



Global Salm-Surv

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

Laboratory Protocols

Level 2 Training Course

Identification of thermotolerant *Campylobacter*

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Contents:

1. Identification of thermotolerant *Campylobacte*r from food, faeces or water

Introduction

The following procedures will guide you through the steps that are necessary to carry out a biochemical identification of *Campylobacter*.

***Campylobacter* are generally identified by:**

- Slender helical or curved gram-negative rods.
- Highly motile by means of a single polar flagellum.
- Optimal oxygen concentration for growth 5-10%.
- Do not ferment or oxidize sugars.
- Do not produce indole (mind the different with hydrolysis of indoxyl acetate!).

According to ISO 10272 (Microbiology of food and animal feeding stuffs – Horizontal method for detection of thermotolerant *Campylobacter*) *Campylobacter* is identified by the following characteristics:

- morphology and motility
- morphology in Gram staining
- oxidase
- glucose
- lactose
- sucrose
- gas

In this course identification and differentiation of strains is performed by:

- morphology and motility
- morphology in Gram staining
- katalase
- oxidase
- hippurate hydrolysis
- hydrolysis of indoxyl acetate

***Campylobacter* from faeces, food or water**

Campylobacter food poisoning occurs in most cases sporadically affecting individuals. Outbreaks due to Campylobacter infections are rare. Outbreaks due to contaminated milk and drinking water are described more often than food borne outbreaks. *Campylobacter jejuni* is the most common cause of human bacterial enteritis but *Campylobacter coli* may also be responsible.

Campylobacter jejuni is commonly isolated from chicken and cattle, and chicken is expected to be one of the major sources of infection for humans.

Pigs commonly carry *Campylobacter coli* In some countries where large quantities of pork are consumed *Campylobacter coli* infections frequently occur.

Campylobacter may also be present in faeces or food in low numbers and they may be injured. To diminish the risk of obtaining false negative results, selective enrichment of a large food sample can be performed:

- Enrichment in selective enrichment broth (*e.g.* Preston).
- Selective plating on CCD-agar plates.

References

1. Nachamkin I. and M. J. Blaser (eds) (2000). *Campylobacter 2nd ed.* ASM Press, Washington, D.C.

2. Identification of thermotolerant *Campylobacter* from food, faeces or water

Materials

Equipment

- Disposable inoculation loops (1 µl and 10 µl)
- Incubators at 37°C/42°C
- Microscope
- Slides
- Cover glass
- Mineral oil
- Paper disc 6 mm
- Pipettes for 0.2 ml (e.g. 1 ml pipettes)
- 200 ml flask
- Forceps
- Eppendorf tubes, 1.5 ml
- Drop counters

Media

- Sterile water
- 3%-H_2O_2
- 1%-hippurate solution
- 3.5%-ninhydrin solution
- 10%-indoxyl acetat solution
- Oxidase sticks
- Gram staining reagents
- Crystal violet
- Gram's iodine
- Ethanol (95%)
- Carbol fuchsine

Bacterial strains:

Campylobacter.lari	ATCC 35221
Campylobacter.coli	ATCC 33559
Campylobacter.jejuni	ATCC 700819
Pseudomonas aeruginasa	ATCC 27853
Enterococcus faecalis	ATCC 29212
Staphylococcus aureus	ATCC 29213

Safety

Carry out all procedures in accordance with the local codes of safe practice.

Procedure

Theory / comments

Identification

Microscopy (morphology and motility)

One drop of sterile saline is placed on a slide. Colonies from the CCD agar plates are mixed with the saline. Place cover glass above the colonies, and place the slide in the microscope.

A striking character of *Campylobacter* is their helical or curved shape. Long spiral forms can resemble spirochaetes superficially, but campylobacters have flagella, usually single, at one or both poles and are highly motile, spinning around their long axes and frequently reversing direction.

Gram staining (morphology)

One small drop of saline is placed on a slide. Colonies from the CCD agar plates are mixed with saline and smeared over the surface of the slide. The smears are allowed to dry thoroughly. The smears are fixed by passing the slide, smear up, quickly through the Bunsen flame three times. After cooling the smears can be stained. Between each staining reagent the smear is washed under a gently running tap, excess of ater tipped off before the next reagent is added.

1. Crystal violet (60 sec)
2. Gram's iodine (60 sec)
3. Ethanol (decolouriser) (60 sec)
4. Carbol fuchsine (60 sec)

Gram negative bacteria (like Campylobacter) are decolourised and stained red by the counter-stain (Carbol fuchsine). Campylobacter are curved or gull shaped forms. Old cultures may contain coccoid bacteria.

Test for catalase

Put a colony at a small spot on a slide (do NOT not make a suspension; just dry). Put one drop of 3%-H_2O_2 on the spot with the bacterial material. Examine immediately for evolution of gas, which indicates catalase activity.

The catalase-enzyme cleaves the hydrogen peroxide $H_2O_2 + H_2O_2 => O_2 + 2H_2O$ The peroxidase is only able to reduce H_2O_2 if an organic substrate is present at the same time and serves as a donor for hydrogen.

Test for oxidase

Transfer one colony to a filter paper. Soak the filter in a oxidase solution. Appearance of a blue color within 10 sec indicates a positive result.

The method is based on the principle that certain phenylen-diamin-derivatives are oxidised by cytochrom C to produce a bluish indophenol. Commercial kits are available.

Hippurate hydrolysis

Suspend a loopful of a growth from an 18-24 hour columbia agar plate containing 5% cattle blood culture in 400 µl of a 1%-hippurate solution (take care not to incorporate agar!). Incubate at 37°C for 2 hours. Then slowly add 200 µl 3.5%-ninhydrin solution to the side of the tube to form an overlay. Reincubate at 37°C for 10 min, and read the reaction. Positive reaction: dark purple/blue.Negative reaction: clear or gray.

Hydrolysis of hippuracid releases benzoeacid. Hippuracid is soluble in excess of an acidic solution of ferrichloride while benzoeacid precipitates.

1%-hippurate solution: freshly prepared or stored at -20 °C for about 6 month.
3.5%-ninhydrin solution: Stable for about one month. Stored at room temperature in a dark bottle.

Identification

Hydrolysis of indoxyl acetate

Add 50 µl of a 10% (w/v) solution of indoxyl acetate in acetone to an absorbent paper disc 6 mm in diameter and allow to dry in air. Apply growth from a *Campylobacter* colony directly to disc and then wet with a drop of sterile distilled water. Appearance of a blue-green color within 5-10 minutes indicates a positive result.

The bacterial enzyme esterase releases indoxyl from indoxyl acetate which spontaneously forms indigo in the presence of oxygen.
Dried discs are stable for at least 12 months if stored at 4ºC in a dark glass bottle with silica gel. Discs should not be used if the color has changed from white, or if the expiration date has passed

Appendix 1. Result sheet
Appendix 2. Photographs of pos. and neg. reactions of biochemical tests on *Campylobacter*

3. Composition and preparation of culture media and reagents

The media and reagents are available from companies like Oxoid, Merck and Difco. The composition of the dehydrated media given below is an example and may vary a little among the different manufacturers. Also the media should be prepared according to the manufacturers description if it differs from the description given here.

Saline solution

Sodium chloride 8.5 g
Water 1000 ml

Preparation:
Dissolve the sodium chloride in the water, by heating if necessary. Adjust pH ~ 7.0 after sterilisation. Dispense the solution into tubes so 4 ml is obtained after autoclaving at 121^{o}C for 20 min.

3,5 % Ninhydrin solution

Ninhydrin ($C_9H_6O_4$) 3,5 g
Acetone (C_3H_6O) 50 ml
Butanol ($C_4H_{10}O$) 50 ml

Dissolve the chemical in the solutions. Stored at + 5^{o}C in dark bottles of 20 ml.

1% Hippurate solution

Natriumhippurat ($C_9H_8NNaO_3$) 1 g
PBS 99 ml

Dissolve the chemical with the solutions. Stored at -20^{o}C in tubes of 15 ml.

Gram-staining

Crystal violet

Crystal violet	2.0
Ethanol 95% (vol/vol)	20.0 ml
Ammonium oxalate	0.8 g
Distilled water	80.0 ml

The crystal violet is first dissolved in the ethanol, then the ammonium oxalate is dissolved in the distilled water. The two solutions are added together. To aid the dissolving process, both mixtures are agitated in a bath of hot water.

Gram's iodine

Iodine crystals	1.0 g
Potassium iodide	2.0 g
Distilled water	200 ml

The iodine crystals and the potassium iodine are ground together in a mortar and the distilled water is added slowly. If necessary the mixture can be agitated in a bath of hot water to aid dissolution.

Decolourizer
Ethanol 95% (vol/vol)

Carbol fuchsine (counterstain)

Concentrated carbol fuchsine	10.0 ml
Distilled water	90.0 ml

10% (wt/vol) Indoxylacetate solution

Indoxylacetat ($C_{10}H_9NO_2$)	10 g
Acetone (C_3H_6O)	90 ml

Dissolve the chemical in acetone. Stored at +4^{o}C in a dark bottle.

Oxidase solution

L(+)-Ascorbicacid	0,03 g
N,N,N´,N´- Tetramethyl-p-Phenylendiamine Dihydrochloride ($C_{10}H_{16}N_2 \cdot 2HCl$)	0,03 g
Sterile water	30 ml

Dissolve the chemicals in water, and store the solution in a dark bottle at +5 ^{o}C for 3 weeks.

References

1. BARROW & FELTHAM (eds.): *Cowan and Steel's Manual for the Identification of Medical Bacteria,* 3 rd edn.
2. NMKL method no. 119, 2nd ed, *Campylobacter Jejuni/Coli detection in foods.* Nordic committee on food analysis.

Record sheet: Quality Control / Batch Control

Date:_____ Init.:_____

Biochemical tests

QC-Strain	***C.jejuni*** **ATCC 700819**	***C.coli*** **ATCC 33559**	***C.lari*** **ATCC 35221**	***E. faecalis*** **ATCC 29212**	***S. aureus*** **ATCC 29213**	***P. aeruginosa*** **ATCC 27853**
Gram staining						
Test for catalase						
Test for oxidase						
Hippurate hydrolysis						
Hydrolysis of indoxyl acetate						

Record sheet: Identification of Campylobacter.

Date:_____ Init.:_____

Biochemical tests

	Strain #	**Strain** #	**Strain** #	**Strain** #	**Strain** #	**Strain** #
Morphology of the cell (microscopy)						
Motility (microscopy)						
Gram staining						
Test for catalase						
Test for oxidase						
Hippurate hydrolysis						
Hydrolysis of indoxyl acetate						
Species:	_____	_____	_____	_____	_____	_____

Record sheet: Isolation and identification of Campylobacter from faeces, food or water.

Date:_____ Init.:_____

Biochemical tests

	Faeces-sample 1	Faeces-sample 2	Food-sample 1	Food-sample 2	Water-sample 1	Water-sample 2
Morphology of the cell (microscopy)						
Motility (microscopy)						
Gram staining						
Test for catalase						
Test for oxidase						
Hippurate hydrolysis						
Hydrolysis of indoxyl acetate						
Species:	_____	_____	_____	_____	_____	_____

APPENDIX 1

Result sheet for identification of *Campylobacter*

	Campylobacter jejuni	***Campylobacter lari***	***Campylobacter coli***
Gram staining	**Gram negative curved rod**	**Gram negative curved rod**	**Gram negative curved rod**
Test for catalase	+	+	+
Test for oxidase	+	+	+
Hippurate hydrolysis	+	-	-
Hydrolysis of indoxyl acetate	+	-	+

Comments: _____

_____

_____

_____

_____

_____

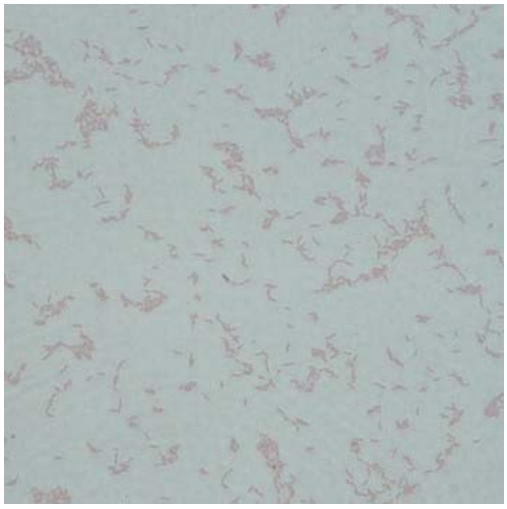
_____

APPENDIX 2

Photographs of positive and negative reactions of biochemical tests on *Campylobacter*.

The positive and negative control strains for the biochemical tests are indicated in brackets.

Gram Straining:

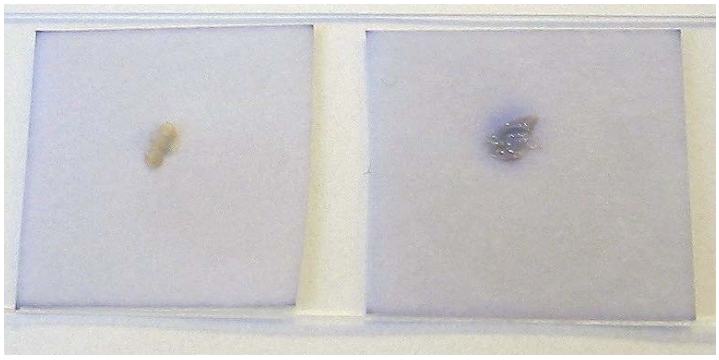


Gram strain of a Campylobacter culture. Note the curved shape (arrowed / seagull). The degenerate appearance shown by many of the cells are typical of ageing cultures.

Katalase Test:

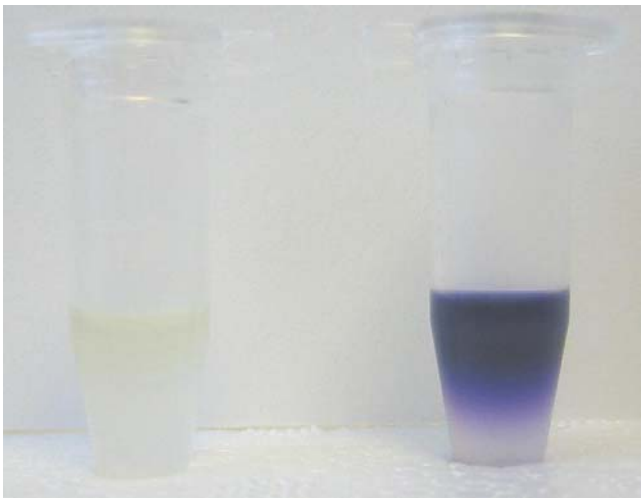
The catalase reaction have nothing to do with the organism being microaerophilic, But many microaerophilic microorganisms are not sensitive towards hydrogen peroxide and as result to this doen´t need the catalase enzyme.
Left: Positive reaction:
Ps. aeruginosa ATCC 27853
Right: Negative reaction:
E.faecalis ATCC 29212

Oxidase test:



Left: Negative reaction:
S. aureus ATCC 29213
Right: Positive reaction:
Ps. aeruginosa ATCC 27853

Hippurate hydrolysis:



Left: Negative reaction: C.coli ATCC 33559
Right: Positive reaction: C. jejuni ATCC 700819

Hydrolysis of Indoxyl acetate:

Left: Negative reaction: C.lari ATCC 35221
Right: Positive reaction: C. jejuni ATCC 700819

Reference:

1. Food-borne Pathogens. Monograph Number 3 Campylobacter, Oxoid Standards.
2. © Institut for Veterinær Mikrobiologi, Den Kgl. Veterinær- og Landbohøjskole, 2000.