NordVal International / NMKL
c/o Danish Technical University
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NordVal International Certificate

Issued for:	Campylobacter real-time PCR
NordVal No:	017
First approval date:	31 June 2005
Renewal date:	1 April 2017
Valid until:	1 April 2019

Campylobacter real-time PCR

Manufactured by:
Eurofins GeneScan GmbH
Engesserstraβe4
79108 DE-Freiburg im Breisgau

Supplied by:
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Eurofins Steins Laboratory, Hjaltesvej 8, DK-7500 Holstebro, Denmark has applied for renewal of the NordVal certificate of the Campylobacter real-time PCR for detection of thermotolerant *Campylobacters* in chicken raw meat, faeces on cloacae swabs and on disposal shoe covers with chicken faces.

After having reviewed the method description and the evaluated results obtained in the validations, NordVal International concludes that it has been satisfactorily demonstrated that this real-time PCR method for *Campylobacter* provides equivalent results to the reference methods EN ISO 10272-1 and NMKL 119.

The validations were carried out according to ISO 16140:2003. In this certificate the results have been re-evaluated according to ISO 16140-2:2016/ NordVal International Protocol 2016.

Date: 31/3 2017

Yours sincerely

Hilde Skår Norli
Chair of NordVal International

Nina Skall Nielsen
NMKL Secretary General

METHOD DESCRIPTION

SCOPE AND FIELD OF APPLICATION.

This real time PCR method is applicable for the detection of the human pathogenic thermotolerant *Campylobacters* (*C. jejuni, C. coli* and *C. lari)* in chicken raw meat, cloacae swabs and faecal samples collected on disposable shoe covers in rearing houses. Thermotolerant *Campylobacters* are qualitatively determined with a sensitivity of 1-10 cfu/ 25 gram chicken raw meat and 100-1000 cfu/ml dilution of cloacae swabs and faecal samples collected on disposable shoe covers.

HISTORY

In 2005, the comparison studies and the interlaboratory study were carried out.

From 2007-2017, the method has been renewed every 2 years without any modifications or technical changes.

In 2017, the results are reviewed according to ISO 16140-2:2016, i.e. acceptability limits for the sensitivity study are included.

PRINCIPLE AND PROCEDURE

For raw chicken: 25 g sample is incubated in 225 ml of Bolton broth according to NMKL No. 119. After 24 hours 1 ml of the enriched meat buffer is transferred to a 1.5 ml Eppendorf tube.

The cloacae swab samples are collected in tubes with 3 ml 0.9% NaCl. 3 ml sample preparation is transferred to a small filter bag. 1 ml is removed from the other side of the filter to a 1.5 ml Eppendorf tube. Alternatively the tube with the swab is slightly centrifuged, in order to spin down remains of faecal material. 1 ml of the supernatant is removed to a 1.5 ml Eppendorf tube.

Disposable shoe covers with faeces samples are weighed and washed in ten times in 0.9% NaCl and homogenized using a stomacher before 1 ml is removed for the preparation of DNA to the real time PCR analysis.

The real time PCR is performed on a part of the prepared volume. The primers are targeting a *C. jejuni* 16S rRNA sequence. An internal amplification control (IAC) is analyzed with the samples to detect false negative responses.

Preparation of DNA

The tube is centrifuged at 16000 x G for 7 minutes. The supernatant is discarded and the pellet is resuspended in 200 µl of lysis buffer and placed in the automated sample handler

The sample and the lysis buffer are continuously mixed for 5 min at room temperature and 75 µl magnetic silica beads are added automatically. If a semi automatic system is used, the pellet is resuspended in 300 µl lysis buffer, and the process is followed from the following step.

The released DNA binds to the silica beads and are transferred to the salt wash buffer and washed at least one time for 5 min at room temperature.

The purified DNA is transferred on the silica-beads to alcohol wash buffer and is washed twice.

The washed DNA is dried on the silica beads for 5 min in air at room temperature.

The DNA is eluted in 100 µl Millipore water (or equivalent purity) for 5 min and the magnetic silica beads are discarded and the DNA is used as template DNA in the real-time PCR reaction described below.

PCR analysis

Prepare a PCR mix, per sample:

7.75 µl PCR H_2O
2.5 µl 10 x PCR Buffer (Roche 1480022)
1.0 µl 10 mM dUTP (Applied Biosystem no. 8080270)
2.5 µl 25 mM $MgCl_2$
2.0 µl Glycerol (87%)
1.1 µl 10 pmol/µl Primer 1 (5' CTG CTT AAC ACA AGT TGA GTA GG 3')
1.2 µl 10 pmol/µl Primer 2 (5' TTC CTT AGG TAC CGT CAG AA 3')
0.25 µl 20 mg/ml BSA (Roche no. 711454)
0.2 µl 500 U T*th* (Roche no. 1480022)
0.25 µl 5 pmol/µl Campy probe (5' *FAM*-TGT CAT CCT CCA CGC GGC GTT GCT GC-*TAMRA* 3')
0.25 µl 6 pmol/µl IAC probe (5'*VIC*-TTC ATG AGG ACA CCT GAG TTG A-*TAMRA* 3')
1.0 µl IAC Internal control ($5*10^3$ copies of IAC 124 bp)
5.0 µl Template DNA
25.0 µl

To avoid variations in the PCR reaction mix between individual tubes a master mix is prepared, i.e. a batch large enough to analyse all samples in the analysis. Dispense the mix in 0.5-ml Eppendorf tubes;-25 µl per tube. Include controls, see below. Start the PCR analysis.

Alternatively the Master mix can be produced by an extern manufactory.

PCR program:

The fluorescence dyes used in the PCR reaction are FAM and VIC/HEX, *alternatively JOE* and the PCR instrument should be set accordingly. The filter for TET/HEX can also be used for VIC *(JOE).*

1 cycle 95°C 3 min,
40 cycles 95°C 15 s, 58°C 1 min

When the program is finished set the baseline and the threshold according to the machine settings. Register the positive and negative results.

Controls

Positive control

A solution containing DNA prepared from a *Campylobacter jejuni, coli* or *lari* strain corresponding to about 1.000 cfu/ml may be used as positive control.

Negative controls

6 µl sterile water +19 µl PCR mix without IAC. (No template control (NTC))

5 µl sterile water + 20 µl PCR mix with IAC. (Internal control positive, target negative (NTC + IAC))

5 µl solution with non campylobacter DNA + 20 µl PCR mix with IAC. (Negative control (NC))*

5 µl sterile water processed as a sample + 20 µl PCR mix with IAC (Preparation blank)

*A solution containing DNA prepared from a non-Campylobacter gram negative strain corresponding to about 1.000 cfu/ml may be used as NC.

READING OF THE RESULTS

The result of a sample is considered positive if the C_t value is below *36* and the controls are as expected.

If the Ct value is between 36 and 40, a retest in duplicate must be done starting with preparation of DNA. If the two new preparations do have Ct values below 40 or one of the preparations has a Ct value below 40 and the other one is negative, the sample is considered positive. If both preparations are negative the sample is considered negative.

If the internal control is not amplified in the negative results, the result may be false negative and the analysis must be repeated.

THE COMPARISON STUDY

The comparative real-time PCR trial was performed in comparison to two reference culture methods (NMKL 119 and EN ISO 10272-1) on naturally contaminated samples, 99 shoe covers, 101 cloacae swabs , 102 neck skins from abattoirs and 100 retail neck skins. Culturing included enrichment in both Bolton and Preston broths followed by isolation on preston agar and mCCDA. The results obtained by the real-time PCR and the reference culture method were as followed:

	No. of samples with indicated result[a]						Relative			
Matrix	PA	NA	FN	TP	FP	Total	Accuracy	Sensitivity	Specificity	Kappa
Shoe covers	18	73	4	0	4	99	96	82	95	0.77
Cloacae swabs	39	55	5	0	2	101	95	88	96	0.86
Neck skin Abattoir	46	55	0	0	1	102	100	100	98	0.98
Retailers	57	43	0	0	0	100	100	100	100	1.00
Total	160	226	9	0	7	402	98	95	97	0.92

[a] PA, positive agreement; NA, negative agreement; TP, true positive; FN, false negative; FP, false positive,

Kappa = The degree of agreement between the alternative method and the "true result", kappa of 0.80 or higher is considered to be very good agreement.

Agreement between the alternative method and the reference method

In the validation, the degree of agreement between the alternative method and the reference method is satisfactory when the statistical entity kappa is no less than 0.80. For all samples except for shoe covers kappa is above 0.80.

Acceptability limit for the sensitivity study

The acceptability limits are not based on statistics, but on experience. For each category in a paired study, (FN - (FP+TP)) should be no more than 3, and (FN+FP+TP) should be no more than 6. For the four categories, the (FN - (FP+TP)) should be no more than 5 and the (FN+FP+TP) should be no more than 12. The results are summarised in the table below.

Acceptability limit of the sensitivity study

	(FN -(FP+TP)	(FN +FP+TP)
Shoe covers	5 – (4+0) = 1	5+4+0 = 9
Cloacae swabs	5 – (2+0) = 3	5+2+0 = 7
Neck skin Abattoir	0 – (1+0) = -1	0+1+0 = 1
Neck skin Retailers	0 – (0+0) = 0	0+0+0 = 0
Total	9 – (7+0) = 2	9+7+0 = 16

For all samples, the differences in the negative and positive deviations are less than or equal to 3, and hence satisfactory. The acceptability limit is not met for shoe covers and cloacae swabs as there are some false positive and false negative.

THE INTERLABORATORY STUDY

Nine laboratories participated in the study analysing pellets from 18 coded 1-ml samples including 6 chicken neck skin samples, 6 shoe cover samples and 6 cloacae swab samples. The samples were spiked in duplicate with C.*jejuni* CCUG 11284 at three levels. The laboratories also got a positive DNA

control, a ready-to-use PCR mixture with added IAC, and reagents for the magnetically based DNA extraction. A second collaborative study, comprising eight participated laboratories, was subsequently performed on shoe cover samples. The results of the studies are given in the table below:

	Specificity %	Sensitivity %		Accuracy %		
Matrix	No spiking	Low	High	No spiking	Low	High
Neck skins	100	100	100	100	100	100
Shoe covers	94	100	100	100	100	100
Cloacal swabs	100	92	100	100	92	100

The results of the studies show that the PCR method performs satisfactory and that there are no statistical significant differences in the results between the methods tested.