Real-time PCR Assays for the Detection of Carbapenemase-Producing *Enterobacteriaceae* (CPE) from Stool Samples

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Background

The main carbapenemases identified in *Enterobacteriaceae* belong to Ambler class A (KPC), class B (VIM, IMP and NDM), and class D (OXA-48). Currently identification of CPE carriers is a difficult task for laboratories.[1]
Conventional methods usually rely on overnight selective culture of screening specimens (rectal swabs or feces). The presence of CPE is then confirmed by additional phenotypic testing performed on colonies including antimicrobial susceptibility testing, Hodge test, colorimetric detection of carbapenem hydrolysis (NP test)[2], or molecular detection of carbapenemase coding genes by PCR.[3] The entire process can take up to 48-72 hours.
In order to reduce the turn around time allowing faster adjustment of infection control measures, molecular tests were developed to detect CPE directly on clinical samples.
We aimed to compare the performance of three commercially designed real-time PCR assays for the screening of CPE carriage.

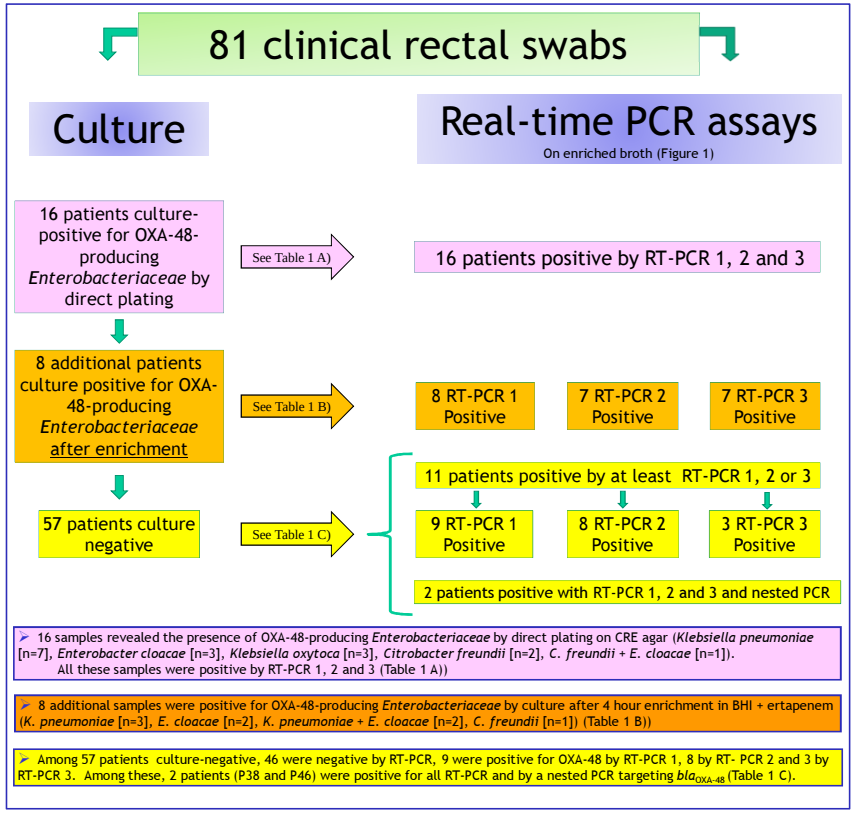
Methods

Screening clinical samples.
Two clinical rectal swabs from 81 patients were collected in semi-solid Stuart's transport medium during an OXA-48 outbreak situation at one hospital. The first swab of each patient was tested within the 4 hours in the collection hospital. The second swab was kept at 4°C and sent to the reference laboratory for enrichment and molecular testing and was cultured within the 48 hours after collection.

Bacterial culturing and enrichment and bacterial identification
The swabs were analyzed by direct plating on Brilliance chromogenic agar (CRE, Oxoïd) at the local laboratory. Following transfer of the specimens to the reference laboratory, the swabs were incubated at 37°C for 4 hours in 2,5 mL of BHI broth supplemented with 0,25 mg/L of ertapenem. Twenty µL of the enriched broth were spread on CRE selective chromogenic agar.
Suspected colonies were selected, identified by MALDI-TOF (Microflex LT, Brucker Daltonics) and antimicrobial susceptibility testing was performed by disc diffusion according to CLSI standards. The presence of carbapenemases was confirmed by end-point PCR targeting OXA-48, KPC, VIM, IMP and NDM coding genes as previously described[3].

Real-time PCR assays
All PCRs were performed on 5 µL of extracted DNA from enriched broth obtained with NucliSENS easyMag (BioMérieux).
•**RT-PCR1: Eppendorf assay targeting KPC and OXA-48** (not commercialized)was performed on Lightcycler LC480 with the following conditions (1X 50°C for 2 min, 1X 95°c for 3 min, 50 X (95 °C 15 sec, 60°C 1 min)).
•**RT-PCR2: Check-Points CP direct targeting KPC, OXA-48, VIM/NDM** was performed according to manufacturer instructions on ABI7500. VIM and NDM are detected with the same fluorophore.
•**RT-PCR3: Progenie Molecular targeting OXA-48 and VIM** was performed according to manufacturer on LC480 after colour compensation.
•**Nested PCR** for bla_{OXA-48} used a first pair of primers (OXA-48 extFW: 5'-TTG CAT TAA GCA AGG GGA C and OXA-48 extRV: 5'-TCG AGC ATC AGC ATT TTG TC PCR) for an amplification of 40 cycles in 50 µL (hybridisation at 57°C). The second step used internal primers OXA-48 intFW: 5'-ATG CGT GTA TTA GCC TTA TCG and OXA-48 intRV: 5'-CAT CCT TAA CCA CGC CCA AAT C. Amplification product was sequenced for confirmation.

Results



➢ 16 samples revealed the presence of OXA-48-producing *Enterobacteriaceae* by direct plating on CRE agar (*Klebsiella pneumoniae* [n=7], *Enterobacter cloacae* [n=3], *Klebsiella oxytoca* [n=3], *Citrobacter freundii* [n=2], *C. freundii* + *E. cloacae* [n=1]). All these samples were positive by RT-PCR 1, 2 and 3 (Table 1 A))

➢ 8 additional samples were positive for OXA-48-producing *Enterobacteriaceae* by culture after 4 hour enrichment in BHI + ertapenem (*K. pneumoniae* [n=3], *E. cloacae* [n=2], *K. pneumoniae* + *E. cloacae* [n=2], *C. freundii* [n=1]) (Table 1 B))

➢ Among 57 patients culture-negative, 46 were negative by RT-PCR, 9 were positive for OXA-48 by RT-PCR 1, 8 by RT- PCR 2 and 3 by RT-PCR 3. Among these, 2 patients (P38 and P46) were positive for all RT-PCR and by a nested PCR targeting bla_{OXA-48} (Table 1 C).

Real-time PCR assays

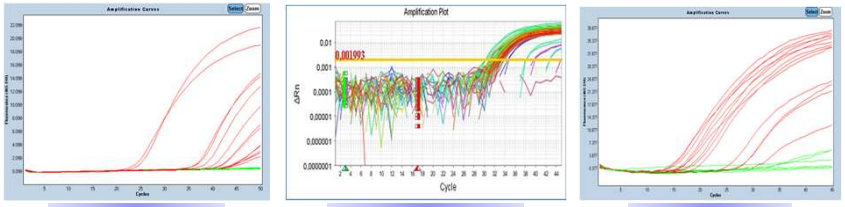


Figure 1. Representative amplification curves for the detection of bla_{OXA-48}. RT-PCR 1: Eppendorf assay targeting OXA-48 and KPC, RT-PCR 2: Check-Points CP Direct targeting OXA-48, KPC, VIM/NDM, RT-PCR 3 : Progenie molecular tagetiing OXA-48 and VIM

A)

Direct culture positive/ RT-PCR OXA-48 positive			
N°	Ct RT-PCR 1	Ct RT-PCR 2	Ct RT-PCR 3
P21	27,4	17,1	20,8
P23	24,8	14,6	18,4
P34	24,2	13,9	17,7
P48	25,2	15,7	19,2
P49	22,4	12,7	14,2
P50	25,3	14,3	16,9
P51	26,5	16,5	19,9
P52	32,9	22,6	25,4
P53	21,6	12,3	15,1
P54	22,5	12,7	15,8
P55	36,2	26,3	27,9
P56	24,9	14,0	16,5
P57	23,0	13,9	16,3
P58	25,2	15,9	18,9
P68	30,1	19,9	24,0
P95	40,0	30,8	31,7
Mean	27,0	17,1	19,9

B)

Direct culture negative/Enriched culture positive/ RT-PCR OXA-48 results for patient			
N°	Ct RT-PCR 1	Ct RT-PCR 2	Ct RT-PCR 3
P32	43,9	-	-
P33	41,8	32,3	33,2
P37	40,5	31,6	31,9
P45	36,8	29,7	27,4
P47	38,8	31,2	29,7
P74	27,7	21,5	18,2
P80	26,9	20,9	17,5
P91	43,9	32,4	36,7
Mean	37,5	28,5	27,8

C)

Culture negative/ RT-PCR OXA-48 positive				
N°	Ct RT-PCR 1	Ct RT-PCR 2	Ct RT-PCR 3	PCR Nested OXA-48
P2	45,0	-	-	+
P18	-	42,1	-	-
P19	43,0	41,0	-	-
P24	45,0	-	-	-
P35	43,3	-	-	+
P36	42,9	38,0	-	+
P38	44,2	29,8	31,2	+
P41	41,8	35,0	-	+
P42	44,2	41,4	-	-
P43	-	43,5	30,5	-
P46	37,5	27,1	30,1	+

Table 1. Cycle threshold (Ct) obtained with the 3 RT-PCR for the detection of OXA-48 coding gene
A) Patients positive by direct culture . B) Patients positive by culture only after enrichment, C) Patients only positive by RT-PCR

➢ For culture positive samples, Ct values observed with RT-PCR-2 and 3 are about 10 Ct lower (better signal) than RT-PCR 1 (Table 1 A) and B))
➢ RT-PCR 1 presents no false negative results whereas RT-PCR 2 and 3 do not detect Patient 32 as OXA-48 carriers (Table 1 B))
➢ Compared to culture, false positive results were observed for several patients with any RT-PCR (Table 1 C))
➢ Each RT-PCR includes a internal control permitting to avoid false negative results because of inhibitors. Expected Ct for RT-PCR 1 is 40, and 30 for RT-PCR 2 and 3 (not shown).

Conclusions

➢ Four hour enrichment in presence of 0,25 µg/mL of ertapenem permits the detection of 30 % additional carriers (8/24 positive by culture).
➢ Low Ct values (strong signal) observed with RT-PCR 2 and 3 suggests to use these tests directly on clinical samples avoiding the preliminary enrichment step and reducing the time to result.
➢ Several positive samples by RT-PCR could not be confirmed by culture, underlining the need of cut-off determination for the risk of inter-patients transmission
➢ RT-PCR 2 targets the most relevant carbapenemase (KPC, OXA-48, VIM and NDM) but does not permit to distinguish between NDM and VIM carriers (same fluorophore for both MBL)
➢ RT-PCR 3 targets only OXA-48 and VIM but a new product version includes now KPC
➢ RT-PCR 1 is not available commercially

References

1. **Nordmann P. *et al.*** 2011. Emerg Infect Dis **17:** 1791-8.
2. **Nordmann P.** et al. 2012b. Emerg Infect Dis **18:**1503-7.
3. **Bogaerts, P. *et al.*** 2013. J Antimicrob Chemother **68:** 1576-82.

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