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Rapid detection of four non-fermenting Gram-negative bacteria directly from cystic fibrosis patient's respiratory samples on the BD MAX™ system

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ABSTRACT

The aim of this study was to develop a multiplex PCR test to detect *Achromobacter xylosoxidans* (AX), *Burkholderia cepacia* (BC), *Pseudomonas aeruginosa* (PSA) and *Stenotrophomonas maltophilia* (SM) directly from CF patient's respiratory samples using the open mode of the BD MAX™ System. A total of 402 CF respiratory samples were evaluated by culture and PCR. Specific sets of primers and probes for each target were designed in-house. Out of 402 samples tested, 196 were identified as negative and 206 as positive by culture for AX, PSA, BC and SM. Among culture positive samples, PCR detected 21/27 AX, 4/5 BC, 138/140 PSA and 29/34 SM. In addition, PCR assay identified 35 samples as positive that were initially negative by culture for those 4 targets. The CF BDM test proved to be an excellent tool to detect AX, BC, PSA and SM by real-time PCR on an automated platform.

1. Introduction

Cystic fibrosis (CF) is a recessive genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator protein (CFTR) [1]. In consequence, transport of chloride and sodium across epithelial cells is compromised resulting in secretions containing a higher concentration of salt and subsequently more viscosity [2]. Dense mucus precludes mucociliary clearance and favors bacterial colonization. As a result, respiratory infection is the most serious complications that CF patients can present and represent the major cause of morbidity and mortality\ on this population [3]. For this reason, CF care involves the use of intensive antimicrobial therapy in younger patients to eradicate initial infection and, in older patients to suppress chronic infection or to treat the intermittent exacerbations of respiratory symptoms characteristic of CF [1].

Pseudomonas aeruginosa (PSA) remains the most common non-fermenting gram-negative bacteria isolated from CF respiratory infections. In the past years, some opportunistic non-fermenting gram-negative bacteria, such as *Burkholderia cepacia* complex (BCC), *Achromobacter xylosoxidans* (AX) and *Stenotrophomonas maltophilia* (SM) have been increasingly reported as causing respiratory infections in these patients [4]. Together, these four microorganisms consist of the most prevalent non-fermenting gram-negative bacteria isolated in respiratory infection of CF patients. Respiratory infections caused by these microorganisms are closely related to loss of the lung function and this fact, added to the emergence of multidrug-resistant bacteria, makes it important to

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rapidly and correctly identify these microorganisms [5].

Culture-based microbiological approaches easily identify pathogens, such as PSA, which are common and prevalent in CF lung infections. On the other hand, it might be challenging for non-pseudomonas bacterial identification, especially in laboratories that are not specialized in CF respiratory examination [6]. For this reason, colonization and infection by these microorganisms can go undetected resulting in negative consequences. In addition, phenotypic techniques are time consuming and results from a traditional culture can take up to 72 h to be available. Early detection of initial respiratory infection, however, allows successful treatment to eradicate or, at least, delay the onset of chronic infections [7,8].

Several techniques have been introduced in the routine of clinical laboratories to accelerate the detection of these pathogens. Polymerase Chain Reaction (PCR) amplification, specifically real-time PCR (qPCR), appears to be an excellent alternative for the diagnosis of bacterial infection directly from respiratory samples of CF patients. Many strategies of qPCR have already been suggested to improve detection [9–11]; none of them, however, use a multiplex test for a concomitant diagnostic of different bacterial strains directly from CF respiratory samples.

The use of automated DNA extraction combined with qPCR avoid the risk of process contamination by reducing sample handling and decrease the test turnaround time which collaborate with early therapy decisions [11]. The BD MAX™ (BDM) System (BD Diagnostic Systems, Sparks, MD) is an automated platform that combines extraction and qPCR on the same instrument. It offers the choice to use BD MAX™ FDA-cleared assays [12] or the open platform mode for user developed tests [13,14].

The aim of this study was to develop a multiplex PCR test to detect *Achromobacter xylosoxidans*, *Burkholderia cepacia* complex, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* directly from CF patient's respiratory samples using the open mode of the BD MAX™ System (BDM).

2. Material and methods

2.1. Cystic fibrosis BD MAX user developed test (CF BDM Test)

2.1.1. Primers and probes

A multiplex real-time PCR was developed to detect AX, BCC, PSA, SM and *Beta-globin* (*BG*, internal control) on the BD MAX System (Table 1). A set of primers and probe to detect PSA, previously described were evaluated in this study [15]. Primers and probes for AX, SM, BCC and BG were designed in-house and were selected based on alignments with sequences collected from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). Sequences were aligned using SeqMan II (DNASTAR 5.0 software) to obtain consensus sequences for each target. The AX alignment included all types of $bla_{OXA-114}$ sequences available on NCBI (types a-n, p-v). The BCC alignment included 9 species from *B. cepacia* complex (*B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthinia* and *B. pyrrocina*). Specific primers and probes were selected by using the Primer3 Program (http://simgene.com/Primer3) and all of them were checked with Oligo-Analyzer 3.0 (http://biotools.idtdna.com.analyzer), an online service of IDT Biotools (Coralville, Iowa) to ensure good parameters (melting temperature, % of Cytosine (C) and Guanine (G) nucleotide, size), minimal self-complementary and to prevent the presence of secondary structures. The binding site was confirmed by Mfold web Server (http://unafold.rna.albany.edu/?q=mfold) to make sure regions that could impede primers and probes to anneal were not present. An NCBI BLAST was performed to check the specificity of the DNA sequences of primers and probes. Primers and probes were synthesized at IDT (Coralville, Iowa) and BG probe at LGT Biosearch Technologies (Petaluma, California).

Table 1
Primers and probes used for the CF BDM Test.

Target	Sequence (5′ to 3′)	Gene
BCC	PF: TCCGGAAAGAAAWCCTTGGY PR: AATGCAGTTCCCAGGTTGAG Pb: **FAM** CGTGCGCAGGCGGTTTGCTA **BHQ1**	16S *rRNA*
AX	PF: CACGAGCCGGTCTGGAA PR: GTGAATACCAGACCACCGAATAC Pb: **JOE** TACCAGCCYGCCTATCCCGACT **BHQ1**	*blaOxa*-114
PSA	PF: ACGACGGTCATGGGCAACT PR: GTGATAGTAGCCGGAGTAGTAGCTGT Pb: **ROX** AAGCTGCTCTCGGAACAGGT **BHQ1**	*regA*
SM	PF: ACTGCGCGTGTARTCGTA PR: GGCATCGATCGKGACACC Pb: **CY5** AAGGCTTCGGCAACAAGCGC **BHQ1**	*metB*
BG	PF: GCAAGGTGAACGTGGATGAA PR: AACCTGTCTTGTAACCTTGATACCAA Pb: **Quasar 705** TTGGTGGTGAGGCCCTGGGC **BHQ3**	*Beta-Globin*

PF: Primer Forward; PR: Primer reverse; Pb: Probe; BHQ 1: Black Hole Quencher 1; BHQ 3: Black Hole Quencher 3.

2.1.2. BD MAX procedure

The new test was performed on the BD MAX open mode System, using the BD MAX™ ExK TNA-2 extraction kit and the BD MAX TNA MMK master mix, along with specific primers and probes for AX, BCC, PSA, SM and *BG* detection. Before testing, thawed samples were transferred into a 500 µL tube of SL (Sputum Liquefying) solution (Copan Diagnostics, California) and incubated for 15 min at room temperature for liquefaction. A 500 µL aliquot of the liquefied sample was treated with 25 µL of Proteinase K (Qiagen, Valencia, CA) at 60 °C for 30 min and at 95 °C for 5 min. A total of 250 µL of each sample was inoculated into the BD MAX TNA-2 extraction kit Sample Buffer Tube. The PCR master mix was distributed in two different tubes snapped into the BD MAX extraction reagent strip. The first tube was the BD MAX TNA MMK PCR reagent mix, which is a lyophilized PCR reagent mix containing dNTPs, $MgCl_2$, Hot Start DNA polymerase and buffers. The second master mix tube was prepared in-house and contained a combination of the five sets of primers and probes (1.8 µM of each primer and 0.4 µM of the probe), 4.25 µL of primer diluent (from BD MAX MMK) and water to complete a 12.5 µL final volume. Primers and probes mix were prepared and added to snap-in tubes at the start of the run. Cycling conditions were as follows: 80 °C for 10 min and 42 cycles of 95 °C for 15 s, 60 °C for 60 s. The PCR gain and threshold were set at 50 and 100 fluorescence detection for AX and BG, 50 and 200 for PSA, 50 and 250 for SM and 50 and 300 for BCC.

2.2. Analytical test

The limit of detection (LoD) for AX, BCC, PSA and SM were evaluated by using the following ATCC strains: *Achromobacter xylosoxidans* ATCC 27061, *Burkholderia cepacia* ATCC 25416, *Pseudomonas aeruginosa* ATCC 27853 and *Stenotrophomonas maltophilia* ATCC 51331. Strains were cultured in BAP and incubated for 24 h at 35 °C. A 0.5 McFarland (1.5×10^8 CFU/ml) suspension of each strain was prepared in ultra-pure water (Sigma-Aldrich, Co. Ltd, Saint Louis, MO, US) followed by seven 10-fold dilutions, also prepared in ultra-pure water. The last PCR positive dilution (i.e. lowest CFU/ml concentration) was cultured on BAP and incubated for 24 h at 35 °C for colony counts. Tests were performed in triplicate to determinate the LoD Ct (cycle threshold), mean Ct and standard deviations (SD). PCR efficiency was calculated from values generated by the standard curve (R^2) values and Specificity tests were carryout by testing species of mycobacteria (n = 29), aerobic bacteria (n = 15) and Candida (n = 7), previously identified at TGH laboratory and commonly isolated from respiratory infections (Table 2).

Table 2
Reference Strains used for analytical sensitivity and specificity validation.

Organism	Reference number	Organism	Reference number
Slow Growing Mycobacteria		*B. pyrrocinia*	TGH Clinical Sample
M. tuberculosis	ATCC 27294	*B, multivorans*	ATCC 11762
M. avium	ATCC 25291	*B. cenocepacia*	TGH Clinical Sample
M. intracellulare	ATCC 13950	*B. stabilis*	TGH Clinical Sample
M. timonense	TGH Clinical Sample	*B. vietnamiensis*	TGH Clinical Sample
M. chimaera	TGH Clinical Sample	*B. dolosa*	TGH Clinical Sample
M. szulgai	TGH Clinical Sample	*Stenotrophomonas maltophilia*	ATCC 51331
M gordonae	TGH Clinical Sample	*Achromobacter xylosoxidans*	ATCC 27061
M. kansasii	TGH Clinical Sample	*Achromobacter denitrificans*	ATCC 15173
M. phocaicum	TGH Clinical Sample	*Pseudomonas aeruginosa*	ATCC 27853
M, nebraskense	TGH Clinical Sample	*Pseudomonas fluorescens*	TGH Clinical Sample
M. marinum	TGH Clinical Sample	*Acinetobacter baumannii*	TGH Clinical Sample
Rapid Growing Mycobacteria		*Klebsiella pneumoniae*	ATCC 700603
M. mucogenicum	*Proteus mirabilis*	*Proteus mirabilis*	TGH Clinical Sample
M. brisbanense	ATCC 49938	*Escherichia coli*	ATCC 25922
M. mageritense	ATCC 700351	*Enterobacter cloacae*	TGH Clinical Sample
M. immunogenum	ATCC 700505	*Neisseria gonorrhoeae*	ATCC 49226
M. abscessus	ATCC 19977	*Neisseria meningitidis*	ATCC 13090
M. bollettii	TGH Clinical Sample	*Haemophilus influenzae*	ATCC 19418
M. massiliense	TGH Clinical Sample	**Gram Positive Bacteria**	
M. chelonae	ATCC 35752	*Staphylococcus epidermidis*	ATCC 14990
M. fortuitum	ATCC 6841	*Staphylococcus aureus*	ATCC 29213
M. porcinum	ATCC 33776	*Streptococcus pneumoniae*	ATCC 19619
M. peregrinum	ATCC 14467	*Streptococcus pyogenes*	ATCC 19615
M. alvei	ATCC 51305	*Enterococcus durans*	ATCC 11516
M. farcinogenes	ATCC 35753	*Enterococcus faecalis*	ATCC 29212
M. boenickei	ATCC 49935	**Candida species**	
M. neworleansense	ATCC 49404	*Candida albicans*	ATCC 14053
M. houstonense	ATCC 49403	*Candida krusei*	ATCC 14243
M. septicum	ATCC 700731	*Candida glabrata*	ATCC MYA 2950
M. senegalense	ATCC BAA-851	*Candida parapsilosis*	ATCC 22019
Gram Negative Bacteria		*Candida tropicalis*	ATCC 750
Burkholderia cepacia	ATCC 25416	*Candida guilliermondi*	TGH Clinical Sample
B. ambifaria	ATCC BAA-244	*Candida dubliniensis*	TGH Clinical Sample
B. anthinia	ATCC BAA-689		

ATCC - American Type Culture Collection ⟨https://www.atcc.org/⟩; TGH - Tampa General Hospital.

To assess the PCR assay's ability to correctly identify more than one target present in a clinical sample, a set of eight clinical samples (known negative by culture and CF BDM test for AX, BCC, SM and PSA) were spiked with 125 µL of McFarland suspension from each ATCC strain control in four different combinations: 1- two samples were spiked with *B. cepacia*, *A. xylosoxidans* and *P. aeruginosa*; 2- two samples with *B. cepacia*, *A. xylosoxidans* and *S. maltophilia*; 3- two samples with *A. xylosoxidans*, *P. aeruginosa* and *S. maltophilia*; 4- two samples with all four ATCC controls.

Statistical analysis for specificity, sensitivity and Cohen's kappa test [16] were performed using the SPSS version 17.0 statistical software to compare the results between phenotypic and CF BD MAX test. Results with a p-value < 0.05 were considered statistically significant

2.3. Clinical specimens

A total of 402 respiratory samples were tested by PCR and culture in this study. Respiratory samples were collected from CF patients attending the Tampa General Hospital (TGH; Florida, USA) and the Children's Healthcare of Atlanta (CHOA; Georgia, USA). At TGH samples were cultured on *Burkholderia cepacia* selective agar (Hardy Diagnostics, Santa Maria, CA), TSA with 5% Sheep Blood (aerobic and anaerobic conditions), MacConkey II Agar and Chocolate II Agar (BD Diagnostic Systems, Sparks, MD). The media used at CHOA was purchased from Remel (Lenexa, KS) and included Chocolate Agar (CHOC), TSA with 5% Sheep Blood (BAP), MacConkey Agar, Colistin Nalidixic Acid Agar, Mannitol Salt Agar and *Burkholderia cepacia* selective Agar. The CHOC and BAP were incubated in CO_2, while the remaining media were incubated in an ambient air incubator. All plates were incubated at 35 °C. Cultured organisms were identified by the VITEK® MS system (BioMérieux, France). Semi-quantitative culture results were reported accordingly to the number of colonies of each species identified, such as “rare”, “light”, “moderate”, “many” or “heavy”. Respiratory residual specimens were stored at − 80 °C until they were tested by the new CF BDM Test.

2.4. Discrepant results samples

Samples with discrepant results between culture and the new CF BDM test were retested by PCR and culture to confirm previous results.

3. Results

3.1. Analytical studies

The LoD, mean Ct, SD, R^2 and efficiency for AX, BCC, PSA and SM targets are described in Table 3. The last PCR positive dilution confirmed by colony count was 10^2 CFU/ml for AX, BCC and PSA and 10^3 CFU/ml for SM.

A total of 52 different species of microorganisms (26 mycobacteria species, 19 non-mycobacteria species and 7 *Candida* species) were used to evaluate the CF BDM test primers and probes specificity. The new CF BDM test probes were specific, didn’t present cross reactivity to related or unrelated organisms and correctly identified the species corresponding to each PCR target. AX set was specific to *A. xylosoxdans* specie only, BCC set was specific to all 9 *Burkholderia* species tested and SM and PSA set was specific to *S. maltophilia* and *P. aeruginosa,* respectively. The 8 negative samples spiked with more than one of the bacterial targets were correctly identified by the CF BDM test.

3.2. Clinical studies

A total of 402 respiratory samples collected from CF patients were included in the study. Of these, 227 were identified as negative and 175 as positive by culture, for at least one of the targets tested by PCR (AX, BCC, PSA and SM). Among culture positive samples,

Table 3
CF BDM Test Limit of Detection (LoD) for BCC, AX, PSA and SM.

Target	Bacteria Control	LoD CFU/ml	LoD CFU/ml repetition Ct	Mean Ct (SD)	R^2	Efficiency (%)
BCC	***B. cepacia* ATCC 25416**	10^2	35 35 35.3	35.1 (0.17)	0.99	93
AX	***A. xylosoxidans* ATCC 27061**	10^2	39.4 38.9 40.8	39.7(0.98)	0.99	98.5
PSA	***P. aeruginosa* ATCC 27853**	10^2	35.5 35.5 36.5	35.8(0.58)	0.99	108
SM	***S. maltophilia* ATCC 51331**	10^3	37 36.5 37	36.8(0.29)	0.99	103

LOD: Limit of Detection; Ct: Cycle Threshold; SD: standard deviations; R^2: coefficient of determination.

Table 4
Comparison of the new CF BDM PCR test and culture results from 402 CF samples.

	CF BDM PCR Negative/ Culture Negative	CF BDM PCR Negative/ Culture Positive	CF BDM PCR Positive/ Culture Negative	CF BDM PCR Positive/ Culture Positive
***P. aeruginosa* (PSA)**	250	2	12	138
***B. cepacia* complex (BCC)**[a]	395	1	2	4
***A. xylosoxidans* (AX)**[b]	367	6	8	21
***S. maltophilia* (SM)**	355	5	13	29

[a] Culture results were identified as *B. cepacia* by the Vitek® MS System.
[b] Culture results were identified as *A. xylosoxidans_denitrificans* by the Vitek® MS System.

111 were positive only for PSA, 21 for SM, 11 for AX, 3 for BCC, 14 for PSA and AX, 11 for PSA and SM, 2 for PSA and BCC and 2 for PSA, SM and AX.

Results from the new CF BDM test were compared to the standard of care CF culture. Four out of five BCC, 21 out of 27 AX, 138 out of 140 PSA and 29 out of 34 SM were correctly identified by the new CF BDM test. This data includes strains isolated from single organism and mixed cultures. In addition, the new CF BDM test was positive for two BCC, eight AX, 12 PSA and 13 SM among culture negative specimens (Table 4).

A total of 49 discrepant results were observed between culture and CF BDM test. Of these, 35 samples were negative by culture and positive by the new CF BDM test for at least one of the four targets tested (two were positive for BCC, eight were positive for AX, 12 were positive for PSA and 13 were positive for SM). Another 14 samples were positive by culture for at least one of the four targets tested and negative by the CF BDM test (one was positive for BCC, six were positive for AX, two were positive for PSA and five were positive for SM). All 49 samples with discrepant results were retested by culture and by the new CF BDM test. On retesting, all PCR results were in agreement with prior PCR results. Results from cultures, however, were different then prior results. All of the cultures were negative after retesting; including the 14 previously culture positive specimens.

After PCR results were compared to culture, the CF BDM test presented a sensitivity and specificity of: 78% and 97% for AX; 80% and 99% for BCC; 99% and 97% for PSA and 85% and 96% for SM, respectively. The Kappa test was 0.73 (moderate level of agreement) for AX; 0.72 (moderate level of agreement) for BCC; 0.92 (almost perfect level of agreement) for PSA and 0.74 (moderate level of agreement) for SM (Table 4). The overall agreement between culture and the new CF BDM Test was 99.3% for BCC, 96.5% for AX, 96.5% for PSA, and 95.5% for SM. All statistics were based on initial results.

4. Discussion

Respiratory infections remain a major threat to CF patients. Rapid detection and correct identification of the bacteria implicated in these infections is critical for therapeutic management of these patients [3,4,7,17].

Cultured based methods still remain the gold standard for CF respiratory infection diagnosis. However, culture-based analysis can be problematic since the process is time-consuming, potentially inaccurate, and requires species-specific selective media [18]. Cultivation allows an assessment of all pathogens present in the specimen evaluated; though, in cases of polymicrobial cultures, commonly found in CF patients, faster growing organisms can overgrow or obscure slower growing organisms, or those present in lesser numbers. More fundamentally, culture might miss organisms that have formed biofilms in vivo and other organisms that may not grow in current culture media incubated in routine clinical environments (atmosphere and temperature) [19–21].

Culture-independent studies using molecular methods have allowed a more precise evaluation of the microbial diversity in the lung of CF patients [19,20,22]. Different approaches applying such methods have been extensively used to study the microbial communities, including real-time PCR, Sanger sequencing, pyrosequencing and lately, next-generation sequencing. The strengths of these methods are that they can be performed directly from clinical samples without any need for culturing [23]. In addition, data generated from these techniques has begun to alter our thinking about the CF microbiota and distinctive features of the CF airway affecting bacterial growth [23].

The present study aimed to develop a multiplex real-time PCR for rapid detection of four important gram-negative pathogens, commonly isolated from CF patient's respiratory samples [18,24,25].

The new test was developed to work on the BD MAX, a fully automated walk-away system, which combines extraction and amplification steps in an open-mode platform and has the advantage of reduced hands-on time and low risk of reaction contamination. Moreover, in contrast to other fully automated systems that can run only one sample at a time, the BD MAX can test up to 24 samples concomitantly [12–14]. The overall agreement between the culture and PCR for 402 respiratory samples analyzed was 99.3% for BCC, 96.5% for AX, 96.5% for PSA, and 95.5% for SM. Of the 49 samples with discrepant results, 35 were negative by culture and positive by the new CF BDM test for at least one of the four targets tested, and 14 were positive by culture and negative by PCR. Culture limitations described above could possibly explain these 35 culture negative discrepant results, due to polymicrobial growth with potential overgrowth with PSA in particular. A limitation of our study is not having a third technique to resolve the

discrepant results between culture and the CF BDM test. In spite of this, the results from this study presented a high overall agreement between the two methods analyzed and it proved to be a sensitive and rapid test to detect four gram-negative bacteria commonly found in the lung of CF patients.

The availability of a rapid test for identification of common bacterial species associated with severe respiratory infections can improve the health and prognosis for the CF patients treated all over the world. In this instance, molecular diagnostic assays may provide the only reliable method to detect the presence of these organisms [19–23]. If results from respiratory samples are available sooner, appropriate antimicrobial treatment can be initiated sooner and the lung damage caused by these infections may be avoided or at least, postponed. In addition, the dissemination of some contagious bacteria around the hospital and among CF patients may be prevented. The clinical importance and infection control implications of specific bacterial organisms such as PSA and BCC in CF patients make rapid and accurate identification imperative. Finally, such testing and targeted treatment may help to delay the need for lung transplantation and allow a better management of the patient after transplantation, since these organisms can be also problematic for post-transplant patients.

It is clear that both, culture-based and molecular methods can contribute to the clinical management of CF respiratory infections, and in many ways, the two methods are complementary. Benefits of culture-based methods include the inexpensive cost and the ability to identify viable organisms that can be archived for further epidemiological and antimicrobial studies [10,18,21]. However, culture independent tests, such as real-time PCR performed directly on CF respiratory samples, appears to offer advantages for detection of polymicrobial infections and uncultivable strains, yielding results more rapidly than culture [20,22]. The new CF BDM test described in this study was able to test up to 24 samples in less than 4 h. Results from culture, on the other hand, took up to 72 h to be available.

To conclude, our study presents a new CF BDM test, which was able to identify directly from respiratory samples four gram-negative pathogens, commonly found in the respiratory tract of CF patients. The new test presented a high overall agreement for all four targets tested, on a walkaway automated molecular platform. The ability to test a direct specimen may aid in guiding the administration of prophylaxis in patients with severe respiratory diseases and in pre- and post-lung transplant patients. This new BD MAX assay for the detection of four major gram-negative organisms in CF is a powerful tool for rapid detection of organisms and will play a pivotal role in adjunct to culture.

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Conflict of interest statement

The authors wish to confirm that there are no known conflicts of interest associated with this publication. There has been no significant financial support for this work that could have influenced its outcome.

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