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Technical Note

Cross-reaction of naturally-produced β-lactamases from *Citrobacter farmeri* and *Citrobacter amalonaticus* with immunological detection of CTX-M enzymes

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ABSTRACT

The NG-Test CTX-M MULTI immunochromatographic assay has been developed to identify CTX-M-type β-lactamases in Enterobacterales, being the most widespread extended-spectrum β-lactamases. We showed here that the chromosomally-encoded ß-lactamases from *Citrobacter farmeri* and *Citrobacter amalonaticus* generated false-positive NG-Test CTX-M MULTI results, compromising the specificity of the test.

Since 2000's, worldwide spread of extended-spectrum-β-lactamases (ESBL) is a main public health concern since acquisition of such enzymes confer at least resistance to expanded-spectrum cephalosporins. The most commonly identified ESBLs are CTX-M-type enzymes being reported in all Enterobacterales species [1,2].

Molecular approaches based on PCR gene amplification and/or sequencing, biochemical identification of ESBL activity, and immunological detection of ESBLs have been developed to rapidly identify ESBL producers [3–7]. Among them, the NG-Test CTX-M MULTI assay (NG Biotech, Guipry, France) allows rapid detection of CTX-M-type ESBLs from bacterial culture and requires minimal hands-on time and no specific equipment. It was evaluated against 172 CTX-M producers and showed excellent sensitivity and specificity for detection of CTX-M-like enzymes [6,8]. Of note, it was previously evaluated with 13 *Citrobacter* spp. strains (8 *Citrobacter freundii*, 2 *Citrobacter farmeri*, 2 *Citrobacter koseri*, and 1 *Citrobacter amalonaticus*), giving 100% specificity and sensitivity including 5 *Citrobacter* spp. producing also CTX-M-type ESBLs [6,7].

Among the twelve characterized *Citrobacter* species [9], *C. freundii, C. youngae, C. murliniae, C. braakii,* and *C. werkmanii* produce a chromosomally-encoded and inducible class C β-lactamase [10–12]. Instead, other *Citrobacter* species produce a class A β-lactamase possessing ESBL activities such as CKO-1 for *C. koseri*, CdiA for *C. amalonaticus*, GIL-1 for *C. gillenii* and SED-1 for *C. sedlakii, C. farmeri*, and *C. rodentium* [13–16]. Noteworthy, CdiA and SED-1 share 95% amino-acid sequence identity and the expression of the corresponding gene was shown to be inducible, unlike that encoding CKO-1 sharing 40% amino-acid sequence identity with the latter 2 enzymes which is constitutively expressed [16,17].

By evaluating the performance of rapid tests for screening carriers of ESBL producers, we identified *C. farmeri* isolate N2252 and *C. amalonaticus* isolate CHUV-445 that turned to be positive with the NG-Test CTX-M MULTI, but remained negative for any $bla_{\text{CTX-M}}$ gene and other ESBL encoding genes (TEM, SHV, PER, VEB) by PCR. Therefore, we aimed to determine the biological mechanisms leading to this false-positivity.

Whole-genome sequencing of *C. farmerii* N2252 (PRJNA837042) and *C. amalonaticus* CHUV-445 (PRJNA837049) were performed by Illumina sequencing platform, and data analysis identified only a single ß-lactamase gene in each isolate, corresponding to the chromosomal class A ß-lactamase genes $bla_{\text{SED-1}}$ and bla_{CdiA}, respectively. This confirmed the false-positive result obtained with the NG-Test CTX-M MULTI.

Recently, OXY-1-type β-lactamases from *Klebsiella oxytoca* were also shown to cross-react with the monoclonal antibodies contained

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Table 1
Evaluation of the NG-Test CTX-M MULTI and MIC results of clinical and reference *Citrobacter* isolates and CdiA/SED-1-producing *E. coli* recombinant strains.

				Minimal inhibitory concentration (MIC)					
Strains	Species	Motif sequence	NG-Test CTX-M MULTI	PIP	PTZ	CTX	CAZ	ATM	IMP
CHUV-445	*Citrobacter amanolaticus*	KKA	+	64	3	1	0.25	16	0.125
N2252	*Citrobacter farmeri*	KKT	+	128	3	8	0.25	>256	0.25
DSM4593	*Citrobacter amalonaticus*	KKA	–	1	1	0.03	0.015	0.25	0.125
DSM17655	*Citrobacter farmeri*	KKT	–	1	1	0.06	0.06	0.5	0.25
SED-1 T10	*Escherichia coli*	KKT	+	>256	3	16	1	>256	0.25
SED-1-mut1 T10 (**K** → **N**)	*Escherichia coli*	NKT	+	>256	3	16	1	>256	0.25
SED-1-mut2 T10 (**KK** → **NA**)	*Escherichia coli*	NAT	–	>256	3	16	1	>256	0.25
CdiA T10	*Escherichia coli*	KKA	+	>256	4	32	1	>256	0.25

E = ESBL phenotype; C =AmpC co-expression; PIP = piperacillin; PTZ = piperacillin-tazobactam; CEF = cephalotin; CTX = cefotaxime; CAZ = ceftazidime; ATM = aztreonam; IMP = imipenem; + = positive result; – = negative result.
C. amalonaticus produces a SED-like ß-lactamase and *C. farmeri* produces a CidA-like ß-lactamase.

in the NG-Test CTX-M MULTI assay, giving rise to false-positive results [18]. Interestingly, OXY-2-type β-lactamases, sharing 89% amino-acid identity with OXY-1-type enzymes, gave negative results with the NG-Test CTX-M MULTI. Experimental results further demonstrated that 3 consecutive residues (KKS) at Ambler positions 101-103 were involved in the false-positive results observed only with OXY-1-type β-lactamases [18].

With the purpose to decipher whether the SED-1 and Cdi β-lactamase production might lead to the false positivity of the NG-Test CTX-M MULTI, cloning of the corresponding genes were performed in the pCR-Blunt II-TOPO recipient vector (https://www.thermofisher.com/ch/) and expression in a same *Escherichia coli* TOP10 background. It confirmed that both SED-1 and CdiA-producing *E. coli* gave positive results with the NG-Test CTX-M MULTI assay (Table 1).

A comparative analysis of the OXY-1, OXY-2, SED-1, CdiA and CTX-M amino acid sequences was performed in-silico by Clusta1W (http://www.ebi.ac.uk/clustalw) (Supplementary Figure 1). Two out of the 3 consecutive residues (KKS) located at positions Ambler 101-103 of the OXY-1 protein sequence previously shown to be responsible for the cross-reactivity with the NG-Test CTX-M MULTI were also identified in the SED-1 (KKT) and CdiA (KKA) sequences (Table 1 and Supplementary Figure 1). Therefore, it might be hypothesized that both residues of the KK doublet, or at least one of them, are targets of the monoclonal antibodies of the NG-Test CTX-M MULTI assay, being located at the surface of the ß-lactamase structure (Supplementary Figure 2).

In order to further explore this hypothesis, site-directed mutagenesis was performed modifying the KK residues of the SED-1-type ß-lactamase using Q5 Site-Directed Mutagenesis Kit (NEB) with a couple of primers generated by NEBaseChanger online tool (http://nebasechanger.neb.com/) and pTOPO-SED-1 recombinant plasmid as template. These residues were replaced with the NA residues present in the OXY-2-1 protein sequence, which showed negative results with NG-Test CTX-M MULTI, as reported previously (Supplementary Figure 1) [18]. This test gave a negative result with the mutated (KK->NA) SED-1-producing *E. coli* recombinant strain. By contrast, the mutated (K->N) SED-1-producing strain gave a positive result, highlighting the cross-reaction observed with the class A β-lactamase produced by some *Citrobacter* spp. Hence, we showed that residue K102 was responsible for this false positivity (Table 1 and Supplementary Figure 1).

Our results therefore showed that SED-1- and CdiA-type ß-lactamases, once their corresponding gene being cloned in a multicopy vector, lead to cross reaction with monoclonal antibodies contained in the NG-Test CTX-M MULTI assay, giving rise to false-positive results. In a previous study, only 2 *C. farmeri* and a single *C. amalonaticus* that did not produce any acquired CTX-M-type ß-lactamase had been tested, and no false-positive results was reported (6). It might be possible that, in that latter case, the expression of the intrinsic class A ß-lactamase gene of those *Citrobacter* strains was poorly expressed.

Overall, the NG-Test CTX-M MULTI assay is now widely used in routine laboratories. It is claimed that this test has an excellent sensitivity and specificity for detection of CTX-M-like ESBLs. However, we demonstrate that false positivity of this assay is possible not only for OXY-1-type-producing *K. oxytoca* strains as previously reported, but also for *C. amalonaticus* and *C. farmeri* strains. This study adds information on the growing list of enterobacterales species that possess an intrinsic ESBL gene which expression may lead to false positivity of immunological detection aimed to detect transferable ESBL gene. This false positivity could lead to inappropriate diagnostic and antibiotic escalation. In addition, it might lead to wrong clinical decision of isolation of patients carrying ESBL producers whereas they are simply carriers of *C. amalonaticus* or *C. farmeri* strains expressing their natural class A ß-lactamase.

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Declaration of competing interest

The authors report no conflicts of interest relevant to this article.

Authors' contributions

PN designed the study. JMOR and MB performed the experiments. GB and DB provided some material. LP and PN wrote the final version of the manuscript. All authors agreed on the final version.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.diagmicrobio.2022.115760.

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