Aquavalens Project

"Protecting the health of Europeans by improving methods for the detection of pathogens in drinking water and water used in food preparation."

Grant agreement number: 311846

Deliverable D3.1

Standard monitoring methods for identification of the targeted bacteria harmonized & Standard Operating Procedures (SOPs) developed

Authors: Manfred Höfle, HZI, Germany; Carla Pruzzo, UNIGE, Italy; Maria Jose Figueras, URV, Spain; Antonio Martinez-Murcia, GPS; Spain.

Version 1, Date July 31, 2013.

Table of contents

1. Introduction

During the first 6 months, protocols for the classical and molecular detection and identification of the major targeted bacterial species were established and harmonized among the partners of WP 3. This included the definition of test strains and reference molecular materials as well the detection and identification technologies. The following major bacterial species were identified in the kick-off meeting as first priorities: *Arcobacter butzleri, Campylobacter coli, Campylobacter jejuni, Vibrio cholerae, Pseudomonas aeruginosa, Salmonella enterica, Escherichia coli*. In addition, two bacterial species of interest to the AQUAVALENS projects were offered by single partners to be included in the work on a voluntary basis. These optional species were *Legionella pneumophila* (HZI) *and Helicobacter pylori* (URV). For the establishment of a solid and well defined detection and identification technology the reference strains have to be identified and, ideally, deposited in a central strain collection. At the kick-off meeting it was decided to do this as much as possible with type strains of the targeted species. This was approached for all species except *S. enterica* and *E. coli* where specific pathovars of the species are the targeted waterborne bacterial pathogen. Gene targets for molecular detection and identification were selected according to the specificity needed to have save discrimination between the next taxonomic neighbors. Therefore, not only 16S rRNA genes were chosen in several cases. The specific details of the targeted bacterial species are summarized in the following table.

Species name	**Reference strain**	**Target gene**	**Detection method**
Arcobacter butzleri	LMG 10828^T	*hsp60*	Plate counts, qPCR
Campylobacter coli	DSMZ 4689^T	*cadF*	MPN, qPCR
Campylobacter jejuni	DSMZ 4688^T	*hipO*	MPN, qPCR
Vibrio cholerae	ATCC 39315	*16S-23S rRNA IS*	Plate counts, qPCR
Pseudomonas aeruginosa	DSMZ 50071^T	*gyrB*	Plate counts, qPCR
Salmonella enterica Typhi	CECT 409	*pfim*	MPN, qPCR
Escherichia coli (O157:H7)	ATCC 47076 (CECT 4076)	*16S rRNA (eae,stx#)*	Plate counts, qPCR
Legionella pneumophila	ATCC 33152^T	*16S rRNA*	Plate counts, qPCR
Helicobacter pylori	ATCC 43504^T	*16S rRNA*	Cultivation, qPCR

Table 1: Overview about all the bacterial species targeted in the AQUAVALENS project in WP3, their respective reference strains, the gene used for detection and the main detection methods used.

Monitoring of specific bacteria in drinking water can be done using classical cultivation techniques with selective agar media or by molecular techniques using environmental DNA obtained from the water sample to be analyzed. For the cultivation based methods of most of the well-known waterborne bacterial pathogens highly standardized methods exist, like ISO procedures. These standard methods were used as basis for the SOPs in WP3 for the quantitative determination of the abundances of the specific bacterial species. For molecular identification of the strains normally full 16S rRNA sequencing is used or a confirmatory PCR targeting a species-specific gene or gene fragment. For the molecular detection and quantification of the targeted bacteria, corresponding to the cultivation-based quantification, currently real-time PCR is using a SYBR Green I or TaqMan approach. In addition, a standard protocol for the extraction of DNA from bulk water is provided which forms the basis of all molecular detection and quantification methods. All protocols represent the current state-of-the art, based on the experience of the WP3 partners and or literature data. They are made for experts and will be subjected to changes and modifications during the course of the project. A final set of approved protocols is given in the following sections.

2. SOP for bulk water filtration and DNA extraction using DNeasy kit

Procedure for bulk water filtration:

1. Drinking water is sampled according to ISO 5667-2, i.e. bulk water is collected after heat sterilization of the faucet and a pre-run of 5 min. The number of replicates and filtered volume may vary according to the specific sampling plan and sampling site (at least **3 replicates with 3 liters** each of drinking water are recommended).
2. Screw and push the rubber stopper into the filtration machine.
3. Screw and push the sterile blue funnel into the rubber stopper.
4. Fix the black rubber ring in the sterile blue funnel with a pincers.
5. Push the glass filter plate for fixation down and make it wet with respective water.
6. Place the nucleopore-filter (90mm diameter, 0.2 µm pore size, Track-etch, Whatman Corp.) with a sterile pincers on the glass filter plate without folds.
7. Place the glass fiber-microfilter (GF/F 90 mm, Whatman Corp. pre-combusted at 450°C for 5h) with a sterile pincers on top of the nucleopore-filter and make it wet.
8. The second rubber ring put on glass fiber-microfilter (GF/F).
9. Screw 1 L glass top into the sterile blue funnel which h is fixed with the suction machine and check if the machine is really closed (handle on the right) and if the filtration is working properly (every inlet valve of the vacuum tubes should be green!)
10. Fill into the glass top about 100ml-200ml water and turn down the apparatus‘ lever down, build up to 300mbar-400mbar vacuum → check the tightness and constant vacuum (maybe close the glass top more)
11. Fill into the glass tops more water in regular time spacing → filter are not allowed to get dry!
12. Stop the vacuum (turn handle right) when the filtration is finished and note the flow time.
13. Screw the glass top and put it to the side, remove the rubber ring.
14. Both filters half fold with sterile pincers and transfer them into an aluminum round foil and storage it at -20°C (for 1 day). For storage at a longer time please keep it at -70°C.

DNA extraction using DNeasy Blood & Tissue Kit (#69506, Qiagen, Germany)

Preparations of solutions:

-prepare *Enzymatic Lyses Buffer (ELB)*:

- 20mM Tris-HCl
- 2mM EDTA
- Adjust to pH 8.0 with 1M NaOH
- autoclave and add .,2 % TX-100 (1,2ml for 10ml buffer)

- before using ELB: add **Lysozyme** to an aliquot at a final concentration of 10mg/ml

Procedure for one sandwich:

- use a scalpel and cut *filter sandwich* in small pieces and put them with a tweezers into a Falcon tube (15 ml)
- add **3ml** *ELB-Buffer* to the filter pieces into the Falcon tube
- close Falcons and vortex

- incubate them for *60 min at 37°C* in a thermo mixer (shaking 500rpm)
- add **75µl** Proteinase K solution
- vortex
- incubate 30min at 56°C, 500rpm
- add **2.7ml** *AL buffer*
- vortex thoroughly
- Incubation: 20 min at 78°C in a Thermo mixer, shaking 500rpm
- Place a cell strainer on a 50 ml Falcon
- Transfer filter pieces with a sterile plastic loop and decant the lysate into the cell strainer
- centrifuge at 3000 rpm (2600 g) for 3 min
- discard the cell strainer and the dry filter pieces
- aliquot the filtrate in **2** x 15ml Falcon Tubes with the same volume and add in each tube half of the total Filtrate volume with ethanol (abs). (e.g. add for 2 ml filtrate 1ml EtOH) and vortex
- unpack Dneasy Spin columns and label them
- cut off the ground of the Falcon's with a scissor, clean it thoroughly with some tissue and press the Dneasy Spin column under the Falcon tubes. Put the Dneasy Spin column which is fixed now with the falcon together on the Wizard vacuum station; open the Falcon's lid carefully, switch on the vacuum pump and absorb the whole volume from the Falcon through the spin column with little pressure (max. 200 mbar).
  Or: centrifuge (1min. 8000rpm) with 500µl filtrate each time until you used all the filtrate; discard the flow through
- wash the spin column with 500µl AW1 (add und centrifuge 1min 8000rpm, discard flow through)
- wash the spin column with 500µl AW2 (add and centrifuge 1min 8000rpm, discard flow through)
- put the spin column into a new 2ml labeled Collection tube
- centrifuge 3min at 13 000 rpm in a table centrifuge
- Transfer the spin column into labeled 1.5ml sterile reaction tubes (Eppendorf) and pipette **30µl AE Puffer** or *sterile water* in the middle of the membrane of the spin column
- **incubate 1min**
- centrifuge the spin columns which is fixed for 1min. at 7000 rpm (8000 g)
- repeat the elution step once

Storage:

- store the 60µl Eluate at -20°C or if you measure short time later into the fridge (4°C)

3.1 SOP for quantification of *Arcobacter butzleri* using cultivation

Principle

For enumeration of *Arcobacter butzleri*, counting by direct plating into a selective medium is used.

Materials

Culture media
Arcobacter-selective isolation agar, containing 24 g L^{-1} Arcobacter-broth (Oxoid, Basingstoke, UK) 12 g L^{-1} Agar Technical No. 3 (Oxoid, Basingstoke, UK) supplemented with 100 mg L^{-1} 5-fluorouracil, 100 mg L^{-1} cycloheximide, 10 mg L^{-1} amphotericin B, 16 mg L^{-1} cefoperazone, 32 mg L^{-1} novobiocin and 64 mg L^{-1} trimethoprim (Sigma, St. Louis, MO, USA)

Reagents

- Saline (sodium chloride (NaCl) 8.5 g L^{-1})
- Gram staining regents
- Oxidase reagent (Becton Dickinson, New Jersey, USA)
- Instagene^{TM} Matrix (Bio-Rad, Hercules, CA, USA)

Sampling

Samples of water (generally 1L) shall be collected in glass, polyethylene or similar containers. If used previously, they shall be cleaned, rinsed with distilled or mains tap water and autoclaved at (121 ± 3) °C for 15 min. Samples should be protected from direct sunlight and transported at ambient temperature in an insulated container. Samples should be examined as soon as possible on the day of collection. In exceptional circumstances, if there is a delay, storage under the above conditions should not exceed 24 hours.

Procedure

1 ml volume of water was pipetted in triplicate into three empty tube wells. Perform 6 serial decimal dilutions (100:900 µl) of the sample original suspension in saline, in triplicate. Inoculate 100 µl of each dilution onto *Arcobacter*-selective isolation agar and incubate the plates at 30°C for 2–3 days under aerobic condition. After the incubation time, count in those plates with 30 – 300 colonies. Identify the colonies using Gram's staining and oxidase reactions. In order to confirm their identity, extract DNA from a colony using the InstageneTM Matrix following the manufacturer instructions, and perform the 16S rRNA-RFLP method as described by Figueras *et al.* (2012).

Expression of results

Express the average results in CFU of *Arcobacter butzleri* in 100 ml, and report the absence of as "not detected" in the volume examined.

References

De Smet S, De Zutter L, Debruyne L, Vangroenweghe F, Vandamme P, Houf K. *Arcobacter* population dynamics in pigs on farrow-to-finish farms. Appl Environ Microbiol. 2011; 77(5):1732-8.
Figueras MJ, Levican A, Collado L. Updated 16S rRNA-RFLP method for the identification of all currently characterised Arcobacter spp. BMC Microbiol. 2012;12:292. doi: 10.1186/1471-2180-12-292.

3.2 SOP for quantification of *Arcobacter butzleri* by Real Time PCR

A 7900HT Fast Real-Time PCR System instrument (Applied Biosystems) is used. Calibration curves are generated by preparing standard DNA dilution series from *A. butzleri* strain LMG 10828^T. The real-time PCR assay uses environmental DNA obtained by the filtration and extraction SOP indicated above or a specific variation of it. A TaqMan-based real-time PCR approach was used based on the TaqMan Universal PCR Master Mix (Applied Biosystems).

1. PCR mixtures:

Reagent	**Quantity (µl)**	**Final concentration into the mix**
PCR grade water	3.4	
Primer prVC-F	1.3	300 nM
Primer prVCM-R	1.3	300 nM
2x GoTaq qPCR Master Mix	10	1x
Template DNA	4	2-10 ng
Total reaction volume	20	

2. Primers:

Target gene	**Primer sequence**	**Amplified fragment (bp)**	**Detectable strains**	**Ref.**
hsp60	**Abutz-F:** CTC TTC ATT AAA AGA GAT GTT ACC AAT TTT **Abutz-R:** CAC CAT CTA CAT CTT CWG CAA TAA TTA CT **Abutz-probe*:** CTT CCT GAT TGA TTT ACT GAT T	89	*A. butzleri*	1

*The FAM (6-carboxy-fluorescein) is used as fluorescent reporter dye and conjugated to 5' ends of each probe. The quencher dye BBQ is attached at the 3'end of the probes.

3. Thermal Cycle:

***Arcobacter butzleri* quantification by Real Time PCR (prvc-F/R primers)**				
Cycles	**1**			
Taq activation	**Step 1**			
Target temp. (°C)	95			
Incubation time	10 min			
Temp. Transition rate (°C/sec)	20			
Acquisition mode	none			
Cycles	**40**			
Amplification	**Step 1**	**Step 2**		
Target temp. (°C)	95	60		
Incubation time	15 s	1 min		
Temp. Transition rate (°C/sec)	20	20		

4. Detection limit according to calibration curve

Detection limit: 10^3 CFU/ml sample

Reference

1. de Boer RF, Ott A, Güren P, van Zanten E, van Belkum A, Kooistra-Smid AM. Detection of Campylobacter species and Arcobacter butzleri in stool samples by use of real-time multiplex PCR. J Clin Microbiol. 2013;51(1):253-9.

4.1 SOP for quantification of *Campylobacter jejuni and Campylobacter coli* using cultivation

Principle

This SOP comprises 2 approaches for the enumeration of *Campylobacter* spp., one is a miniaturized most probable number (mMPN) method as described by Chenu *et al*. (2006) and the other in the ISO 17995:2005 that specifies a method for detection and semi-quantitative enumeration of thermotolerant *Campylobacter* species (**Health Protection Agency, 2007;** ISO 17995:2005).

Sampling

Samples of water (generally 1 L) shall be collected in glass, polyethylene or similar containers. If used previously, they shall be cleaned, rinsed with distilled or mains tap water and autoclaved at (121 ± 3) °C for 15 min. Samples should be protected from direct sunlight and transported at ambient temperature in an insulated container. Samples should be examined as soon as possible on the day of collection. In exceptional circumstances, if there is a delay, storage under the above conditions should not exceed 24 hours.

Method 1: Miniaturized most probable number method (Chenu *et al*., 2013)

Materials

Culture media

mBFBB (modified blood free Bolton broth, containing 25 mg/l sulfamethoxazole (SMX, Sigma Aldrich, Castle Hill, Australia) dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich, Castle Hill, Australia).
mCCDA (modified Cefoperazone Charcoal Deoxycholate Agar; Oxoid, Basingstoke, UK)
Mueller Hinton agar 5% sheep blood plates (ready plates, Becton Dickinson, New Jersey, USA)

Reagents

- Bacteriological agar (Agar No. 1 ; Oxoid, Basingstoke, UK)
- 2,3,5-triphenyltetrazolium solution (TTC; Sigma, St. Louis, MO, USA).
- Gram staining regents
- Oxidase reagent (Becton Dickinson, New Jersey, USA)
- Hydrogen peroxide (for catalase reaction)
- Hippurate hydrolysis reagents DIETABS (Rosco Diagnostica, Taastrup, Denmark)
- Sensi-Disc cephalothin 30 µg (Becton Dickinson, New jersey, USA)
- Sensi-Disc nalidixic acid 30 µg (Becton Dickinson, New jersey, USA)
- Instagene™ Matrix (Bio-Rad, Hercules, CA, USA)

Procedure

1 ml volume of water was pipetted in triplicate into three empty tube wells. Perform serial decimal dilutions (100:900 µl) of the original sample in single-strength mBFBB to generate the 3-tube MPN. Mixed with repeated aspiration. The triplicate dilutions were incubated for 24–48 h at 42 °C under microaerobic conditions. Transfer 50 µl aliquots from each of the dilutions in plasma tubes post-incubation, into a V-bottomed microtiter plate (Greiner bio-one, Gloucestershire, UK) with each

dilution in a subsequent row (e.g. 10^{-1} in A1 to A3, 10^{2} in B1 to B3, to a theoretical maximum dilution of 10^{-8} in row H1 to H3). The V-bottomed microtiter plates should be pre-filled with 100 µl per well of complete mBFBB + 0.3% bacteriological agar and 150 µg ml^{-1} 2,3,5-triphenyltetrazolium solution. Cover the plate with an adhesive plastic film (Seal Plate®; Excel Scientific Inc, Victorville, CA, USA) and incubate at 42 °C 24 h, under microaerobic condition.

Red color development in a well is considered a presumptive positive result for the presence of *Campylobacter* spp. Confirm by sub-culture onto mCCDA each 3 × 3 MPN tube. Incubate the plates in microaerobic conditions at 42°C for 48 hours. After incubation, examine the plates for presumptive *Campylobacter* colonies and confirm their identity using morphological and biochemical tests, including Gram's staining, oxidase and catalase reactions, the absence of aerobic growth on blood agar plates, hippurate hydrolysis, and susceptibility to cephalothin and nalidixic acid summarized in the following Table.

Table: Biochemical test of *C. jejuni* and *C. coli*

Characteristic	***C. jejuni***	***C. coli***
Catalase	+	+
Oxidase	+	+
Aerobic growth	-	-
Motility (wet mount)	+	+
Hippurate hydrolysis	+	-
Resistance to nalidixic acid	S	S
Resistance to cephalothin	R	R

Method 2: MPN UK Health Protection Agency (2007) standard method, based on ISO/DIS 17995-2005

Materials

Culture media

Preston Broth (Oxoid, Basingstoke, UK).
mCCDA (modified Cefoperazone Charcoal Deoxycholate Agar; Oxoid, Basingstoke, UK)
Columbia agar 5% sheep blood plates (ready plates, Becton Dickinson, New jersey, USA)
Mueller Hinton agar 5% sheep blood plates (ready plates, Becton Dickinson, New jersey, USA)

Reagents

- Gram staining regents
- Oxidase reagent (Becton Dickinson, New jersey, USA)
- Hydrogen peroxide (for catalase reaction)
- Hippurate hydrolysis reagents DIETABS (Rosco Diagnostica, Taastrup, Denmark)
- Sensi-Disc cephalothin 30 µg (Becton Dickinson, New jersey, USA)
- Sensi-Disc nalidixic acid 30 µg (Becton Dickinson, New jersey, USA)

- Instagene™ Matrix (Bio-Rad, Hercules, CA, USA)

Procedure

Add 10 mL water to 90 mL of Preston broth (dilution 1/10). Perform serial decimal dilutions (1:9 ml) of the 1/10 dilution in Preston broth to generate the 3-tube MPN. Mix well and place the inoculated broth in an incubator at 37°C ± 1°C for 22 hours ± 2 hours then at 41.5°C ± 1°C for a further 22 hours ± 2 hours, under microaerobic condition (80% N2,10% H2 ,10% CO2). Sub-culture the broth onto mCCDA. Incubate the plates under microaerobic condition in an incubator at 41.5°C for 44 ± 4 hours. After incubation, examine the plates for presumptive *Campylobacter* colonies and confirm their Identity using morphological and biochemical tests, including Gram's staining, oxidase and catalase reactions, the absence of aerobic growth on blood agar plates, hippurate hydrolysis, and susceptibility to cephalothin and nalidixic acid (Table 1).

Confirmation of the identity for method 1 and 2

In order to confirm the identity of the isolates, extract DNA from a colony using the Instagene™ Matrix, following the manufacturer instructions. Sequence the *rpoB* gene using the primers, conditions and criteria described by Korczak *et al*. (2006).

Expression of results

Express the results in MPN *Campylobacter jejuni* and/or *Campylobacter coli* in 100ml, and report the absence as "not detected" in the volume examined.

References

Chenu JW, Pavic A, Cox JM. A novel miniaturized most probable number method for the enumeration of *Campylobacter* spp. from poultry-associated matrices. J Microbiol Methods. 2013; 93(1):12-9.

Health Protection Agency. W8 - Detection of *Campylobacter* species in water London: Health Protection Agency; 2007

ISO 17995:2005 – Water quality – Detection and enumeration of thermotolerant Campylobacter species.

Korczak BM, Stieber R, Emler S, Burnens AP, Frey J, Kuhnert P. Genetic relatedness within the genus *Campylobacter* inferred from *rpoB* sequences. Int J Syst Evol Microbiol. 2006; 56:937-945.

4.2 SOP for quantification of *Campylobacter coli* by Real Time PCR

A 7900HT Fast Real-Time PCR System instrument (Applied Biosystems) is used. Calibration curves are generated by preparing standard DNA dilution series from *C. coli* strain DSMZ 4689^T. The real-time PCR assay will use environmental DNA obtained by the filtration and extraction SOP indicated above or a specific variation of it. A TaqMan-based real-time PCR approach was used based on the TaqMan Universal PCR Master Mix (Applied Biosystems).

1. PCR mixtures:

Reagent	**Quantity (µl)**	**Final concentration into the mix**
PCR grade water	3.4	
Primer prVC-F	1.3	900 nM
Primer prVCM-R	1.3	900 nM
2x GoTaq qPCR Master Mix	10	1x
Template DNA	4	2-10 ng
Total reaction volume	20	

2. Primers:

Target gene	**Primer sequence**	**Amplified fragment (bp)**	**Detectable strains**	**Ref.**
cadF	**CC_cadF-F:** GAGAAATTTTATTTTTATGGTTTAGCTGGT **CC_cadF-R:** ACCTGCTCCATAATGGCCAA **CC_cadF-probe*:** CCTCCACTTTTATTATCAAAAGCGCCTTTAGAAA	103	*C. coli*	1

*The FAM (6-carboxy-fluorescein) is used as fluorescent reporter dye and conjugated to 5' ends of each probe. The quencher dye BBQ is attached at the 3'end of the probes.

3. **Thermal Cycle:**

***Campylobacter coli* quantification by Real Time PCR (prvc-F/R primers)**				
Cycles	**1**			
Taq activation	**Step 1**			
Target temp. (°C)	95			
Incubation time	10 min			
Temp. Transition rate (°C/sec)	20			
Acquisition mode	None			
Cycles	**45**			
Amplification	**Step 1**	**Step 2**		
Target temp. (°C)	95	60		
Incubation time	15 s	1 min		
Temp. Transition rate (°C/sec)	20	20		

4. Detection limit according to calibration curve
Detection limit: $10^2 – 10^3$ CFU/ml sample

Reference

1. Toplak N, Kovač M, Piskernik S, Možina SS, Jeršek B. Detection and quantification of *Campylobacter jejuni* and *Campylobacter* coli using real-time multiplex PCR. J Appl Microbiol. 2012;112(4):752-64.

5.1 SOP for quantification of *Campylobacter jejuni* by Real Time PCR

A 7900HT Fast Real-Time PCR System instrument (Applied Biosystems) is used. Calibration curves are generated by preparing standard DNA dilution series from *C. jejuni* strain DSMZ 4688^T. The real-time PCR assay will use environmental DNA obtained by the filtration and extraction SOP indicated above or a specific variation of it. A TaqMan-based real-time PCR approach was used based on the TaqMan Universal PCR Master Mix (Applied Biosystems).

1. PCR mixtures:

Reagent	**Quantity (µl)**	**Final concentration into the mix**
PCR grade water	3.4	
Primer prVC-F	1.3	900 nM
Primer prVCM-R	1.3	900 nM
2x GoTaq qPCR Master Mix	10	1x
Template DNA	4	2-10 ng
Total reaction volume	20	

2. Primers:

Target gene	**Primer sequence**	**Amplified fragment (bp)**	**Detectable strains**	**Ref.**
hipO	**CJ_hipO-F:** AATGCACAAATTTGCCTTATAAAAGC **CJ_hipOR:** TNCCATTAAAATTCTGACTTGCTAAATA **CJ_hipO_probe*:** ACATACTACTTCTTTATTGCTTG	123	*C. jejuni*	1

*The FAM (6-carboxy-fluorescein) is used as fluorescent reporter dye and conjugated to 5' ends of each probe. The quencher dye BBQ is attached at the 3'end of the probes.

3. Thermal Cycle:

***Campylobacter jejuni* quantification by Real Time PCR (prvc-F/R primers)**				
Cycles	**1**			
Taq activation	**Step 1**			
Target temp. (°C)	95			
Incubation time	10 min			
Temp. Transition rate (°C/sec)	20			
Acquisition mode	None			
Cycles	**45**			
Amplification	**Step 1**	**Step 2**		
Target temp. (°C)	95	60		
Incubation time	15 s	1 min		
Temp. Transition rate (°C/sec)	20	20		

4. Detection limit according to calibration curve

Detection limit: $10^2 – 10^3$ / ml sample

Reference

1. Toplak N, Kovač M, Piskernik S, Možina SS, Jeršek B. Detection and quantification of Campylobacter jejuni and Campylobacter coli using real-time multiplex PCR. J Appl Microbiol. 2012;112(4):752-64.

6.1 SOP for quantification of *Vibrio cholerae* by cultivation based on US-EPA/600/R-10/139

Principle

V. cholerae O1 and O139 can be identified in water samples using selective media and biochemical and serological analysis. An enrichment phase in alkaline peptone water (APW) is performed. APW tubes are streaked onto thiosulfate citrate bile salts sucrose (TCBS) agar and incubated. TCBS plates are examined for large yellow colonies typical of *V. cholerae* O1 and O139. Isolated, presumptive colonies are sub-cultured onto tryptic soy agar (TSA) and submitted to biochemical characterization and serological confirmation. Quantitation of *V. cholerae* O1 and O139 samples is determined using the most probable number (MPN) technique. A filtration based method is also described for sample qualitative analysis, according to Huq et al. (2012).

Materials

Culture media
Thiosulfate Citrate Bile Salts Sucrose (TCBS) Agar (dehydrated medium, Biogenetics); **Tryptic Soy Agar (TSA)** (dehydrated medium, Oxoid); **Alkaline Peptone Water (APW)** (dehydrated medium, Fluka).

Reagents
Phosphate Buffered Saline (PBS) (Monosodium phosphate (NaH_2PO_4) 0.58 g, Disodium phosphate (Na_2HPO_4) 2.50 g, Sodium chloride 8.50 g, Reagent-grade water 1.0 L); **Saline, physiological** (NaCl 0.85% w/v); ***V. cholerae* O1** (Difco™ 210603 or equivalent) **and O139 antisera** (Difco™ 210431 or equivalent); **Biochemical test strip** (bioMérieux API 20E® or equivalent); **Oxidase reagent** (BBL™ DrySlide™ 231746 or equivalent);

Sampling

Uncap pre-sterilized plastic bottle, submerge bottle to fill. Fill to half of volume of bottle, re-cap, shake to rinse, and discard. Repeat 3 to 5 times. Rinse downstream of water sample collection site if possible. Remove the sample bottle from the water. Cap, leaving enough air in the bottle for agitation and mixing. Process samples promptly upon receipt, allowing no more than six hours to elapse from the time of sample collection to sample analysis. Samples should not be placed on ice or held at <10°C. Alternatively, samples can be kept in the dark at ambient air temperature (22◦-25◦C) after collection for up to 24 hr (Huq et al. 2012).

Procedure

Qualitative Sample Analyses: Filter 100 to 1000 ml water through 0.22-µm pore size polycarbonate filter. Add the filter with attached bacteria to a 50-ml centrifuge tube with 12 ml of sterile PBS. Vortex vigorously for 5 min. Spread 100 to 1000 µl of the PBS containing detached bacterial cells onto TCBS. Incubate TCBS plates at 30 to 35°C for 24 ± 2 hr. Add 1ml PBS containing detached bacterial cells to an enrichment flask containing 25 ml APW. Incubate the APW enrichment flask statically for 16 hr at 30 to 35°C. After enrichment in APW, collect surface growth from the enrichment flask with an inoculating loop and streak onto TCBS and incubate the plates as above. After incubation, proceed to section 1.3.

Quantitative Sample Analyses: A multiple-tube assay incorporating differential sample volumes is used to estimate *V. cholerae* O1 and O139 densities in undiluted or diluted samples. Bring APW to

36.0°C ± 1.0°C prior to inoculation. Arrange APW tubes in three rows (5 mL of 5X, 10 mL of 2X, 10 mL of 1X) of five tubes each. Inoculate the first row of tubes (5 mL of 5X APW) with 20 mL of the undiluted sample. Inoculate 10 mL of the undiluted sample into each of the tubes in the second row (10 mL of 2X APW). Inoculate 1 mL from the initial sample into each of the tubes in the third row (10 mL of 1X APW). If high levels of *V. cholerae* are suspected, additional serial dilutions should be used. If low levels of *V. cholerae* are suspected the analysis can be performed with samples concentrated by 0.22 µm filtration as described above. Incubate tubes for 16 hr at 30 to 35°C. Perform primary isolation on all APW tubes exhibiting growth. Obtain inoculum 2 – 5 mm from the top of the tube (pellicle) and streak for isolation onto TCBS plates. Incubate TCBS plates at 30 to 35°C for 24 ± 2 hr. After incubation, proceed to section 1.3. A range of incubation temperatures can be used, as V. cholerae is known to grow between 12 and 42°C. Lower incubation temperatures for longer periods of time may allow for more V. cholerae cells to grow while also allowing for other competitive bacteria to grow as well. Higher incubation temperatures may inhibit growth of some environmentally stressed V. cholerae cells but will also inhibit growth of competitive bacteria species. It may be beneficial to consider testing two incubation temperatures and choosing which yields better results for your sample area of interest.

Isolation of TCBS grown colonies on TSA Plates: Typical *V. cholerae* colonies on TCBS are large and yellow. Streak a single typical colony for isolation onto a TSA plate from each TCBS plate. Incubate the plates at 36.0°C ± 1.0°C for 24 ± 2 hours. Use the TSA plates for serological and biochemical analyses.

Serological and Biochemical Analyses: Well-isolated colonies from each of the TSA plates are analyzed using *V. cholerae* O1 or O139 antisera (as appropriate). Agglutination reaction indicates a positive result. *V. cholerae* O1 is agglutination-positive for O1 antiserum and *V. cholerae* O139 is agglutination-positive for O139 antiserum. Results should be compared with those for positive and negative controls analyzed at the same time. Use a single colony from each TSA plate for biochemical analysis (*Test Strips API 20E® or equivalent)* and for oxidase analysis (*Oxidase Test, BBL™ DrySlide™ 231746 or equivalent)*. APW tubes (MPN and qualitative analysis) may be subjected to real-time polymerase chain reaction (PCR) confirmation in place of biochemical and serological confirmation.

Expression of Results

Quantitative results are obtained by using MPN technique: estimation of bacterial densities is determined based on the number of tubes positive for *V. cholerae* O1 or O139 either by biochemical and serological confirmation or PCR.

Table1. Positive and Negative Result Descriptions and *Vibrio cholerae* O1 and O139 Results

Medium/Test	*V.cholerae* O1/O139 results	Positive control results and description	Negative control results and description
TCBS	Positive	Large, yellow colonies	Pink to red colonies
APW	Positive	Growth at alkaline pH (pellicle or turbidity)	No growth
Oxidase	Positive	Purple to violet color change within 20 sec	Colorless or very light pink color change over time
Biochemical test strip	Consult manufacturers' instruction		
O1/O139 antiserum	Positive	Agglutination	No agglutination

References

EPA 600/R-10/139: Standard Analytical Protocol for *Vibrio cholerae* O1 and O139 in Drinking Water and Surface Water. | October 2010 | www.epa.gov/ord

Huq A, Haley BJ, Taviani E, Chen A, Hasan NH, Colwell RR. Detection, Isolation, and Identification of *Vibrio cholerae* from the Environment. *Current Protocols in Microbiology* 6A.5.1-6A.5.51 2012.

6.2 SOP for quantification *Vibrio cholerae* by Real Time PCR

A Light Cycler 1.5 instrument (Roche) is used. Calibration curves are generated by preparing standard DNA dilution series from *V. cholerae* N16961 strain (ATCC 39315). The real-time PCR assay uses environmental DNA obtained by the filtration and extraction SOP indicated above or a specific variation of it. A SYBR green-based real-time PCR approach was applied using the Go Taq qPCR (Promega) chemistry.

1. **PCR mixtures:**

Reagent	**Quantity (µl)**	**Final concentration in the mix**
PCR grade water	2.4	
Primer prVC-F	1.3	0.65 µM
Primer prVCM-R	1.3	0.65 µM
GoTaqqPCR Master Mix (2x)	10	1x
Template DNA	5	2-10 ng environmental DNA
Total reaction volume	20	

2. **Primers:**

Target gene	**Primer sequence**	**Amplified fragment(bp)**	**Detectable strains**	**Ref.**
16S-23S rDNA ISR	**prVC-F**5'- TTAAGCGTTTTCGCTGAGAATG - 3' **prVCM-R**5' - AGTCACTTAACCATACAACCCG - 3'	295	*V. cholerae*	1

3. **Thermal Cycle:**

***V. cholerae* quantification by Real Time PCR (prvc-F/R primers)**				
Cycles	**1**			
Taq activation	**Step 1**			
Target temp. (°C)	95			
Incubation time (min)	2			
Temp. Transition rate (°C/sec)	20			
Acquisition mode	None			
Cycles	**45**			
Amplification	**Step 1**	**Step 2**	**Step 3**	
Target temp. (°C)	95	58	72	
Incubation time (sec)	15	30	45	
Temp. Transition rate (°C/sec)	20	20	20	
Acquisition mode	None	None	Single	
Cycles	**1**			
Melting curve and cooling	**Step 1**	**Step 2**	**Step 3**	**Step 4**
Target temp. (°C)	72	55	98	40
Incubation time (sec)	30	60	1	5
Temp. Transition rate (°C/sec)	20	20	0.5	20
Acquisition mode	None	None	Step	None

4. Detection limit according to calibration curve

Detection limit: 45 gene copies per assay.

Reference: 1. Chun J, Huq A, Colwell RR. Analysis of 16S-23S rRNA intergenic spacer regions of *Vibrio cholerae* and *Vibrio mimicus*. Appl Environ Microbiol. 1999; 65(5):2202-8.

7.1 SOP for quantification of *Pseudomonas aeruginosa* by cultivation based on ISO 16266:2006

Principle

Bacteria, including *P. aeruginosa*, in the water sample are concentrated by membrane filtration. The filter is subsequently transferred onto a plate of agar medium selective for *P. aeruginosa* and incubated. ISO 16266 provides a standardized procedure to isolate and quantify P. aeruginosa in water. This procedure is performed by membrane filtration on Cetrimide agar medium with nalidixic acid, a highly selective medium which enhances the production of pyocyanin and pyoverdin, two characteristic pigments of *P. aeruginosa*. After this presumptive identification, the isolates are tested on two media: acetamide broth, to evaluate its capacity to produce ammonium from acetamide; and King B agar, which improves the production of pyoverdin. Moreover, the Oxidase test is also performed. In addition, ISO 16266 recommends complementary assays at distinct growing temperatures (4 °C and 42 °C) on nutrient agar in order to confirm presumptive *P. aeruginosa* isolates.

Materials

Culture media

P. aeruginosa-selective **Cetrimide agar medium with nalidixic acid (CN) agar** contains the following (g/l): Gelatin Peptone 16.00, Cetrimide 0.20, Hydrolyzed Casein 10.00, Nalidixic Acid 0.015, Anhydrous Potassium Sulfate 10.00, Bacteriological Agar 13.00, Anhydrous Magnesium Chloride 1.40. Final pH is adjusted to 7.1 ± 0.2 at 25ºC. **Nutrient agar** contains the following: Peptone 5.0 g meat extrakt 1.0 g; yeast extrakt 2.0 ;,NaCl 5,0 g and agar 15,0 g.

Reagents

- Oxidase reagent (Becton Dickinson, New Jersey, USA)
- King's B agar medium (Sigma-Aldrich, St. Louis, USA)
- Acetamide solution (Roth, Karlsruhe, Germany)
- Nessler Reagent (Sigma-Aldrich, St. Louis, USA)

Sampling

The volume of sample collected depends upon the nature of water system and the purpose of the examination. Details of the origin and volume of the sample, the temperature of the water at the time of sampling as well as the presence and nature of any biocide shall be recorded and given to the laboratory with the samples as an aid to examination. For both safety and analytical reasons it is not advisable to examine samples of unknown origin or of cooling and process waters unless they are accompanied by adequate information that will include information about the chemical additives used in or likely contaminants that are present as a result of the process.

Samples of water (generally 1 litre) shall be collected in glass, polyethylene or similar containers. If used previously, they shall be cleaned, rinsed with distilled or mains tap water and autoclaved at (121 ± 3) °C for 15 min. Deliver the samples to the laboratory as soon as possible preferably within 1 d but not more than 2 d. If analysed the same working day, transport samples at ambient temperature protected from sunlight. Otherwise cool samples, ideally (5 ± 3) °C, during transport. It is recommended that the time interval between collection of the sample and its filtration in these circumstances is ideally within 24 h and should not exceed 2 d.

Procedure

Filter 100ml of drinking water sample onto a sterile black membrane filter with a counting grid (47 mm diameter, 0.45 µm pore size, cellulose acetate, Millipore). *P. aeruginosa*-selective CN agar is used for the identification of *P. aeruginosa* by the membrane filtration technique based on pyocyanin production. It is a modification of Pseudomonas P Agar (King A Medium - Cat. 1531). This medium is recommended by UNE-EN 12780 and EN ISO 16266. The membrane is placed on dishes containing the medium and it is incubated at 36 ± 2°C for 40 - 48 hours in containers that prevent moisture loss. The colonies are examined to verify their growth after 20 - 24 and 40 - 48 hours. The colonies that produce a green-blue color and fluoresce under UV light are considered presumptive *Pseudomonas aeruginosa*. Presumptive colonies must be confirmed with the biochemical tests and should be transferred as many as possible to nutrient agar and are incubated for 22±2h at 36±2°C. Reddish brown colonies should be texted for oxidase. Oxidase-positive reddish brown should be sub-cultured on King's B medium for up to 5 d at 36±2°C and checked for fluoresce under UV light. To assess ammonia production from acetamide, acetamide solution is inoculated from the subcultures on nutrient media and incubated for 22±2h at 36±2°C. 1- to 2 drops of Nessler reagents are added to check for the production of ammonia by the development of a yellow to red colour.

A summary of the biochemical features to be tested and their interpretation are given in the following table.

Description of colony on CN agar	**Formation of ammonia from acetamide**	**Oxidase positive**	**Fluorescence on King's B agar**	**Confirmation of *P. aeruginosa***
Blue-green	NA	NA	NA	yes
Fluorescing (not blue green)	+	NA	NA	yes
Reddish brown	+	+	+	yes
Different colors	NA	NA	NA	no

NA = not assessed

Reference

ISO 16266:2006 Water quality - Detection and enumeration of Pseudomonas aeruginosa -- Method by membrane filtration

7.2 SOP for quantification of *Pseudomonas aeruginosa* by Real Time PCR

A Light Cycler 480 instrument (Roche Applied Science) is used. Calibration curves are generated by preparing standard DNA dilution series from *Pseudomonas aeruginosa* (DSM 50071). The real-time PCR assay uses environmental DNA obtained by the filtration and extraction SOP indicated above or a specific variation of it. A SYBR Green based real-time PCR approach was applied using the Roche Applied Science chemistry.

1. PCR mixtures:

Reagent	**Volume (µl)**	**Final concentration in the mix**
PCR grade water	3	
Primer Pa722-F	1	500 nM
Primer Pa899-R	1	500 nM
LC480 SYBR Green Master Mix (2x)	10	1x
Template DNA	5	
Total reaction volume	20	

2. Primers:

Target gene	**Primer sequence**	**Amplified fragment (bp)**	**Detectable strains**	**Ref.**
gyrB	**Pa722-F** 5'- GGCGTGGGTGTGGAAGTC - 3' **Pa899-R** 5' – TGGTGGCGATCTTGAACTTCTT- 3'	190	*P. aeruginosa*	1

3. Thermal Cycle:

***Pseudomonas aeruginosa* quantification by Real Time PCR (Pa722-F, Pa899-R)**				
Cycles	**1**			
Taq activation	**Step 1**			
Target temp. (°C)	95			
Incubation time (min)	5			
Temp. Transition rate (°C/sec)	4.4			
Acquisition mode	None			
Cycles	**40**			
Amplification	**Step 1**	**Step 2**	**Step 3**	
Target temp. (°C)	95	60	72	
Incubation time (sec)	10	30	30	
Temp. Transition rate (°C/sec)	4.4	2.2	4.4	
Acquisition mode	None	None	Single	
Cycles	**1**			
Melting curve and cooling	**Step 1**	**Step 2**	**Step 3**	**Step 4**
Target temp. (°C)	95	65	98	40
Incubation time (sec)	10	60	-	10
Temp. Transition rate (°C/sec)	4.4	2.2	0.08	1.5
Acquisition mode	None	None	continuous	None

4. Detection limit according to calibration curve

Detection limit: 330 copies of gyr B per assay.

Reference: 1. Lee CS, Wetzel K, Buckley T, Wozniak D, Lee J (2011) Rapid and sensitive detection of *Pseudomonas aeruginosa* in chlorinated water and aerosols targeting gyrB gene using real-time PCR. J Appl Microbi 111(4):893–903

8.1 SOP for quantification of *Salmonella enterica* subsp. *enterica*, serovar Typhi by cultivation

Principle

This SOP is for the identification, confirmation, and quantitation of *Salmonella enterica* subspecies *enterica* serotype Typhi (referred to as "*S.* Typhi" in the following) in drinking water samples, using selective and non-selective media followed by biochemical and serological confirmation. Bacterial densities can be estimated using the most probable number (MPN) approach.

Materials

Culture media
In general, commercially prepared media are recommended. Universal Pre-enrichment **(UP)** Broth (Difco™ 223510 or equivalent), Bismuth Sulfite (**BS**) Agar (Difco™ 273300 or equivalent), Miller-Mallinson (**MM**) Agar (ready plates, NorthEast Laboratories # P1424), Tryptic Soy Agar (TSA) (Difco™ 236950 or equivalent) and Selenite Cystine Broth (**SCB**) (Difco™ 268740 or equivalent).

Reagents

- *Salmonella* Vi antiserum (BD™ 228271 or equivalent)
- Biochemical test strip (bioMérieux API 20E® or equivalent)
- Oxidase reagent (BD™ DrySlide™ 231746 or equivalent)

Procedure

For quantitative results, samples are analyzed as received. Samples are analyzed using a 15-tube MPN. Inoculated UP broth tubes are incubated at 35.0°C ± 0.5°C for 24 ± 2 hours. One mL of each UP broth culture (MPN and qualitative analyses tubes) with positive growth (turbidity) is transferred to selenite cystine broth (SCB). Tubes are incubated at 35.0°C ± 0.5°C for 18 ± 2 hours. Tubes with growth are streaked onto bismuth sulfite (BS) and Miller-Mallinson (MM) agars. Plates are incubated at 35.0°C ± 0.5°C for 24 – 48 hours. Isolated, typical colonies are sub-cultured onto tryptic soy agar (TSA) and submitted to serological and biochemical confirmation. Serological typing is by agglutination using Vi antiserum, followed by biochemical characterization using commercially available test strips (e.g., API 20E® or equivalent) or with a group of selected individual biochemical tests. UP broth tubes (MPN and qualitative analysis) exhibiting growth (turbidity) or growth from agar plates (BS or MM), may be confirmed by real-time polymerase chain reaction (PCR) in place of serological and biochemical confirmation.

Quantitative Sample Analyses

A multiple-tube assay incorporating differential sample volumes is used to estimate *S.* Typhi densities in undiluted or diluted samples. If low levels of *S.* Typhi are suspected, larger sample volumes (20.0 mL of original sample) should be used to inoculate the first row of tubes in the series. If high levels of *S.* Typhi are suspected, additional serial dilutions should be used. A minimum sample volume of 156 mL is required if 20 mL volumes are used to inoculate the first row of tubes.

Sample inoculation: Arrange UP broth tubes in three rows (10 mL of 3X, 5 mL of 3X, and 10 mL of 1X) of five tubes each. Inoculate the first row of tubes (10 mL of 3X UP broth) with 20 mL of the undiluted sample. Inoculate 10 mL of the undiluted sample into each of the tubes in the second row (5 mL of 3X UP broth). Inoculate 1 mL from the initial sample into each of the tubes in the third row (10 mL of 1 UP broth). Samples may require serial dilution prior to inoculation due to high levels of *S.*

Typhi. If analyzing serially diluted samples, 1.0 mL of each dilution will be used to inoculate each tube of 1X UP broth, as appropriate. Incubate tubes at 35.0°C ± 0.5°C for 24 ± 2 hours. Proceed to Section SCB cultivation for selective enrichment of *S.* Typhi.

Selenite Cystine Broth Culture: For each tube with growth, gently swirl the tube to mix and transfer 1.0 mL to a set of tubes with 10 mL of SCB. Incubate at 35.0°C ± 0.5°C for 18± 2 hours. Proceed with isolation on BS and MM agars.

Isolation on Bismuth Sulfite and Miller-Mallinson Agar Plates: Select all SCB tubes with growth (qualitative and quantitative) and streak for isolation onto BS and MM plates using a sterile inoculation loop (20 µL). Incubate plates for 24 – 48 hours at 35.0°C ± 0.5°C. Typical *S.* Typhi colonies are green-black with metallic sheen on BS agar and black on MM agar.

Isolation on Tryptic Soy Agar Plates: Examine plates at 24 ± 2 hours. For each BS and MM plate with typical colonies, streak a single typical colony onto TSA and incubate at 35.0°C ± 0.5°C. If no typical colonies are observed, continue incubation at 35.0°C ± 0.5°C for a total of 48 ± 3 hours. Re-examine plates, streak typical colonies onto TSA, and incubate at 35.0°C ± 0.5°C.

Seal the BS and MM plates with Parafilm® and store at <10°C and above freezing for use as backup plates. Use the TSA plates for serological and biochemical analyses.

Summary of cultivation and test results:

Medium/Test	*S.* Typhi Results	Positive Control Result and Description	Negative Control Result and Description
SCB	Positive	growth	No growth
BS	Positive	Green-black colonies with metallic sheen	No growth
MM	Positive	Black colonies	No growth
Oxidase	Negative	Purple to violet color change within 20 seconds	Colorless or very light pink color

Reference

EPA 600/R-10/133 Standard Analytical Protocol for *Salmonella* Typhi in Drinking Water | October 2010 | www.epa.gov/ord

8.2 SOP for quantification of *Salmonella enterica* subsp. *enterica*, serovar Typhi by Real Time PCR

A BIO-RAD Chromo 4 Real Time PCR System is used. Calibration curves are generated by preparing standard DNA dilution series from *Salmonella enterica* subsp. *enterica*, serovar Typhi (CECT 409). The real-time PCR assay uses environmental DNA obtained by the filtration and extraction SOP indicated above or a specific variation of it. A TaqMan-based real-time PCR approach was used with a homemade Master Mix (QIAGEN components).

1. PCR mixtures:

Reagent	Quantity (µl)	Final concentration into the mix
PCR grade water	11.3	
Primer pfim-**forward** 10µM	1	0.4 µM
Primer pfim-**reverse** 10µM	1	0.4 µM
pfim-probe 5µM	0.75	0.15 µM
TaqMan Master Mix 10x	2.5	1x
- MgCl2 25mM	3.5	5 mM (1.5 mM from buffer and add 3.5 mM more)
- dNTPs	-	0.2 mM each
- Hot Start Taq DNA pol. (QIAGEN)	-	1 U
Template DNA	5	From 1 to 10^5 copies/µl
Total reaction volume	25	

2. Primers:

Target gene	Primers and probe sequences	Amplified fragment(bp)	Detectable strains	Ref.
pfim (putative fimbrial-like adhesin protein)	pfim-**forward** cgcgaagtcagagtcgacatag pfim-**reverse** aagacctcaacgccgatcac pfim-**probe** catttgttctggagcaggctgacgg	175	*S.enterica* serovar Typhi	1

*The FAM (6-carboxy-fluorescein) is used as fluorescent reporter dye and conjugated to 5' ends of each probe. The quencher dye TAMRA is attached at the 3'end of the probes.

3. Thermal cycle

***Salmonella enterica* subsp. *enterica* serovar Typhi quantification by Real Time PCR (*pfim* gene)**				
Cycles	**1**			
Taq activation	**Step 1**			
Target temp. (°C)	95			
Incubation time (min)	15			
Temp. Transition rate (°C/sec)	3			
Acquisition mode	None			
Cycles	**45**			
Amplification	**Step 1**	**Step 2**	**Step 3**	
Target temp. (°C)	95	60	72	
Incubation time (sec)	30	30	30	
Temp. Transition rate (°C/sec)	3	3	3	
Acquisition mode	None	None	Single	

4. Detection limit according to calibration curve

Detection limit: 50 gene copies per assay.

Reference

1. Tran Vu Thieu Nga, Abhilasha Karkey, Sabina Dongol, Hang Nguyen Thuy, Sarah Dunstan, Kathryn Holt, Le Thi Phuong Tu, James I Campbell, Tran Thuy Chau, Nguyen Van Vinh Chau, Amit Arjyal, Samir Koirala, Buddha Basnyat, Christiane Dolecek, Jeremy Farrar and Stephen Baker. *The sensitivity of real-time PCR amplification targeting invasive Salmonella serovars in biological specimens. BMC Infectious Diseases* 2010, **10**:125

9.1 SOP for quantification of *Escherichia coli by cultivation* based on ISO 9308-1:2000 and ISO 9308-1:2000/Cor.1:2007

Principle

There are many methods for enumerating *E. coli* based either on membrane filtration or liquid enrichment broth and MPN. Several methods use fluorogenic reagents like MUG or other chromogenic substrates. The reported protocol is based on ISO 9308-1:2000 Part 1. The method is based on membrane filtration and consists of two parts, the reference Standard Test and the optional Rapid Test, which can be performed in parallel. The Standard Test includes incubation of the membrane on a selective medium with subsequent further biochemical characterization of the typical lactose positive colonies, leading to the detection and enumeration of coliform bacteria and *E. coli* within 2 d to 3 d. The Rapid Test consists of two incubation steps allowing the detection and enumeration of *E. coli* within 21 ±3 h. If both tests are performed in parallel, the final result for *E. coli* shall be the higher of the two. The Standard and Rapid Test can be applied to waters in which suspended matter or background flora does not interfere with filtration, culture and counting

Materials

Culture media

Lactose TTC agar with sodium heptadecylsulfate

Basal medium: (Lactose 20 g, Peptone 10 g, Yeast extract 6 g, Meat extract 5 g, Bromothymol blue 0,05 g, Agar (in powder or flake form) 15 g to 25 g, DW 1000 ml); TTC solution: (2,3,5-Triphenyltetrazolium chloride (TTC) 0,05 g, DW 100 ml); Sodium heptadecylsulfate solution: (Sodium heptadecylsulfate 0,2 g, DW 100 ml); Complete medium (Basal medium 100 ml, TTC solution 5 ml, Sodium heptadecylsulfate solution 5 ml)

Tryptophan broth (Tryptic digest of casein 10 g, L-Tryptophan 1g, Sodium chloride 5 g, DW 1000 ml)

Tryptone Soy Agar (TSA) (Tryptic digest of casein 15 g, Soy peptone 5 g, Sodium chloride 5 g, Agar (in powder or flake form) 15 g to 25 g, DW to 1 000 ml

Tryptone Bile Agar (TBA) (Tryptone 20 g, Bile salts 1,5 g, Agar (in powder or flake form) 15 g to 25 g, DW to 1 000 ml)

MUG Broth (Tryptone 15 g, Lactose 5 g, Sodium chloride 5 g, Bile salts Mixture 1.5 g, Dipotassium Phosphate 4 g, Monopotassium Phosphate 1.5 g, 4-methyl-β-D-umbelliferyl glucuronide [MUG] 0.1g, DW to 1000 ml)

Reagents

Kovacs' Reagent for indole test, Standard Test (*p*-Dimethylaminobenzaldehyde 5 g, Amyl or butyl alcohol (free from organic bases) 75 ml, Hydrochloric acid 25 ml). Dissolve the aldehyde in the alcohol. Add the concentrated acid with care. Protect from light and store at 5 ± 3°C. Carry out the preparation work in an exhaust protection cabinet. Use protective gloves and avoid skin contact with *p*-dimethylaminobenzaldehyde.

Indole reagent, Rapid Test (*p*-Dimethylaminobenzaldehyde 0,5 g, Hydrochloric acid 100 ml). Dissolve the *p*-dimethylaminobenzaldehyde in the hydrochloric acid. Carry out the preparation work in an exhaust protection cabinet. Use protective gloves and avoid skin contact with *p*-dimethylaminobenzaldehyde. Store the reagent in a non-translucent flask at 5 ± 3°C. The reagent should be light yellow in colour and shall not be used if the colour becomes brownish yellow.

Oxidase reagent (Tetramethyl-*p*-phenylenediamine hydrochloride 0,1 g, DW 10 ml). This reagent is not stable and shall be freshly prepared each time it is needed. Tetramethyl-*p*-phenylenediamine dihydrochloride is carcinogenic. The preparation work must be done in a fume cupboard. Use protective gloves and avoid skin contact.

Sampling

Water samples are collected in sterile glass, polyethylene or similar containers. Ice or refrigerate water samples at a temperature of 1-4°C during transit to the laboratory. Analyze samples as soon as possible after collection. Drinking water samples should be analyzed within 30 h of collection. Do not hold source water samples longer than 6 h between collection and initiation of analyses, and the analyses should be complete within 8 h of sample collection.

Procedure

Filter 100 ml (or higher volumes) of the sample to be studied using a membrane filter composed of cellulose esters, usually about 47 mm or 50 mm in diameter, with filtration characteristics equivalent to a rated nominal pore diameter of 0.45 µm and preferably with grids.

Standard Test: Place the filter on the Lactose TTC agar plate ensuring that no air is trapped underneath and incubate at 36 ± 2°C for 21 ± 3 h. Examine the membranes and count as lactose-positive bacteria all characteristic colonies, irrespective of size, which show a yellow colour development in the medium under the membrane. For oxidase and indole tests, subculture preferentially all, or a representative number (at least ten), of the characteristic colonies obtained onto nonselective agar and in tryptophan broth, respectively. Incubate the nonselective agar at 36 ± 2°C for 21 ± 2 h and carry out an oxidase test as follows: place two to three drops of freshly prepared oxidase reagent on a filter paper; with a glass rod, wooden applicator stick, plastics or platinum (not Nichrome) wire loop, smear part of the colony on the prepared filter paper and regard the appearance of a deep blue-purple colour within 30s as a positive reaction. Incubate the tryptophan broth tube at 44.0±0.5°C for 21 ± 3 h and examine for the production of indole by adding 0.2 ml to 0.3 ml of Kovacs' reagent. Development of a cherry-red colour at the surface of the broth confirms the production of indole. Count all colonies giving a negative oxidase reaction as coliform bacteria. Count all colonies giving a negative oxidase and a positive indole reaction as *E. coli*. Some strains of *Klebsiella oxytoca* give a positive indole reaction. To prevent these false-positive results, 9308-1:2000/Cor.1:2007 recommends to carry out β-glucuronidase test in addition (inoculum in MUG broth and incubation at 44.5± 0.2°C for 24 h; observance of bright blue fluorescence when subjected to 366 nm UV light indicates a positive test for *E. coli*).

Rapid test: After filtration, place the membrane on TSA medium and incubate at 36 ± 2°C for 4 - 5 h. Thereafter, place the membrane on TBA medium and incubate at 44.0± 0.5°C for 19 h to 20 h. The two agar media can be combined into one double-layer plate. In that case, place the membrane on a freshly prepared double layer plate consisting of TSA and TBA and incubate at 36±2°C for 4 h to 5 h followed by incubation at 44.0±0.5°C for 19 - 20 h. After incubation, place the membrane on a filter pad saturated with indole reagent and irradiate with an ultraviolet lamp for 10 - 30 min depending on the colour development. All red colonies on the membrane filter are counted as *E. coli*.

Expression of results

From the numbers of characteristic colonies counted on the membrane and taking into account the results of the confirmatory tests performed, calculate the numbers of *E. coli*, coliform bacteria and, if necessary, lactose positive bacteria present in 100 ml of the sample. In case both tests (Standard Test and Rapid Test) are used in parallel, the final result is the higher of the two.

Reference

ISO 9308-1 and ISO 9308-1:2000/Cor.1:2007 (Technical corrigendum 1) Water quality — Detection and enumeration of *Escherichia coli* and coliform bacteria —Part 1: Membrane filtration method

9.2 SOP for quantification of *Escherichia coli* by Real Time PCR using the 16S rRNA gene

A Light Cycler 1.5 instrument (Roche) is used. Calibration curves are generated by preparing standard DNA dilution series from *E. coli* strain K12 (ATCC 47076). The real-time PCR assay will use environmental DNA obtained by the filtration and extraction SOP indicated above or a specific variation of it. A SyBr green-based real-time PCR approach was used based on the Go Taq qPCR (Promega) chemistry.

1. PCR mixtures:

Reagent	**Quantity (µl)**	**Final concentration into the mix**
PCR grade water	4.6	
Primer F395	0.2	0.1 µM
Primer R490	0.2	0.1 µM
2x GoTaq qPCR Master Mix	10	1x
Template DNA	5	
Total reaction volume	20	

2. Primers:

Target gene	**Primer sequence**	**Amplified fragment (bp)**	**Detectable strains**	**Reference**
16S rDNA	**F395:** 5'-CATGCCGCGTGTATGAAGAA-3' **R490:** 5'-CGGGTAACGTCAATGAGCAAA-3'	95	*E. coli*	1, 2

3. Thermal Cycle:

***E. coli* quantification by Real Time PCR (F395/R490 primers)**				
Cycles	**1**			
Taq activation	**Step 1**			
Target temp. (°C)	95			
Incubation time (min)	2			
Temp. Transition rate (°C/sec)	20			
Acquisition mode	None			
Cycles	**40**			
Amplification	**Step 1**	**Step 2**	**Step 3**	
Target temp. (°C)	95	60	60	
Incubation time (sec)	15	30	30	
Temp. Transition rate (°C/sec)	20	20	20	
Acquisition mode	None	None	Single	
Cycles	**1**			
Melting curve and cooling	**Step 1**	**Step 2**	**Step 3**	**Step 4**
Target temp. (°C)	72	55	98	40
Incubation time (sec)	30	30	1	5
Temp. Transition rate (°C/sec)	20	20	0.5	20
Acquisition mode	None	None	Step	None

4. Detection limit according to calibration curve

Detection limit: 140 gene copies per assay.

References

1. **Penders, J., Vink, C., Driessen, C., London, N., Thiis, C., Sobberingh, E.E.,** 2005. Quantification of *Bifidobacterium spp*., *Escherichia coli* and *Clostridium difficile* in fecal samples of breast-fed and formula-fed infants by Real Time PCR. FEMS Microbiol. Ecol. 24, 141–147.
2. **Luna GM, Dell'Anno A, Pietrangeli B, Danovaro R.** A new molecular approach based on qPCR for the quantification of fecal bacteria in contaminated marine sediments. Journal of Biotechnology 157 (2012) 446–453.

9.3 SOP for quantification of pathogenic *Eschrichia coli* O157:H7 by cultivation based on US- EPA/600/R-10/056.

Principle

E. coli O157:H7 can be identified in a variety of water samples using selective media, and biochemical and serological analysis. For qualitative results, samples are diluted in double-strength modified buffered peptone water (mBPW) and incubated. For quantitative results, samples are analyzed using the most probable number (MPN) technique in mBPW and incubated. Broth cultures (MPN and qualitative analyses tubes) with positive growth are submitted to immunomagnetic separation (IMS) and sub-cultured onto tellurite cefixime sorbitol MacConkey (TC-SMAC) and Rainbow® agars. TC-SMAC plates are examined for colourless/gray colonies typical of *E. coli* O157:H7. On Rainbow® plates, typical colonies are black/gray. Isolated typical colonies are submitted to serological confirmation and biochemical characterization. Quantification of *E. coli* O157:H7 is determined using the MPN technique.

Materials

Culture media
Tryptic Soy Agar (TSA)(dehydrated medium, BBL™211043), **Tellurite Cefixime Sorbitol MacConkey Agar (TC-SMAC)** (dehydrated medium, Oxoid CM0813) with cefixime and potassium tellurite (CT) supplement (Oxoid SR172), **Rainbow® Agar** (dehydrated medium, Biolog 80101); **Modified Buffered Peptone Water (mBPW)1X** (Peptone 10.0 g, Sodium chloride 5.0 g, Disodium phosphate (Na_2HPO_4) 3.6 g, Monopotassium phosphate (KH_2PO_4) 1.5 g, Casamino acids 5.0 g, Yeast extract 6.0 g, Lactose 10.0 g, Pyruvate 2.0 g, Reagent-grade water 1.0 L).

Reagents
Phosphate buffered dilution water (add 1.25 mL stock phosphate buffer solution [Monopotassium phosphate (KH_2PO_4) 68g/L] and 5.0 mL stock magnesium chloride solution [Magnesium chloride hexahydrate ($MgCl_2*6H_2O$) 81.1g/L] to 1.0 L of reagent-grade water); **Phosphate Buffered Saline (PBS)** (Monosodium phosphate (NaH_2PO_4) 0.58 g, Disodium phosphate (Na_2HPO_4) 2.50 g, Sodium chloride 8.50 g, Reagent-grade water 1.0 L); **IMS wash buffer** (50 µL Tween® 20, 100 mL PBS); **Saline, physiological** (NaCl 0.85% w/v), ***E. coli* O157 IMS beads** (Invitrogen™ 71004 or equivalent), ***E. coli* O157 latex agglutination reagent** (Oxoid DR0620M or equivalent), ***E. coli* H7 latex agglutination reagent** (Wellcolex® *E. coli* O157:H7 Rapid Latex Agglutination Test or equivalent), **Biochemical test strip** (bioMérieux API 20E® or equivalent), **Oxidase reagent** (BD™ DrySlide™ 231746 or equivalent).

Sampling

Process samples promptly upon receipt, allowing no more than six hours to elapse from the time of sample collection to the start of sample processing, which should be completed within two hours.

Procedure

Qualitative Sample Analysis: Add a sample volume to an equal volume of double-strength mBPW. Incubate at 36.0°C ± 1.0°C for 2.0 – 2.5 hours. Transfer samples to 42.0°C ± 0.5°C and continue incubation, for a total of 20 – 24 hours. After incubation, proceed to Section 1.3 for selective separation and concentration of *E. coli* O157:H7.

Quantitative Sample Analysis: A multiple-tube assay incorporating differential sample volumes is used to estimate *E. coli* O157:H7 densities in undiluted or diluted samples. Arrange mBPW tubes in three rows (5 mL of 5X, 10 mL of 2X, and 10 mL of 1X) of five tubes each. Inoculate the first row of tubes (5 mL of 5X mBPW) with 20 mL of the undiluted sample. Inoculate 10 mL of the undiluted sample into each of the tubes in the second row (5 mL of 3X mBPW). Inoculate 1 mL from the initial sample into each of the tubes in the third row (1X mBPW). If low levels of *E. coli* O157:H7 are suspected, the analysis could be performed with either larger water samples or samples concentrated by 0.22 µm filtration. If high levels of *E. coli* O157:H7 are suspected, additional serial dilutions should be used. Incubate tubes at 36.0°C ± 1.0°C for 2.0 – 2.5 hours. Transfer samples to 42.0°C ± 0.5°C and continue incubation, for a total of 20 – 24 hours. After incubation, proceed to Section 1.3 for selective separation and concentration of *E. coli* O157:H7.

Immunomagnetic Separation and Concentration: From each tube with growth, conduct IMS as follows: gently swirl contents of mBPW tubes to mix, remove 1.0 mL of mBPW culture, add to 20 µL of re-suspended *E. coli* O157:H7 beads. Mix for 10 minutes at room temperature. Place tubes in magnetic holder, inverting tubes several times. Allow beads to settle for 3 minutes. Aspirate liquid and discard. Re-suspend beads in 1.0 mL IMS wash buffer. Place tubes in magnetic holder and allow beads to settle for 3 minutes. Aspirate wash buffer and re-suspend the beads in 100 µL of IMS buffer.

Isolation on Selective Agars: Spread a 50-µL aliquot of the beads over one-third of the plate of Rainbow® agar and streak for isolation on the remaining portion of the plate. Repeat this procedure with the remaining 50 µL onto TC-SMAC. Incubate Rainbow® and TC-SMAC plates for 18 – 24 hours at 42.0°C ± 0.5°C. On TC-SMAC, typical *E. coli* O157:H7 colonies are colorless (2-3 mm in diameter). On Rainbow® agar, typical E. coli O157:H7 colonies are black/gray.

Serological Analyses: Well-isolated typical colonies from each of the positive TC-SMAC and/or Rainbow® plates are selected for serological confirmation using *E. coli* O157 latex agglutination kit and H7 latex agglutination reagent.

Isolation on TSA Plates: Streak one O157 serological-positive isolate onto a TSA plate from each serology-positive TC-SMAC or Rainbow® plate. For spiked samples with no background, a single serological-positive isolate from each dilution should be streaked onto a TSA plate. Incubate the plates at 36.0°C ± 1.0°C for 24 ± 2 hours.

Biochemical & molecular Analyses: Use a single, isolated, large colony from each TSA plate for biochemical test strip (API 20E® or equivalent) and oxidase analysis (BD™ DrySlide™ 231746 or equivalent). Broth cultures (MPN and qualitative analysis tubes) concentrated by IMS may be subjected to real-time polymerase chain reaction (PCR) confirmation in place of biochemical and serological confirmation.

Expression of results: Quantitative results are obtained by using MPN technique: estimation of bacterial densities may be determined based on the number of tubes positive for *E.coli* O157:H7 by biochemical and serological confirmation or PCR.

Table 1. Positive and Negative Control, and *E. coli* O157:H7 Results

Medium/Test	*E. coli* O157:H7 results	Positive control results and description	Negative control results and description
TC-SMAC	Positive	Colorless colonies (sorbitol not fermented)	Pink to red colonies (sorbitol fermented)
Rainbow	Positive	Black to gray colonies (glucoronidase-negative)	Pink to magenta colonies (glucoronidase-positive)
Oxidase	Negative	Purple to violet color change within 20 sec	Colorless or very light pink color change over time
Biochemical test strip	Consult manufacturers' instruction		
O157 antiserum	Positive	Agglutination	No agglutination

Reference: EPA 600/R-10/056 Standard Analytical Protocol for *E. coli* O157:H7 in Water. | September 2010 | www.epa.gov/ord

9.4 SOP for quantification of pathogenic *Escherichia coli* by Real Time PCR

A Light Cycler 1.5 instrument (Roche) is used. Calibration curves are generated by preparing standard DNA dilution series from *E.coli* strain O157:H7 (CECT 4076). The real-time PCR assay will use environmental DNA obtained by the filtration and extraction SOP indicated above or a specific variation of it. A TaqMan-based real-time PCR approach was used based on the Light Cycler TaqMan Master Mix (Roche) chemistry. The reported protocol is modified from the multiplex protocol described in the reference (1) in order to perform three different reactions, each with a single set of primers.

1. PCR mixtures:

***stx*1 primers**

Reagent	**Quantity (µl)**	**Final concentration into the mix**
PCR grade water	9.4	
Primer **stx1-forward**	0.6	0.3 µM
Primer **stx1-reverse**	0.6	0.3 µM
stx1-probe	0.4	0.2 µM
TaqMan Master Mix 5x	4	1x
Template DNA	5	2-10 ng
Total reaction volume	20	

***stx*2 primers**

Reagent	**Quantity (µl)**	**Final concentration into the mix**
PCR grade water	4.6	
Primer **stx2-forward**	3	1.5 µM
Primer **stx2-reverse**	3	1.5 µM
stx2-probe	0.4	0.2 µM
TaqMan Master Mix 5x	4	1x
Template DNA	5	2-10 ng
Total reaction volume	20	

***eae*$_{(O157:H7)}$ primers**

Reagent	**Quantity (µl)**	**Final concentration into the mix**
PCR grade water	9.4	
Primer **eae-forward**	0.6	0.3 µM
Primer **eae-reverse**	0.6	0.3 µM
eae-probe	0.4	0.2 µM
TaqMan Master Mix 5x	4	1x
Template DNA	5	2-10 ng
Total reaction volume	20	

2. Primers:

Target gene	Primers and probe sequences	Amplified Frag. (bp)	Detectable strains	Ref.
stx1	**stx1-forward** GACTGCAAAGACGTATGTAGATTCG **stx1-reverse** ATCTATCCCTCTGACATCAACTGC ***stx1-probe** TGAATGTCATTCGCTCTGCAATAGGTACTC	150	Shiga-toxin1 producing *E.coli*	1
stx2	**stx2-forward** ATTAACCACACCCCACCG **stx2-reverse** GTCATGGAAACCGTTGTCAC ***stx2-probe** CAGTTATTTTGCTGTGGATATACGAGGGCTTG	200	Shiga-toxin2 producing *E.coli*	1
eae (O157:H7)	**eae-forward** GTAAGTTACACTATAAAAGCACCGTCG **eae-reverse** TCTGTGTGGATGGTAATAAATTTTTG ***eae-probe** AAATGGACATAGCATCAGCATAATAGGCTTGCT	106	*E.coli* EHEC O157:H7	1

*The FAM (6-carboxy-fluorescein) is used as fluorescent reporter dye and conjugated to 5' ends of each probe. The quencher dye BBQ is attached at the 3'end of the probes.

3. Thermal Cycle:

***E.coli* O157:H7 quantification by Real Time PCR (*stx1*, *stx2*, *eae* genes)**				
Cycles	**1**			
Taq activation	**Step 1**			
Target temp. (°C)	95			
Incubation time (min)	10			
Temp. Transition rate (°C/sec)	20			
Acquisition mode	None			
Cycles	**40**			
Amplification	**Step 1**	**Step 2**	**Step 3**	
Target temp. (°C)	95	55	72	
Incubation time (sec)	20	30	1	
Temp. Transition rate (°C/sec)	20	20	20	
Acquisition mode	None	None	Single	
Cycles	**1**			
Cooling	**Step 1**			
Target temp. (°C)	25			
Incubation time (sec)	30			
Temp. Transition rate (°C/sec)	20			
Acquisition mode	None			

4. Detection limit according to calibration curve

Detection limit: 20 gene copies per assay (similar in all three assays given).

Reference

1. Sharma VK, Dean-Nystrom EA. Detection of enterohemorrhagic *Escherichia coli* O157:H7 by using a multiplex real-time PCR assay for genes encoding intimin and Shiga toxins. *Veterinary microbiology* 93 (2003) 247-260.

10.1 SOP for quantification of *Legionella pneumophila* by cultivation based on ISO 11731-2

Principle

Bacteria, including *Legionella*, in the water sample are concentrated by membrane filtration. After filtration, the filter is treated with acid buffer added directly into the funnel to reduce the growth of non-Legionella organisms. The filter is subsequently transferred onto a plate of agar medium selective for *Legionella* and incubated. Presumptive colonies are confirmed as *Legionella pneumophila* by subculture to demonstrate their growth requirement for L-cysteine and iron.

Materials

Culture media

Legionella Buffered Charcoal Yeast Extract **BCYE agar** (Ready plates, Oxoid), **GVPC agar** (Ready plates, Oxoid) and **BCYE-Agar without L-Cystein** (Ready plates, Oxoid). Composition of BYCE agar (g/l): Yeast extract (bacteriological grade) 10.0 g, Activated charcoal 2.0 g, α-Ketoglutarate, mono-potassium salt 1.0 g, ACES buffer (N-2-acetamido-2-aminoethane sulfonic acid) 10.0 g, Potassium hydroxide (KOH) (pellets) 2.8 g, L-cysteine hydrochloride monohydrate 0.4 g, Iron(III) pyrophosphate [Fe4(P2O7)3] 0.25g Agar 12.0g.

Reagents

- Acid buffer (filter sterilized)
  Prepare a 0.2mol solution of hydrochloric acid (HCL) (Solution A).
  Prepare a 0.2mol/l solution of potassium chloride (KCL) (Solution B). To prepare acid buffer mix 3.9ml of Solution A and 25ml of Solution B. Adjust to pH2.2 ±0.2 by addition of a 1mol/l solution of potassium hydroxide (KOH). Store in a stopped glass container in the dark at room temperature for no longer than 1 month.

- Solution A: 0.2mol/l HCL
  Add 17.4ml of concentrated HCL (p= 1.18 minimum assay 35.4%) or 20ml HCL (p= 1.16 minimum assay 31.5%) to 1l distilled water. Sterilise by autoclaving at (121±3)°C for (15±1) min.

- Solution B: 0.2mol/l KCL
  Dissolve 14.9g of KCL in 1l of distilled water. Sterilise by autoclaving (121±3)°C for (15±1) min.

- Page´s saline

Sodium chloride (NaCl)	0.120 g
Magnesium sulfate (MgSO4·7H2O)	0.004 g
Calcium chloride (CaCl2·2 H2O)	0.004 g
Disodium hydrogenphosphate (Na2HPO4)	0.142 g
Potassium dihydrogenphosphate (KH2PO4)	0.136 g
Distilled water 1 000 ml	

Add the chemicals to the distilled water. Allow to dissolve, mix well and autoclave at (121 ± 3) °C for (15 ± 1) min. To aid accurate preparation, prepare a 10 l volume of Page's saline.

Sampling

The volume of sample collected depends upon the nature of water system and the purpose of the examination. Details of the origin and volume of the sample, the temperature of the water at the time of sampling as well as the presence and nature of any biocide shall be recorded and given to the laboratory with the samples as an aid to examination. For both safety and analytical reasons it is not advisable to examine samples of unknown origin or of cooling and process waters unless they are accompanied by adequate information that will include information about the chemical additives used in or likely contaminants that are present as a result of the process.

Sample containers
Samples of water (generally 1 litre) shall be collected in glass, polyethylene or similar containers. If used previously, they shall be cleaned, rinsed with distilled or mains tap water and autoclaved at (121 ± 3) °C for 15 min.

Sample transportation and storage
Deliver the samples to the laboratory as soon as possible preferably within 1 d but not more than 2 d. If analysed the same working day, transport samples at ambient temperature protected from sunlight. Otherwise cool samples, ideally (5 ± 3) °C, during transport. It is recommended that the time interval between collection of the sample and its filtration in these circumstances is ideally within 24 h and should not exceed 2 d.

Procedure

Filter 100ml of drinking water sample onto a sterile black membrane filter with a counting grid (47 mm, 0.45 µm pore size, cellulose nitrate, Millipore). After filtration, add 30ml of acid buffer on top of the membrane and leave for 5min. Rinse the filter with 20ml Page´s saline. Remove membrane from the stand with sterile forceps and place it (up side up!) on agar plate. Use 5 replicates of BCYE and/ or GVPC agar plates. Incubate the plates upside down at (36 ± 2)°C for 10 d. Check plates twice at day 3 or 4 during 10 days. Do final reading after 10d with description of colonies. Select at least 5 colonies characteristic for legionella at random for each positive sample and subculture onto BCYE and BCYE-cysteine. Incubate 36±2°C for at least 2 days. Regard as *Legionella* those colonies which grow on BCYE but fail to grow on BCYE-Cys medium. Record the results for each plate. Isolate at least 5 positive (better more) and identity in molecular terms (not mandatory). When the numbers of individual sero-groups or species of Legionella are to be reported, always confirm at least three representative colonies of each colonial type of subculture according to ISO 11731.

Expression of results
The purpose is to estimate the number of Legionella present or demonstrate the absence of *Legionella* in the sample. Report the confirmed estimated number of *Legionella* present, as CFU of *Legionella* species or, as *L. pneumophila* in case of molecular confirmation, in the volume examined (100ml), and report the absence of Legionella as “not detected” in the volume examined.

Reference

ISO 11731-2:2004: Water quality — Detection and enumeration of Legionella —Part 2: Direct membrane filtration method for waters with low bacterial counts.

10.2 SOP for quantification of *Legionella pneumophila* by Real Time PCR

A LightCycler 480 instrument (Roche) is used. Calibration curves are generated by preparing standard DNA dilution series from *L. pneumophila* strain Philadelphia (ATCC 33152). A SYBR-green based real-time PCR approach was performed using the SYBR Master Kit (Roche). All calibration standards, samples and non-template controls are performed in triplicates.

1. PCR mixtures:

Reagent	**Quantity (µl)**	**Final concentration into the mix**
PCR grade water	3	
L2-F (5 µM)	1	250 nM
L2-R (5 µM)	1	250 nM
LC480 SYBR master (2x)	10	1x
Template DNA	5	0.1 – 2 ng env. DNA
Total reaction volume	20	

2.Primers:

Target gene	**Primer sequence**	**Amplified fragment (bp)**	**Detectable strains**	**Reference**
16S rDNA	**L2F** 5' - ATGGCCGATACAGAGGGCG - 3' **L2R** 5' - GAACGTATTCACCGCGACA - 3'	144	*L. pneumophila*	In house

3.Thermal Cycle:

***L pneumophila* quantification by Real Time PCR (L2F/R primers)**				
Cycles	**1**			
Taq activation	**Step 1**			
Target temp. (°C)	95			
Incubation time	5 min			
Temp. Transition rate (°C/sec)	4.4			
Acquisition mode	None			
Cycles	**45**			
Amplification	**Step 1**	**Step 2**	**Step 3**	
Target temp. (°C)	95	60	72	
Incubation time	10 sec	20 sec	1 sec	
Temp. Transition rate (°C/sec)	4.4	2.2	4.4	
Acquisition mode	None	None	Single	
Cycles	**1**			
Melting curve and cooling	**Step 1**	**Step 2**	**Step 3**	**Step 4**
Target temp. (°C)	95	65	97	40
Incubation time	5 sec	1 min	1 sec	10 sec
Temp. Transition rate (°C/sec)	4.4	2.2	0.08	1.5
Acquisitions (per °C)	0	0	7	0
Acquisition mode	None	None	Continuous	None

4. Detection limit according to calibration curve

Detection limit: 15 - 30 gene copies per assay (equals 5 - 10 genome units).

11.1 SOP for quantification of *Helicobacter pylori* by cultivation

Principle

For enumeration of *Helicobacter pylori*, counting by direct plating into a selective medium is used.

Materials

Culture media

Brucella agar (Oxoid, Basingstoke, UK); campylobacter selective supplement (Merck, Darmstadt, Germany), Urea broth Christensen (Oxoid, Basingstoke, UK).

Reagents

- Saline (sodium chloride (NaCl) 8.5 g L^{-1})
- Trimethoprim (0.25mg/L, (Sigma, Saint Louis, Missouri, USA),
- Amphotericin B (Sigma, Saint Louis, Missouri, USA),
- Sheep blood
- Foetal calf serum (Sigma, Saint Louis, Missouri, USA)
- Gram staining reagents
- Oxidase reagent (Becton Dickinson, New jersey, USA)
- Instagene^{TM} Matrix (Bio-Rad, Hercules, CA, USA)

Sampling

Samples of water (generally 1 L) shall be collected in glass, polyethylene or similar containers. If used previously, they shall be cleaned, rinsed with distilled or mains tap water and autoclaved at (121 ± 3) °C for 15 min. Samples should be protected from direct sunlight and transported at ambient temperature in an insulated container. Samples should be examined as soon as possible on the day of collection. In exceptional circumstances, if there is a delay, storage under the above conditions should not exceed 24 hours.

Procedure

1 ml volume of water was pipetted in triplicate into three empty tube wells. Perform 6 serial decimal dilutions (100:900 µl) of the sample original suspension in saline, in triplicate. Inoculate 100 µl of each dilution onto Brucella agar containing campylobacter selective supplement plus 5mg L^{-1} trimethoprim, 0.25mg L^{-1} amphotericin B, sheep blood (5%), and 7% foetal calf serum. After 72 h incubation at 37 ºC in microaerophilic condition, count in those plates with 30 – 300 colonies. Identify the colonies using Gram's staining, oxidase and urease reactions. In order to confirm identity of the isolates, extract DNA from a colony using the InstageneTM DNA Purification Matrix following the manufacturer instructions. Sequence the *rpoB* gene using the primers, conditions and criteria described by Korczack *et al.* (2006).

Expression of results

Express the average results in CFU of *Helicobacter pylori* in 100ml, and report the absence of as "not detected" in the volume examined.

References

Bahrami AR, Rahimi E, Ghasemian Safaei H. (2013) Detection of *Helicobacter* pylori in city water, dental units' water, and bottled mineral water in Isfahan, Iran. ScientificWorldJournal. 31;2013:280510. doi: 10.1155/2013/280510.

Korczak BM, Stieber R, Emler S, Burnens AP, Frey J, Kuhnert P. Genetic relatedness within the genus *Campylobacter* inferred from *rpoB* sequences. Int J Syst Evol Microbiol. 2006; 56:937-945.

11.2. SOP for quantification of *Helicobacter pylori* by Real Time PCR

A 7900HT Fast Real-Time PCR System instrument (Applied Biosystems) is used. The real-time PCR assay will use environmental DNA obtained by the filtration and extraction SOP indicated above or a specific variation of it. A TaqMan-based real-time PCR approach was used based on the TaqMan Universal PCR Master Mix (Applied Biosystems).

1. PCR mixtures:

Reagent	**Quantity (µl)**	**Final concentration into the mix**
PCR grade water	5.4	
Primer prVC-F	1.3	500 nM
Primer prVCM-R	1.3	500 nM
2x GoTaq qPCR Master Mix	10	1x
Template DNA	2	2-10 ng
Total reaction volume	20	

2. Primers:

Target gene	**Primer sequence**	**Amplified fragment (bp)**	**Detectable strains**	**Ref.**
16S rRNA	**16S_rRNA-F:** TGC GAA GTG GAG CCA ATC TT **16S_rRNA-R:** GGA ACG TAT TCA CCG CAA CA **16S_rRNA-probe*:** CCT CTC AGT TCG GAT TGT AGG CTG CAA C	118	*H. pylori*	1

*The FAM (6-carboxy-fluorescein) is used as fluorescent reporter dye and conjugated to 5' ends of each probe. The quencher dye BBQ is attached at the 3'end of the probes.

3. Thermal Cycle:

***H. pylori* quantification by Real Time PCR (prvc-F/R primers)**				
Cycles	**1**			
Taq activation	**Step 1**			
Target temp. (°C)	95			
Incubation time	10 min			
Temp. Transition rate (°C/sec)	20			
Acquisition mode	None			
Cycles	**50**			
Amplification	**Step 1**	**Step 2**		
Target temp. (°C)	95	60		
Incubation time	15 s	1 min		
Temp. Transition rate (°C/sec)	20	20		

4. Detection limit according to calibration curve
Detection limit: 10 gene copies per assay.

References

1. Yamazaki S, Kato S, Matsukura N, Ohtani M, Ito Y, Suto H, Yamazaki Y, Yamakawa A, Tokudome S, Higashi H, Hatakeyama M, Azuma T. Identification of Helicobacter pylori and the cagA genotype in gastric biopsies using highly sensitive real-time PCR as a new diagnostic tool. FEMS Immunol Med Microbiol. 2005; 44(3):261-8.
2. Böckelmann U, Dörries HH, Ayuso-Gabella MN, Salgot de Marçay M, Tandoi V, Levantesi C, Masciopinto C, Van Houtte E, Szewzyk U, Wintgens T, Grohmann E. Quantitative PCR monitoring of antibiotic resistance genes and bacterial pathogens in three European artificial groundwater recharge systems.Appl Environ Microbiol. 2009;75(1):154-63.