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Validation of carbapenemase and extended-spectrum β-lactamase multiplex endpoint PCR assays according to ISO 15189

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Objectives: To validate and accredit a set of three multiplex endpoint PCR assays, targeting the most important carbapenemase and minor extended-spectrum β-lactamase (ESBL) resistance genes, according to the international ISO 15189 particular requirements for the quality and competence of medical laboratories.

Methods: Specific primers targeting ESBLs and carbapenemases were collected from the literature or designed internally. The multiplex PCRs were validated for sensitivity, specificity, intra- and inter-run reproducibility and accuracy by means of external quality control (EQC) using a collection of 137 characterized and referenced isolates. For each multiplex PCR assay, the presence of an extraction control ruled out false-negative results due to PCR inhibition or extraction faults. Amplicons were separated by capillary electrophoresis (QIAxcel system, Qiagen). The protocols and validation files were reviewed in the setting of an external audit conducted by the Belgian organization for accreditation (BELAC).

Results: Sensitivity, specificity and reproducibility for each targeted gene were 100%. All isolates from the three EQC panels were correctly identified by each PCR assay (accuracy 100%). The validation files were controlled by BELAC, and the PCR protocols were accepted as accredited according to ISO 15189.

Conclusions: Three home-made multiplex PCRs targeting the major carbapenemases and four minor class A ESBL genes encountered in Gram-negative bacteria were accredited according to the ISO 15189 standards. This validation scheme could provide a useful model for laboratories aiming to accredit their own protocols.

Keywords: accreditation, minor ESBLs, molecular detection

Introduction

The worldwide spread of genes conferring resistance to broad spectrum β-lactams including carbapenems in Gram-negative bacteria is a source of global concern.[1–3] Class A extended-spectrum β-lactamase (ESBL)-encoding genes such as bla_{TEM}, bla_{SHV} and especially bla_{CTX-M} have largely disseminated worldwide among Enterobacteriaceae. Other *bla* genes encoding minor ESBLs (bla_{BEL}, bla_{VEB}, bla_{GES} and bla_{PER}) are more rarely reported, although they have also been observed worldwide especially among Gram-negative non-fermenters.[4]

Even more worrying is the recent emergence and spread of genes encoding carbapenemases.[3] Although class D carbapenem-hydrolysing β-lactamases of group OXA-23, OXA-24, OXA-58 or OXA-143 type are almost exclusively reported in *Acinetobacter baumannii*,[5] OXA-48 (and related types) seems to be exclusively and widely expressed in Enterobacteriaceae.[6] Besides OXA-48, the spread of carbapenemases of class A (KPC and some GES variants such as GES-2 and GES-5) and of class B (VIM, IMP and NDM) have been reported, albeit at differing frequencies, in Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter* spp.[1,7,8]

Rapid and reliable detection methods are important for the early implementation of infection control measures and for preventing the subsequent dissemination of ESBLs and of carbapenemases. Although a promising, rapid and easy detection method based on the antibiotic hydrolytic properties of the expressed β-lactamases has been recently published,[9,10] confirmation and identification of the precise types of β-lactamase genes involved still need the use of molecular PCR-based methods.

There is a plethora of PCR methods described in the literature that are often validated only internally.[11–15] Nevertheless, quality standards requirements for medical laboratories are

now evolving as a general rule, and compliance with the international ISO 15189 requirements for the quality and competence of medical laboratories is becoming mandatory.[16] The accreditation process according to ISO 15189 represents a highly challenging task for medical laboratories. One particular difficulty is related to the fact that these requirements have to be fulfilled, but there are no universal ways or hints about how to reach them.

We present here the validation scheme for three multiplex endpoint PCR assays targeting some of the most epidemiologically relevant carbapenemases (PCR CARBA targeting bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{KPC} and bla_{OXA-48} and PCR OXACARBA targeting bla_{OXA-23}, bla_{OXA-24} and bla_{OXA-58}) and the minor ESBLs (PCR MINESBL targeting bla_{BEL}, bla_{GES}, bla_{PER} and bla_{VEB}). The latter two tests were particularly important for our activity of reference centre as no commercial assay is currently available for these targets. These PCR assays were validated and accredited according to the international ISO 15189 standards after an external audit performed by the Belgian accreditation body BELAC. This validation scheme could be useful for implementing accredited home-made PCRs in the routine laboratory.

Materials and methods

Bacterial isolates (Table 1)

A collection of 137 Gram-negative clinical isolates obtained from the European FP7 TEMPOtest-QC consortium Number 241742 (http://www.tempotest-qc.eu/newweb/index.php?pageId=12) was used to validate the three multiplex PCR assays (82 isolates for PCR CARBA, 79 for PCR MINESBL and 68 for PCR OXACARBA). This collection comprised 56 Enterobacteriaceae isolates [*Klebsiella pneumoniae* ($n=21$), *Escherichia coli* ($n=8$), *Citrobacter* spp. ($n=8$), *Enterobacter* spp. ($n=6$), *Klebsiella oxytoca* ($n=3$), *Serratia marcescens* ($n=2$), *Providencia* spp. ($n=2$), *Proteus* spp. ($n=2$), *Hafnia alvei* ($n=1$), *Morganella morganii* ($n=1$), *Salmonella enterica* ($n=1$) and *Aeromonas hydrophila* ($n=1$)] and 81 Gram-negative non-fermenters [*Pseudomonas* spp. ($n=41$), *Acinetobacter* spp. ($n=39$) and *Alcaligenes xylosoxidans* ($n=1$)] expressing various resistance genes.

Design of primers (Table 2)

For each gene family, all alleles referenced on the Lahey Clinic web site (http://www.lahey.org/Studies/) were uploaded from GenBank databases and aligned using the ClustalX software version 2.0. Primers were designed within the common coding region of the published alleles. An additional primer pair that targets the chromosomal AmpC of *A. baumannii* (bla_{ADC}) was used as an internal PCR/extraction control. All the primers apart from the forward primer targeting bla_{VEB}[17] were designed in our laboratory.

DNA extraction and multiplex PCR assays

A single colony was suspended in 200 μL of distilled water, and 10 μL of a McFarland 3 turbidity standard of *A. baumannii* ATCC 19606 was added to the suspension before extraction as an internal extraction control (95°C for 10 min in a dry bath). The 25 μL amplification mixture contained 2 μL of DNA extract, 12.5 μL of 2 × master mix multiplex PCR Kit (Qiagen Benelux, Antwerp, Belgium) and 200 μM of each primer (except for IMP, for which the concentration of the primers was raised to 600 μM). PCR was performed on a ABI 2720 thermocycler (Life Technologies Europe BV, Gent, Belgium) under the following conditions: 15 min at 95°C and 30 cycles of 30 s denaturation at 94°C; 90 s annealing at 57°C and 90 s elongation at 72°C; and a final elongation step at 72°C for 10 min.

Each PCR run had to include three PCR controls: a positive resistance gene control including each of the targeted genes, a DNA extraction positive control including only the internal extraction control suspended in water, and a negative control (only water, no DNA). The amplicons were visualized by capillary electrophoresis on a QIAxcel instrument (Qiagen Benelux) using the QIAxcel high-resolution kit, QX DNA size marker 100–2500 bp and QX alignment markers 15/5000 bp according to the manufacturer's recommendations. The whole process, including extraction and electrophoresis takes <4 h. A negative result could only be technically validated when the band corresponding to the internal extraction control was present. In case of a positive result for any of the targeted genes, the presence of the internal control does not need to be taken into account.

Validation process

The validation process was based on the procedure proposed by Rabenau *et al.*[18] for home-made qualitative nucleic acid testing. This process includes the control of the specificity, sensitivity, reproducibility and accuracy of each PCR multiplex assay. For sensitivity, at least 10 different isolates positive for the gene to be detected were tested once. In cases where the minimum number of isolates harbouring the targeted genes was not available in the library, the available isolates were extracted twice or more so as to reach 10 different sample preparations. For specificity, at least 20 isolates known to be negative for the targeted genes, but possibly expressing other resistance genes representative of the current β-lactamase epidemiology, were tested once. For reproducibility, one isolate positive for each resistance gene was tested three times intra-run and three times inter-run. Finally, accuracy was certified by testing a panel prepared and sent by an external laboratory [external quality control (EQC) process] comprising three positive isolates for each target to be tested and three negative ones. This panel equally comprised referenced isolates from the TEMPOtest-QC collection.

Results and discussion

The three multiplex PCR assays were validated with a panel of 137 characterized and referenced Gram-negative clinical isolates (Table 1) according to the validation protocol presented in the 'Materials and methods' section. This collection includes 30 metallo-β-lactamase-expressing isolates (10 VIM-, 10 IMP- and 10 NDM-expressing isolates), 10 OXA-48-expressing isolates, 10 KPC-expressing isolates, 31 OXA-23, OXA-24 or OXA-58-expressing isolates, 41 minor ESBL-expressing isolates (BEL, VEB, GES and PER), 27 TEM-expressing isolates, 26 SHV-expressing isolates and 14 CTX-M-expressing isolates. In addition, 33 isolates expressing additional β-lactamases not targeted by the PCR assays were used for specificity testing.

For each multiplex PCR assay, the presence of an extraction control ruled out false-negative results due to PCR inhibition or extraction faults. In the positive resistance gene control, the presence of each band at the expected size confirms the ability of the PCR to detect up to five resistance genes in a single PCR (Figure 1). The positive extraction PCR control has to be performed in a separate well as the corresponding 1059 bp amplicon tends to disappear in the presence of another resistance gene. It highlights that the extraction/inhibition control does not interfere with the detection of the targeted resistance genes generating smaller amplicons.

Table 1. Collection of characterized clinical isolates used for multiplex PCR assay validation

Species	Number	bla_{VIM}, bla_{IMP} or bla_{NDM}	bla_{OXA-48}	bla_{KPC}	bla_{OXA-23}, bla_{OXA-24} or $bla_{OXA-58\ groups}$	bla_{BEL}, bla_{GES}, $bla_{PER\ or}$ bla_{VEB}	bla_{TEM}[a]	bla_{SHV}[a]	bla_{CTX-M} of G1, 2 and 9[a]	Other β-lactamases[b]
Klebsiella pneumoniae	21	5	4	10			11	20	3	10
Escherichia coli	8	2	2				6	2	3	9
Enterobacter cloacae	4	2	2				1	3	4	1
Klebsiella oxytoca	3	1	1			1				
Citrobacter freundii	3		1			1	1			
Serratia marcescens	2	1					1		1	
Enterobacter aerogenes	1						1	1		
Enterobacter asburiae	1									
Other Enterobacteriaceae[c]	14	4				3	4		3	2
Acinetobacter baumannii	34	2			27	10	1			1
Acinetobacter spp. other than *A. baumannii*	5	1			4		1			
Pseudomonas aeruginosa	39	10				26				10
Pseudomonas spp. other than *P. aeruginosa*	2	2								
Total	137	30	10	10	31	41	27	26	14	33

[a]Including both ESBLs and non-ESBLs.
[b]Including plasmidic AmpC, carbenicillinases, oxacillinases and SPM metallo-β-lactamase.
[c]Including *Proteus vulgaris*, *Aeromonas hydrophila*, *Citrobacter* spp., *Hafnia alvei*, *Morganella morganii* and *Providencia* spp.

Table 2. Primer sequences and amplicon sizes

PCR name	Targeted gene		Primer sequence (5′ to 3′)[a]	Amplicon size
CARBA	bla_{NDM}	Forward	ACT TGG CCT TGC TGT CCT T	603 bp
		Reverse	CAT TAG CCG CTG CAT TGA T	
	bla_{VIM}	Forward	TGT CCG TGA TGG TGA TGA GT	437 bp
		Reverse	ATT CAG CCA GAT CGG CAT C	
	bla_{IMP}	Forward	ACA YGG YTT RGT DGT KCT TG	387 bp
		Reverse	GGT TTA AYA AAR CAA CCA CC	
	bla_{KPC}	Forward	TCG CCG TCT AGT TCT GCT GTC TTG	353 bp
		Reverse	ACA GCT CCG CCA CCG TCA T	
	bla_{OXA-48}	Forward	ATG CGT GTA TTA GCC TTA TCG	265 bp
		Reverse	CAT CCT TAA CCA CGC CCA AAT C	
OXACARBA	$bla_{OXA-23\ group}$	Forward	CCC CGA GTC AGA TTG TTC AAG G	330 bp
		Reverse	TAC GTC GCG CAA GTT CCT GA	
	$bla_{OXA-24/143\ group}$	Forward	GCA GAA AGA AGT AAA RCG GGT	271 bp
		Reverse	CCA ACC WGT CAA CCA ACC TA	
	$bla_{OXA-58\ group}$	Forward	GGG GCT TGT GCT GAG CAT AGT	688 bp
		Reverse	CCA CTT GCC CAT CTG CCT TT	
MINESBL	bla_{PER}	Forward	AGT GTG GGG GCC TGA CGA T	725 bp
		Reverse	GCA ACC TGC GCA ATR ATA GCT T	
	bla_{GES}	Forward	CTG GCA GGG ATC GCT CAC TC	600 bp
		Reverse	TTC CGA TCA GCC ACC TCT CA	
	bla_{BEL}	Forward	CGA CAA TGC CGC AGC TAA CC	448 bp
		Reverse	CAG AAG CAA TTA ATA ACG CCC	
	bla_{VEB}	Forward	CGA CTT CCA TTT CCC GAT GC	376 bp
		Reverse	TGT TGG GGT TGC CCA ATT TT	
Inhibition control	bla_{ADC}[b]	Forward	GTA CCT CAA TTT ATG CGG RCA ATA C	1059 bp
		Reverse	TGC GYT CTT CAT TTG GAA TAC G	

[a]For degenerate primers: D = A, G or T; R = A or G; Y = C or T; K =G or T; W = A or T.
[b]ADC, *Acinetobacter*-derived cephalosporinase used as an inhibition/extraction control in each of the three multiplex PCRs.

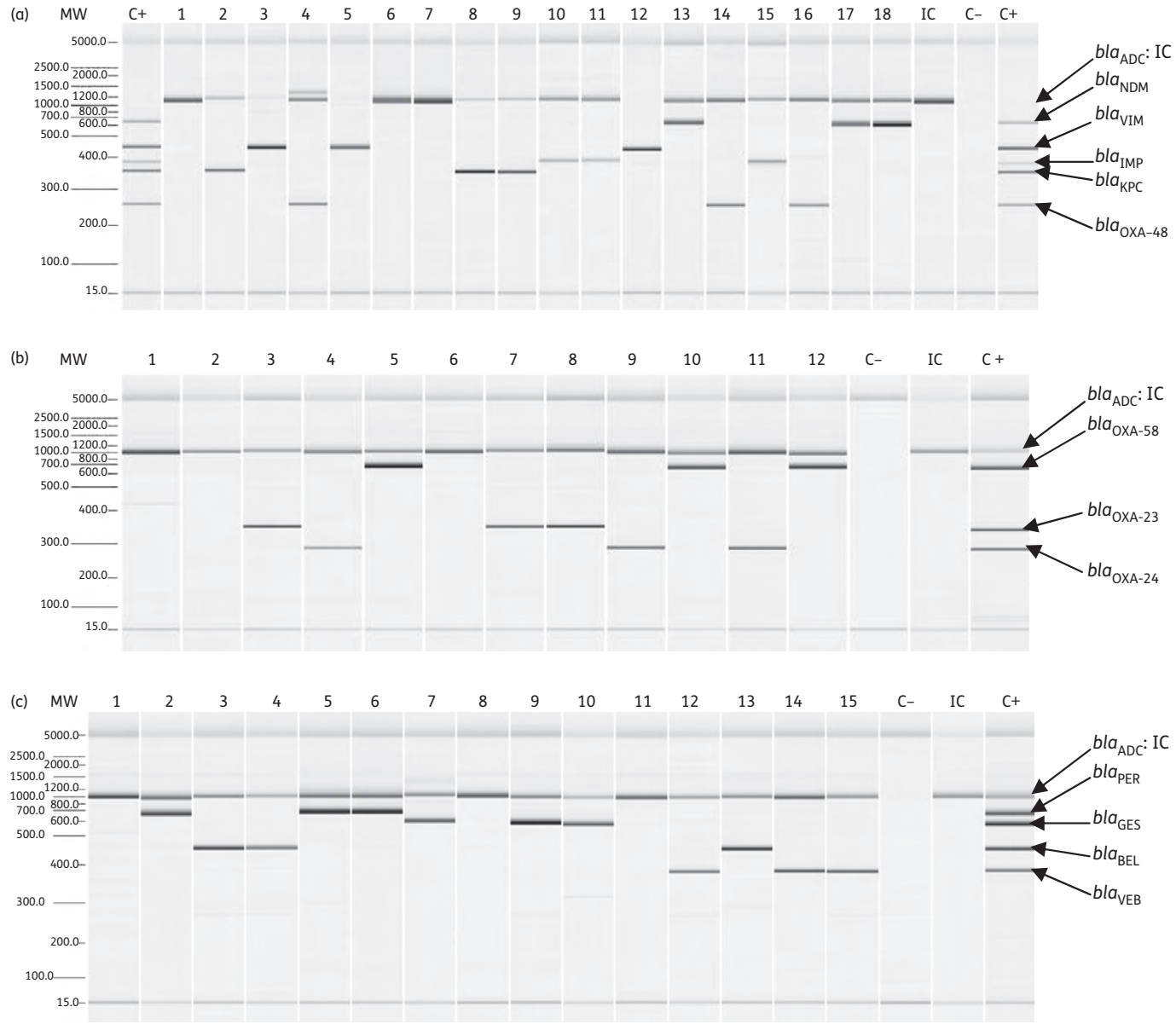

Figure 1. Capillary electrophoresis of amplicons obtained for accuracy testing (EQC ring test) of the three multiplex PCR assays. MW, molecular weight (bp); IC, internal control, corresponding to bla_{ADC} of *Acinetobacter baumannii*; C+, positive control consisting of a nucleic acid extracted from a mixture of strains expressing the resistance genes to be targeted; C-, negative control (water only). (a) PCR CARBA targeting $bla_{VIM,}$ $bla_{IMP,}$ $bla_{NDM,}$ bla_{KPC} and bla_{OXA-48}: lanes 1, 6 and 7, negative strains (*Acinetobacter radioresistens* OXA-23, *A. baumannii* PER-1 and *Pseudomonas aeruginosa* BEL-1); lanes 2, 8 and 9, KPC-producing strains (*Klebsiella pneumoniae* KPC-2); lanes 3, 5 and 12, VIM-producing strains (*Citrobacter braakii* VIM-1, *P. aeruginosa* VIM-4 and *P. aeruginosa* VIM-2); lanes 4, 14 and 16, OXA-48-producing strains (*Enterobacter cloacae* OXA-48, *K. pneumoniae* OXA-48 and *E. coli* OXA-48); lanes 10, 11 and 15, IMP-producing strains (*P. aeruginosa* IMP-13, *P. aeruginosa* IMP-13 and *P. aeruginosa* IMP-7); and lanes 13, 17 and 18, NDM-producing strains (*K. pneumoniae* NDM-1). (b) PCR OXACARBA targeting $bla_{OXA-23,}$ bla_{OXA-24} and bla_{OXA-58}: lanes 1, 2 and 6, negative strains (*Citrobacter freundii* TEM-1, *Proteus mirabilis* CTX-M-2 and *P. aeruginosa* SPM); lanes 3, 7 and 8, OXA-23-producing strains (*A. radioresistens* OXA-23 and *A. baumannii* OXA-23); lanes 4, 9 and 11, OXA-24-like producing strains (*A. baumannii* OXA-72); and lanes 5, 10 and 12, OXA-58-producing strains (*Acinetobacter haemolyticus* OXA-58, *A. baumannii* OXA-58 and *Acinetobacter pittii* OXA-58). (c) PCR MINESBL targeting $bla_{BEL,}$ $bla_{GES,}$ bla_{PER} and bla_{VEB}: lanes 1, 8 and 11, negative strains (*C. braakii* VIM-1, *P. aeruginosa* VIM-2 and *P. aeruginosa* IMP-7); lanes 2, 5 and 6, PER-producing strains (*A. baumannii* PER-1); lanes 3, 4 and 13: BEL-producing strains (*P. aeruginosa* BEL-1); lanes 7, 9 and 10: GES-producing strains (*A. baumannii* GES-12, *P. aeruginosa* GES-1 and *P. aeruginosa* GES-18); and lanes 12, 14 and 15, VEB-producing strains (*P. aeruginosa* VEB-1b, 1a, 1).

Sensitivity, specificity and reproducibility (including the extraction step) for each of the targeted genes were 100% (data not shown). The resistance genes of the isolates included in the three EQC panels were correctly identified by each PCR assay (accuracy 100%; Figure 1). Regarding specificity, non-specific amplifications generating fragments of unexpected sizes were

observed in about 2% of the cases, and have to be interpreted as negative results (Figure 1b, lane 1). These non-specific results were often related to an *Enterobacter asburiae* isolate (data not shown). No explanation could be found to explain this phenomenon, although it was less frequently observed when the primers were HPLC purified and was not observed when the PCR was performed in simplex for each individual target. The validation files were controlled by BELAC during an external audit held in UCL Mont-Godinne on 19 March 2012, and the three PCR protocols were accepted for accreditation according to ISO 15189 in July 2012 (BELAC Certificate 431-MED).

It is important to point out that, according to ISO 15189, an efficient separation between the different work areas must be provided in order to efficiently avoid cross-contamination. All the processes described here only represent the analytical part of the accreditation process. Pre-analytical and post-analytical stages must also follow ISO 15189 standards, but these are more difficult to export from one laboratory to another. Many excellent home-made PCRs (endpoint or real-time) have already been published in the literature (e.g. Dallenne *et al.*,[11] Huang *et al.*,[12] Naas *et al.*,[13] Poirel *et al.*,[14] Swayne *et al.*[15] and Naas *et al.*,[19] although many other publications exist). Regarding other multiplex PCRs for carbapenemases, each method presents its own characteristics. For example, Huang *et al.*[12] proposed a real-time TaqMan multiplex targeting different class D carbapenemases from *A. baumannii*, while Swayne *et al.*[15] published a TaqMan PCR targeting five class A and D carbapenemases encountered in Enterobacteriaceae. The three multiplex PCR assays presented by Poirel *et al.*[14] are endpoint PCRs targeting the largest panel of carbapenemase genes (11 targets). The implementation of PCR in a laboratory will depend on its particular needs, local epidemiology, technical resources and quality requirements. Most methods are indeed efficient and have already been peer-reviewed, but they would most probably not be accepted as such by external auditors as conforming to ISO 15189. Major non-conformities with ISO 15189 standards are the lack of an internal control able to rule out false-negative results and the absence of accuracy testing by participating in at least one annual EQC.

Testing accuracy is optimally achieved by participation in external quality assurance schemes organized by independent bodies such as Quality Control for Molecular Diagnostics or the United Kingdom National External Quality Assessment Service. Unfortunately, these bodies do not yet organize EQC evaluations for the detection of resistance genes. In such situations, ISO 15189 accepts that laboratories organize a so-called ring test (a blind exchange of an EQC proficiency panel) to evaluate the accuracy of their own methods. This is what was performed with the strains obtained from the TEMPOtest-QC consortium.

In summary, we report here the successful accreditation process of three home-made multiplex PCR assays according to the ISO 15189 standards. We believe that this validation scheme should constitute a valuable tool for laboratories in order to accredit their own protocols. Moreover, as these three multiplex PCR assays are already accredited, they could easily be implemented in other diagnostic laboratories through a lighter verification procedure.[18]

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Transparency declarations

None to declare.

References

1 Bush K, Fisher JF. Epidemiological expansion, structural studies, and clinical challenges of new β-lactamases from Gram-negative bacteria. *Annu Rev Microbiol* 2011; **65**: 455–78.

2 Canton R, Lumb J. Emerging resistance in Gram-negative pathogens and implications for clinical practice. *Future Microbiol* 2010; **6**: 19–22.

3 Nordmann P, Cornaglia G. Carbapenemase-producing Enterobacteriaceae: a call for action! *Clin Microbiol Infect* 2012; **18**: 411–2.

4 Naas T, Poirel L, Nordmann P. Minor extended-spectrum β-lactamases. *Clin Microbiol Infect* 2008; **14** Suppl 1: 42–52.

5 Poirel L, Bonnin RA, Nordmann P. Genetic basis of antibiotic resistance in pathogenic *Acinetobacter* species. *IUBMB Life* 2011; **63**: 1061–7.

6 Poirel L, Potron A, Nordmann P. OXA-48-like carbapenemases: the phantom menace. *J Antimicrob Chemother* 2012; **67**: 1597–606.

7 Cuzon G, Naas T, Truong H *et al*. Worldwide diversity of *Klebsiella pneumoniae* that produce β-lactamase bla_{KPC-} *gene. Emerg Infect Dis* 2010; **16**: 1349–56.

8 Queenan AM, Bush K. Carbapenemases: the versatile β-lactamases. *Clin Microbiol Rev* 2007; **20**: 440–58.

9 Dortet L, Poirel L, Nordmann P. Rapid Identification of carbapenemase types in Enterobacteriaceae and *Pseudomonas* spp. by using a biochemical test. *Antimicrob Agents Chemother* 2012; **56**: 6437–0.

10 Nordmann P, Dortet L, Poirel L. Rapid detection of extended-spectrum-β-lactamase-producing Enterobacteriaceae. *J Clin Microbiol* 2012; **50**: 3016–22.

11 Dallenne C, Da Costa A, Decre D *et al*. Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae. *J Antimicrob Chemother* 2010; **65**: 490–5.

12 Huang XZ, Cash DM, Chahine MA *et al*. Development and validation of a multiplex TaqMan real-time PCR for rapid detection of genes encoding four types of class D carbapenemase in *Acinetobacter baumannii*. *J Med Microbiol* 2012; **61**: 1532–7.

13 Naas T, Ergani A, Carrer A *et al*. Real-time PCR for detection of NDM-1 carbapenemase genes from spiked stool samples. *Antimicrob Agents Chemother* 2011; **55**: 4038–43.

14 Poirel L, Walsh TR, Cuvillier V *et al*. Multiplex PCR for detection of acquired carbapenemase genes. *Diagn Microbiol Infect Dis* 2011; **70**: 119–23.

15 Swayne RL, Ludlam HA, Shet VG *et al*. Real-time TaqMan PCR for rapid detection of genes encoding five types of non-metallo- (class A and D) carbapenemases in Enterobacteriaceae. *Int J Antimicrob Agents* 2011; **38**: 35–8.

16 ISO 15189:2012. *Medical Laboratories – Requirements for Quality and Competence*. http://www.iso.org/iso/home/store/catalogue_ics/

catalogue_detail_ics.htm?csnumber=56115 (2 January 2013, date last accessed).

17 Naas T, Poirel L, Karim A *et al*. Molecular characterization of In50, a class 1 integron encoding the gene for the extended-spectrum β-lactamase VEB-1 in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 1999; **176**: 411–9.

18 Rabenau HF, Kessler HH, Kortenbusch M *et al*. Verification and validation of diagnostic laboratory tests in clinical virology. *J Clin Virol* 2007; **40**: 93–8.

19 Naas T, Cotellon G, Ergani A *et al*. Real-time PCR for detection of bla_{OXA-48} genes from stools. *J Antimicrob Chemother* 2012; **68**: 101–4.