture, swabs of skin/superficial sites, deep sites/abscesses, wounds (not otherwise specified) and genital swabs. Pure anaerobic bacterial isolates were obtained from blood culture and sterile sites; the

presence of anaerobes from polymicrobial or non-sterile sites is indicated by a zone of inhibition around a metronidazole (5µg) disc as per laboratory protocol. Using the Bruker matrix-assisted laser desorption/ionization-time of flight (MALDI-TOFMS) instrument, anaerobic bacteria were identified to species and genus level based on log(score) ≥2.0 and log(score) 1.7-2.0, respectively.

Susceptibility testing:

Gradient diffusion MIC determination. A 1 MacFarland standard suspension of a 48hr growth culture was made and inoculated with a swab onto Brucella Agar supplemented with blood (5%), 5mg/L haemin and 1mg/L vitamin K (Oxoid PP2459). E-test strips (bioMérieux) were then applied and the plates incubated at 35^{O}C, under anaerobic conditions using an atmosphere generation system (AnaeroGen, Oxoid, AN0035A). *Bacteroides fragilis* ATCC25285 was tested against each new lot number of Etest strips as quality control, as per the manufacturer's instructions. Controls for anaerobiasis included organism controls, *Bacteroides fragilis* ATCC25285 and *Pseudomonas aeruginosa* ATCC25668, and a chemical resazurin redox indicator (Anaerobic Indicator, Oxoid, BR0055B). Each isolate was tested against the antibiotics benzylpenicillin, amoxycillin-clavulanate, clindamycin, metronidazole and meropenem. MIC was read at 100% growth inhibition after 24 and 48hours of incubation. Results obtained at 48hours were considered final. The MIC values were interpreted according to both the CLSI(8) and EUCAST(9) clinical breakpoints. The amoxycillin-clavulanate E test strips contained amoxycillin and clavulanic acid in a 2:1 ratio, therefore only CLSI breakpoints were applied for interpretation. Slower growing anaerobic bacteria and those which could not be identified reliably by Maldi-TOF MS were excluded from this study.

Results/Discussion

Four hundred and sixteen anaerobic isolates were collected during the study period. Clinical sources for these bacteria included blood culture(n=68), swabs of skin and superficial sites(n=77), deep collection/abscesses (n=37), wounds (not otherwise specified) (n=216), and genital swabs(n=19). The anaerobic bacteria collected and tested are shown in Table 1. *Bacteroides fragilis* (n=120) and *Peptostreptococcus anaerobius* (n=79) were the most commonly isolated anaerobes. The MIC range, MIC_{50}, MIC_{90} and percentage susceptibility according to CLSI and EUCAST breakpoints for the Gram negative and Gram positive anaerobes are shown in Tables 2.

Consistent with known data, all isolates in the *Bacteroides fragilis* group and most *Prevotella* isolates (83.7%) were penicillin non-susceptible. 99.1% and 94% *B. fragilis* isolates were susceptible to metronidazole and meropenem, respectively. Most Gram positive anaerobic cocci (GPAC), except *Peptostreptococcus anaerobius* (64.6% penicillin-susceptible), remained susceptible to penicillin. 100% *Propionebacterium* sp. tested (n=17) were metronidazole-resistant and susceptible to penicillin and clindamycin. All *Clostridium perfringens* isolates tested were penicillin, metronidazole and meropenem susceptible. Clindamycin susceptibility varied across all groups of anaerobic bacteria. When both CLSI and EUCAST MIC breakpoints were applied, the overall categorical agreement for penicillin, clindamycin, metronidazole and meropenem were 97.6%, 92.3%,99.0% and 98.3%, respectively.

Anaerobic bacteria form part of the normal human indigenous microflora.(18) Research into the remarkable diversity of the human microbiome, including anaerobic bacteria in health and disease states has flourished in recent years, with particular emphasis on gut microbiome.(18, 19) Anaerobic bacteria are also

opportunistic pathogens, causing bacteraemia and sepsis(20), necrotizing skin infections,(21) and rarely endocarditis.(22) The mortality rate associated with anaerobic bacteraemia has been reported to be 1-19%.(20, 23) Publications on antimicrobial resistance trends among anaerobic bacteria in North America(24, 25), Europe(2, 26, 27), and Australasia(28, 29) are prolific. Overall there has been increase in cfiA gene encoded chromosomal zinc metallo-β-lactamase enzyme mediated carbapenem resistance and resistance to clindamycin and metronidazole.

According to our study, metronidazole, meropenem and amoxycillin-clavulanate were found to be active against almost all isolates tested, making them ideal agents for empirical therapy. Of the 120 *B. fragilis* isolates tested, 6 (5%) and 1 (0.83%) were meropenem and metronidazole non-susceptible, respectively. Clindamycin susceptibility in anaerobes other than the GPAC was approximately 75% and therefore should not be used as empirical treatment in the absence of susceptibility testing. Of note, 100% of *Propionebacterium* spp. was found to be metronidazole-resistant; this resistance profile may reliably be used as a supplementary for organism identification; conversely, in cases of *Propionebacterium* spp. post-operative shoulder joint or central nervous system shunt infections, metronidazole should not be used as a therapeutic agent.

The use of a metronidazole (5µg) disc for screening and detection of anaerobes in polymicrobial and non-sterile samples has been part of laboratory practice for decades. While this method is cheap and simple, it biases towards isolation of susceptible strains and will inherently miss metronidazole resistant strains. Thus, the proportion of metronidazole-resistant anaerobic bacterial isolates from non-sterile sites in this study is likely underestimated.

Gradient diffusion MIC determination using commercially available products E tests (bioMérieux) and MIC Evaluator (M.I.C.E., Thermo Fisher Scientific) strips are easy to setup and use without the need of specialized equipment. According to our experience, MIC determination of the commonly encountered anaerobes at 100% growth inhibition read at 48 hours was straightforward, with minimal interobserver variability. Therefore, this method is uniquely placed for susceptibility testing of anaerobic bacteria in routine clinical microbiology practice, and should be more commonly utilized. In this study, carbapenem and metronidazole resistant isolates by E test was not confirmed with agar dilution or molecular detection of resistance genes.

This study provides MIC data on the current local resistance patterns of commonly encountered anaerobes in Australia by gradient diffusion. Considering the global trend of antibiotic resistance among aerobic bacteria, routine susceptibility testing of anaerobic bacteria, particularly isolates from critical sites, as well as surveillance of local resistance trends is strongly encouraged.

Acknowledgements:

Contribution statement: LD and JT jointly conceived the study idea and design. JT performed susceptibility testing on all isolates. JT and LD contributed to data analysis, manuscript writing and review of manuscript. The authors would like to acknowledge all microbiology scientists at Australian Clinical Labs for assistance in collection of isolates.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors**.**

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1 Table 1: List of anaerobic bacteria isolated between January 2015-January 2018

Anaerobic bacteria	**No. of isolates**
Actinomyces spp.	
A.europeus	2
A.turiciensis	1
Anaerococcus spp.	
A.hydrogenalis	6
A.murdochii	2
A.octavius	1
A.vaginalis	10
Bacteroides spp.	
B. caccae	3
B. cellulosilyticus	1
B. faecis	3
B. fragilis	83
B. ovatus	7
B. pyogenes	6
B. stercoris	2
B. thetaiotaomicron	16
B. uniformis	3
B. vulgatus	5
Bifidobacterium dentium	1
Clostridium spp.	
C. paraputrificum	2
C. perfringens	30

C. ramosum	2
C. septicum	4
C. sporogenes	3
C. tertium	2
Disulfovibrio desulfuricans	1
Eggerthia catenaformis	1
Finegoldia magna	47
Murdochiella asaccharolytica	1
Odoribacter splanchnicus	1
Parvimonas micra	2
Peptoniphilus harei	38
Peptostreptococcus anaerobius	79
Prevotella sp	
P. bivia	24
P. buccae	1
P. disiens	4
P. nanceiensis	1
P. timonensis	1
Propionebacterium spp.	17
Ruminococcus gnavus	1
Staphylococcus saccharolyticus	2
Total	416

3

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Table 2: Susceptibility patterns of anaerobic Gram-negative isolates to penicillin, clindamycin, metronidazole and meropenem.

Isolate (N tested) and antimicrobial agent	MIC(µg/mL)			CLSI (µg/mL)				EUCAST (µg/mL)			
				Clinical breakpoint		Susceptibility (%)**		Clinical breakpoint		Susceptibility (%)**	
	Range	MIC_{50}	MIC_{90}	S	R	S	R	S	R	S	R
Bacteroides fragilis group[1] (120)											
penicillin	0.38->256	>256	>256	≤0.5	≥2	0.83	98.33	≤0.25	>0.5	0	99.17
amoxicillin/clavulanate*	0.125-32	0.5	4	≤4/2	≥16/8	92.5	0.83	≤4	>8	-	-
clindamycin	<0.016->256	2	>256	≤2	≥8	60.83	20.83	≤4	>4	76.67	23.33
metronidazole	0.047-16	0.5	1	≤8	≥32	99.17	0	≤4	>4	99.17	0.83
meropenem	0.0125->32	0.094	0.5	≤4	≥16	94.16	4.17	≤2	>8	93.33	5.83
Peptostreptococcus anaerobius (79)											
penicillin	0.023-16	0.094	12	≤0.5	≥2	64.56	35.44	≤0.25	>0.5	64.56	35.44
amoxicillin/clavulanate*	0.032->256	0.25	128	≤4/2	≥16/8	67.09	30.38	≤4	>8	-	-
clindamycin	0.016-16	0.38	1	≤2	≥8	98.73	1.27	≤4	>4	98.73	1.27
metronidazole	<0.016-0.5	0.19	0.38	≤8	≥32	100	0	≤4	>4	100	0
meropenem	0.003-6	0.125	2	≤4	≥16	98.73	0	≤2	>8	93.67	0

Finegoldia magna (47)											
penicillin	0.016-0.019	0.047	0.094	≤0.5	≥2	100	0	≤0.25	>0.5	100	0
amoxicillin/clavulanate*	0.047-0.25	0.125	0.19	≤4/2	≥16/8	100	0	≤4	>8	-	-
clindamycin	0.016->256	0.5	3	≤2	≥8	85.11	6.38	≤4	>4	93.62	6.38
metronidazole	0.032-1.5	0.19	0.5	≤8	≥32	100	0	≤4	>4	100	0
meropenem	0.003-0.19	0.023	0.047	≤4	≥16	100	0	≤2	>8	100	0
Peptoniphilus harei (38)											
penicillin	<0.016-0.5	0.032	0.047	≤0.5	≥2	100	0	≤0.25	>0.5	97.37	0
amoxicillin/clavulanate*	<0.016-0.064	0.016	0.047	≤4/2	≥16/8	100	0	≤4	>8	-	-
clindamycin	<0.016->256	0.094	0.75	≤2	≥8	97.37	2.63	≤4	>4	97.37	2.63
metronidazole	0.016-3	0.5	1	≤8	≥32	100	0	≤4	>4	100	0
meropenem	<0.002-0.003	<0.002	0.002	≤4	≥16	100	0	≤2	>8	100	0
Prevotella spp.[2] (31)											
penicillin	0.032-32	6	16	≤0.5	≥2	16.13	77.42	≤0.25	>0.5	16.13	83.87

amoxicillin/clavulanate*	0.032-64	1	2	≤4/2	≥16/8	96.77	3.23	≤4	>8	-	-
clindamycin	<0.016->256	0.032	>256	≤2	≥8	74.19	25.81	≤4	>4	74.19	25.81
metronidazole	0.38-12	1	2	≤8	≥32	93.55	0	≤4	>4	90.32	9.68
meropenem	0.012-1	0.064	0.094	≤4	≥16	100	0	≤2	>8	100	0
Clostridium perfringens (30)											
penicillin	0.032-0.25	0.064	0.125	≤0.5	≥2	100	0	≤0.25	>0.5	100	0
amoxicillin/clavulanate*	<0.016-0.25	0.047	0.125	≤4/2	≥16/8	100	0	≤4	>8	-	-
clindamycin	0.064->256	1	4	≤2	≥8	76.67	3.33	≤4	>4	96.67	3.33
metronidazole	0.5-3.0	1.5	2	≤8	≥32	100	0	≤4	>4	100	0
meropenem	<0.002-0.094	0.006	0.012	≤4	≥16	100	0	≤2	>8	100	0
Anaerococcus spp(19)											
penicillin	<0.016-0.5	0.023	0.19	≤0.5	≥2	100	0	≤0.25	>0.5	94.74	0
amoxicillin/clavulanate*	<0.016-0.38	0.016	0.094	≤4/2	≥16/8	100	0	≤4	>8	-	-
clindamycin	<0.016->256	0.023	2	≤2	≥8	89.47	10.53	≤4	>4	89.47	10.53

metronidazole	<0.016-0.75	0.047	0.75	≤8	≥32	100	0	≤4	>4	100	0
meropenem	<0.002-0.064	0.006	0.047	≤4	≥16	100	0	≤2	>8	100	0
Propionebacterium spp.(17)											
penicillin	<0.016-0.64	0.016	0.023	≤0.5	≥2	100	0	≤0.25	>0.5	100	0
amoxicillin/clavulanate*	<0.016-0.19	0.032	0.094	≤4/2	≥16/8	100	0	≤4	>8	-	-
clindamycin	0.016-0.094	0.032	0.064	≤2	≥8	100	0	≤4	>4	100	0
metronidazole	64->256	>256	>256	≤8	≥32	0	100	≤4	>4	0	100
meropenem	0.006-0.064	0.012	0.047	≤4	≥16	100	0	≤2	>8	100	0
Other Clostridium spp.[3] (13)											
penicillin	0.016-2	0.064	1	≤0.5	≥2	76.92	7.69	≤0.25	>0.5	76.93	23.07
amoxicillin/clavulanate*	0.016-1	0.125	0.5	≤4/2	≥16/8	100	0	≤4	>8	-	-
clindamycin	0.064-64	2	32	≤2	≥8	53.85	46.15	≤4	>4	53.85	46.15
metronidazole	0.032-16	1	4	≤8	≥32	92.31	0	≤4	>4	92.31	7.69
meropenem	0.012-0.38	0.094	0.38	≤4	≥16	100	0	≤2	>8	100	0
Other Gram negative anaerobic bacteria[4](11)											
penicillin	<0.016-	1	>256	≤0.5	≥2	38.46	46.15	≤0.25	>0.5	38.46	61.54

	>256										
amoxicillin/clavulanate*	<0.016-4	0.125	0.75	≤4/2	≥16/8	100	0	≤4	>8	-	-
	<0.016-										
clindamycin	>256	0.016	2	≤2	≥8	92.31	7.69	≤4	>4	92.31	7.69
metronidazole	<0.016-1	0.094	0.75	≤8	≥32	100	0	≤4	>4	100	0
meropenem	<0.002-0.19	0.016	0.19	≤4	≥16	100	0	≤2	>8	100	0
Other Gram positive anaerobic bacteria[5](11)											
	<0.016-										
penicillin	0.094	0.016	0.064	≤0.5	≥2	100	0	≤0.25	>0.5	100	0
	<0.016-										
amoxicillin/clavulanate*	0.125	0.023	0.064	≤4/2	≥16/8	100	0	≤4	>8	-	-
clindamycin	0.016->256	0.064	1.5	≤2	≥8	90.91	9.09	≤4	>4	90.91	9.09
metronidazole	0.016->256	1	>256	≤8	≥32	54.54	45.45	≤4	>4	54.55	45.45
meropenem	0.003-0.047	0.016	0.047	≤4	≥16	100	0	≤2	>8	100	0

[1]*Bacteroides fragilis* (n=83), *Bacteroides thetaiotaomicron* (n=16), *Bacteroides ovatus* (n=7), *Bacteroides vulgatus* (n=5), *Bacteroides caccae* (n=3), *Bacteroides uniformis* (n=3), *Bacteroides stercoris* (n=2*), Bacteroides cellulosilyticus*(n=1); [2]*Prevotella bivia* (n=24), *Prevotella disiens* (n=4), *Prevotella buccae* (n=1), *Prevotella nanceiensis* (n=1), *Prevotella timonensis* (n=1), [3]*Clostridium septicum* (n=4), *Clostridium sporogenes*

(n=3), *Clostridium tertium* (n=2), *Clostridium ramosum* (n=2), *Clostridium paraputrificum* (n=2), [4]*Bacteroides faecis*(n=3), *Bacteroides pyogenes* (n=6), *Odoribacter splanchnicus*(n=1), *Disulfovibrio desulfuricans*(n=1); [5]*Actinomyces europeus* (n=2), *Actinomyces turiciensis* (n=1*), Bifidobacterium dentium* (n=1), *Eggerthia catenaformis* (n=1), *Murdochiella asaccharolytica* (n=1), *Parvimonas micra* (n=2), *Ruminococcus gnavus* (n=1), *Staphylococcus saccharolyticus* (n=1); *Amoxycillin/clavulanate Etest strips in 2:1 ratio; **Organisms classified as intermediate according to the CLSI method and EUCAST methods are not explicitly presented.