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Antimicrobial Susceptibility and Sub-MIC Biofilm Formation of *Moraxella catarrhalis* Clinical Isolates under Anaerobic Conditions

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Abstract

A medium was developed to support the anaerobic growth and antimicrobial susceptibility testing of clinical *Moraxella catarrhalis* isolates. The MICs of clinical *Moraxella catarrhalis* isolates under anaerobic conditions were, in general, decreased as compared to atmospheric or capnophilic conditions, while the MBCs for all conditions were within a 2 fold concentration dilution. Biofilm formation was affected by the presence of sub-MIC concentrations of azithromycin and the tested quinolones with the exception of levofloxacin.

Keywords

Anaerobic, Nitrate, Otitis Media, Sinusitis, Pneumonia, Biofilm, Sub-MIC, Anaerobic Respiration, *Moraxella catarrhalis*

1. Introduction

The vast majority of infections (60% to 80%) are biofilm associated [1] [2]. Biofilms are comprised of organisms embedded in an extracellular matrix [3]. It is well established that biofilms form an environment that protects its inhabitants from the actions of both host factors and antimicrobials [1] [2] [4]. This protective function of biofilm and its subsequent inhibitory effect on antimicrobial activity is attributed, in part, to the inability of the drug to penetrate the biofilm in concentrations sufficient for activity [4]-[6]. The presence of sub-inhibitory antibiotic concentrations can further exacerbate the situation by enhancing the levels of biofilm formation [7]-[10]. An additional factor that can contribute to the phenotypic antimicrobial resistance of bacteria in a biofilm is the physiologic status of the organisms, including whether the organism is undergoing aerobic or anaerobic res-

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piration. Growth in reduced oxygen conditions is associated with decreased antimicrobial susceptibility [11]. Thus, bacteria can exhibit *de facto* increased intrinsic biofilm-associated resistance as a result of the combination of decreased levels of oxygen, and sub-MIC antibiotic levels which further enhances phenotypic resistance [7] [8].

M. catarrhalis is a significant pathogen of the respiratory tract [12]-[14]. In addition to causing sinusitis, acute exacerbation of chronic obstructive pulmonary disease and pneumonia, it is the third most common bacterial cause of otitis media (OM). While long thought of as a strict aerobe, recent studies have shown that laboratory strains of *M. catarrhalis* are capable of anaerobic respiration via the nitrate reductase pathway, a factor that could play a role in treatment failure [15]-[17]. In pneumonia, sinusitis and otitis media (OM), the levels of available oxygen vary depending on the amount of oxygen diffusing from the mucosa and amount of microbial biofilm present [11] [18]. In OM with effusion, the Eustachian tube is hypoxic with the extent of anaerobic areas dependent on the levels of biofilm present [11] [18]. Clinically, treatment failure for OM, as well as sinusitis and other respiratory tract diseases, may be related to altered growth in response to variations in oxygen levels. To date, growth of *M. catarrhalis* clinical isolates under anaerobic conditions has not been studied. In addition, the effect of oxygenation on clinical isolates antimicrobial susceptibility and biofilm formation in response to sub-MICs of antibiotics is not known. The focus of this study was to develop a medium for the anaerobic culture of clinical *M. catarrhalis* isolates and determine their susceptibility and biofilm formation under various atmospheric conditions.

2. Materials and Methods

2.1. Bacterial Isolates

Clinical isolates of *M. catarrhalis* (n = 14) were prepared as previously described [19]. The isolates used were kindly provided by J. Tjhio (Loyola University Stritch School of Medicine, Maywood, IL). All isolates tested elaborated a beta-lactamase by nitrocefin assay (data not shown).

2.2. Development of Media for Anaerobic Growth of Clinical *M. catarrhalis* Isolates

All commercial media were prepared per label directions. Supplements were prepared individually, and in combination (as indicated) then filter sterilized (**Table 1**). For maximal level of achievable growth and generation time, each medium was inoculated with *M. catarrhalis* to a final concentration of ~10^5 CFU/ml then incubated (35˚C) aerobically and anaerobically (Whitley Anaerobic Workstation A35). After incubation (48 h), samples were removed for standard viability count (Muller Hinton: MH agar; aerobic conditions). The optimal medium for anaerobic growth was determined after testing five media alone and with seven different supplements, in various combinations.

Table 1. Media with and without supplements tested for their ability to support the anaerobic growth of *Moraxella catarrhalis* clinical isolates (n = 14).

		Supplements[a]										
		A	B	C	D	E	F	G				
Broth Medium with $NaNO_3$ (10 mM)[b]	Medium alone	YNB[a]	BSA (0.5%)[d]	Yeast Extract 0.10%	Glycerol 0.20%	Glucose 0.20%	Casein 0.10%	Bovine Hemoglobin 0.20%	A-C[e]	A-E	A-G	Aerobic Growth
Muller Hinton	-[c]	-	-	-	-	-	-	-	-	-	-	++[b]
Brain Heart Infusion (BHI)	-	-	-	-	-	-	-	-	-	+[f]	++	++[f]
Middlebrook	-	-	-	-	-	-	-	-	-	+	+	ND[g]
Brucella	-	-	-	-	-	-	-	-	-	-	-	ND
Luria-Bertoni	-	-	-	-	-	-	-	-	-	-	-	ND

[a]All media were tested with all combinations of supplements; all supplements were made in yeast nitrogen base medium without amino acids or ammonium chloride, pH 7.0, Difco; [b]Muller Hinton and Brain Heart Infusion with supplements A-E (BHI-S) were also tested aerobically with and without $NaNO_3$; no significant differences in final $absorbance_{600nm}$ or CFU/ml in MH and BHI-S media with and without nitrate were measured; [c]Minus sign (-) indicates no growth; plus sign (++) indicates ~1-3 × 10^9 CFU/ml; (+) indicates ~1-2 × 10^8 CFU/ml; [d]Bovine Serum Albumin fraction IV; [e]Indicates combination of indicated supplements, e.g. A-C = A, B, C; [f]Medium used for anaerobic growth of *M. catarrhalis* for all subsequent experiments; [g]Not Determined.

2.3. Antimicrobial Testing

The MIC and MBC for antimicrobials used in the treatment of *M. catarrhalis* otitis media, pneumonia and sinusitis were measured using a microdilution method [14] [20]. Each drug was tested in sextuplicate and repeated once (*M. catarrhalis*, n = 8; 35˚C, 48 h; 5% CO_2 in air; normal atmospheric conditions; or anaerobic conditions) [19].

2.4. Biofilm Formation in Sub-MICs of Antibiotics

The effect sub-MICs have on *M. catarrhalis* biofilm formation was determined, as previously described, by the simple expedient of emptying, washing and staining the 96 well plate wells after growth in the presence and absence of antibiotics (PBS, 3x wash; crystal violet, stain; absolute ethanol elutant) [21]. Biofilm levels were determined as a measure of crystal violet staining (Beckman EIA reader; Abs_{540}). The measurements were compared against a control 96 well plate that contained only drug (negative control) or only organisms (positive control). Significance testing was determined by ANOVA, with Tukey post hoc test (GraphPad Software, San Diego, CA).

3. Results

3.1. Development of Medium for Anaerobic Growth of Clinical Isolates

Previous studies report the growth of laboratory-adapted *M. catarrhalis* in brain heart infusion medium (BHI) with 10 mM $NaNO_3$ [22] [23]. This medium did not support the growth of any *M. catarrhalis* clinical isolates tested (n = 14). This was probably because clinical isolates can exhibit growth characteristics that are different from laboratory-adapted strains [24]. Therefore, a requisite first step focused on development of a medium that supported the anaerobic growth of *M. catarrhalis* clinical isolates (n = 14) to levels similar to that measured for the isolates' aerobic growth in Mueller Hinton broth, the standard medium used for antibiotic testing (**Table 1**). Of the media tested, only BHI with supplements A-E (BHI-S) or A-G consistently supported growth of all isolates; the level of anaerobic and aerobic growth was similar to growth measured in MH incubated aerobically (1 - 3 × 10^9 CFU/ml). Middlebrook medium with supplements A-E or A-G also supported the anaerobic growth of *M. catarrhalis*, but to a lesser extent (~8% - 13% less) than that measured for BHI-S. BHI-S was used for all subsequent determinations of the effects aerobic, capnophilic and anaerobic growth conditions have on antimicrobial susceptibility and biofilm formation.

3.2. MIC and MBC of Clinical Isolates under Various Levels of Oxygenation

The anaerobic MIC for all antimicrobials tested ranged from significantly less to within 2 fold dilution of that measured for aerobic and capnophilic conditions (**Table 2**). Anaerobically grown *M. catarrhalis*' MICs in the presence of clarithromycin, and the quinolones nalidixic acid and ciprofloxacin were the most sensitive as compared to aerobic/capnophilic growth (31-250; 508-1016; 8-31 fold less MIC, respectively). However, the MBC range for these drugs was within 2 fold dilution for all growth conditions. This pattern of the MBC ranges overlapping regardless of growth conditions vs. the MIC range registering significantly less under anaerobic growth occurred for all compounds with the exception of ceftriaxone where the MBC was 4 fold less than that measured under atmospheric conditions and 2 fold below capnophilic conditions. No discernible pattern of susceptibility was noted for the individual isolates with the exception of *M. catarrhalis* isolate 6 which exhibited the highest MIC and MBC to macrolide-azide drugs clarithromycin and azithromycin under all growth conditions.

3.3. Biofilm Formation

Biofilm formation in positive controls (organism alone) was unaffected by the lack of oxygen or increased concentration of CO_2 (**Table 2**). Of the antimicrobials tested, sub-MIC levels of azithromycin and the quinolones, with the exception of levofloxacin, significantly ($p < 0.05$) affected biofilm formation. Nalidixic acid exhibited a bimodal effect on biofilm formation, inhibiting biofilm formation under anaerobic growth conditions while promoting biofilm levels under capnophilic and aerobic conditions. Norfloxacin and ofloxacin increased the biofilm levels under aerobic and capnophilic conditions only. Ciprofloxacin only affected biofilm levels under anaerobic growth (1.8 fold above positive growth control). Azithromycin promoted biofilm formation for one isolate (iso-

Table 2. Effect of atmospheric air, capnophilic (5% CO_2 in air), and anaerobic environments on *Moraxella catarrhalis* (n = 8) response to antibiotics (MIC and MBC) and biofilm formation.

Antibiotic	Growth Condition[1]	MIC (µg/ml)	MBC (µg/ml)	Biofilm (No. of Isolates Affected)	Peak Biofilm Level Abs_{540}[2]	Drug Concentration (µg/ml) at Peak Biofilm Level[3] (Maximum Drug Concentration Affecting Biofilm)	Ratio (Test Biofilm Level /Control Biofilm Level)[5]
Amoxicillin-clavulanate[6]							
	Aero	0.125 - 2.0	0.125 - 2.0	0	NE[4]	NE	NE
	CO_2	0.125 - 2.0	0.125 - 2.0	0	NE	NE	NE
	Anaero	0.032 - 1.0	0.063 - 1.0	0	NE	NE	NE
Cefaclor[7]							
	Aero	16.0 - 256.0	16.0 - 256.0	0	NE	NE	NE
	CO_2	4.0 - 64.0	4.0 - 128.0	0	NE	NE	NE
	Anaero	0.063 - 32.0	1.0 - 32.0	0	NE	NE	NE
Cefdinir[7]							
	Aero	0.5 - 8.0	0.5 - 16.0	0	NE	NE	NE
	CO_2	0.5 - 4.0	1.0 - 4.0	0	NE	NE	NE
	Anaero	0.008 - 1.0	0.25 - 1.0	0	NE	NE	NE
Cefixime[7]							
	Aero	0.125 - 2.0	0.5 - 4.0	0	NE	NE	NE
	CO_2	0.125 - 2.0	0.5 - 4.0	0	NE	NE	NE
	Anaero	0.002 - 0.25	0.125 - 2.0	0	NE	NE	NE
Ceftriaxone[7]							
	Aero	0.063 - 4.0	0.063 - 4.0	0	NE	NE	NE
	CO_2	0.063 - 2.0	0.063 - 2.0	0	NE	NE	NE
	Anaero	0.063 - 0.25	0.032 - 1.0	0	NE	NE	NE
Cefuroxime[7]							
	Aero	2.0 - 16.0	2.0 - 16.0	0	NE	NE	NE
	CO_2	2.0 - 16.0	2.0 - 16.0	0	NE	NE	NE
	Anaero	0.5 - 8.0	0.032 - 1.0	0	NE	NE	NE
Azithromycin[8]							
	Aero	0.063 - 0.5	0.125 - 1.0	0	NE	NE	NE
	CO_2	0.25 - 1.0	1.0 - 2.0	1	0.38 ± 0.002	0.25 (0.25)	2.21
	Anaero	0.004 - 0.25	0.5 - 2.0	0	NE	NE	NE
Clarithromycin[8]							
	Aero	0.5 - 4.0	0.5 - 8.0	0	NE	NE	NE
	CO_2	0.5 - 4.0	0.5 - 4.0	0	NE	NE	NE
	Anaero	0.016 - 0.016	0.5 - 4.0	0	NE	NE	NE

Continued

Nalidixic Acid[9]							
	Aero	32.0 - 64.0	32.0 - 64.0	4	0.15 ± 0.001	32.0 (64.0)	1.28
	CO_2	32.0 - 64.0	32.0 - 64.0	4	0.13 ± 0.001	64.0 (64.0)	1.25
	Anaero	0.063 - 0.063	16.0 - 128.0	3	0.09 ± 0.002	16.0 (16.0)	0.80
Ciprofloxacin[9]							
	Aero	0.031 - 0.125	0.031 - 0.125	0	NE	NE	NE
	CO_2	0.063 - 0.125	0.063 - 0.125	0	NE	NE	NE
	Anaero	0.004 - 0.004	0.031 - 0.25	1	0.22 ± 0.003	1.0 (1.0)	1.80
Norfloxacin[9]							
	Aero	1.0 - 4.0	2.0 - 8.0	4	0.15 ± 0.001	0.13 (8.0)	1.47
	CO_2	4.0 - 8.0	4.0 - 16.0	3	0.16 ± 0.002	4.0 (8.0)	1.51
	Anaero	0.008 - 4.0	1.0 - 8.0	0	NE	NE	NE
Levofloxacin[9]							
	Aero	0.125 - 0.25	0.25 - 0.5	0	NE	NE	NE
	CO_2	0.125 - 0.25	0.125 - 0.5	0	NE	NE	NE
	Anaero	0.004 - 0.125	0.125 - 0.25	0	NE	NE	NE
Ofloxacin[9]							
	Aero	0.125 - 0.25	0.125 - 0.5	4	0.21 ± 0.001	2.0 (4.0)	1.60
	CO_2	0.125 - 0.25	0.125 - 0.5	8	0.18 ± 0.003	4.0 (4.0)	1.54
	Anaero	0.004 - 0.25	0.25 - 1.0	0	NE	NE	NE

[1]Growth conditions: Aero = aerobic-atmospheric oxygen; CO_2 = 5% CO_2 in air; Anaero = anaerobic. [2]Peak biofilm Formation-Maximum level of biofilm measured across sub-MIC antibiotic concentrations tested. [3]Maximum [Drug] affecting Biofilm: The maximum concentration of antimicrobial causing a significant ($p < 0.05$) alteration in biofilm formation. [4]NE = No effect. [5]Maximum level of biofilm measured across sub-MIC antibiotic concentrations tested (Abs_{540})/Maximum level of biofilm measured for positive antibiotic-free control (Abs_{540}) where biofilm levels were significantly ($p < 0.05$) affected. [6]Penicillin class. [7]Cephalosporin class. [8]Macrolide class. [9]Quinolone class.

late 3; 2.21 fold increase above positive control) to the greatest extent under 5% CO_2 in air. The response of the isolates was highly variable with none responding to more than one of the compounds; isolate 9 biofilm formation was unaffected, regardless of growth condition.

4. Discussion

Moraxella catarrhalis is a significant cause of diseases of the upper airways, ear and lungs [12] [17] [25]-[28]. Collectively, these diseases rank as significant causes of morbidity and mortality that are associated with treatment failure [17]. Biofilm formation is linked to varying extents with each of these diseases [2] [4] [5] [28]. To survive in diverse host environments *M. catarrhalis* must be able to grow both aerobically and anaerobically. Studies with *Neisseria gonorrhoeae* and *Pseudomonas* spp., in model biofilms systems, have shown that biofilms in aerobic environments have areas where the oxygen threshold is sufficiently low that the organism transitions to anaerobic respiration, enabled by the elevated levels of nitrate from oxidation of nitric oxide produced during the inflammatory process [11] [29]-[31]. Although it is known that pathogens growing anaerobically typically exhibit alterations in antimicrobial susceptibility (less susceptible), clinical laboratory testing of most bacteria, including *M. catarrhalis*, is performed aerobically [32] [33]. With the newly developed medium, the anaerobic MBC measured was similar to that determined either aerobically or in the presence of increased CO_2 levels, although the MIC was typically significantly reduced. This indicates that presence or absence of oxygen is not an essential factor dictating *M. catarrhalis* susceptibility. However, the combination of atmospheric environment

and sub-MIC of antimicrobial can alter *M. catarrhalis* expression of biofilm.

We are only beginning to understand *M. catarrhalis* biofilms as they relate to antibiotic sub-MICs and microbe physiologic state [22] [23] [34] [35]. To an extent, advancement was hampered by the differences in nutritional requirements of *M. catarrhalis* clinical isolates vs. laboratory-adapted strains. Pharmacodynamic data show that antibiotic sub-MICs occur during the course of treatment [9]. Antibiotic sub-MICs can affect biofilm formation [7] [9]. The effect of the antimicrobials on *M. catarrhalis* biofilm formation was dependent on the combination of sub-MICs of the antimicrobial, growth condition, and clinical isolate tested, with levels of biofilm formed either enhanced or reduced in comparison to antibiotic-free controls. The study also showed that biofilm formation in response to sub-MICs of antibiotics, particularly with respect to biofilm enhancement by certain quinolones, is isolate dependent, as has been reported for other microbes [7] [9]. The findings in this study of clinical isolate specificity in environmental response may provide an alternative avenue for determining the molecular factors involved with enhanced phenotypic antimicrobial resistance associated with clinical isolates.

In summary, this study reports a novel medium that supports the anaerobic growth of *M. catarrhalis* clinical isolates, and that for anaerobically grown cells the MBC is the best indicator of susceptibility. In addition, the findings herein show that environmental conditions in combination affect elaboration of biofilm in an isolate-specific manner.

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