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Method Article

Comparative analysis of the standard PCR-Based Replicon Typing (PBRT) with the commercial PBRT-KIT

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ABSTRACT

Plasmids are the main vectors of resistance and virulence genes in Enterobacteriaceae and plasmid typing is essential for the analysis of evolution, epidemiology and spread of antibacterial resistance. The PCR-Based Replicon Typing (PBRT), developed by Carattoli et al. in 2005, was an efficient method for plasmid identification and typing in Enterobacteriaceae. The 2005 PBRT scheme detected 18 replicons in 8 PCR reactions. Recently, the identification of novel replicons and plasmid types requested an update of the PBRT scheme. A commercial PBRT-KIT was devised for the identification of 28 different replicons in 8 multiplex PCRs. Here we report sensitivity and specificity of the PBRT-KIT carried out in comparison with the 2005 PBRT. The analysis of plasmid content was performed on forty-two enterobacterial strains from different sources, containing different replicon content. The 2005 PBRT identified replicons in 76.2% of the strains. The PBRT-KIT detected replicons in 100% of the analyzed strains, demonstrating increasing sensitivity and specificity of the commercial test with respect to the former 2005 PBRT scheme.

1. Introduction

Antimicrobial resistance is a serious health problem and large conjugative plasmids carrying relevant resistance genes have been described as the most frequent mechanism of acquisition and spread of resistance in Enterobacteriaceae (Carattoli, 2011; Hawkey and Jones, 2009). The ability to recognize plasmids allows the identification of the same plasmid in different strains but also helps to trace the transfer of plasmids among different bacterial species. Plasmid methods based on plasmid DNA purification and comparative analysis of restricted fragments, or on incompatibility assays performed by conjugation are laborious, not standardized, and cannot be performed on a large number of strains (Couturier et al., 1988; Novick, 1987). Plasmid identification is very often complicated by the presence of multiple plasmids within the same cell, making difficult to determine the whole plasmid content within a bacterial strain. For this reason, the PCR-Based Replicon Typing (PBRT), based on PCR amplification of replicons has been in the last ten years worldwide adopted (>1300 citations, Google Scholar Oct. 2016) as the method for plasmid identification and typing (Carattoli et al., 2005). It has been estimated that >10,000 strains have been tested using this scheme, allowing the identification of the so-called "epidemic" plasmids, which disseminated in Enterobacteriaceae beyond species boundaries, at very distant geographical sites (Carattoli, 2013; Hansen et al., 2016; Liakopoulos et al., 2016). However, about 10% of plasmids reported in the 2005 PBRT citation list, especially in the most recent literature, were described as untypable, resulting negative for all the replicons tested.

The first PBRT scheme was devised in 5 multiplex and 3 simplex PCRs, targeting the HI1, HI2, I1-αγ, X, L/M, N, FIA, FIB, W, Y, P, FIC, A/C, T, FIIAs, F, K, B/O replicons, namely those of the plasmids classified by conjugation in the major 18 Incompatibility groups (Carattoli et al., 2005). PBRT had the value to trace back the molecular targets identified by PCR into the existing Incompatibility typing scheme. The replicon content defined by PBRT has been used for microbiological investigation of outbreak clones and for tracing the spread of specific resistant determinants in sets of epidemiologically link, but genetically unrelated, bacterial isolates (Carattoli, 2013).

Recently, novel replicons and plasmid types have been identified in clinically relevant enterobacteria, mostly by Whole Genome Sequencing (WGS) analysis. Consequently, the PBRT method has been continuously upgraded. In particular, in 2009 two new replicons from IncU and IncR

Abbreviations: PBRT, PCR-Based Replicon Typing; WGS, Whole Genome Sequencing.

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plasmids were included in the PBRT scheme, to trace some plasmids carrying *qnr* genes identified in *Salmonella* (García-Fernández et al., 2009).

In 2010, replicons belonging to the IncF plasmid family were revised and PBRT updated. In particular, the IncF replicon sequence typing (RST) scheme was proposed for IncF plasmid subtyping (Villa et al., 2010). New PCRs for FIA, FIB, FIC were devised and specific FII PCRs were proposed to discern the FIIK of *Klebsiella*, FIIY of *Yersinia* and FIIS of *Salmonella* from the most common FII replicon of plasmids constitutively resident in almost all *Escherichia coli* and *Shigella* strains. These FII replicons discriminates the huge IncF plasmid family, identifying specific plasmids more frequently occurring in their respective bacterial species (Villa et al., 2010).

In 2012, the IncX plasmid family was studied. The analysis revealed that this family possessed a highly syntenic plasmid backbone quite divergent at nucleotide sequence level, redefining the IncX group in four IncX subgroups (IncX1-IncX4) on the base of the *taxC* sequence (Johnson et al., 2012). In the same period, a new plasmid called pNDM-MAR, encoding the emergent metallo beta-lactamase NDM-1, the Extended Spectrum Beta-Lactamase CTX-M-15 and the quinolone resistance QnrB1, harbored two novel replicons, one related to FIB replicon (FIB-M) and one to HI1B (HIB-M). For both specific primer pairs were included in the PBRT scheme (Villa et al., 2012).

More recently, other important plasmids involved in the dissemination of influential drug resistance genes such as $bla_{CTX\text{-}M\text{-}55}$, $bla_{CMY\text{-}2}$ and the plasmid-located colistin resistance *mcr*-1 genes have been studied and associated to the IncI2 group, which was not included in the 2005 PBRT scheme (Carattoli, 2009; Lv et al., 2013). Furthermore, a more specific discrimination within the IncI1-αγ and IncL/M plasmid families became epidemiologically relevant. In particular, IncI1-α plasmids associated with bla_{CTX} and bla_{CMY} genes encoding extended spectrum resistance to cephalosporins (Bortolaia et al., 2010; Cloeckaert et al., 2010; Folster et al., 2010; Literak et al., 2010; Moissenet et al., 2010; Smet et al., 2010), needed to be discerned from the IncI1-γ plasmids found in porcine enterotoxigenic *Escherichia coli* (ETEC) isolates (Johnson et al., 2011). The epidemic IncL plasmid associated with the worldwide spread of the class D OXA-48 carbapenemase was not previously detected and PBRT was updated (Carattoli et al., 2015). In fact, the 2005 PBRT scheme was devised on the highly related IncM plasmids, which had a different epidemiology, being associated with other carbapenemases like KPC, NDM and IMP, but not with OXA-48 (Bonnin et al., 2013; Carattoli et al., 2015; Di Pilato et al., 2014; Galimand et al., 2005; Gołebiewski et al., 2007; Ho et al., 2011; Partridge et al., 2012; Poirel et al., 2012; Smet et al., 2010; Villa et al., 2000; Zhu et al., 2009).

A PBRT-KIT has been developed and is commercially available since 2011. The last version of the PBRT-KIT includes all the modifications added to the 2005 PBRT along the years. It was devised for the identification of 28 different replicons (HI1, HI2, I1-α, M, N, I2, B/O, FIB, FIA, W, L, P, X3, I1-γ, T, A/C, FIIS, U, X1, R, FIIK, Y, X2, FIC, K, HIB-M, FIB-M, and FII) in 8 multiplex PCRs.

The scope of this work is the validation of sensitivity and specificity of the PBRT-KIT, comparing the results with those obtained using the standard 2005 PBRT scheme.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Forty-two gram negative Enterobacteriaceae, including 20 clinical isolates and 22 *E. coli* K12 transformants, carrying plasmids belonging to different incompatibility groups, were available from the collection of the Istituto Superiore di Sanità (ISS) in Rome, Italy. Among the strains chosen for the analysis, 10 carried plasmids used as references for Incompatibility testing by conjugation (Couturier et al., 1988). Whole Genome Sequencing (WGS) data available for three clinical isolates (strains LS6, 55873, 51712) and 14 transformants obtained from clinical isolates in previous studies, were used to detect the replicon content by the PlasmidFinder tool at the https://cge.cbs.dtu.dk/service/PlasmidFinder/ and results were compared with those obtained by PBRT (Carattoli, 2013; Carattoli et al., 2015, 2014; García-Fernández et al., 2012; Johnson et al., 2012; Norman et al., 2008; Sherburne et al., 2000; Villa et al., 2013, 2012).

Twenty-seven strains were previously typed for plasmid content by 2005 PBRT, 8 of them resulting untypable. All strains were kept as frozen stock cultures in Microbank vials (Biolife, Milano, Italy) at −80 °C, reactivated by plating on Luria-Bertani (LB) agar and by incubating overnight at 37 °C.

2.2. PCR assay

Total DNA of the reactivated strains has been obtained by boiling an isolated colony in 0.1 ml of distilled water. Lysate preparations have been clarified by centrifugation (15,000 ×*g* for 10 min) and 1 μl of the supernatant has been tested in PCR assays.

Two PCR-based assays were used in this study: the 2005 PBRT as previously described (Carattoli et al., 2005) and the "PBRT kit - PCR based replicon typing" (Ver.13/03/2016) (Diatheva, Fano, Italy).

The 2005 PBRT consisted of five PCRs in multiplex format, each of which amplifies three different replicon types, and three simplex-PCRs for F, K and B/O targets (Table 1). Primer sequences, amplification and cycle conditions and positive controls have been kept constant with respect of the reference work (Carattoli et al., 2005). The amplification products were analyzed by 2.5% agarose gel electrophoresis.

The PBRT-KIT is composed of 8 multiplex PCRs, each of which amplifies three or four targets, allowing to detect a total of 28 replicons (Table 1). The kit has been used following manufacturer's instructions. This system contains the specific mixtures of positive control plasmids for all the 8 multiplex PCRs.

1 μl of DNA was added to 24 μl of each PCR mix solution. All amplification products were resolved on 2.5% agarose gel, stained with GelRed

Table 1
PCRs organization and replicon targets in the 2005 PBRT and PBRT-KIT schemes.

2005 PBRT			PBRT-KIT		
Multiplex	Target name	Amplicon size (bp)	Multiplex	Target name	Amplicon size (bp)
M1	HI2	644	M1	HI1	534
	HI1	471		HI2	298–308
	I1-αγ	139		I1-α	159
M2	L/M	785	M2	M	741
	N	559		N	514
	X	376		I2	316
M3	FIB	702		B/O	159
	FIA	462	M3	FIB	683
	W	242		FIA	462
M4	Y	765		W	242
	P	534	M4	L	854
	FIC	262		P	534
M5	T	750		X3	284
	A/C	465		I1-γ	161
	$FIIA_S$	270	M5	T	750
S1	F	270		A/C	418
S2	K	160		FIIS	259–260
S3	B/O	159	M6	U	843
				X1	370
				R	251
				FIIK	142–148
			M7	Y	765
				X2	376
				FIC	262
				K	160
			M8	HIB-M	570
				FIB-M	440
				FII	258–262

M: multiplex-PCR; S: simplex-PCR.

Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA) and visualized using the Gel Doc 2000 system (Bio-Rad, Hercules, CA, USA).

3. Results and discussion

Forty-two enterobacterial strains were analyzed for replicon content using both the 2005 PBRT scheme and the current PBRT-KIT in commerce, and when available, results compared with those obtained by WGS and PlasmidFinder.

The 2005 PBRT detected replicons in 32 of 42 strains (76.2%), while 10 bacterial strains were untypable using this scheme (23.8%). The PBRT-KIT identified replicons in 100% of the analyzed strains (Table 2). 2005 PBRT and PBRT-KIT gave a partially overlapping result in 30 on 42 strains. These data considered the correspondence among FrepB, X and L/M results obtained by 2005 PBRT and the FII, X2 and M equivalent replicons identified by the PBRT-KIT, respectively.

In strains 172.23, pX52, LS6, F22, 55873 and 51712 of Table 2, PBRT-KIT identified more replicons than the 2005 PBRT scheme, including the FIIK, R, I2, L, HIB-M and FIB-M. With the former scheme, replicons in two *K. pneumoniae* isolates (strains 55873 and 51712) were not detected (X3) or gave a mistaken result (FIIS with 2005 PBRT was FIIK with PBRT-KIT) (Table 2). These two strains have been fully sequenced in 2014 and plasmid content predicted *in silico*, matched with results obtained with the PBRT-KIT, being positive for pKpQIL (FIIK), pKpN-IT (FIIK) and pSHV-IT (X3) plasmids (García-Fernández et al., 2012). In these two strains, the FIIS primers of the 2005 PBRT demonstrated to

Table 2
Characteristics of the strains analyzed in this study and results of the replicon content by 2005 PBRT and PBRT-KIT schemes.

Strain[a]	Origin[b]	Relevant resistance gene content	Inc typing Ref[c]	Confirmed by WGS and PlasmidFinder[d]	2005 PBRT	PBRT-KIT[f]
NO003T	*K. oxytoca*	bla_{SHV-12}			A/C, FIA, FIB, FIC, FrepB	A/C, FIA, FIB, FIC, FII
A2-VLA (TR)	*E. coli*	bla_{DHA-1}			FIA, FIB, FrepB	FIA, FIB, FII
Pn137 (TR)	*E. coli*	$bla_{CTX-M-32}$			FIB, FIC, FrepB	FIB, FIC, FII
R27 (TR)	*S. typhi*	*tetA*	Yes	HI1A, HI1B, FIA(HI1)	HI1	HI1
31038	*E. coli*	bla_{CMY-2}, $bla_{CTX-M-1}$			HI1, I1-αγ, N	HI1, I1-α, N
C1090	*E. coli*	$bla_{CTX-M-1}$			N, FIB, Y, FrepB	N, FIB, Y, FII
Rts1 (TR)	*P. vulgaris*	*aphA*	Yes	T_1	T	T
pULB2426 (TR)	*E. coli*	ND	Yes		W	W
pNDM-CIT (TR)	*C. freundii*	bla_{NDM-1}, *armA*, *aadA2*, *dfrA12*		HI1A(CIT), HI1B(CIT)	HI1	HI1
pZM3 (TR)	*S. virchow*	bla_{OXA-1}, *aadA1*	Yes		FIA, FIB, FIC, FrepB	FIA, FIB, FIC, FII
TP160 (TR)	*S. virchow*	bla_{OXA-1}, *aadA1*	Yes		FIA, FIB, FrepB	FIA, FIB, FII
366D2 (TR)	*S. typhimurium*	*aadB*, *catB3*, bla_{OXA-1}, *aadA1*		FIA_1, FIB_1, FII_1	FIA, FIB, FrepB	FIA, FIB, FII
4204 (TR)	*S. typhimurium*	bla_{CMY-2}		A/C2_1	A/C	A/C
p2731-1 (TR)	*S. virchow*	$bla_{CTX-M-9}$			HI2	HI2
IN21Rtc13	*E. coli*	*qnrB1*, bla_{TEM-1}			HI2	HI2
TcK147	*K. pneumoniae*	*qnrA*, bla_{SHV-12}		A/C2[e] (TR-TcK147)	A/C, HI2, P	A/C, HI2, P
1382-D	*E. coli*	$bla_{CTX-M-9}$			I1-αγ, HI2	I1-α, HI2
1338-D	*E. coli*	$bla_{CTX-M-9}$			HI2, FIB, FrepB	HI2, FIB, FII
128.12	*S. kentucky*	*qnrS1*, bla_{TEM-1}			N	N
TP245 (TR)	ND	*aphA*	Yes		HI2	HI2
pNGX2 (TR)	*E. coli*	*qnrS1*, bla_{TEM-1}		X2_1	X	X2
R69 (TR)	*Providencia* spp.	*tetA*, bla_{TEM-1}	Yes	L/M(pMU407)	L/M	M
R621a (TR)	*S. typhimurium*	*tetA*	Yes		I1-αγ	I1-γ
82/10	*S. enteriditis*	ND			I1-αγ, FIIS	I1-α, FIIS
20224 (TR)	*S. typhimurium*	bla_{SHV-5}, *aacA4*, *aacC1*		L/M(pMU407)	L/M	M
17830	*K. pneumoniae*	bla_{OXA-1}, $bla_{CTX-M-15}$		R_1[e] (TR-17830)	FrepB	FII, **R, FIIK**
172.23	*S. montevideo*	*qnrS1*			FIA, FIB, FIC, FrepB	FIA, FIB, FIC, FII, **R**
pX52	*E. coli*	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{OXA-1}, *qnrs1*, *aac6-1b*			Y	Y, **I2**
LS6	*K. pneumoniae*	bla_{KPC-3}		FIIK_1, FIB(K)_1, A/C2_1, FIB(pQIL)_1, ColRNAI_1	A/C	A/C, **FIIK**
F22	*K. pneumoniae*	bla_{OXA-48}			A/C	A/C, **L, HIB-M, FIB-M**
55873	*K. pneumoniae*	bla_{KPC-3}		FIIK_1, X3_1, FIB(K)_1, FIB(pQil), ColRNAI_1	**FIIS**	**FIIK, X3**
51712	*K. pneumoniae*	bla_{KPC-3}		FIIK_1, X3_1, FIB(K)_1, FIB(pQil), ColRNAI_1	**FIIS**	**FIIK, X3**
TP114 (TR)	*E. coli*	ND	Yes		No results	**I2**
A37 (TR)	*A. punctata*	*qnrS2*			No results	**U**
pOLA52 (TR)	*E. coli*	bla_{TEM-1}, *oqxAB*		X1_1	No results	**X1**
MV128	*E. coli*	*qnrS1*			No results	**X1**
MV111	*E. coli*	*qnrS1*			No results	**X1**
R471a (TR)	*S. marcescens*	bla_{TEM-1}	Yes	L/M(pOXA-48)	No results	**L**
F2	*K. pneumoniae*	bla_{OXA-48}			No results	**L, FIIK**
F200	*S. marcescens*	bla_{OXA-48}			No results	**L, R**
pNDM-MAR (TR)	*K. pneumoniae*	bla_{NDM-1}, $bla_{CTX-M-15}$, *qnrB1*, bla_{OXA-1}, *aac6-1b*		HI1B(Mar), FIB(Mar)	No results	**HIB-M, FIB-M**
pOM26 (TR)	*E. coli*	bla_{NDM-7}		X3_1	No results	**X3**

[a] TR: *E. coli* K12 transformant obtained from the original clinical strain.
[b] Abbreviations: *E.*: *Escherichia*; *C.*: *Citrobacter*; *S.*: *Salmonella*; *S. marcescens*: *Serratia marcescens*; *K.*: *Klebsiella*, *P.*: *Proteus*; *A.*: *Aeromonas*; ND: not determined.
[c] Plasmids used as reference for incompatibility groups performed by conjugation (Datta and Hedges 1971).
[d] Replicon content determined by Whole Genome Sequencing (WGS) and PlasmidFinder (Carattoli et al. 2014).
[e] Result obtained by WGS of the transformant strain indicated in parenthesis. WGS of the respective clinical isolate was not performed.
[f] Results that were different using two PBRT schemes are highlighted in bold.

cross-react on the IncFIIK plasmid of *K. pneumoniae*. However, PBRT-KIT failed to detect the second FIB replicon on plasmids pKpN-IT (FIB-K) and pKpQIL (FIB-pQil) and the small ColE-like plasmid previously identified in these strains (García-Fernández et al., 2012; Villa et al., 2013).

The 10 strains that were untypable by the 2005 PBRT and carried the new replicons I2, X3, R, U, X1, L, HIB-M, FIIK and FIB-M, were correctly identified by the PBRT-KIT. In particular, PBRT-KIT detected IncL plasmids associated with the spread of bla_{OXA-48} and the R471 plasmid, which is the reference plasmid for the IncL group (Carattoli et al., 2015). This group is epidemiologically relevant and was completely missed by the former PBRT scheme (Poirel et al., 2012).

PBRT-KIT efficiently distinguished the IncI1α and IncI1γ subgroups, whereas the 2005 PBRT scheme produced a unique result constituted by the two plasmids type together (IncI1-αγ).

No cross-reactions, false positive or false negative results were obtained, also in strains carrying 5 different replicons.

The number of PCR reactions remained the same, but the comparison highlighted the benefits of the current PBRT-KIT scheme in terms of increased sensitivity and specificity of replicon detection and typing, with respect to the former PBRT scheme. However, undetectable replicons belonging to new or rare plasmids can be expected using a wider range of non-selected Enterobacteriaceae of different origin and sources, despite PBRT-KIT strongly improved the detection of plasmid content and replicon diversity with respect to 2005 PBRT. WGS and PlasmidFinder can be helpful to supplement PBRT results if the evaluation of complete replicon content beside the 28 chosen replicons is required.

PBRT-KIT is an open system able to hold new targets gathered from epidemiological studies.

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