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Evaluation of NG-Test CARBA 5 version 2, Cepheid Xpert Carba-R, and carbapenem inactivation methods in comparison to whole-genome sequencing for the identification of carbapenemases in non-fermenting Gram-negative bacilli

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ABSTRACT NG-Test CARBA 5 (NG-Biotech) is a rapid *in vitro* multiplex immunoassay for the phenotypic detection and differentiation of the "big five" carbapenemase families (KPC, OXA-48-like, VIM, IMP, and NDM). Version 2 of this assay was evaluated alongside the Xpert Carba-R assay (Cepheid, Inc.), the modified carbapenem inactivation method (mCIM), and the CIMTris assay, with a collection of carbapenem-resistant non-fermenting Gram-negative bacilli comprising 138 *Pseudomonas aeruginosa* and 97 *Acinetobacter baumannii* isolates. Whole-genome sequencing (WGS) was used as the reference standard. For *P. aeruginosa*, NG-Test CARBA 5 produced an overall percentage agreement (OPA) with WGS of 97.1%, compared with 92.8% forXpert Carba-R and 90.6% for mCIM. For *A. baumannii*, as OXA-type carbapenemases (non-OXA-48) are not included, both the NG-Test CARBA 5 and Xpert Carba-R only had an OPA of 6.2%, while the CIMTris performed well with an OPA of 99.0%. The majority of *A. baumannii* isolates (95.9%) tested falsely positive for IMP on NG-Test CARBA 5; no IMP genes were found on WGS. No clear cause was found for this phenomenon; a cross-reacting protein antigen unique to *A. baumannii* is a possible culprit. NG-Test CARBA 5 performed well for carbapenemase detection in *P. aeruginosa*. However, results from *A. baumannii* isolates should be interpreted with caution.

KEYWORDS NG-Test CARBA 5, Xpert Carba-R, whole-genome sequencing, mCIM, CIMTris, carbapenemase, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, rapid diagnostics

Carbapenemase detection from carbapenemase-producing Gram-negative bacilli (CP-GNB) has become a critical component of clinical and public health microbiology laboratory workflows for the purpose of infection prevention and control, patient care, and epidemiologic surveillance. The identification of carbapenemases in CP-GNB isolates typically involves the initial detection of carbapenem non-susceptibility, followed by the detection of carbapenemase production by a phenotypic method [e.g., a carbapenem hydrolysis assay such as the Rapidec CarbaNP test, or various forms of carbapenem inactivation method (CIM)] and/or detection of a specific carbapenemase gene(s) by PCR-based assays. Despite continued refinement of both genotypic and phenotypic assays, there remains room for improvement in balancing performance characteristics, labor intensity, test complexity, turnaround time, and cost (1–4).

NG-Test CARBA 5 is a rapid *in vitro* multiplex immunoassay for the phenotypic detection and differentiation of the "big five" common carbapenemase families (KPC,

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OXA-48-like, VIM, IMP, and NDM) directly from bacterial colonies. The assay is a rapid diagnostic test (<15 min), is simple to perform, and has been validated in several studies for detection of carbapenemase production by *Enterobacterales* and *Pseudomonas aeruginosa* (4–6). Thus far, Ambler class A, B, and D carbapenemases have been identified in *P. aeruginosa*, with class B enzymes being the most prevalent, whereas class D OXA-type carbapenemases are rarely identified (7); in Singapore specifically, IMP and NDM metallo-β-lactamases occur most frequently (8). Few data, however, exist for the use of NG-Test CARBA 5 on other non-fermenting Gram-negative bacilli such as the *Acinetobacter calcoaceticus-baumannii* (ACB) complex, which is an important reservoir for carbapenemases, besides other resistance determinants for multiple drug classes. The carbapenemases most commonly produced by the *Acinetobacter* spp. (predominantly Ambler class D OXA-type enzymes, which are non-OXA-48-like) are not included in NG-Test CARBA 5, which somewhat limits the assay's utility for this genus. Nevertheless, depending on local epidemiology, the ACB complex may carry NDM-type enzymes [which were thought to have originated in this complex (9)], or less commonly other metallo-β-lactamases such as IMP and VIM (10). Horizontal gene transfer may also occur between the ACB complex and other endogenous or environmental flora, which presents similar infection control ramifications as that seen with carbapenemase-producing *Enterobacterales*. Therefore, the use of rapid assays such as NG-Test CARBA 5 on *A. baumannii* isolates remains relevant in evaluating patients for CP-GNB infection or colonization, even if this is outside of the manufacturer's intended use.

The Xpert Carba-R is validated for use on *A. baumannii* and *P. aeruginosa*. The mCIM is a CLSI method that has been validated on *P. aeruginosa*, whereas the CIMTris, while currently not a CLSI method, has been studied and is a promising method for *A. baumannii* (3). We conducted a method comparison study to evaluate the performance of NG-Test CARBA 5, Xpert Carba-R, mCIM (for *P. aeruginosa*), and CIMTris (for *A. baumannii*) for the detection of carbapenemases with whole-genome sequencing (WGS) as the reference standard on isolates of both carbapenem-resistant *A. baumannii* (CRAB) and carbapenem-resistant *P. aeruginosa* (CRPA).

MATERIALS AND METHODS

Bacterial isolates

We studied 138 CRPA and 97 CRAB archived isolates from 128 and 85 unique patients, respectively. These were obtained between July 2019 and May 2022 from patients admitted to our institution (Tan Tock Seng Hospital and the National Centre for Infectious Diseases), a 1,700-bed teaching hospital in Singapore. These isolates were obtained via a mix of surveillance rectal swabs (done to identify CP-GNB carriage) and clinical samples; all CRPA isolates were from surveillance samples, while the CRAB isolates consisted of 53 clinical and 44 surveillance samples.

Rectal swabs (Copan, ESwab) collected for routine surveillance of CP-GNB in our hospital were cultured on chromID CARBA SMART Agar (CARB/OXA) (bioMérieux, France). We identified the species of colonies grown on chromID CARBA SMART using MALDI-TOF mass spectrometry (Bruker, Germany). Carbapenem resistance was confirmed using meropenem disk diffusion (Oxoid/ThermoScientific, USA). For clinical isolates, routine susceptibility testing was performed by either the VITEK system (bioMérieux, France) or disk diffusion testing, as per usual clinical workflows. Interpretive criteria for susceptibility were as per CLSI M100-ED32:2022 (11). Isolates were received from our hospital's clinical microbiology laboratory as pure subcultures and stored in cryovials (Microbank, Pro-Lab Diagnostics) at −80°C in our research laboratory.

The assays studied (NG-Test CARBA 5, Xpert Carba-R, mCIM, CIMTris, WGS) were performed from a fresh second subculture from frozen stock onto TSA with 5% sheep blood agar. No selection with a carbapenem disk was performed on subcultures, except for two isolates of *P. aeruginosa* where re-testing was required and a 10-µg meropenem

disk was used. As it was not possible to perform all tests simultaneously, second passage subcultures from frozen stock were performed as needed.

NG-Test CARBA 5

Using a 1-µL loop, three colonies were touched and inoculated into the manufacturer-supplied 1.5 mL microcentrifuge tube containing five drops of extraction buffer. As the assay was originally designed for *Enterobacterales*/*P. aeruginosa*, use on *A. baumannii* necessitated an inoculum of about six to seven colonies, due to the smaller colony size of *A. baumannii*. After brief vortexing, 100 µL of mixture was loaded into the cassette's sample well marked with "S" using the manufacturer-supplied disposable transfer pipette. The test cassette was visually examined 15 min later for the presence or absence of the control and test lines. To avoid biased result interpretation, two separate study team members visually examined the NG-Test CARBA 5 results, with at least one member being blinded to the genotype of the isolate (if already available). Isolates with discrepancies between the two readers (expected to be due to faint test lines) were re-tested immediately for adjudication with a higher bacterial inoculum (8–10 colonies). Quality control (QC) was performed every day of testing and upon receiving a new lot of test kits. This included a negative control (*Escherichia coli* ATCC 25922) and one positive control for each target (OXA-48/NDM/KPC/IMP/VIM).

Xpert Carba-R

A 0.5 MacFarland suspension (approximately 1 to 2 × 10^8 CFU/mL) of the test isolate was prepared. Ten microliters of the bacterial suspension were added into 5 mL of sample reagent and vortexed vigorously for 10 s. Next, 1.7 mL of the earlier discussed preparation was loaded into an Xpert Carba-R cartridge and run on the GeneXpert instrument. QC was performed every day of testing and included a negative control (*E. coli* ATCC 25922) and two positive controls (one for OXA-48/NDM/KPC, and one for IMP/VIM).

Carbapenem inactivation methods

mCIM was performed and interpreted according to CLSI M100-ED32:2022 (11) on CRPA isolates, while the CIMTris assay was performed on CRAB isolates. This assay is a further modification of the CIM, primarily differing from mCIM in its use of 0.5 M Tris-HCl (pH 7.6) buffer for improved carbapenemase extraction from *Acinetobacter* species (3).

Briefly, for each test isolate, a 10-µL loopful of bacteria from an overnight blood agar plate was emulsified in 2 mL of tryptic soy broth (mCIM) or 400 µL of 0.5M Tris-HCl buffer (CIMTris). After vortexing for 10–15 seconds, a 10-µg meropenem disk (BD, USA) was immersed in the suspension and incubated at 35°C ± 2°C in ambient air for 4 h (mCIM) or 2 h (CIMTris), before being placed on a Mueller-Hinton agar (MHA) plate lawned with *E. coli* ATCC 25922. MHA plates were then inverted and incubated at 35°C ± 2°C in ambient air for 18 h, following which zones of inhibition were measured as per the routine disk diffusion method. These were interpreted as carbapenemase positive (zone diameter of 6–15 mm or presence of pinpoint colonies within a 16–18 mm zone), negative (zone diameter of ≥19 mm), or indeterminate (zone diameter of 16–18 mm or zone diameter of 19 mm and the presence of pinpoint colonies within the zone; counted as positive for the purposes of this study). QC was performed every day of testing using one positive and one negative control isolate. Details of bacterial isolates used for QC for all three tests may be found in the supplementary appendix.

WGS and genome assembly

WGS was performed on the Illumina platform to obtain 2 × 150 base pair (bp) paired-end reads. Quality control for Illumina reads was performed using FastQC (v0.11.7) followed by adapter trimming and quality filtering using BBDuk (v38.11) with parameters "ktrim = r k = 23 mink = 11 hdist = 1 qtrim = rl trimq = 30 minavgquality = 30" (12, 13). *De novo* assembly of the short-read sequences was performed using SPAdes (v3.13.0;

parameter: --careful) and contigs with size <1,000 bp were excluded from downstream analysis (14).

Genome analyses

Multilocus sequence type (MLST; v2.19.0) was used for species assignment and determination of ST profiles (15). All assembled genome sequences were screened for antibiotic resistance genes by a stand-alone local version of NCBI AMRFinderPlus (v3.10.21; default parameters) (16). In the instances where co-carriage of carbapenemase genes was detected, we used the SRST2 tool (v0.2.0; default parameters) to confirm the gene's presence. All genomic analyses were conducted by separate investigators who were blinded to the results of testing by NG-Test CARBA 5 and Xpert Carba-R.

Statistical analysis

All data were analyzed using Vassarstats (http://vassarstats.net/) software for positive percentage agreement (PPA), negative percentage agreement (NPA), and/or overall percentage agreement (OPA) as appropriate, with WGS as the comparator. Approximate upper- and lower-bound 95% confidence intervals (95% *CIs*) were also calculated.

RESULTS

The results of testing by WGS, Xpert Carba-R, NG-Test CARBA 5, and mCIM/CIMTris are summarized in Table 1. In all, there was disagreement between both readers over the initial NG-Test CARBA 5 result of four CRAB isolates and one CRPA isolate; all cases were due to faint test lines, and all were resolved after repeating the test once. No quality control failures occurred.

Pseudomonas aeruginosa

WGS identified carbapenemase genes in 117 of 138 CRPA isolates; the remaining 21 were non-carbapenemase-producing. A single isolate harbored two carbapenemase genes ($bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$). Only two isolates harbored a carbapenemase outside the "big five" carbapenemase types (in this case $bla_{\text{GES-5}}$).

NG-Test CARBA 5 produced concordant results with WGS for 134 out of 138 isolates. All four discordant results were false negatives; the two isolates with $bla_{\text{GES-5}}$ could not be detected as GES-5 is not included in the assay, while only NDM was detected in the NDM-1/OXA-48 co-producer. NG-Test CARBA 5 also failed to detect NDM in another isolate. PPA and NPA compared to WGS as the reference standard were 96.6% (95% CI, 91.0% to 98.9%) and 100.0% (95% CI, 80.8% to 100.0%), respectively. If the NDM-1/OXA-48 co-producing isolate (where only NDM was detected) were counted as a true positive (as there would be no downstream infection control impact from this discrepant result in a surveillance specimen), the PPA and NPA would be 97.4% and 100.0%, respectively.

Xpert Carba-R produced concordant results with WGS for 128 out of 138 isolates. All 10 discordant results were false negatives; as with NG-Test CARBA 5, Xpert Carba-R did not detect the off-panel $bla_{\text{GES-5}}$ and only detected bla_{NDM} in the $bla_{\text{NDM-1}}/bla_{\text{OXA-48}}$ co-carrier. Xpert Carba-R also failed to detect all $bla_{\text{IMP-7}}$ and $bla_{\text{IMP-13}}$. PPA and NPA compared to WGS as the reference standard were 91.5% (95% CI, 84.5% to 95.6%) and 100.0% (95% CI, 80.8% to 100.0%), respectively. If the $bla_{\text{NDM-1}}/bla_{\text{OXA-48}}$co-carrying isolate (where only bla_{NDM} was detected) were counted as a true positive (such as described earlier for NG-Test CARBA 5), the PPA would rise to 92.3%; if, in addition, isolates harboring $bla_{\text{IMP-7}}$ and $bla_{\text{IMP-13}}$ were excluded from the analysis as these were not expected to be detected by the Xpert assay, the PPA would rise further to 98.2%.

mCIM produced concordant results with WGS in detecting/not detecting carbapenemase for 125 out of 138 isolates resulting in an OPA of 90.6%; it detected 89.7% of carbapenemase-producing isolates. The majority of mCIM false negatives occurred in CRPA isolates harboring $bla_{\text{VIM-2}}$. mCIM detected carbapenemase in all isolates harboring

TABLE 1 Results of testing by NG-Test CARBA 5, Xpert Carba-R, and mCIM/CIMTris compared with WGS as the reference method

Bacterial species	Genotype by WGS[a]		No. of isolates	No. (%) concordant on NG-Test CARBA 5	No. (%) concordant on Xpert Carba-R	No. (%) concordant by mCIM/ CIMTris[b]
	CP[c] status	Carbapenemase gene(s)				
Pseudomonas aeruginosa	Non-CP	None	21	21 (100)	21 (100)	20 (95.2)
	CP	$bla_{\text{NDM-1}}$	71	70 (98.6)	71 (100)	71 (100)
		$bla_{\text{IMP-1}}$	18	18 (100)	18 (100)	18 (100)
		$bla_{\text{IMP-7}}$[d]	6	6 (100)	0 (0)	6 (100)
		$bla_{\text{IMP-13}}$[d]	1	1 (100)	0 (0)	1 (100)
		$bla_{\text{VIM-2}}$	17	17 (100)	17 (100)	6 (35.3)
		$bla_{\text{VIM-6}}$	1	1 (100)	1 (100)	1 (100)
		$bla_{\text{GES-5}}$	2	0 (0)	0 (0)	1 (50)
		$bla_{\text{NDM-1}}$, $bla_{\text{OXA-48}}$	1	0 (0)[e]	0 (0)[e]	1 (100)
		On-panel (Xpert/ CARBA 5) "big five" CP types	115	113 (98.3)	107 (93.0)	104 (90.4)
		All CP types	117	113 (96.6)	107 (91.5)	105 (89.7)
		Total	138	134 (97.1)	128 (92.8)	125 (90.6)
Acinetobacter baumannii complex	CP	$bla_{\text{OXA-23}}$ family,[f] $bla_{\text{OXA-51}}$ family[g]	88	0 (0)[h]	0 (0)	88 (100)
		$bla_{\text{OXA-23}}$ family[f], $bla_{\text{OXA-51}}$ family[g], $bla_{\text{OXA-72}}$	1	0 (0)[h]	0 (0)	1 (100)
		$bla_{\text{OXA-51}}$ family[g]	2	0 (0)[h]	0 (0)	1 (50)
		$bla_{\text{NDM-1}}$, $bla_{\text{OXA-51}}$ family[g], $bla_{\text{OXA-58}}$	2	2 (100)	2 (100)	2 (100)
		$bla_{\text{NDM-1}}$, $bla_{\text{OXA-23}}$ family[f], $bla_{\text{OXA-51}}$ family[g]	1	1 (100)	1 (100)	1 (100)
		$bla_{\text{NDM-1}}$, $bla_{\text{OXA-51}}$ family[g]	1	1 (100)	1 (100)	1 (100)
		$bla_{\text{NDM-1}}$, $bla_{\text{OXA-58}}$	1	1 (100)[h]	1 (100)	1 (100)
		$bla_{\text{NDM-1}}$, $bla_{\text{OXA-23}}$ family[f], $bla_{\text{OXA-58}}$, $bla_{\text{OXA-51}}$ family[g]	1	1 (100)[h]	1 (100)	1 (100)
		All CP types	97	6 (6.2)	6 (6.2)[i]	96 (99.0)

[a]By multilocus sequence typing (MLST), the following STs were present: for CRPA, STs 111, 234, 235, 244, 253, 262, 307, 308, 313, 357, 377, 381, 399, 446, 606, 621, 823, 827, 1119, 1123, 1232, 1816, 2434, 3118; for CRAB, STs 1, 2, 25, 103, 149, 150, 164, 218, 782.
[b]mCIM performed on CRPA isolates; CIMTris performed on CRAB isolates. All indeterminate results counted as positive.
[c]CP, carbapenemase-producing.
[d]The Xpert Carba-R package insert dated July 2020 (Table 19) states that IMP-7/13/14 genes were not detectable in a prior study of performance characteristics and were not predicted to be detected by *in silico* analysis.
[e]Both NG-Test CARBA 5 and Xpert Carba-R detected NDM, but did not detect OXA-48.
[f]AMRFinderPlus was unable to identify the exact OXA-23 family variant for four isolates, while all other isolates carried $bla_{\text{OXA-23}}$.
[g]OXA-51 family genes identified in our study cohort included $bla_{\text{OXA-51}}$, $bla_{\text{OXA-64}}$, $bla_{\text{OXA-65}}$, $bla_{\text{OXA-66}}$, $bla_{\text{OXA-69}}$, $bla_{\text{OXA-70}}$, $bla_{\text{OXA-91}}$, $bla_{\text{OXA-104}}$, and $bla_{\text{OXA-121}}$. 85 isolates carried either $bla_{\text{OXA-66}}$, $bla_{\text{OXA-69}}$, or $bla_{\text{OXA-91}}$. AMRFinderPlus was unable to identify the exact OXA-51 family variant for another four isolates.
[h]CARBA five testing returned false-positive results for IMP on nearly all the test isolates (see Table 2 and Discussion), including two which were also true positive for NDM.
[i]Xpert Carba-R detected 100% of on-panel targets (KPC/OXA-48/NDM/IMP/VIM).

$bla_{\text{NDM-1}}$ and bla_{IMP} genes. mCIM also detected carbapenemase in 3 out of 4 isolates that tested false negative by NG-Test CARBA 5.

Acinetobacter baumannii

All test isolates were classified as carbapenemase-producing by WGS, which detected the various OXA beta-lactamases commonly harbored by CRAB. $bla_{\text{NDM-1}}$ was the only "big five" carbapenemase present in the test population. All but one of the CRAB isolates tested positive on CIMTris.

A breakdown of the results of NG-Test CARBA 5, Xpert Carba-R, and WGS performed on the study cohort of 97 CRAB isolates is shown in Table 2. NG-Test CARBA 5 correctly identified 100% of the isolates harboring $bla_{\text{NDM-1}}$, and also falsely detected IMP in 93 of 97 isolates. Thirty of these had faint test lines. All IMP detections by NG-Test CARBA 5 were deemed to be false positives as both Xpert Carba-R and WGS did not detect any bla_{IMP} genes in the test isolate genomes (Table 2). PPA of both NG-Test CARBA 5 and Xpert Carba-R was low at 6.2%, which was expected because the OXA-type

TABLE 2 Breakdown of 97 CRAB isolates comparing NG-Test CARBA 5, Xpert Carba-R, and WGS results

Number of isolates (n = 97)	NG-Test CARBA 5 result	Xpert Carba-R result	Carbapenemase genes identified on WGS ("big five")	Carbapenemase genes identified on WGS (other)
2	NDM	NDM	*bla*$_{NDM-1}$	*bla*$_{OXA-51}$ family[a], *bla*$_{OXA-58}$
1	NDM	NDM	*bla*$_{NDM-1}$	*bla*$_{OXA-23}$ family[b], *bla*$_{OXA-51}$ family[a]
1	NDM	NDM	*bla*$_{NDM-1}$	*bla*$_{OXA-51}$ family[a]
1	NDM + IMP[c]	NDM	*bla*$_{NDM-1}$	*bla*$_{OXA-58}$
1	NDM + IMP[c]	NDM	*bla*$_{NDM-1}$	*bla*$_{OXA-23}$ family[b], *bla*$_{OXA-58}$, *bla*$_{OXA-51}$ family[a]
2	IMP[c]	Not detected	None	*bla*$_{OXA-51}$ family[a]
1	IMP[c]	Not detected	None	*bla*$_{OXA-23}$ family[b], *bla*$_{OXA-51}$ family[a], *bla*$_{OXA-72}$
88	IMP[c]	Not detected	None	*bla*$_{OXA-23}$ family[b], *bla*$_{OXA-51}$ family[a]

[a]OXA-51 family genes identified in our study cohort included *bla*$_{OXA-51}$, *bla*$_{OXA-64}$, *bla*$_{OXA-65}$, *bla*$_{OXA-66}$, *bla*$_{OXA-69}$, *bla*$_{OXA-70}$, *bla*$_{OXA-91}$, *bla*$_{OXA-104}$, and *bla*$_{OXA-121}$. 85 isolates carried either *bla*$_{OXA-66}$, *bla*$_{OXA-69}$, or *bla*$_{OXA-91}$. AMRFinderPlus was unable to identify the exact OXA-51 family variant for another four isolates.
[b]AMRFinderPlus was unable to identify the exact OXA-23 family variant for four isolates, while all other isolates carried *bla*$_{OXA-23}$.
[c]All IMP detections by NG-Test CARBA 5 were deemed false positives.

carbapenemases present were off-panel for both tests. PPA for the "big five" carbapenemases was 100%. NPA could not be calculated as no non-carbapenemase-producing CRAB were present.

Given the earlier discussion, further efforts were undertaken to detect or exclude systematic error for IMP detection with the NG-Test CARBA 5. First, all 97 test isolates were re-tested with a different lot of NG-Test CARBA 5 kits using a lower bacterial inoculum (three to four colonies). 61 of 97 isolates tested positive for IMP on NG-Test CARBA 5, which was substantively similar to initial testing, which resulted in a high degree of false-positive IMP results with *A. baumannii*. The isolates which re-tested negative were mostly different from those which produced faint test lines for IMP in the initial test run. Second, a different set of 64 CRAB isolates from our collection of clinical and surveillance samples was tested with NG-Test CARBA 5 (using the originally employed bacterial inoculum of six to seven colonies); 63 of these tested positive for IMP. WGS did not detect *bla*$_{IMP}$ in any of these isolates. Third, we reviewed the beta-lactam resistome (see Table S5) generated from WGS data to determine if any beta-lactam resistance genes or types of genes in particular were responsible for cross-reacting with the NG-Test CARBA 5 assay. No clear correlation was found. Finally, multi-locus sequence typing (MLST) data were obtained to likewise look for correlation with the false-positive IMP results. Again, no clear correlation between STs and false-positive IMP results was observed.

In addition, because the *A. baumannii* complex intrinsically harbors *bla*$_{OXA-51}$, which is promoted by the IS*Aba1* insertion sequence, we examined our isolates for the presence of IS*Aba1*/*bla*$_{OXA-51}$ via WGS (see Supplementary material). Of the 97 CRAB isolates, 96 carried an OXA-51 family variant. Based on mapping of the unassembled reads, IS*Aba1* was detected in 95 isolates (99%) with 100% coverage and less than 1% nucleotide divergence. Ninety-four of 96 OXA-51-family-harboring isolates also harbored either NDM-1 or OXA-23. For the two remaining CRAB isolates where an OXA-51-like gene was the only carbapenemase detected by WGS, IS*Aba1* was detected in one isolate; this isolate was also positive on CIMTris. The second isolate (in which IS*Aba1* was not detected by WGS) was CIMTris negative.

DISCUSSION

Rapid, point-of-care multiplex immunoassays such as NG-Test CARBA 5 have the potential to bring about welcome improvements to the diagnostic armamentarium available for carbapenemase detection. Rapid carbapenemase detection and differentiation immediately after carbapenem resistance is detected is critical to guide subsequent antibiotic therapy, as the activity of novel β-lactam-β-lactamase inhibitor combinations

depends on the carbapenemase type (notably, currently approved novel β-lactam-β-lactamase inhibitors have no activity against metallo-β-lactamases) (17). This is a task for which rapid immunoassays, with turnaround times even shorter than multiplex PCRs such as Xpert Carba-R, seem particularly well-suited. Furthermore, should they be able to match the diagnostic reliability of genotypic detection methods, they do so at a lower cost and complexity.

NG-Test CARBA 5 was recently evaluated in a multicenter study of 309 *Enterobacterales* and *Pseudomonas aeruginosa* isolates by Jenkins et al. (4), where it demonstrated excellent performance for detecting and differentiating carbapenemase-producing *Enterobacterales* and *P. aeruginosa* isolates, attaining 100% PPA and NPA compared to the composite reference method. Our results for NG-Test CARBA 5's performance on CRPA isolates are comparable to theirs and other prior validation studies, accounting for the improvements made to version 2 of the assay, which increase its ability to detect genetically diverse IMP variants (frequently carried by *P. aeruginosa*) (18); this improved version of NG-Test CARBA 5 was used for all tests performed in our study. Of note, the Xpert Carba-R package insert states that IMP-7 and IMP-13 genes were not detectable in a prior study of its performance characteristics and were not predicted to be detected by *in silico* analysis. In contrast, IMP-7 and IMP-13 are included in the list of IMP variants expected to be detected by NG-Test CARBA 5 in its package insert. It is therefore not surprising that NG-Test CARBA 5 was able to detect IMP-7 or IMP-13 in six isolates where Xpert Carba-R could not (see Table 1). Thus, the utility of NG-Test CARBA 5 for carbapenemase detection in CRPA has been conclusively demonstrated, especially in a setting with a higher prevalence of IMP producers.

NG-Test CARBA 5 is not developed nor marketed for intended use on *Acinetobacter* spp., presumably because it does not target the most common carbapenemases produced by this genus. Nevertheless, in 2019 Potron et al. (19) evaluated the older version 1 of NG-Test CARBA 5 with a collection of 107 carbapenemase-producing lactose-non-fermenting Gram-negative bacillus isolates (of which 51 were *Acinetobacter* species), as well as 61 carbapenemase-negative isolates. Notably, all 61 non-carbapenemase-producing isolates and all 33 isolates producing a carbapenemase apart from those targeted by NG-Test CARBA 5 gave negative test results, demonstrating no cross-reactivity between OXA-type carbapenemases encountered in *Acinetobacter* spp. and OXA-48-like enzymes identified in *Enterobacterales*. A subsequent study by Volland et al. (18) evaluated version 2, but only included three isolates of *Acinetobacter* species. NG-Test CARBA 5v2 gave similar results to NG-Test CARBA 5v1, except for increased detection of IMP variants (which was verified by WGS). The median time for a positive signal was comparable between the two assays, indicating that the addition of novel antibodies did not interfere with the other targets. Neither study reported false-positive results, and in general the reported false-positive rate for NG-Test CARBA 5 in published literature has been negligible (4, 5, 18–20). It was remarkable that the assay displayed multiple false-positive detections for IMP in *Acinetobacter* in our study; to our knowledge, this is the first description of such a phenomenon, and should be verified with studies with more isolates from different locales.

Xpert Carba-R, mCIM, and CIMTris performed as expected in our study. Xpert Carba-R detected carbapenemase genes from the "big five" carbapenemase families with a high degree of accuracy, though there is room for improvement in the detection of bla_{IMP} genes and some increasingly encountered off-panel carbapenemases. The performance of mCIM appears to vary somewhat across different studies (3, 21–24), and poorer performance in VIM- and GES-producers has occasionally been reported (3, 24), similar to our own findings. CIMTris has emerged as a reliable phenotypic test for carbapenemase detection (3, 23, 24), in most cases outperforming mCIM (especially for non-fermenters), though these were not directly compared in our study as they were performed on different bacterial species.

The strengths of our study include the use of WGS as the reference standard, as well as the use of blinding to allow for unbiased result interpretation and accounting

for potential inter-observer variability. Regarding the unexpected false-positive IMP detections, an exhaustive effort was undertaken attempting to pinpoint a cause of interference. This did not seem to be a batch issue of the test kits, and while we were not able to specifically determine an underlying cause, we hypothesize that this is likely due to a cross-reacting protein antigen in *A. baumannii*, as an overwhelming majority of isolates tested had a false-positive IMP result. Studies in other locales and collections will be required to further evaluate this issue.

Our study has several limitations. Our exclusive use of version 2 of NG-Test CARBA 5, which has been specifically modified for improved IMP detection, precludes any conclusions about whether version 1 would have avoided the problem of false positives, especially considering the results of the study by Potron et al. (19). The performance of NG-Test CARBA 5 for the detection of other "big five" carbapenemases in CRAB cannot be determined from our study, as NDM-1 was the only "big five" carbapenemase carried by our population of CRAB isolates. This reflects known epidemiology as it is much less common for *A. baumannii* to harbor other "big five" carbapenemases. The epidemiology of carbapenemase spread and carriage may be different in other geographic regions, which may preclude generalization to some extent. Finally, we did not evaluate NG-Test CARBA 5 on bacterial colonies recovered from other culture media.

In summary, NG-Test CARBA 5 performed extremely well relative to established genotypic methods for carbapenemase detection and differentiation in CRPA, as well as for the detection of NDM expression by CRAB. Unfortunately, we unexpectedly encountered many false-positive IMP detections in CRAB isolates. To our knowledge, such a phenomenon has not been reported to date. Thus, results of NG-Test CARBA 5 performed "off-label" on CRAB (in particular IMP-positive results) should be interpreted with caution, until this issue is further elucidated and resolved.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental figure and tables (JCM00316-23-S0001.pdf). Tables S1 to S5, Figure S1, ISAba1 insertion sequence analysis.

REFERENCES

1. Tamma PD, Simner PJ. 2018. Phenotypic detection of carbapenemase-producing organisms from clinical isolates. J Clin Microbiol 56:e01140-18. https://doi.org/10.1128/JCM.01140-18
2. van der Zee A, Roorda L, Bosman G, Fluit AC, Hermans M, Smits PHM, van der Zanden AGM, Te Witt R, Bruijnesteijn van Coppenraet LES, Cohen Stuart J, Ossewaarde JM. 2014. Multi-centre evaluation of real-time multiplex PCR for detection of carbapenemase genes OXA-48, VIM, IMP, NDM and KPC. BMC Infect Dis 14:27. https://doi.org/10.1186/1471-2334-14-27
3. Uechi K, Tada T, Shimada K, Kuwahara-Arai K, Arakaki M, Tome T, Nakasone I, Maeda S, Kirikae T, Fujita J. 2017. A modified carbapenem inactivation method, cimtris, for carbapenemase production in *Acinetobacter* and *Pseudomonas* species. J Clin Microbiol 55:3405–3410. https://doi.org/10.1128/JCM.00893-17
4. Jenkins S, Ledeboer NA, Westblade LF, Burnham CA, Faron ML, Bergman Y, Yee R, Mesich B, Gerstbrein D, Wallace MA, Robertson A, Fauntleroy KA, Klavins AS, Malherbe R, Hsiung A, Simner PJ. 2020. Evaluation of NG-test carba 5 for rapid phenotypic detection and differentiation of five common carbapenemase families: results of a multicenter clinical evaluation. J Clin Microbiol 58:e00344-20. https://doi.org/10.1128/JCM.00344-20
5. Zhu Y, Jia P, Li X, Wang T, Zhang J, Zhang G, Duan S, Kang W, Xu Y, Yang Q. 2021. Carbapenemase detection by NG-test CARBA 5-a rapid Immunochromatographic assay in carbapenem-resistant *Enterobacterales* diagnosis. Ann Transl Med 9:769. https://doi.org/10.21037/atm-20-8216
6. Yoon J, Kim CH, Yoon S-Y, Lim CS, Lee CK. 2021. Application of a multiplex immunochromatographic assay for rapid identification of

carbapenemases in a clinical microbiology laboratory: performance and turn-around-time evaluation of NG-test carba 5. BMC Microbiol 21:260. https://doi.org/10.1186/s12866-021-02309-9
7. Yoon E-J, Jeong SH. 2021. Mobile carbapenemase genes in *Pseudomonas aeruginosa*. Front Microbiol 12:614058. https://doi.org/10.3389/fmicb.2021.614058
8. Teo J-M, Tang CY, Lim JC, Lee S-Y, Tan SH, Koh T-H, Sim J-C, Tan T-T, Kwa A-H, Ong R-H. 2021. Genomic characterization of carbapenem-non-susceptible *Pseudomonas aeruginosa* in Singapore. Emerg Microbes Infect 10:1706–1716. https://doi.org/10.1080/22221751.2021.1968318
9. Bonnin RA, Poirel L, Nordmann P. 2014. New Delhi metallo-B-lactamase-producing *Acinetobacter baumannii*: a novel paradigm for spreading antibiotic resistance genes. Future Microbiol 9:33–41. https://doi.org/10.2217/fmb.13.69
10. Potron A, Poirel L, Nordmann P. 2015. Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: mechanisms and epidemiology. Int J Antimicrob Agents 45:568–585. https://doi.org/10.1016/j.ijantimicag.2015.03.001
11. CLSI. 2022. Performance standards for antimicrobial susceptibility testing. 32nd ed. Clinical and Laboratory Standards Institute, USA.
12. Andrews S. 2010. Fastqc: a quality control tool for high throughput sequence data. Available from: https://www.bioinformatics.babraham.ac.uk/projects/fastqc
13. Bushnell B. 2014. Bbmap: A fast, accurate, splice-aware Aligner. LBNL Department of Energy Joint Genome Institute Walnut Creek, CA. Available from: http://1ofdmq2n8tc36m6i46scovo2e.wpengine.netdna-cdn.com/wp-content/uploads/2013/11/BB_User-Meeting-2014-poster-FINAL.pdf
14. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. Spades: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477. https://doi.org/10.1089/cmb.2012.0021
15. Seemann T. 2016. Scan contig files against Pubmlst typing schemes. Available from: https://github.com/tseemann/mlst
16. Seemann T. 2017. Abricate. mass screening of contigs for antimicrobial resistance or virulence genes. Available from: https://github.com/tseemann/abricate
17. Mojica MF, Rossi M-A, Vila AJ, Bonomo RA. 2022. The urgent need for metallo-B-lactamase inhibitors: an unattended global threat. Lancet Infect Dis 22:e28–e34. https://doi.org/10.1016/S1473-3099(20)30868-9
18. Volland H, Girlich D, Laguide M, Gonzalez C, Paris V, Laroche M, Oueslati S, Dortet L, Simon S, Naas T. 2019. Improvement of the immunochromatographic NG-test Carba 5 assay for the detection of IMP variants previously undetected. Antimicrob Agents Chemother 64:e01940-19. https://doi.org/10.1128/AAC.01940-19
19. Potron A, Fournier D, Emeraud C, Triponney P, Plésiat P, Naas T, Dortet L. 2019. Evaluation of the immunochromatographic NG-test Carba 5 for rapid identification of carbapenemase in nonfermenters. Antimicrob Agents Chemother 63. https://doi.org/10.1128/AAC.00968-19
20. Hopkins KL, Meunier D, Naas T, Volland H, Woodford N. 2018. Evaluation of the NG-test CARBA 5 multiplex Immunochromatographic assay for the detection of KPC, OXA-48-like, NDM, VIM and IMP carbapenemases. J Antimicrob Chemother 73:3523–3526. https://doi.org/10.1093/jac/dky342
21. Lisboa LF, Turnbull L, Boyd DA, Mulvey MR, Dingle TC. 2018. Evaluation of a modified carbapenem inactivation method for detection of carbapenemases in *Pseudomonas aeruginosa*. J Clin Microbiol 56:e01234-17. https://doi.org/10.1128/JCM.01234-17
22. Simner PJ, Johnson JK, Brasso WB, Anderson K, Lonsway DR, Pierce VM, Bobenchik AM, Lockett ZC, Charnot-Katsikas A, Westblade LF, Yoo BB, Jenkins SG, Limbago BM, Das S, Roe-Carpenter DE. 2018. Multicenter evaluation of the modified carbapenem inactivation method and the carba NP for detection of carbapenemase-producing *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. J Clin Microbiol 56:e01369-17. https://doi.org/10.1128/JCM.01369-17
23. Kuchibiro T, Komatsu M, Yamasaki K, Nakamura T, Nishio H, Kimura K, Niki M, Kida K, Ohama M, Fukuda N, Sawa K, Nakamura A, Nishi I. 2021. Comparison of the performance of three carbapenem inactivation methods for the detection of carbapenemase-producing gram-negative bacilli. J Infect Chemother 27:1634–1638. https://doi.org/10.1016/j.jiac.2021.07.021
24. Ferjani S, Maamar E, Ferjani A, Kanzari L, Boubaker IBB. 2022. Evaluation of three carbapenemase-Phenotypic detection methods and emergence of diverse VIM and GES variants among *Pseudomonas aeruginosa* isolates in Tunisia. Antibiotics (Basel) 11:858. https://doi.org/10.3390/antibiotics11070858