Note

Development of an allele-specific PCR for *Escherichia coli* B2 sub-typing, a rapid and easy to perform substitute of multilocus sequence typing Olivier Clermont^{a,b}, Julia K. Christenson^c, Anne-Sophie Daubié^{a,d}, David M. Gordon^c, Erick Denamur^{a,b,*}^a INSERM, IAME, UMR 1137, F-75018 Paris, France^b Univ Paris Diderot, IAME, UMR 1137, Sorbonne Paris Cité, F-75018 Paris, France^c Division of Evolution, Ecology & Genetics, Research School of Biology, The Australian National University, Canberra ACT 0200, Australia^d Univ Paris Nord, IAME, UMR 1137, Sorbonne Paris Cité, F-75018 Paris, France

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ABSTRACT

We developed and validated an allele-specific PCR method for detecting the nine main *Escherichia coli* phylogroup B2 lineages involved in extra-intestinal infections, which could be used as a substitute for multilocus sequence typing in studies for which the greater resolution at the sequence type level is not needed.

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Escherichia coli is not only a commensal of the gut of the vertebrates (Tenaillon et al., 2010) but also a major intestinal and extra-intestinal pathogen (Croten and Finlay, 2010; Lefort et al., 2011). It has a strong clonal population structure (Selander and Levin, 1980; Desjardins et al., 1995), with the delineation of robust phylogenetic groups and sub-groups also called lineages or sequence type complexes (STC) (Jolley and Maiden, 2010) in reference to multi-locus sequence typing (MLST) (Lecointre et al., 1998; Reid et al., 2000; Wirth et al., 2006; Le Gall et al., 2007; Gordon et al., 2008; Jauregui et al., 2008). One of the major *E. coli* phylogenetic groups is the B2 phylogroup. At least 10 phylogenetic sub-groups (I–X) or lineages, in addition to the enteropathogenic *E. coli* (EPEC)–1 lineage, have been identified in the B2 group (Le Gall et al., 2007). Recently, due to the increased use of MLST in epidemiologic analyses, it appears that some of these sub-groups are found preferentially in the various extra-intestinal pathologies (Bidet et al., 2007; Bert et al., 2010; Mahjoub-Messai et al., 2011; Bengtsson et al., 2012; Gibreel et al., 2012; Alhashash et al., 2013). Thus, identification of the clinically significant B2 sub-groups or STC is desirable. However, MLST is still a costly and time-consuming approach. There is clearly the need for the development of a rapid and easy to perform method that can be used world-wide in classical bacteriology laboratories

performing molecular typing. With these objectives in mind, we developed an allele-specific PCR method for detecting the main *E. coli* B2 STC.

Based on the literature (Bidet et al., 2007; Bert et al., 2010; Clermont et al., 2011; Mahjoub-Messai et al., 2011; Bengtsson et al., 2012; Gibreel et al., 2012; Alhashash et al., 2013), we decided to focus on the 9 previously reported sub-groups that have been found to be repeatedly involved in various extra-intestinal infections of humans, i.e. sub-groups I to VII, IX and X. So as to be easily recognized by the scientific community using the Achtman MLST scheme, we also named these 9 sub-groups or lineages as STC followed by the number of the most common [which is not always the most central genotype (Jolley and Maiden, 2010)] ST according to Achtman (<http://mlst.ucc.ie/mlst/dbs/Ecoli>).

We designed allele-specific primers that could amplify specific sub-group strains but not strains from the other sub-groups within the B2 group, using the nucleotide sequence alignments of the MLST data. These primers were also designed so that the PCR amplified products would give distinct resolvable fragment lengths. We thus decided to use 2 panels of primers, corresponding to 2 independent multiplex PCRs (Table 1). In the first panel, sub-groups II (STC73), III (STC127), VI (STC12), VII (STC14), and IX (STC95) are identified, whereas the second panel identifies sub-groups I (STC131), IV (STC141), V (STC144) and X (STC372) (Fig. 1). In both panels, we included primers for an internal control present in all B2 strains (*chuA* gene) that allow checking of the quality of the DNA. A band weaker than the control band should not be considered as positive. Each PCR reaction was

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Table 1

List of the primers used in the study and sizes of the PCR products.

Primer designation	Primer sequences	Target	Sub-group	Size of PCR product (bp)
Panel 1				
pabBgpII.f	5'-GAGTCACTGCCAGAAATTGCA-3'	pabB	II	415
pabBgpII.r	5'-GGCGAAAGGCTTAAAAATGCACT-3'			
trpAgpIII.f	5'-GACGCGCTGGAATTAGGCTC-3'	trpA	III	255
trpAgpIII.r	5'-ATCGGCAACCAGCACCGAAT-3'			
dinBgpVI.f	5'-CAGCGGTGGAGATGCGCGAT-3'	dinB	VI	652
dinBgpVI.r	5'-TCGTCAATGCCCTGACTACA-3'			
icdgpVII.f	5'-GCCGTATTGCTCTCTCTGAAT-3'	icd	VII	810
icdgpVII.r	5'-CAATTAAATCAGCCGCTTCG-3'			
aesgpIX.f	5'-CCTGGCTGCAACGGGAG-3'	aes	IX	160
aesgpIX.r	5'-TCTGGCTGCGGATAAAAGAG-3'			
chuAgene.1	5'-CGATACGGTCGATGCAAAAG-3'	chuA	Internal control	1013
chuAgene.2	5'-TTGGACAACATCAGGTCATC-3'			
Panel 2				
putPgpl.f	5'-GGTATCGCTTACTTTAACGG-3'	putP	I	373
putPgpl.r	5'-ACCACCGGACCAAAACGCC-3'			
trpAgpIV.f	5'-TGCCAGTGGAAAGAGTCCGCT-3'	trpA	IV	261
trpAgpIV.r	5'-CCGGGGCGGAAATACCAAAG-3'			
polBgpV.f	5'-GCCGTTTCGCCGAAGATAAA-3'	polB	V	530
polBgpV.r	5'-TAATGATCTTCAGCGCCTGT-3'			
aesgpX.f	5'-GACCGTTGTGAATACTCTTCA-3'	aes	X	713
aesgpX.r	5'-TATAACAGGGCGGCACATTT-3'			
chuAgene.1	5'-CGATACGGTCGATGCAAAAG-3'	chuA	Internal control	1013
chuAgene.2	5'-TTGGACAACATCAGGTCATC-3'

Fig. 1. Profiles obtained by the allele-specific PCR B2 sub-grouping. M indicates the molecular weight marker (1 kb plus DNA-Ladder, Invitrogen). The first four lanes from the left in (A) and (B) correspond to examples of unassignable (UA) strains with our assay. Strain ED1a [sub-group (SG) VIII] amplifies only with the *chuA* internal control in both panels (the higher molecular band). Strain 102-Ach030 [ungrouped (UG) by MLST] amplifies both sub-groups VI (panel 1) and IV (panel 2). Strain TA014 (UG) amplifies both sub-groups II and IX in panel 1 and the internal control in panel 2. Strain IAI53 (UG) amplifies the internal control in panel 1 and both sub-groups IV and V in panel 2. The remaining lanes in (A) correspond to examples of strains of sub-groups IX (S88 and UTI89, STc95), III (536 and F11, STc127), II (CFT073 and ECOR57, STc73), VI (ECOR60 and J96, STc12) and VII (ECOR64 and C1845, STc14). The remaining lanes in (B) correspond to examples of strains from sub-groups IV (IAI74 and H223, STc141), I (ECOR66 and EC7372, STc131), V (S107 and H176, STc144) and X (IAI64 and A0 34/86, STc372). Note, the absence or the faint amplification of the internal control *chuA* (indicated by an arrow) is due to the competition between *chuA* and the allele-specific targets, the sizes of the latter being smaller.

carried out in a 20- μ l volume containing 2 μ l of 10 $\times$ buffer (supplied with Taq polymerase) giving a final MgCl₂ concentration at 1.5 mM, 20 pmol of each primer, 200 μ M each dNTP, 2 U of Taq polymerase (Promega, Charbonnières-les-Bains, France), and 3 μ l of bacterial lysate supernatant (10 colonies in 500 μ l of water boiled, chilled in ice and centrifuged) or 2 μ l of DNA (approximately 200 ng). PCR was performed with an Eppendorf Mastercycler with MicroAm tubes in the following conditions: 4 min denaturation at 94 °C, 30 cycles for 5 s at 94 °C and 20 s at 63 °C, and a final extension step of 5 min at 72 °C. The primers used for panel 1 were: pabBgplI.f, pabBgplI.r, trpAgpIII.f, trpAgpIII.r, dinBgplI.f, dinBgplI.r, icdgpVII.f, icdgpVII.r, aesgpIX.f, aesgpIX.r, chuAgene.1 and chuAgene.2. The primers used for panel 2 were: putPgpl.f, putPgpl.r, trpAgpIV.f, trpAgpIV.r, polBgplV.f, polBgplV.r, aesgpX.f, aesgpX.r, chuAgene.1 and chuAgene.2 (Table 1). The chuAgene.1 and chuAgene.2 primers amplify the *chuA* gene in all the B2 phylogroup strains (Clermont et al., 2013), which was used as an internal control. PCR products were loaded on 2% agarose gel with SYBR® Safe DNA gel stain (Invitrogen, Cergy Pontoise, France). After electrophoresis, the gels were photographed under UV light.

We considered strains as unassigned when (i) they only yielded a band for the *chuA* internal control or (ii) they gave 2 bands (excepting the *chuA* control) after screening with the two PCR panels. One hundred and fifty three *E. coli* phylogroup B2 strains belonging to several representative collections of *E. coli* isolated world-wide since the 1980's from a variety of vertebrate species, and that include both fecal and clinical isolates (Ochman and Selander, 1984; Picard et al., 1999; Gordon and Cowling, 2003; Gordon et al., 2005; Clermont et al., 2011) and spanning the B2 ST diversity based on an extensive MLST (13 genes, 9807 nucleotides) were studied (Table S1 and Fig S1). The sensitivity of the assay was very good. All strains (n = 57) of sub-groups II, III, IV, V, VI, VII, IX and X were correctly assigned to their respective sub-groups by our allele-specific PCR. For sub-group I strains, 5 of the twenty strains tested were unassigned: 2 strains did not give any PCR product except for the internal control (TA216 and B1098), 2 closely related strains (B103 and E2062) yielded PCR products corresponding to sub-groups V and X, and one strain (M605) yielded sub-group I and X PCR products.

Inspection of the nucleotide sequence data revealed the reasons for the discrepancies observed between the MLST data and the PCR-based sub-typing method. Strain M605 not only exhibits the polymorphism in *putP* corresponding to sub-group I, but also has the sub-group X *aes* polymorphism, an outcome that probably represents a horizontal gene transfer event. Strain TA216 exhibits a *putP* allele of unknown origin, while strain B1098 exhibits a *putP* allele corresponding to sub-group IV. Horizontal gene transfer likely also explains the results for strains B103 and E2062, as these strains exhibit a *putP* allele corresponding to sub-group III and divergent alleles for the *polB* and *aes* genes. In fact, sub-group I is highly diverse and encompasses more STs than the classical ST131, which is already heterogeneous. Indeed, ST131 is composed at least of O25b (NA114 and EC7372) and O16 (SE15) clones (Matsumura et al., 2012; Dahbi et al., 2013; Johnson et al., 2014).

The 3 EPEC-1 strains and the 3 sub-group VIII strains (Clermont et al., 2008), as expected, were unassignable with our PCR assay. Of the 70 strains that could not be assigned using the MLST data to subgroups I–X, that is the ungrouped strains, 61 were unassignable using PCR panels I and II. Among the 9 remaining strains, 3 strains closely related to sub-group IV, including the AIEC strain LF82, amplify with the primers that we have designed for targeting sub-group IV strains (Fig. S1). We were not able to find single nucleotide polymorphisms in the sequences that could discriminate between these two closely related groups of strains. This represents a limitation of our assay, but on another hand does allow these adherent-invasive *E. coli* (AIEC) strains to be identified. Six strains were mis-assigned to sub-groups II (1 strain), VII (1 strain), IX (3 strains) and X (1 strain). In all cases, we observed in the targeted sequences of *pabB*, *icd* and *aes* the polymorphisms specific of sub-

Table 2Sensitivity and specificity of the allele specific PCR assay for the B2 sub-grouping of *E. coli*.

B2 sub-group	STc designation according to Achtman scheme ^a	No. of tested strains	Specific PCR-positive strains	Sensitivity (%)	Specificity (false positive/no of tested strains)
I	131	28	23	82.1	0/215
II	73	20	20	100	1/215
III	127	11	11	100	0/215
IV	141	10	10	100	3 ^b /215
V	144	3	3	100	0/215
VI	12	10	10	100	0/215
VII	14	12	12	100	1/215
IX	95	37	37	100	3/215
X	372	5	5	100	1/215
Total		137	132	96.3	9/215

^a Wirth et al. (2006).^b These 3 strains correspond to the group closely related to sub-group IV that encompasses the AIEC LF82 strain.

groups II, VII, IX and X, respectively, indicating the presence of horizontal gene transfer.

We also screened 62 B2 strains characterized using the Institut Pasteur MLST scheme (Jaureguy et al., 2008) and assigned using the MLST data to a sub-group: sub-group I (n = 8), sub-group II (n = 8), sub-group III (n = 8), sub-group IV (n = 5), sub-group V (n = 1), sub-group VI (n = 3), sub-group VII (n = 7), sub-group VIII (n = 1), sub-group IX (n = 17), sub-group X (n = 2) and ungrouped (n = 2) strains. All strains belonging to sub-groups I to VII, IX and X were correctly assigned, whereas the sub-group VIII and the ungrouped strains, as expected, were unassignable. A summary of the sensitivity/specificity of our assay on all the tested strains is given in Table 2.

Lastly, we characterized 283 B2 phylogroup bacteremia clinical isolates recovered from humans living in Australia and France using our sub-typing assay. Among the strains, only 4.9% were unassigned and the majority of the strains were found to belong to the IX (STc95) and II (STc73) sub-groups (28.2 and 27.9%, respectively), followed by the sub-group I (STc131) strains (14.1%). Strains belonging to all the other sub-groups were identified, but at a level below 10%. Interestingly, the prevalence of the sub-groups was similar in both countries (Table S2).

We have developed a robust, rapid, easy to perform and non-costly assay that allows B2 strains responsible of human extra-intestinal infections to be accurately typed to the 9 main sub-groups or lineages. While we were focused on the detection of the sub-groups implicated in extra-intestinal infections, our assay can be developed to identify additional sub-groups according to the epidemiological purpose.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.mimet.2014.03.008>.

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