HHS Public Access

Author manuscript

J Microbiol Methods. Author manuscript; available in PMC 2017 September 19.

Published in final edited form as:

J Microbiol Methods. 2017 September ; 140: 1–4. doi:10.1016/j.mimet.2017.06.005.

Multiplex polymerase chain reaction for identification of *Escherichia coli, Escherichia albertii* and *Escherichia fergusonii*

Rebecca L. Lindsey[a,*], **L. Garcia-Toledo**[a,b], **D. Fasulo**[c], **L.M. Gladney**[a,d], and **N. Strockbine**[a]

[a]Enteric Diseases Laboratory Branch, Centers for Disease Control and Prevention, Atlanta, Georgia

[b]Oak Ridge Institute for Science and Education, TN, USA

[c]Pattern Genomics, LLC, CT, USA

[d]IHRC, Inc., Atlanta, GA, USA

Abstract

Escherichia coli, Escherichia albertii, and *Escherichia fergusonii* are closely related bacteria that can cause illness in humans, such as bacteremia, urinary tract infections and diarrhea. Current identification strategies for these three species vary in complexity and typically rely on the use of multiple phenotypic and genetic tests. To facilitate their rapid identification, we developed a multiplex PCR assay targeting conserved, species-specific genes. We used the Daydreamer™ (Pattern Genomics, USA) software platform to concurrently analyze whole genome sequence assemblies (WGS) from 150 *Enterobacteriaceae* genomes (107 *E. coli*, 5 *Shigella* spp., 21 *E. albertii*, 12 *E. fergusonii* and 5 other species) and design primers for the following species-specific regions: a 212 bp region of the cyclic di-GMP regulator gene (*cdgR*, AW869_22935 from genome K-12 MG1655, CP014225) for *E. coli/Shigella*; a 393 bp region of the DNA-binding transcriptional activator of cysteine biosynthesis gene (EAKF1_ch4033 from genome KF1, CP007025) for *E. albertii*; and a 575 bp region of the palmitoleoyl-acyl carrier protein (ACP)-dependent acyltransferase (EFER_0790 from genome ATCC 35469, CU928158) for *E. fergusonii*. We incorporated the species-specific primers into a conventional multiplex PCR assay and assessed its performance with a collection of 97 *Enterobacteriaceae* strains. The assay was 100% sensitive and specific for detecting the expected species and offers a quick and accurate strategy for identifying *E. coli, E. albertii*, and *E. fergusonii* in either a single reaction or by *in silico* PCR with sequence assemblies.

*Corresponding author. Rebecca.Lindsey@cdc.hhs.gov (R.L. Lindsey).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2017.06.005.

Author contributions

RL designed this project, RL, DF, LGT and LMG contributed to the analysis and interpretation of the work. RL drafted the manuscript and RL, LGT, NS, DF and LMG and reviewed the manuscript for intellectual content.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention. Use of trade names is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention or by the U.S. Department of Health and Human Services. DF is the founder and President of Pattern Genomics, LLC, Connecticut, USA.

Keywords

Escherichia coli; *albertii*; *fergusonii*; PCR; Multiplex

1. Introduction

Escherichia coli, Escherichia albertii and *Escherichia fergusonii* may be present in animals or humans as commensals or pathogens. To begin to understand the role these organisms may play in disease, it is important to quickly and accurately identify them. Foodborne illnesses associated with *E. coli* and *Shigella*, including Shiga toxin-producing *E. coli*, are of significant public health concern (Luna-Gierke et al., 2014). *E. albertii* is an emerging pathogen, and it was determined to be the causative agent in a human gastroenteritis outbreak at a restaurant in Japan (Ooka et al., 2013). *E. albertii* frequently carries the intimin gene causing it to be mistakenly identified as enteropathogenic *E. coli* (EPEC) or enterohemorrhagic *E. coli* (EHEC) (Ooka et al., 2012). *E. albertii* can carry Shiga toxin 2f gene (*stx2f*) which is associated with mild clinical symptoms (Friesema et al., 2014; Murakami et al., 2014; Ooka et al., 2012). *E. fergusonii* is a sporadic human pathogen responsible for urinary tract infections, wound infections, and diarrhea (Gaastra et al., 2014). Multidrug-resistant forms of *E. fergusonii* have been isolated from humans with acute cystitis (Gaastra et al., 2014; Lagace-Wiens et al., 2010; Savini et al., 2008).

Determination of species is an important first step in identification of organisms in a clinical laboratory. *Shigella* spp. are considered to be pathotypes of *E. coli* (Pupo et al., 2000; Lan et al., 2004). Traditional phenotypic testing for identification of *Escherichia* spp. is time-consuming and includes multiple biochemical tests (Edwards and Ewing, 1986). Polymerase Chain Reaction (PCR) or MLST based tests for specific genes for identification of *Escherichia* spp. have been described (Ooka et al., 2013, Maheux et al., 2014, Maheux et al., 2014). A PCR that detects the *uidA* gene (encoding the b–glucuronidase enzyme) is frequently used to identify *E. coli* (Bej et al., 1991, (Frahm and Obst, 2003), Pavlovic et al., 2010). Traditionally, *E. albertii* was detected with a multiplex PCR for the presence of *clpX, lysP*, and *mdh* genes but this does not detect all *E. albertii* (Hyma et al., 2005, Murakami et al., 2014, Lindsey et al., 2015). Recently, Ooka et al. developed a nested PCR to detect *E. albertii* (Ooka et al., 2015); however, this assay does not detect additional *Escherichia* species. There are also *rpoB* gene sequencing based methods or multi locus sequencing of whole genome sequence (WGS), but these methods are time consuming and take days to complete (Hyma et al., 2005, Murakami et al., 2014, Lindsey et al., 2015). The wealth of WGS data provides an opportunity to develop a much needed multiplex polymerase chain reaction (PCR) assay to quickly speciate the common *Escherichia* species in a single reaction.

2. Materials and methods

2.1. Primer design and in silico PCR test

Daydreamer™ (Pattern Genomics, USA) software platform was used to concurrently analyze 150 genome assemblies (107 *E. coli*, 5 *Shigella* spp., 21 *E. albertii*, 12 *E. fergusonii*

and 5 other *Enterobacteriaceae*) that were generated by Illumina sequencing or downloaded from NCBI (Supplemental Table 1). The workflow for analyzing these sequences is illustrated in Fig. 1. First, genomic regions that are conserved in, and unique to, each species were identified. Sequences that were not specific due to any blast hit to non-target organisms on NCBI were removed and PCR primers were designed for the remaining sequences. Next, the target sequences were annotated using BLAST. For all three species, genes highly conserved at the nucleotide level and primers that had 100% accuracy and 100% coverage to our training dataset as reported by the Daydreamer™ *in silico* PCR tool were selected for further analysis. Primers were tested by *in silico* PCR with 324 assemblies, 280 *E. coli* (139 serogroups), 6 *Shigella* spp., 21 *E. albertii*, 12 *E. fergusonii* and 5 other *Enterobacteriaceae*. All 150 sequence assemblies that were used in primer design were also used to in testing of *in silico* PCR (Supplementary Table 1). The primers had 100% accuracy and 100% coverage to our expanded training dataset. The primers were purchased from the Biotechnology Core Facility Branch, CDC (GA, USA) and diluted to 5 μM concentration (Table 1).

2.2. Escherichia isolate identification and storage

Isolates were characterized to genus and species (Table 2) using traditional phenotypic methods (Edwards and Ewing, 1986) or *rpoB* analysis (Lindsey et al., 2015). Isolates were stored as stocks at −80 °C in Trypticase soy broth with 20% glycerol (Becton, Dickinson and Company, USA). Isolates were streaked for single colonies onto blood agar (Becton, Dickinson and Company, USA) and incubated overnight (12–16 h) at 37C. Single colonies were used for DNA extraction.

2.3. DNA extraction, PCR amplification and electrophoresis

DNA was isolated from a collection of 68 *Escherichia*, 10 *Shigella*, and 19 other *Enterobacteriaceae* isolates from pure cultures using one of two methods: the ArchivePure DNA Cell/Tissue Kit (5 Prime, USA), which was performed according to the manufacturer's protocol, or the boiled lysis method. For the boiled lysis method, a 1 μl loop-full of bacterial growth was suspended in 300 μl of water and boiled at 100 °C for 10 min.

2.4. PCR amplification and electrophoresis

Isolated DNA samples were amplified using Veriti® Thermal Cycler (Applied Biosystems, ThermoFisher, USA) and PCR kits supplied by HotStar *Taq* Master Mix Kit (Qiagen, USA). The final reaction mixture contained 2 μl of boiled lysate, 100 μM of each dNTP, 0.375 μM of each primer, 1.5 mM $MgCl_2$, and 1.25 units of HotStar *Taq*. The PCR amplification was performed as follows: one cycle at 95 °C for 10 min; 30 cycles of 92 °C for 1 min, 57 °C for 1 min, and 72 °C for 30 s; and one final cycle at 72 °C for 5 min. PCR products (4 μl each) or 2 μl of Low DNA Mass Ladder (Invitrogen, ThermoFisher, USA) were diluted in 2 μl of 5 × bromophenol blue dye and electrophoresed on a 2% agarose gel prepared with TAE (40 mM Tris-acetate, pH 8.3, 1 mM EDTA) buffer. Electrophoresis was performed at a constant voltage of 100 V for 45 min and the agarose gel was stained with GelRed™ Nucleic Acid Gel Stain (VWR, USA). Agarose gels were imaged under UV light using the Gel Doc™ XR + system (Bio-Rad, USA) and analyzed for band sizes. Fig. 2 is a representative agarose gel, positive controls are the following standard strains; *E. coli* (EDL933), *E. albertii* (97–3260 = type strain Albert 19982) and *E. fergusonii* (ATCC 35472) (Albert et al., 1991).

3. Results

3.1. Primer generation and in silico PCR results

Primers were generated with the Daydreamer™ (Pattern Genomics, USA) software platform (Fig. 1), by analyzing the WGS assemblies of 150 *Enterobacteriaceae* genomes. The target genes and expected amplicon sizes for each species are summarized in Table 1. Primers were tested by *in silico* PCR with 324 WGS assemblies (Supplementary Table 1) and found to be 100% accurate with 100% coverage for each species. *In silico* results are 280 *E. coli* and 6 *Shigella* spp. were positive with targets at 212 bp, 21 *E. albertii* were positive with targets at 393 bp, 12 *E. fergusonii* were positive with targets at 575 bp and 5 other *Enterobacteriaceae* were negative with no targets detected.

3.2. Results of conventional multiplex PCR

Primers (Table 1) were validated in the laboratory with a strain set of 97 previously characterized isolates that included 27 *E. coli*, 10 *Shigella*, 23 *E. albertii*, 14 *E. fergusonii*, and 23 other *Enterobacteriaceae* spp. (Table 2). All targeted *Escherichia* spp. yielded a single amplicon of the expected size; other *Enterobacteriaceae* spp. included in the validation did not yield a product.

4. Discussion

4.1. Isolates and sequences used in this study

Isolates used in this study were previously identified to genus and species using traditional phenotypic methods or as identified by submitters to the *Escherichia* and *Shigella* Reference Unit at the Centers for Disease Control and Prevention (CDC) or the National Center for Biotechnology Information (NCBI).

4.2. Genome targets for PCR

Genome targets were determined by NCBI BLAST search of representative amplicon sequences and are listed in Table 1. The Enteric Disease Laboratory Branch reference and PulseNet laboratories routinely sequence *Escherichia* isolates that have been confirmed by traditional methods and release the Illumina raw reads to the NCBI short read archive (SRA), under the publicly available Bioproject PRJNA218110. We used sequence available at Bioproject PRJNA218110, as well as other publicly available locations, to generate *Escherichia* specific primers which were 100% specific when tested *in silico*. We developed a rapid multiplex polymerase chain reaction to detect and differentiate *E. coli, E. albertii* and *E. fergusonii* in a single tube. Of the 405 total sequences or strains used in this study only twenty-two strains were used for all three purposes: primer design, *in silico* PCR testing and conventional PCR in the laboratory (Supplementary Table 1). Primers were 100% specific when tested in the laboratory on 97 known isolates of *Enterobacteriaceae* species.

The availability of extensive quantities of sequence data for the *Escherichia* spp. and software tools allowed us to successfully generate primers to differentiate these species. Traditional PCRs for *E. albertii* targeting only a few genes (*clpX*, *lysP* and *mdh*) were based on the analysis of a limited number of isolates and were unable to detect all *E. albertii*

(Hyma, Lacher et al. 2005, Murakami, Etoh et al. 2014, Lindsey, Fedorka-Cray et al. 2015). The sequences used to design primers for *E. albertii* in this study included human and previously published chicken isolates (Lindsey, Fedorka-Cray et al. 2015). *E. coli* and *Shigella* sequences were also diverse and represented 48 different serogroups.

5. Conclusions

This *Escherichia* species PCR showed 100% concordance with the results of the traditional culture methods, and it is much faster, more sensitive, and less labor intensive than the existing methods for detection of *E. coli, E. albertii*, and *E. fergusonii*. We routinely use these primers *in silico* to quickly identify these three *Escherichia* species from sequence assemblies. With the increased availability of WGS surveillance data these primers can be used by others for the rapid detection of these three species *in silico*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are very grateful to Devon Stripling and Haley Martin for phenotypic reference characterization, to Sung Im and Lee Katz for assembly of genomes and to Heather Carleton for critical review of this manuscript.

Funding

This work was funded by Federal appropriations to the Centers for Disease Control and Prevention, through the Advanced Molecular Detection Initiative line item.

References

Albert MJ, Alam K, Islam M, Montanaro J, Rahaman AS, Haider K, Hossain MA, Kibriya AK, Tzipori S. Hafnia alvei, a probable cause of diarrhea in humans. Infect Immun. 1991; 59(4):1507–1513. [PubMed: 2004829]

Bej AK, McCarty SC, Atlas RM. Detection of coliform bacteria and *Escherichia coli* by multiplex polymerase chain reaction: comparison with defined substrate and plating methods for water quality monitoring. Appl Environ Microbiol. 1991; 57(8):2429–2432. [PubMed: 1768116]

Edwards, PR., Ewing, WH. Edwards and Ewing's Identification of Enterobacteriaceae. 4th. Elsevier; New York: 1986. p. 7-142.

Frahm E, Obst U. Application of the fluorogenic probe technique (TaqMan PCR) to the detection of *Enterococcus* spp. and *Escherichia coli* in water samples. J Microbiol Methods. 2003; 52(1):123–131. [PubMed: 12401234]

Friesema I, van der Zwaluw K, Schuurman T, Kooistra-Smid M, Franz E, van Duynhoven Y, van Pelt W. Emergence of *Escherichia coli* encoding Shiga toxin 2f in human Shiga toxin-producing *E. coli* (STEC) infections in the Netherlands, January 2008 to December 2011. Euro Surveill. 2014; 19(17): 26–32. [PubMed: 24821123]

Gaastra W, Kusters JG, van Duijkeren E, Lipman LJ. Escherichia fergusonii. Vet Microbiol. 2014; 172(1–2):7–12. [PubMed: 24861842]

Hyma KE, Lacher DW, Nelson AM, Bumbaugh AC, Janda JM, Strockbine NA, Young VB, Whittam TS. Evolutionary genetics of a new pathogenic *Escherichia* species: *Escherichia albertii* and related *Shigella boydii* strains. J Bacteriol. 2005; 187(2):619–628. [PubMed: 15629933]

Lagace-Wiens PR, Baudry PJ, Pang P, Hammond G. First description of an extended-spectrum-beta-lactamase-producing multidrug-resistant *Escherichia fergusonii* strain in a patient with cystitis. J Clin Microbiol. 2010; 48(6):2301–2302. [PubMed: 20410344]

Lan R, Alles MC, Donohoe K, Martinez MB, Reeves PR. Molecular evolutionary relationships of enteroinvasive *Escherichia coli* and *Shigella* spp. Infect Immun. 2004; 72(9):5080–5088. [PubMed: 15322001]

Lindsey RL, Fedorka-Cray PJ, Abley M, Turpin JB, Meinersmann RJ. Evaluating the occurrence of *Escherichia albertii* in chicken carcass rinses by PCR, Vitek analysis, and sequencing of the rpoB gene. Appl Environ Microbiol. 2015; 81(5):1727–1734. [PubMed: 25548040]

Luna-Gierke RE, Griffin PM, Gould LH, Herman K, Bopp CA, Strockbine N, Mody RK. Outbreaks of non-O157 Shiga toxin-producing *Escherichia coli* infection: USA. Epidemiol Infect. 2014; 142(11):2270–2280. [PubMed: 24398154]

Maheux AF, Boudreau DK, Bergeron MG, Rodriguez MJ. Characterization of *Escherichia fergusonii* and *Escherichia albertii* isolated from water. J Appl Microbiol. 2014; 117(2):597–609. [PubMed: 24849008]

Murakami K, Etoh Y, Tanaka E, Ichihara S, Horikawa K, Kawano K, Ooka T, Kawamura Y, Ito K. Shiga toxin 2f-producing *Escherichia albertii* from a symptomatic human. Jpn J Infect Dis. 2014; 67(3):204–208. [PubMed: 24858610]

Ooka T, Seto K, Kawano K, Kobayashi H, Etoh Y, Ichihara S, Kaneko A, Isobe J, Yamaguchi K, Horikawa K, Gomes TA, Linden A, Bardiau M, Mainil JG, Beutin L, Ogura Y, Hayashi T. Clinical significance of *Escherichia albertii*. Emerg Infect Dis. 2012; 18(3):488–492. [PubMed: 22377117]

Ooka T, Tokuoka E, Furukawa M, Nagamura T, Ogura Y, Arisawa K, Harada S, Hayashi T. Human gastroenteritis outbreak associated with *Escherichia albertii, Japan*. Emerg Infect Dis. 2013; 19(1): 144–146. [PubMed: 23260717]

Ooka T, Ogura Y, Katsura K, Seto K, Kobayashi H, Kawano K, Tokuoka E, Furukawa M, Harada S, Yoshino S, Seto J, Ikeda T, Yamaguchi K, Murase K, Gotoh Y, Imuta N, Nishi J, Gomes TA, Beutin L, Hayashi T. Defining the genome features of *Escherichia albertii*, an emerging enteropathogen closely related to *Escherichia coli*. Genome Biol Evol. 2015; 7(12):3170–3179. [PubMed: 26537224]

Pavlovic M, Huber I, Skala H, Konrad R, Schmidt H, Sing A, Busch U. Development of a multiplex real-time polymerase chain reaction for simultaneous detection of enterohemorrhagic *Escherichia coli* and enteropathogenic *Escherichia coli* strains. Foodborne Pathog Dis. 2010; 7(7):801–808. [PubMed: 20156086]

Pupo GM, Lan R, Reeves PR. Multiple independent origins of *Shigella* clones of *Escherichia coli* and convergent evolution of many of their characteristics. Proc Natl Acad Sci U S A. 2000; 97(19): 10567–10572. [PubMed: 10954745]

Savini V, Catavitello C, Talia M, Manna A, Pompetti F, Favaro M, Fontana C, Febbo F, Balbinot A, Di Berardino F, Di Bonaventura G, Di Zacomo S, Esattore F, D'Antonio D. Multidrug-resistant *Escherichia fergusonii*: a case of acute cystitis. J Clin Microbiol. 2008; 46(4):1551–1552. [PubMed: 18256229]

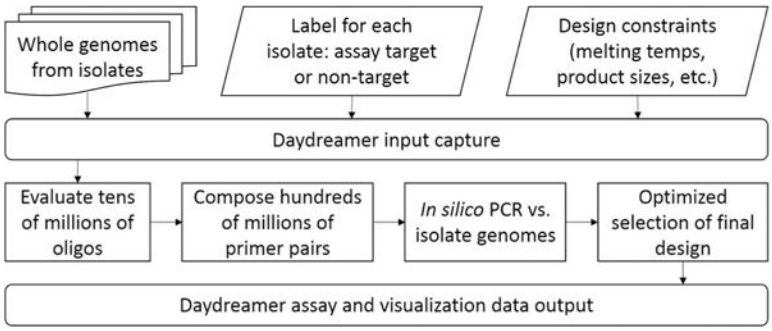

Fig. 1.
Daydreamer™ workflow.

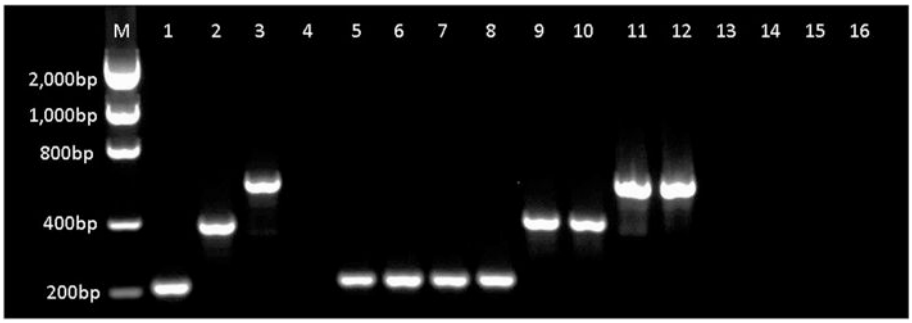

Fig. 2.

Agarose gel electrophoresis of amplicons from the *Escherichia* species conventional multiplex PCR. M: Low DNA Mass Ladder. 1–3, Standard strains, in order: EDL933, *E. coli* (212 bp), 97–3260, *E. albertii* (393 bp), ATCC 35472, *E. fergusonii* (575 bp). 4. No DNA, water control. 5–8: *E. coli/Shigella* (ATCC BAA-460, ATCC 25922, 06–3873, 2013C-3760). 9–10: *E. albertii* (97–3261, 97–3762), 11–12 *E. fergusonii* (ATCC 35470, ATCC 35471) 13–16: Negative controls include *Escherichia hermannii* (90–3390), *Escherichia vulneris* (ATCC 33821), *Salmonella enterica* (ATCC 6962), and *Salmonella enteriditidis* (ATCC 13076).

Table 1

Target genes and primers used in this study for each *Escherichia* species and amplicon size.

***Escherichia* species**	**Target gene**	**Primer name**	**Primer sequence (5′ to 3′)**	**Amplicon size**
E. coli	Cyclic di-GMP regulator gene (*cdgR*, AW869_22935 from MG1655, CP014225)	EC_F EC_R	CCAGGCAAAGAGTTTATGTTGA GCTATTTCCTGCCGATAAGAGA	212 bp
E. albertii	DNA-binding transcriptional activator of cysteine biosynthesis gene (EAKF1_ch4033 from genome KF1, CP007025)	EA_F EA_R	GTAAATAATGCTGGTCAGACGTTA AGTGTAGAGTATATTGGCAACTTC	393 bp
E. fergusonii	Palmitoleoyl-acyl carrier protein (ACP)-dependent acyltransferase (EFER_0790 from genome ATCC 35469, CU928158)	EF_F EF_R	AGATTCACGTAAGCTGTTACCTT CGTCTGATGAAAGATTTGGGAAG	575 bp

Table 2

Sensitivity of conventional multiplex PCR.

Species tested	Number tested	*E. coli* positive	*E. albertii* positive	*E. fergusonii*
Escherichia albertii	23	0	23	0
Escherichia coli	27	27	0	0
Escherichia fergusonii	14	0	0	14
Shigella sonnei	2	2	0	0
Shigella boydii	2	2	0	0
Shigella flexneri	2	2	0	0
Shigella dysenteriae	1	1	0	0
Citrobacter freundii	2	0	0	0
Enterobacter cancerogenus	1	0	0	0
Enterobacter cloacae	1	0	0	0
Escherichia hermannii	2	0	0	0
Escherichia vulneris	2	0	0	0
Klebsiella pneumoniae	1	0	0	0
Leminorella richardii	1	0	0	0
Morganella morganii	1	0	0	0
Pragia fontium	1	0	0	0
Provisional Shigella	3	3	0	0
Providencia rettgeri	1	0	0	0
Salmonella arizonae	2	0	0	0
Salmonella enterica	2	0	0	0
Salmonella enteritidis	1	0	0	0
Salmonella paratyphi B	2	0	0	0
Salmonella typhi	1	0	0	0
Yersinia enterocolitica	2	0	0	0
Total	97	37	23	14