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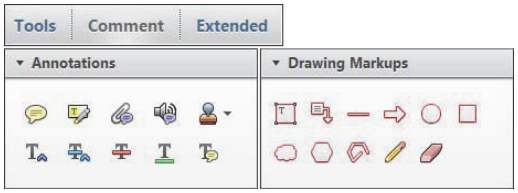

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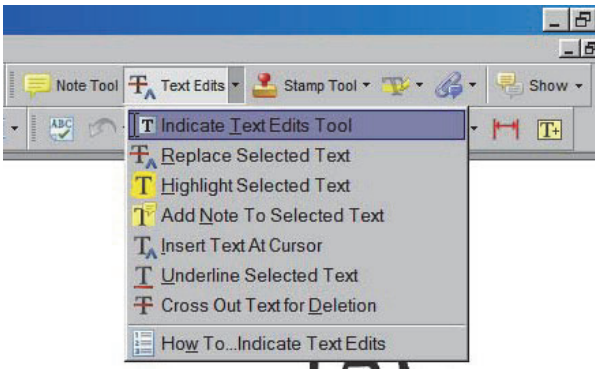

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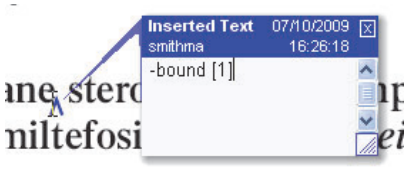

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Evaluation of two new commercial immunochromatographic assays for the rapid detection of OXA-48 and KPC carbapenemases from cultured bacteria

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Background: Rapid detection and confirmation of carbapenemases remains very challenging for diagnostic laboratories.

Objectives: The objective of this study was to assess the performance of two new immunochromatographic (IC) commercial assays for the rapid detection of OXA-48-producing and KPC-producing Enterobacteriaceae in pure bacterial isolates.

Methods: A panel of 92 bacterial isolates predominantly including carbapenem-non-susceptible Enterobacteriaceae with previously defined carbapenem resistance mechanisms was tested. Then, 342 consecutive carbapenem-non-susceptible Enterobacteriaceae isolates referred to the reference laboratory were investigated prospectively in parallel with other phenotypic tests and with multiplex PCR and sequencing as the gold standard.

Q1 **Results:** In the collection panel, each of the two IC assays correctly detected all 30 OXA-48-like-producing isolates and 25 KPC-producing isolates, whatever the species, their association with other β-lactamases and the level of resistance to carbapenems. All other carbapenemase producers and all non-carbapenemase-producing isolates yielded negative results with both tests. In the prospective evaluation, all OXA-48-like-producing Enterobacteriaceae isolates ($n=130$) and KPC-producing Enterobacteriaceae isolates ($n=33$) were correctly detected by the individual IC assays, while 179 non-OXA-48-producing and non-KPC-producing strains (137 non-carbapenemase producers and 42 isolates belonging to other carbapenemase family types) yielded negative results. Thus, both assays yielded 100% sensitivity and 100% specificity for the detection of OXA-48-like and KPC enzymes at 15 min.

Q2 Q3 **Conclusions:** The two IC assays allow rapid and reliable direct confirmation of OXA-48 and KPC carbapenemases from culture colonies and appear to be very useful additions to the existing tests, obviating the need for more costly characterization by molecular amplification methods.

Introduction

The global dissemination of MDR Gram-negative bacteria with acquired and transferable carbapenemase genes constitutes a major public health concern worldwide.[1–3] Active public health surveillance programmes and rapid reliable identification of these strains by clinical microbiology laboratories at a local level are considered essential measures for effective infection control and for optimizing individual patient management.[4,5] MS and molecular genotyping can be used to detect enzymatic resistance to carbapenems and to rule out non-susceptibility to carbapenems due to impermeability mechanisms.[6] These techniques do, however, require specific equipment and trained personnel with expertise and thus cannot be implemented in all laboratories. Moreover, PCR-based techniques have the limitation, besides their inherent higher cost, of detecting only genes encoding known enzymes.[7] In recent years, several phenotypic testing methods have been proposed and described for the identification of carbapenemase-producing Enterobacteriaceae (CPE), rendering their diagnosis more accessible. These methods include the modified Hodge test (MHT),[8] biochemical colorimetric tests detecting carbapenem hydrolytic activity[9,10] and carbapenemase inhibitor-based disc tests.[11,12]

While the MHT has been found useful for the phenotypic detection of carbapenemase production, this test cannot discriminate between carbapenemase types and can give false-positive results

among non-carbapenemase-producing strains.[1,7,13] The Carba NP test has proven to be a very accurate biochemical test for the screening of carbapenemase production in Enterobacteriaceae and in *Pseudomonas* spp.,[10] and a ready-to-use version (RAPIDEC® Carba NP test) well adapted for routine use in laboratories has been recently commercialized.[14] This test followed by the use of molecular techniques can accurately differentiate types of carbapenemases.[15]

The usefulness of combination disc tests with boronic acid derivatives and EDTA or dipicolinic acid as carbapenemase inhibitors in disc potentiation tests has been demonstrated for the differentiation of KPC producers from those producing metallo-β-lactamases (MBLs).[11,12] On the other hand, OXA-48-like carbapenem-hydrolysing class D enzymes (CHDLs) have proven particularly difficult to detect, both because of their relatively low inherent MICs and because of the lack of specific inhibitor compounds to use in confirmatory tests.

We recently developed lateral flow immunochromatographic (IC) assays (OXA-48 *K*-SeT® Coris BioConcept, Gembloux, Belgium) for direct confirmation of OXA-48-like carbapenemase based on monoclonal antibodies that were generated by immunization in mice.[16] In the present study, we aimed to evaluate in parallel with the OXA-48 IC assay the performance of another IC assay, KPC-*K*-SeT® (Coris BioConcept), to differentiate accurately OXA-48-type and KPC-type-possessing Enterobacteriaceae isolates from those either possessing carbapenemases belonging to other families (i.e. MBLs such as NDM, VIM or IMP) or exhibiting reduced carbapenem susceptibility due to ESBL or AmpC production combined with decreased permeability of the outer membrane proteins.

Materials and methods

Clinical isolates

First, the evaluation of the test was performed with a collection of 92 Enterobacteriaceae and non-glucose-fermenting Gram-negative collection isolates with resistance mechanisms to β-lactam agents that had been previously characterized. These isolates comprised 63 carbapenemase producers, among which 30 were confirmed to belong to the OXA-48 type, 25 to KPC types and 14 to other major carbapenemase families (i.e. NDM, VIM and IMP) that were carbapenem non-susceptible by CLSI interpretative criteria.[8] Six of the isolates from the collection panel harboured two different carbapenemase-encoding genes, including either bla_{KPC} or bla_{OXA-48} as one of these genes (Table S1, available as Supplementary data at *JAC* Online). In a second step, 342 non-duplicate consecutive Enterobacteriaceae clinical isolates that were referred to the national reference centre from April to August 2015 for suspected carbapenemase production were tested (Table S2).

All received clinical isolates were re-identified using MALDI-TOF MS (Microflex LT, Bruker Daltonik, Leipzig, Germany) with MALDI Biotyper software (version 3.0).

Antimicrobial susceptibility testing and algorithms used for the detection of carbapenemases

Inhibition zone diameters of meropenem (10 μg), ertapenem (10 μg) and temocillin (30 μg) paper discs (Bio-Rad) tested according to CLSI disc diffusion methodology were measured for all isolates. Screening cut-off values for detecting putative carbapenemase producers were based on the criteria defined by EUCAST (zone <25 mm for ertapenem)[17] and modified for meropenem (zone <28 mm) according to a previous evaluation performed, by the French and the Belgian national reference centres, in two geographical areas with high prevalence of OXA-48 producers.[18] ertapenem, meropenem and temocillin were determined by the broth microdilution method according to current CLSI guidelines.[8] For temocillin, for which no CLSI interpretative guidelines are reported, high-level resistance was arbitrarily defined as an MIC >128 mg/L. In isolates displaying a positive screening for carbapenemase, a diameter zone of temocillin of <12 mm was considered highly suggestive of the presence of OXA-48 and led to the direct confirmation of the presence of this carbapenemase by means of the OXA-48 IC assay.

Phenotypic confirmation tests

The Carba NP test used in our laboratory as routine screening test was performed in parallel with the prospective assessment of the performance of the OXA-48 and KPC IC assays according to the procedure initially published by Nordmann *et al.*[9] In the algorithm used for detection of carbapenemases in this survey, the Carba NP test was performed either as a first-line confirmatory test for isolates with a temocillin zone size ≥12 mm or as a second-line test for those isolates with a zone size <12 mm after ruling out the presence of OXA-48 carbapenemase by a negative result of the OXA-48 IC assay. In this algorithm, isolates with a negative Carba NP test result were not further processed and were considered to be non-carbapenemase producers.

The presence of MBLs was assessed by means of inhibitor-based disc tests using a Total MBL Confirm Kit (98016; Rosco Diagnostica, A/S, Taastrup, Denmark) in compliance with the manufacturer's instructions (http://www.rosco.dk/). Detection of MBLs by inhibitor-based disc tests was carried out selectively in the subset of isolates that showed a positive Carba NP test result but were found negative for OXA-48 and KPC by IC assays, according to the algorithm shown in Figure 1.

Molecular testing for β-lactamase genes

β-Lactamase genes were amplified using an in-house validated multiplex PCR targeting bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{KPC} and bla_{OXA-48}.[19] Other β-lactamase-encoding genes, including plasmid-mediated AmpCs and

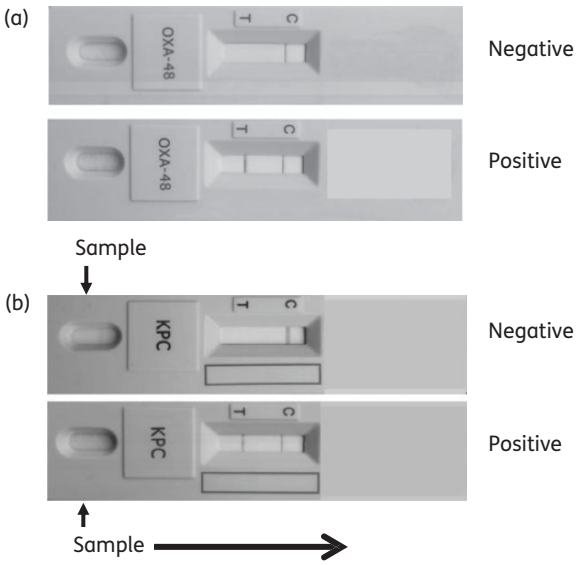

Figure 1. IC assays for the detection of OXA-48-type (a) and KPC-type (b) carbapenemases. For negative results, a single line appears at the position of the control line (C) only. For positive results, another line also appears at the position of the test line (T) in addition to the control line. The arrow indicates the direction of lateral flow.

ESBLs (SHV, TEM and CTX-M), were detected by a DNA microarray PCR-ligase method as previously reported.[20,21] The PCR products were subjected to direct sequencing using an external service company (Macrogen, Seoul, South Korea). The sequence obtained was compared with the genes present in GenBank and was aligned with the reference gene cited at the Lahey Clinic web site (http://www.lahey.org/Studies).

Immunochromatography

The OXA-48 *K*-SeT® and KPC-*K*-SeT® assays developed by Coris BioConcept are based on monoclonal antibodies that were generated by immunization in mice. Both assays rely on immunological capture of two epitopes specific to the OXA-48 and KPC enzymes, using colloidal gold nanoparticles bound to a nitrocellulose membrane within a lateral flow device.[16] For OXA-48, capture and detection antibodies were selected (on more than 20 different monoclonal antibodies raised) among those binding all current CHDL OXA-48-like variants (OXA-48, OXA-162, OXA-181, OXA-204, OXA-232 and OXA-244). Antibodies reacting with OXA-48-like non-CHDL variants, such as OXA-163 or other related variants (OXA-247 and OXA-405), were not retained, in order to detect only OXA-48 variants with carbapenemase activity.

The two assays are currently commercialized as single separate tests for detection of OXA-48-like and KPC carbapenemases directly from bacterial culture. Kits used in the present evaluation were kindly provided by Coris BioConcept and used according to the manufacturer's instructions. The time to apparition of the OXA-48 or of the KPC signal was recorded by one technologist, and the final results were confirmed after 15 min by another independent technologist. A negative result was confirmed when the control band appeared while the OXA-48- or KPC-corresponding bands were not observed. Each strain from the collection was tested in triplicate. Prospectively, the isolates were tested only once.

Sensitivity, specificity and statistical analysis

The performance of the two IC assays was evaluated by using our in-house multiplex PCR method and/or commercial molecular assays as the gold standard. The sensitivity of the assays was calculated from the number of OXA-48- and KPC-producing organisms that were correctly determined, while the specificity was calculated from the number of non-OXA-48- and non-KPC-producing organisms that were correctly determined. The overall sensitivity and specificity were calculated on all tested strains, while positive and negative predictive values were calculated on consecutive isolates referred to the reference laboratory.

Results

Evaluation of the performance of the IC assays for detection of OXA-48-like and KPC carbapenemases on 92 strains selected from the Belgian reference laboratory is presented in Table S1. Overall, the OXA-48 IC assay detected all 30 OXA-48-like carbapenemases, including OXA-48 and different variants (OXA-162, OXA-181, OXA-204, OXA-232, OXA-244), whatever the carbapenem MIC range and independently of the association of the OXA-48 protein with other β-lactamases. As anticipated, the OXA-48 IC assay did not detect OXA-163 or OXA-405, two variants of OXA-48 with hydrolytic activity against expended-spectrum cephalosporins (ESBL-like) but lacking any significant activity against carbapenems (data not shown). This finding further confirms the excellent specificity of the assay for the detection of carbapenemase variants of OXA-48 only. The KPC IC assay detected all 25 KPC-producing organisms (KPC-2, KPC-3 and KPC-4 variants), including four KPC-positive isolates that co-produced an OXA-48 or an NDM-1 carbapenemase. Visible bands for a positive test systematically appeared between 15 and 360 s after inoculation of the strip, with no significant difference in intensity observed between the different OXA-48 or KPC variants and/or the Enterobacteriaceae species. On the other hand, both IC assays yielded negative results at 15 min for all non-OXA-48- and non-KPC-producing strains, comprising a diverse panel of carbapenemase-producing organisms from types other than the OXA-48 family, as well as 24 non-carbapenemase-producing isolates. Overall, the sensitivity and the specificity of the assays on the collection panel were 100% for the detection of OXA-48-like and KPC carbapenemases. Examples of positive and negative results for the rapid detection of OXA-48 and KPC by these IC assays are shown in Figure 1.

In the prospective study, 205 (59.9%) of the 342 putative carbapenem-non-susceptible Enterobacteriaceae isolates that were referred to the reference laboratory harboured carbapenemase genes, and 103 (50.2%) of these isolates co-produced one ESBL or several ESBLs. Carbapenemase genes were mostly present in *Klebsiella pneumoniae* ($n=145$), followed by *Enterobacter cloacae* ($n=21$), *Escherichia coli* ($n=14$), *Citrobacter freundii* ($n=12$) and miscellaneous other species ($n=13$). Among the 205 CPE isolates, 130 (63.4%) had positive results for OXA-48-group carbapenemase [OXA-48 ($n=123$), OXA-181 ($n=5$) and OXA-232 ($n=2$)], 33 (16.1%) for KPC [KPC-3 ($n=31$) and KPC-2 ($n=2$)], 22 (10.7%) for NDM [NDM-1 ($n=21$) and NDM-5 ($n=1$)], 21 (10.2%) for VIM [VIM-1 ($n=13$) and VIM-4 ($n=8$)] and 1 (0.5%) for IMP-1. Two *K. pneumoniae* isolates simultaneously harboured an OXA-48 variant (OXA-232) and an NDM-1-encoding gene (Table S2).

The OXA-48 IC assay yielded a positive test result in all 130 OXA-48 producers. Likewise, the 33 KPC-producing isolates were detected by the KPC IC assay. On the other hand, 42 MBL-producing isolates, as well as the 137 carbapenemase-negative Enterobacteriaceae isolates, yielded a negative result after 15 min.

Equivocal or indeterminate results were not recorded in any of the strains tested, while inter-observer agreement for the reading of the test was obtained in all cases. The results were thus fully consistent with those of PCR analysis for bla_{OXA-48} and for bla_{KPC} genes, showing 100% sensitivity and 100% specificity and thus indicating reliability of the IC assays for the detection and identification of these two carbapenemases. On the other hand, the Carba NP test yielded positive results for all class A (KPC) and class B (MBLs) enzymes, but failed to detect the presence of a weakly expressed carbapenemase in 7 of 130 OXA-48-producing isolates, three of which were found, by sequencing, to correspond to the OXA-181 variant (Table S2).

The algorithm for confirmation of CPE based on the results obtained in this prospective evaluation is shown in Figure 2. The presence of a carbapenemase could be ruled out by PCR in all 53 Enterobacteriaceae isolates with diameter inhibition zones of ertapenem of ≥25 mm, hence validating the screening cut-off value proposed by EUCAST. For meropenem, none of the isolates with a zone diameter ≥28 mm could be confirmed as CPE, indicating that either of these criteria could be used as first screening for excluding strains from further analysis.

All 130 OXA-48-like CPE isolates had a temocillin zone <12 mm, supporting the hypothesis that this cut-off level could be considered as a first screening step before realization of the OXA-48 IC assay for confirmation of this resistance mechanism.

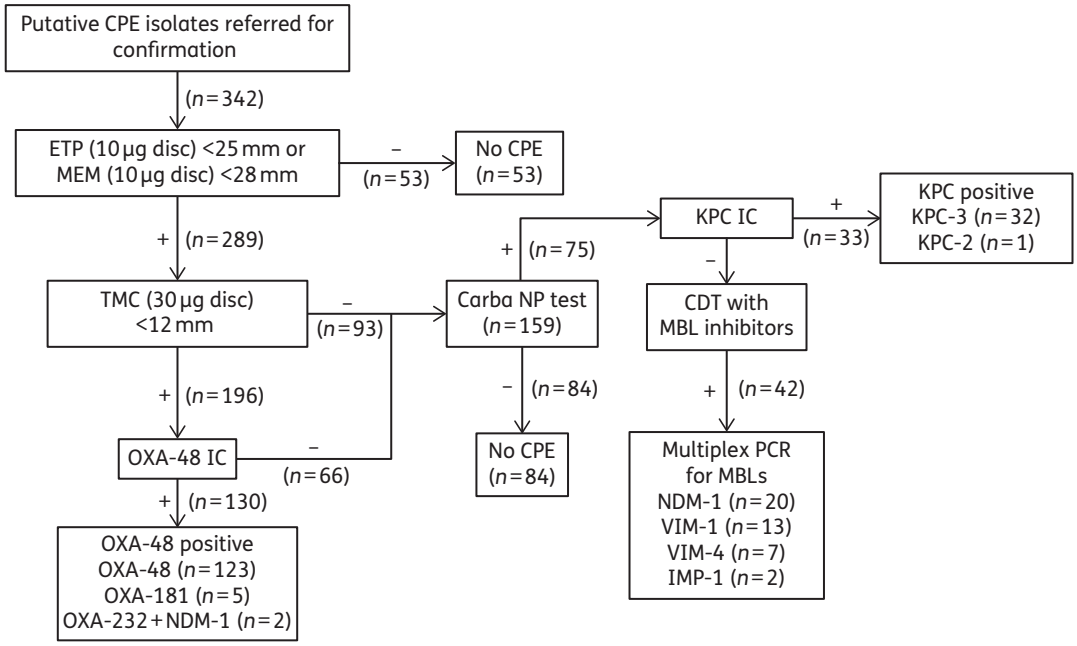

Figure 2. Algorithm for the detection and characterization of carbapenemases in 342 putative CPE isolates using OXA-48 and KPC IC assays in combination with other phenotypic and molecular confirmatory tests. ETP, ertapenem; MEM, meropenem; TMC, temocillin; CDT, combined disc test (DPA/EDTA used as MBL inhibitory compounds).

Of the 159 putative CPE isolates either yielding a negative result by the OXA-48 IC assay ($n=66$) or displaying a temocillin zone ≥12 mm ($n=93$), 75 were found positive by the Carba NP test. Among these, 33 *K. pneumoniae* isolates were identified as KPC positive by the IC assay (none was positive for OXA-48). The 42 remaining isolates (all with a positive inhibitor-based synergy disc test result for MBLs) were confirmed as MBL producers by our in-house multiplex PCR assay (Figure 2). Thus, in our setting, almost 80% of the CPE isolates could have been directly identified (KPC and OXA-48-like producers) in a few minutes from culture colonies, without the need for any further testing.

Discussion

Nowadays, carbapenem-non-susceptible Enterobacteriaceae possessing various carbapenemases or other broad-spectrum β-lactamases are rapidly disseminating in several countries and large geographical areas.[2,3] More specifically, Enterobacteriaceae producing KPC-type and MBL-type carbapenemases are very widespread worldwide, while broad-spectrum β-lactamases, such as ESBLs and AmpCs usually combined with porin loss, also sporadically contribute to reduced levels of susceptibility to carbapenems. Recent epidemiologic data have highlighted that the prevalence and incidence of OXA-48 variants are rapidly increasing and that OXA-48 is currently becoming the predominant carbapenemase type in Enterobacteriaceae in many countries in Europe,[2,3] as well as in other areas such as North Africa, Turkey and the Middle East.[2,3,22–24] In view of the apparently uncontained spread and the concern for undetectable low-level resistance to carbapenems,[18,25] there is good justification for clinical laboratories to test for OXA-48-like carbapenemases. Detection of this carbapenemase family appears, however, to be very challenging because a substantial proportion of OXA-48-producing Enterobacteriaceae strains, especially *E. coli* and *K. pneumoniae*, show low-level resistance or even susceptibility to carbapenems. Moreover, all currently used phenotypic methods are suboptimal and there are no single tests apart from molecular methods that allow the direct positive confirmation of this carbapenemase.

We report here the evaluation of two novel IC assays for the direct detection of OXA-48 or KPC carbapenemases in organisms exhibiting reduced susceptibility to carbapenems. Both tests were found to be convenient, easy to perform and highly accurate for rapid confirmation of OXA-48 or KPC carbapenemases. One major advantage of these assays is that they allow the accurate identification of these two carbapenemase families in a few minutes directly from bacterial culture, obviating the need to perform the MHT or carbapenemase inhibitor-based disc tests, which are much more complex to implement in the routine workflow of a clinical microbiology laboratory and in any case do not allow the precise identification of the carbapenemase type. Preliminary personal experience (unpublished data) indicates that both tests may also prove efficient for the rapid detection of OXA-48 or KPC carbapenemases directly from clinical specimens (i.e. urine and blood specimens), which may constitute another major advantage.

Further, it should be noted that besides the detection of the OXA-48 enzyme itself, the IC assay also allowed the detection of several OXA-48 allelic variants (OXA-181, OXA-204, OXA-232, OXA-244), some of which were missed by the Carba NP test. Moreover, the assay did not react with OXA-48-like non-CHDL variants, such as OXA-163 or OXA-405, which exhibit a typical ESBL profile (resistance to expanded-spectrum cephalosporins,

susceptibility to carbapenems) but no carbapenemase activity. Consequently, the OXA-48 IC assay besides its usefulness for infection control purposes may also be of great interest for therapeutic management of infected patients.

In areas with higher endemicity of OXA-48, we suggest an algorithm in which the OXA-48 IC assay would be performed as a first step on all putative CPE isolates (i.e. those strains displaying resistance or reduced susceptibility to carbapenems and high-level resistance to temocillin). Following a negative result by the test, an imipenem hydrolysis-based test may eventually be realized as second-line testing. Alternatively, the two IC assays could be carried out as a definitive confirmatory test for identification of KPC and/or OXA-48 on putative CPE isolates displaying a positive result by a rapid carbapenem hydrolysis test such as the Carba NP test. Relying on the IC assays for rapid confirmation of OXA-48 and KPC would thus obviate the need for PCR or other tedious confirmatory testing.

Adoption of these tests should contribute to more effective control of the spread of organisms possessing these resistance mechanisms in outbreak settings. The potential benefits would possibly also include better patient management and a reduction in the escalation of antibiotic resistance through better infection control.

In conclusion, the two new IC assays are considered to be very valuable tests for direct confirmation of OXA-48-like and KPC carbapenemases in routine clinical microbiology testing, with advantages in cost (retail price of 6€/test), short processing time, reliability and convenience. In particular, they do not require any special equipment and obviate the need for molecular amplification methods, which are both costly and frequently unavailable in most laboratories.

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Transparency declarations

SQ4 [redacted] and P. M. are currently employed at Coris BioConcept, as project leader and head of the research and development department, respectively. T. L. is CEO and Scientific Director of Coris BioConcept. All other authors: none to declare.

The OXA-48 and KPC-*K*-SeT assays have been patented under the patent number EP15163286.6: 'Method and device for detecting a carbapenemase-producing Enterobacteriaceae'.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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