European Journal of Clinical Microbiology & Infectious Diseases (2020) 39:2037–2044
https://doi.org/10.1007/s10096-020-03937-1

ORIGINAL ARTICLE

Multicenter evaluation of the RAPIDEC® CARBA NP assay for the detection of carbapenemase production in clinical isolates of Enterobacterales and *Pseudomonas aeruginosa*

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Received: 11 February 2020 / Accepted: 29 May 2020 / Published online: 23 June 2020

Abstract

Carbapenem-resistant Gram-negative bacilli are a major public health problem. Accurate and rapid detection of carbapenemase-producing organisms can facilitate appropriate infection prevention measures. The objective was to evaluate the performance of the RAPIDEC® CARBA NP assay (RAPIDEC), a screening assay that utilizes a pH indicator to detect carbapenem hydrolysis within 2 h. A multicenter study evaluated 306 clinical bacterial strains of Enterobacterales ($n = 257$) and *Pseudomonas aeruginosa* ($n = 49$). The RAPIDEC was compared to a composite reference standard—the Clinical Laboratory Standards Institute (CLSI) Carba NP assay, PCR for specific carbapenemase genes (bla_{KPC}, bla_{NDM}, $bla_{OXA\text{-}48\text{-}like}$, bla_{VIM} and bla_{IMP}), and phenotypic carbapenem susceptibility testing. The assay was evaluated using two culture incubation times for the bacterial isolates: "routine"(cultures incubated 18-24 h) and "short" (cultures incubated 4-5 h). For the routine incubation, the overall percent agreement was 98.7% with a positive percent agreement (PPA) of 99.6% and a negative percent agreement (NPA) of 97.4%; there were five false positives and one false negative. For the short incubation, the overall percent agreement was 98.0% with a PPA of 98.5% and a NPA of 97.3%; there were five false positives and four false negatives. RAPIDEC results for the *P. aeruginosa* isolates were 100% concordant with the reference standard for both incubation times. The RAPIDEC assay is an accurate and rapid (≤ 2 h) assay for the detection of the most common carbapenemases in clinical isolates. Growth from a short incubation culture may be used to reliably detect carbapenemase production in clinical strains.

Keywords Carba NP · CP-GNB · *Pseudomonas* · Carbapenemase production · Enterobacterales

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10096-020-03937-1) contains supplementary material, which is available to authorized users.

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Introduction

Carbapenemase-producing Gram-negative bacilli (CP-GNB) are a major public health problem [1]. Carbapenems are broad-spectrum antimicrobials and frequently represent "drugs of last resort" for the treatment of severe infections. Carbapenemases, enzymes that degrade carbapenems and other β-lactams, are encoded by genes located on mobile genetic elements, facilitating spread in healthcare facilities.

There are numerous carbapenemase genes that have been identified in Enterobacterales and non-fermenting Gram-negative bacteria including *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. These genes are classified according to their molecular properties and in the USA most frequently include the *Klebsiella pneumoniae* carbapenemase (bla_{KPC}) (Ambler class A), New Delhi metallo-β-lactamase (bla_{NDM}) (Ambler class B), and OXA-48-like ($bla_{OXA\text{-}48\text{-}like}$)

(Ambler class D) [2, 3]. Additionally, carbapenem resistance can be conferred by mechanisms other than carbapenemases, such as an AmpC β-lactamase combined with a porin mutation [4–7]. The ability to differentiate between CP-GNB and other resistance mechanisms is important for infection control, treatment and epidemiological surveillance [8].

Multiple tests have been developed to facilitate the accurate detection of carbapenemases in bacterial isolates. The modified Hodge test (MHT), which was historically recommended by the Clinical Laboratory Standards Institute (CLSI) for carbapenemase confirmation, has limitations for both the analytical sensitivity and specificity. For example, isolates producing the NDM carbapenemase have been shown to yield a false-negative result [9, 10]. As a result, CLSI no longer recommends the usage of MHT and currently endorses the use of the Carba NP test and the modified carbapenem inactivation method (mCIM) [11]. The Carba NP test is a colorimetric assay that detects the hydrolysis of the β-lactam ring of imipenem [12]. Like the MHT, this assay also has limitations including low sensitivity for the detection of the OXA-48-like enzyme and its variants [9]. The mCIM measures an isolate's ability to degrade meropenem utilizing readily available materials in a clinical laboratory. Recent studies have shown that this method has high sensitivity and specificity [9, 10, 13, 14]. The method has also been modified for the detection of carbapenemase production in *P. aeruginosa* [15–17].

Adapted from the Carba NP test, the RAPIDEC® CARBA NP assay (bioMérieux) is a commercially available, FDA-cleared assay for the phenotypic detection of the five most common carbapenemases in Enterobacterales and *P. aeruginosa* with results available in 30 min to 2 h. The objective of this multicenter study was to evaluate the analytical performance characteristics of the RAPIDEC® CARBA NP assay.

Materials and methods

Clinical evaluation sites

The evaluation of the bioMérieux RAPIDEC® CARBA NP was performed at five sites: Cleveland Clinic (Cleveland, OH), International Health Management Associates (IHMA) (Schaumburg IL), New York Presbyterian Queens (Flushing, NY), University of California- Los Angeles (UCLA) Health (Los Angeles, CA), and Washington University School of Medicine (St. Louis, MO). Institutional review board (IRB) approval was obtained at each study site prior trial initiation.

Quality control

Quality control (QC) organisms included *K. pneumoniae* ATCC BAA-1705 (bla_{KPC} positive), *K. pneumoniae* ATCC BAA-1706 (bla_{KPC} negative), and *K. pneumoniae* ATCC 700603 (ESBL-positive, carbapenemase-negative). QC organisms were tested on each day of testing at all clinical trial sites and were tested at a minimum of 20 replicates on the RAPIDEC CARBA NP test. No more than five replicates of each isolate were tested per day. QC organisms were tested using both the routine and short incubation procedures.

Clinical isolates

Clinical isolates of Enterobacterales and *P. aeruginosa* were tested from both fresh and stock isolates. A fresh clinical isolate was defined as one that had been isolated from a clinical specimen in the past 7 days and stored on an agar plate or slant at room temperature or refrigerated. A stock isolate was defined as an isolate that had been stored (typically frozen at − 70 °C) for future sub-culture. Stock isolates were previously characterized at each testing site as carbapenemase-positive by the site's methodologies and represented various enzyme types (KPC, NDM, VIM, OXA-48-like, and/or IMP) (Table 1). For RAPIDEC CARBA NP testing, two culture incubations were evaluated: routine (18 to 24 h) and short (4 to 5 h). For the routine culture incubation, fresh clinical isolates were sub-cultured onto trypticase soy agar with 5% sheep blood and incubated in ambient air for 18 to 24 h prior to testing. Frozen stock and challenge isolates were passaged a second time on sheep blood agar and incubated for 18 to 24 h prior to testing. For the short culture incubation, fresh clinical isolates were first sub-cultured onto MacConkey agar and incubated for 18 to 24 h then sub-cultured to sheep blood agar and incubated for 4 to 5 h to allow for sufficient growth to perform testing. Frozen stock and challenge isolates were sub-cultured twice before being tested with an initial sub-culture to sheep blood agar and incubated for 18 to 24 h. The isolates were then sub-cultured to MacConkey agar and incubated for 18 to 24 h followed by a final sub-culture to sheep blood agar and incubated for 4-5 h.

Challenge isolates

A challenge set consisting of 151 well-characterized isolates (sourced from bioMérieux internal stock collection and the FDA-CDC AR bank) was evaluated at two sites, University of California—Los Angeles (bioMérieux isolates) and International Health Management Associates (FDA-CDC AR bank isolates). Challenge isolates were incubated using both the short and routine culture incubation times as described for the clinical isolates. All challenge isolates were tested at a single site.

Reproducibility

Ten well-characterized isolates were tested in triplicate for a total of 6 days by two different operators at three different

Table 1 Description of isolates evaluated (clinical and challenge organisms)

Carbapenemase	Total tested	Enterobacterales	*P. aeruginosa*	Site 1	Site 2	Site 3	Site 4	Site 5
Routine culture incubation								
Negative	192	149	20	32	41	45	37	37
Positive	265	245	43	21	114	24	89	17
KPC	147	145[1]	3	20	33	24	56	17
NDM	52	51	1	0	37	0	12	3
VIM	26	15	11	0	19	0	7	0
IMP	17	12	5	0	13	0	4	0
OXA-48-like	23	23[1]	0	1	12	0	10	0
Total	457	394	63	53	155	69	126	54
Short culture incubation								
Negative	188	148	40	29	40	45	37	37
Positive	261	244	17	20	111	24	89	17
KPC	146	144[a]	3	19	33	24	56	17
NDM	52	51	1	0	37	0	12	3
VIM	23	15	8	0	16	0	7	0
IMP	17	12	5	0	13	0	4	0
OXA-48-like	23	23[1]	0	1	12	0	10	0
Total	449	392	57	49	151	69	126	54

KPC Klebsiella pneumoniae carbapenemase, *NDM* New Delhi metallo-β-lactamase, *VIM* Verona integrin-encoded metallo-β-lactamase, *IMP* imipenem-resistant *Pseudomonas*, *OXA-48* oxacillinase-48

[a] One isolate was positive for both KPC and OXA-48

clinical trial sites. Reproducibility was performed for both the routine and short culture incubation procedures.

RAPIDEC® CARBA NP test

The RAPIDEC® CARBA NP assay was performed according to the manufacturer's guidelines. One hundred (100) microliters of API suspension medium was dispensed into each of three wells: (a), (b), and (c). With the lid on the strip, the strip was incubated for 4–10 min at room temperature (15–25 °C). Next, the contents in well (b) were gently mixed using the provided stick. Using a new stick, several colonies of the test organism were picked up and deposited into well (c) and mixed until the turbidity was at least equivalent to that of well (b). The strip was then incubated at room temperature for 30 min. After incubation, 25 μL from well (c) was transferred to both wells (d) and (e). Next, 25 μL from well (a) was transferred to wells (d) and (e). The strip was then incubated at 33–38 °C for 30–40 min. Reading of test results was performed by comparing the colors in well (d) and (e) using the provided reading guide. A test was positive when a significant variation in color was observed between the two wells. For a negative or indeterminate reaction, the test was re-incubated for an additional 90 min (total test time was not to exceed 2 h). For mucoid strains, 100 μL of the API suspension medium was added to well (a) (well (b) was not used). The strip was incubated for 4–10 min at room temperature. During this incubation time, 150 μL of the API suspension medium was added to a 1.-mL tube with two 3-mm diameter glass beads. A 10-μL loop full of bacterial colonies was added to the tube using the stick provided in the kit. The tube was vortexed until the mucoid appearance of the suspension had disappeared enough for the suspension to be easily pipetted; 100 μL of this suspension was dispensed into well (c) and incubated for 30 min at room temperature. The remaining test procedure was followed as shown above.

Reference standard

The composite reference standard for this study was based on three different tests for carbapenemase determination: the CLSI Carba NP test, PCR, and carbapenem MIC testing (imipenem (IMP), meropenem (MEM), and ertapenem (ETP) for Enterobacterales and imipenem, meropenem, and doripenem (DOR) for *P. aeruginosa*) (Supplemental Table). Methods for PCR testing are described in detail in the Supplemental Methods. MIC testing was performed using the GN-71 (IMP, MER, and ERT) and GN-68 (DOR) cards on the VITEK2 instrument (bioMérieux) per manufacturer's instructions using the FDA-approved breakpoints (CLSI

M100-S20). Isolates were considered carbapenem not susceptible if they tested as either intermediate or resistant to at least one of the carbapenems tested.

Statistical analysis

Clinical trial results obtained from the RAPIDEC® CARBA NP test were compared to the test results obtained from the composite reference methods. Positive and negative percent agreement data were calculated. For discrepant results, a major error was considered for any isolate that was positive by the RAPIDEC® CARBA NP assay, but negative by the composite reference method and a very major error occurred when the RAPIDEC® CARBA NP assay was negative, but the isolate was positive for a carbapenemase by the composite reference method. Any sample in which the RAPIDEC® CARBA NP result was not interpretable was excluded from the study (< 5%).

Results

Bacterial isolates

A total of 485 unique isolates were tested. For the routine and short culture incubations, 306 and 300 clinical isolates and 151 and 149 challenge isolates, respectively, were included in the final analyses. Isolates excluded from the final analyses included those which were determined to have a carbapenemase enzyme other than KPC, NDM, OXA-48-like, VIM, and IMP ($n = 22$); a hyper-mucoid phenotype ($n = 3$); and isolates which were determined to have lost the resistance mechanism during serial sub-culture ($n = 3$). For the short culture incubation, an additional eight isolates were excluded due to insufficient growth for testing following the short incubation.

Quality control testing

All quality control results ($n = 478$ results for routine culture incubation and $n = 501$ results for short culture incubation) for both culture incubation times generated expected results for all isolates and at all sites performing testing.

Reproducibility studies

For the routine culture incubation, 98.2% (884/900) of the tests agreed with the expected results. The reproducibility rate was slightly higher at 99.1% (892/900) using the short culture incubation. All results that were in disagreement occurred with the same isolate at two different sites, which was linked to partial loss of expected enzyme activity. Reproducibility for that isolate ranged from 60.0 to 86.7% at the different sites with the different culture incubations.

Challenge studies

Routine culture incubation

With the routine culture incubation period (18–24 h before testing), the overall percent agreement was 99.3% with a positive percent agreement (PPA) of 99.1% and a negative percent agreement (NPA) of 100.0% (Table 2). There were no false-positive results and only one false-negative result (Table 4). The false-negative result occurred with a KPC positive *Proteus mirabilis*. For this isolate, only imipenem (MIC = 16.0 μg/mL) was resistant using the Vitek 2; MEM (MIC = 0.5 μg/mL) and ETP (MIC = 1.0 μg/mL) were susceptible. The KPC-positive result was determined using PCR.

Short culture incubation

Using the shorter culture incubation time provided similar results to the routine culture incubation. The overall percent agreement was 97.9%, with a PPA of 97.3% and an NPA of 100.0% (Table 2). Again, there were no false-positive results (Table 3), but there were three false-negative results. The false-negative results occurred with the same *P. mirabilis* from the routine culture incubation in addition to an *Enterobacter cloacae* (VIM) and a *Klebsiella pneumoniae* (NDM) isolate (Table 5). The *E. cloacae* (MEM > 16 μg/mL, IMP > 16 μg/mL, ETP > 8 μg/mL) and *K. pneumoniae* (MER >8 μg/mL, IMP 32 μg/mL, ETP > 8 μg/mL) isolates had very high MICs to the carbapenems tested.

Clinical isolate studies

Routine culture incubation

The overall percent agreement was 98.4% with a positive percent agreement (PPA) of 100.0% and a negative percent agreement (NPA) of 96.8% (Table 2). There were five (3.2%) false-positive results (Table 3). Four of the false-positive isolates were negative by all other methods (VITEK® 2 susceptibility testing, PCR and CLSI Carba NP), but one isolate was positive for the bla_{KPC} gene by PCR, but negative for the presence a carbapenemase by the other two methods. All errors occurred with Enterobacterales (Tables 3 and 4). False-positive errors occurred in *Escherichia coli* (3), *K. pneumoniae* (1), and *Serratia marcescens* (1) isolates. There was 100% agreement for all *P. aeruginosa* isolates tested (Table 4).

Table 2 Analytical performance characteristics of the RAPIDEC® CARBA NP test

	Total	Negative	Positive	Overall percent agreement (%)	PPA (%)	NPA (%)
Routine culture incubation						
Clinical	306	155	151	98.4 (301/306)	100.0 (151/151)	96.8 (150/155)
Challenge	151	37	114	99.3 (150/151)	99.1 (113/114)	100.0 (37/37)
Combined	457	192	265	98.7 (451/457)	99.6 (264/265)	97.4 (187/192)
Short culture incubation						
Clinical	300	152	148	98.0 (294/300)	99.3 (147/148)	96.7 (147/152)
Challenge	149	36	113	97.9 (146/149)	97.3 (110/113)	100 (36/36)
Combined	449	188	261	98.0 (440/449)	98.5 (257/261)	97.3 (183/188)

PPA positive percent agreement, *NPA* negative percent agreement

Short culture incubation

The overall percent agreement was 98.0%, with a PPA of 99.3% and an NPA of 96.7% (Table 2). Again, there were five (3.3%) false-positive results (Table 3), all of which also provided false-positive results with the routine culture incubation (Table 3). In addition, there was one false-negative result. The false-negative result occurred with an *E. cloacae* isolate with an OXA-48-like enzyme (MICs: MER 4 μg/mL, IMP 8 μg/mL, ETP > 8 μg/mL (Table 4).

This study also evaluated the performance of the RAPIDEC® CARBA NP assay using fresh (< 7 days) and stock (frozen) isolates. For the clinical isolates, four out of the five false-positive errors occurred with stock isolates (routine and short culture incubations), and the false-negative result occurring with the short culture incubation period also occurred in a stock isolate (Table 5). Only one error (false negative) occurred using a fresh isolate. All challenge isolates were stock isolates and had four false negatives.

Discussion

The emergence and rapid spread of CP-GNB is a world-wide public health concern. KPC-producing organisms are endemic in the USA, with other carbapenemases being isolated at an

Table 3 Analytical performance characteristics by enzyme group

Enzyme group	Number of samples	True positive	False negative	True negative	False positive
Routine					
IMP	17	17	0		
KPC	147	146	1		
NDM	52	52	0		
VIM	26	26	0		
OXA 48-like	23	23	0		
Carbapenemase enzymes	**265**	**264**	**1**		
Non-carbapenemase enzymes	**192**			**187**	**5**
All enzymes	**457**	**264**	**1**	**187**	**5**
Short					
IMP	17	17	0		
KPC	146	145	1		
NDM	52	51	1		
VIM	23	22	1		
OXA 48-like	23	22	1		
Carbapenemase enzymes	**261**	**257**	**4**		
Non-carbapenemase enzymes	**188**			**183**	**5**
All Enzymes	**449**	**257**	**4**	**183**	**5**

KPC Klebsiella pneumoniae carbapenemase, *NDM* New Delhi metallo-β-lactamase, *VIM* Verona integrin-encoded metallo-β-lactamase, *IMP* imipenem-resistant *Pseudomonas*, *OXA-48* oxacillinase-48

Table 4 Description and evaluation of discrepant results

Culture incubation	Organism	Clinical sample type	Isolate Type	RAPIDEC Interp	MIC IMP	MIC MEM	MIC ETP	MIC Interp	CarbaNP result	PCR result	PCR Interp	Sample status	Discrepancy
Routine, short	*S. marcescens*	Stock	Clinical	POS	> 16	> 16	> 8	NS	Negative	Negative for all enzymes tested	Negative	NEG	Major error
Routine, short	*E. coli*	Fresh, < 7 days	Clinical	POS	4	> 16	4	NS	Negative	Negative for all enzymes tested	Negative	NEG	Major error
Routine, short	*K. pneumoniae*	Stock	Clinical	POS	2	> 16	> 8	NS	Negative	Negative for all enzymes tested	Negative	NEG	Major error
Routine, short	*E. coli*	Stock	Clinical	POS	< 0.25	0.5	4	NS	Negative	Negative for all enzymes tested	Negative	NEG	Major error
Routine, short	*E. coli*	Stock	Clinical	POS	< 0.25	< 0.25	< 0.5	S	Negative	KPC	Positive	NEG	Major error
Short	*E. cloacae*	Stock	Clinical	NEG	8	4	> 8	NS	Negative	OXA-48-like	Positive	POS	Very major error
Routine, short	*P. mirabilis*	Stock	Challenge	NEG	16	0.5	1	NS	N/A	KPC	Positive	POS	Very major error
Short	*E. cloacae*	Stock	Challenge	NEG	> 16	> 16	> 8	NS	Invalid	VIM	Positive	POS	Very major error
Short	*K. pneumoniae*	Stock	Challenge	NEG	32	> 8	> 8	NS	N/A	NDM	Positive	POS	Very major error

POS positive, *NEG* negative, *NS* not susceptible, *S* susceptible, *KPC Klebsiella pneumoniae* carbapenemase, *VIM* Verona integrin-encoded metallo-β-lactamase, *OXA-48* oxacillinase-48

increasing frequency [2, 18]. In 2018, the CDC has added carbapenemase-producing Enterobacterales (CP-CRE; *E. coli*, *Enterobacter* spp. and *Klebsiella* spp.) as a National Notifiable Disease [19]. Laboratories now have a greater need for a rapid and simple method to detect the expression of these enzymes in clinical isolates. The RAPIDEC® CARBA NP assay is FDA-cleared for the detection of carbapenemases in Enterobacterales and *P. aeruginosa* and is simple to perform and less expensive as compared to PCR. The clinical trial data presented here from five different sites shows that the assay has an overall PPA of 99.6% and NPA of 97.4% using both clinical and challenge isolates with a routine culture incubation period (18–24 h). Additionally, the assay was evaluated using a short culture incubation (4–5 h) and demonstrated a PPA of 98.5% and NPA of 97.3%.

The results of our study are similar to previous evaluations of the assay testing Enterobacterales and *P. aeruginosa* [9, 20–27]. Overall, in all isolates tested, there were five false-positive and one false-negative result. All incorrect results occurred with Enterobacterales isolates. It is possible that the *Serratia marcescens* isolate, which was considered a false positive based on the reference method utilized in this study, may actually contain a SME carbapenemase which has been previously described in *S. marcescens* isolates but was not specifically tested for herein [28, 29]. For the five carbapenemase enzymes detected by the reference methods, there was one false-negative isolate for each KPC, NDM, OXA-48-like, and VIM enzyme with the short culture incubation period and only one false-negative result for the KPC enzyme with the routine culture incubation. The shortened culture incubation time may not have allowed for full expression of the enzymes and therefore was not detected. False-negative results using the shorter culture incubation period were also reported in a previous study [21].

Previous studies examining tests for carbapenemase detection have shown lower sensitivities for isolates containing OXA-48-like enzymes [9, 30–34]. Our study examined 23 OXA-48-like containing isolates with only one false-negative result. That isolate was correctly detected using the routine culture incubation but was missed with the short culture incubation. This again may be due to decreased expression of the carbapenemase in the shortened culture incubation time. Other studies examining this assay have also demonstrated the need for the longer culture incubation time for the detection of the OXA-48-like enzyme [21]. Previous studies have shown that the RAPIDEC® CARBA NP assay has higher sensitivity for the detection of OXA-48-like enzyme as compared to other colorimetric assays [9, 22, 24], but several studies using the RAPIDEC® CARBA NP assay did report false-negative results for isolates containing the OXA-48-like enzyme [9, 23, 25]. The increased sensitivity for OXA-48-like enzymes may be, at least partially, due to

Table 5 Percent agreement for organism groups tested

Organism group	Number of samples	True positive	False negative	True negative	False positive	PPA (%)	NPA (%)
Routine							
Enterobacterales	245	244	1			99.6	
Pseudomonas aeruginosa	20	20	0			100.0	
All positive organisms	**265**	**264**	**1**			**99.6**	
Enterobacteriales	149			144	5		96.6
Pseudomonas aeruginosa	43			43	0		100.0
All negative organisms	**192**			**187**	**5**		**97.4**
Short							
Enterobacteriales	244	240	4			98.4	
Pseudomonas aeruginosa	17	17	0			100.0	
All positive organisms	**261**	**257**	**4**			**98.5**	
Enterobacteriales	148			143	5		96.6
Pseudomonas aeruginosa	40			40	0		100.0
All negative organisms	**188**			**183**	**5**		**97.3**

the increased inoculum size (10 μL) of the RAPIDEC® CARBA NP assay as compared to the CLSI Carba NP assay (1 μL) [22].

Strengths of this study include a large number of isolates evaluated from five different clinical sites, representing multiple different genera and carbapenemase enzymes. Limitations of this study include a relatively low number of isolates containing OXA-48-like, VIM, and IMP carbapenemase-containing isolates evaluated. Additionally, isolates expressing carbapenemases other than KPC, OXA-48-like, NDM, VIM, and IMP were not represented. Although not an issue in this current study, others have reported some difficulty in the interpretation of the color variation for determining the final results, especially for weaker carbapenemases (weak positives) [35].

In conclusion, the RAPIDEC® CARBA NP assay is an FDA-cleared test that can be utilized to confirm the presence of carbapenemases in Enterobacterales and *P. aeruginosa*. It is an accurate and rapid assay that can be utilized by clinical laboratories as part of infection control and antimicrobial stewardship programs for CP-GNB.

Funding information This study was funded by bioMérieux. These data were submitted to the US FDA for IVD clearance of this assay.

Compliance with ethical standards

Conflict of interest A.R.M. has received speaker fees from bioMerieux, Roche and Cepheid. V.L. Speaker Fees: bioMerieux. S.S.R. has been an employee of bioMerieux since August 12, 2019. When employed at Cleveland Clinic, she received research funding from bioMerieux, BD Diagnostics, Roche, Diasorin, Accelerate, and Affinity Biosensors. C.-A.B. Consulting: Monsanto, Thermo Fisher, Research Support: Cepheid, Luminex, bioMerieux, Theravance, Aperture Bio, Speaker Fees: Bruker, BD.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Ethical approval retrospective studies Although retrospective studies are conducted on already available data or biological material (for which formal consent may not be needed or is difficult to obtain), ethical approval may be required dependent on the law and the national ethical guidelines of a country. The authors should check with their institution to make sure they are complying with the specific requirements of their country.

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