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Rapid Identification of OXA-48 and OXA-163 Subfamilies in Carbapenem-Resistant Gram-Negative Bacilli with a Novel Immunochromatographic Lateral Flow Assay

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We assessed a novel immunochromatographic lateral flow assay for direct identification of OXA-48-like carbapenemases and accurate differentiation of allele variants with distinct substrate profiles (OXA-48 or OXA-163 subfamilies). The assay allowed rapid (less than 4 min) and reliable direct confirmation of OXA-163- and/or OXA-48-like enzymes (with 100% sensitivity and 100% specificity) from cultured colonies that were recovered from both solid medium and spiked blood culture bottles.

The emergence and spread of *Enterobacteriaceae* that produce plasmids encoding class A (KPC, GES, Sme, NMC-A), B (IMP, VIM, NDM), and D (OXA-48-like) carbapenemases are worldwide public health threats. To prevent the spread of carbapenemase producers and define an appropriate empirical antimicrobial therapy, the rapid detection of carbapenemase-producing organisms has become imperative (1). One of the major concerns for controlling the spread of OXA-48-like producers is the absence of reliable phenotypical tests that might contribute to their easy recognition. This is due in part to relatively low carbapenem MICs, a lack of suitable inhibitor compounds for use in confirmatory tests, and the very low expression of carbapenemase activity which cannot be detected readily by recently developed biochemical methods (1–5). Recently, a novel means of detecting OXA-48-like enzymes via an antibody-mediated approach was developed (6). The OXA-48 K-SeT test (Coris BioConcept, Belgium) relies on immunological capture of two epitopes specific to the OXA-48 variants OXA-48, OXA-181, OXA-204, OXA-232, and OXA-244 using colloidal gold nanoparticles bound to a nitrocellulose membrane within a lateral flow device (6). The reported sensitivity and specificity were both 100%, with the result obtained in less than 10 min (6–10). Noteworthy is that new allelic variants of OXA-48 have emerged, namely, OXA-163 and the related variants OXA-247, OXA-405, and OXA-438 (11–14), which are not recognized by the OXA-48 K-SeT test (6, 8, 10). OXA-163, which is the only variant of this subfamily that has spread to several hospitals in Argentina and Egypt (15, 16), differs from OXA-48 by a single amino acid substitution and a four-amino-acid deletion. In earlier studies, OXA-163 exhibited almost undetectable carbapenemase activity with a substrate profile that included broad-spectrum cephalosporins (11). However, recent reports indicate that this allele might produce enhanced carbapenemase activity in the presence of carbonates (13, 17), suggesting that its activity is strongly influenced by the rate of carboxylation of the active site, as observed for other carbapenem-hydrolyzing class D β-lactamases (18, 19). Additionally, *in vivo* data suggested that OXA-163 might cause carbapenem treatment failures in critically ill patients (12, 15, 20) or favor intrapatient selection of new OXA-48 variants (12), which highlights the urgent need for efficient identification tests.

Definitive confirmation of OXA-163- and/or OXA-48-like producers currently relies on molecular assays and gene sequencing for the assignment of allelic variants. However, these methods are of limited practical use for daily application in most clinical laboratories due to the high cost and requirement for significant expertise to interpret test results. We recently developed a new immunochromatographic lateral flow assay, the OXA-163/48 Duo K-SeT test (Coris BioConcept), for direct identification and accurate differentiation of OXA-48- and OXA-163-like subfamilies, in which two monoclonal anti-OXA-48 antibodies are selected as specific capture reagents on two lines for the test (21). The test included (i) a first antibody directed against all current OXA-48-like variants (OXA-48, OXA-162, OXA-181, OXA-204, OXA-232, and OXA-244) but not the OXA-163 subfamily (first line, labeled 48), and (ii) a second antibody directed against another epitope present in all OXA-48-like enzymes, which also binds the OXA-163 subfamily (second line, labeled 163) (Fig. 1). If the sample contains OXA-48, it will remain bound to the first capture antibody (anti-OXA-48). If the sample contains OXA-163, it will not react with the first antibody but will bind to the second capture antibody (anti-OXA-163 and anti-OXA-48). If the sample contains a large amount of OXA-48, the second line may give a faint signal from a portion of OXA-48 that is not captured by the first line. Finally, a third antibody directed against a third

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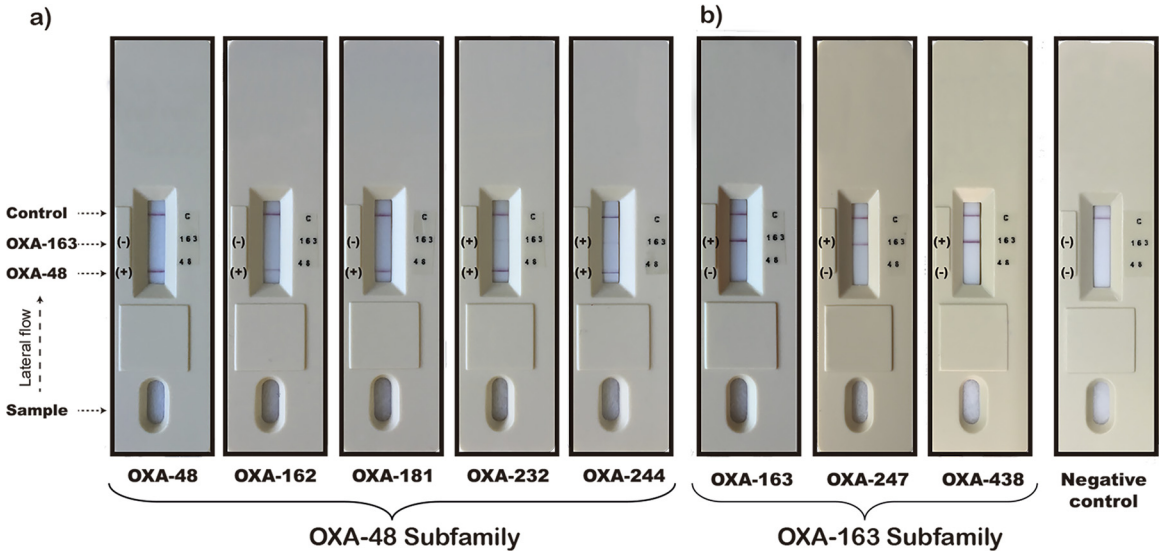

FIG 1 OXA-163/48 Duo K-SeT assays for the detection of OXA-48-type (a) and OXA-163-type (b) enzymes. For positive results, in addition to a reddish-purple band at the control line (C), a visible band at test line position 48 corresponds to a sample containing OXA-48 (or closely related variants, but not OXA-163), regardless of the presence or absence of a faint signal at the 163 test line. If the only positive test line is line 163, the sample contains the OXA-163 variant (or closely related variants). For negative results, a single line appears at the position of the control line (C) only. The vertical arrow indicates the direction of lateral flow.

epitope present in all OXA-48-like variants (including the OXA-163 subfamily) was chosen as a detection reagent. In this study, we challenged the performance of the Duo K-SeT assay for the detection of OXA-48-like and OXA-163-like enzymes against a collection of Gram-negative bacilli.

The evaluation of the test was performed with a panel of 75 clinical isolates. PCR and DNA sequencing of entire *bla* alleles were considered the gold standard for β-lactamase characterization (4). The selected isolates comprised 64 carbapenemase producers, among which 50 were confirmed to belong to the OXA-48 family (Table 1). The isolates included were found to be nonclonal by pulsed-field gel electrophoresis (PFGE) (data not shown). Species identification was confirmed using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF [BD Biotyper 3.1; Bruker, Germany]). MICs were obtained using an Etest (bioMérieux; Marcy l'Étoile, France) or by agar dilution and interpreted using Clinical and Laboratory Standards Institute guidelines (22).

The OXA-163/48 Duo K-SeT test was performed according to the manufacturer's instructions. Briefly, strains were grown overnight on Mueller-Hinton, Trypticase soy, or Columbia agar with 5% sheep blood plates. A single colony was resuspended in 10 drops of LY-A buffer (Tris-HCl, NaN_3 [pH 7.5]). Using colonies representative of each of the OXA-163 and OXA-48 variants, we verified that a colony size of ≥0.2 mm was required for the test to perform well, although Gram-negative bacteria grown overnight in standard culture medium generally exceeded this limit. Subsequently, 3 drops of the homogenized solution were applied to the sample well. Tests were read by eye within 15 min. The time to appearance of the OXA-48 or OXA-163 signal was recorded.

A band at the test position for OXA-48 (first line) was visible in all 17 OXA-48-like carbapenemases, including OXA-48 and different variants (OXA-162, OXA-181, OXA-232, and OXA-244), confirming the high sensitivity for these alleles (6–10) (Table 1). A faint signal on the OXA-163 test line also appeared in one *Klebsiella pneumoniae* OXA-232 isolate and one *Escherichia coli* OXA-242 isolate (Fig. 1). In these cases, the test results were interpreted as OXA-48 positive and OXA-163 negative, as explained above. A positive signal in only the OXA-163 test line was observed for all 33 OXA-163-like producers, including OXA-163 and different related variants (OXA-247 and OXA-438) (Table 1). These findings highlight the excellent specificity of the assay to differentiate the two subfamilies of OXA-48-like enzymes. Visible bands for a positive test systematically appeared between 15 and 240 s after inoculation of the strip. In contrast, the OXA-163/48 Duo K-SeT test yielded negative results at 15 min for all OXA carbapenemases typically found in *Acinetobacter* spp., susceptible isolates with narrow-spectrum OXA-type enzymes, isolates producing other major carbapenemase families (classes A and B), and those exhibiting reduced carbapenem susceptibility due to extended-spectrum β-lactamase (ESBL) or AmpC production combined with decreased permeability (overall sensitivity and specificity were 100%) (Table 1). Colonies representative of each OXA-163 and OXA-48 variant taken from culture plates and stored at 4°C for up to 60 days were tested weekly using the OXA-163/48 Duo K-SeT test. All tests yielded positive results at all different culture ages.

The limit of detection (LoD) of the OXA-163/48 Duo K-SeT test was calculated using two approaches, (i) by determining the lower viable colony counts (CFU/ml) with a positive signal using *E. coli* M15260 (OXA-48), *K. pneumoniae* M11969 (OXA-163), *K. pneumoniae* M13888 (OXA-247), and *E. coli* M17244 (OXA-438) isolates (13), as described previously (7), and (ii) on the basis of purified recombinant OXA proteins. Briefly, plasmid-borne $bla_{OXA-163}$, $bla_{OXA-247}$, $bla_{OXA-438}$, and bla_{OXA-48} were cloned from the indicated donors (or transconjugants) in *E. coli* BL21. OXA enzymes were produced and purified for homogeneity as described previously (13, 23), yielding concentrations of 0.41 (OXA-48), 0.49 (OXA-163), 0.625 (OXA-247), and 0.645 mg/ml (OXA-438). Serial $\log_{10}$ or $\log_2$ dilutions of the homogenates were made in phosphate-buffered saline (PBS) (pH 7.5). About 3 drops of

TABLE 1 Detection of OXA-48-like-producing Gram-negative bacilli using the OXA-163/48 Duo K-SeT test

Group/β-lactamase	No. of isolates	Bacterial species included (No. of isolates)	Nonsusceptibility (%)[a]		Positive isolates with OXA-163/48 Duo assay (*n* [%])			
					From solid medium		Direct from blood culture	
			Imipenem	Meropenem	OXA-48 band (first line)	OXA-163 band (second line)	OXA-48 band (first line)	OXA-163 band (second line)
OXA-48 subfamily								
OXA-48	7	*K. pneumoniae* (3), *E. coli* (3), *K. oxytoca* (1)	57	57	7 (100)	0 (0)	7 (100)	0 (0)
OXA-162	1	*K. pneumoniae* (1)	100	100	1 (100)	0 (0)	NT[b]	NT
OXA-181	4	*K. pneumoniae* (4)	75	75	4 (100)	0 (0)	4 (100)	0 (0)
OXA-232	3	*K. pneumoniae* (3)	100	100	3 (100)	1 (33)	3 (100)	0 (0)
OXA-244	2	*E. coli* (2)	100	100	2 (100)	1 (50)	2 (100)	0 (0)
Total	17		76	76	17 (100)	2 (12)	16 (100)	0 (0)
OXA-163 subfamily								
OXA-163	29	*K. pneumoniae* (12), *Enterobacter cloacae* (5), *Providencia stuartii* (4), *Citrobacter freundii* (3), *E. coli* (3), *Kluyvera georgiana* (1), *Klebsiella ozaenae* (1)	34	69	0 (0)	29 (100)	0 (0)	29 (100)
OXA-247	2	*E. coli* (1), *K. pneumoniae* (1)	50	50	0 (0)	2 (100)	0 (0)	2 (100)
OXA-438	2	*E. coli* (2)	50	50	0 (0)	2 (100)	0 (0)	2 (100)
Total	33		36	67	0 (0)	33 (100)	0 (0)	33 (100)
Other carbapenemase producers								
OXA-23	1	*A cinetobacter baumannii* (1)	100	100	0 (0)	0 (0)	0 (0)	0 (0)
OXA-58	1	*A. baumannii* (1)	100	100	0 (0)	0 (0)	0 (0)	0 (0)
OXA-72	1	*A. baumannii* (1)	100	100	0 (0)	0 (0)	0 (0)	0 (0)
OXA-143	1	*A. baumannii* (1)	100	100	0 (0)	0 (0)	0 (0)	0 (0)
KPC-2	1	*K. pneumoniae* (1)	100	100	0 (0)	0 (0)	0 (0)	0 (0)
KPC-3	1	*K. pneumoniae* (1)	100	100	0 (0)	0 (0)	0 (0)	0 (0)
Sme	1	*Serratia marcescens* (1)	100	0	0 (0)	0 (0)	0 (0)	0 (0)
IMI	1	*E. cloacae* (1)	100	0	0 (0)	0 (0)	0 (0)	0 (0)
GES-5	1	*K. pneumoniae* (1)	100	100	0 (0)	0 (0)	0 (0)	0 (0)
IMP-8	1	*E. coli* (1)	0	0	0 (0)	0 (0)	0 (0)	0 (0)
NDM-1	1	*K. pneumoniae* (1)	100	100	0 (0)	0 (0)	0 (0)	0 (0)
VIM-1	1	*E. coli* (1)	100	100	0 (0)	0 (0)	0 (0)	0 (0)
VIM-2	1	*K. pneumoniae* (1)	0	0	0 (0)	0 (0)	0 (0)	0 (0)
SPM-1	1	*Pseudomonas aeruginosa* (1)	100	100	0 (0)	0 (0)	0 (0)	0 (0)
Total	14		77	71	0 (0)	0 (0)	0 (0)	0 (0)
Non-carbapenemase producers								
OXA-1	1	*Proteus mirabilis* (1)	0	0	0 (0)	0 (0)	0 (0)	0 (0)
OXA-3	1	*E. coli* (1)	0	0	0 (0)	0 (0)	0 (0)	0 (0)
OXA-4	1	*E. coli* (1)	0	0	0 (0)	0 (0)	0 (0)	0 (0)
OXA-5	1	*P. aeruginosa* (1)	0	0	0 (0)	0 (0)	0 (0)	0 (0)
OXA-6	1	*E. coli* (1)	0	0	0 (0)	0 (0)	0 (0)	0 (0)
OXA-7	1	*E. coli* (1)	0	0	0 (0)	0 (0)	0 (0)	0 (0)
OXA-9	1	*E. coli* (1)	0	0	0 (0)	0 (0)	0 (0)	0 (0)
CMY-2	1	*E. coli* (1)	0	0	0 (0)	0 (0)	0 (0)	0 (0)
GES-1 + OXA-2	1	*P. aeruginosa* (1)	0	0	0 (0)	0 (0)	0 (0)	0 (0)
AmpC + porins[c]	1	*E. cloacae* (1)	100	0	0 (0)	0 (0)	0 (0)	0 (0)
CTX-M + porins[c]	1	*K. pneumoniae* (1)	0	100	0 (0)	0 (0)	0 (0)	0 (0)
Total	11		9	9	0 (0)	0 (0)	0 (0)	0 (0)

[a] All carbapenemase-producer and nonproducer isolates with extended-spectrum β-lactamase (ESBL)/AmpC production combined with decreased permeability were ertapenem nonsusceptible.
[b] NT = not tested.
[c] Decreased permeability of the outer membrane proteins.

each dilution were applied (without prior lysis) to the sample well. Independent tests were performed in triplicate, and the LoD was calculated as recommended (24). Using viable colony counts, the LoDs of the assay were 9.2×10^6 (OXA-163), 4.6×10^7 (OXA-247), and 2.1×10^7 CFU/ml (OXA-438), while for OXA-48, the LoD was 1.9×10^6 CFU/ml, which was similar to that of a previous report (2.41×10^6 CFU/ml [7]). Purified proteins were detected at up to 0.4 (OXA-48), 0.49 (OXA-163), and 0.625 ng/ml (OXA-247 and OXA-438), similar to the LoD indicated by the manufacturer for another K-SeT version (OXA-48 K-SeT; 0.125 ng/ml).

Finally, the ability of the OXA-163/48 Duo K-SeT test to directly detect OXA-163- and OXA-48-producing isolates recovered from blood culture was assessed with spiked bottles using the BD Bactec FX culture system (BD, Durham, NC). Blood cultures (Plus Aerobic/F, 30-ml bottles) were made from 10 ml sterile whole human blood inoculated with 0.1 ml of a suspension containing 10^2 CFU of each strain. Bottles were incubated until the blood culture was flagged positive by the system (from 6 to 11 h). Aliquots of a 0.1-ml volume were harvested in 10 drops of LY-A buffer, and subsequently, 3 drops of the mix were applied to the sample well (7). We confirmed that the final inoculum of positive cultures was higher than the LoD by performing serial-diluted subcultures. A band at the test position for OXA-48 was visible in all isolates with OXA-48-like enzymes (average inoculum, 8.7×10^8 CFU/ml), while a band at the test position for OXA-163 was observed for all OXA-163-like subfamily isolates (average inoculum, 3.1×10^9 CFU/ml) (Table 1). Negative results were obtained for non-OXA-163-like and non-OXA-48-like strains (average inoculum, 6.4×10^9 CFU/ml). Overall, the sensitivity and specificity for direct detection of OXA-48-like strains from blood cultures were both 100%.

We developed the OXA-163/48 Duo K-SeT test for the rapid identification of enzymes from the OXA-48 and OXA-163 subfamilies (21). The duo test was conceived to enable the detection of several OXA-48 allelic variants (OXA-48, OXA-181, OXA-204, OXA-232, and OXA-244), since this carbapenemase family is currently becoming the predominant carbapenemase type in *Enterobacteriaceae* in many countries in Europe and Africa (2). Additionally, the objective was to expand the detection portfolio to include other OXA-48-like enzymes with complex therapeutic management (12, 15, 20), such as OXA-163, which are becoming endemic in many hospitals in the Middle East and Argentina (15, 16). On the basis of the number of isolates submitted for confirmation of carbapenemases to the National Reference Laboratory (NRL), an estimated annual increase in the prevalence of *K. pneumoniae*-producing OXA-163 from 0.4% (2013) to 2.6% (2014) was revealed, suggesting that this variant contributes to bacterial dissemination. The OXA-163/48 Duo K-SeT test differentiates between subfamilies with distinct substrate profiles, such as OXA-48 and OXA-163, obviating the need for more costly and lengthy characterization with molecular amplification methods. The assay was highly sensitive and specific and detected the presence of OXA-48-producing strains within seconds to minutes, similar to results previously reported for the OXA-48 subfamily and KPC carbapenemases (7–10, 25). No significant difference in band intensities was observed between the different subfamilies and/or variants tested or between the bacterial species, in carbapenem MICs, or in associations of the OXA protein to other β-lactamases (data not shown). The 100% detection performance of the test was assessed on the basis of bacterial cultures from a representative panel of clinical bacterial isolates submitted to the NRL in Argentina. Due to the low limits of detection observed for key enzymes (10^6 to 10^7 CFU/ml), the OXA-163/48 Duo K-SeT tests were also proven to be efficient for the rapid detection of OXA-48 and OXA-163 subfamilies from clinical specimens, such as positive blood cultures. Users of blood culture medium brands other than that used here should validate the performance of this test with their systems. The average time for a positive result was less than 9 h after inoculation of the bottles, which may confer another major advantage for rapid therapeutic management of infected patients and for infection control purposes.

In conclusion, the OXA-163/48 Duo K-SeT assay is an ideal tool for routine identification of OXA-48-like-producing *Enterobacteriaceae* due to its accuracy, simplicity, and short turn-around time to result. The inclusion of targets for new worldwide-disseminated OXA-48-like enzymes enables broader surveillance of bacteria with potential resistance to carbapenem treatment.

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