Identification of Animal Pathogenic and Zoonotic *Escherichia coli* by Polymerase Chain Reaction (PCR) ECL-PON-002

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Appendices

1. Aim

Animal Pathogenic and Zoonotic *E. coli* (APZEC) are *E. coli* carrying genes encoding virulence factors enabling them to cause disease in animals or humans. APZEC are subdivided into the ETEC, EPEC, STEC/VTEC, and ExPEC pathotypes. ETEC carry one or more of the genes encoding enterotoxins LT, STa, and STb. ETEC in pigs mostly carry the genes encoding F4 or F18 fimbriae. EPEC carry the *eae* gene encoding Eae or Intimin. STEC/VTEC carry genes encoding VT1/Stx1 and/or VT2/Stx2 and STEC/VTEC potentially pathogenic to humans often carry the *eae* gene. Most ExPEC carry the genes encoding aerobactin, and often carry the genes encoding Tsh, CNF, or P fimbriae.

The aim of this procedure is to detect by multiplex PCR the presence of genes in *E. coli* cultures for their identification as ETEC, EPEC, STEC, or ExPEC. In addition, the determination of the presence of the genes encoding F4 or F18 in ETEC will permit their assignation as definitive agents of diarrhea in pigs. This procedure can be applied to the identification of APZEC in LB enrichment broth cultures originating from clinical samples or colonies isolated on MacConkey or other solid medium.

2. Principle

The method is based on PCR amplification of specific DNA regions from a template DNA, with oligonucleotides triggering the start of the PCR reaction. The PCR reaction is carried out using specific primers (Appendix 1).

Abbreviations
EtBr: Ethidium Bromide
EPEC: Enteropathogenic *Escherichia coli*
ETEC: Enterotoxigenic *Escherichia coli*
ExPEC*:* Extraintestinal *Escherichia coli*
STEC/VTEC: Shiga toxin producing *Escherichia coli*
LB: LuriaBertani Broth
PCR: Polymerase Chain Reaction

3. Methodology

3.1. Template preparation

Cultures streaked onto solid media (e.g. TSA, blood Agar or MacConkey Agar) are processed as follows:

- Take a streak from the first quadrant with a loop and inoculate in a tube of 5 ml of LB broth.
- Swabs from feces or tissues are placed in a tube of 5 ml of LB broth.
- Incubate overnight at 37±1°C.

3.2. DNA template

(Adapted from the method used by the Laboratory for Foodborne Zoonoses MFLP-86)

- Identify the sample by a code on the cap of the 1.5 ml tube, centrifuge 1 ml of LB broth culture incubated overnight at 37±1°C, at 12 000 RPM for 2 minutes.
- Discard the supernatant in a Corning bottle reserved for this use.
- Add 1 ml PBS or FA buffer (Difco) to the pellet, mix well (vortex or up and down with pipetter).
- Centrifuge at 12 000 RPM for 2 minutes, remove the supernatant and resuspend the pellet in 0.5 ml of sterile water Milli-Q (vortex or up and down with pipetter).
- Heat at 100°C for 10 minutes, centrifuge at 12 000 RPM for 2 minutes. Transfer the supernatant to another tube, and identify the tube. When preparing a large number of tubes, ensure that the tubes containing DNA are kept on ice or cold.
- Conserve sample DNA tubes at -20°C if not tested immediately.

3.3. Setting up the PCR reaction

For each sample, set up a 25 µl PCR reaction mixture (Appendix 2). The volume of the reagents can be scaled according to the final volume of reaction. MilliQ water must be used for PCR reactions. In each PCR assay, a positive and two negative controls must be included. The positive controls are DNA templates obtained from *E. coli* strains possessing the virulence genes tested, whereas one negative control is the DNA from a non-pathogenic *E. coli* isolate (no virulence genes harbored) and the other consists of a sample without template added. The PCR reaction mixtures are incubated in a thermal cycler programmed with the thermal profile described (Appendix 2).

3.4. Agarose gel electrophoresis

Prepare a 1.8% (w/v) agarose gel in 1X Tris/EDTA (Appendix 3). Each well of the gel is loaded with 15 µl of appropriate PCR reaction products with loading dye added at 1X final concentration. Run the samples in 1X running buffer (Tris/EDTA) at constant voltage (96V). Use a molecular weight marker suitable for assignment of the correct molecular weights to the amplicons produced (refer to the table in Appendix 1). Take into consideration that a correct band assignment is a crucial point in the assessment of the presence of the virulence genes. Make sure that the bands produced by the reference strains match exactly the expected molecular weight.

Ethidium Bromide should be added to agarose gels to allow the visualisation of DNA. This reagent is a DNA intercalating agent commonly used as a nucleic acid stain in molecular biology laboratories. When exposed to ultraviolet light, it will fluoresce with a red-orange color. EtBr should be added at a final concentration of 0.5 µg/ml before pouring the agarose gel in the electrophoresis gel cast. Alternatively, the agarose gel can be stained after electrophoresis in a 0.5 µg/ml ethidium bromide aqueous solution.

Abbreviations

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LB: LuriaBertani Broth
PCR: Polymerase Chain Reaction

4. Equipment and instruments

- Laminar flow hood for PCR
- Bacteriology loops
- 100 ml Corning bottles
- 37°C+/-1°C incubator
- Micropipettes
- Sterile micropipette tips
- 1.5 ml microcentrifuge tubes
- 0.2 or 0.5 ml PCR tubes
- Thermal cycler
- MilliQ deionizer
- Electrophoresis apparatus
- U.V. transilluminator
- Microwave oven

5. Reagents and media

- Agar plates (blood Agar and MacConkey Agar)
- Luria-Bertani Broth
- FA buffer or PBS
- PCR Reaction Mixture (Appendix 2)
  - dNTP mix stock solution
  - 10X PCR buffer stock solution (BIOTOOLS or equivalent)
  - $MgCl_2$ stock solution 50 mM (BIOTOOLS or equivalent)
  - Taq DNA polymerase (BIOTOOLS or equivalent)
- Synthetic oligonucleotide (primer)
- Electrophoresis running buffer
- Molecular weight DNA marker
- Agarose
- Loading buffer
- EtBr solution

6. Safety and protection devices

Some STEC/VTEC strains can infect humans at a very low infectious dose and can cause severe disease. Laboratory acquired infections have been reported. Consequently, working with VTEC requires good laboratory practices and the use of protection devices. EtBr is a mutagen and toxic agent; therefore it should be used in compliance with the safety sheet provided and with protection devices (lab-coats and nitrile gloves). The U.V. light may cause damage to the eyes, thus, the use of plexiglass shields and protective glasses is mandatory.

7. Reference strains

The control templates can be prepared in advance and stored in 50 µl ready to use aliquots at −20°C for eight months. Data sheets for the reference strains used at the EcL are found in Appendix 4.

8. Interpretation of the results

Samples showing amplification fragments of the expected size (Appendix 1) are considered as positive for appropriate target genes. Positive and negative controls must be included in each reaction and give positive and negative results, respectively.

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EtBr: Ethidium Bromide
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Appendix 1. Primers used at EcL for detection of the ETEC, EPEC, STEC and ExPEC pathotypes

Virulence factor	Gene name	Accession no	Primer sequence	Amplicon size (bp)	TM	Control strain	Reference
LT	*eltB*	J01646	for 5' TTA CGG CGT TAC TAT CCT CTC TA	275	60°	ECL 7805	Furrer et al., 1990. *Lett. Appl. Microbiol.* 10: 31-34.
LT	*eltB*	J01646	rev 5' GGT CTC GGT CAG ATA TGT GAT TC	275	60°	ECL 7805	Furrer et al., 1990. *Lett. Appl. Microbiol.* 10: 31-34.
STa	*estA*	M58746	for 5' TCC CCT CTT TTA GTC AGT CAA CTG	163	60°	ECL 7805	Ngeleka et al., 2003. *J. Vet. Invest.* 15: 242-252.
STa	*estA*	M58746	rev 5' GCA CAG GCA GGA TTA CAA CAA AGT	163	60°	ECL 7805	Ngeleka et al., 2003. *J. Vet. Invest.* 15: 242-252.
STb	*estB*	M35586	for 5' GCA ATA AGG TTG AGG TGA T	368	60°	ECL 7805	Lortie et al., 1991. *J. Clin. Micro.* 29: 656-659.
STb	*estB*	M35586	rev 5' GCC TGC AGT GAG AAA TGG AC	368	60°	ECL 7805	Lortie et al., 1991. *J. Clin. Micro.* 29: 656-659.
F4 K88ab1 and K88ab2	*faeG*	M29374	for 5' ATC GGT GGT AGT ATC ACT GC	601	60°	ECL 7805	Ojeniyi et al., 1994. *Zentralbl. Veterinarmed*. 41: 49-59.
F4 K88ab1 and K88ab2	*faeG*	M29374	rev 5' AAC CTG CGA CGT CAA CAA GA	601	60°	ECL 7805	Ojeniyi et al., 1994. *Zentralbl. Veterinarmed*. 41: 49-59.
Stx1	*stxA*	M19437	for 5' TTA GAC TTC TCG ACT GCA AAG	531	60°	ECL 6611	Woodward et al., 1992. *Vet. Micro.* 31: 251-261.
Stx1	*stxA*	M19437	rev 5' TGT TGT ACG AAA TCC CCT CTG	531	60°	ECL 6611	Woodward et al., 1992. *Vet. Micro.* 31: 251-261.
Stx2all Shiga-like toxin typeII subunit A and B	*stx2A*	X07865	for 5' TTA TAT CTG CGC CGG GTC TG	327	60°	ECL 6611	Woodward et al., 1992. *Vet. Micro.* 31: 251-261.
Stx2all Shiga-like toxin typeII subunit A and B	*stx2A*	X07865	rev 5' AGA CGA AGA TGG TCA AAA CG	327	60°	ECL 6611	Woodward et al., 1992. *Vet. Micro.* 31: 251-261.
EAE (Intimin)	*eae*	U66102	for 5' CAT TAT GGA ACG GCA GAG GT	791	60°	ECL 6611	Beaudry et al., 1996. *J. Clin. Microbiol.* 34: 144-148.
EAE (Intimin)	*eae*	U66102	rev 5' ATC TTC TGC GTA CTG CGT TCA	791	60°	ECL 6611	Beaudry et al., 1996. *J. Clin. Microbiol.* 34: 144-148.

Virulence factor	Gene name	Accession no	Primer sequence	Amplicon size (bp)	TM	Control strain	Reference
CNF(multiplex)	*cnf*	U01097	for 5' TTA TAT AGT CGT CAA GAT GGA	634	60°	ECL 13421	Toth et al., 2003. *J. Clin. Microbiol.* 41: 4285-4291.
CNF(multiplex)	*cnf*	U01097	rev 5' CAC TAA GCT TTA CAA TAT TGA C	634	60°	ECL 13421	Toth et al., 2003. *J. Clin. Microbiol.* 41: 4285-4291.
P (PapC)	*papC*	X61239 Y00529	for 5' GAC GGC TGT ACT GCA GGG TGT GGCG	328	60°	ECL 13421	Daigle et al., 1994. *Can. J. Microbiol.* 40: 286-291.
P (PapC)	*papC*	X61239 Y00529	rev 5' ATA TCC TTT CTG CAG GGA TGC AAT A	328	60°	ECL 13421	Daigle et al., 1994. *Can. J. Microbiol.* 40: 286-291.
Aerobactin	*iucD*	M18968	for 5' AAG TGT CGA TTT TAT TGG TGT A	778	60°	ECL 3110	Herrero et al., 1988. *J. Bacteriol.* 170: 56-64.
Aerobactin	*iucD*	M18968	rev 5' CCA TCC GAT GTC AGT TTT CTG	778	60°	ECL 3110	Herrero et al., 1988. *J. Bacteriol.* 170: 56-64.
Tsh	*tsh*	AF218073 L27423	for 5' GGT GGT GCA CTG GAG TGG	640	55°	ECL 3110	Dozois et al., 2000. *Infect. Immun.* 68: 4145-4154.
Tsh	*tsh*	AF218073 L27423	rev 5' AGT CCA GCG TGA TAG TGG	640	55°	ECL 3110	Dozois et al., 2000. *Infect. Immun.* 68: 4145-4154.

Appendix 2. Preparation of PCR reaction mixtures

1. General procedure (http://fermentas.com/techinfo/pcr/dnaamplprotocol.htm)

Reagent	Final concentration	Quantity for 25 µl of PCR sample mixture
Sterile deionized water		Variable
10X PCR buffer	1X	2.5 µl
2 mM dNTP mix	0.2 mM of each	2.5 µl
Primer forward	0.1-1 µM	Variable
Primer reverse	0.1-1 µM	Variable
Taq DNA Polymerase	2 U/50 µl	Variable
50 mM $MgCl_2$	2mM	1µl
Template DNA	10 pg-1 µg	Variable

2. Procedure for LT:STa:STb:F4 Multiplex PCR

The following is a formula for preparing a master mix for 10 reaction tubes. The final volume is 200µl.

10X PCR buffer	25 µl
2 mM $MgCl_2$	10 µl
2 mM dNTP mix	25 µl
Solution of 10 µM primer LT_{for}	10 µl
Solution of 10 µM primer LT_{rev}	10 µl
Solution of 5 µM primer STa_{for}	10 µl
Solution of 5 µM primer STa_{rev}	10 µl
Solution of 10 µM primer STb_{for}	12.5 µl
Solution of 10 µM primer STb_{rev}	12.5 µl
Solution of 10 µM primer $F4_{for}$	12.5 µl
Solution of 10 µM primer $F4_{rev}$	12.5µl
H_2O Milli-Q, sterile, kept at room temperature	48 µl
2 U Taq DNA Polymerase (5 U/µl)	2 µl
For a total of	**200 µl**

- Centrifuge for a few seconds in the micro-centrifuge.
- Prepare tubes of this mixture, at a volume of 20 µl per PCR tube. A number of tubes can be prepared ready for use and kept at -20°C for up to 2 months.
- Shake the DNA tube, add 5 µl of DNA template to 20 µl of this mixture in a PCR tube.
- Centrifuge a few seconds.
- Add to Thermal cycler and choose the cycle 60.

Positive control is *E. coli* strain ECL7805. Negative control is *E. coli* strain ECL3463. See Appendix 4 for strain data sheets.

3. Procedure for Stx1:Stx2 Multiplex PCR

The following is a formula for preparing a master mix for 10 reaction tubes. The final volume is 200 µl.

10X PCR buffer	25 µl
2 mM $MgCl_2$	10 µl
2 mM dNTP mix	25 µl
Solution of 10 µM primer $Stx1_{for}$	12.5 µl
Solution of 10 µM primer $Stx1_{rev}$	12.5 µl
Solution of 10 µM primer $Stx2_{for}$	12.5 µl
Solution of 10 µM primer $Stx2_{rev}$	12.5 µl
H_2O Milli-Q, sterile, kept at room temperature	88 µl
2 U Taq DNA Polymerase (5 U/µl)	2 µl
For a total of	**200 µl**

- Centrifuge for a few seconds in the micro-centrifuge.
- Prepare tubes of this mixture, at a volume of 20 µl per PCR tube. A number of tubes can be prepared ready for use and kept at -20°C for up to 2 months.
- Shake the DNA tube, add 5 µl of DNA template to 20 µl of this mixture in a PCR tube.
- Centrifuge for a few seconds.
- Place in Thermal cycler and choose the cycle 60.

Positive control is *E. coli* strain ECL6611. Negative control is *E. coli* strain ECL3463. See Appendix 4 for strain data sheets.

4. Procedure for single virulence gene PCR

All virulence genes can be tested in this way, taking into consideration the reaction temperature. The following is a formula for preparing a master mix for 10 reaction tubes. The final volume is 200 µl.

10X PCR buffer	25 µl
2 mM $MgCl_2$	10 µl
2 mM dNTP mix	25 µl
Solution of 10 µM primer $_{for}$	12.5 µl
Solution of 10 µM primer $_{rev}$	12.5 µl
H_2O Milli-Q, sterile, kept at room temperature	113 µl
2 U Taq DNA Polymerase (5 U/µl)	2 µl
For a total of	**200 µl**

- Centrifuge a few seconds in the micro-centrifuge.
- Prepare tubes of this mixture, at a volume of 20 µl per PCR tube. A number of tubes can be prepared ready for use and kept at -20°C for up to 2 months.
- Shake the DNA tube, add 5 µl of DNA template to 20 µl of this mixture in a PCR tube.
- Centrifuge a few seconds.
- Place in Thermal cycler and choose the cycle for the specific gene.

Positive control is an *E. coli* strain possessing the virulence gene tested, whereas one negative control is the DNA from a non-pathogenic *E. coli* isolate (no virulence genes). See Appendix 4 for strain data sheets.

5. Program temperature cycles

- Cycle 60 EcL Laboratory

1. 95°C for 2 minutes
2. 94°C for 30 seconds
3. 60°C for 30 seconds
4. 72°Cfor 30 seconds
5. Repeat from 2 for 24 more cycles
6. 4°C until the end

- Cycle 55 EcL Laboratory

1. 95°C for 2 minutes
2. 94°C for 30 seconds
3. 55°C for 30 seconds
4. 72°C for 30 seconds
5. Repeat from 2 for 24 more cycles
6. 4°C until the end

Appendix 3. Buffers

Buffers, the Milli-Q water, pipetters, pipette tips, and all materials coming into contact with the samples should be autoclaved before use, unless otherwise indicated.

1. Buffer 50XE Tris/EDTA

Recipe for 1000 ml	
2 M Tris	242 g Tris base
Glacial acetic acid	57.1 ml
0.05 M EDTA	100 ml EDTA 0.5 M pH 8
H_2O Milli-Q	Up to 1000 ml

Autoclave at 121°C for 15 minutes.

Buffer solution **1 XE**:

- 50 ml of buffer 50 XE
- 2450 ml H_2O Milli-Q

2. Loading buffer

Recipe for 10 ml	
0.25% bromophenol blue	0.025 g
0.25% xylene cyanole	0.025 g
40% (w/v) sucrose	4 g
H_2O Milli-Q	10 ml

Filter the solution with a syringe filter 0.45 µm.

Note. If you want to have the buffer without a bottom line, remove the bromophenol blue in the preparation (for amplicons around 100 bp, use loading buffer without bromophenol blue).

3. Phosphate buffer pH 7.2

- Solution A: $NaH_2PO_4.H_2O$ (0.2 M) 27.6 g/litre d'H_2O Milli-Q
- Solution B: $Na_2HPO_4.7H_2O$ (0.2 M) 53.6 g/litre d'H_2O Milli-Q

  - 140 ml solution A + 360 ml solution B
  - Add 500 ml of H_2O Milli-Q
  - Add 8.5 g NaCl
  - Adjust to pH 7.2
  - Divide in 100 ml bottles or in glass tubes
  - Autoclave at 121°C for 15 minutes

Appendix 4. *Escherichia coli* strain datasheets

Escherichia coli strain datasheet

EcL number	ECL3110
Other designation	89-7098-92-143
Serotype	O78
Virotype	Aerobactin;Tsh[1]
Isolated from (species, age, origin, disease state)	Chicken, unknown, unknown, colibacillosis
Geographical origin (country, state/province)	Canada, Quebec
History	1989, EcL Laboratory
Growth conditions	Tryptic soy broth
References	

[1] Strain tested by colony hybridization for the following virulence factors: LT, STa, STb, Stx1, Stx2, Eae, P, CNF, Aerobactin, EAST1, AFA, Paa, Aida, Tsh, F4, F18, F5, F6, F41, F17

Escherichia coli strain datasheet

EcL number	ECL3463
Other designation	82-862B2
Serotype	O115
Virotype	Negative[1]
Isolated from (species, age, origin, disease state)	Pig, two weeks, ileum, diarrhea
Geographical origin (country, state/province)	Canada, Quebec
History	1982, EcL Laboratory
Growth conditions	Tryptic soy broth
References	Ngeleka M, Jacques M, Martineau-Doize B, Daigle F, Harel J and Fairbrother JM (1993). Pathogenicity of an *Escherichia coli* O115:K"V165" mutant negative for F165(1) fimbriae in septicemia of gnotobiotic pigs. *Infection and Immunity* 61:836-843.

[1] Strain tested by colony hybridization for the following virulence factors: LT, STa, STb, Stx1, Stx2, Eae, P, CNF, Aerobactin, EAST1, AFA, Paa, Aida, Tsh, F4, F18, F5, F6, F41, F17

Escherichia coli strain datasheet

EcL number	ECL6611
Other designation	94-2127-175
Serotype	O111
Virotype	Stx1:Stx2:Eae:EAST1:Paa:EhxA:EFA-1[1]
Isolated from (species, age, origin, disease state)	Bovine, unknown, ileum, unknown
Geographical origin (country, state/province)	Canada, Quebec
History	1994, EcL Laboratory
Growth conditions	Tryptic soy broth
References	

[1] Strain tested by colony hybridization or by PCR for the following virulence factors: LT, STa, STb, Stx1, Stx2, Eae, P, CNF, Aerobactin, EAST1, AFA, Paa, Aida, Tsh, F4, F18, F5, F6, F41, F17

Escherichia coli strain datasheet

EcL number	ECL7805
Other designation	97-2554B1
Serotype	O149:H10
Virotype	LT:STa:STb:EAST1: Paa:F4(K88):[1]
Isolated from (species, age, origin, disease state)	Pig, six weeks, ileum, diarrhea
Geographical origin (country, state/province)	Canada, Quebec
History	1997, EcL Laboratory
Growth conditions	Tryptic soy broth
References	Bekal S, Brousseau R, Masson L, Préfontaine G, Fairbrother J and Harel J (2003). Rapid identification of *Escherichia coli* pathotypes by virulence gene detection with DNA microarrays. *Journal of Clinical Microbiology* 41: 2113-2125. Ngeleka M, Pritchard J, Appleyard G, Middleton DM and Fairbrother JM (2003). Isolation and association of *Escherichia coli* AIDA-I/STb, rather than EAST1 pathotype, with diarrhea in piglets and antibiotic sensitivity of isolates. *Journal of Veterinary Diagnostic Investigation* 15: 242-252.

[1] Strain tested by colony hybridization for the following virulence factors: LT, STa, STb, Stx1, Stx2, Eae, P, CNF, Aerobactin, EAST1, AFA, Paa, Aida, Tsh, F4, F18, F5, F6, F41, F17

Escherichia coli strain datasheet

EcL number	ECL13421
Other designation	2005-B089-1
Serotype	
Virotype	P:CNF[1]
Isolated from (species, age, origin, disease state)	Pig, 4 weeks, unknown, unknown
Geographical origin (country, state/province)	Canada, Quebec
History	2005, EcL Laboratory
Growth conditions	Tryptic soy broth
References	

[1] Strain tested by colony hybridization for the following virulence factors: LT, STa, STb, Stx1, Stx2, Eae, P, CNF, Aerobactin, EAST1, AFA, Paa, Aida, Tsh, F4, F18, F5, F6, F41, F17