Global Salm-Surv

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

Laboratory Protocols

Level 4 Training Course

Isolation and identification of enterohaemorrhagic Escherichia coli O157.

1st Ed. February 2003

Edited by: Rene S. Hendriksen (DFVF)

Foreword:

Enterohaemorrhagic strains of *Escherichia coli* , especially *E. coli* O157, have emerged as important enteric pathogens in recent years. Various serotypes have been implicated in human disease, but O157 is the most common. The H7 flagellum is frequently but not always present on O157 isolates.

The isolates produce a toxin almost identical to that of *Shigella dysenteriae* and this is responsible for the gastroenteritis, which ranges in severity from mild to bloody diarrhoea and haemorrhagic colitis. Some patients develop haemolytic uraemic syndrome (HUS) with anaemia and acute renal failure. Children are most at risk of developing HUS.

The main source of O157 appears to be cattle but other animal species (wild birds, sheep, deer etc.) have also been implicated. O157 are commonly found among pigs, but appear rarely to produce verotoxins. Human infections commonly derive from beef products, un pasteurised milk and contaminated water.

Detection is by direct culture or selective enrichment followed by plating on sorbitol MacConkey agar. The sensitivity can be improved by immuno-magnetic separation.

Contents

1. Isolation and identification of *Escherichia coli O157* from faeces and food.

Introduction

The following procedures will guide you through the steps necessary to isolate *Escherichia coli O157* confidently from faeces and food and carry out a short biochemical identification of *Escherichia coli O157*:

1. Isolation of *Escherichia coli O157* from food and faeces.
2. Biochemical confirmation of *Escherichia coli O157* suspect colonies (after isolation from faeces or food).

Escherichia coli O157 is generally identified as being a non-sorbitole fermenting, (NLFs) Gram negative rod shaped organism, ranging 0.7 to 1.5 x 2 to 5 μm in size, oxidase negative, catalase positive and indole positive. Some of these characteristics are used for biochemical confirmation of *Escherichia coli O157*.

Isolation of *Escherichia coli O157* from animal faeces and food
Some farm animals mainly cattle are infected with *Escherichia coli O157* without showing signs of the illness, i.e. they are subclinically infected. Faeces from these herds of cattle may contain *Escherichia coli O157* in low numbers. In food, *Escherichia coli O157* may also be present in low numbers in addition to a lot of other micro-organisms, and they may be injured.
To diminish the risk of obtaining false negative results, an enrichment of a large faeces or food sample, in combination with immunomagnetic beads and two selective media are performed:

- Enrichment in modified tryptone soya broth containing novobiocin (mTSB+N).
- Separation and concentration by the use of immunomagnetic beads coated with antibodies to *Escherichia coli O157*.
- Subcultivation on cefixime tellurite sorbitol MacConkey agar (CT-SMAC) and cefixime rhamnose sorbitol MacConkey agar (CR-SMAC) or another selective agar media.

The ISO-16654 standard is applicable to products intended for human consumption or feeding of animals. The procedures for isolation of *Escherichia coli O157* from food and animal faeces given in this protocol follows the ISO-16654:2001 standard (ref. 1). An overview of the procedure of isolating *Escherichia coli O157* according to other standards is given in Appendix 1. Instead of CR-SMAC plates you may use other selective agar plates, e.g. Sorbitol MacConkey agar, Sorbitol MacConkey agar with 4 methylumbelliferyl-β-D-glucuronide, Violet Red Bile agar with 4 methylumbelliferyl-β-D-glucuronide, Haemorrhagic colitis (HC) agar, Sorbitol MacConkey agar with chromogen 5-bromo-4-chloro-3-indolyl-β-D-glucuronide agar and CHROMagar (ref. 2).
The separation step in this manual using immunomagnetic beads is based on the Dynadeads® (ref.4). Therefore the procedure and method described in this manuals could differ if the using another manufacturer or brand of immunomagnetic beads.

Biochemical confirmation and serotyping of *Escherichia coli O157.*
Subsequently it is confirmed with biochemical tests whether the colonies resembling *Escherichia coli O157* on CT-SMAC and CR-SMAC are *Escherichia coli O157*. The ISO-16654 standard (ref. 1) recommends using the Indole tests in this order. The test is described in this manual. In addition to the Indole test, the *Escherichia coli O157* colonies are serotyped. The biochemical confirmation of *Escherichia coli O157* and the serotyping may be performed at the same time.

Safety

Several countries follow the CDC/NIH biosafety recommendations indicated in "Biosafety in Microbiological and Biomedical Laboratories", 4th Edition, 1999 (ref. 4) that recommend Biosafety Level 2 practices for all the *Escherichia coli O157* work.

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

References

1. ISO 16654 :2001. 1st ed. Microbiology – Horizontal method for the detection of *Escherichia coli O157,* International Organization for Standardization, Geneve, Switzerland.
2. Post, D.E. Food-borne pathogens monograph number 5 *Escherichia coli* and *Shigella species*. Oxoid Limited, wade Road, Basingstoke, Hampshire RG24, UK.
3. CDC/NIH. Biosafety in Microbiological and Biomedical Laboratories (BMBL) - 4th edition, US Government Printing Office, Washington. http://www.cdc.gov/od/ohs/biosfty/biosfty.htm
4. Dynal – Simply Magnetic prod. no.: 710.03 rev.no.:003

Isolation of *Escherichia coli O157* from food and animal faeces.

Materials

Equipment

- Erlenmeyer flasks (500 ml) etc. sterile (for enrichment)
- Disposable inoculation loops (1 µl)
- Graduated pipettes. 1000µl:
- Balance
- Incubators at 37°C and 41.5°C
- Bunsen burner
- Pipette tips for Graduated pipettes 1000ul
- Wood spatulas.
- Cotton swabs.
- Eppendorf tubes. 1.8 ml
- Magnet holder.
- Sample mixer for magnet holder.
- Racks for tubes
- Laboratory coats:
- Waste containers:
- Bottle of 70% ethanol

Media

- Modified tryptone soya broth containing novobiocin (mTSB+N) 225 ml.
- immunomagnetic beads coated with antibodies to *Escherichia coli O157*.
- Wash buffer: modified phosphate buffer 0.01mol/l ph 7,2
- Cefixime tellurite sorbitol MacConkey agar (CT-SMAC)
- Cefixime rhamnose sorbitol MacConkey agar (CR-SMAC)
- Nutrient agar plates
- Kovacs reagens
- Tryptone/tryptophan medium for indol

Bacterial strains

- Food samples
- Faeces samples
- E.coli CCUG 29889
- P.aeruginosa ATCC 27853
- E.coli ATCC 25922

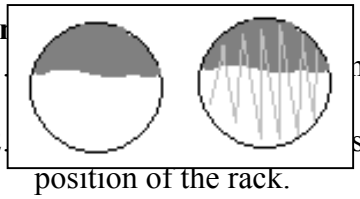
Safety

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

Procedure

Day 1:
Enrichment:
Weigh out 25 g food or faeces with a sterile wooden spatula, put it into an Erlenmeyer flask etc. and add 225 ml modified tryptone soya broth containing novobiocin (pre-warmed to 41,5°C) to obtain 1 part sample + 9 part buffer. Mix. Incubate at 41,5°C for 6 hours.
After 6 hours incubation a sample is taken followed by immunomagnetic separation. The initial suspensions continue the incubation and addition samples is taken every 6 hours up to a total of 24 hours.

I



1. magnet from the
2. s in the right position of the rack.
3. Resuspend or mix the immunomagnetic beads until the pellet in the buttom disappears.
4. Dispense 20 µl of the beads into the tubes.
5. Transfer 1 ml of pre-warmed mTSB+N enrichment broth into the eppendorf-tubes. Eventual a tube lock could be used.
6. Invert the tubes a few times to insure the content is mixed.
7. Incubate at room temperature for 10-30 minutes with continuously weak agitation. If a sample mixer is used the agitation should be about 12-20 rpm.
8. Insert the magnet into the rack and invert it slowly 3-4 times to insure a pellet is formed at the side up to the magnet. Allow the immunomagnetic beads to recover for 3 minutes.
9. Open the lid of the tube and discard the supernatant by pipette from the opposite side of the magnet. Be careful NOT to remove the beads, as this would affect the result of the recovery. Avoid emptying the tube completely.
10. Remove the magnet from the rack.
11. Add 1 ml of wash buffer PBS/Tween 20. Invert the rack / tubes a few times to

Procedure

Theory / comments

The volume of the faeces investigated determines the sensitivity of detection, the larger the volume the higher sensitivity. Volumes ranging from a swab sample of less than 1 gram to 25 grams are commonly applied. It is critical that the relationship between faeces and enrichment broth remains 1 part faeces to 9 part buffer for all volumes. The more faeces the higher is the sensitivity
This ratio also applies to pooled samples, for instance 5x5 grams of faeces to 225 ml enrichment broth.

When testing food samples it is recommended to use a stomacher to reduce the interference of food with immunomagnetic beads. Only non frozen samples that are likely to have a high background of other bacteria e.g. raw meat should be tested after 6 h incubation oly. Others should be tested after both 6 h and 24 h.

A 6 h incubation followed by immunomagnetic separation and plateing onto selective agars can yield a presumptive positive result which can become negative after further 18 h incubation as heavy growth of other bacteria may rise and make it difficult to find the *Escherichia coli O157*.

The instruction next to this is from the manufacturers that produces the Dynal beads. If you use beads from another manufacturer disregard this and follow the instruction followed by the product.

Dynabeads react with all types of *Escherichia coli O157both.*

Use an aseptic technique to avoid aerosoles.

This procedure could be difficult to apply to fat products or cheese.

Sensitivity is increased by concentration cells from enrichment culture by immunomagnetic separation. However the dynabeads might record a false neg. rate between 2-10 % depending on the inoculum level and background flora.

Theory / comments

resuspend the beads.

12. Repeat step 8 to 11.
13. Repeat step 8 to 10.
14. Resuspend the beads in each tube with 100 µl of wash buffer PBS/Tween 20.
15. Mix the content of the tube and transfer 50 µl of the beads onto each of the two selective agar plates.

In the same sample without immunomagnetic separation this false rate is significatly increased and is often more than 25%.

Spread on selective agar plates:
Spread with a swab the 50 µl of beads onto the first half of the CT-SMAC and CR-SMAC). Streak with a loop from the first half of the plates to the second half. (see figure 1.).

One should follow the recommended swab-streak technique when plating as this will results in a better isolated colony formation on the media.

1. Swab. 2. Streak with a loop.
Figure 1.

Incubate the plates at 37^{o}C overnight (18-24 hours).

Day 2: Subcultivation of presumptive colonies of *Escherichia coli O157*.

Read the CT-SMAC plates:
A typical *Escherichia coli O157* colony are slightly transparent, almost colourless with a weak pale brownish appearance but otherwise typical for *Escherichia coli with a diameter of 1mm.*
Read the CR-SMAC plates:
Typical *Escherichia coli O157* colonies on a CR-SMAC agar plate appear as on the CT-SMAC agar plates almost coloured as a straw. To determined the right morphology on the media for *Escherichia coli O157* you could see the Appendix 2.

Some sorbitol positive *Escherichia coli O157* are NOT detected on CT-SMAC plates.

Use any other solid selective media of your own choice complementary to CT-SMAC agar and aimed for isolation of *Escherichia coli O157*

When investigating samples for *Escherichia coli O157,* CR-SMAC offers a media that is effective at differentiating the bacgground flora, and when used in conjunction with CT-SMAC allows a high level of recovery of O157

Pick five *Escherichia coli O157* suspect colonies from the CR-SMAC agar plates and the CT-SMAC agar plates. Streak the colonies onto non-selective media, e.g. nutrient agar plates for biochemical confirmation and

Procedure

agglutination. Incubate the plates at 37°C for 18-24 hours. Continue with the biochemical confirmation on pure cultures.

Day 3: Biochemical confirmation.
From a pure culture on nutrient agar plates, inoculate the media:

- Tryptone/tryptophane medium for indole, (use a 1 µl loop full).

Incubate the biochemical tests at 37°C for 18 to 24 hours (overnight).

Day 4: Read biochemical tests
Add reagents to the following tests:
Indole: Add 1 ml of the Kovacs reagent to the medium.

Read the results of the biochemical tests according to appendix 2 and write the results in the record sheets to identify *Escherichia coli O157.*
Escherichia coli O157 should then be agglutinated according to the procedure in the Protocol.

Theory / comments

Some indole negative mutations have been found.

Commerial available biochemical identification kits and latex agglutinations kit for *Escherichia coli O157could be use for confirmation.*

Furter characterization of virulence factors (VT1 or VT2) could be investigated.

2. Composition and preparation of culture media and reagents

The media and reagents are available from several companies including Oxoid, Merck, Dynal 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. Refer to Appendix 2 for a colour presentation of growth of *Escherichia coli O157* on selective agar media and positive and negative reaction of the biochemical test.

Modified tryptone soya broth supplemented with Novobiocin. (mTSB+N) (ref. 1)

Pancreatic digest of casein	17.0 g
Papaic digest of soybean meal	3.0 g
Sodium chloride	5.0 g
Di-potassium hydrogen phosphate	4.0 g
Glucose	2.5 g
Bile salts	1.5 g
Novobiocin solution	4.0 ml
Water	1000 ml

Preparation:
Dissolve the dehydrated medium in the water by heating if necessary. Transfer into a bottle and autoclave at 121°C for 15 min. Allow the media to cool to 50°C before adding the novobiocin supplement as appropriate. Adjust pH to ~7.4 -/+ 0.2 at 25°C after sterilisation.

Novobiocin solution. (ref. 2)

Novobiocin	0.5 g
Water	100 ml

Preparation:
Dissolve the novobiocin in the water and sterilise by membrane filtration. Do not store the solution for more that one day.

Wash buffer: Modified phosphate buffer 0.01mol/l of ph 7,2 (ref. 2)

Sodium chloride	8.0 g
Potassium chloride	0,2 g
Dissodium hydrogen phosphate (anhydrous)	1,44 g
Potassium dihydrogen phosphate (anhydrous)	0,24 g
Polyoxyethylene sorbitan monolaurate (Tween 20)	0,2 ml
Water	1000 ml

Preparation:
Dissolve the components in the water. Transfer into a bottle and autoclave at 121°C for 15 min. Adjust pH to ~7.2 -/+ 0.2 at 25°C after sterilisation. The solusion may appear cloudy but clears up.

Cefixime Tellurite Sorbitol MacConkey agar. (CT-SMAC) (ref. 3)

Peptone	20.0 g
Agar	15.0 g
Sorbitol	10.0 g
Bile salts No.: 3	1.5 g
Sodium chloride	5.0 g
Neutral red	0.03 g
Crystal violet	0.001 g
Potassium tellurite solution	1.0 ml
Cefixime solution	1.0 ml
Water	1000 ml

Preparation:
Dissolve the dehydrated medium in the water by heating if necessary. Transfer into a bottle and autoclave at 121°C for 15 min. Allow the media to cool to 44-47°C before adding the potassium tellurite and cefixime supplement as appropriate. Adjust pH to ~7.1 -/+ 0.2 at 25°C after sterilisation. The final plates could be stored at 1-5°C for 14 days.

Potassium tellurite solution. (ref. 2)

Potassium tellurite	0.25 g
Water	100 ml

Preparation:
Dissolve the Potassium tellurite in the water and sterilise by membrane filtration. The solution could be stored at room-temperature for up till 1 month.

Cefixime solution. (ref. 2)

Cefixime	**5.0 mg**
Water	100 ml

Preparation:
Dissolve the Potassium tellurite in 2 ml ethanol and 98 ml of water and sterilise by membrane filtration. The solution could be stored at 1-5°C for up till 1 week.

Cefixime Rhamnose Sorbitol MacConkey agar. (CR-SMAC) (ref. 3)

Peptone	20.0 g
Agar	15.0 g
Sorbitol	10.0 g
Bile salts No.: 3	1.5 g
Sodium chloride	5.0 g
Neutral red	0.03 g
Crystal violet	0.001 g
Rhamnose	5.0 g
Cefixime solution	1.0 ml
Water	1000 ml

Preparation:
Dissolve the dehydrated medium in the water by heating if necessary. Transfer into a bottle and autoclave at 121°C for 15 min. Allow the media to cool to 44-47°C before adding the potassium

tellurite and cefixime supplement as appropriate. Adjust pH to ~7.1 -/+ 0.2 at 25°C after sterilisation. The final plates could be stored at 1-5°C for 14 days.

Potassium tellurite solution. (ref. 2)

Potassium tellurite	0.25 g
Water	100 ml

Preparation:
Dissolve the Potassium tellurite in the water and sterilise by membrane filtration. The solution could be stored at room-temperature for up till 1 month.

Nutrient agar (ref. 2)

Meat extract	3.0 g
Peptone	5.0 g
Agar	12 g to 18 g[1)]
Water	1000 ml

[1)] Depending on the gel strength of the agar.

Preparation:
Dissolve the dehydrated medium in the water by heating if necessary. Adjust pH to ~7.0 after sterilisation, transfer into bottles and autoclave at 121°C for 20 min.

Kovacs reagent for indole reaction (ref. 2)

4-Dimethylaminobenzaldehyde	5 g
Hydrochloric acid, ρ = 1.18 - 1.19 g/ml	25 ml
2-Methylbutan-2-ol	75 ml

Preparation:
Mix the components.

Tryptone/tryptophane medium for indole reaction (ref. 2)

Tryptone	10 g
Sodium chloride	5 g
DL-Tryptophane	1 g
Water	1000 ml

Preparation:
Dissolve tryptone and chemicals in the water at 100°C. Adjust pH to ~ 7.5 after sterilisation. Dispense 5 ml of medium into tubes and autoclave at 121°C for 15 min.

Description
The media is used for testing the liberation of indole from tryptophane. When Kovacs reagent containing amyl alcohol and p-dimethylaminobenzaldehyde is added, indole can be extracted into the amyl alcohol layer by shaking a little. Indole and p-dimethylaminobenzaldehyde produces a red/pink colour.

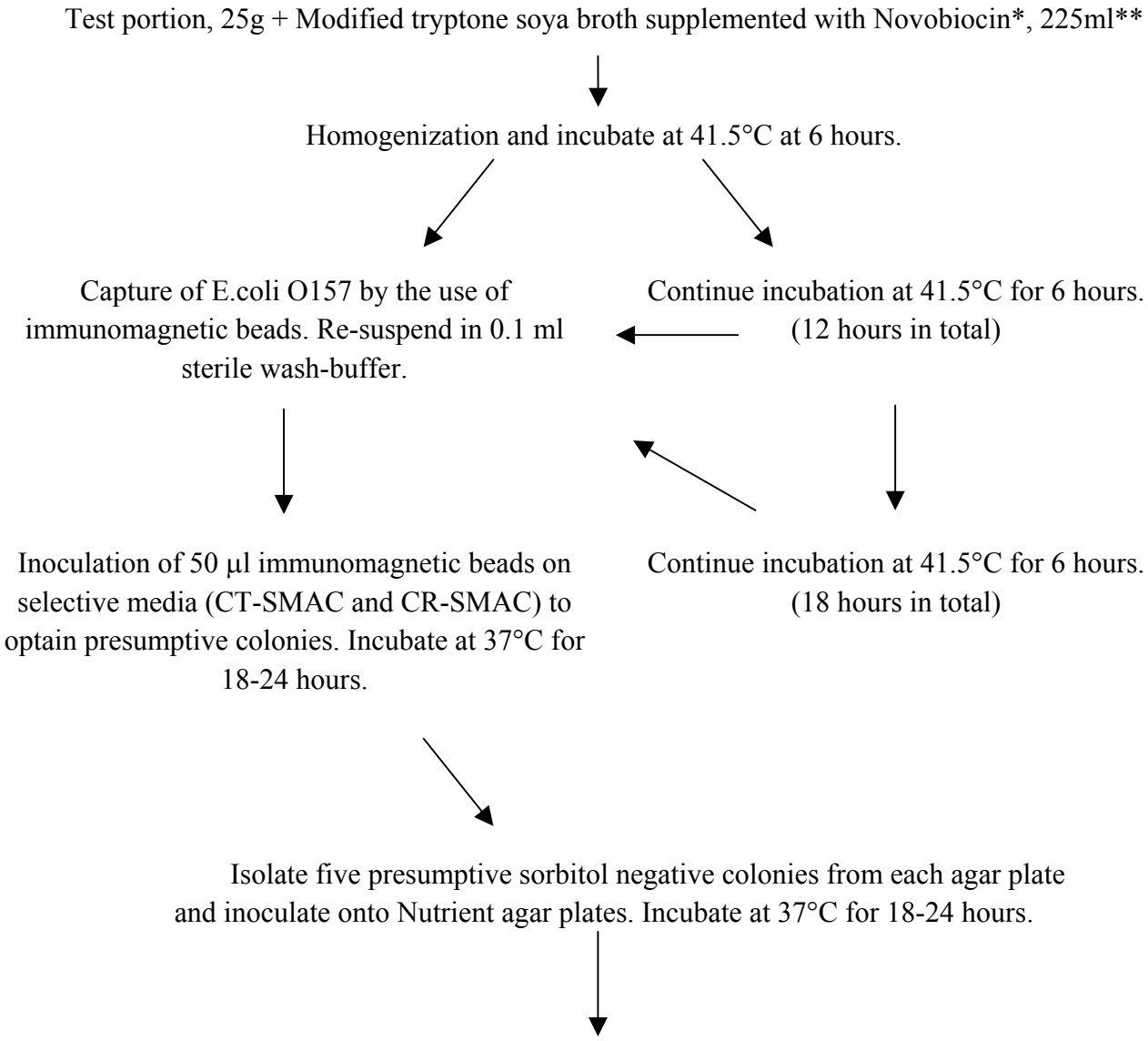
References

1. Oxoid Limited, wade Road, Basingstoke, Hampshire RG24, UK. Folio 762 /M335
2. ISO 16654 :2001. 1st ed. Microbiology – Horizontal method for the detection of *Escherichia coli O157,* International Organization for Standardization, Geneve, Switzerland..
3. Oxoid Limited, wade Road, Basingstoke, Hampshire RG24, UK. Folio 801 /M353.

APPENDIX 1

An overview of different standards for *Escherichia coli O157* isolation procedures

Figure 1. ISO *Escherichia coli O157* Isolation Procedure

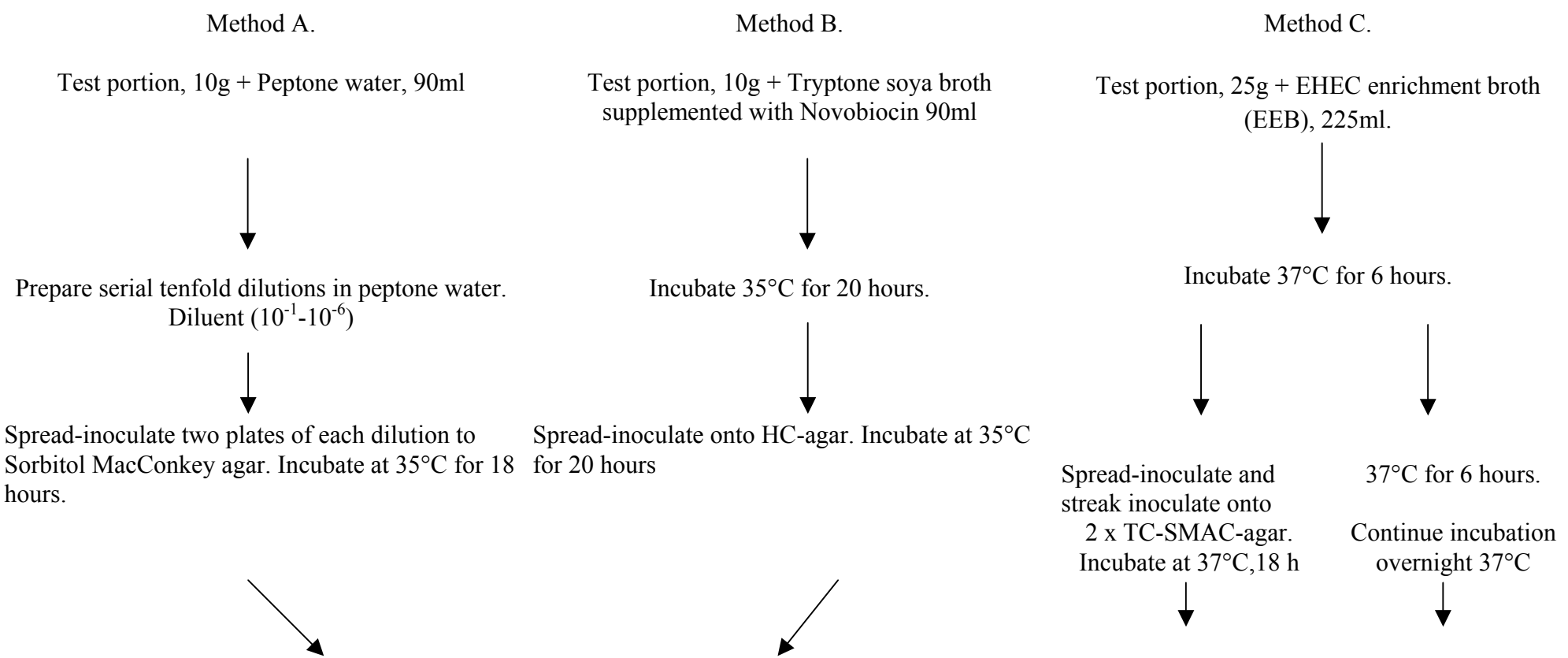


* The broth should be pre-warmed to 41.5°C before use.
** If the sample weight is less than 25 grams use the necessary quantity of medium to give a 1/10 dilution (weight to volume).

References:

ISO 16654 :2001. 1st ed. Microbiology – Horizontal method for the detection of *Escherichia coli O157,* International Organization for Standardization, Geneve, Switzerland

Figure 1. FDA/ BAM *Escherichia coli O157* Isolation Procedure.



References:

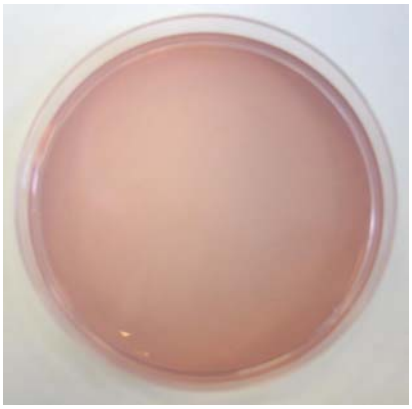
FDA Bacteriological Analytical Manual, 8th Ed.1995 Chapter 4, AOAC International, Arlington Va.USA

APPENDIX 2

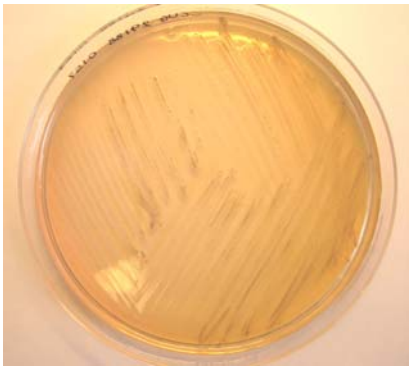
Photographs of *Escherichia coli O157* growth on various media and positive and negative reactions of the biochemical test.

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

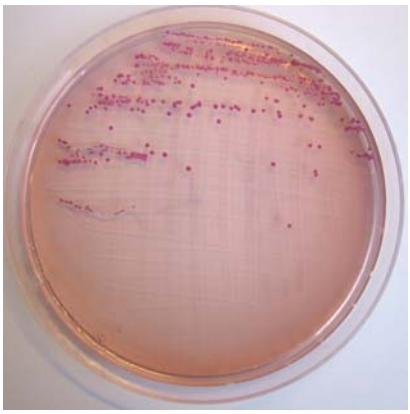
Cefixime Tellurite Sorbitol MacConkey agar. (CT-SMAC).



The picture shows an uninoculated plate.

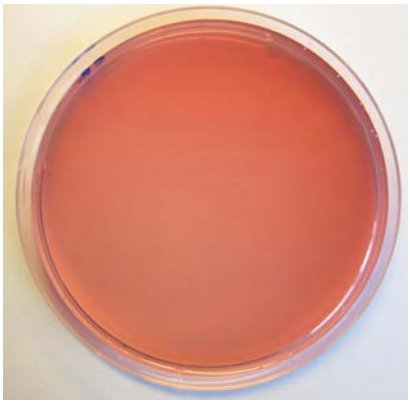


Escherichia coli O157 on CT-SMAC plates. The colonies are transparent / colourless with a weak pale brownish appearance because the bacterium does not ferment sorbitol.
Positive control: *Escherichia coli O157* (CCUG 29889)

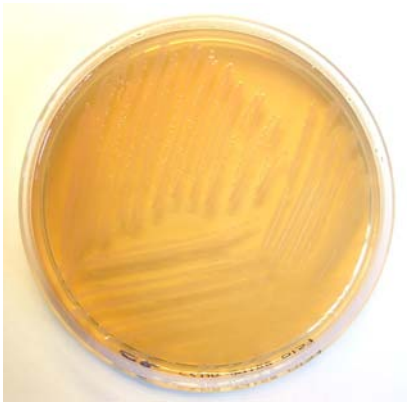


Escherichia coli on CT-SMAC plates. The colonies has a pink or red appearance because the bacterium does ferment sorbitol.
Negative control: *Escherichia coli* (ATCC 25922)

Cefixime Rhamnose Sorbitol MacConkey agar. (CR-SMAC)



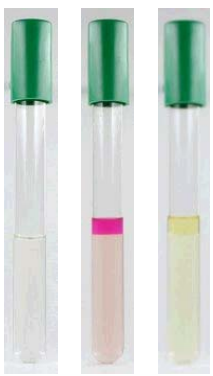
The picture shows an uninoculated plate.



Escherichia coli O157 on CR-SMAC plates. The colonies are transparent / colourless with a weak pale brownish appearance because the bacterium does not ferment sorbitol.

Escherichia coli on CR-SMAC plates. The colonies has a pink or red appearance because the bacterium does ferment sorbitol.
Negative control: *Escherichia coli* (ATCC 25922)

Indol Test.



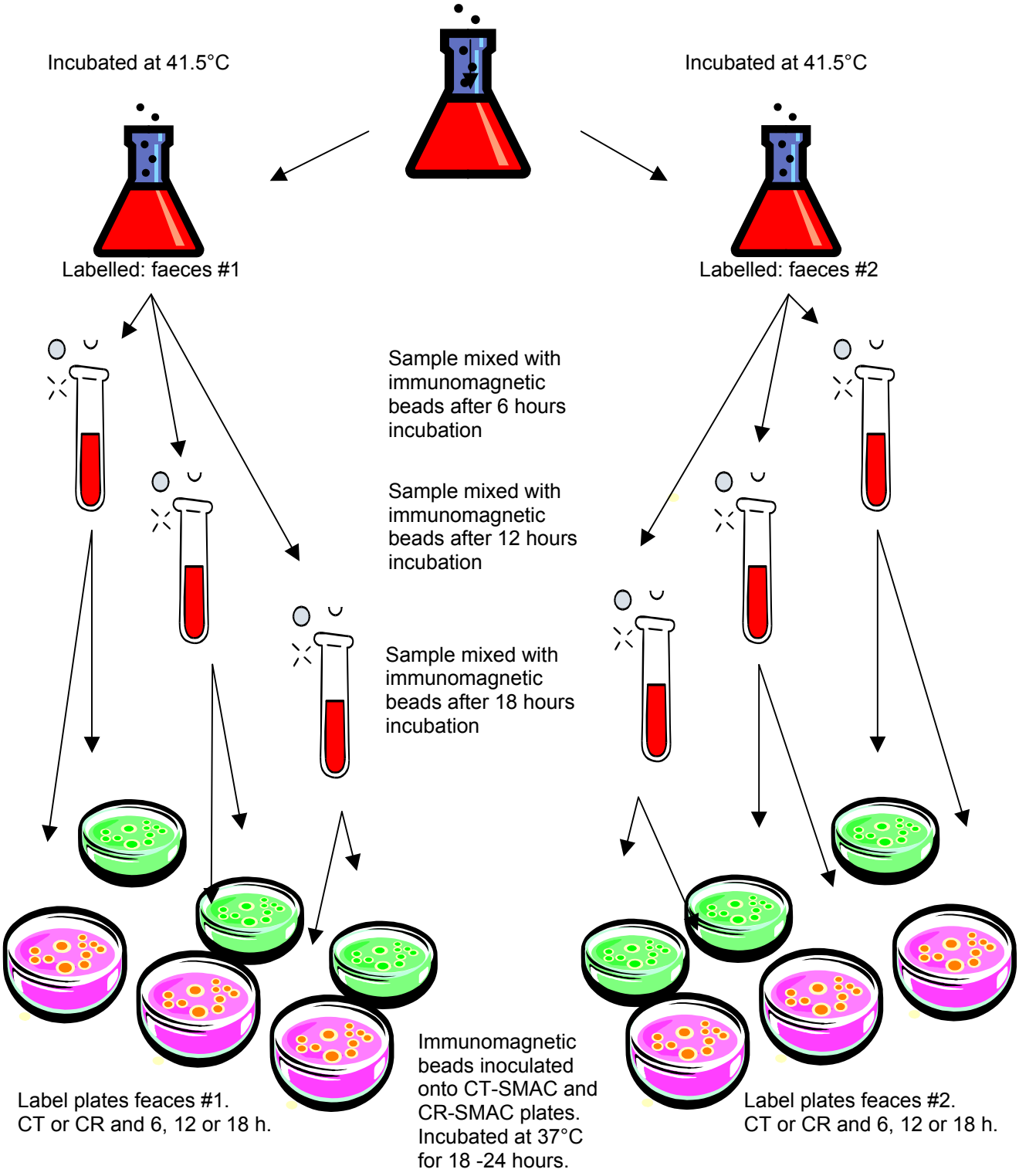
#1 #2 #3

1. Uninoculated medium.
2. Positive reaction: *Escherichia coli*. (*Escherichia coli* ATCC 25922)
3. Negative reaction: *Ps*. *aeruginosa.* (*Ps. aeruginosa* ATCC 27853)

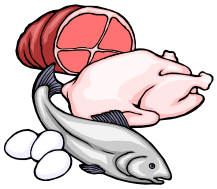
This test is used to determine whether an organism can split indole from tryptophan. After incubation in tryptophan or peptone broth, Kovac's reagent is added.

Flowdiagram – faeces part.

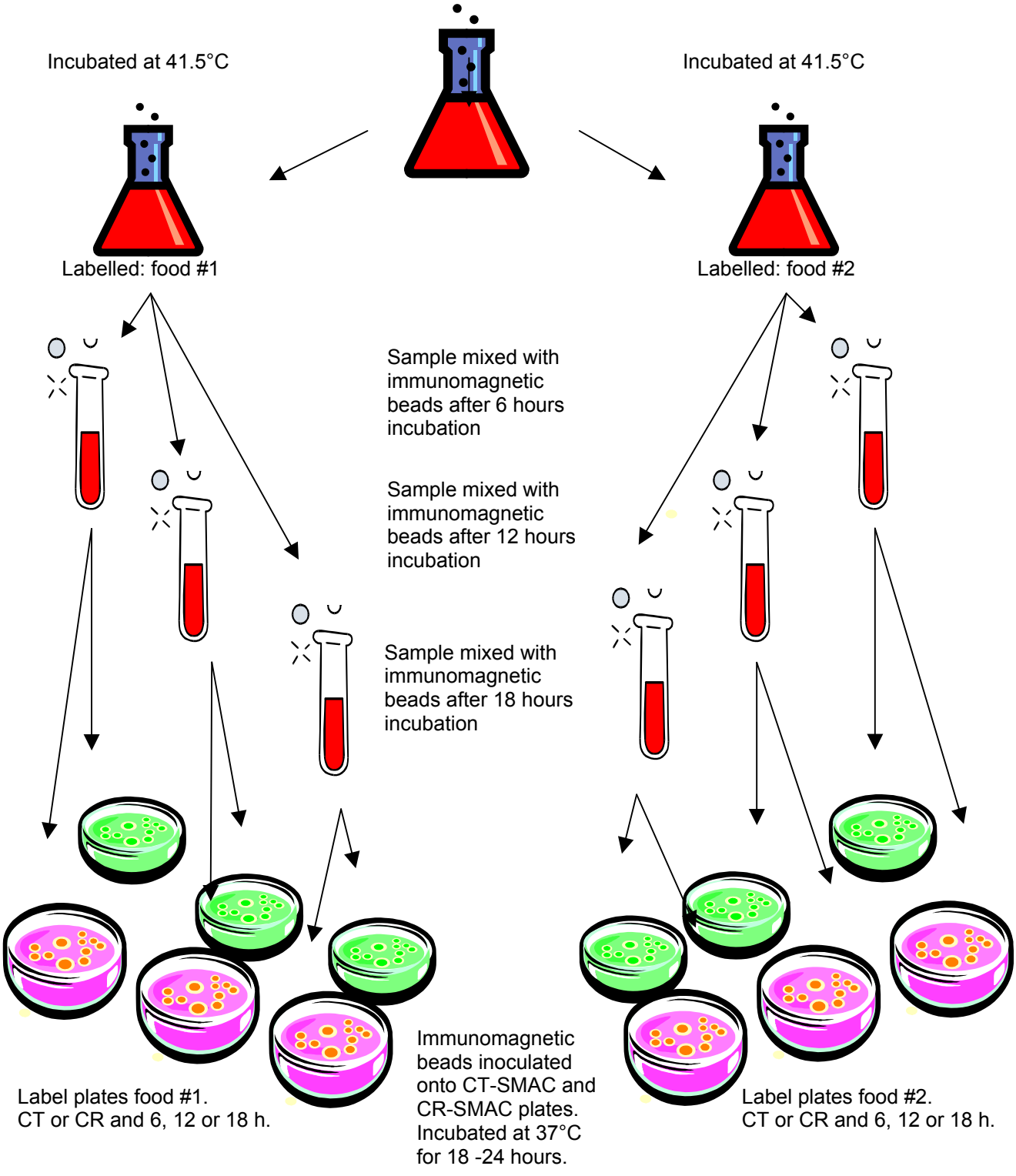
Weight 25g of faeces to 225ml of Modified tryptone soya broth supplemented with Novobiocin.



Flowdiagram – food part.



Weight 25g of food to 225ml of Modified tryptone soya broth supplemented with Novobiocin.



Date: _____

Initials:_____

Recordsheet:

Isolation of *Escherichia coli O157* from faeces and food.

Morphology on selective agar plates

Sample: Faeces #1

	Colour	**Results**	**Comments**
Morphology of colonies on CT-SMAC			
Morphology of colonies on CR-SMAC			

Sample: Faeces #2

	Colour	**Results**	**Comments**
Morphology of colonies on CT-SMAC			
Morphology of colonies on CR-SMAC			

Sample: Food #1

	Colour	**Results**	**Comments**
Morphology of colonies on CT-SMAC			
Morphology of colonies on CR-SMAC			

Sample: Food #2

	Colour	**Results**	**Comments**
Morphology of colonies on CT-SMAC			
Morphology of colonies on CR-SMAC			

Date: _____

Initials:_____

Recordsheet:

Isolation of *Escherichia coli O157 a* from faeces and food.

Biochemical tests

Indole test:

QC-strains	Colour:
E.coli ATCC 25922	
Ps. aeruginosa ATCC 27853	

Result of QC on the media:

Sample: _____

	Colour	**Results**	**Comments**
Indole reaction			

Overall result: _____

Sample: _____

	Colour	**Results**	**Comments**
Indole reaction			

Overall result: _____