



Global Salm-Surv

A global *Salmonella* surveillance and laboratory support project
of the World Health Organization

Laboratory Protocols

Level 4 Training Course

Multiplex PCR for differentiation of *C. coli* and *C. jejuni*.

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Contents

	Page
1. Introduction to the multiplex PCR for differentiation of <i>C. coli</i> and <i>C. jejuni</i>	2
2. Multiplex PCR for differentiation of <i>C. coli</i> and <i>C. jejuni</i>	3
3. Composition and preparation of media and reagents.....	8
Laboratory record sheets.....	10
Appendix 1 (laboratory safety)	12
Appendix 2 (example of PCR set-up).....	17

1. Introduction

In general, discrimination between *C. jejuni* and *C. coli* is performed based on the hippuricase activity. This gene encodes the enzyme *N*-benzoylglycine amidohydrolase, which presence can be demonstrated by hydrolysis of hippurate. However the results of this biochemical test are not always clear. Therefore several PCR based tests were developed.

In the assay described in this protocol, specific PCR amplification of *C. jejuni* and *C. coli* was performed with primers based on the nucleotide sequences of monospecific probes, selected for specificity from *C. jejuni* and *C. coli* DNA fragment libraries (see reference).

Reference

- Giessen A. van de, J. Tilburg, W. Ritmeester, J. van de Plas. Reduction of Campylobacter infections in broiler flocks by application of hygiene measures. Epidemiol. Infect 1998; 121:57-66.
- Vandamme P., L.J. van Doorn, S.T. Al Rashid, W.G.V. Quint, J. van der Plas, V.L. Chan and S.L.W. On, Campylobacter hyoilei Alderton et al. 1995 and Campylobacter coli Veron and Cahatelain 1973 are subjective synonyms, Int. J. Syst. Bact., 1997, 1055-1060.

2. Multiplex PCR for the detection of *C. jejuni* and *C. coli*

Materials

Equipment

- PCR Thermocycler
- Pipettes for 1 µl to 1000 µl
- Electrophoresis unit
- Microwave or autoclave
- Photo camera
- Eppendorf centrifuge
- UV-transilluminator
- Water bath 50°C

Materials

- Molecular Marker (Lambda ladder 100bp)
- Ethidium bromide solution (1%)
- Loading dye
- Staining bath
- Electrophoresis buffer TAE or TBE (see composition of media and reagents)
- Eppendorf tubes
- Tips (filter) for pipettes 1 µl to 1000 µl
- Agarose
- Primer Col1: 5' AGG CAA GGG AGC CTT TAA TC 3'
- Primer Col2: 5' TAT CCC TAT CTA CAA ATT CGC 3'
- Primer Jun3: 5' CAT CTT CCC TAG TCA AGC CT 3'
- Primer Jun4: 5' AAG ATA TGG CAC TAG CAA GAC 3'
- MgCl₂ (25 mM) (Perkin Elmer)
- 10* PCR-buffer II (Perkin Elmer) (see composition of media and reagents)
- dNTP's (2mM)
- Taq polymerase 0.5 U/µl (Applied Biosystems)
- Water pure
- DNA template
- TE buffer (Tris:EDTA 10:1)
- PBS buffer
- Crushed ice
- Mineral oil

Strains

- *C. jejuni* ATCC 29428: Positive control
- *C. coli* ATCC 33559 : Negative control
- *C. lari* ATCC 35221: Negative control

Safety

Campylobacter is considered as human pathogenic. Therefore, carry out all procedures in accordance with the local codes of safe practice.

For staining the DNA, ethidiumbromide is used. This dye is carcinogenic. Therefore gloves and proper clothes should be worn (appendix 1: laboratory safety).

Visualisation of the stained DNA is done by use of UV transilluminator. UV light is harmful for skin and eyes. Therefore proper protection (facemask, glasses) should be worn (appendix 1: laboratory safety).

Procedure

Preparation of the samples

1. 1 ml of PBS is transferred to a 1.5-ml Eppendorf tube. Using a disposable inoculation loop (white; 1 μ l), a loop full of bacteria is picked from a plate and transferred to the Eppendorf tube.
2. Centrifuge at 14.000 rpm for 5 min. Supernatant is discarded and the pellet is re-suspended in 100 μ l TE 10:1.
3. Boil the suspension (or heat at 95°C) for 5-10 minutes and transfer directly to ice. Dilute the lysed DNA 10 fold in TE 10:1

Preparation of the mix

1. Check the number of samples and calculate the amount of PCR master mix needed.
2. Prepare the PCR master mix in a tray of crushed ice as mentioned in appendix 2.
3. Aliquot the PCR master mix to the required number of PCR tubes (24 μ l per tube). Depending on the PCR machine that is used, one drop of mineral oil should be added.

Theory / comments

Make a homogeneous suspension in an Eppendorf tube, by use of a loop or cotton swab. Shake or vortex suspension just before use. Only very little cell mass is needed. If too many bacteria are used an inhibitory effect is seen.

TE is used because it contains EDTA that binds divalent ions needed by enzymes.

Boiling breaks down the bacterial cell wall and allows release of DNA.
A ventilation hole is made in the lid of the Eppendorf tube using a needle.

Always prepare mix for at least 2 additional samples ($n \text{ mix} = n \text{ samples} + 2$). Finalise the mix by adding the Taq-polymerase, since this is the most expensive content of the mix. Mix should always be prepared fresh prior to use or freeze at -20°C without the Taq-polymerase. Crushed ice is used if the PCR master mix is prepared at temperatures above 25°C as this may affect the result.

Oil is used as a lid to avoid the mixture to vaporise and condensate in the lid of the tubes

Procedure

Theory / comments

Running the PCR

1. Add 1 µl of water to the negative control tube and close the lid.
2. Add 1 µl of sample to the sample tube and close the lid.
3. Finish the procedure by adding the positive control DNA and close the lid.
4. Place the tubes into the PCR Thermocycler.
5. Program the PCR Thermocycler (or select the requested program) as mentioned in appendix 2 (Example of PCR set-up).
6. Run the program.

As the negative control is sterile water, it is advised to close the lid as soon as possible in order to avoid contaminations.

Always end the set up with the positive control DNA to avoid contaminations of the test tubes, causing false positive results.

For PCR a positive control and a negative control (template = sterile water) should be taken along.

Make sure DNA is dispensed below the oil phase.

Preparation of the agarose gel

1. Assemble the gel tray and make a proper set-up.
2. Prepare a 1% agarose solution by boiling the solution a few minutes till completely solved by the use of a water bath or microwave oven.
3. Cool the agarose to 40-50°C in a water bath and pour the agarose into the gel tray.
4. Let the gel solidify for 15-30 minutes.
5. Prepare a staining bath containing a final concentration of 5 µg/ml ethidiumbromide.

Wear suitable protection for working with ethidiumbromide (gloves, clothes). Appendix 1: laboratory safety.

Procedure

Assembling the results

1. Put the gel into the electrophoresis unit and if necessary refill with buffer.
2. Mix 10 ul of each PCR sample with approximately 3 ul loading dye.
3. Load the samples into the wells of the gel. Finish off by loading at least 1 molecular marker.
4. Replace the lid of the unit and run the gel by starting the electrophoresis process.
5. Remove the lid of the unit after a complete run of 30-45 minutes and place the gel in a straining-bath for about 30 minutes. Rinse shortly in water before visualising the gel / bands.
6. Place the gel / tray on top of the UV-transilluminator.
7. Visualise the results by switching on the UV-lamp.
8. Look for the presence of specific bands. For *C. jejuni* this should be 773 bp and for *C. coli* 364 bp. Other types of Campylobacter will be negative in this PCR (Appendix 2: Example of PCR set up).

Theory / comments

TBE contains boric acid. See Appendix 1 for safety sheet.

The loading dye makes the PCR sample visual and heavier due to the higher density compared to the buffer.

It is important to use a proper marker in order to notice whether the PCR product has the right size.

Electrophoresis can be done at different voltages/amperages. Normally, 70mA seems to be fine. Running time depends on several parameters like buffer composition, resistance, current.

UV light is harmful for skin and eyes. Wear proper protection (facemask) see app.: 1 laboratory safety

The whole PCR process is very sensitive towards contaminations and can affect the result as false positive results. It is therefore recommended to perform the different steps, if possible, in different rooms e.a.:

Room 1: Preparing the PCR master mix into tubes.

Room 2: Adding the samples to the tubes and run the samples in the PCR Thermocycler.

Room 3: Running the electrophoresis and visualising the DNA.

3. Composition and preparation of media and reagents

Reagents can be made as described below and/or are commercially available from companies like Invitrogen Life Technologies and Roche Applied Science.

TAE (Tris-Acetate EDTA) buffer

Working solution

- 0.04 M Tris Acetate
- 0.001 M EDTA

Concentrated stock solution (50*)

Per liter

- Tris base 242 g
- Glacial acetic acid 57.1 ml
- 0.5 M EDTA (pH 8.0) 100 ml

TBE (Tris-Borate EDTA) buffer

Working solution

- 0.089 M Tris borate
- 0.089 M boric-acid
- 0.002 M EDTA

Concentrated stock solution (5*)

Per liter

- Tris base 54 g
- Boric acid 27.5 g
- 0.5 M EDTA (pH 8.0) 20 ml

10* PCR buffer II

- 100 mM Tris-HCl
- 500 mM KCl (pH 8.3)

Phosphate buffered saline (PBS) (pH: 7,3)

- Sodium Chloride 28,8 g.
- Potassium di-hydrogen phosphate 1,72 g.
- di-Sodium hydrogen phosphate anhydrous 5,92 g.
- Water 4000ml

Tris-EDTA buffer (10:1) 1 L (pH 8)

- 1 M Tris-HCl (pH 8) 10 ml.
- 0,5 M EDTA (pH 8.0) 2 ml.
- Water 1000 ml.

Loading dye (Blue Juice 10ml)

- 40% Sucrose 4 g.
- 0,25% Bromphenolblue 25 g.
- Xylene Cyanol 25 mg.
- Tris –EDTA 10:1 10 ml.

Ethidium bromide (10mg/ml)

- Add 1 g of ethidium bromide to 100 ml of H₂O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer to a dark bottle and store at 4°C.

Date: _____

**Recordsheet:
PCR results**

Initials: _____

Calculating PCR mix:

Date: _____

No. of samples:	1	5	Number of samples + 2
PCR H ₂ O	15.5 ul	77.5 ul	
10 X PCR buffer II	2.5 ul	12.5 ul	
dNTP mix (2mM)	1.0 ul	5.0 ul	
MgCl ₂ (25mM)	2.5 ul	12.5 ul	
Primer Col1 (100pmol/ul)	0.5 ul	2.5 ul	
Primer Col2 (100pmol/ul)	0.5 ul	2.5 ul	
Primer Jun3 (100pmol/ul)	0.5 ul	2.5 ul	
Primer Jun4 (100pmol/ul)	0.5 ul	2.5 ul	
Taq polymerase (0.5 U/ul)	0.5 ul	2.5 ul	
Total volume	24 ul	120 ul	

PCR Program:

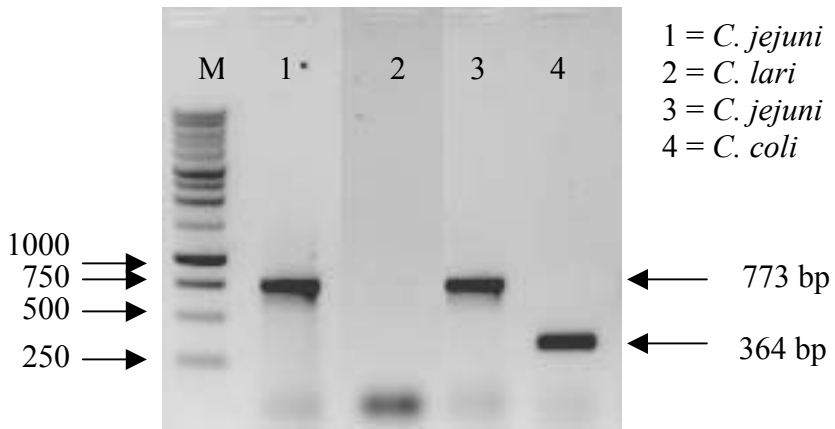
5 min. 94°C
1 min. 94°C, 1 min. 64°C, 1 min 72°C, 2*
1 min. 94°C, 1 min. 62°C, 1 min 72°C, 2*
1 min. 94°C, 1 min. 60°C, 1 min 72°C, 2*
1 min. 94°C, 1 min. 58°C, 1 min 72°C, 2*
1 min. 94°C, 1 min. 56°C, 1 min 72°C, 2*
1 min. 94°C, 1 min. 54°C, 1 min 72°C, 30*
10 min. 72°C
hold 4°C

PCR product size:

- *C. jejuni* 773 bp
- *C. coli* 364 bp

Samples:

M: Molecular marker 100 bp
1 H ₂ O
2 <i>C. jejuni</i> ATCC 29428 (Pos.)
3 <i>C. coli</i> ATCC 33559 (Pos.)
4 <i>C. lari</i> ATCC 35221 (Neg.)
5
6
7
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9
10
11



APPENDIX 1

Safe Work Procedure ETHIDIUM BROMIDE

Use

Ethidium bromide is added to electrophoresis gels for visualisation of nucleic acids.

Hazards

Class 6 – Toxic. Potent mutagen.

Risk Control Measures

Only use Ethidium bromide (EtBr) after receiving safety training (Laboratory Induction / Authorisation). Wear safety glasses when using ethidium bromide. Avoid skin contact, ethidium bromide may be absorbed through the skin. Wear Latex gloves, laboratory coat. Always dispose of gloves after use. Don't touch equipment, door handles, phone, keyboard, etc.

Weighing solid - Powder may cause irritation when inhaled - wear dust mask and use in ventilated area. Use designated micropipette only when dispensing the liquid.

Engineering / Ventilation Controls

Ensure access to a safety shower and eye wash in areas where ethidium bromide is used. Preferably weigh the solid in a fume hood.

Storage Requirements

Store in a cool, dry place away from strong oxidizing agents. Keep containers tightly closed. Use with adequate ventilation.

First Aid / Spill Control Procedures

Wash off immediately with copious amounts of cold water (at least 10 minutes). Ethidium bromide is absorbed through the skin so follow the cold water washing with a thorough washing with warm water and soap. Contaminated clothing should be removed as soon as possible and thoroughly washed.

In case of contact with eyes, immediately flush eyes with copious amounts of water for at least 15 minutes (eye wash).

Seek medical attention.

If the spill is on equipment, use ultraviolet light (wear appropriate eye protection) to locate spill, then use the decontamination procedure outlined below.

Wear protective clothing.

Small spill:

If in solution, absorb freestanding liquid using vermiculite or Polyzorb from Spill Kit. Use ultraviolet light to locate spill. Follow instructions on Spill Kit.

Large Spill: Notify others in the area of spill. Evacuate area. Barricade area with tape (in Spill Kits) to prevent entry until arrival of response personnel. Provide assistance and information to spill clean up crew.

Waste

Store waste: Liquid - In proper waste container

Solid - In proper waste container

Ethidium Bromide Liquid Disposal

1. Add 10g activated charcoal per 2.5L waste.
2. Leave for 1 hour, with occasional shaking.
3. Filter contents through Whatman Number 1 filter paper.
4. Filtrate may be disposed of down the sink.
5. Charcoal & paper is treated as solid hazardous waste and disposed in the EtBr Solid Waste Bucket.

If Using 'Green Bag' (Bio-101 Cat. No. 2350-200):

- 1 For 10mg Ethidium Bromide (max) add 1 'Green Bag' to the waste bottle with a magnetic flea.
2. Place waste bottle onto a magnetic stirrer and mix the solution for 24hours.
3. Dispose of the 'Green Bag' in the dry Ethidium Bromide waste. The remaining solution may be disposed of in the sink.

Staining gels

During electrophoresis - Add EtBr after boiling up the agarose - let it cool down before adding EtBr

Afterwards -Soak gel in a well marked plastic container - Put name and date on container as it is possible to re-use the staining solution.

Applicable Standards and Regulations

[University of Melbourne Environment and Safety Manual - Poisons and Drugs](#)

[University of Melbourne Environment and Safety Manual - Carcinogens](#)

Occupational Health and Safety (Hazardous Substances) Regulations 1999

Safe Work Procedure ULTRA VIOLET SOURCE

Ultraviolet Light

Ultraviolet Radiation is that portion of the electromagnetic spectrum that falls in the region of 100 to 400nm. This spectrum has been divided into three regions:-

A: 400nm to 315nm known as Near UV or UV-A,

B: 315nm to 280nm known as Mid UV or UV-B,

C: 280nm to 100nm known as Far UV or UV-C.

Hazards

Two categories of hazard are involved in the use of high intensity UV lamps: those inherent in the radiation itself and those associated with operation of the lamps. All radiation of wavelength shorter than 250 nm should be considered dangerous.

- Damage to eyes and skin caused by exposure to UV radiation. Repeated overexposure of skin to UV has been linked with premature aging, wrinkles and most seriously, skin cancer. Eye damage can result in corneal scarring and cataract formation.
- Burns caused by contact with a hot UV lamp.
- Fire ignited by hot UV lamp.
- Interaction of other nearby chemicals with UV radiation.

Damage caused to apparatus placed close to UV lamp

Risks

Damage to vision is likely following exposure to high intensity UV radiation.

Who is likely to be injured?

The user or anyone exposed to the UV light as a result of faulty procedure. Injuries may be slight to severe.

Control Measures Operating Precautions

Lab-coats, gloves and safety glasses or other appropriate eye/skin protection such as UV protective glasses or a UV protective face shield must be worn.

Reactions using UV lamps: external irradiation sources.

- These operations must never be attempted by an untrained person.
- These operations must never be attempted by a single person.
- These operations must never be attempted out of normal working hours.
- Use of UV lamps must be carried out in a fume hood with boarded up windows.
- As far as possible, the UV source should be contained in a closed radiation box.
- The fume hood sash must remain closed while the UV lamp is switched on.

- The fume hood may contain only the UV lamp and associated apparatus and chemicals. No other chemicals are to be stored in the fume hood and no other reactions are to be performed in the fume hood.
- Reaction vessels containing flammable solvents must be at least 20 cm away from the lamp to avoid excessive heating.
- Flammable equipment (*e.g.* rubber/plastic tubing) must be positioned at least 10 cm away from the lamp.
- After the UV lamp is switched off, unless the reaction mixture requires immediate attention, the fume hood sash should remain closed for 30 minutes to allow the UV lamp to cool.

Reactions using UV lamps: low/medium pressure Hg lamps in an immersion well.

- These operations must never be attempted by an untrained person.
- These operations must never be attempted by a single person.
- Low/ Medium pressure lamps are to be used ONLY in approved, water-cooled immersion well apparatus.
- The UV lamp power supplies must incorporate an electrical cut-out that activates in the event of disruption to cooling water.
- The UV lamp must not be switched on until:
 - The glassware is shrouded in Al foil,
 - The immersion well set-up is shielded by the appropriate metal case
 - The boarded up fume-hood doors are closed.
- The UV lamp must NEVER be switched on/connected outside of the shrouded immersion well apparatus.

Training

For the use of high intensity UV sources, new users must be trained by another member of the laboratory who, in the opinion of the member of staff in charge of the laboratory, is sufficiently competent to give instruction on the correct procedure. Newly trained users should be overseen for some time by a competent person.

Emergency Procedures

UV exposure: Call a First Aider.

Burns: See Basic First Aid. Call a First Aider

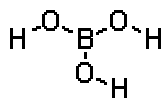
Hazard sheet **BORIC ACID**

Synonyms: Boric Acid, MB Grade (1.12015); Boron; Boricacidhighpurity; Boricacidwhitextl

Molecular Formula: H_3BO_3

Formula Weight: 61.83

Registry number: 10043-35-3



Registry number: 10043-35-3

Density: 1.43

Melting point: 169 °C

Hazard Symbol



Toxic

Risk Description

R60 May impair fertility.

Safety Description

S45 In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

S53 Avoid exposure - obtain special instructions before use.

IR

Analysis

Result

Miscellaneous	545 (29.51); 641 (30.64); 813 (26.79); 1193 (21.05); 1456 (17.55); 3215 (15.75)
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Appendix 2

Example of PCR set up

Calculating PCR mix:

Date:

No. of samples:	1	5	Number of samples + 2
PCR H ₂ O	15.5 ul	77.5 ul	
10 X PCR buffer II	2.5 ul	12.5 ul	
dNTP mix (2mM)	1.0 ul	5.0 ul	
MgCl ₂ (25mM)	2.5 ul	12.5 ul	
Primer Col1 (100pmol/ul)	0.5 ul	2.5 ul	
Primer Col2 (100pmol/ul)	0.5 ul	2.5 ul	
Primer Jun3 (100pmol/ul)	0.5 ul	2.5 ul	
Primer Jun4 (100pmol/ul)	0.5 ul	2.5 ul	
Taq polymerase (0.5 U/ul)	0.5 ul	2.5 ul	
Total volume	24 ul	120 ul	

PCR Program:

5 min. 94°C 1 min. 94°C, 1 min. 64°C, 1 min 72°C, 2* 1 min. 94°C, 1 min. 62°C, 1 min 72°C, 2* 1 min. 94°C, 1 min. 60°C, 1 min 72°C, 2* 1 min. 94°C, 1 min. 58°C, 1 min 72°C, 2* 1 min. 94°C, 1 min. 56°C, 1 min 72°C, 2* 1 min. 94°C, 1 min. 54°C, 1 min 72°C, 30* 10 min. 72°C hold 4°C
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PCR product size:

- <i>C. jejuni</i> 773 bp - <i>C. coli</i> 364 bp
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Samples:

M: Molecular marker 100 bp
1 H ₂ O
2 <i>C. jejuni</i> ATCC 29428 (Pos.)
3 <i>C. coli</i> ATCC 33559 (Pos.)
4 <i>C. lari</i> ATCC 35221 (Neg.)
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