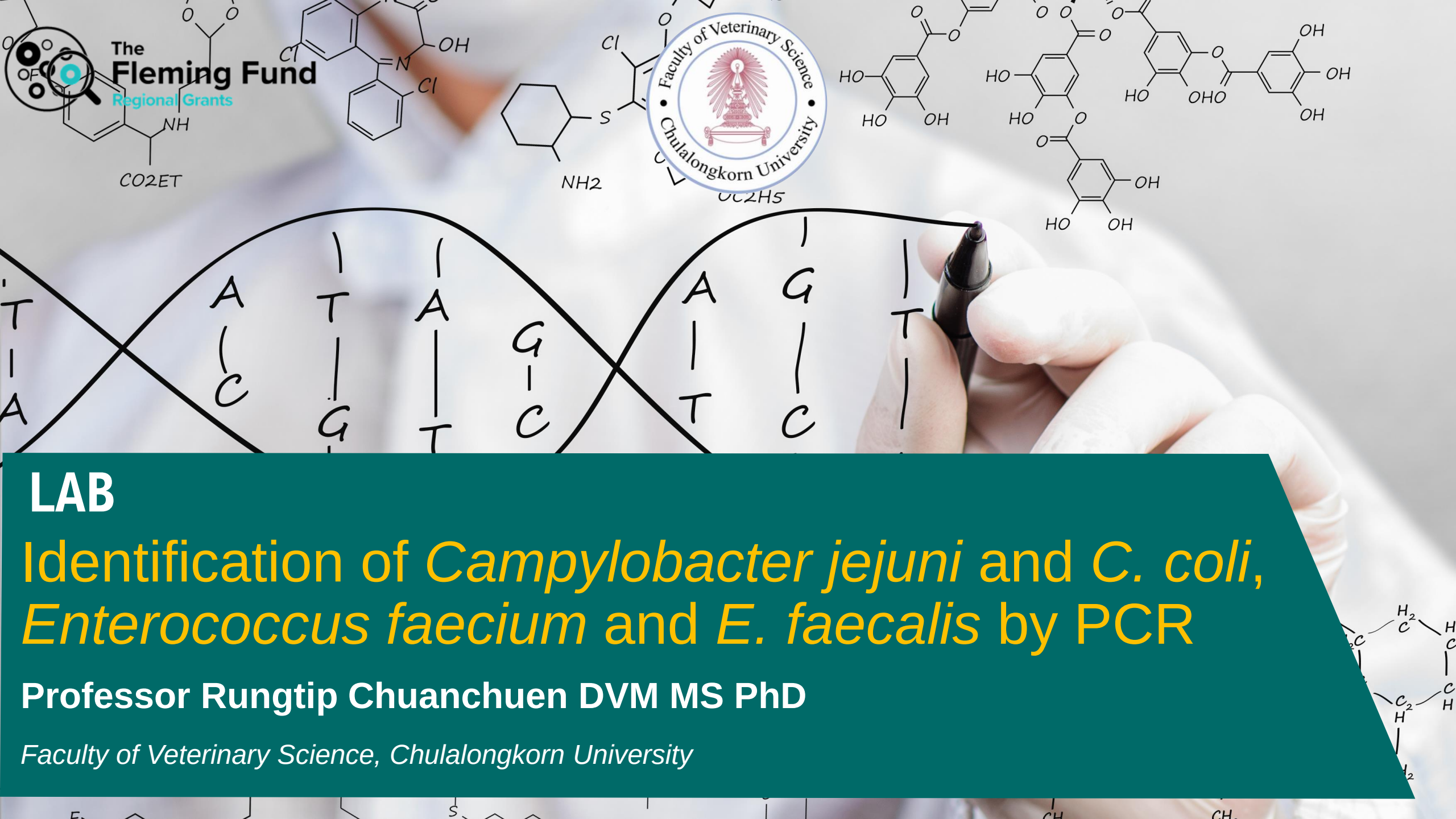


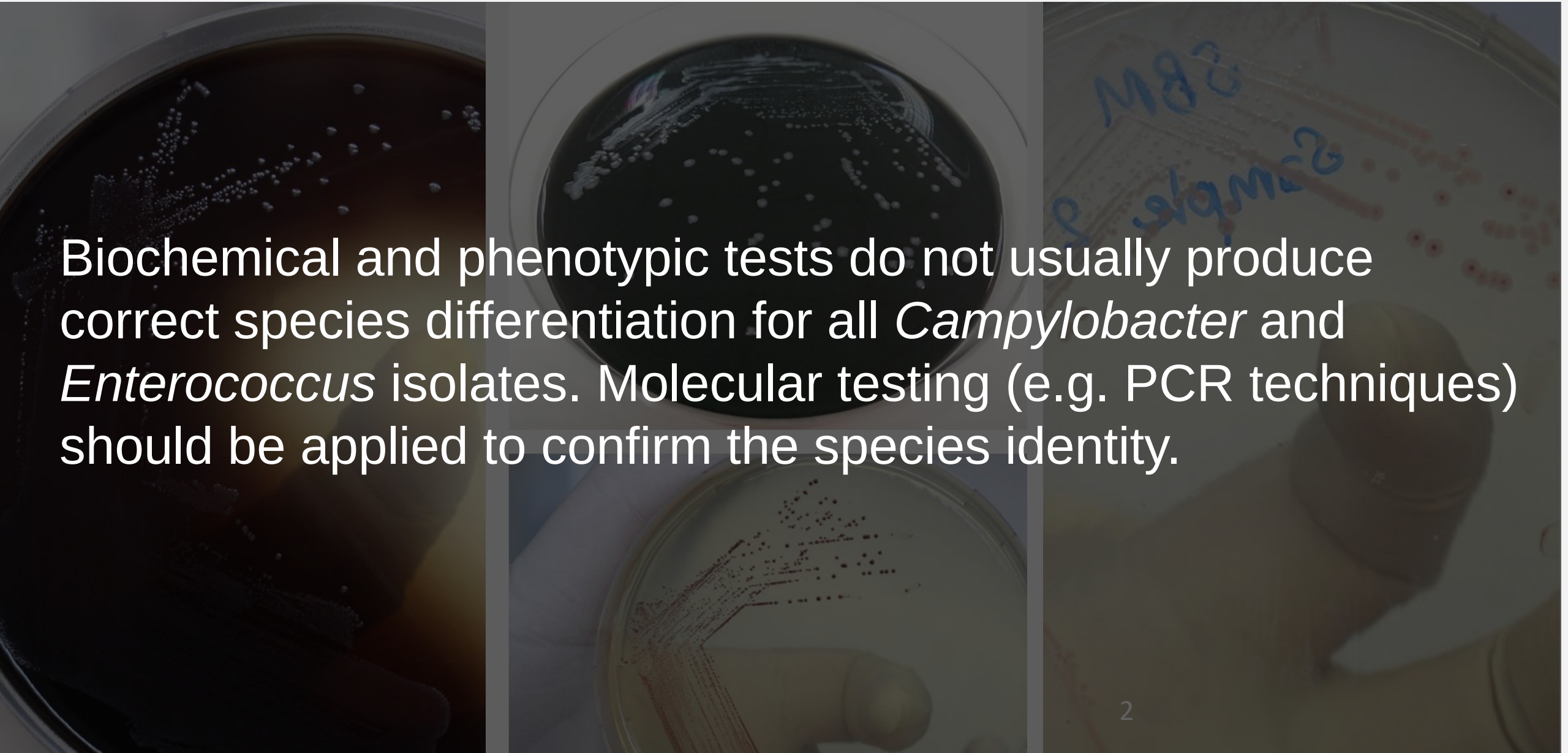
LAB

Identification of *Campylobacter jejuni* and *C. coli*, *Enterococcus faecium* and *E. faecalis* by PCR

Professor Rungtip Chuanchuen DVM MS PhD

Faculty of Veterinary Science, Chulalongkorn University

Why PCR for *Campylobacter* and *Enterococcus*?



Biochemical and phenotypic tests do not usually produce correct species differentiation for all *Campylobacter* and *Enterococcus* isolates. Molecular testing (e.g. PCR techniques) should be applied to confirm the species identity.

PCR identification of *E. faecalis & E. faecium*

Amplification of *ddl* (D-alanine-D-alanine ligase) gene **(Dutka-Malen et al. (1995)**

Target	Primer	PCR product (bp)
E. faecalis	FW 5'-ATCAAGTACAGTTAGTCTT-3' RV 5'-ACGATTCAAAGCTAACTG-3'	941
E. faecium	FW 5'-GCAAGGCTTAGAGA-3' RV 5'-CATCGTGTAAGCTAACTTC-3'	550

1. Pre-denaturation	94°C	2 min
2. Denaturation	94°C	1 min
3. Annealing	54°C	1 min
4. Extension	72°C	1 min
Repeat 2 – 4 for 30 cycles		
5. Final extension	72°C	10 min
6. Storage	4°C	∞

PCR identification of *E. faecalis & E. faecium*

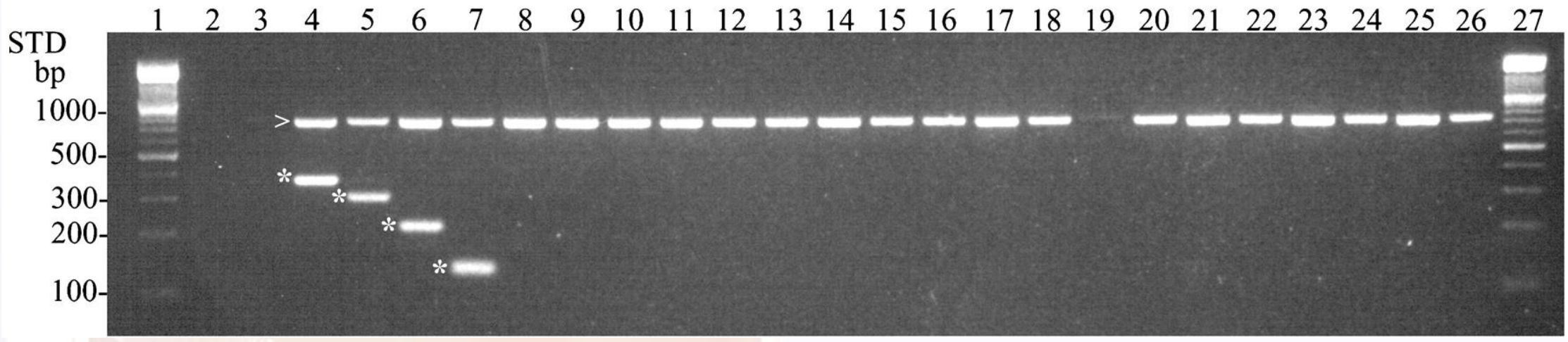
Amplification of ***sodA*** (superoxide dismutase) gene (**Jackson et al. (2004**)

Target	Primer	PCR product (bp)
E. faecalis	FW 5'-ACTTATGTGACTAACTTAACC-3' RV 5'-TAATGGTGAATCTTGGTTTGG-3'	360
E. faecium	FW 5'-GAAAAAACAATAGAAGAATTAT-3' RV 5'-TGCTTTTTTGAATTCTTCTTTA-3'	215

1. Pre-denaturation	95°C	4 min
2. Denaturation	95°C	30 sec
3. Annealing	55°C	1 min
4. Extension	72°C	1 min
Repeat 2 – 4 for 30 cycles		
5. Final extension	72°C	7 min
6. Storage	4°C	∞

PCR identification of *E. faecalis & E. faecium*

Identification of *Enterococcus* species using multiplex PCR (Jackson et al., 2004)



Lanes 4 to 7: L4 = *E. faecalis* (360 bp); L5 = *E. durans* (295 bp);
L6 = *E. faecium* (215 bp); L7 = *E. malodoratus* (134 bp)

>, enterococcus genus-specific amplicon based on 16S rRNA primers (733 bp)

PCR for identification of *Campylobacter* species

Multiplex PCR for *Campylobacter* genus, *C. jejuni*,and *C. coli* (**Wang et al., 2002**)

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

1. Pre-denaturationc	95°C	6 min
2. Denaturation	95°C	30 sec
3. Annealing	57°C	30 sec
4. Extension	72°C	30 sec
Repeat 2 – 4 for 30 cycles		
5. Final extension	c	7 min
6. Storage	4°C	∞

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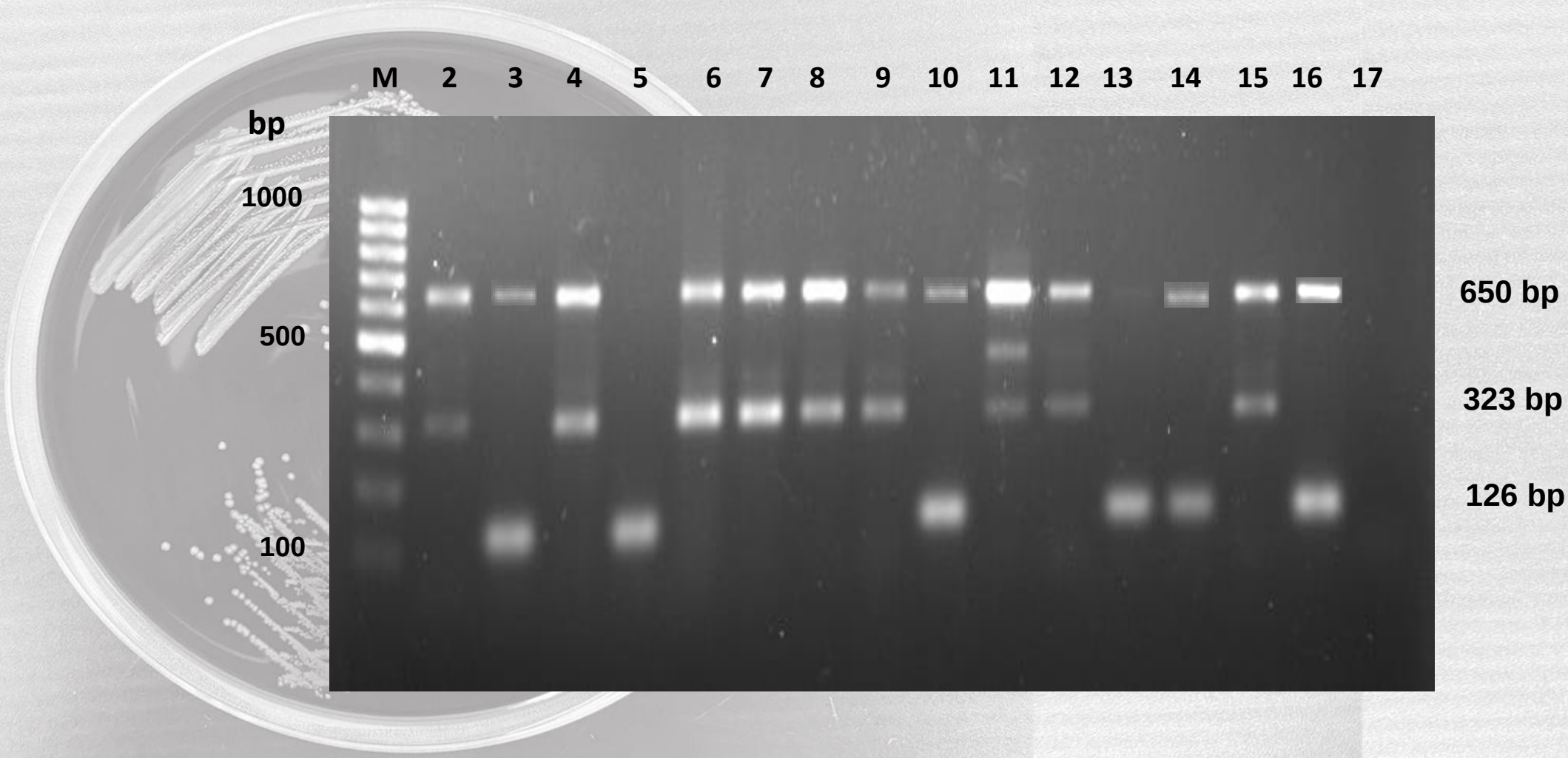
PCR for identification of *Campylobacter* species



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Conventional PCR

Carry out PCR under sterile conditions.

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Flow of a conventional PCR experiment

1

Make a plan

2

Prepare PCR components

3

Set PCR reaction

4

Run thermocycler

5

Run electrophoresis

6

PCR product visualization

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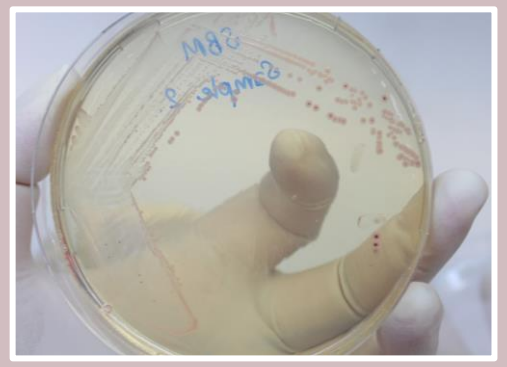
TODAY assignment

1 Identification of *Campylobacter* species

1.Sample C1
2.Sample C2

2 Identification of *Enterococcus* species



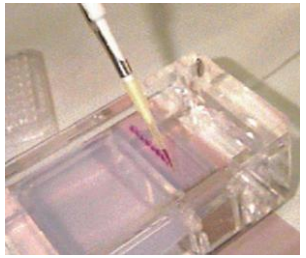
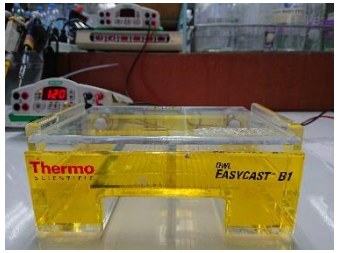
1.Sample E1
2.Sample E2

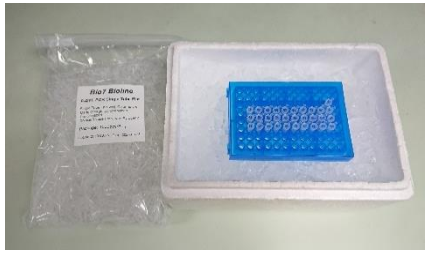
Make a plan for your PCR

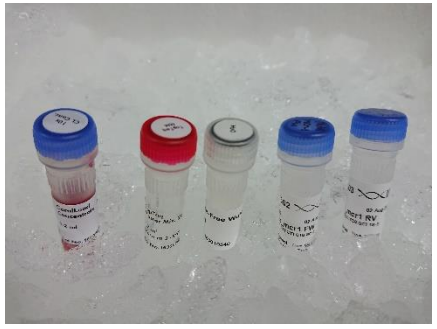
Materials

- Molecular marker (Ladder 100bp)
- Electrophoresis buffer (TAE or TBE)
- Tips (filter) for pipettes 1 µL to 1000 µL
- Agarose
- PCR components
- TE buffer
- Tris HCl buffer
- Crushed ice
- Mineral oil (if necessary)
- etc





Equipment

- PCR thermocycler
- Pipettes for 1 µL to 1000 µL
- Eppendorf tubes
- PCR tubes
- Electrophoresis unit
- Microwave
- Autoclave
- Eppendorf centrifuge (PCR tubes)
- Photo camera
- UV-transilluminator
- Water bath

etc

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Flow of a conventional PCR experiment

1

Make a plan

2

Prepare PCR components

3

Set PCR reaction

4

Run thermocycler

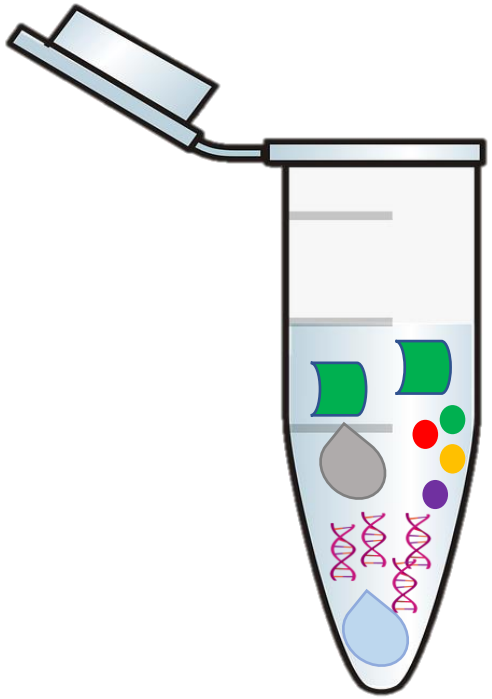
5

Run electrophoresis

6

PCR product visualization

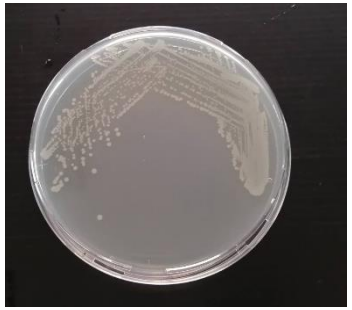
Components of a PCR reaction



- Template DNA
- Thermostable DNA polymerase
- Primers
- dNTPs
- Buffer and salts (KCl, $MgCl_2$)
- PCR grade water
- Optional: DMSO, Formamide

Template DNA preparation

Whole cell boiling methods



✓ Suspend a loopful of culture in 100 µl of sterile DW.

✓ Boiled 10 min at 100ºC

✓ Place on ice

✓ Centrifuged 5 min at 12,000-13,000xg

✓ Transfer supernatant to a new tube

✓ Store at -20ºC

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Flow of a conventional PCR experiment

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PCR product visualization

Setting PCR reaction

Keep and thaw all reagents on ice.

Assemble reaction mix into 50 µL volume in a thin walled 0.2 mL PCR tubes.

Add reagents in following order:

Water → buffer → dNTPs → $MgCl_2$, → template DNA → primers → Taq polymerase.

↓

Gently mix by tapping tube. Briefly centrifuge to settle tube contents.

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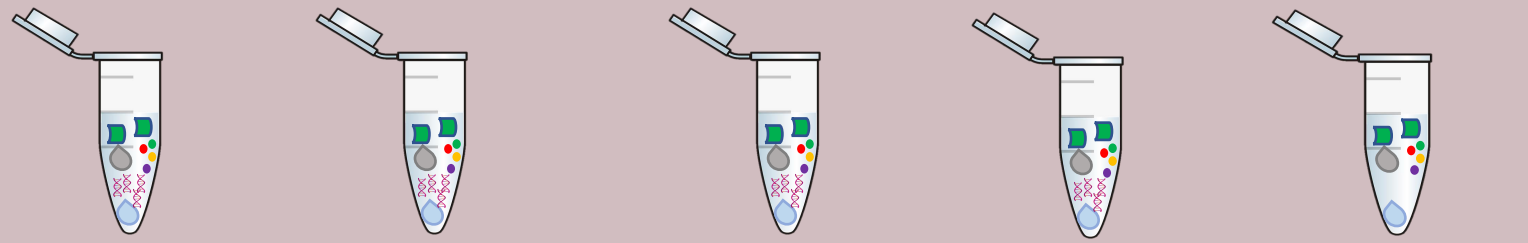
Setting PCR reaction

1 *Enterococcus* species



Sample E1 | Sample E2 | Faecium | Faecalis | Negative control

Positive control

2 *Campylobacter* species

Sample C1 | Sample C2 | *C. jejuni* | *C. coli* | Negative control

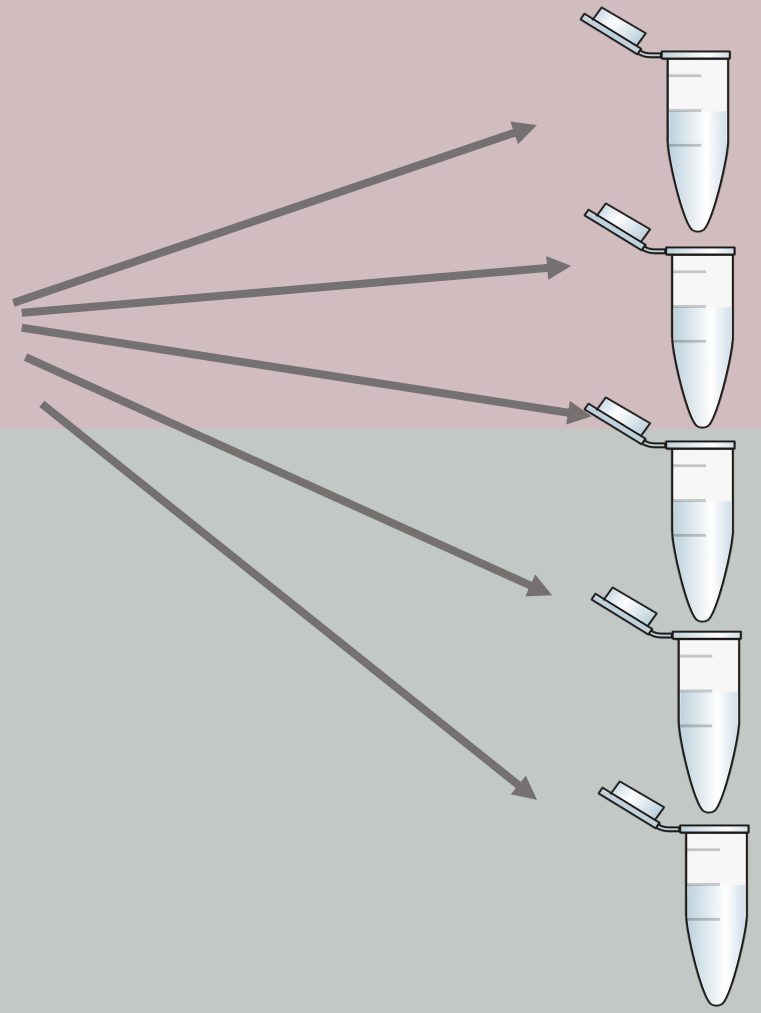
Positive control

Setting PCR reaction

PCR cocktail

DNA
polymerase
dNTPs
reaction buffer
water

Aliquot into PCR tubes



Add DNA as the last step

Setting PCR reaction

Primers for multiplex PCR

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

Wang et al. (2002)

Setting PCR reaction

Primers for multiplex PCR

Target	Primer	PCR product (bp)
E. faecalis	FW 5'-ACTTATGTGACTAACTTAACC -3'	360
	RV 5'-TAATGGTGAATCTTGGTTTGG-3'	
E. faecium	FW 5'-GAAAAAACAATAGAAGAATTAT-3'	215
	RV 5'-TGCTTTTTTGAATTCTTCTTTA-3'	

Jackson et al. (2004)

Setting PCR reaction

1 *Enterococcus* spp.

Component	Stock conc.	Volume in 1 rxn (µL)	No. of rxn	Volume in cocktail (6rxn)	Final conc. in 1 rxn (1ul)
Sterile DW		25.75	6	154.5	Add to 50 µl
PCR buffer	10x	5	6	30	1x
dNTPs	10mM	1	6	6	200 µM
$MgCl_2$	25mM	3	6	18	1.5 mM
E. faecalis Forward primer	10 nmol/µL (10 µM)	2.5	6	15	0.5 µM
E. faecalis Reverse primer	10 nmol/µL (10 µM)	2.5	6	15	0.5 µM
E. faecium Forward primer	10 nmol/µL (10 µM)	2.5	6	15	0.5 µM
E. faecium Reverse primer	10 nmol/µL (10 µM)	2.5	6	15	0.5 µM
Taq polymerase	5 unit/µL	0.25	6	1.5	1.25 units
DNA template	25 ng/µL	5	-	-	50 ng
Total	-	50	-	45x6=270	-

Setting PCR reaction

2 *Campylobacter* species

Component	Stock conc.	Volume in 1 rxn (µL)	No. of rxn	Volume in cocktail (6rxn)	Final conc. In 1 rxn
Sterile DW		18.75	6	112.5	Add to 50 µl
PCR buffer	10x	5	6	30	1x
dNTPs	10mM	1	6	6	200 µM of each
$MgCl_2$	25mM	3	6	18	1.5 mM
C. jejuni Forward primer	10 nmol/µL (10 µM)	2.5	6	15	0.5 µM
C. jejuni Reverse primer	10 nmol/µL (10 µM)	2.5	6	15	0.5 µM
C. coli Forward primer	10 nmol/µL (10 µM)	5	6	30	1 µM
C. coli Reverse primer	10 nmol/µL (10 µM)	5	6	30	1 µM
23SrRNA Forward primer	10 nmol/µL (10 µM)	1	6	6	0.2 µM
23SrRNA Reverse primer	10 nmol/µL (10 µM)	1	6	6	0.2 µM
Taq polymerase	5 unit/µL	0.25	6	1.5	1.25 units
DNA template	25 ng/µL	5	-	-	125 ng
Total	-	50 µL	-	45 x6 = 270µL	-

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Flow of a conventional PCR experiment

1

Make a plan

2

Prepare PCR components

3

Set PCR reaction

4

Run thermocycler

5

Run electrophoresis

6

PCR product visualization

Set PCR cycle and run PCR

1 PCR condition for *Enterococcus*

PCR step	Temp	Time
1. Pre-denaturation	95°C	4 minutes
2. Denaturation	95°C	30 secs
3. Annealing	55°C	1 minute.
4. Extension	72°	1 minute
Repeat step 2 to 4 for 30 cycles		
5. Final extension	72°	7 minute

Set PCR cycle and run PCR

2 PCR condition for *Campylobacter*

PCR step	Temp	Time
1. Pre-denaturation	95°C	6 minutes
2. Denaturation	95°C	30 sec
3. Annealing	59°C	30 sec
4. Extension	72°	30 sec
Repeat step 2 to 4 for 24-30 cycles		
5. Final extension	72°	7 minute

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Set PCR cycle and run PCR

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Flow of a conventional PCR experiment

1

Make a plan

2

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Set PCR reaction

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Run thermocycler

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Run electrophoresis

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PCR product visualization

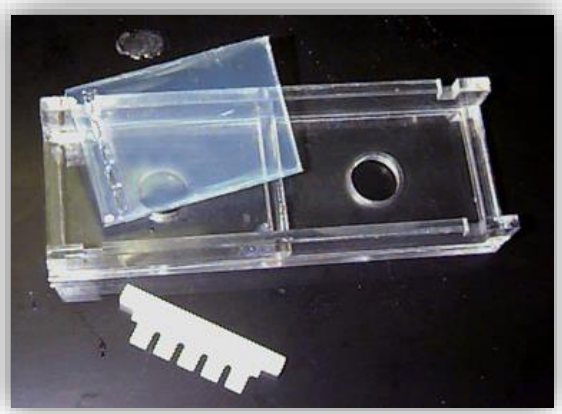
Preparation of agarose gel

reparation of agarose gel

- Weigh out the appropriate amount of agarose needed

- Pour the agarose into a flask
- Fill a graduated cylinder with the appropriate volume of TAE buffer.
- Pour the solution into a small flask

- Heat the solution to dissolve agarose for intervals of 15-20 seconds in a microwave oven.
- Gently swirl to disperse the contents after each interval

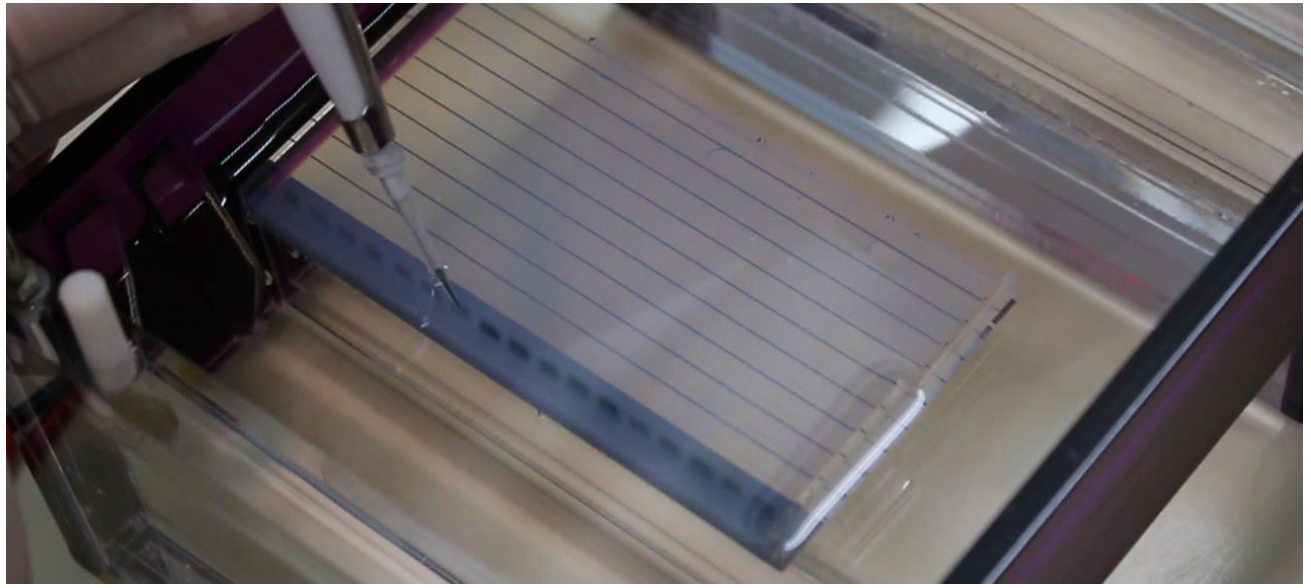


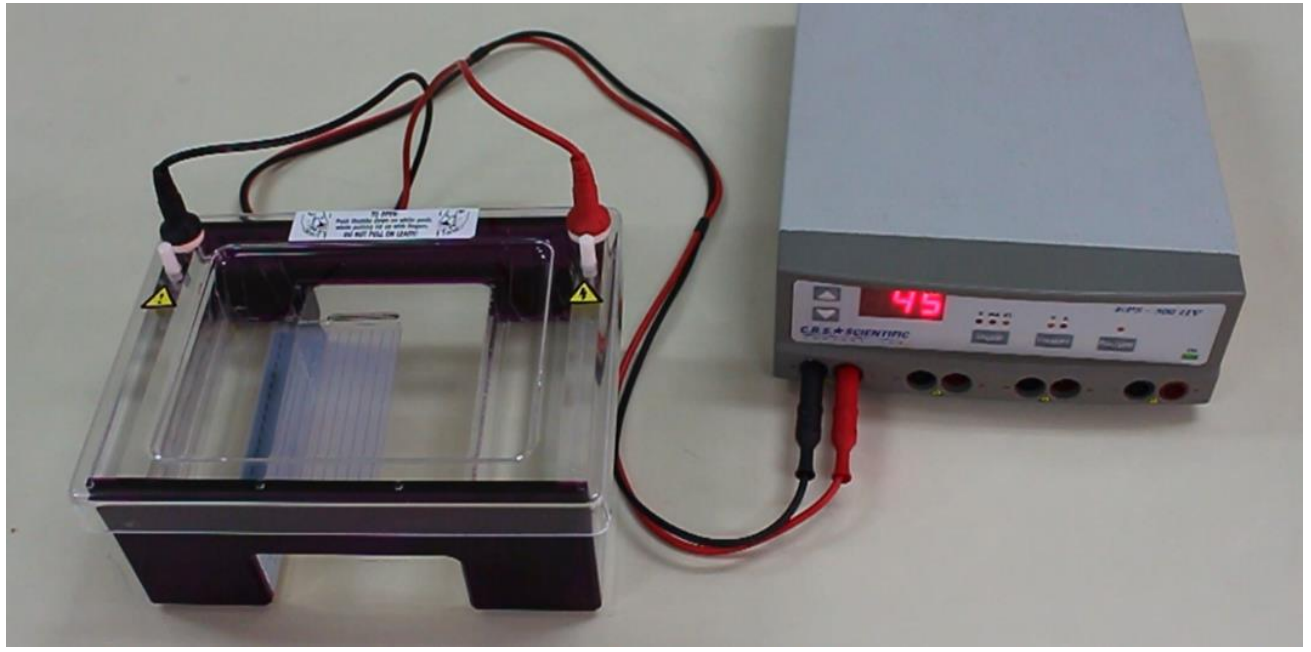
- Pour agarose gel solutions into the casting tray.
- Wait until the gel is set.
- Do not move the casting platform until the gel sets.

Preparing gel

Running PCR products on agarose gel

- Mix DNA with loading dye
- Load to a gel.
- Apply electricity(Run DNA on gel)
- Staining
- Visualize DNA
- PCR products are visible as different “bands”.





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Flow of a conventional PCR experiment

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Prepare PCR components

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Run electrophoresis

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PCR product visualization

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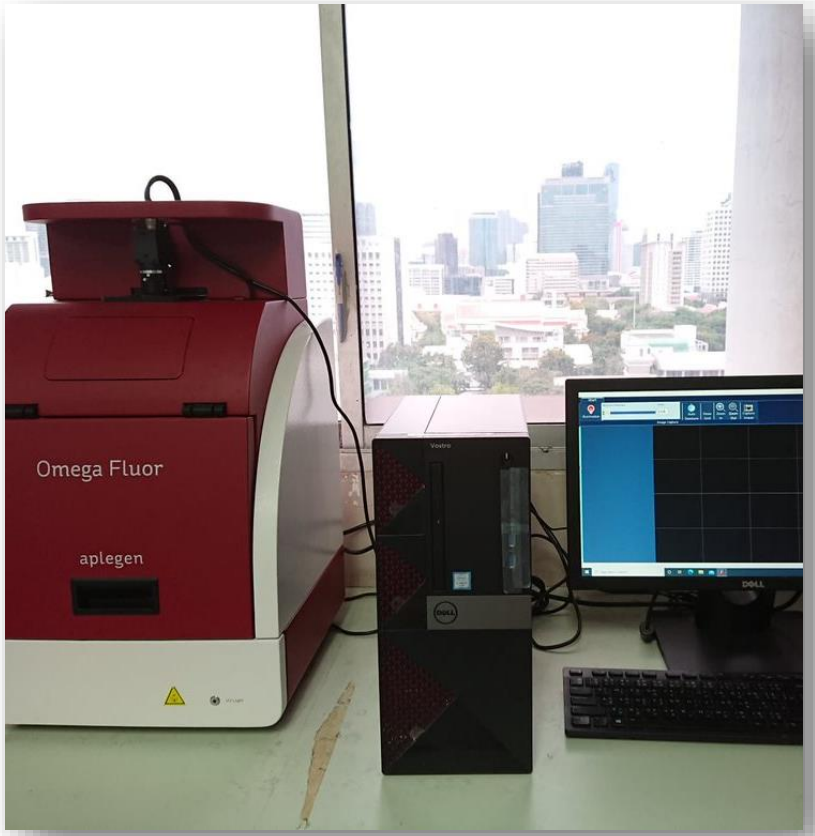
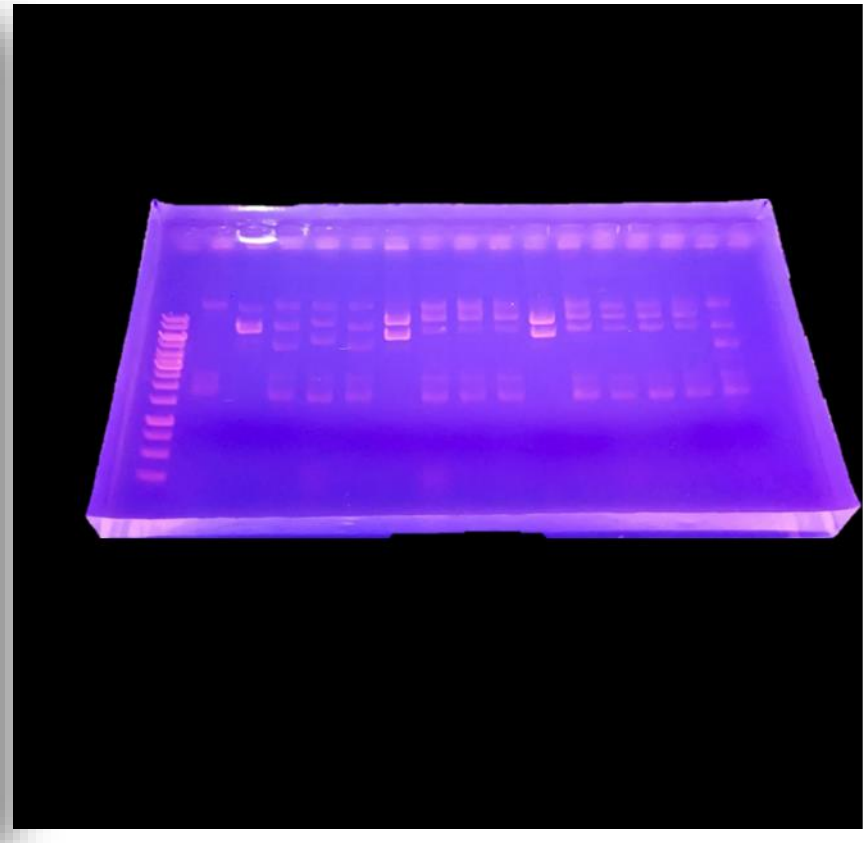
Visualizing PCR products



UV transilluminator



DNA gel

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Visualizing PCR products

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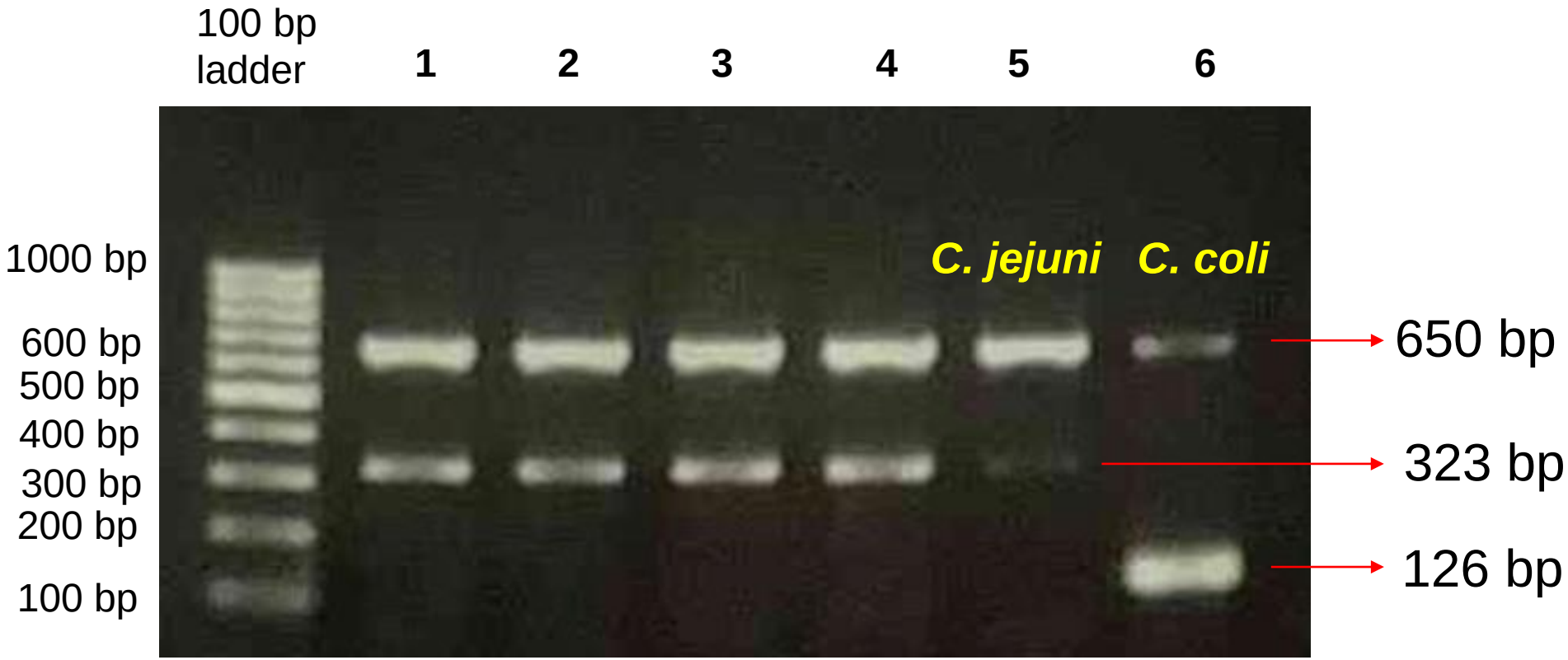
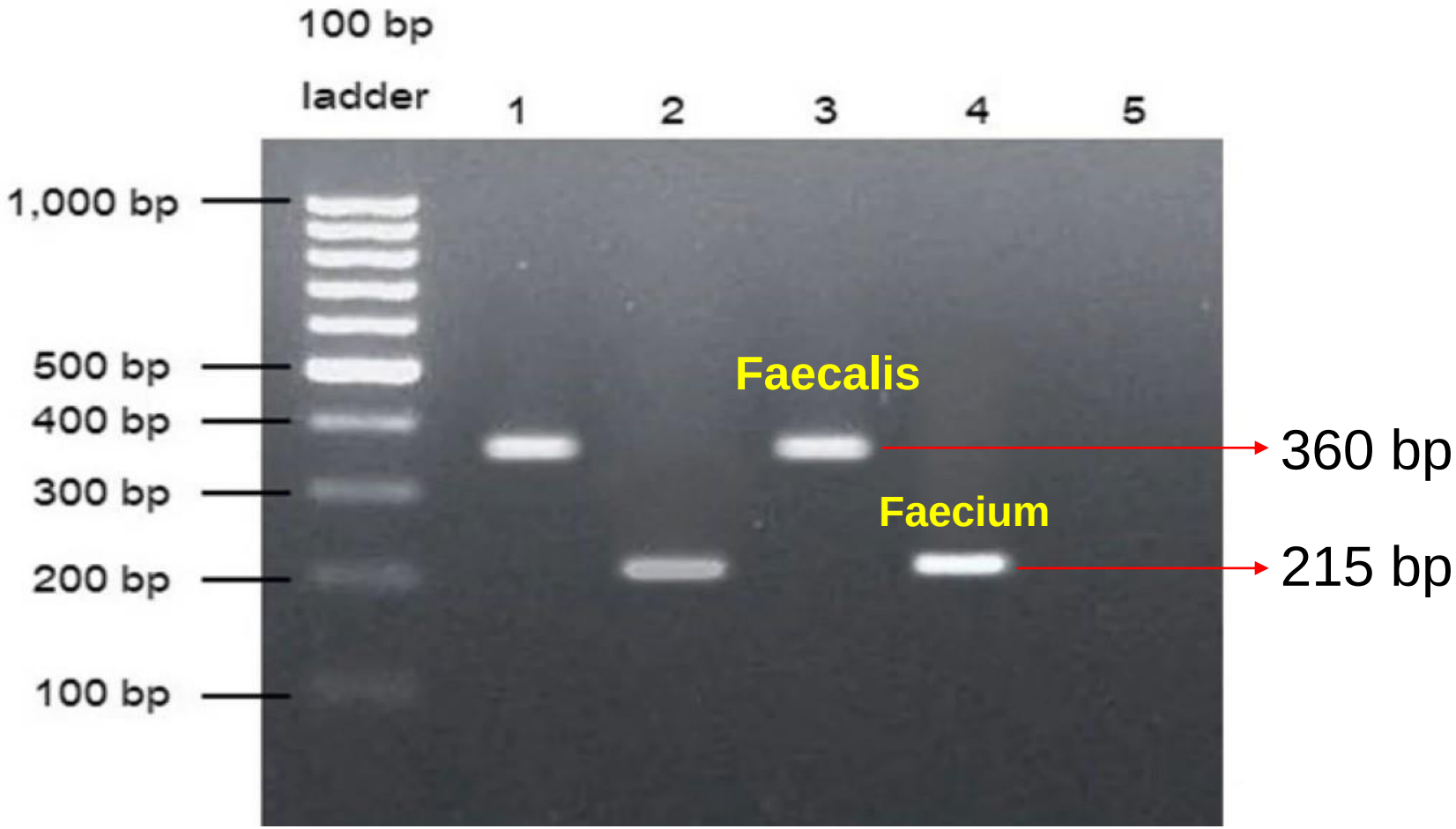
Visualizing PCR products



PCR results from identification of *Campylobacter* spp.

Visualizing PCR products



PCR results from identification of *Enterococcus* spp.

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Thank you

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The views expressed do not necessarily reflect the UK Government's official policies.