Isolation and Identification of *Campylobacter*

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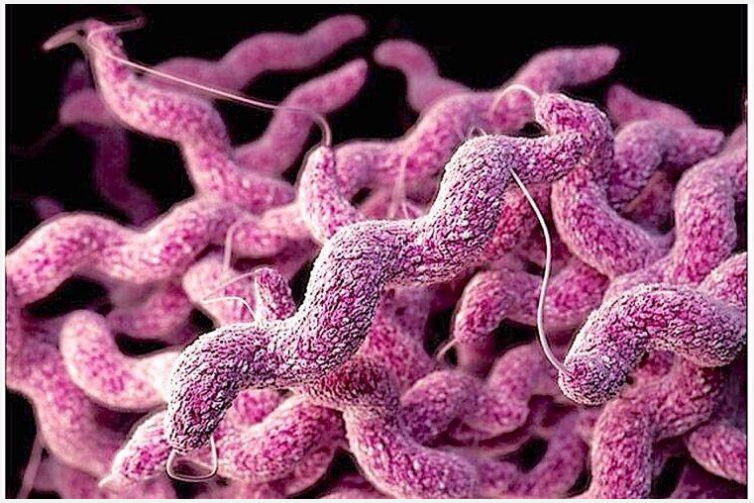
Campylobacter spp.

- A leading cause of foodborne gastroenteritis in humans worldwide
- *C. jejuni* (around 85%) followed by *C. coli* and other *Campylobacter* species
- Commonly present in the intestinal tract of animals, especially poultry
- Strictly microaerophilic (5% O_2 and 10% CO_2)
- Grows well at 37 °C – 42 °C (Thermophilic *Campylobacter*)



(https://about-campylobacter.com/)

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Standard methods for *Campylobacter* isolation

- **WOAH (OIE)**
  - Farm samples (e.g. fresh fecal droppings, cloacal swabs, or boot-swab samples) and cecal content
- **ISO**
  - ISO 10272-1:2006(E) focused on food samples
  - ISO 10272-1:2017(E) focused on food and farm samples

    *ISO 10272-1:2017/Amendment 1:2023(E)
- **FDA-BAM**
  - Food and water samples (e.g. shellfish, egg, milk, cheese, other dairy products, etc.)

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Standard methods for *Campylobacter* isolation

- Similarities
  - Incubation atmosphere: microaerobic conditions (5% O_2 and 10% CO_2)
  - Incubation temperature: 37 °C or 42 °C
  - Incubation time: 24 – 48 hours
- Differences
  - Different types of enrichment broth and agar
  - Non-selective blood agar for passive filtration method as recommended by WOAH

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Selective agents commonly used in *Campylobacter* selective enrichment broth

Table 1. Selective agents incorporated in some *Campylobacter* selective enrichment broth (concentrations in mg/l)

Medium	Cefoperazone	Vancomycin	Trimethoprim	Polymyxin B	Rifampicin	Cycloheximide	Amphotericin B
Park-Sanders broth	32	10	10			100	
Bolton broth	20	20	20				10
Hunt & Radle broth	30	10	12.5			100	
Preston broth			10	5,000 i.u.	10	100	

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Media commonly used for *Campylobacter* isolation

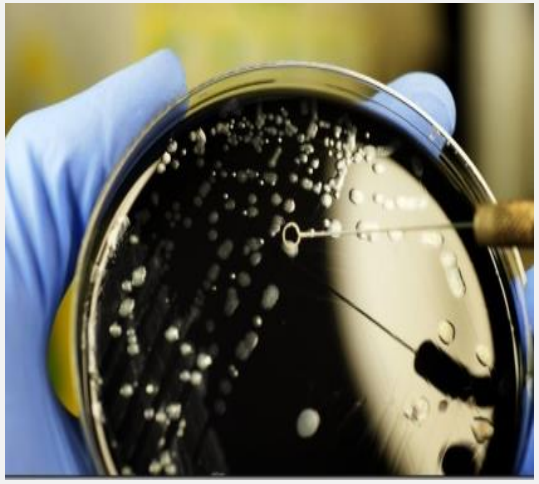
Table 2. Agar media commonly used for *Campylobacter* isolation

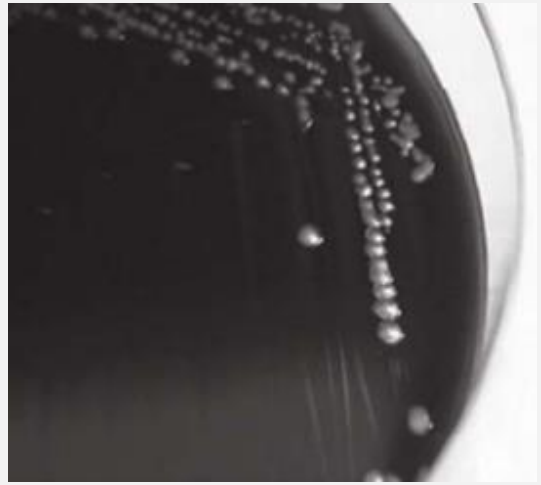
Charcoal-based media	Blood-containing media
mCCDA (modified charcoal cefoperazone deoxycholate agar)	Preston agar
Karmali agar or CSM (charcoal-selective medium)	Skirrow agar
CAT agar (cefoperazone, amphotericin and teicoplanin)	Campy-cefex agar

Note: Oxygen quencher commonly used in *Campylobacter* selective agar: charcoal, blood, or FBP (ferrous sulfate, sodium metabisulfite, and sodium pyruvate)

Campylobacter colonies on media commonly used for *Campylobacter* isolation

mCCDA

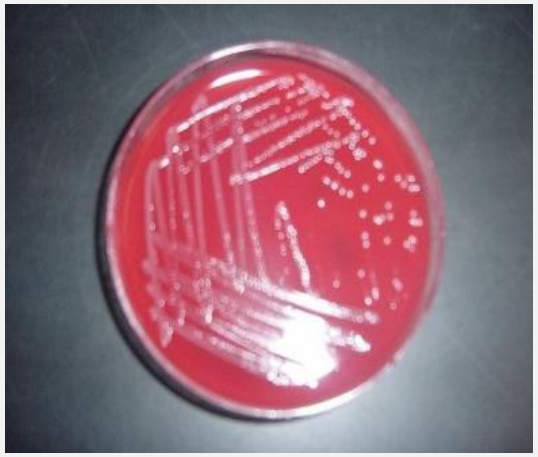


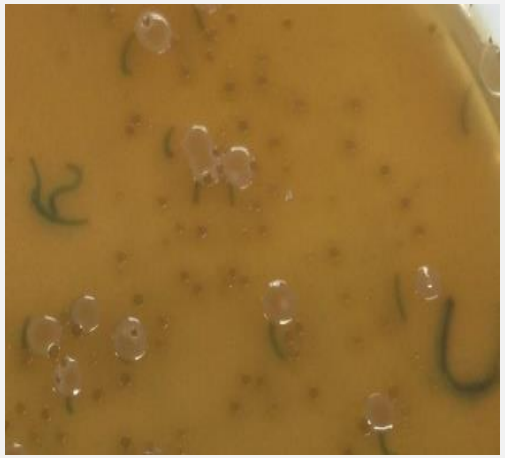



Typical colonies of *Campylobacter* on mCCDA are greyish with metallic sheen, flat and moist with a tendency to spread.

Campylobacter colonies on media commonly used for *Campylobacter* isolation

Other media



Campylobacter on blood-based agar can appear as greyish with metallic sheen or brown to orange in color.

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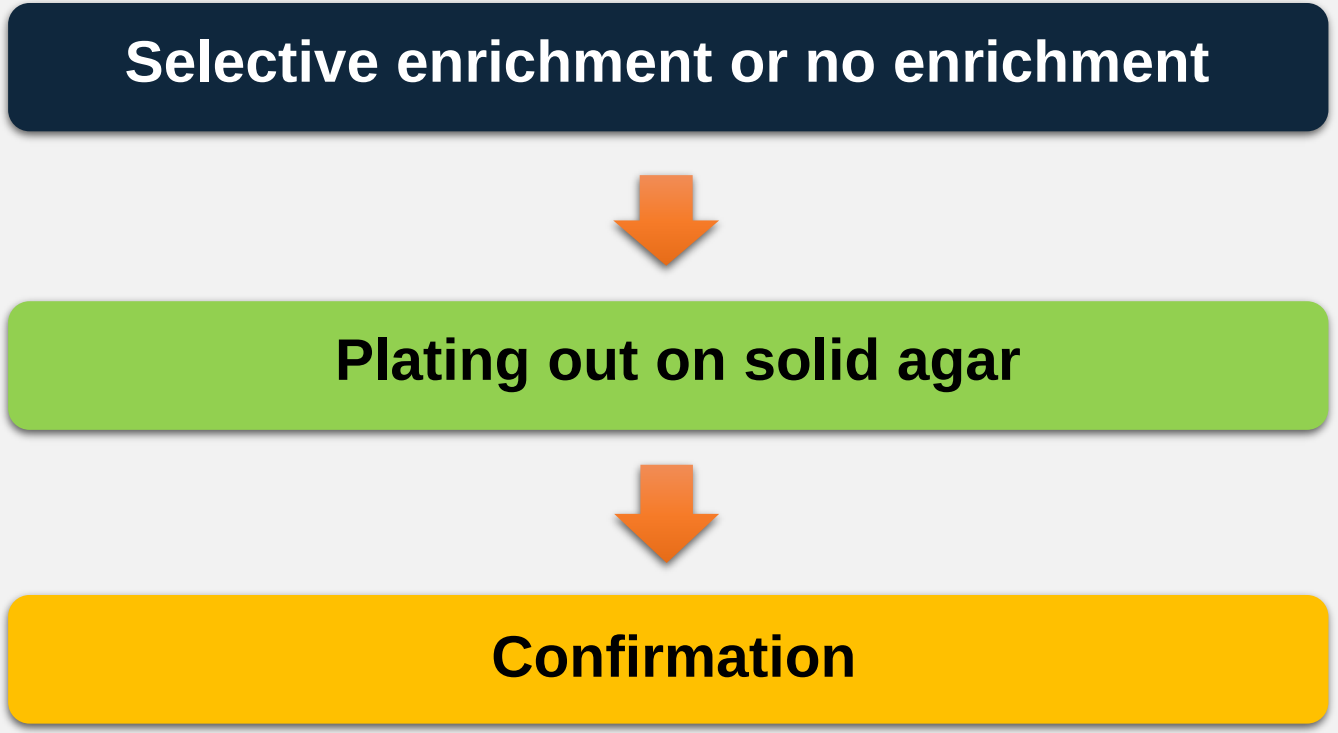
ISO protocol for *Campylobacter* isolation

- **ISO 10272-1:2017(E)**



Campylobacter detection by ISO 10272-1:2017(E)

Three different detection procedures depending on sample type

- **Procedure A**: Samples with low numbers of *Campylobacter* and low level of background microflora (selective enrichment with Bolton broth)
- **Procedure B**: Samples with low numbers of *Campylobacter* and high level of background microflora (selective enrichment with Preston broth)
- **Procedure C**: Samples with high numbers of *Campylobacter* (direct plating method)

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Initial process of sample preparation

Sample preparation for *Campylobacter* isolation

Carcass sample

- Sample + Buffered peptone water (BPW) at 1:9 ratio
- 28 g of sample + 252 ml of BPW, then take 250 ml, 10 ml, 10 ml, and 10 ml for *Salmonella*, *Campylobacter*, *Enterococcus*, and *E. coli*, respectively

Campylobacter detection by ISO 10272-1:2017(E)

Procedure A

Sample 10 g or 10 ml + Bolton broth 90 ml (1:9 ratio)

↓

Incubate in a microaerobic atmosphere at 37°C for 4 to 6 h
and then at 41.5°C for 44 h ± 4 h

↓

Subculture onto mCCDA and 2nd medium; incubate at
41.5°C for 44 h ± 4 h in a microaerobic atmosphere

Campylobacter detection by ISO 10272-1:2017(E)

Procedure B

Sample 10 g or 10 ml + Preston broth 90 ml (1:9 ratio)

↓

Incubate in a microaerobic atmosphere at 41.5°C for 24 h ± 2 h

↓

Subculture onto mCCDA and incubate at 41.5°C for 44 h ± 4 h
in a microaerobic atmosphere

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Campylobacter detection by ISO 10272-1:2017(E)

Procedure C

Direct plating on mCCDA and 2nd medium (optional)

Incubate in a microaerobic atmosphere

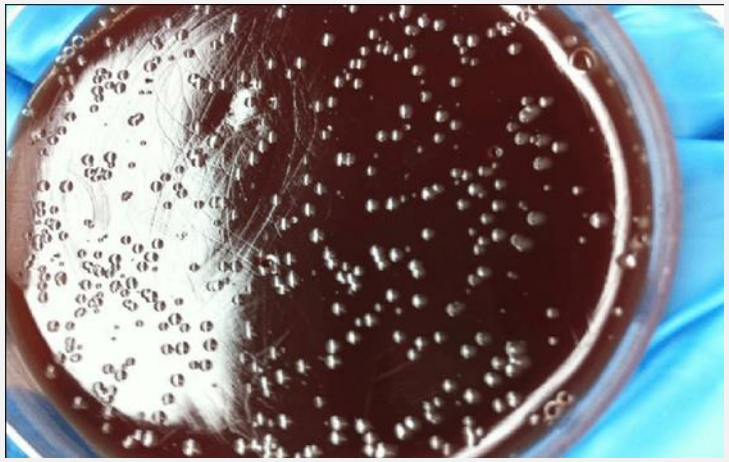
at 41.5°C for 44 h ± 4 h

Campylobacter detection by ISO 10272-1:2017(E)

Confirmation of *Campylobacter*

Selection of colonies for confirmation

- Test at least 1 typical colony; if negative, test up to 4 more suspect colonies
- Subculture onto a non-selective blood agar plate, e.g. Columbia blood agar, then incubate the agar plate in a microaerobic atmosphere at 41.5°C for 24 h to 48 h



https://www.researchgate.net/figure/Colonies-of-Campylobacter-on-blood-agar_fig1_335840996

Campylobacter detection by ISO 10272-1:2017(E)

Confirmation of *Campylobacter*

***Campylobacter* confirmation tests**:

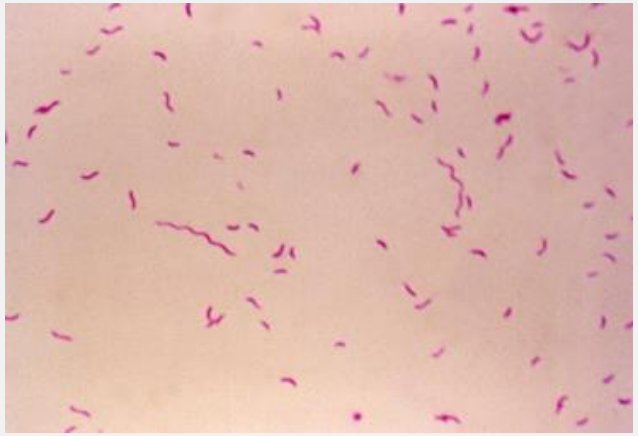
- Examination of morphology and motility
- Absence of aerobic growth at 25°C
- Detection of oxidase activity
- Other alternative or additional tests, e.g. PCR, MALDI-TOF-MS, serological test, etc.

Campylobacter detection by ISO 10272-1:2017(E)

Confirmation of *Campylobacter*

Examination of morphology and motility

- Suspend one colony from the blood agar plate in 1 ml of liquid medium
- Examine for morphology and motility using a microscope
- *Campylobacter* are curved bacilli with a corkscrew motility.

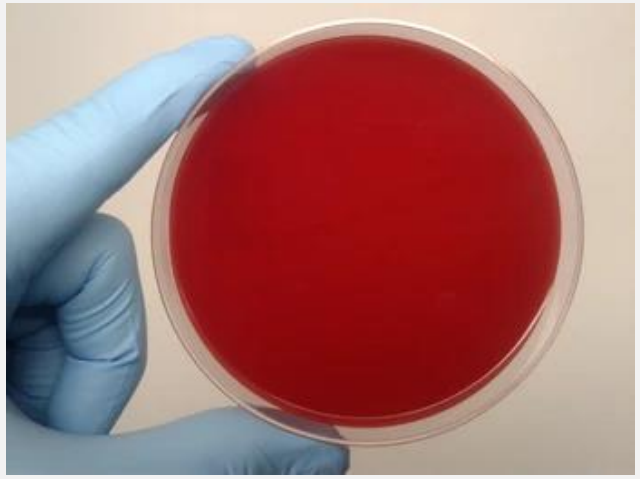


(Whitehouse et al., 2018)

Campylobacter detection by ISO 10272-1:2017(E)

Confirmation of *Campylobacter*

Study of aerobic growth at 25°C



https://www.shutterstock.com/search/blood-agar

Subculture suspect *Campylobacter* colonies on a non-selective blood agar plate

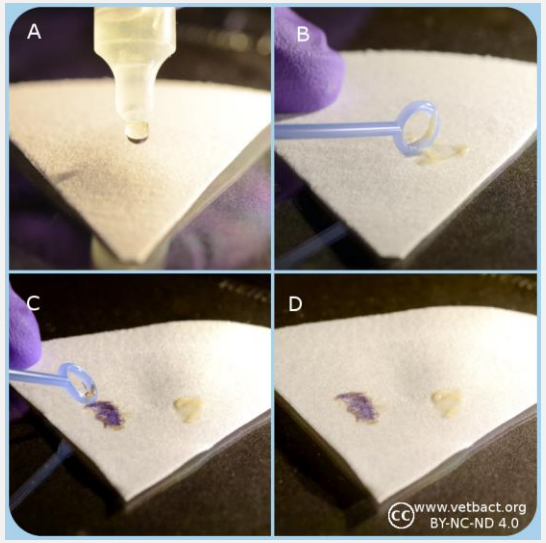
Incubate the plate aerobically at 25 °C for 44 h ± 4 h

Campylobacter detection by ISO 10272-1:2017(E)

Confirmation of *Campylobacter*

Detection of oxidase activity

- Streak a portion of a well-isolated colony onto a filter paper moistened with the oxidase reagent
- Violet or deep purple/blue color within 10 s indicates positive reaction.
- *Campylobacter* are oxidase positive.



http://www.vetbact.org/index.php?displayextinfo=33

Campylobacter detection by ISO 10272-1:2017(E)

Table 3. Confirmation tests for *Campylobacter* spp.

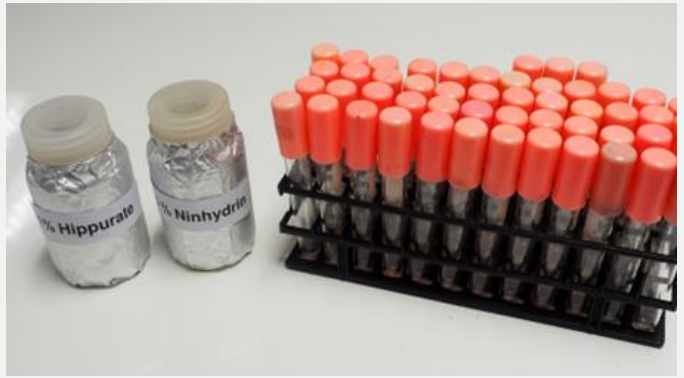
Characteristics of *Campylobacter* spp.	
Morphology	Small curved bacilli
Motility	Corkscrew motility
Aerobic growth at 25°C	-
Oxidase activity	+

Campylobacter detection by ISO 10272-1:2017(E)

Identification of *Campylobacter* species (optional)

Differentiation of *Campylobacter* species

- Detection of catalase activity
- Hippurate hydrolysis test
- Indoxyl acetate hydrolysis test
- No more nalidixic acid sensitivity test

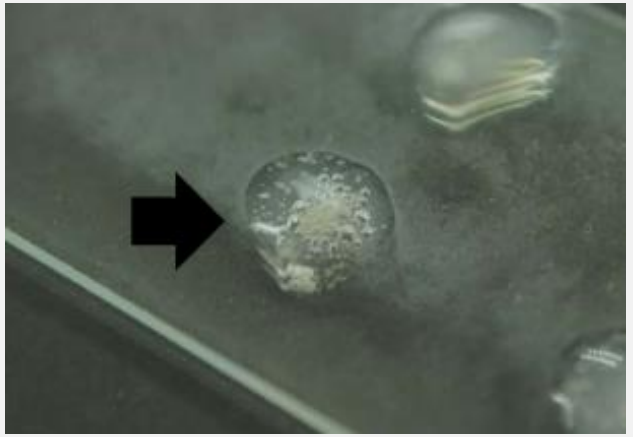


Campylobacter detection by ISO 10272-1:2017(E)

Identification of *Campylobacter* species (optional)

Catalase test

- Deposit a loopful of culture into a drop of hydrogen peroxide solution
- Bubbles within 30 s indicates positive reaction.
- Most *Campylobacter* are catalase positive.

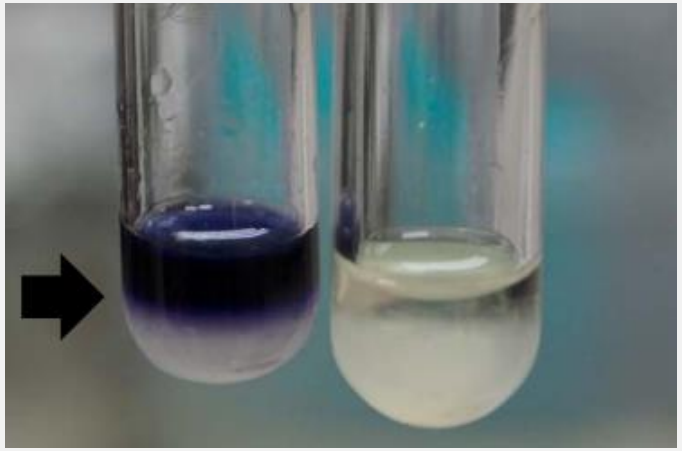


Campylobacter detection by ISO 10272-1:2017(E)

Identification of *Campylobacter* species (optional)

Hippurate hydrolysis test

- Ability to hydrolyze sodium hippurate
- A dark purple color indicates a positive reaction.
- A pale violet color or no color change indicates a negative reaction.



Detection of hippurate hydrolysis

Inoculate a loopful of culture into a tube containing
0.4 ml of sodium hippurate solution (heavy inoculum)

Mix thoroughly and then incubate for 2 h in a water bath set at 37°C
or 4 h in an incubator set at 37°C

Add 0.2 ml of 3.5% ninhydrin solution on top of sodium hippurate solution. Do not shake. Incubate in a water bath or an incubator set at 37°C for 10 min.

Interpret the results

Positive = Dark purple color; Negative = Pale violet color or no color change

Campylobacter detection by ISO 10272-1:2017(E)

Identification of *Campylobacter* species (optional)

Indoxyl acetate hydrolysis test

- Ability to hydrolyze indoxyl acetate
- Place a loopful of colony on an indoxyl acetate disc, then add a drop of sterile distilled water on the disc
- If indoxyl acetate is hydrolyzed, a color change to dark blue will occur within 5 – 10 min.

Campylobacter detection by ISO 10272-1:2017(E)

Table 4. Phenotypic characteristics of thermophilic *Campylobacter* species

Characteristics	*C. jejuni*	*C. coli*	*C. lari*
Catalase	+	+	+
Hippurate hydrolysis	+[a]	-	-
Indoxyl acetate hydrolysis	+	+	-

[a] Some hippurate-negative *C. jejuni* strains have been reported.

Protocol for *Campylobacter* detection by WOAH

Isolation of *Campylobacter*

- Selective media for isolation
- Passive filtration
- Incubation (atmosphere, temperature, and time)

Confirmation of *Campylobacter*

- Identification of *Campylobacter* colonies on solid medium
- Microscopic examination of morphology and motility
- Detection of oxidase
- Aerobic growth at 25 °C
- Latex agglutination test

Identification of *Campylobacter* to the species level

- Detection of hippurate hydrolysis
- Detection of indoxyl acetate hydrolysis

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WOAH protocol for *Campylobacter* detection

Passive filtration

Fecal sample + PBS (approximately 1/10 dilution)

↓

Add 100 µl of the suspension onto a 0.45 or 0.65 µm filter which has been previously placed on top of a non-selective blood agar plate

↓

Wait for 30 - 45 minutes at 37 °C or room temperature to allow the bacteria to migrate through the filter and then remove the filter

↓

Incubate the agar plate at 42 °C for 48 hours under a microaerobic atmosphere

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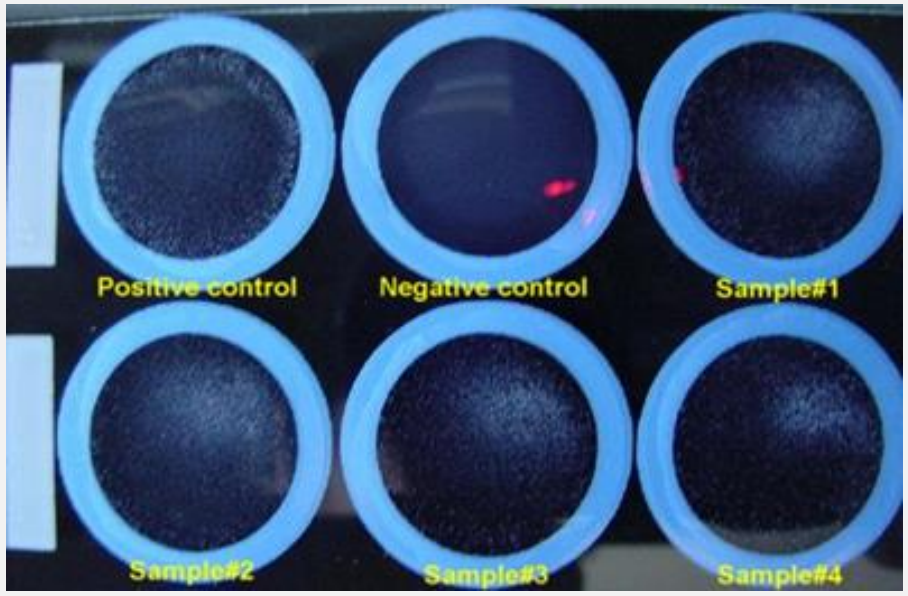
WOAH protocol for *Campylobacter* detection

Confirmation of *Campylobacter* using latex agglutination test



https://microgenbioproducts.com/microgen-latex-agglutination-kits/

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Campylobacter isolation methods for samples from broiler farms and slaughterhouses

Table 5. Recommended *Campylobacter* isolation methods for different sample types

Purpose	Target sample	Method	Media
Detect infection status of broiler farms (before slaughtering)	Boot-swab	Direct plating	mCCDA and Preston
Detect colonization status of broiler flocks (after slaughtering)	Cecal content	Direct plating	mCCDA and Preston or mCCDA
Detect contamination of broiler meat	Neck/breast skin	Selective enrichment	Preston broth and mCCDA

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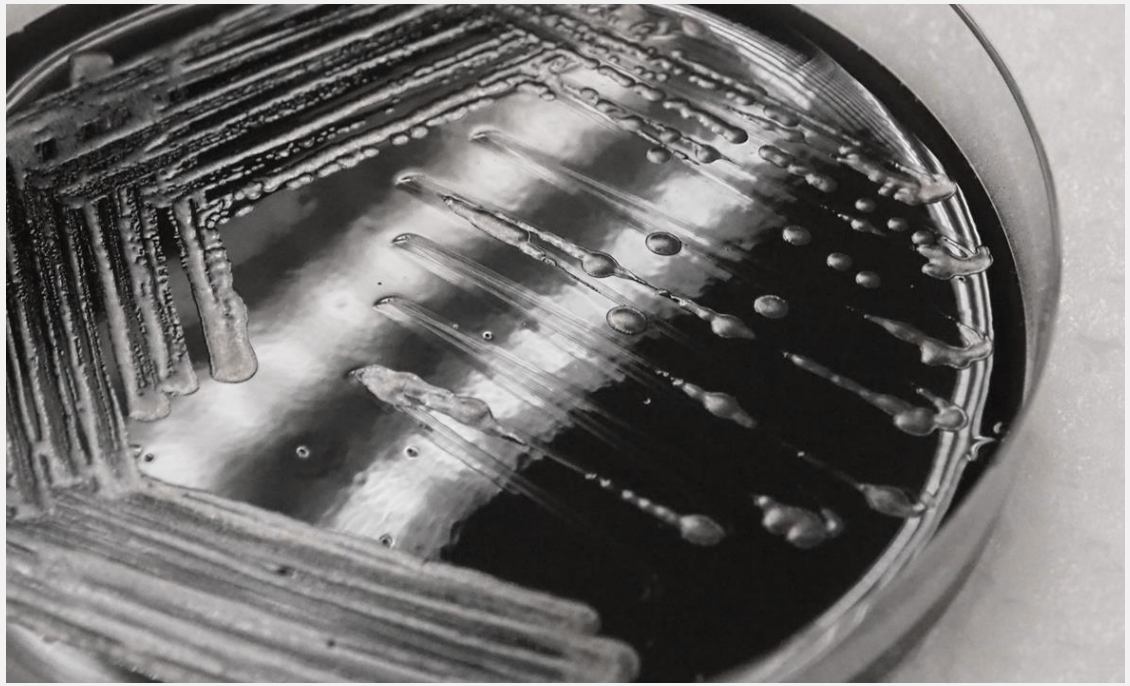
Campylobacter colonies on mCCDA



Typical colonies of *Campylobacter* on mCCDA

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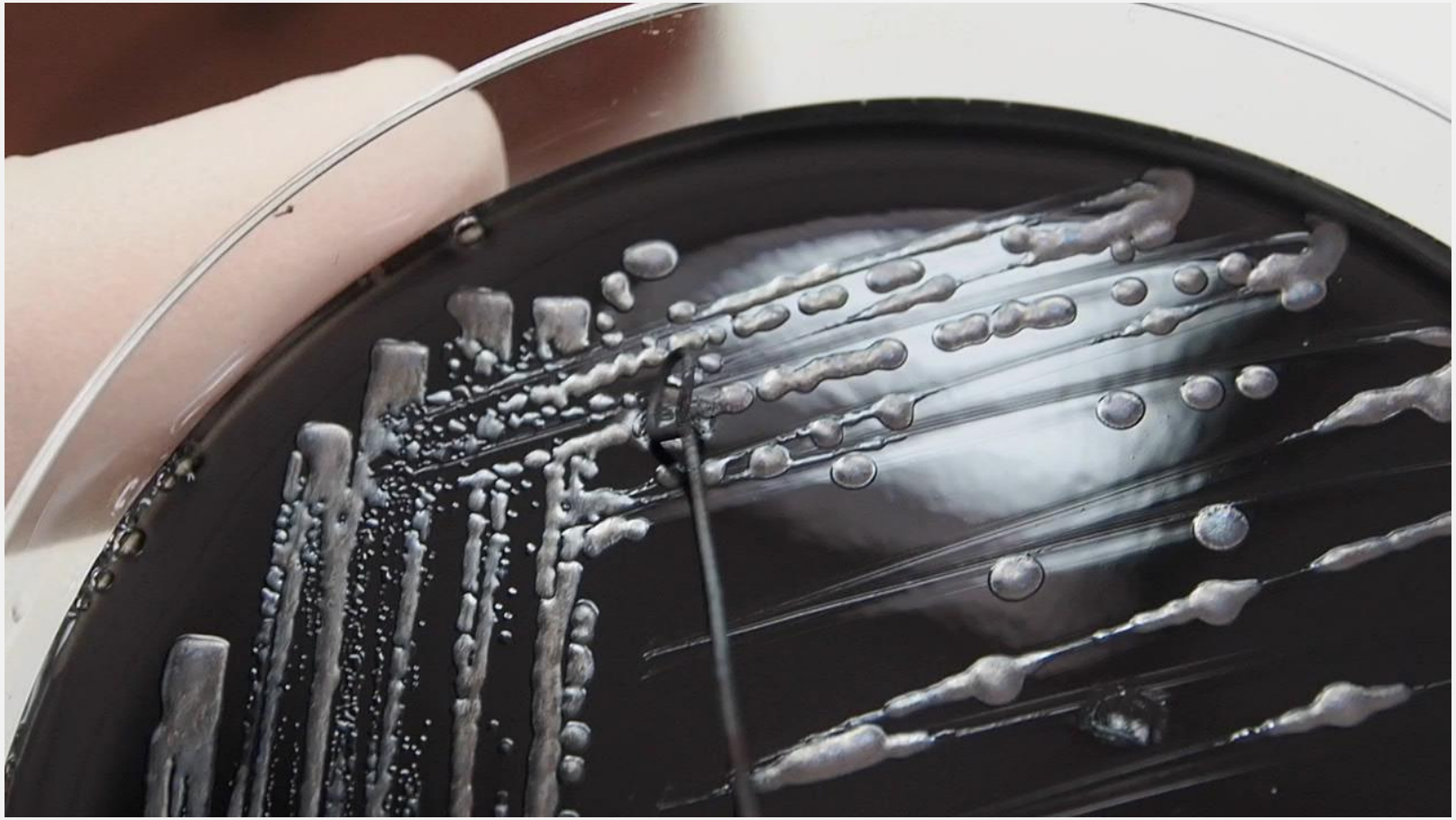
Campylobacter colonies on mCCDA



Sticky colonies with pink and/or peach in color when they are scraped by a loop

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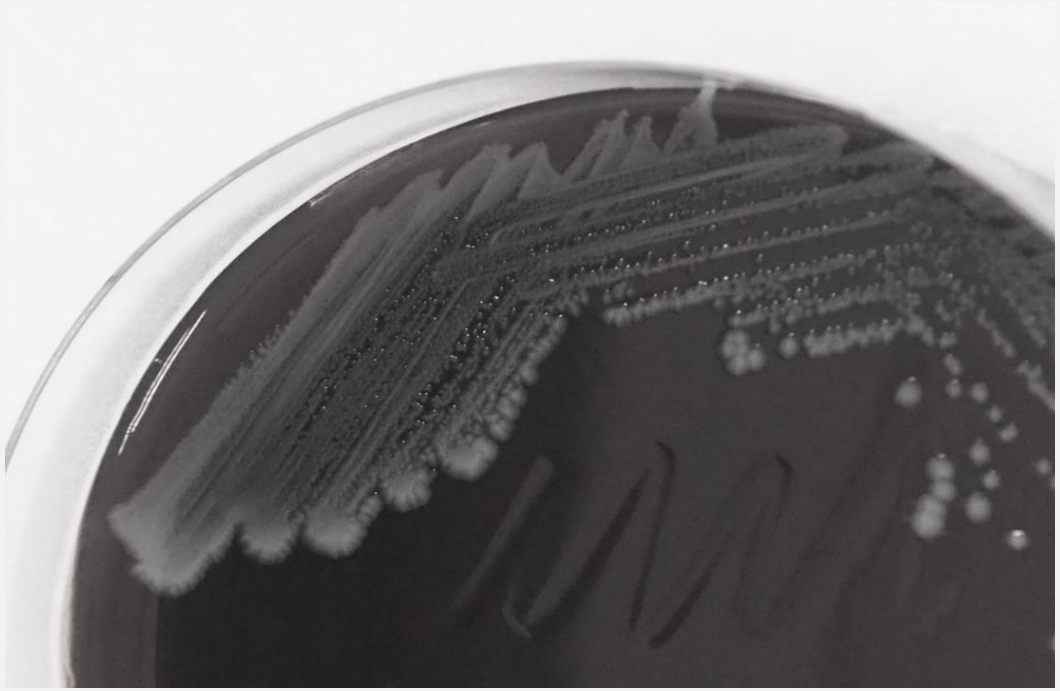
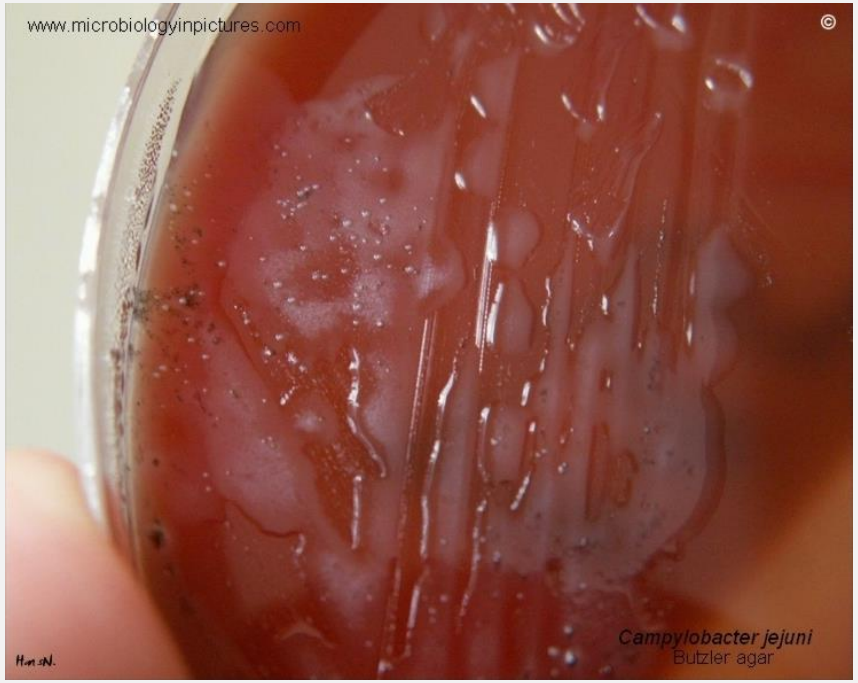
E. coli colonies on mCCDA



Non-sticky colonies with white/creamy in color when they are scraped by a loop

Campylobacter colonies on blood agar

Campylobacter colonies on Preston agar



https://microbiologyinpictures.com/bacteria-photos/campylobacter-jejuni-photos/butzler-agar.html

Typical colonies of *Campylobacter* on blood-based agar

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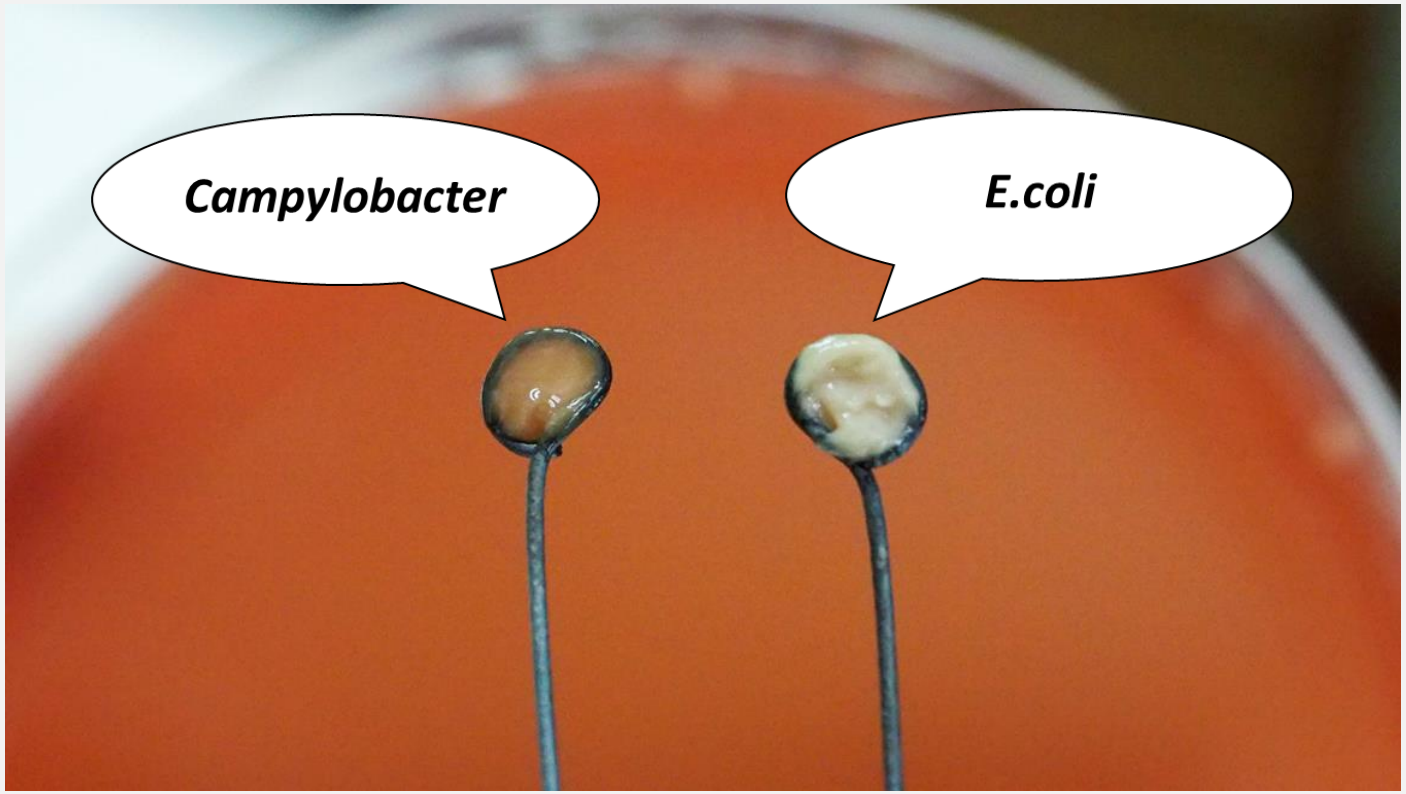
Campylobacter vs. *E. coli* on Preston agar



Brown to orange in color for *Campylobacter* vs. white/creamy in color for *E. coli*

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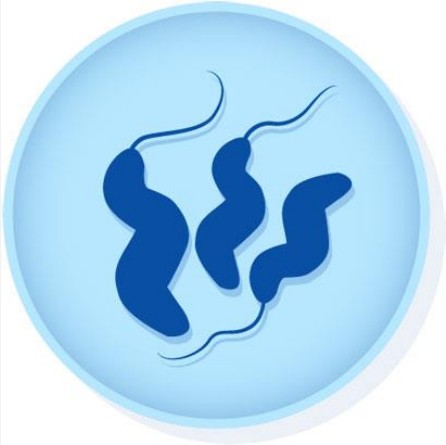
Tips for *Campylobacter* isolation

- **To successfully isolate *Campylobacter* spp.**
  - Protection of samples from light, desiccation, and extreme temperature
  - Process samples as soon as possible (same day)
  - Refrigerate samples only when they cannot be processed on the same day
  - Leave samples at room temperature before processing
  - Recommended transport media: Amies, Cary-Blair, or Stuart



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Tips for *Campylobacter* isolation

❖ To successfully isolate *Campylobacter* spp. (cont.)

- No enrichment of cecal samples
- Use gas pak or gas mix to generate microaerobic conditions (follow manufacturer's instructions)
- Change gas pak or refill gas mix after opening the jar
- Include *Campylobacter* control strain subcultured on an agar plate in the jar
- Incubate samples at 42 °C for 48 hrs.
- Test pure colonies with different phenotypic morphologies

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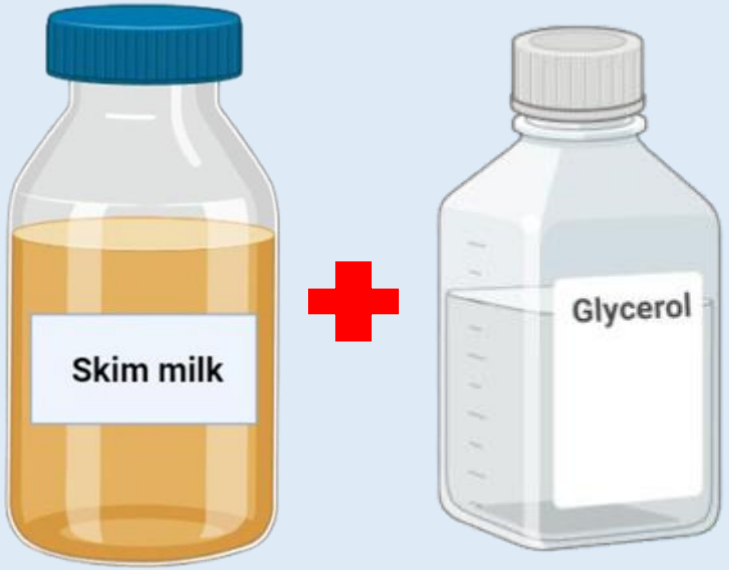
Preservation of *Campylobacter*

- **Preparation of storage medium (Skim milk with glycerol)**



Prepare 10 – 20% skim milk (70 ml) and glycerol (30 ml) in a separate bottle and autoclave at 121 °C for 15 minutes

Mix skim milk and glycerol thoroughly to make a culture storage medium

Distribute 1 ml of the storage medium containing skim milk and 30% glycerol (v/v) into a cryovial tube

Preservation of *Campylobacter*

- **Preparation of bacterial stock culture**

STEP 1 Subculture a single colony of *Campylobacter* onto a blood agar plate and then incubate the plate at 42 °C for 24 hr. under microaerobic conditions

STEP 2 Harvest the fresh overnight culture of *Campylobacter* on the blood agar plate (the entire lawn of bacterial growth)

STEP 3 Transfer the collected *Campylobacter* culture to a cryovial tube containing skim milk and 30% glycerol (v/v), mix thoroughly, and then freeze the stock culture at -80 °C

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Tips for *Campylobacter* preservation

❖ **To successfully preserve *Campylobacter* spp.**

- Good growth of *Campylobacter* before preservation (At least 1 – 2 loopful of culture per cryovial tube)
- Mix the culture and storage medium well before freezing
- Always keep the stock culture at -80 °C
- Avoid repeatedly freezing and thawing the stock culture
- Place the stock culture on ice during the re-subculturing to prevent the stock from completely defrosting

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Molecular detection of *Campylobacter*

PCR-based method for confirmation and identification of *Campylobacter* species

Multiplex PCR

- Previously published protocol (Wang et al., 2002) with some modifications
- Three pairs of primer specific for *Campylobacter* genus, *C. jejuni*, and *C. coli*
- Annealing temperature at 57 °C instead of 59 °C

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Molecular detection of *Campylobacter*

Multiplex PCR for *Campylobacter* confirmation and identification

Target	Primer	PCR product (bp)
Campylobacter spp. (23S rRNA)	FW 5'-TATACCGGTAAGGAGTGCTGGAG-3'	650
	RV 5'-ATCAATTAACCTTCGAGCACCG-3'	
C. jejuni (*hipO*)	FW 5'-ACTTCTTTATTGCTTGCTGC-3'	323
	RV 5'-GCCACAACAAGTAAAGAAGC-3'	
C. coli (*glyA*)	FW 5'-GTAAAACCAAAGCTTATCGTG-3'	126
	RV 5'-TCCAGCAATGTGTGCAATG-3'	

Molecular detection of *Campylobacter*

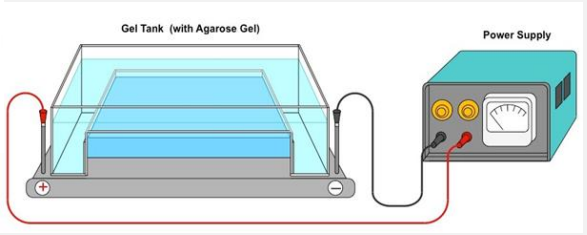
Multiplex PCR for *Campylobacter* confirmation and identification

Multiplex PCR conditions

- Pre-denaturation: 95°C for 6 min.
- Denaturation: 95°C for 30 sec.
- Annealing: 57°C for 30 sec.
- Extension: 72°C for 30 sec.

} 30 cycles

- Final extension: 72°C for 7 min.



https://microbenotes.com/agarose-gel-electrophoresis/

Then, check PCR products in 1.2 – 1.5% agarose gel @ 90 volts for 45 min.

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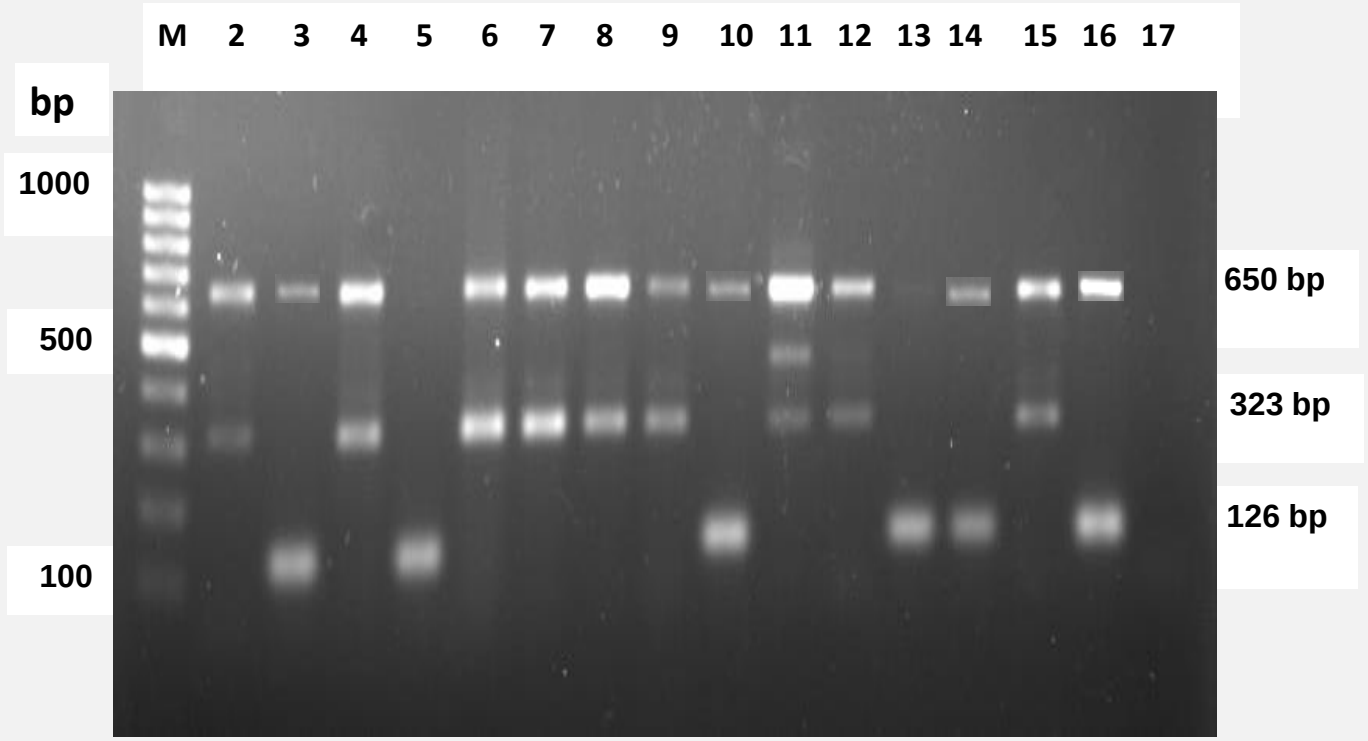
Molecular detection of *Campylobacter*

Multiplex PCR for *Campylobacter* confirmation and identification



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