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Discrimination between Native and Tn*6010*-Associated *oqxAB* in *Klebsiella* spp., *Raoultella* spp., and other *Enterobacteriaceae* by Using a Two-Step Strategy

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We developed a two-step PCR-based strategy to detect genes encoding OqxAB, allowing a specific assignment of Tn*6010*-associated *oqxAB* in *Enterobacteriaceae*. Chromosomal location in this setup was confirmed by hybridization with I-*Ceu*I-restricted genomes. This approach led us to find that *Klebsiella* sp. and *Raoultella* sp. reference strains chromosomally carried *oqxAB*.

Four plasmid-mediated quinolone resistance (PMQR) mechanisms have been described in *Enterobacteriaceae* so far: Qnr, AAC(6′)-Ib-cr, QepA, and OqxAB (1–4). OqxAB belongs to the resistance-nodulation-cell-division (RND) family multidrug efflux pump, and OqxA and OqxB have shown high homology with the AcrAB pump from *Escherichia coli* (4). It has been shown that OqxAB-encoding genes may be located in *Enterobacteriaceae* on the conjugative plasmid pOLA52, within a composite transposon Tn*6010* flanked by IS*26* (5), but also natively on the chromosome in *Klebsiella pneumoniae* without association to a transposon (6, 7).

As for other PMQR, OqxAB alone confers low-level resistance to fluoroquinolones (8). Although the increased MIC of ciprofloxacin remains below the critical concentration, categorizing the enterobacterial isolate as susceptible, it has been shown that PMQR pose a challenge to antimicrobial treatments (9). Moreover, differentiation between plasmid-borne and chromosomal locations of these genes has a stake in preventing the spread of such resistance markers (10). All together, these data underscore the importance of setting up molecular detection assays. The aim of this study was to develop a reliable PCR-based method to detect not only *oqxA* and *oqxB* but also any associations with IS*26*, allowing the detection of the transposon-associated *oqxAB* (Fig. 1A). The mobile version of *oqxAB*, which is embedded in Tn*6010*, has been almost exclusively reported on plasmids (10–13). To our best knowledge, only two *Salmonella* isolates carrying a chromosomally encoded OqxAB associated with IS*26* have been reported so far (14). It is noteworthy that the transposon-borne version is associated with boosted expression because of the lack of *rarA* that normally downregulates *oqxR* and the presence of several plasmid copies of the plasmid-borne transposon version (7).

First, we carried out a multiplex conventional PCR to detect *oqxA*, *oqxB*, and an amplification product covering IS*26* and *oqxA*. DNA extractions were all performed using the NucliSens easyMAG (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's recommendations. Multiplex PCRs were run using the MyCycler thermocycler (Bio-Rad, Marnes-la-Coquette, France). Amplicons of 222 bp, 127 bp, and 746 bp targeting *oqxA*, *oqxB*, and the region spanning the IS*26* right end and *oqxA* 5′ terminus, respectively (Fig. 1A), were generated in a 50-μl PCR that contained 5 μl of target DNA, 5 μl of PCR buffer, 0.5 μl of $MgCl_2$ (25 mM), 0.5 μl of deoxynucleoside triphosphate (dNTP) (25 mM), 25 or 100 pmol of each primer (Table 1), 0.5 μl of *Taq* polymerase (5 U/μl), and 26.5 μl of sterile water. The PCRs were run using the following temperature-time profile: 95°C for 3 min, 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min, followed by 72°C for 5 min. PCR products of the expected size were verified by electrophoresis in a 2% agarose gel. The control strains used for setting up the multiplex PCR were *E. coli* CSH26/pOLA52 (4), a clinical isolate of *K. pneumoniae* (KP10887) that we previously detected as positive for *oqxAB* (personal data), and *E. coli* CIP 54.8^T as a negative control. An IS*26*-*oqxA* fragment specific to Tn*6010* was amplified only in the plasmid-borne control *E. coli* CSH26/pOLA52 (Fig. 1B). The hybridization of I-*Ceu*I pulsed-field gel electrophoresis (PFGE) profiles using an *oqxB* probe showed a chromosomal location only for *K. pneumoniae* KP10887 (15).

Second, we succeeded in setting up a simplex real-time PCR targeting each amplicon described above. These assays were carried out with the LightCycler 480 (Roche Molecular Diagnostics, Germany) using the same primers as described above (Table 1), with a PCR mix as follows: 2 μl of target DNA, 10 μl of 1× Kapa SYBR Fast quantitative PCR (qPCR) master mix (Kapa Biosystems, Wilmington, MA, USA), 0.4 μl of each primer, and 7.2 μl of sterile water. The PCR thermocycling program was as follows: 95°C for 7 min, followed by 40 cycles of 95°C for 10 s, 52°C for 30 s, and then melting resolution from 60°C to 98°C. As shown in Fig.

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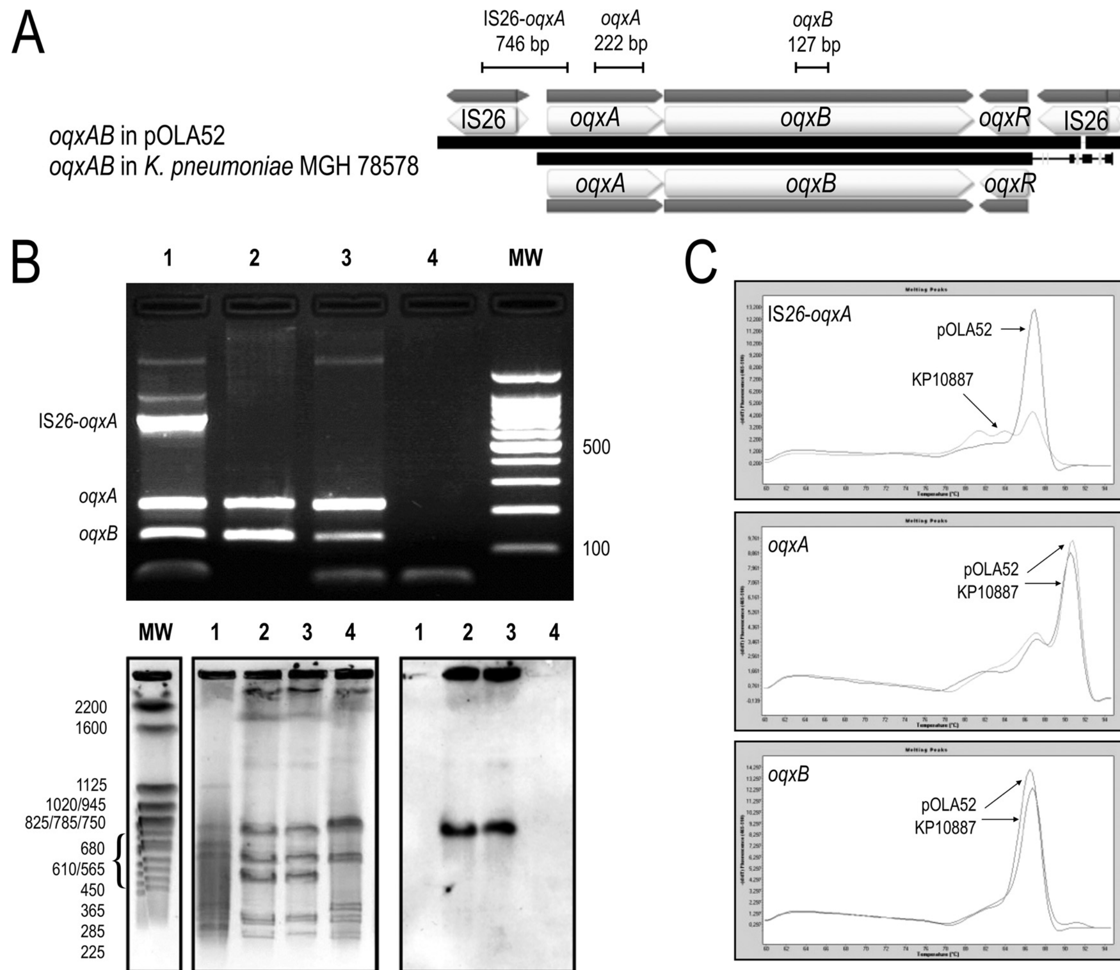

FIG 1 (A) Schematic representation of *oqxAB* carried in pOLA52 and *K. pneumoniae* strain MGH 78578 (GenBank accession no. NC_009648.1). Lines indicate the size of the PCR fragments. (B) Top, agarose gel electrophoresis (2%) used for the separation of multiplex PCR products. Lane 1, *E. coli* CSH26/pOLA52; lanes 2 and 3, *K. pneumoniae* KP10887; lane 4, *E. coli* CIP 54.8^T; MW, molecular weight marker (100-bp ladder; Invitrogen, Cergy-Pontoise, France). Bottom, PFGE migration profiles of I-*Ceu*I restricted whole-cell DNAs. Lane 1, *E. coli* CSH26/pOLA52; lanes 2 and 3, *K. pneumoniae* KP10887; lane 4, *E. coli* CIP 54.8^T; MW, *Saccharomyces cerevisiae* chromosomes (Bio-Rad Laboratories, Hercules, CA, USA) used as a molecular weight marker. The set on the right shows hybridization performed with a specific probe for the *oqxB* gene. (C) Derivative fluorescence melting curve (melting peak) for IS*26*-*oqxA*, *oqxA*, and *oqxB* in SYBR green real-time PCR with LC480. The melting temperatures (T_m) were 86.86°C, 90.79°C, and 86.16°C for IS*26*-*oqxA*, *oqxA*, and *oqxB*, respectively.

1C, distinct peaks were clearly distinguished for each amplicon except IS*26*-*oqxA* for *K. pneumoniae* KP10887, demonstrating the absence of a neighboring IS*26*.

According to the prevalence of *oqxAB* (6, 16, 17), we proposed to combine our assays into a two-step strategy, with screening of *oqxAB* by detecting *oqxB* in real-time PCR and then determination of *oqxAB* location only on strains that were positive by multiplex PCR. This method was applied to (i) 25 *E. coli*, 25 *K. pneumoniae*, and 3 *Raoultella ornithinolytica* strains prospectively isolated from urine samples from patients hospitalized in our emergency room in Decem-

TABLE 1 Primers used in this study

Target	Primer	Primer sequence (5′ to 3′)[a]	T_m (°C)	Concn (pmol/reaction)	Product size (bp)
IS*26*-*oqxA*	IS26-oqxA_Fm2	GTTTTTCCATTTCAGGCGCATA	60	100[b]	746
	IS26-oqxA_Rm	TTCACTTTATCAATGTATCCCGAGAC	60	100[b]	
oqxA	oqxA_Fm5	GCCAAAACRCAGGCCAGYCT	60	25	222
	oqxA_Rm3	TCARCGCSCGGCTGGCGCG	60	25	
oqxB	oqxB_Fm3	GGCTGGATTTTCCGTCCGTT	60	25	127
	oqxB_Rm4	GCGGCRCARAGCAGCAG	60	25	

[a] Degenerate residues are underlined. All primers were designed using Geneious 7.1 (Biomatters Ltd., Auckland, New Zealand).

[b] These primer concentrations were used for the multiplex PCR, while all primers were used at 25 pmol/reaction in the simplex real-time PCR.

ber 2014 and April 2015, (ii) 38 *E. coli* and 35 *K. pneumoniae* strains whose epidemiological links were studied previously (18), and (iii) the reference strains of the related genera *Klebsiella* and *Raoultella* (19) (*Klebsiella oxytoca* CIP 103434^T, *K. pneumoniae* subsp. *ozaenae* CIP 52.211^T, *K. pneumoniae* subsp. *pneumoniae* CIP 82.91^T, *K. pneumoniae* subsp. *rhinoscleromatis* CIP 52.210^T, *Klebsiella michiganensis* CIP 110787^T, *Klebsiella singaporensis* CIP 108642^T, *Klebsiella variicola* CIP108585^T, *R. ornithinolytica* CIP 103364^T, *Raoultella planticola* CIP 100751^T, and *Raoultella terrigena* CIP 80.7^T). For all the strains with no detection of IS*26-oqxA* using our method, we confirmed the chromosomal location of *oqxAB* by amplifying a 1.3 kb-long fragment overlapping the upstream region of *oqxA* that matches with the chromosome and *oqxA*.

Among the *E. coli* clinical strains, we did not detect any *oqxAB* genes. However, all the clinical *K. pneumoniae* isolates (61/61) carried *oqxAB* without detection of Tn*6010*. Interestingly, all the *Klebsiella* sp. and *Raoultella* sp. reference strains carried *oqxAB* in their chromosome, indicating normal housekeeping functions in these specimens.

Norman et al. (5) have proposed that *K. pneumoniae* may be a reservoir for *oqxAB* and that an *oqxAB*-carrying plasmid, such as pOLA52, may have arisen by capture from the chromosome of *K. pneumoniae* recruited by IS*26*. Nonetheless, some studies failed to detect *oqxAB* in all the *K. pneumoniae* isolates studied (6, 16, 17). This raised the question of the chromosomal location of *oqxAB* in *K. pneumoniae*, which might be particular not only for capture from the chromosome but also for its ability to lose *oqxAB*. However, it is noteworthy that a lack of detection in previous studies might have resulted from inadequate primers.

The method we describe here led us to show that (i) all *Klebsiella* spp. (clinical, $n = 61$; reference, $n = 7$) and *Raoultella* spp. (reference, $n = 3$) strains harbored *oqxAB* as part of their native chromosome, and (ii) the degenerate primers used in the present study allowed the detection of *oqxAB* in all cases. Moreover, our findings highlight the hypothesis stated by Norman et al. (5) and also demonstrate the importance of discriminating native and Tn*6010*-associated *oqxAB* regarding the overexpression of the mobile version.

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