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Mycobacterium avium ssp. avium ATCC 700898 (QC) Culture Differences Demonstrate Variable MIC Susceptibility Results in the Sensititre® SLOMYCO Plate

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ABSTRACT - Revised

Background: Clinical strains of the *M. avium* Complex have been reported to produce multiple morphotypes upon subculture[1]. The recommended quality control (QC) organism for testing is *M. avium* ATCC 700898 (CLSI M-24A). Four different stocks of the QC isolate were evaluated for various morphotypes and minimum inhibitory concentrations (MICs). Since morphotype ratio and MIC values varied between isolates tested, an attempt was made to correlate the morphotype with MIC values. The SLOMYCO is a 96-well microtiter dry plate designed for testing slow-growing non-tuberculosis mycobacteria spp. The plate contains doubling dilutions for 13 antimicrobics and uses Mueller-Hinton broth with OADC for final inoculation.
Methods: Four different stocks of the QC isolate were evaluated between two user sites; one stock per site was purchased new from ATCC. Morphotypes were isolated after 3 - 4 weeks using 7H10 flasks and categorized by colony shape, elevation, margin, and density. Up to eight different types were tested on the SLOMYCO plate. Data analysis was performed on four cultures and two morphotypes, smooth-domed and flat-transparent, for a total of 136 data points.
Results: Susceptibility results for QC isolates agreed with what has been previously reported for clinical strains, the smooth-domed morphotype was most susceptible and flat-transparent was most resistant[1]. MIC's that were most affected by these morphotypes were with Ciprofloxacin, Clarithromycin, Doxycycline, Isoniazid, and Rifampin with up to a 8 fold difference and Amikacin, Linezolid, Moxifloxacin, Streptomycin, and Trimethoprim/Sulfamethoxazole having up to a 4 fold difference in MIC. MIC's of each isolate correlated to the MIC's of the most resistant morphotype in that culture.
Conclusions: The flat-transparent morphotype is most commonly seen in low passage clinical strains[1] and likely present in low passage QC isolates. When using *M. avium* ATCC 700898 for QC testing, labs should observe for morphotype variability between cultures, or routinely purchase new QC isolates of low passage, to maintain consistent results. Future testing includes optimizing growth conditions to favor the most resistant morphotype for QC testing.

INTRODUCTION

Morphotypes have been observed in many *Mycobacterium* species, including clinical strains of *M. avium* complex[1]. They may be categorized by colony shape, elevation, margin, density, etc. Some of the most commonly reported morphotypes for clinical *M. avium* strains are (1) a smooth, opaque, and domed type; (2) a smooth, transparent, and flat type; and (3) a rough type[1]. Although morphotypes may be common, their ratio and MIC values are reported to vary between cultures tested. The recommended quality control (QC) organism for testing is *M. avium* ATCC 700898 (CLSI M-24A). In this study, *M. avium* ATCC 700898 stocks were tested to a) observe for morphotypes and b) possibly correlate morphotype with MIC values for thirteen different antimicrobics. Four different stocks of *M. avium* ATCC 700898 were cultured between two user sites. One stock per site was purchased new from ATCC. Mixed colony growth was tested for all four cultures. Up to eight different morphotypes were observed in three of the cultures. They were tested on the SLOMYCO plate, alongside cultures, for a total of 155 data points. Data analysis was performed on cultures and two morphotypes, smooth-domed and flat-transparent, for a total of 136 data points. Additional testing completed after submission of the abstract increases the total number of data points for cultures, smooth-domed, and flat-transparent to 143. Several data points from the remaining 6 morphotypes are not reported here. Plates were read manually using a mirror box, and MICs were recorded and compared to previously observed in-house values.

MATERIALS & METHODS

Culture Preparation

- Mixed colony growth was prepared from four different cultures. The confluent portion of 7 day growth in a 7H10 flask (Hardy Diagnostics) was swabbed, emulsified in a 5ml Sensititre® demineralized water (TREK Diagnostic Systems), adjusted to a 0.5 McFarland Standard, and vortexed.
- Cultures were diluted to 50 to 100 CFU's and grown in a 7H10 flask to check for purity and morphotype. Each morphotype was counted and ratios of predominant culture morphotype were estimated from these counts.
- Individual colony morphotypes were picked from agar with a sterile 1µl loop after 3-4 weeks of growth in 7H10 flasks, emulsified in 5ml Sensititre® demineralized water, adjusted to a 0.5 McFarland Standard, and vortexed.

SLOMYCO Plate Inoculation

- The SLOMYCO plate (TREK Diagnostic Systems) is a 96-well dry mibrobroth dilution plate designed to test slow-growing non-tuberculosis mycobacteria spp.
- The SLOMYCO plate contains the following antibiotics and concentrations (ug/ml): Amikacin (1-64), Ciprofloxacin (0.12-16), Clarithromycin (0.06-64), Doxycycline (0.12-16), Ethambutol (0.5-16), Ethionamide (0.3-20), Isoniazid (0.25-8), Linezolid (1-64), Moxifloxacin (0.12-8), Rifabutin (Ansamycin) (0.25-8), Rifampicin (0.12-8), Streptomycin (0.5-64) and Trimethoprim / sulphamethoxazole (0.12/2.38-8/152).
- Fifty microliters (50µl) of the organism suspension was added to 10mL of Sensititre® Mueller-Hinton broth with 5% v/v OADC growth supplement (TREK Diagnostic Systems). Tubes were inverted 8-10 times.
- One hundred microliters (100µl) of the organism dilution was manually pipetted into the SLOMYCO plate for a final target inoculum of $5x10^5$ CFU/mL.

MATERIALS & METHODS cont.

SLOMYCO Plate Inoculation cont.

- After inoculation of the plate, CFU counts were performed by sampling the positive growth control well using a sterile 1µl loop, mixing with 50µl of Sensititre® demineralized water (TREK Diagnostic Systems) and inoculating onto a 7H10 flask (Hardy Diagnostics). Flasks were incubated up to 3-4 weeks at 34°C to 36°C. Colony counts and morphotype observations were recorded.
- Plates were covered with an adhesive seal and incubated at 34°C to 36°C in a non-CO_2 incubator for 7 days.
- Results were read manually with a mirror box.

RESULTS

The four cultures contained the following morphotype ratios: approximately 90% smooth-dome (Culture A); approximately 70% flat-transparent (Culture B); and two cultures purchased new from ATCC (Culture C and D) contained roughly an even distribution of each morphotype. Susceptibility results for the QC isolates agreed with what has been previously reported for clinical strains, the smooth-domed morphotype was most susceptible and flat-transparent was most resistant[1]. However, one culture requires further testing for better interpretation of the data. Culture B, which contained approximately 70% of the flat-transparent morphotype, demonstrated an MIC of >8 for Isoniazid whereas the flat-transparent morphotype testing demonstrated an MIC of 2. Since other morphotypes were present in Culture B (approximately 30%) additional testing is necessary to determine how each type attributed to the MIC of >8. Examples of Smooth-Domed and Flat-Transparent colonial variants of *M. avium* ATCC 700898 were photographed at 3-4 weeks growth with under and overhead lighting with a magnification of x20 to x40 (See figures 1 through 4).

The majority of MIC's on the SLOMYCO plate for each isolate correlated to the MIC's of the most prevalent or resistant morphotype in that culture. Tables 1 and 2 report MICs for the cultures and morphotypes, and indicate the number of isolates tested and the distribution of each to its corresponding MIC value. A few data points (n=4) were considered invalid due to skipped wells or single well contamination. One plate demonstrated a invalid positive control well due to a seal issue. This data is not included in the results. The antibiotics Ciprofloxacin, Clarithromycin, Doxycycline, Isoniazid, and Rifampin had MIC's most affected by morphotype with up to an eight fold difference between the highest and lowest MIC recorded. Doxycycline and Rifampin demonstrated the most significant difference in MIC where no overlap of smooth-dome and flat-transparent values were observed. MIC's associated with Amikacin, Linezolid, Moxifloxacin, Streptomycin, and Trimethoprim/Sulfamethoxazole were less affected by morphotype differences, having up to a four fold difference between highest and lowest recorded MIC values. Data for the three remaining antimicrobics (Ethambutol, Ethionamide, and Rifabutin (Ansamycin)) on the SLOMYCO plate were not included in the results tables. Ethambutol and Ethionamide MIC's were least affected by different morphotypes, having only a two fold difference and the MIC for *M. avium* ATCC 700898 for Rifabutin (Ansamycin) was outside the dilution range.

Table 1 MIC Results for Antibiotics with Up to an 8 Fold Difference Between MICs.
Number of isolates with corresponding MIC value

Ciprofloxacin	Plate Range ↦	0.12	0.25	0.5	1	2	4	8	16	>16
Culture A	~90% Smooth-Domed					28	10			
Culture B	~70% Flat-Transparent							3	3	
Culture C	~equal % of each type						20	9		
Culture D	~equal % of each type					4	13	20		1
Total Cultures						32	43	32	3	1
Morphotype	Smooth-Domed				5	14	1			
	Flat-Transparent						3	9		

Clarithromycin	Plate Range ↦	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
Culture A	~90% Smooth-Domed			17	19	2						
Culture B	~70% Flat-Transparent							6				
Culture C	~equal % of each type				1	28						
Culture D	~equal % of each type			2	3	33						
Total Cultures				19	23	63		6				
Morphotype	Smooth-Domed			11	8	1						
	Flat-Transparent				1	3	5	3				

Doxycycline	Plate Range ↦	0.12	0.25	0.5	1	2	4	8	16	>16
Culture A	~90% Smooth-Domed						20	17	1	
Culture B	~70% Flat-Transparent									6
Culture C	~equal % of each type								22	7
Culture D	~equal % of each type						3	1	19	15
Total Cultures							3	21	17	42	28
Morphotype	Smooth-Domed					1	10	9			
	Flat-Transparent								2	10

Isoniazid	Plate Range ↦	0.25	0.5	1	2	4	8	>8
Culture A	~90% Smooth-Domed				28	3		7
Culture B	~70% Flat-Transparent							6
Culture C	~equal % of each type			5	23	1		
Culture D	~equal % of each type			2	34	1		
Total Cultures				7	85	5		13
Morphotype	Smooth-Domed				20			
	Flat-Transparent			2	10			

Rifampin	Plate Range ↦	0.12	0.25	0.5	1	2	4	8	>8
Culture A	~90% Smooth-Domed				34	4			
Culture B	~70% Flat-Transparent							2	4
Culture C	~equal % of each type					1	27	1	
Culture D	~equal % of each type						17	17	
Total Cultures				1	37	5	44	20	4
Morphotype	Smooth-Domed			9	11				
	Flat-Transparent						2	9	1

Table 1 Key: Culture Modal Range (≥ 40%) Total Culture Modal Range (≥25%) Morphotype Modal Range (≥40%)

RESULTS cont.

Figure 1. Smooth-Domed and Flat-Transparent with over light

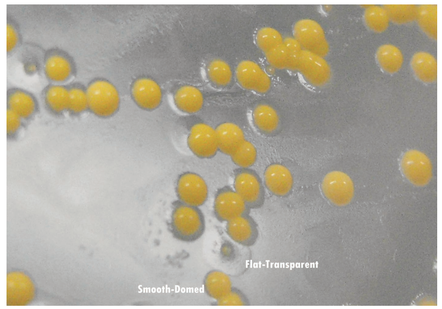

Figure 2. Smooth-Domed and Flat-Transparent with under light

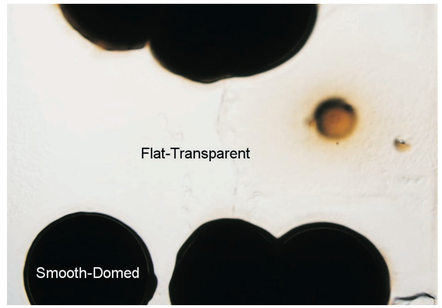

Table 2 MIC Results for Antibiotics with Up to a 4 Fold Difference Between MICs.
Number of isolates with corresponding MIC value

Amikacin	Plate Range ↦	1	2	4	8	16	32	64	>64
Culture A	~90% Smooth-Domed			26	12				
Culture B	~70% Flat-Transparent				3	2	1		
Culture C	~equal % of each type				27	2			
Culture D	~equal % of each type			4	34				
Total Cultures				30	76	4	1		
Morphotype	Smooth-Domed			11	7	2			
	Flat-Transparent				2	10			

Linezolid	Plate Range ↦	1	2	4	8	16	32	64	>64
Culture A	~90% Smooth-Domed			3	20	14	1		
Culture B	~70% Flat-Transparent						5	1	
Culture C	~equal % of each type					28	1		
Culture D	~equal % of each type			1	2	24	11		
Total Cultures				4	22	66	18	1	
Morphotype	Smooth-Domed			3	10	6	1		
	Flat-Transparent					1	10	1	

Moxifloxacin	Plate Range ↦	0.12	0.25	0.5	1	2	4	8	>8
Culture A	~90% Smooth-Domed		6	32					
Culture B	~70% Flat-Transparent				4	2			
Culture C	~equal % of each type			27	2				
Culture D	~equal % of each type		3	19	16				
Total Cultures			9	78	22	2			
Morphotype	Smooth-Domed		8	12					
	Flat-Transparent				8	4			

Streptomycin	Plate Range ↦	0.5	1	2	4	8	16	32	64	>64
Culture A	~90% Smooth-Domed					23	15			
Culture B	~70% Flat-Transparent							2	3	
Culture C	~equal % of each type						28	1		
Culture D	~equal % of each type					4	28	4		
Total Cultures						27	71	7	3	
Morphotype	Smooth-Domed				3	12	5			
	Flat-Transparent							12		

Trimethoprim / sulfamethoxazole	Plate Range ↦	0.12	0.25	0.5	1	2	4	8	>8
Culture A	~90% Smooth-Domed			37	1				
Culture B	~70% Flat-Transparent				1	3	2		
Culture C	~equal % of each type			21	8				
Culture D	~equal % of each type			38					
Total Cultures				97	12	2			
Morphotype	Smooth-Domed		2	18					
	Flat-Transparent				11	1			

Table 1 Key: Culture Modal Range (≥ 40%) Total Culture Modal Range (≥25%) Morphotype Modal Range (≥40%)

RESULTS cont.

Figure 3. Flat-Transparent with over light

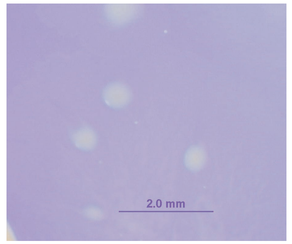

Figure 4. Flat-Transparent with under light

DISCUSSION and CONCLUSIONS

Cultures of QC organism *M. avium* ATCC 700898 show variable MIC susceptibility results in the Sensititre® SLOMYCO plate (TREK Diagnostic Systems) due to differences in colonial morphotype ratios. This finding corresponds to previously reported *M. avium* clinical strain colony variants and antimicrobial susceptibility trends of different morphotypes[1]. Consequently, due to the variability of *M. avium* ATCC 700898, the parameters of MIC values for QC testing are wider for some antimicrobics than they are for others such as was found here, when both the most resistant (flat-transparent) and susceptible (smooth-domed) morphotypes are considered.

Since a wide range of MIC values can be obtained with *M. avium* ATCC 700898 for specific antimicrobics, it is not only important for QC testing to limit variability between cultures in order to maintain consistent results, but also to perform QC with a culture containing the most resistant morphotype (flat-transparent). There is published evidence that based on both macrophage and animal studies that this variant is more virulent and thus more clinically important[1, 3]. However, selecting for the flat-transparent morphotype can be a challenge.

It has been reported, and observations here agree, that the flat-transparent variant grows slower on agar and is less obvious because of its transparency than pigmented variants such as the smooth-domed[1, 2]. In this study, observation of individual morphotypes required dilution and 2-4 weeks growth. Also, selection of the flat-transparent morphotype can be difficult since it is not entirely stable with a reported rate of transparent to pigmented transition of 10^{-4} to 10^{-5} per bacterium per generation[1, 3]. During testing it was noted that the CFU counts obtained from specific morphotypes contained at least two or more morphotypes. This supports previous reports of morphotype transformation[1, 3]. However, the rate of the transition has previously been found to be temperature dependent[3]. Better optimization of flat-transparent growth may be achieved by increasing temperature and/or length of incubation. This testing is currently ongoing.

Until optimization of growth of the flat-transparent morphotype can be determined, laboratories using *M. avium* ATCC 700898 for QC testing, should observe for morphotype variability between cultures, or routinely purchase new QC isolates of low passage, to maintain consistent results. The flat-transparent morphotype is most commonly seen in low passage clinical strains[1] and likely present in low passage QC isolates.

REFERENCES

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