D-JRP9-2.2

WP2

Responsible Partner: NVI

Contributing partners: DTU, IZSAM, VRI, PIWET

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Leader	Task leader: Gro S. Johannessen, NVI
Other contributors	All other participants
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D-JRP9-2.2 STANDARD OPERATING PROCEDURE FOR AIR SAMPLING

Standard Operating Procedure (SOP) for Air Sampling

1. Introduction

The aim of the AirSample project has been to evaluate an alternative sampling method for the detection of *Campylobacter* spp. in broiler houses. This deliverable describes the standard operating procedure for collecting and analyzing air samples. The results from the project suggest that air filters analysed by real-time PCR is a way forward. However, there might be circumstances where a cultivation is beneficial. In this SOP, both real-time PCR (procedure 1), as well as a mixed real-time and cultivation according to ISO 10272-1:2017 are described (procedure 2).

For collecting air samples, please see, https://www.youtube.com/watch?v=FhumPlmZDPw for demonstration of the actual sampling.

1.1. Aim

The aim of the air sampling is to detect *Campylobacter* spp. in broiler houses, employing either real-time PCR or a mixed procedure of real-time PCR and cultivation for analysis.

Normally only one air filter is collected. The filter is split in two halves that are analysed separately, either both halves by real-time PCR, or one half by PCR and the second half by culture.

1. Material, equipment and reagents

Materials

- Tube racks (appropriate sizes)
- Sterile tips, volume from 0.5 ul to 1000ul, DNase and RNase free
- Eppendorf tubes (0.2 ml, 0.5ml, 1.5ml, 2ml)
- Pipettes volume from 0.2 ul to 1000ul, DNase and RNase free
- Inoculation loops (10 µl)
- Gloves

Equipment

- Laminar air flow cabinet class II
- Refrigerator +4˚C±2˚C
- Safety cabinet with UV light
- Real time PCR instrument
- Centrifuge 0-20,000 RPM
- Vortex mixer
- Freezer (-20 and -80°C)

- Equipment for incubation in a microaerophilic atmosphere
- Incubators

Reagents

For real-time PCR:

- Nuclease free water
- DNA extraction and purification kit: DNeasy Blood&Tissue Kit
- Campylobacter control DNA
- Real time PCR master mix
- Oligonucletides specific primers and probes from Josefsen et al. 2010:

Name	Sequence
camp F2	5'- CACGTGCTACAATGGCATAT -3'
camp R2	5'- GGCTTCATGCTCTCGAGTT -3'
camp P2 probe	5'- FAM-CAGAGAACAATCCGAACTGGGACA-BHQ -3'

- Kit Exogenous IPC

For cultivation:

- Bolton or Preston broth as appropriate
- mCCDA agar plates and plates of own choice if appropriate
- blood agar or other non-selective agar for sub-culturing of colonies

1.2. Analysis of air filters by PCR

For PCR analysis of the air filters, see flow diagram 1. The air filter is split in two parts. DNA is extracted from each half separately as described in the Consensus Protocol for DNA extraction method from gelatine filters.

1. **Pre-treatment of the filters:**
   a) Place a half of the gelatine filter in a suitable tube.
   b) Dissolve half of filter in 3.5 ml of double distilled (e.g. Milli-Q) water tempered to room temperature).
   c) Add 100 ul of an alkaline protease (kept at 4ºC) (e.g. Protex 6L, Genencor International, AE Leiden, The Netherlands).
   d) Vortexed thoroughly until filter totally dissolve in water.
   e) Pipette two equal volumes of the mixture (1.8ml x2) into two Eppendorf tubes.
   f) Incubate Eppendorf tubes in a thermal shaker for 6 min at 37ºC at 1000 x g.
   g) Centrifuge the solution at 8,000xg for 5 min at 4ºC.
   h) Discarded the supernatant.
   i) The two pellets are ready for DNA extraction.

2. DNA extraction by DNeasy Blood&Tissue Kit, QIAGEN, according to manufacturer's instruction with several modifications:

a) Perform all centrifugation steps at room temperature (15–25°C).
b) Re-dissolve any precipitates in Buffer AL and Buffer ATL.
c) Add ethanol to Buffer AW1 and Buffer AW2 concentrates.
d) Prepare TE buffer with EDTA (0.1mM), RNase A (100mg/ml) and Proteinase K.
e) Equilibrate frozen pellets to room temperature.
f) Preheat an incubator to 56°C.

3. Preparation Gram-negative bacteria:

a) Re-suspend the bacterial pellet in 180 µl ATL buffer.
b) Add 25 µl proteinase K.

c) Incubate at 56 °C for 1 hour with shaking.

4. Isolation of DNA:

a) After the 56 °C incubation step let the sample cool down to 40 °C.
b) Add 4 µl RNase A (100 mg/ml), vortex and incubate the samples at room temp for 5 min.
c) Vortex for 15 s.
d) Add 200 µl Buffer AL to the sample, and mix thoroughly by vortexing.
e) Add 200 µl ethanol (96-100 %) to the sample and mix thoroughly.

It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples.

f) Pipette the mixture into a DNeasy spin column placed in a collection tube.
g) Centrifuge at 10000 x g for one min. Discard the flow-through and the collection tube.
h) Place the spin column in a new collection tube and add 500 µl Buffer AW1.
i) Centrifuge at 10000 x g 1 min. Discard the flow-through and the collection tube.
j) Place the spin column in a new collection tube and add 500 µl Buffer AW2.
k) Centrifuge at 10000 x g 1 min. Discard the flow-through and the collection tube.
l) Place the spin column in a new collection tube.
m) Centrifuge at 10000 x g 3 min. Discard the flow-through and the collection tube.
n) Place the spin column in a 1.5 ml Eppendorf tube and add 100 µl 70 °C TE buffer with EDTA (0.1 mM) to the centre of the spin column membrane.
o) Incubate the samples at room temperature for 1 min.
p) Centrifuge at 10000 x g 1 min.
q) Discard the spin column.
r) Collect the 100 µl of DNA in TE buffer with EDTA (0.1 mM) from each Eppendorf tubes to the one Eppendorf tube.
s) Store the 200 µl of DNA at -20 °C. Long-time storage at 80 °C

6. Real-time PCR

a) Run real-time PCR (preferably) with the primers and probe described by Josefsen et al. 2010, and record the results. The cycle threshold (Ct) is recommended as previously established where Ct < 36 is considered positive.

1.3. Analysis of air filters by real-time PCR and cultivation

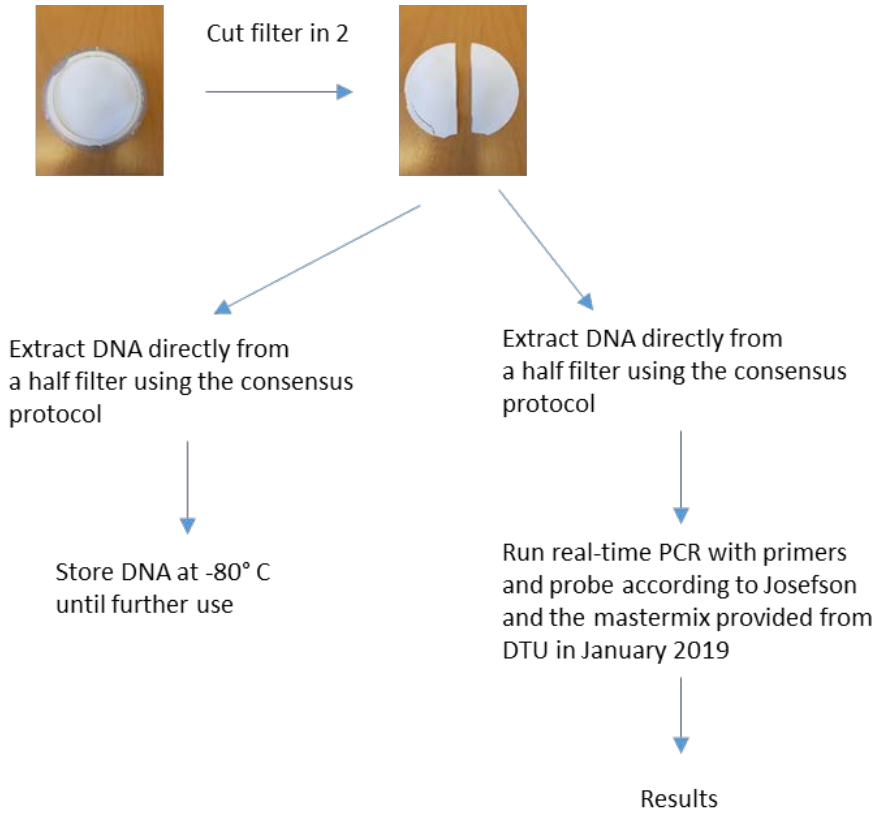
In some circumstances it might be desirable to run real-time PCR and attempt isolation of the bacteria at the same time. This can be obtained by using one half of the filter for PCR and the second half of the filter for cultivation as described below (see flow diagram 2).

Protocol for mixed procedure with real-time PCR and cultivation

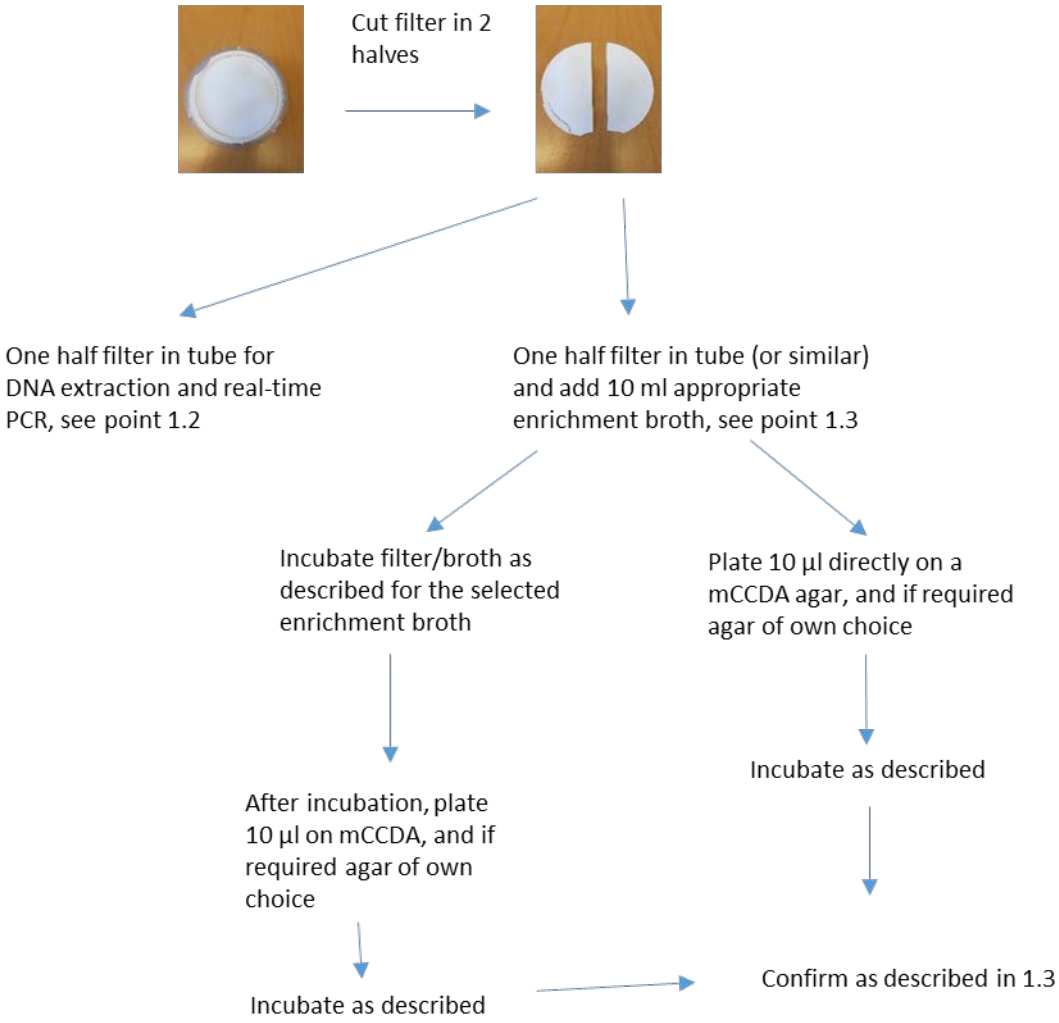
1. The air filter is split in two parts, each halv transferred to a suitable container. One of the halves is treated and analysed as described in 1.2 Analysis of air filters by PCR.
2. To the second half, add 10 ml of either Bolton broth or Preston broth as appropriate for the sample. For the second half, continue with the real-toem PCR as described above.
3. Prior to incubation of the enrichment broth, plate 10 µl (blue loop) directly on a mCCDA plate and another plate of own choice from the Bolton, or on mCCDA from the Preston broth. Incubate the plates a at 41.5°C in a microaerophilic atmosphere for 44 ± 4 hrs .
4. Incubate the broths in a microaerophilic atmosphere as described in ISO 10272, with Bolton at 41.5°C for 44±4 hrs and Preston at 41.5°C for 24±2 hrs.
5. After enrichment, plate 10 µl from the Bolton broth on mCCDA as well as a plate of own choice, or 10 µl of the broth on mCCDA if Preston broth is used.
6. Incubate the plates at 41.5°C in a microaerophilic atmosphere for 44 ± 4 hrs.

7. Read the plates for typical and suspicious colonies. Confirm by picking and pure-culturing a total of three colonies from each step, direct and after enrichment from both Preston and Bolton enrichment, onto a non-selective blood agar and incubate for 24-48 hrs at 41.5°C in a microaerophilic atmosphere.
8. First, confirm one colony from each step, using the lab's routine confirmation (PCR or Maldi-TOF). If the first colony does not belong to *Campylobacter* spp., continue with the remaining colonies.

Flow diagram 1. PCR air filters.



Flow diagram 2: Mixed procdure with real-time PCR and cultivation.



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