

# Pathogenic microorganisms

## [Summary of Pathogenic microorganisms]

Inspections of pathogenic microorganisms are performed for feed ingredient and formula feed in order to understand actual situations of *Salmonella* contamination.

### «Start of inspection»

In July 1976, to solve problems on increase of the use of feed additives and diversification of producer countries of feed ingredients along with development of feeding technologies for livestock and poultry, The Law Concerning Safety Assurance and Quality Improvement of Feeds (Feed Safety Law. Partial amendment of the Law Concerning Quality Improvement of Feed.) was enforced. In accordance with the amended law which was additionally aiming at safety of feed, the detection method of *Salmonella* was developed and then the inspection started mainly for feed ingredients.

In 1996 and 1997, mass food poisoning broke out due to enterohemorrhagic *Escherichia coli* O157 and the need of understanding the situation of contamination in feed arose. Thus, the detection method of enterohemorrhagic *Escherichia coli* O157 was developed and the inspection started for feed ingredient and formula feed.

### «Regulations on microorganism»

Article 23 of the Feed Safety Law prohibits production of feed or feed additives which is contaminated or suspected to be contaminated with pathogenic microorganisms. The standards of production, usage and storage of feed and feed additives contain regulations such that ingredients which are contaminated or suspected to be contaminated with pathogenic microorganisms must not be used.

On the other hand, there is no specifications concerning the types and amount of pathogenic microorganism in feeds. However, pathogenic microorganism in the Feed Safety Law is considered to include each and every various infectious bacteria for livestock and poultry.

At present, the Analytical Standards of Feeds stipulate the methods to detect *Salmonella* and *Escherichia coli*.

### «Situations revolving around feeds»

The following factors are considerably critical subjects to be solved: high incidence of food poisoning through animal products, including chicken eggs contaminated with *Salmonella*, mass food poisoning caused by enterohemorrhagic *Escherichia coli* O157, milk contaminated with staphylococcal; and production and supply of safe food of animal origin in the background of anxiety of consumers on food related to BSE occurred in September 2001 and raise of interest on food safety. Especially, it is pointed out that human food poisoning is related to feed contaminated with *Salmonella*. In foreign countries, cases of food poisoning ascribed to animal feed have been reported. Based on these facts, *Salmonella* is defined to be the most critical pathogenic microorganism in feed.

### «Guidelines on salmonella control and Survey project»

In June 1998, the Ministry of Agriculture, Forestry and Fisheries of Japan notified “Guidelines on salmonella control in feed production” (notified by Director of Commercial Feed Division, Livestock Industry Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan, as of June 30, 1998), in order to promote control measures against *Salmonella* contamination in feed and promote settlement of the

concept about new manufacturing and quality control methods.

To implement the guidelines, FAMIC (Fertilizer and Feed Inspection Services, at that time) enforced a survey project for safety assurance of feed from 1998. To survey and verify HACCP in feed manufacturing while understanding how manufacturers were actually doing it in cooperation with feed manufacturers, this project figured out the actual situations of *Salmonella* contamination in the stage of feed manufacturing, sought for causes of contamination and examined measures for prevention of contamination and rapid detection methods.

FAMIC is now promoting settlement of the guideline by performing monitoring for feed ingredient and formula feed. *Salmonella* contamination in feed shows a decreasing trend for years now, which can be evaluated as successful results of the project.

# 1 *Salmonella*

## [Summary of *Salmonella*]

Taxonomically, *Salmonella* is a generic name of Gram-negative bacillus belonging to *Salmonella* genus in the family Enterobacteriaceae. Based on its biochemical properties, it is classified into three bacteria species (*Salmonella enterica*, *Salmonera bongori* and *Salmonella subterranea*) and *Salmonella enterica* is classified into 6 sub-species. And, based on the combination of O antigen and H antigen, it is further classified into more than 2500 serotypes. Most of them possess peritrichous mobility, use citric acid as a carbon source, and produce acid and gas by decomposing glucoses, with some exceptions. *Salmonella* does not decompose lactose and sucrose and does not produce indole. It usually produces hydrogen sulfide and shows positive in the lysine decarboxylase reaction, with some exceptions.

As one of the sources of *Salmonella* infection, contamination in feed has been pointed out for years and countermeasures against *Salmonella* contamination in feed have been promoted. (See [Summary of Pathogenic microorganism].)

Reference (on *Salmonella* contamination in feed)

Kiyoshi Sugano, et al.: Inspection results from *Salmonella* contamination in feed, Studies on livestock; 29 (1985)

Mitsuaki Kinoshita, et al.: Inspection results from *Salmonella* contamination in animal feed ingredients and studies on livestock; Studies on livestock, 43, 721 (1989)

Shizuo Sato: Feed and *Salmonella* 1. Distribution of *Salmonella* and contamination factors, Journal of the Japanese Society on Poultry Diseases, 26, 85 (1990)

Haruyoshi Harada, et al: Situations of *Salmonella* contamination in feed (from 1979 to 1997), Research Report of Animal Feed; 23, 161 (1998)

Katsunori Yoneda: Results of the project for safety assurance of feed from 1998 to 1999, Journal of the veterinary medicine, 54, 568 (2001)

Shizuo Sato: *Salmonella* contamination in feed and countermeasures, Journal of the Japanese Society on Poultry Diseases, 39, 113 (2003)

Fumio Kojima, et al.: Summary of monitoring results on *Salmonella* in feed – Survey results on project for safety assurance of feed, Research Report of Animal Feed, 29, 236 (2004)

## [Method listed in the Feed Analysis Standards]

1 *Salmonella* confirmation method (Group O, H antigen) [Analytical Standards of Feeds, Article 1, Chapter 18]

Use sterilized water, reagents and instruments as required.

Use hydrochloric acid solution (1 mol/L) or sodium hydroxide solution (1 mol/L) to adjust pH of medium.

### A. Reagent preparation

- 1) Surfactant solution.<sup>[1]</sup> Autoclave surfactant solution\*<sup>1</sup> (10 v/v%) at 121 °C for 15 minutes.
- 2) Iodine-iodide solution. Dissolve 20 g of potassium iodide with 50 mL of water. Add 12.5 g of

iodine and dissolve it thoroughly. Then, dilute to 100 mL with water.

3) Saline. Autoclave sodium chloride solution (0.9 w/v%) at 121 °C for 15 minutes.

4) Buffered peptone water.<sup>[2]</sup> Dissolve 10 g of peptone, 5 g of sodium chloride, 9 g of disodium hydrogen phosphate-12-water and 1.5 g of potassium dihydrogen phosphate with 1,000 mL of water and adjust the pH to 6.9 to 7.1.

Dispense 250 mL portions of the solution into 500 mL culture bottles<sup>[3]</sup> and autoclave them at 121 °C for 15 minutes. Cool them down below 45 °C. Then, add 15 mL of surfactant solution to the cooled solution and shake to mix.

5) Hajna tetrathionate broth.<sup>\*2[4]</sup> Dissolve 18 g of peptone, 2 g of yeast extract, 0.5 g of glucose, 2.5 g of mannitol, 5 g of sodium chloride, 26 g of sodium thiosulfate (anhydrous), 0.5 g of sodium deoxycholate, 25 g of calcium carbonate and 10 mg of brilliant green with 1,000 mL of water by heating in a water bath, then adjust the pH to 7.3 to 7.5.

Cool it down below 45 °C. Then, add 40 mL of iodine-iodide solution and dispense 100 mL portions into 200 mL culture bottles<sup>[5]</sup> while stirring the broth.

6) Rappaport-Vassiliadis broth.<sup>\*3[6]</sup> Dissolve 5 g of soya peptone, 8 g of sodium chloride, 1.6 g of potassium dihydrogen phosphate, 40 g of magnesium chloride six hydrate and 40 mg of malachite green oxalate with 1,000 mL of water by heating in a water bath, then adjust the pH to 5.1 to 5.3.

Dispense 100 mL portions of the broth into 200 mL culture bottles<sup>[5]</sup>.

7) DHL agar medium.<sup>\*4[7]</sup> Dissolve 20 g of peptone, 3 g of beef extract, 10 g of sucrose, 10 g of lactose monohydrate, 10 g of lactose monohydrate, 1 g of trisodium citrate dihydrate, 1 g of ammonium ferric citrate, 2.2 g of sodium thiosulfate (anhydrous), 1 g of sodium deoxycholate, 30 mg of neutral red and 15 g of agar with 1,000 mL of water by heating in a water bath, then adjust the pH to 7.2 to 7.4.

As required, add 20 mg of novobiocin sodium<sup>[8]</sup> to 1,000 mL of medium.

Pour 20 mL of the medium evenly on Petri dishes<sup>[9]</sup> and allow to solidify. Then, turn them over and slightly slide the lids and leave them at 35 to 37 °C for one hour until the surface of the agar is dry<sup>[10]</sup>.

8) Brilliant green agar medium.<sup>\*5[11]</sup> Dissolve 10 g of peptone, 3 g of yeast extract, 10 g of sucrose, 10 g of lactose monohydrate, 5 g of sodium chloride, 80 mg of phenol red, 12.5 mg of brilliant green and 15 g of agar with 1,000 mL of water by heating in a water bath, then adjust the pH to 6.8 to 7.0.

As required, add 20 mg of novobiocin sodium<sup>[8]</sup> to 1,000 mL of medium.

Then, do the same procedure as in 7).

9) CHROMagar Salmonella medium.<sup>\*6[12]</sup> Dissolve 7 g of mixture of peptone and yeast extract, 12.9 g of selective agent and chromogenic enzyme substrate mixture<sup>\*7</sup> and 15 g of agar with 1,000 mL of water by heating in a water bath, then adjust the pH to 7.5 to 7.7.

Then, do the same procedure as in 7).

10) TSI agar medium.<sup>\*8[13]</sup> Dissolve 15 g of peptone, 4 g of beef extract, 10 g of sucrose, 10 g of lactose monohydrate, 1 g of glucose, 5 g of sodium chloride, 0.4 g of sodium sulfite, 80 mg of sodium thiosulfate (anhydrous), 0.2 g of ferrous sulfate heptahydrate, 20 mg of phenol red and 15 g of agar with 1,000 mL of water by heating in a water bath, then adjust the pH to 7.3 to 7.5.

Dispense 4 mL portions of the medium into small test tubes and autoclave them at 121 °C for 15

minutes. Then, allow to set in a sloping position to solidify in a way of slant with butt (Slant is upper 1/3 and butt is lower 2/3 depth).<sup>[14]</sup>

11) SIM agar medium.<sup>\*8[16]</sup> Dissolve 30 g of peptone, 3 g of beef extract, 0.5 g of ammonium ferric citrate, 50 mg of sodium thiosulfate (anhydrous), 0.2 g of L-cysteine hydrochloride monohydrate and 5 g of agar with 1,000 mL of water by heating in a water bath, then adjust the pH to 7.3 to 7.5.

Dispense 4 mL portions of the medium into small test tubes and autoclave them at 121 °C for 15 minutes. Then, allow to set in a vertical position to solidify in a way of butt<sup>[17]</sup>.

12) Lysine decarboxylation test medium.<sup>\*8[17]</sup> Dissolve 5 g of peptone, 3 g of yeast extract, 1 g of glucose, 5 g of L-lysine hydrochloride and 20 mg of bromocresol purple with 1,000 mL of water by heating in a water bath, then adjust the pH to 6.7 to 6.9.

Dispense 4 mL portions of the medium into small test tubes and autoclave them at 121 °C for 15 minutes<sup>[18]</sup>.

### B. Cultivation<sup>[19]</sup>

Pre-enrichment. Weigh 25 g of analysis sample<sup>[20]</sup> into a culture bottle of buffered peptone water and shake it well. Then, incubate it at 35 to 37 °C for 18 to 24 hours.<sup>[21]</sup>

Selective enrichment. Transfer a 10-mL aliquot of the pre-enrichment culture solution into Hajna tetrathionate broth, and additional 10-mL aliquot into Rappaport-Vassiliadis broth.<sup>[23]</sup> Shake both of broths to mix well, then incubate them at 41 to 43 °C for 18 to 24 hours.<sup>\*5[24]</sup>

Selective isolation. Streak<sup>[25]</sup> a loopful of the selective enrichment culture solution of Hajna tetrathionate broth on plates of DHL agar medium and brilliant green agar medium. Repeat with 2 mm loopful of the selective enrichment culture solution of Rappaport-Vassiliadis broth. Then, turn the plates over and incubate them at 35 to 37 °C for 18 to 24 hours.

If needed, do the same procedure for CHROMagar Salmonella medium<sup>\*6</sup>.

Pure isolation.<sup>[26]</sup> Pick with needle<sup>[28]</sup> three typical or suspicious colonies of *Salmonella*<sup>[27]</sup>, if present, each from the surface of DHL agar medium, brilliant green agar medium and CHROMagar Salmonella medium having growth. Dilute each colony with 0.3 mL of saline<sup>[29]</sup>. Streak a loopful of each diluted solution on plates of DHL agar medium, brilliant green agar medium and CHROMagar Salmonella medium.<sup>[30]</sup> Turn the plates over and incubate them at 35 to 37 °C for 18 to 24 hours.

Confirmation. Pick with needle a typical or suspicious colony of *Salmonella*, if present, each from the surface of DHL agar medium, brilliant green agar medium and CHROMagar Salmonella medium having growth. Inoculate TSI agar medium by stabbing the butt and streaking the agar slant surface with the needle. After inoculating TSI, inoculate with the needle, SIM agar medium and lysine decarboxylation test medium by stabbing in a row.<sup>[31]</sup> Then, incubate them at 35 to 37 °C for 18 to 24 hours.

### C Identification

Identification. Identify *Salmonella* by judging from the following biochemical properties<sup>[32]</sup> observed from the above three types of confirmation medium.

In most cases, *Salmonella* exhibits following properties: lactose/sucrose fermentation (-), glucose fermentation (gas formation) (+), hydrogen sulfide (+), motility (+), IPA<sup>[33]</sup> (-), indole<sup>[34]</sup> (-) and lysine decarboxylation (+).

Serotyping.<sup>\*9[35]</sup> Place each one drop of saline and *Salmonella* polyvalent somatic (O) antiserum on a slide glass. Pick a small amount of colony on TSI agar slant which exhibits the properties of *Salmonella*. Mix it with saline, then with polyvalent O antiserum.<sup>[36]</sup> Tilt the slide glass back and forth and observe whether an agglutination occurs.

Test the colony agglutinated with polyvalent O antiserum using each individual group O antiserum<sup>[37]</sup> in the same way to determine the O group<sup>[37]</sup>.

Test the colony which has been determined its O group for agglutination with flagellar (H) antiserum using the test tube method and determine the H antigen<sup>[39]</sup>.

Determine the serotype of *Salmonella* by reference to the Kauffmann and White classification scheme.

- \* 1 Tween 80 (Atlas Powder) or equivalent
- 2 For this medium, a commercially available dry powder medium having the same compositions, for example Hajna tetrathionate broth (Eiken Chemical), can also be used.
- 3 For this medium, a commercially available dry powder medium having the same compositions, for example Rappaport-Vasilliadis (RV) Enrichment Broth (Oxoid), can also be used.
- 4 For this medium, a commercially available dry powder medium having the same compositions, for example DHL agar (Pearl Core; Eiken Chemical), can also be used.
- 5 For this medium, a commercially available dry powder medium having the same compositions, for example Brilliant Green Agar (Difco), can also be used.
- 6 For this medium, a commercially available dry powder medium having the same compositions, for example CHROMagar *Salmonella* (CHROMagar), can also be used.
- 7 Mixture of chromogenic substrate in purple which is decomposed through the enzyme activities specific to *Salmonella*.
- 8 For these media, commercially available dry powder media having the same compositions can also be used.
- 9 Agglutinations with polyvalent or individual group O antiserum are judged to be positive when they firmly agglutinate within one minute.

#### «Summary of analysis method»

Standard Test Method developed by the National Institute of Health Sciences in 2009 is the typical example for the test method of *Salmonella* in food. The method listed in analytical standards of feeds, however, targets feed contaminated by *Salmonella* and is characterized by the pre-enrichment culture using buffered peptone water so that the method can be applied to the cases having few numbers of *Salmonella*.

Figure 18.1-1 outlines this method.

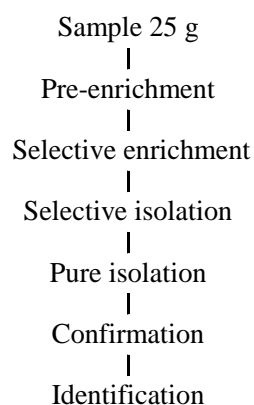


Figure 18.1-1 Summary of *Salmonella* Detection Method

References (concerning *Salmonella* detection methods)

Shizuo Sato, et al. : Detecting *Salmonella* from feed; 6, New Technology (Vol. 18); Agriculture, Forestry and Fisheries Research Council (1980)

Riichi Sakazaki, Kazumichi Tamura : Intestinal bacteria <Vol. 1> Summary; *Salmonella Genus*; Kindai Shuppan Co, Ltd. (1992)

Comprehensive Dictionary of Food Safety Editorial Board edition: Food toxic microorganism; Sanchoh Publishing Co., Ltd. (1997)

Japanese Society on Poultry Diseases edition: Series of *Salmonella* in chicken eggs and poultry; Livestock Industry Promotion Association of Japan (1998)

Toyoko Kusama: Detection method for *Salmonella* for feed; Poultry Pathology Working Group, Newsletter (Japanese Society of Veterinary Science) , 5, 5 (2001)

Japanese Society on Poultry Diseases: *Salmonella* detection method; Japanese Society on Poultry Diseases, Newsletter, 37, 14 (2001)

International Organization for Standardization EN ISO 6579: Microbiology of food and animal feeding stuffs. Horizontal method for the detection of *Salmonella* spp. (2002)

Tetsuo Chihara, et al.: Comparison of selective isolation medium performance in *Salmonella* inspection for feed; Livestock research, 57, 780 (2003)

Edition of the Ministry of Health, Labour and Welfare: Microorganisms listed in Food Safety Inspection Guidelines, Japan Food Hygiene Association (2004)

AOAC International: AOAC Official Method 995.20, *Salmonella* in Raw, Highly Contaminated Foods and Poultry Feed, AOAC Official Method of Analysis, Chapter 17, 113 (2005)

Edition of Pharmaceutical Society of Japan: Standard Methods of Analysis for Hygienic Chemists 2005, Kanehara & Co., Ltd. (2005)

U.S. Food and Drug Administration: Bacteriological Analytical Manual, Chapter 5 *Salmonella* (2006)

National Institute of Health Sciences: *Salmonella* Standard Test Methods, NIHSJ-01-ST4 (090218); <http://www.nihs.go.jp/fhm/kensa/sal/Salmonells%20ST4-091014F.pdf> (2009)

«Notes and precautions»

[1] Added so as to disperse fatty components in the sample and obtain homogeneous culture solution.

Since the surfactant (Tween 80) is hard to dissolve in low-temperature water, dissolve it by heating.

The chemical name of Tween 80 is polyoxyethylene sorbitan monooleate and it is also called as polysorbate 80.

- [2] Buffered peptone water (BBL, Difco, Merck, Oxoid, Eiken Chemical, Kyokuto Pharmaceutical Industrial or Nissui Pharmaceutical) or equivalent dry powder medium can be used.
- [3] Equip a culture bottle (glass) with an aluminum cap to keep ventilation. You can use a sterilized stomacher bag in place of a culture bottle. In this case, be careful not to damage the bag with a fish bone. When using a stomacher bag, dispense 250 mL of the given solution autoclaved in advance. Then, add 15 mL of surfactant solution to the bag and shake it well.
- [4] As calcium carbonate contained in this broth becomes deposited (insoluble), take due considerations on homogeneous dispensing into culture bottles. Because calcium carbonate may transform by heating, heat it in a water bath for 10 minutes or so. Do not autoclave the broth.
- [5] Use a commercially available sterilized tissue culture flask (made of synthetic resin) or a glass culture bottle. When using a glass culture bottle, sterilize it with an aluminum cap by dry heating in advance.
- [6] Prepare this broth in the same way of Hajna tetrathionate broth. In addition, you may autoclave the broth at 115 °C for 15 minutes.
- [7] Prepare the medium by heating in a water bath until the agar dissolves completely, by heating for several times in a microwave oven until the agar dissolves completely, or by autoclaving at 110 °C for 10 minutes. DHL stands for Desoxycholate Hydrogen sulfide Lactose.
- [8] Novobiocin is effective for samples possibly contaminated with enterobacteria such as *Citrobacter* or *Proteus* in a high level because the substance suppresses growth of these bacteria.

Dissolve novobiocin sodium (Sigma Aldrich, Kanto Chemical, Wako Pure Chemical Industries, or equivalent.) with water to make 2 mg/mL solution and filter and sterilize it using a membrane filter. Then, dispense it in small test tubes up to 2 mL and store the test tubes in a freezer. Use the appropriate volume depending on the medium preparation amount.

- [9] Use a sterilized Petri dish of glass or synthetic resin with diameter of 90 mm and height of 15 mm.

- [10] As shown in Figure 18.1-2, turn over the Petri dish and dry it in the incubator until the surface of the medium will be free of waterdrops. If the medium is not completely dried, bacteria spreads over waterdrops and grows there when it is streaked, possibly resulting in failure of generating appropriate colonies. If the medium is dried too much, the surface of the medium is likely to be cracked.

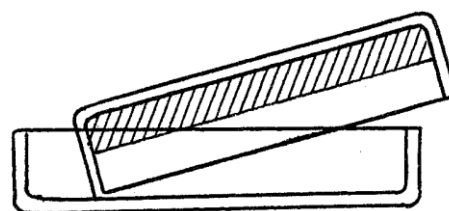


Figure 18.1-2 Drying the agar plate

- [11] Prepare the medium by heating in a water bath or heating for several times in a microwave oven until the agar dissolves completely, or by autoclaving at 110 °C for 10 minutes.
- [12] Prepare the medium by heating in a water bath or heating for several times in a microwave oven until the agar dissolves completely.

Since this medium is likely to transform by heating, do not autoclave.



[13] TSI agar medium (Eiken Chemical) or equivalent dry powder medium can be used. Prepare the medium by heating in a water bath or heating in a microwave oven for several times until the agar dissolves completely. TSI stands for Triple Sugar Iron.

[14] Dispense the medium in small test tubes (with diameter of 10 mm and length of 120 mm) up to 3 to 4 mL using a measuring pipette, Komagome pipette or a dispenser. Equip each test tube with an aluminum cap and autoclave them. Then, tilt the test tube to solidify the medium in a way of slant with butt as shown in Figure 18.1-3.

[15] SIM agar medium (Eiken Chemical) or equivalent dry powder medium can be used. Prepare the medium by heating in a water bath for 10 minutes or heating in a microwave oven for several times until the agar dissolves completely. SIM stands for Sulfide Indole Motility.

[16] Dispense the medium in small test tubes (with diameter of 10 mm and length of 120 mm) up to 3 to 4 mL using a measuring pipette, Komagome pipette or a dispenser. Equip each test tube with an aluminum cap and autoclave them. Then, stand the test tube vertically to solidify the medium in the way of butt as shown in Figure 18.1-4.

[17] Lysine Decarboxylase Broth (Difco or Eiken Chemical) or equivalent dry powder medium can be used. Prepare the medium by heating in a water bath or heating in a microwave oven for several times until the ingredients dissolve completely.

[18] After autoclave, leave the test tubes until the medium cool down to the room temperature.

[19] After incubation, be sure to autoclave culture bottles, Petri dishes and others contaminated with enterobacteria.

[20] Samples to be tested are animal-derived feed ingredients including fish meal and poultry by-products, plant-derived feed ingredients including soybean meal and rapeseed meal and formula feeds.

To take a sample of feed on-site, use sampling bags and sampling shovels which have been sterilized (through radiation sterilization, gaseous sterilization, etc.) and wear sterilized gloves. If you cannot prepare these things, sterilize your fingers and sampling shovels with alcohol pads.

To weigh samples, use buffered peptone water which is kept at 35 to 37 °C, sterilized charta (with gaseous sterilization or ultraviolet sterilization, etc.) and medicine spoons sterilized with alcohol pad or dry-heat sterilized and prevent contamination through contact to other samples.

[21] Figure 18.1-6 shows an example of pre-enrichment culture using buffered peptone water.

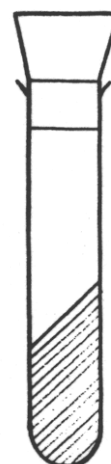


Figure 18.1-3 Medium (Slant with butt)

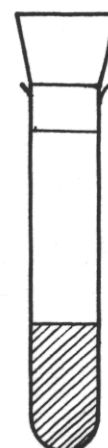


Figure 18.1-4 Medium (Butt)

[22] Use the selective enrichment broth kept at 41 to 43 °C. Add the pre-enrichment culture solution to the selective enrichment broth using a Komagome pipette.

After use, put a Komagome pipette in a container with antiseptic solution and then autoclave it.

[23] Figure 18.1-7 shows an example of selective enrichment culture. (Note that actual cases may show a color different from the one in the example.) Compared to culture at 35 to 37 °C, this method can suppress increase of enterobacteria such as *Citrobacter* and *Proteus*, with a trend of slight raise in *Salmonella* detection rate.

[24] There are several methods to streak the culture solution onto the selective isolation medium. One of them is described below. As shown in Figure 18.1-5, hold a Petri dish at your left hand and reciprocate the inoculating 3-mm loop which has sampled the culture medium within the narrow range surrounded by three edges of the Petri dish to apply the culture medium. Then, gradually move the inoculating loop to other areas.

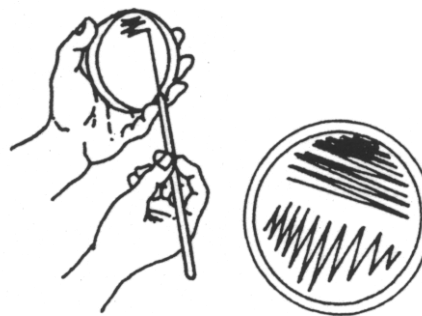


Figure 18.1-5 Method of streaking onto selective isolation medium

[25] When a colony suspected to be *Salmonella* can be obviously distinguished from other colonies and it can be identified as the pure *Salmonella* colony, you can omit this procedure. However, if the results of confirmation culture are suspicious, other bacteria may contaminate the colony. Thus, be sure to perform the pure isolation.

[26] The characteristics of *Salmonella* colonies on respective selective isolation media are shown as below (see Figure 18.1-8).

- DHL agar medium

Forms a colony in which the center is black (In case of non-hydrogen sulfide-producing *Salmonella*, it is colorless.) and translucent.

- Brilliant green agar medium

Forms a pinkish opaque colony. The medium around the colony shows in vivid red.

- CHROMagar *Salmonella* medium

Forms a purple colony.

Table 18.1-1 shows comparisons of properties between *Salmonella* and a part of other enterobacteria cultured with DHL agar medium, brilliant green agar medium and CHROMagar *Salmonella* medium. For details, see the following references:

Eiken Manual (10<sup>th</sup> version): Eiken Chemical (1996)

Riichi Sakazaki, et al.: Lecture on new bacteria medium study, Second Vol., Kindai Shuppan (1988)

The Oxoid Manual 8<sup>th</sup> Edition: Oxoid (1998)

Difco<sup>™</sup> & BBL<sup>™</sup> Manual:

<http://www.bd.com/ds/technicalCenter/inserts/difcoBblManual.asp>

Table 18.1-1 Properties of *Salmonella* and other enterobacteria on several selective isolation media

| Medium                  | DHL agar medium   | Brilliant green agar medium                                   | CHROMagar Salmonella medium                                     |
|-------------------------|---|---|---|
| <i>Salmonella</i>       | Black colony (H <sub>2</sub> S produced)<br>Peripheral of colony is translucent or opaque and the center shape is rather uneven.                | Pink or red colony<br>The medium around the colony turns red. | Purple colony<br>The medium around the colony remains the same. |
| <i>Citrobacter</i>      | Black colony (H <sub>2</sub> S produced)<br>The colony is round and its peripheral is redish. (It disappears after being left for a long time.) | Pink or red colony<br>The medium around the colony turns red. | Blue colony   |
| <i>Proteus</i>          | Black colony (H <sub>2</sub> S produced)<br>The medium around the colony is brownish red.   | Almost no growth  | Colorless colony  |
| <i>Escherichia coli</i> | Red colony  | Mud greenish yellow colony                                    | Blue colony   |

[27] Pick up colony using a platinum needle or a sterilized toothpick. Most of bacteria remain alive even if they are not growing on the medium. Thus, be careful not to touch other area than the target colony.

[28] You may dilute the colony using sterilized microtitration plate (about 300 µL per well).

[29] Divide each DHL agar medium, brilliant green agar medium and CHROMagar Salmonella medium into 8 sectors, and streak 2 ~ 3 mm loopful of diluted solution within a sector. You can also use the CHROMagar Salmonella medium which is easier to differentiate *Salmonella*.

[30] Pick up colony using a platinum needle and stab it deeply into the center of the butt of TSI agar medium. Remove the needle gently so that the medium will not be damaged, and streak onto the slant. Then, stab the same needle into the SIM agar medium in the same way and inoculate it into the lysine decarboxylation test medium.

[31] The biochemical properties of *Salmonella* on each confirmation medium are explained below:

· TSI agar medium

Since *Salmonella* does not decompose lactose/sucrose, the slant becomes red. (Bacteria which decompose lactose/sucrose will become yellow.) Since *Salmonella* decomposes glucose, the butt becomes yellow. However, since most *Salmonella* produce hydrogen sulfide, the butt becomes black. It also produces gas, which causes bubbles or cracks in the butt.

· SIM agar medium

Since most *Salmonella* is motile, it grows throughout the medium and makes the medium turbid. (Non-motile bacteria will grow only in the line of needling.) Since most *Salmonella* produce hydrogen sulfide, the entire medium becomes black. (Non-motile bacteria will make only the line of needling black.) IPA reaction (see [32]) is negative and the indole test (see [33]) is also negative for *Salmonella*.

· Lysine decarboxylation test medium

Most *Salmonella* produce decarboxylase. The decarboxylation of lysine turns the medium into alkaline and the medium becomes purple. (Non-decarboxylase-producing bacteria become yellow (acidic).)

However, a part of *Salmonella* is non-hydrogen-sulfide-producing or negative in the lysine decarboxylation test. Therefore, for those for which the slant of TSI agar medium is red and the butt is yellow or black, or for those which are negative in the IPA reaction, perform the following agglutination test using polyvalent *Salmonella* O antiserum. If the test shows a positive result, the sample is judged to be *Salmonella*.

In addition, by inspecting other biochemical properties (such as malonate utilization test and PYR test), as required, the sample is judged to be *Salmonella*.

Table 18.1-2 shows comparison of biochemical properties between *Salmonella* and other enterobacteria on TSI agar medium, SIM agar medium and lysine decarboxylation test medium.

Table 18.1-2 Biochemical properties of *Salmonella* and other enterobacteria on various confirmation media

| Medium                  | TSI agar medium |                 |     |                  | SIM agar medium |          |     | Lysine decarboxylase test medium |
|-------------------------|-----------------|-----------------|-----|------------------|-----------------|----------|-----|----------------------------------|
|                         | Slant           | Butt            | Gas | Hydrogen sulfide | Indole          | Motility | IPA |                                  |
| <i>Salmonella</i>       | Red             | Yellow or black | +   | +                | -               | +        | -   | +                                |
| <i>Citrobacter</i>      | Yellow or red   | Yellow or black | +   | +                | -               | +        | -   | -                                |
| <i>Proteus</i>          | Yellow or red   | Yellow or black | +   | + ~ -            | + ~ -           | +        | +   | -                                |
| <i>Escherichia coli</i> | Yellow          | Yellow          | +   | -                | +               | +        | -   | + ~ -                            |

[32] IPA (Indolepyruvic acid) reaction is a phenomenon in which indolepyruvic acid produced by deamination of tryptophan contained in peptone forms a chelate with  $Fe^{3+}$  of ammonium ferric citrate and creates a brown belt in the upper edge of the medium. This is a reaction specific to *Proteus* and related bacteria.

[33] This is the test to check for production of indole. After stratifying 1 mL of chloroform over the SIM agar medium and then drop 1 mL of the following Kovač's reagent on the medium and shake it gently. If the chloroform layer becomes red, the sample is judged to be positive.

To prepare Kovač's reagent, dissolve 5 g of *p*-dimethylaminobenzaldehyde with 75 mL of isoamyl alcohol on a water bath at about 50 °C and then add 25 mL of hydrochloric acid. Store the reagent in a brown bottle.

[34] *Salmonella* serotypes are distinguished by identifying O antigen, which is somatic surface antigen (somatic antigen), and H antigen, which is flagellar antigen.

Note that 67 types of O antigen and 80 types of H antigen are known.

[35] Use *Salmonella* antisera O polyvalent and O1 polyvalent (Denka Seiken) or equivalents.

[36] Use *Salmonella* antisera O2 group, O4 group, O7 group, O8 group, O9 group, O9,46 group, O3,10 group, O1,3,19 group, O11 group, O13 group, O6,14 group, O16 group, O18 group, O21 group and O35 group (Denka Seiken) or equivalent.

More than 95 % of *Salmonella* isolated from feed in tests conducted by FAMIC so far agglutinated with O group antisera of O4, O7, O8, O3,10, O1,3,19, O13 and O18.

- [37] Divide a slide glass into 10 areas using a glass pencil. Place a drop of saline and each polyvalent O antiserum to each area. Scoop a small amount of colony which show properties of *Salmonella* from the slant of TSI agar medium using a platinum needle or loop and mix it with saline. Then, mix it with the polyvalent O antiserum. When tilting the slide glass back and forth, and granular agglutination of bacteria and antiserum is found, the sample is judged to be positive. If some questions on agglutination arise, see the manual attached to the purchased antiserum.
- [38] To determine the H antigen, follow the descriptions in the appended item.



Figure 18.1-6 Example of pre-enrichment culture using buffered peptone water  
(Left: before use Right: after cultivation)



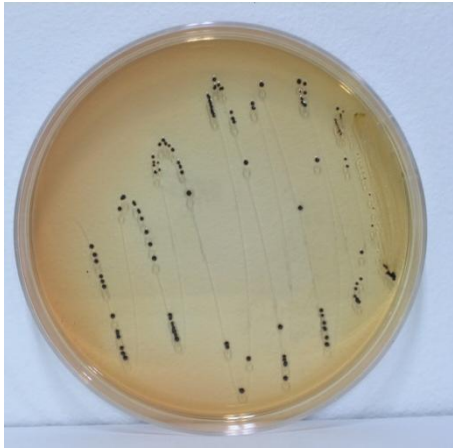
Figure 18.1-7 Example of selective enrichment culture (1: Hajna tetrathionate broth)  
(Left: before use Right: after cultivation)

Depending on the condition of the enriched enterobacteria, the color of the broth changes from pale blue to pale yellow.

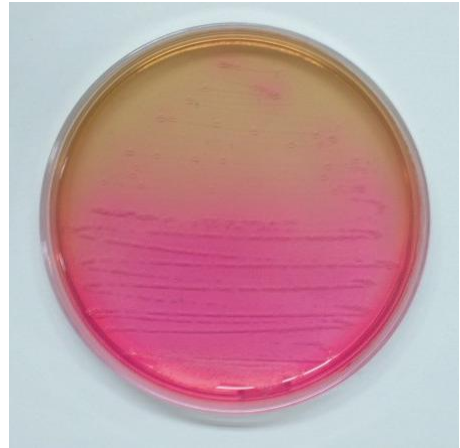


Figure 18.1-7 Example of selective enrichment culture (2: Rappaport-Vassiliadis broth)  
(Left: before use Right: after cultivation)

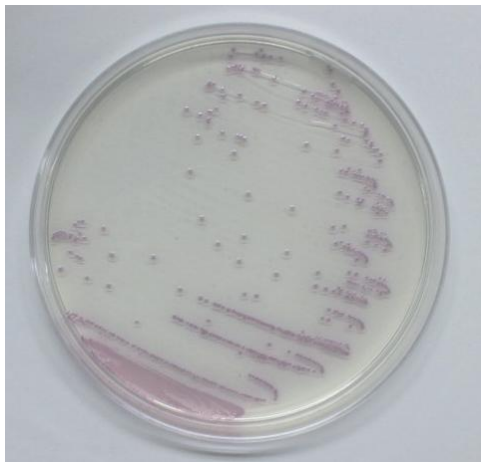
Depending on the condition of the enriched enterobacteria, the color of the broth changes from blue, pale green to pale yellow.



DHL agar medium



Brilliant green agar medium



CHROMagar Salmonella medium

Figure 18.1-8 *Salmonella* colonies on selective isolation media



**(Appended item)**

**Determining the H antigen (flagellar antigen)**

Like O antigens, H antigens are classified into many types. Most of *Salmonella* have two types of H antigens (phase II). However, both types coexist rarely. Thus, after identification of the first H antigen (phase 1), it is required to artificially derivate the second H antigen (phase 2) and identify it.

To identify H antigens, check the aggregation agglutination in the test tube using serum.

**1 Preparation of medium**

**1) Tryptic soy broth**

Dissolve 30 g of tryptic soy broth (Eiken Chemical or equivalent) with 1,000 mL of water and dispense 10 mL each in middle test tubes. Then cap the test tubes with silicone resin stoppers and autoclave them at 121 °C for 15 minutes.

**2) Semisolid agar medium**

Dissolve 10 g of peptone (Difco or equivalent), 5 g of sodium chloride and 3 g of yeast extract (Difco or equivalent) with 1,000 mL of water, adjust the pH to 7.1 to 7.3 and dissolve completely by heating. Then, dispense 1.8 mL each in small test tubes, cap them with aluminum caps and autoclave them at 121 °C for 15 minutes.

**2 Identification of *Salmonella* H antigen (phase 1)**

1) Take a loopful of *Salmonella* from the slant of the TSI agar medium (or a brain heart infusion agar medium onto which *Salmonella* is subcultured as required), inoculate it into 10 mL of tryptic soy broth and incubate it statically at 35 to 37 °C overnight.

2) After incubation, add 10 mL (the same volume of tryptic soy broth) of saline containing 1 v/v% formaldehyde to the test tube, shake it well and then leave it at room temperature over half day. (Define this as the antigen solution\*<sup>1</sup>.)

3) Put two drops of each H antiserum (Denka Seiken or equivalent) in small test tubes. Add 0.5 mL of antigen solution to each test tube, shake them well and leave them in a 50 °C incubator for 30 to 60 minutes to promote reaction. Judge the samples by checking for agglutination.

Observe the agglutinations without shaking the test tubes because clumps of agglutination with H antiserum are fragile. Judge as positive when an agglutination is obviously visible.

**3 Identification of *Salmonella* H antigen (phase 2)**

1) Put the autoclaved semisolid agar medium in a 50 °C incubator to lower the temperature. Add 0.1 mL of phase induction antiserum (Denka Seiken or equivalent) for the H antigen judged as phase 1 to the medium aseptically and mix them gently without bubbles.

2) Stand the sterilized Craigie tube (inner diameter 2 to 3 mm, length 50 to 70 mm, Nichiden-Rika Glass or equivalent) aseptically in the center of the

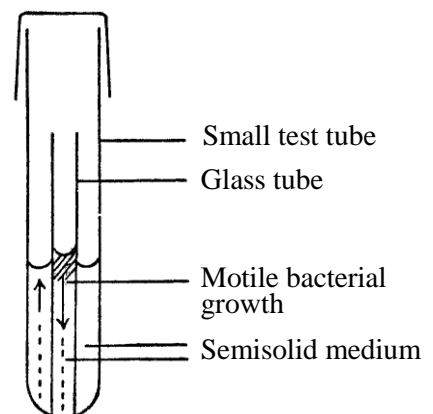


Figure 18.1-9 Craigie tube

medium and solidify the agar (see Figure 18.1-9).

- 3) Pick with platinum needle *Salmonella* from the slant of the TSI agar medium (or the brain heart infusion agar medium onto which *Salmonella* is subcultured as required), inoculate it shallowly on the medium surface (3 mm depth from the surface) within the Craigie tube and incubate it at 35 to 37 °C.
- 4) Incubated *Salmonella* pass through the glass tube in about one or two days, reach the medium surface between the glass tube and the test tube and having growth. As in 2.1), cultivate these bacteria using 10 mL of tryptic soy broth\*<sup>2</sup>.
- 5) As in 2.2), add 10 mL of saline containing 1 v/v% formaldehyde to the test tube and leave it at room temperature over half day.
- 6) Perform the same procedure as in 2.3) and judge H antigen (phase 2)\*<sup>3</sup>.
- 7) When O antigen, H antigen (phase 1) and H antigen (phase 2) have been identified, determine the serotype of *Salmonella* by reference to the Kauffmann and White classification scheme.

#### 4 Example of *Salmonella* identification

- 1) Determine O group. ....O7 group is determined.
- 2) To identify H antigen (phase 1), test with all 17 types of H antisera. ....An agglutination is found in z<sub>10</sub> antiserum.
- 3) Induce phase 2 of H antigen using the phase induction antiserum for z<sub>10</sub>.
- 4) Check the combinations of O7 group – z<sub>10</sub> in the Kauffmann and White classification to narrow the possible phase 2 (1, 2, 5, 6, 7, e, n, x, z, l, w, z<sub>6</sub>, z<sub>35</sub>, z<sub>15</sub>) and test with these antisera.....An agglutination is found in en antiserum.
- 5) Test with en factor antiserum x and z<sub>15</sub>.....An agglutination is found in z<sub>15</sub> factor antiserum.

The following summarizes the above results:

|                     |                       |
|---------------------|-----------------------|
| O antigen           | O7 group              |
| H antigen (phase 1) | z <sub>10</sub>       |
| H antigen (phase 2) | e, n, z <sub>15</sub> |

Thus, the serotype is determined to be *S. Mbandaka* based on the Kauffmann and White classification.

- \* 1 Store the antigen solution at room temperature. It will be valid for about 10 days.
- 2 If *Salmonella* does not grow on the medium, do either of the following operations.
- i Reduce the volume of phase inducing antiserum to 0.05 mL and operate 1) to 3) again.
  - ii Perform the following operation once or twice to activate flagellar of *Salmonella*.  
Inoculate *Salmonella* on the semisolid agar medium without phase inducing antiserum in which Craigie tube stands, then incubate it so that *Salmonella* passes through the glass tube.
- 3 If phase 1 and 2 are the same, it is possibly because masking of phase 1 has been insufficient. Increase the volume of phase inducing antiserum to 0.2 mL and operate 1) to 6) again.

## [Other analysis methods]

### 2 *Salmonella* test kits

Since the standard method for surveillance of *Salmonella* using cultures listed in the Analytical Standards of Feeds takes 5 days until the results are proved, it is unpractical for feed factories that require quick results. Thus, many feed factories apply other test kits that ensure quickness.

Recently, a variety of quick test kits are commercially available. FAMIC performs comparison tests for a part of these kits in accordance with the standard method. The test details are released in the following references. Table 18.1-10 summarizes comparison of quick test kits which have obtained 94 % or more accordance with the standard method.

Each commercially available quick inspection kit has its own features, some of which may not be applied to feeds. Thus, it is preferable to perform the comparison test with the standard method before using a kit.

#### Reference (concerning quick test kits to be used for feeds)

- Toyoko Kusama, et al.: Evaluation of Rapid Detection Kit “KAKUSAN Test *Salmonella*” for Detection of *Salmonella* in Feeds, Research Report of Animal Feed, 25, 51 (2000)
- Tetsuo Chihara, et al.: Evaluation of Rapid Detection Kit “1-2 Test” for Detection of *Salmonella* in Feeds, Research Report of Animal Feed, 25, 61 (2000)
- Seishi Araki, et al.: Evaluation of Rapid Detection Kit “Oxoid *Salmonella* Rapid Kit” for Detection of *Salmonella* in Feeds, Research Report of Animal Feed, 26, 78 (2001)
- Hisashi Kudo, et al.: Evaluation of Rapid Detection Kit “SUNCOLI *Salmonella*” for Detection of *Salmonella* in Feeds, Research Report of Animal Feed, 26, 88 (2001)
- Jun Noguchi, et al.: Evaluation of Rapid Detection Kit “Path-Stik Rapid *Salmonella* Test” for Detection of *Salmonella* in Feeds, Research Report of Animal Feed, 26, 96 (2001)
- Hisashi Kudo, et al.: Evaluation of the Rapid Detection Kit “SALMOCHECK” for Detection of *Salmonella* in Feeds, Research Report of Animal Feed, 27, 112 (2002)
- Seishi Araki, et al.: Evaluation of Rapid Detection Kit “Locate” for Detection of *Salmonella* in Feeds, Research Report of Animal Feed, 27, 122 (2002)
- Seishi Araki, et al.: Evaluation of Rapid Detection Kit “Spectate” for Detection of *Salmonella* in Feeds, Research Report of Animal Feed, 27, 133 (2002)
- Masayuki Shimomura, et al.: Evaluation of Rapid Detection Kit “*Salmonella* Check (MB)” for Detection of *Salmonella* in Feeds, Research Report of Animal Feed, 27, 143 (2002)
- Jun Noguchi, et al.: Evaluation of Rapid Detection Kit “Reveal for *Salmonella* Test System” for Detection of *Salmonella* in Feeds, Research Report of Animal Feed, 28, 99 (2003)
- Tetsuo Chihara, et al.: Studies on a Rapid Method for Detecting *Salmonella* in Animal Feed Using the QUALIBAX<sup>TM</sup> System, Japanese Journal of Food Microbiology, 25, 109 (2008)

FAMIC also examines the PCR method targeting invasion gene of *Salmonella* (*invA*) and the method targeting *Salmonella* plasmid virulence (*SpvC*), a gene related to pathologic expressions.

Reference (concerning the PCR (POLYMERASE CHAIN REACTION) method for feed)

Toyoko Kusama: Detection of *Salmonella* sp. in Animal Feed by Polymerase Chain Reaction, Research Report of Animal Feed, 31, 163 (2006)

Yasutoshi Sugimoto, et al.: Rapid Identification of Six Serotypes of *Salmonella* by PCR, Research Report of Animal Feed, 31,172 (2006)

Table 18.1-3 Comparisons of rapid test kits for *Salmonella* in feeds

| Kit name<br>(Manufacturer)                    | Principles of detection | No. of tested samples |                        | Accordance with<br>standard method<br>(%) | False positive rate *1<br>(%) | False negative rate *2<br>(%) | Required time *3<br>(h) |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |                             |     |    |    |      |     |   |    |  |  |
|---|-------------------------|-----------------------|------------------------|---|-------------------------------|-------------------------------|-------------------------|---|------------------------|----|----|------|-----|-----|----|----|----|---|------------------------|----|----|------|-----|-----|----|----|----|---|------------------------|----|----|------|-----|-----|----|----|----|---|------------------------|----|----|------|-----|-----|----|----|----|-----------------------------|-----|----|----|------|-----|---|----|--|--|
|   |                         | Upper: Formula feed   | Lower: Feed ingredient |   |                               |                               |                         |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |                             |     |    |    |      |     |   |    |  |  |
| Spectate<br>(Rhône-Poulenc Diagnostics)       | Latex<br>agglutination  | 26                    | 26                     | 94.1                                      | —                             | 5.9                           | 55                      |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |                             |     |    |    |      |     |   |    |  |  |
|   |                         | 92                    | 92                     |   |                               |                               |                         | Kakusan-test Salmonella<br>(Fasmac Co., Ltd.) | DNA probing            | 31 | 31 | 95.1 | 1.0 | 3.9 | 51 | 72 | 72 | Locate<br>(Rhône-Poulenc Diagnostics)   | ELISA                  | 26 | 26 | 96.6 | 1.7 | 1.7 | 56 | 92 | 92 | 1-2 Test<br>(BioControl Systems)        | Immunodiffusion        | 28 | 28 | 96.7 | 1.7 | 0.8 | 62 | 92 | 92 | Oxioid Salmonella quick kit<br>(Oxioid) | Latex<br>agglutination | 31 | 31 | 97.5 | 0.8 | 1.7 | 49 | 90 | 90 | QualiBAX system<br>(DuPont) | PCR | 28 | 28 | 97.4 | 1.3 | — | 32 |  |  |
| Kakusan-test Salmonella<br>(Fasmac Co., Ltd.) | DNA probing             | 31                    | 31                     | 95.1                                      | 1.0                           | 3.9                           | 51                      |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |                             |     |    |    |      |     |   |    |  |  |
|   |                         | 72                    | 72                     |   |                               |                               |                         | Locate<br>(Rhône-Poulenc Diagnostics)         | ELISA                  | 26 | 26 | 96.6 | 1.7 | 1.7 | 56 | 92 | 92 | 1-2 Test<br>(BioControl Systems)        | Immunodiffusion        | 28 | 28 | 96.7 | 1.7 | 0.8 | 62 | 92 | 92 | Oxioid Salmonella quick kit<br>(Oxioid) | Latex<br>agglutination | 31 | 31 | 97.5 | 0.8 | 1.7 | 49 | 90 | 90 | QualiBAX system<br>(DuPont)             | PCR                    | 28 | 28 | 97.4 | 1.3 | —   | 32 |    |    | 50                          | 50  |    |    |      |     |   |    |  |  |
| Locate<br>(Rhône-Poulenc Diagnostics)         | ELISA                   | 26                    | 26                     | 96.6                                      | 1.7                           | 1.7                           | 56                      |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |                             |     |    |    |      |     |   |    |  |  |
|   |                         | 92                    | 92                     |   |                               |                               |                         | 1-2 Test<br>(BioControl Systems)              | Immunodiffusion        | 28 | 28 | 96.7 | 1.7 | 0.8 | 62 | 92 | 92 | Oxioid Salmonella quick kit<br>(Oxioid) | Latex<br>agglutination | 31 | 31 | 97.5 | 0.8 | 1.7 | 49 | 90 | 90 | QualiBAX system<br>(DuPont)             | PCR                    | 28 | 28 | 97.4 | 1.3 | —   | 32 |    |    | 50                                      | 50                     |    |    |      |     |     |    |    |    |                             |     |    |    |      |     |   |    |  |  |
| 1-2 Test<br>(BioControl Systems)              | Immunodiffusion         | 28                    | 28                     | 96.7                                      | 1.7                           | 0.8                           | 62                      |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |                             |     |    |    |      |     |   |    |  |  |
|   |                         | 92                    | 92                     |   |                               |                               |                         | Oxioid Salmonella quick kit<br>(Oxioid)       | Latex<br>agglutination | 31 | 31 | 97.5 | 0.8 | 1.7 | 49 | 90 | 90 | QualiBAX system<br>(DuPont)             | PCR                    | 28 | 28 | 97.4 | 1.3 | —   | 32 |    |    | 50                                      | 50                     |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |                             |     |    |    |      |     |   |    |  |  |
| Oxioid Salmonella quick kit<br>(Oxioid)       | Latex<br>agglutination  | 31                    | 31                     | 97.5                                      | 0.8                           | 1.7                           | 49                      |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |                             |     |    |    |      |     |   |    |  |  |
|   |                         | 90                    | 90                     |   |                               |                               |                         | QualiBAX system<br>(DuPont)                   | PCR                    | 28 | 28 | 97.4 | 1.3 | —   | 32 |    |    | 50                                      | 50                     |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |                             |     |    |    |      |     |   |    |  |  |
| QualiBAX system<br>(DuPont)                   | PCR                     | 28                    | 28                     | 97.4                                      | 1.3                           | —                             | 32                      |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |                             |     |    |    |      |     |   |    |  |  |
|   |                         | 50                    | 50                     |   |                               |                               |                         |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |   |                        |    |    |      |     |     |    |    |    |                             |     |    |    |      |     |   |    |  |  |

- \* 1 Negative by Analytical Standards of Feeds; Positive by rapid test kit  
 2 Positive by Analytical Standards of Feeds; Negative by rapid test kit  
 3 Time required for each kit to enrich culture and prepare test solution

## 2 *Escherichia coli*

### [Summary of *Escherichia coli*]

*Escherichia coli* is gram-negative facultative anaerobic nonsporeforming bacteria which taxonomically belong to the genus *Escherichia* of the family Enterobacteriaceae. These bacteria reside in intestines of humans and animals and are distributed in nature environments via feces. Many of them do not show pathogenicity in normal humans. Those which exhibit pathogenicity such as enteritis are called *E. coli*-induced diarrhea and are classified into 4 types: enteropathogenic *E. coli* [EPEC], enteroinvasive *E. coli* [EIEC], enterotoxigenic *E. coli* [ETEC] and verotoxin-producing *E. coli* (or enterohemorrhagic *E. coli* [EHEC]).

The enteropathogenic *E. coli* are specific serotype bacteria which induce diarrhea to infants. About 40 serotypes are currently known. The enteroinvasive *E. coli* break into the colonic epithelial cells and induce diarrhea and febrile symptoms similar to dysentery. About 20 serotypes are currently known. The enterotoxigenic *E. coli* are bacteria which produce heat-labile or heat-stable enterotoxin as the diarrhea-causing toxins. About 30 types of this serotype are currently known. The verotoxin-producing *E. coli* are bacteria which produce verotoxin. About 200 serotypes are currently known. Among them, bacteria which induce serious hemorrhagic colitis to humans, such as O157:H7, are called enterohemorrhagic *E. coli*.

Reference: Riichi Sakazaki, Kazumitsu Tamura: Intestinal bacteria Second vol., *Escherichia* Genus, Kindai Shuppan (1992)  
Edition of Pharmaceutical Society of Japan: Standard Methods of Analysis for Hygienic Chemists with Commentary 2000, Kanehara & Co (2000)

### [Method listed in the Analytical Standards of Feeds]

1 Confirmation method of enterohemorrhagic *Escherichia coli* O157:H7 [Analytical Standards of Feeds, Article 2.1, Chapter 18]

#### «Summary of Enterohemorrhagic *Escherichia coli* O157:H7»

Enterohemorrhagic *Escherichia coli* O157:H7 are bacteria which possess O157 as somatic antigen, a type of *Escherichia coli* which produce verotoxin and induce hemorrhage colitis. According to H antigen, 2 serotypes are known: O157:H7 and O157:H<sup>-</sup>. In 1996, a large scale of food poisoning occurred in Japan. Since O157 cause critical symptoms to humans, it was designated as the official disease in 1998. It is known that O157 exists in intestines of ruminants such as cattle and sheep.

Use sterilized water, reagent and instrumentations, as required.

#### A. Reagent preparation

- 1) Surfactant solution.<sup>[1]</sup> Autoclave surfactant solution (10 v/v%) at 121 °C for 15 minutes.
- 2) Saline. Autoclave sodium chloride solution (0.9 w/v%) at 121 °C for 15 minutes.
- 3) Phosphate buffered saline (hereinafter referred to as PBS).<sup>[2]</sup> Dissolve 1.15 g of disodium hydrogen phosphate 12 water, 1.2 g of potassium dihydrogen phosphate, 8 g of sodium chloride, 0.2 g of

potassium chloride and 0.5 g of surfactant<sup>\*1</sup> in 750 mL of distilled water. Adjust the pH to 7.3 to 7.5. Then, add water to make the total volume 1,000 mL and autoclave at 121 °C for 15 minutes.

- 4) Buffered peptone water.<sup>[3]</sup> Dissolve 10 g of peptone, 5 g of sodium chloride, 9 g of disodium hydrogen phosphate 12 water and 1.5 g of potassium dihydrogen phosphate with 1,000 mL of water, and adjust the pH to 6.9 to 7.1.

Dispense 250 mL of this solution into 500 mL culture bottles<sup>[4]</sup> and autoclave them at 121 °C for 15 minutes. Then cool it down below 45 °C. Add 15 mL of surfactant solution to the bottle and shake it well.

- 5) mEC broth with novobiocin.<sup>\*2[5]</sup> Dissolve 20g of peptone, 1.12g of sodium deoxycholate, 5 g of lactose monohydrate, 4 g of dipotassium hydrogen phosphate, 1.5 g of potassium dihydrogen phosphate and 5 g of sodium chloride with 1,000 mL of water and autoclave at 121 °C for 15 minutes. Then, adjust the pH to 6.8 to 7.0. Cool it down below 45 °C and then add 20 mg of novobiocin sodium.

- 6) CT-SMAC agar medium.<sup>\*2[6]</sup> Dissolve 20 g of peptone, 1.5 g of sodium deoxycholate, 10 g of sorbitol, 5 g of sodium chloride, 30 mg of neutral red, 1 mg of crystal violet and 15 g of agar with 1,000 mL of water and autoclave at 121 °C for 15 minutes. Then, adjust the pH to 7.1 to 7.3. Cool it down below 45 °C and then add 50 µg of cefixime and 2.5 mg of potassium tellurite.

Pour 20 mL of the medium evenly on Petri dishes<sup>[9]</sup> and allow to solidify. Then, turn them over and slightly slide the lids and leave them at 35 to 37 °C for one hour until the surface of the agar is dry<sup>[7]</sup>.

- 7) CHROMagar O157 agar medium.<sup>\*2[8]</sup> Dissolve 5 g of peptone, 3 g of yeast extract, 1 g of meat extract, 5 g of sodium chloride, 1 g of mixture of selective agent and chromogenic substrate<sup>\*3</sup> and 15 g of agar with 1,000 mL of water by heating in a boiling water bath, then adjust the pH to 6.7 to 6.9.

Then, do the same procedure as in 6).

- 8) CLIG agar medium.<sup>\*2[9]</sup> Dissolve 10 g of peptone, 1 g of lactose monohydrate, 10 g of D-cellobiose, 0.1 g of L-tryptophan, 5 g of sodium chloride, 25 mg of phenol red, 20 mg of 4-methylumbelliferyl-β-D-glucuronide and 15 g of agar with 1,000 mL of water by heating in a boiling water bath, then adjust the pH to 7.3 to 7.5.

Dispense 4 mL portions of the medium into small test tubes and autoclave them at 115 °C for 15 minutes. Then, allow to set in a sloping position to solidify in a way of slant with butt (Slant is upper 1/3 and butt is lower 2/3 depth).<sup>[10]</sup>

- 9) SIM agar medium.<sup>[11]</sup> Dissolve 30 g of peptone, 3 g of beef extract, 0.5 g of ammonium ferric citrate, 50 mg of sodium thiosulfate (anhydrous), 0.2 g of L-cysteine hydrochloride monohydrate and 5 g of agar with 1,000 mL of water by hearing in a boiling water bath, then adjust the pH to 7.3 to 7.5.

Dispense 4 mL of the solution into small test tubes and autoclave them at 121 °C for 15 minutes. Then, solidify them in a way of butt<sup>[12]</sup>.

## B. Cultivation<sup>[13]</sup>

Pre-enrichment. Weigh<sup>[15]</sup> 25 g of analysis sample<sup>[14]</sup> into buffered peptone water and shake it well. Then, incubate the solution at 35 to 37 °C for 18 to 24 hours.

Selective enrichment.<sup>\*4</sup> Add 10 mL of pre-enrichment culture to mEC broth with novobiocin<sup>[16]</sup>, and shake it well. Then, incubate the solution at 41 to 43 °C for 18 to 24 hours<sup>[17]</sup>.

Capture of bacteria by immunomagnetic beads.<sup>\*5[18]</sup> Place 1 mL of selective enrichment culture in a plastic centrifugation tube (with capacity of 1.5 mL). Add 20 µL of immunomagnetic beads combined with antibody of *Escherichia coli* O157 to the tube and shake it gently for 15 minutes<sup>\*6[19]</sup>. Mount the tube to magnet particle concentrator<sup>[20]</sup>, sometimes shake it gently, leave the tube for 3 minutes and remove the liquid<sup>[21]</sup>. Remove the tube from magnet particle concentrator, add 1 mL of PBS to the residue and shake it gently for 5 minutes<sup>\*6[19]</sup>. Mount the tube to magnet particle concentrator, sometimes shake the tube gently, leave it for 3 minutes, and remove the liquid.

Remove the tube from magnet particle concentrator, add 1 mL of PBS to the residue and repeat the procedures described above twice. Then, add 100 µL of PBS to suspend immunomagnetic beads.

Selective isolation. Add 50 µL of immunomagnetic beads suspension each to CT-SMAC agar medium and to CHROMagar O157 medium and smear the suspension using a cotton-tipped stick. Then, streak<sup>[22]</sup> with a inoculating loop, turn the plate over and incubate it at 35 to 37 °C for 18 to 24 hours.

Pure isolation.<sup>[23]</sup> Pick with a needle<sup>[25]</sup> five typical or suspicious colonies<sup>[24]</sup> of *Escherichia coli* O157, if present, from each surface of CT-SMAC agar medium and of CHROMagar O157 medium having growth. Dilute<sup>[26]</sup> each colony with 0.3 mL of saline. Streak<sup>[27]</sup> a loopful of each diluted solution on CHROMagar O157 medium, turn the plate over and incubate it at 35 to 37 °C for 18 to 24 hours.

Confirmation. Pick with a needle a typical or suspicious colony of *Escherichia coli* O157, if present, from the surface of CHROMagar O157 medium having growth. Inoculate CLIG agar medium by stabbing the butt and streaking the agar slant surface with the needle. After inoculating CLIG, inoculate with the needle, SIM agar medium by stabbing<sup>[28]</sup>. Then, incubate them at 35 to 37 °C for 18 to 24 hours.

### C. Identification

Identification. Identify *Escherichia coli* O157 by judging from the following biochemical properties<sup>[29]</sup> observed from the CLIG agar medium and the SIM agar medium.

In most cases, *Escherichia coli* O157 exhibits following properties: lactose fermentation (+), oxidase<sup>[30]</sup> (-), β-glucuronidase activity (-), cellobiose utilization (-), hydrogen sulfide (-), motility (+), IPA<sup>[31]</sup> (-) and indole<sup>[32]</sup> (+).

Serotyping.<sup>\*7[33]</sup> Pick a small amount of colony which exhibits the properties of *Escherichia coli* O157, mix with O157 antiserum<sup>[34]</sup> dropped on a slide glass. Tilt the slide glass back and forth and observe whether an agglutination occurs. Then, in the same way, test heat-treated colony at 100 °C for 30 minutes with O157 antiserum whether an agglutination occurs<sup>[35]</sup>.

Test the colony agglutinated with O157 antiserum for agglutinations with H antiserum<sup>[37]</sup> using the test tube method<sup>[36]</sup> and determine the H antigen.

Detection of verotoxin gene.<sup>[38]</sup> Pick a small amount of colony which exhibits the properties of *Escherichia coli* O157. Mix it with 200 µL of saline in a 1.5 mL plastic centrifugation tube, shake the tube and centrifuge it at 7,000×g for 5 minutes. Then, remove the supernatant. Add 100 µL of water to the residue, shake the tube, heat it at 98 °C for 5 minutes and cool it down rapidly.

Centrifuge the heat-treated bacteria solution at 7,000×g for 10 minutes. Use the supernatant<sup>[39]</sup> for PCR<sup>\*8[40]</sup> to test for the verotoxin gene.

Verotoxin productivity test. Pick a small amount of colony which exhibits the properties of *Escherichia coli* O157, mix it with latex particles<sup>\*9</sup> sensitized to verotoxins on a plate to observe agglutinations.

- \* 1 Tween 20 (Kanto Chemical) or equivalent
- 2 For these media, commercially available dry powder media having the same composition as those can be used. Use hydrochloric acid solution (1 mol/L) or sodium hydroxide solution (1 mol/L) to adjust the pH.
- 3 A chromogenic substrate consisting of structure of  $\beta$ -glucuronide and  $\beta$ -glucoside which is decomposed by the enzyme activity specific to O157 and becomes mauve color. This is contained in CHROMagar O157 (CHROMagar) or equivalent.
- 4 When the following PCR method proves that there is no verotoxin-producing *E. coli* gene in the selective enrichment culture solution, the sample is judged to be negative for *E. coli* O157, and the sections after “Capture of bacteria with immunomagnetic beads” will be omitted.

(PCR method)

Place 1 mL of selective enrichment culture solution in a plastic centrifugation tube (capacity: 1.5 mL), centrifuge the tube at 7,000×g for 5 minutes and remove the supernatant. Add 500  $\mu$ L of saline to the residue, shake the tube and centrifuge it at 7,000×g for 5 minutes. Then, remove the supernatant. Add 100  $\mu$ L of water to the residue and shake the tube. Heat it at 98 °C for 5 minutes and then cool it down rapidly.

Use the heat-treated bacteria solution in accordance with the section of “Detection of verotoxin gene” and test for the verotoxin genes.

- 5 Dynabeads anti-E. coli O157 (Dyna) or equivalent
- 6 Use a shaker with frequency of 60 rpm of rotation or vibration.
- 7 Judge a firm agglutination with O157 antiserum found within one minute to be positive.
- 8 O-157 (verotoxin gene) One Shot PCR Screening Kit Ver.2 (Takara Bio) or equivalent
- 9 VTEC-RPLA “SEIKEN” (Denka Seiken) or equivalent

#### «Summary of analysis method»

This method is to enrich bacteria in the sample using buffered peptone water and mEC broth with novobiocin, test for verotoxin gene using PCR, then if the result is positive, capture *E. coli* O157 with immunomagnetic beads, isolate using CT-SMAC agar medium and CHROMagar O157 medium, and then test for biochemical properties, serotype and verotoxin productivity.

Target samples are formula feed and feed ingredients of animal-origin such as fish meal and meat-and-bone meal, and plant-origin such as soybean meal and maize.

Figure 18.2-1 shows the flow sheet of detection of *Escherichia coli* O157 in feed.



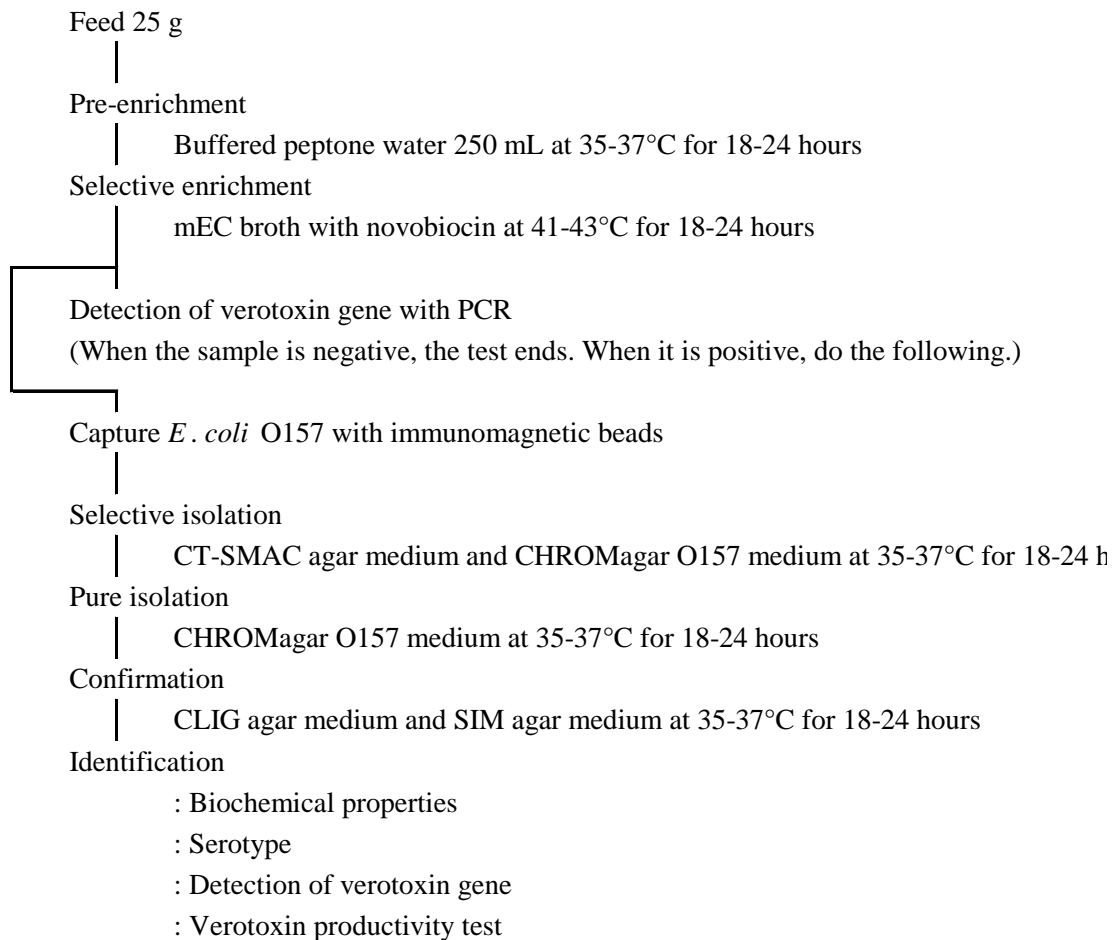


Figure 18.2-1 Flow sheet for detecting enterohemorrhagic *Escherichia coli* O157 in feed

References: Toyoko Kusama, Motomu Suginaka, Research Report of Animal Feed, 23, 128 (1998)

#### «Notes and precautions»

- [1] Added so as to disperse fatty components in the sample and obtain homogeneous culture solution. Since the surfactant (Tween 80) is hard to dissolve in low-temperature water, dissolve it by heating. The chemical name of Tween 80 is polyoxyethylene sorbitan monooleate and it is also called polysorbate 80.
- [2] Phosphate-buffered saline containing surfactant which is used to capture bacteria with immunomagnetic beads. Phosphate Buffered Saline (pH 7.4) (Sigma Aldrich) or equivalent reagent can be used.
- [3] Buffered peptone water (Difco, Oxoid, Eiken Chemical or Nissui Pharmaceutical) or equivalent dry powder medium can be used.
- [4] Equip a glass culture bottle with an aluminum cap to keep ventilation.
- [5] mEC broth with novobiocin (Eiken Chemical, Kyokuto Pharmaceutical Industrial, Oxoid, Merck), mEC broth (Eiken Chemical, Nissui Pharmaceutical, Kyokuto Pharmaceutical Industrial, Oxoid) or equivalent dry powder medium can be used. When using the mEC broth with novobiocin, autoclave the broth and cool it down rapidly. When using the mEC broth, autoclave the broth and cool it down. Then, add 20 mg of novobiocin sodium (Sigma, Kanto Chemical, Wako Pure Chemical Industries or equivalent) to 1,000 mL of the broth. Before use, dissolve novobiocin sodium with water to make 2

- mg/mL solution and then filter and sterilize. Novobiocin has effects to suppress enrichment of enterobacteria such as *Citrobacter* spp. and *Proteus* spp. mEC stands for modified *Escherichia coli*.
- [6] You may use Sorbitol MacConkey Agar (Oxoid, Difco, Merck, Eiken Chemical, Nissui Pharmaceutical, Kyokuto Pharmaceutical Industrial) or equivalent dry powder medium and add CT-supplement (Oxoid, Dynal or Merck) or equivalent to the medium.
- CT stands for cefixime and tellurite. Cefixime has effects to suppress enrichment of *Proteus* spp. and, in combination with tellurite, to selectively enrich *Escherichia coli* O157. SMAC stands for Sorbitol MacConkey. *Escherichia coli* O157 does not degrade sorbitol in this medium so that it is able to be distinguished from general *E. coli*.
- [7] As shown in Figure 18.1-2, turn over the Petri dish and dry it in the incubator until the surface of the plate will be free of waterdrop. If the medium is not completely dried, bacteria spreads over waterdrop and grows there when it is streaked, possibly resulting in failure of generating appropriate colonies. If the medium is dried too much, the surface of the medium is likely to be cracked.
- [8] CHROMagar O157 (CHROMagar) or equivalent dry powder medium can be used. Heat the medium in a water bath until the agar completely dissolves. Since this medium is likely to transform by heating, do not autoclave. Glycolysis and  $\beta$ -glucuronidase activity of *Escherichia coli* O157 enables it to be distinguished from general *E. coli* in this medium.
- [9] CLIG agar medium (Kyokuto Pharmaceutical Industrial) or equivalent dry powder medium can be used. CLIG stands for Cellobiose Lactose Indole  $\beta$ -Glucuronidase. This medium enables to confirm the cellobiose utilization, lactose fermentation and  $\beta$ -glucuronidase activity.
- [10] Dispense the medium in small test tubes (with diameter of 10 mm and length of 120 mm) up to 3 to 4 mL using a measuring pipette, Komagome pipette or a dispenser. Equip each test tube with an aluminum cap and autoclave. Then, tilt the test tube to solidify the medium in a way of slant with butt as shown in Figure 18.1-3.
- [11] SIM agar medium (Eiken Chemical) or equivalent dry powder medium can be used. Heat in a water bath for about 10 minutes until the agar completely dissolves. SIM stands for Sulfide Indole Motility. This medium enables to confirm the production of hydrogen sulfide and indole, motility and IPA reactions.
- [12] Dispense the medium in small test tubes (with diameter of 10 mm and length of 120 mm) up to 3 to 4 mL using a measuring pipette, Komagome pipette or a dispenser. Equip each test tube with an aluminum cap and autoclave. Then, stand the test tube vertically to solidify the medium in a way of butt as shown in Figure 18.1-4.
- [13] After incubation, be sure to autoclave culture bottles, Petri dishes and others contaminated with enterobacteria and then discard or wash them.
- [14] To take a sample of feed on-site, use sampling bags and sampling shovels which have been sterilized (through radiation sterilization, gaseous sterilization, etc.) and wear sterilized gloves.. If you cannot prepare these equipments, sterilize your fingers and sampling shovels with alcohol pad.
- [15] To weigh samples, use sterilized charta (with gaseous sterilization or ultraviolet sterilization) and medicine spoons sterilized with alcohol pad or dry-heat sterilized and prevent contamination through contact to other samples.

- [16] Use a Komagome pipette sterilized. After use, put the pipette in a container with antiseptic solution and autoclave it.
- [17] Compared to incubation at 35 to 37 °C, incubation at 41 to 43 °C enables to suppress enrichment of general *Escherichia coli* and enterobacteria such as *Proteus* spp. except *E. coli* O157, which shows a trend of slightly increase in the detection rate of *E. coli* O157.
- [18] This is a polymer particle of 2 or 3 μm to which the antibody of *Escherichia coli* O157 is adsorbed. It selectively combines with O157 through the antigen-antibody reaction. Since the immunomagnetic beads, after combined with O157 in the culture, are magnetized by closing a magnet and sucked in towards that magnet, this method enables to capture O157 in the culture solution.
- [19] Mix immunomagnetic beads and bacteria gently by rotating at 60 rpm or shaking moderately and combine them through the antigen-antibody reaction. Since strong shaking may detach the bacteria from beads, do not stir using a test tube mixer.
- [20] MPC (Magnetic Particle Concentrator) manufactured by Dynal or equivalent. MPC is a tool which securely holds ten 1.5 mL plastic centrifugation tubes and able to attach or remove a strong magnets on its sides.
- [21] Remove the supernatant using a fine-tipped plastic tip or a Pasteur pipette, with attention not to suck in the immunomagnetic beads magnetized and attached to the side wall of the plastic centrifugation tube. To avoid contamination with bacteria between samples, use the tip or Pasteur pipette sterilized and replace it with a new one for every operation.

[22] As shown in Figure 18.2-2, streak a half surface of the plate using a sterilized cotton-tipped stick. Then, streak at right angles using a sterilized inoculating loop, and turn over the inoculating loop and streak at right angles to the first traces. By streaking in 3 steps in this way, colonies can be separated regardless of the volume of bacteria.

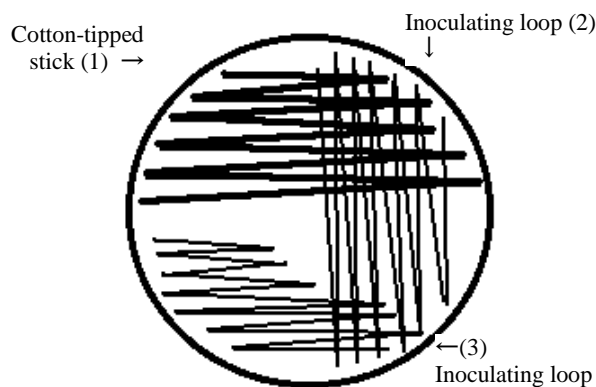


Figure 18.2-2 Streaking immunomagnetic beads

- [23] This process can be omitted when a colony suspected to be *Escherichia coli* O157 is obviously separated from other colonies.
- [24] The characteristics of colonies of *Escherichia coli* O157 and those of other enterobacteria on each selective isolation medium are as described below.

Since the color of the colony on the CT-SMAC agar medium may fade after more than 24 hours of cultivation, avoid long-time cultivation and check the colony soon after cultivation of specified time.

· CT-SMAC agar medium

*Escherichia coli* O157: Generate white colonies due to non-degradability of sorbitol.

Verotoxin-producing *E. coli* other than O157: Generate red colonies in many cases due to the degradability of sorbitol, but also generate white colonies rarely.

General *E. coli*, *Salmonella* spp., *Citrobacter* spp., *Klebsiella* spp.: Generate red colonies due to the degradability of sorbitol.

*Proteus* spp.: Generate white irregular colonies if CT does not suppress enrichment of the bacteria.

· CHROMagar O157 medium

*Escherichia coli* O157, *Salmonella* spp.: Generate mauve-colored colonies due to negative activity of  $\beta$ -glucuronidase.

Verotoxin-producing *E. coli* other than O157: Generate blue colonies, and mauve-colored colonies rarely.

General *E. coli*: Generate blue colonies due to the positive activity of  $\beta$ -glucuronidase.

*Proteus* spp.: Generate white or transparent colonies.

*Escherichia hermanii*: Generate pale green colonies.

[25] Pick up colony using a platinum needle or a sterilized toothpick. Most of bacteria remain alive even if they are not growing on the medium. Thus, be careful not to touch the areas other than the target colony.

[26] You may dilute the colony using a sterilized microtitration plate (about 300  $\mu$ L per well).

[27] Divide a CHROMagar O157 medium into 8 sectors and streak a 2 mm loopful of diluted solution within a sector. You can substitute a medium having relatively low selectivity such as the DHL agar medium (see Article 1 “*Salmonella*”, 1-A-7)).

[28] Pick up colony using a platinum needle and stab into the butt of the CLIG agar medium and streak the bacteria on the slant. Then, stab the same needle into the SIM agar medium.

[29] The biochemical properties of *Escherichia coli* O157 on each confirmation medium are as described below:

· CLIG agar medium

In most cases, *Escherichia coli* O157 decomposes lactose and produces acid, so that the butt of the medium becomes yellow. The slant of the medium becomes red due to non-degradability of cellobiose. O157 does not show fluorescence under 365 nm ultraviolet irradiation due to negative activity of  $\beta$ -glucuronidase while general *E. coli* show fluorescence.

· SIM agar medium

*Escherichia coli* O157 has motility and grows throughout the medium, so that the medium gets muddy. O157:H- has no motility and grows only along the line of needling. Since they do not produce hydrogen sulfide, the color of the entire medium does not change. The IPA reaction (see [31] below) is negative and the indole test (see [32] below) is positive.

Table 18.2-1 shows biochemical properties of *E. coli* O157 and some other enterobacteria.

Table 18.2-1 EHEC O157 and other enterobacteria

| Bacteria type                      | Medium | CLIG agar medium |        |             |         | SIM agar medium  |          |     |        |
|------------------------------------|--------|------------------|--------|-------------|---------|------------------|----------|-----|--------|
|                                    |        | Slant            | Butt   | Fluorescent | Oxidase | Hydrogen sulfide | Motility | IPA | Indole |
| <i>Escherichia coli</i>            |        |                  |        |             |         |                  |          |     |        |
| EHEC O157                          |        | Red              | Yellow | —           | —       | —                | + (—)    | —   | +      |
| Verotoxin-producing <i>E. coli</i> |        | Red              | Yellow | + ~ —       | —       | —                | + (—)    | —   | + (—)  |
| General <i>E. coli</i>             |        | Red              | Yellow | +           | —       | —                | + (—)    | —   | + (—)  |
| <i>Escherichia hermannii</i>       |        | Yellow           | Yellow | —           | —       | —                | +        | —   | +      |
| <i>Salmonella</i> spp.             |        | Red              | Red    | —           | —       | +                | +        | —   | —      |
| <i>Citrobacter</i> spp.            |        | Yellow           | Yellow | —           | —       | +                | +        | —   | —      |
| <i>Proteus</i> spp.                |        | Red              | Red    | —           | —       | + ~ —            | +        | +   | + (—)  |
| <i>Klebsiella</i> spp.             |        | Yellow           | Yellow | —           | —       | —                | —        | —   | —      |
| <i>Enterobacter</i> spp.           |        | Yellow           | Yellow | —           | —       | —                | +        | —   | —      |

Note) + ~ — : Positive or negative

+ (—) : Most of samples are positive

[30] This is a test to confirm the cytochrome oxidase activity. When streaking the culture on the CLIG agar medium onto a piece of moistened oxidase test paper (Eiken Chemical) and the paper turns deep blue within one minute, the culture is judged to be positive. This test is used to distinguish *Escherichia coli* O157 from *Aeromonas* spp. and *Plesiomonas* spp.

[31] IPA (Indolepyruvic acid) reaction is a phenomenon in which indolepyruvic acid produced by deamination of tryptophan contained in peptone forms a chelate with Fe<sup>3+</sup> of ammonium ferric citrate and generates a brown belt in the upper edge of the medium. This is a reaction specific to *Proteus* and related bacteria.

[32] This is a test to check for production of indole. After stratifying 1 mL of chloroform over the SIM agar medium, drop 1 mL of the following Kovač's reagent on the medium and shake it gently. If the chloroform layer becomes red, the sample is judged to be positive.

To prepare Kovač's reagent, dissolve 5 g of *p*-dimethylaminobenzaldehyde with 75 mL of isoamyl alcohol on a water bath at about 50 °C and then add 25 mL of hydrochloric acid. Store the reagent in a brown bottle.

[33] Separation by serotype is performed by identifying the O antigen (somatic antigen), a bacteria surface antigen with the H antigen, a flagellar antigen. Two serotypes of the enterohemorrhagic *Escherichia coli* O157:H7 are well known: those having H7 antigen (O157:H7) and those for which mobility is negative (O157:H-).

[34] O157 antiserum in Pathogenic *E. coli* antisera (Denka Seiken) or equivalent can be used.

Instead of the immune serum, the latex labeled with antibody such as *Escherichia coli* O157 detection reagent "UNI" (Oxoid or equivalent) can be used. However, keep in mind that the judgement time of agglutination may be different.

[35] The agglutination results may be different between the viable bacteria and the heat-treated bacteria. Usually, judgment is based on the agglutinaitons of heat-treated bacteria. However, if either bacteria group shows agglutination in this method, it is preferable to detect verotoxin genes for further confirmation.

It is known that enterobacteria other than *E. coli*, such as *Citrobacter* spp., agglutinate with the O157 antiserum rarely. If a bacteria agglutinate with the O157 antiserum but no verotoxin gene is detected from them, refer to the citric acid utilization and VP reactions («Notes and precautions»

[28] of Article 3 “Other *Escherichia coli* confirmation test methods” in this chapter) and check whether the bacteria will be identified with *E. coli*.

[36] For H antigen agglutination reactions in the test tube, see «**Notes and precautions**» [39] and (**appended item**) of Article 1.1, *Salmonella*, in this chapter.

Since, in most cases, *E. coli* shows less clear H agglutination reactions than *Salmonella*, it is preferable to pass the bacteria several times through the semisolid agar medium in which a Craigie tube stands, and then test the agglutination reaction.

As the test requires a couple of days, you should detect the verotoxin genes first.

[37] H7 antiserum of Pathogenic *E. coli* antisera (Denka Seiken) or equivalent can be used.

[38] As the *Escherichia coli* verotoxin genes, type 1 (VT1), type 2 (VT2) and a variation of type 2 (VT2 variant) are known.

[39] DNA separated from bacteria through heat treatment and centrifuging are dispersed in this supernatant.

[40] PCR (Polymerase Chain Reaction) is a method to detect the presence of the target gene quickly with high sensitivity by amplifying the specific gene segment millionfold in several hours. Detection of *Escherichia coli* verotoxin gene with PCR is described in the appended item.

**(Appended item)**

Detection of *Escherichia coli* verotoxin gene with PCR

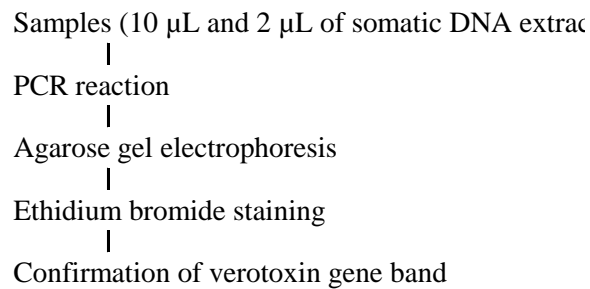


Figure 18.2-3 Flowsheet of detection of verotoxin gene with PCR

1 Devices required for the test

- 1) Thermal cycler  
TaKaRa PCR Thermal Cycler MP (Takara Bio) or equivalent
- 2) Electrophoresis apparatus  
Mupid-2 (Advance) or equivalent
- 3) Ultracentrifuge (applicable with 1.5 mL and 0.2 mL tubes and capable of centrifuging at 12,000 rpm)
- 4) Polaroid camera
- 5) UV transilluminators at 365 nm or 312 nm (Cosmo Bio)

2 Reagent preparation

Use sterilized ultrapure water by autoclaving as required.

- 1) Verotoxin gene detection kit  
O157 (Verotoxin gene) One Shot PCR Screening Kit Ver.2 (Takara Bio) or equivalent  
This kit is designed to detect the verotoxin genes type 1, type 2 and a variation of type 2 at the same time. To identify the genotype, use O157 PCR Typing Set (Takara Bio)
- 2) Electrophoresis buffer (TAE buffer)  
Dissolve 4.8 g of tris(hydroxymethyl)aminomethane, 0.7 g of disodium dihydrogen ethylenediaminetetraacetate and 1.14 mL of acetic acid with water to make 1,000 mL of solution in total.
- 3) Agarose gel  
Dissolve 2.5 g of high purity agarose for electrophoresis with 100 mL of buffer for electrophoresis by heating and cool it down to 50 to 60 °C. Then, pour the gelled solution into the gel mold so that the gel becomes 3 to 4 mm thick. Carefully insert the comb into the gel so that no bubble generates between the gel and the comb. Leave the gel at room temperature to solidify it.
- 4) Dye solution  
Dissolve 25 mg of bromophenol blue, 25 mg of xylene cyanol and 3 g of glycerin with water to make 10 mL of solution in total. Autoclave the solution at 121 °C for 15 minutes.
- 5) DNA size markers (50 to 1,000 bp) (Takara Bio)
- 6) Ethidium bromide solution  
Dissolve 10 mg of ethidium bromide (Aldrich Chem.) with 500 μL of water to prepare the ethidium bromide stock solution. When using, dissolve 25 μL of this solution with 1,000 mL of buffer for

electrophoresis. (Prepare when it is needed.)

As the ethidium bromide stock solution has photodegradability, refrigerate it in light-blocking environments. As the ethidium bromide is carcinogenic, be sure to wear gloves whenever handling. Upon completion of the test, discard the ethidium bromide solution after passing through a filter containing absorbent. Soak the ethidium bromide stained agarose gel in the 5 % sodium hypochlorite solution, expose the gel to direct sunlight to photodegrade and then discard it.

### 3 Test method

#### 1) PCR reaction

- i Add 40  $\mu\text{L}$  of water and 10  $\mu\text{L}$  of heat-treated bacteria solution to an One Shot PCR solution tube (O157 One Shot PCR Screening Kit Ver.2.) Add 48  $\mu\text{L}$  of water and 2  $\mu\text{L}$  of heat-treated bacteria solution in other One Shot PCR solution tube. Use them as the sample solutions for PCR reaction.
- ii Add 50  $\mu\text{L}$  of water to an One Shot PCR solution tube. Use this as a negative control.
- iii Put the sample solution and negative control in the thermal cycler. Perform procedures for the PCR reaction under the following condition: [at 94  $^{\circ}\text{C}$  for one minute  $\rightarrow$  at 55  $^{\circ}\text{C}$  for one minute  $\rightarrow$  at 72  $^{\circ}\text{C}$  for one minute] (35 cycles)  $\rightarrow$  at 72  $^{\circ}\text{C}$  for 10 minutes

#### 2) Electrophoresis

- i For preparation, put the agarose gel in the electrophoresis tank containing the buffer for electrophoresis and apply constant voltage at 100 V for 15 minutes for pre-electrophoresis.
- ii Add about 1.5  $\mu\text{L}$  of dye solution to a 7.5  $\mu\text{L}$  size marker and mix it. Pour the total volume into the slot of agarose gel.
- iii Add 1.5  $\mu\text{L}$  of dye solution to 10  $\mu\text{L}$  of the sample solution finished PCR and add the same amount of dye solution to 10  $\mu\text{L}$  of the negative control and mix each of them. Pour the total volume of each solution to the slot of agarose gel.
- iv Apply constant voltage of 100 V and perform electrophoresis until the bromophenol blue moves by 3 to 4cm from the slot.

#### 3) Ethidium bromide staining

Soak the agarose gel finished with electrophoresis in the ethidium bromide solution for about 10 minutes.

#### 4) Confirmation of verotoxin genes

- i Place the agarose gel on the UV transilluminater and irradiate ultraviolet rays of 365 nm or 312 nm. Obtain the size of DNA fragment of sample solution by comparing its band position with those of the size marker.
- ii Take a Polaroid picture using a black-and-white film.
- iii Judge the samples as below:
  - When a 171 bp (base pair) DNA band is detected from the sample solution, it is judged that the verotoxin gene is positive in that sample.
  - When a 685 bp positive control band is detected from the sample solution and a 171 bp band is not detected, it is judged that the verotoxin gene is negative in that sample.
  - When a 171 bp band is detected in the negative control, it may possibly be contamination. Re-test is required.



- When neither 685 bp nor 171 bp band is detected from the sample solution, the PCR reaction may not be conducted normally. Re-test is required.

## 2 Confirmation method of verotoxin-producing *Escherichia coli* [Analytical Standards of Feeds: Article 2-2, Chapter 18]

### «Summary of Verotoxin-producing *Escherichia coli*»

Verotoxin-producing *Escherichia coli* is a generic name of *Escherichia coli* which produce verotoxin (also called shiga-like toxin) and 200 or more serotypes have been reported. Among them, *E. coli* which induces enteritis to humans is called enterohemorrhagic *E. coli* [EHEC]. Besides O157 described in Article 2-1 of this chapter, O26, O111, O128 and O145 are main serotypes. Even these serotypes may contain non-verotoxin producing strains. Therefore, the final identification is based on the test of verotoxin productivity.

Use sterilized water, reagent and instrumentations, as required.

#### A. Reagent preparation

- 1) Surfactant solution.<sup>[1]</sup> Autoclave surfactant solution (10 v/v%) at 121 °C for 15 minutes.
- 2) Saline. Autoclave sodium chloride solution (0.9 w/v%) at 121 °C for 15 minutes.
- 3) Buffered peptone water.<sup>[2]</sup> Dissolve 10 g of peptone, 5 g of sodium chloride, 9 g of disodium hydrogen phosphate 12 water and 1.5 g of potassium dihydrogen phosphate with 1,000 mL of water, then adjust the pH to 6.9 to 7.1.

Dispense 250 mL of this solution into 500 mL culture bottles<sup>[3]</sup> and autoclave them at 121 °C for 15 minutes. Cool it down below 45 °C. Add 15 mL of surfactant solution and shake it well.

- 4) mEC broth with novobiocin.<sup>[4]</sup> Dissolve 20 g of peptone, 1.12 g of sodium deoxycholate, 5 g of lactose monohydrate, 4 g of dipotassium hydrogen phosphate, 1.5 g of potassium dihydrogen phosphate and 5 g of sodium chloride with 1,000 mL of water and autoclave at 121 °C for 15 minutes, then adjust the pH to 6.8 to 7.0. Cool it down below 45 °C and then add 20 mg of novobiocin sodium.

Dispense 100 mL of the solution into 200 mL of culture bottles<sup>[3]</sup>.

- 5) CT-SMAC agar medium.<sup>[5]</sup> Dissolve 20 g of peptone, 1.5 g of sodium deoxycholate, 10 g of sorbitol, 5 g of sodium chloride, 30 mg of neutral red, 1 mg of crystal violet and 15 g of agar with 1,000 mL of water and autoclave at 121 °C for 15 minutes, then adjust the pH to 7.1 to 7.3. Cool it down below 45 °C and then add 50 µg of cefixime and 2.5 mg of potassium tellurite.

Pour 20 mL of the medium evenly on Petri dishes and allow to solidify. Then, turn them over and slightly slide the lids and leave them at 35 to 37 °C for one hour until the surface of agar is dry<sup>[6]</sup>.

- 6) Rainbow agar O157 medium.<sup>\*[7]</sup> Dissolve 6 g of peptone, 35.6 g of sugar, 0.4 g of chromogenic substrate<sup>\*2</sup> and 14 g of agar with 1,000 mL of water by heating in a boiling water bath, then adjust the pH to 6.7 to 6.9.

Pour 20 mL of the medium evenly on Petri dishes and allow to solidify. Then, turn them over and slightly slide the lids and leave them at 35 to 37 °C for one hour until the surface of agar is dry<sup>[6]</sup>.

7) CLIG agar medium.<sup>[8]</sup> Dissolve 10 g of peptone, 1 g of lactose monohydrate, 10 g of D-cellobiose, 0.1 g of L-tryptophan, 5 g of sodium chloride, 25 mg of phenol red, 20mg of 4-methylumbelliferyl- $\beta$ -D-glucuronide and 15 g of agar with 1,000 mL of water by heating in a boiling water bath, then adjust the pH to 7.3 to 7.5.

Dispense 4 mL portions of the medium into small test tubes and autoclave them at 115 °C for 15 minutes. Then, allow to set in a sloping position to solidify in a way of slant with butt (Slant is upper 1/3 and butt is lower 2/3 depth).<sup>[9]</sup>

8) SIM agar medium.<sup>[10]</sup> Dissolve 30 g of peptone, 3 g of beef extract, 0.5 g of ammonium ferric citrate, 50 mg of sodium thiosulfate (anhydrous), 0.2 g of L-cysteine hydrochloride monohydrate and 5 g of agar with 1,000 mL of water by heating in a water bath, then adjust the pH to 7.3 to 7.5.

Dispense 4 mL portions of the medium into small test tubes and autoclave them at 121 °C for 15 minutes. Then, allow to set in a vertical position to solidify in a way of butt<sup>[11]</sup>.

### B. Cultivation<sup>[12]</sup>

Pre-enrichment. Weigh<sup>[13]</sup> 25g of analysis sample<sup>[14]</sup> into buffered peptone water and shake it well. Then, incubate the solution at 35 to 37 °C for 18 to 24 hours.

Selective enrichment.<sup>\*3</sup> Transfer a 10-mL aliquot of pre-enrichment culture solution to mEC broth with novobiocin<sup>[15]</sup>, and shake it well. Then, incubate the solution at 41 to 43 °C for 18 to 24 hours<sup>[16]</sup>.

Selective isolation. Streak a loopful of the selective enrichment culture solution on plates of CT-SMAC agar medium and rainbow agar O157 medium, turn the plates over and incubate them at 35 to 37 °C for 18 to 24 hours.

Pure isolation.<sup>[17]</sup> Pick with needle<sup>[19]</sup> five typical or suspicious colonies<sup>[18]</sup> of verotoxin-producing *Escherichia coli*, if present, from each surface of CT-SMAC agar medium and of rainbow agar O157 medium. Dilute<sup>[20]</sup> each colony with 0.3 mL of saline. Streak<sup>[21]</sup> a loopful of each diluted solution on plates of rainbow agar O157 medium, turn the plates over and incubate them at 35 to 37 °C for 18 to 24 hours.

Confirmation. Pick with needle a typical or suspicious colony of verotoxin-producing *E. coli*, if present, from the surface of rainbow agar O157 medium having growth. Inoculate CLIG agar medium by stabbing the butt and streaking the agar slant surface with the needle. After inoculating CLIG, inoculate with the needle, SIM agar medium by stabbing.<sup>[22]</sup> Then, incubate them at 35 to 37 °C for 18 to 24 hours.

### C. Identification

Identification. Identify verotoxin-producing *Escherichia coli* by judging from the following biochemical properties<sup>[23]</sup> observed from the CLIG agar medium<sup>[23]</sup> and the SIM agar medium.

In most cases, verotoxin-producing *E. coli* exhibits the following properties: lactose fermentation (+), oxidase<sup>[24]</sup> (-),  $\beta$ -glucuronidase activity (+/-), cellobiose utilization (-), hydrogen sulfide (-), motility (+), IPA<sup>[25]</sup> (-) and indole<sup>[26]</sup> (+/-).

Serotyping.<sup>\*4[27]</sup> Pick a small amount of colony which exhibits the properties of verotoxin-producing *E. coli*. Mix it with pathogenic *Escherichia coli* O-group antiserum<sup>[28]</sup> dropped on a slide glass, determine whether it coagulate or not by shaking the slide glass. Tilt the slide glass back and forth and observe

whether an agglutination occurs<sup>[29]</sup>. Then, in the same way, test heat-treated culture at 100 °C for 30 minutes with O157 antiserum whether an agglutination is produced.

Test the colony agglutinated with O group mixed antiserum using each each group O antiserum<sup>[28]</sup> in the same way<sup>[28]</sup> to determine the O group.

Test the colony which has been determined its O group for agglutination with H antiserum using the test tube method<sup>[30]</sup> and determine the H antigen.

Detection of verotoxin gene.<sup>[31]</sup> Pick a small amount of the colony which exhibits the properties of verotoxin-producing *E. coli*. Mix it with 200 µL of saline in a 1.5 mL plastic centrifugation tube, shake the tube and centrifuge it at 7,000×g for 5 minutes. Then, remove the supernatant. Add 100 µL of water to the residue, shake the tube, heat it at 98 °C for 5 minutes and cool it down rapidly.

Centrifuge the heat-treated bacteria solution at 7,000×g for 10 minutes. Use the supernatant<sup>[32]</sup> for PCR<sup>\*[33]</sup> to test for the verotoxin gene.

Verotoxin productivity test. Pick a small amount of colony which exhibits the properties of verotoxin-producing *E. coli*, mix it with latex particles sensitized to verotoxins on a plate to observe agglutinations.

- \* 1 For these media, commercially available dry powder media having the same compositions as those can be used. Use hydrochloric acid solution (1 mol/L) or sodium hydroxide solution (1 mol/L) to adjust the pH.
- 2 A chromogenic substrate which is decomposed by the enzyme activity specific to the verotoxin-producing *E. coli* and becomes black to dark gray or blue to red-purple. The one contained in Rainbow agar O157 (Biolog) or equivalent.
- 3 When the following PCR method proves that there is no verotoxin-producing *E. coli* gene in the selective enrichment culture solution, the sample is judged to be negative for verotoxin-producing *E. coli*, and the sections after “Selective isolation” will be omitted.

(PCR method)

Place 1 mL of selective enrichment culture solution in a plastic centrifugation tube (capacity: 1.5 mL), centrifuge the tube at 7,000×g for 5 minutes and remove the supernatant. Add 500 µL of saline to the residue, shake the tube and centrifuge it at 7,000×g for 5 minutes. Then, remove the supernatant. Add 100 µL of water to the residue and shake the tube. Heat it at 98 °C for 5 minutes and then cool it down rapidly.

Use the heat-treated bacteria solution in accordance with the section of “Detection of verotoxin gene” and test for the verotoxin genes.

- 4 Judge a firm agglutination with O group mixed antiserum or individual O group antiserum found within one minute to be positive.

#### «Summary of analysis method»

This method to enrich bacteria in the sample using the buffered peptone water and mEC broth with novobiocin, test for verotoxin gene using PCR, then if the result is positive, isolate verotoxin-producing *Escherichia coli* using CT-SMAC agar medium and rainbow agar O157 medium, and then test for biochemical property, serotype and verotoxin productivity. The difference from the test method of

*Escherichia coli* O157 described in the previous article is in the points that there is no capture with immunomagnetic beads and that the rainbow agar O157 medium is used as the selective isolation medium.

Target samples are formula feed and feed ingredients of animal-origin such as fish meal and meat-and-bone meal, and plant-origin such as soybean meal and maize.

Figure 18.2-4 shows the flow sheet of detecting verotoxin-producing *Escherichia coli* in feed.

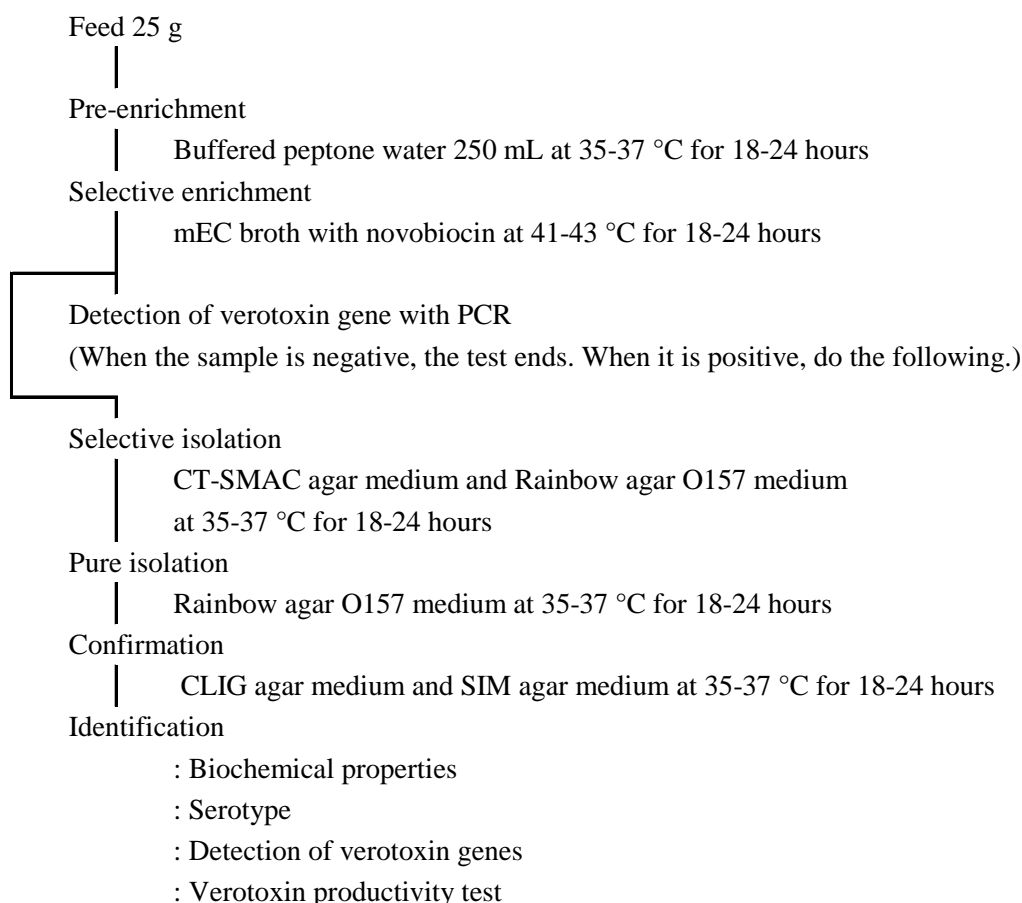


Figure 18.2-4 Flowsheet of detecting verotoxin-producing *Escherichia coli* in feed

References: Toyoko Kusama and Motomu Suginaka, Research Report of Animal Feed, 23, 128 (1998)

#### «Notes and precautions»

- [1] Added so as to disperse fatty components in the sample and obtain homogeneous culture solution. Since the surfactant (Tween 80) is hard to dissolve in low-temperature water, dissolve it by heating. The chemical name of Tween 80 is polyoxyethylene sorbitan monooleate and it is also called polysorbate 80.
- [2] Buffered peptone water (Difco, Oxoid, Eiken Chemical, or Nissui Pharmaceutical) or equivalent dry powder medium can be used.
- [3] Equip a glass culture bottle with an aluminum cap to keep ventilation.
- [4] mEC broth with novobiocin (Eiken Chemical, Kyokuto Pharmaceutical Industrial, Oxoid, Merck), mEC broth (Eiken Chemical, Kyokuto Pharmaceutical Industrial, Oxoid,) or equivalent dry powder medium can be used. When using the mEC broth with novobiocin, autoclave and cool it down

rapidly. When using the mEC broth, autoclave and cool it down. Then, add 20 mg of novobiocin sodium (Sigma, Kanto Chemical, Wako Pure Chemical Industries or equivalent) to 1,000 mL of the broth. Before use, dissolve novobiocin sodium with water to make 2 mg/mL solution and then filter and sterilize. Novobiocin has effects to suppress enrichment of enterobacteria such as *Citrobacter* spp. and *Proteus* spp. mEC stands for modified *Escherichia coli*.

[5] You may use Sorbitol MacConkey Agar (Oxoid, Difco, Merck, Eiken Chemical, Nissui Pharmaceutical, Kyokuto Pharmaceutical Industrial) or equivalent dry powder medium and add CT-supplement (Oxoid, Dynal or Merck) or equivalent to the medium.

CT stands for cefixime and tellurite. Cefixime has effects to suppress enrichment of *Proteus* spp. and, in combination with tellurite, to selectively enrich *Escherichia coli* O157. SMAC stands for Sorbitol MacConkey. *Escherichia coli* O157 does not degrade sorbitol in this medium so that it is able to be distinguished from general *E. coli*.

[6] As shown in Figure 18.1-2, turn over the Petri dish and dry it in the incubator until the surface of the plate will be free of waterdrop. If the medium is not completely dried, bacteria spreads over waterdrop and grows there when it is streaked, possibly resulting in failure of generating appropriate colonies. If the medium is dried too much, the surface of the medium is likely to be cracked.

[7] Rainbow agar O157 (Biolog) or equivalent. *Escherichia coli* O157, verotoxin-producing *E. coli*, general *E. coli* and other enterobacteria are distinguished on this medium depending on their  $\beta$ -glucuronidase activity and the  $\beta$ -galactosidase activity.

[8] CLIG agar medium (Kyokuto Pharmaceutical Industrial) or equivalent dry powder medium can be used. CLIG stands for Cellobiose Lactose Indole  $\beta$ -Glucuronidase. This medium enables to confirm the cellobiose utilization, lactose fermentation and  $\beta$ -glucuronidase activity.

[9] Dispense the medium in small test tubes (with diameter of 10 mm and length of 120 mm) up to 3 to 4 mL using a measuring pipette, Komagome pipette or a dispenser. Equip each test tube with an aluminum cap and autoclave. Then, tilt the test tube to solidify the medium in a way of slant with butt as shown in Figure 18.1-3.

[10] SIM agar medium (Eiken Chemical) or equivalent dry powder medium can be used. Heat the medium in a water bath for about 10 minutes until the agar completely dissolves. SIM stands for Sulfide Indole Motility. This medium enables to confirm the production of hydrogen sulfide and indole, motility and IPA reactions.

[11] Dispense the medium in small test tubes (with diameter of 10 mm and length of 120 mm) up to 3 to 4 mL using a measuring pipette, Komagome pipette or a dispenser. Equip each test tube with an aluminum cap and autoclave. Then, stand the test tube vertically to solidify the medium in a way of butt as shown in Figure 18.1-4.

[12] After incubation, be sure to autoclave culture bottles, Petri dishes and others contaminated with enterobacteria and then discard or wash them.

[13] To take a sample of feed on-site, use sampling bags and sampling shovels which have been sterilized (through radiation sterilization, gaseous sterilization, etc.) and wear sterilized gloves. If you cannot prepare these equipments, sterilize your fingers and sampling shovels with alcohol pad.

[14] To weigh samples, use sterilized charta (with gaseous sterilization or ultraviolet sterilization) and

medicine spoons sterilized with alcohol pad or dry-heat sterilized and prevent contamination through contact to other samples.

[15] Use a Komagome pipette sterilized. After use, put the pipette in a container with antiseptic solution and autoclave it.

[16] Compared to incubation at 35 to 37 °C, incubation at 41 to 43 °C enables to suppress enrichment of general *Escherichia coli* and enterobacteria such as *Proteus* spp. except *E. coli* O157, which shows a trend of slightly increase in the detection rate of *E. coli* O157.

[17] This process can be omitted when a colony suspected to be verotoxin-producing *Escherichia coli* is obviously separated from other colonies.

[18] The characteristics of colonies of verotoxin-producing *Escherichia coli* and those of other enterobacteria on Rainbow agar O157 medium are as described below.

*Escherichia coli* O157: Generate black colonies because of  $\beta$ -glucuronidase-negative and  $\beta$ -glucuronidase-positive.

Verotoxin-producing *E. coli* other than O157: Generate blue to red-purple colonies since  $\beta$ -glucuronidase is produced much more than  $\beta$ -glucuronidase.

Other *E. coli*: Generate red to red-purple colonies because of  $\beta$ -glucuronidase-positive.

Enterobacteria other than *E. coli*: Generate white to pale yellow colonies.

The characteristics of colonies on CT-SMAC agar medium are as described below.

*Escherichia coli* O157: Generate white colonies due to non-degradability of sorbitol.

Verotoxin-producing *E. coli* other than O157: Generate red colonies in many cases due to the degradability of sorbitol, but also generate white colonies rarely.

General *E. coli*, *Salmonella* spp., *Citrobacter* spp., *Klebsiella* spp.: Generate red colonies due to the degradability of sorbitol.

*Proteus* spp.: Generate white irregular colonies if CT does not suppress enrichment of the bacteria.

Since, the color of the colony on the CT-SMAC agar medium may fade after more than 24 hours of cultivation, avoid long-time cultivation and check the colony soon after cultivation of specific time.

[19] Pick up colony using a platinum needle or a sterilized toothpick. Most of bacteria remain alive even if they are not growing on the medium. Thus, be careful not to touch the areas other than the target colony.

[20] You may dilute the colony using a sterilized microtitration plate (about 300  $\mu$ L per well).

[21] Divide a Rainbow O157 medium into 8 sectors and streak a 2 mm loopful of diluted solution within a sector. You can substitute a medium having relatively low selectivity such as the DHL agar medium (see Article 1 “*Salmonella*”, 1-A-7)), SMAC agar medium or EMB agar medium.

[22] Pick up colony using a platinum needle and stab into the butt of the CLIG agar medium and streak the bacteria on the slant. Then, stab the same needle into the SIM agar medium.

[23] The biochemical properties of verotoxin-producing *Escherichia coli* on each confirmation medium are as described below.

The verotoxin-producing *E. coli* contains various serotypes and their biochemical properties drastically vary. Thus, it is difficult to identify the verotoxin-producing *E. coli* based on the biochemical properties, except a part of bacteria such as *Escherichia coli* O157.

To distinguish *E. coli* from other enterobacteria, test for the citrate utilization (–) and VP reaction (–) in addition to the above-mentioned items. (See Article 3: Confirmation method of other *Escherichia coli*, «Notes and precautions» [25].)

· CLIG agar medium

In most cases, verotoxin-producing *Escherichia coli* decomposes lactose and produces acid, so that the butt becomes yellow. The slant of the medium becomes red due to non-degradability of cellobiose.  $\beta$ -Glucuronidase activity is positive or negative, so that fluorescence under radiation of 365 nm UV is variable in occurrence. General *E. coli* shows positive for  $\beta$ -glucuronidase activity and becomes fluorescent.

· SIM agar medium

Since most of verotoxin-producing *Escherichia coli* has motility, it grows throughout the medium. Those having no motility grow only along the line of needling. Since it does not produce hydrogen sulfide, the color of the entire medium does not change. The IPA reaction (see [25] below) is negative and the indole test (see [26] below) is positive or negative.

[24] This is a test to confirm the cytochrome oxidase activity. When streaking the culture on the CLIG agar medium onto a piece of moistened oxidase test paper (Eiken Chemical) and the paper turns deep blue within one minute, the culture is judged to be positive. This test is used to distinguish verotoxin-producing *Escherichia coli* from *Aeromonas* spp. and *Plesiomonas* spp.

[25] The IPA (Indolepyruvic acid) reaction is a phenomenon in which indole pyruvic acid produced by deamination of tryptophan contained in peptone forms a chelate with  $\text{Fe}^{3+}$  of ammonium ferric citrate and generates a brown belt in the upper edge of the medium. This is a reaction specific to the *Proteus* and related bacteria.

[26] This is a test to check for production of indole. After stratifying 1ml of chloroform on the agar medium, drop 1 mL of the following Kovač's reagent on the medium and shake it gently. If the chloroform layer becomes red, the sample is judged to be positive.

To prepare Kovač's reagent, dissolve 5 g of *p*-dimethylaminobenzaldehyde with 75 mL of isoamyl alcohol on a water bath at about 50 °C and then add 25 mL of hydrochloric acid. Store the reagent in a brown bottle.

[27] Serotyping is performed by identifying the O antigen (somatic antigen), which is a bacteria surface antigen, and the H antigen, which is a flagellar antigen.

The verotoxin-producing *Escherichia coli* detected from humans have the following main serotypes: O26:H11, O26:H–, O111:H–, O128:H2, O128:H–, O145:H–, O157:H7, and O157:H–. However, field strains have many other serotypes, which cannot be identified with commercially available antisera.

[28] Pathogenic *Escherichia coli* antisera (Denka Seiken) or equivalent can be used.

[29] The agglutination results may be different between the viable bacteria and the heat-treated bacteria. When using this test for serotyping, judgement is based on the agglutinations of heat-treated bacteria. However, if agglutination is found in either bacteria type, the detection of verotoxin genes should be performed.

[30] For H antigen agglutination reactions in the test tube, see «Notes and precautions» [39] and

(**appended item**) of Article 1.1, *Salmonella*, in this chapter, and «**Notes and precautions**» [36] of Section 1, Confirmation method of enterohemorrhagic *Escherichia coli* O157:H7 in this article.

[31] As the *Escherichia coli* verotoxin genes, type 1 (VT1), type 2 (VT2) and a variation of type 2 (VT2 variant) are known.

[32] DNA separated from bacteria through heat treatment and centrifuging are dispersed in this supernatant.

[33] PCR (Polymerase Chain Reaction) is a method to detect the presence of the target gene quickly with high sensitivity by amplifying the specific gene segment millionfold in several hours. Detection of *Escherichia coli* verotoxin gene with PCR conforms to appended item of «**Notes and precautions**» [40], Section 1, Confirmation method of enterohemorrhagic *Escherichia coli* O157:H7 in this article.

### 3 Confirmation method of other *Escherichia coli* [Analytical Standards of Feeds, Article 2.3, Chapter 18]

Use sterilized water, reagent and instrumentations, as required.

#### A. Reagent preparation

- 1) Surfactant solution.<sup>[1]</sup> Autoclave surfactant solution (10 v/v%) at 121 °C for 15 minutes.
- 2) Saline. Autoclave sodium chloride solution (0.9 w/v%) at 121 °C for 15 minutes.
- 3) Buffered peptone water.<sup>[2]</sup> Dissolve 10 g of peptone, 5 g of sodium chloride, 9 g of disodium hydrogen phosphate 12 water and 1.5 g of potassium dihydrogen phosphate with 1,000 mL of water, then adjust the pH to 6.9 to 7.1.

Dispense 250 mL of this solution into 500 mL culture bottles<sup>[3]</sup> and autoclave them at 121 °C for 15 minutes. Cool it down below 45 °C. Add 15 mL of surfactant solution and shake it well.

- 4) mEC broth with novobiocin.<sup>[4]</sup> Dissolve 20 g of peptone, 1.12 g of sodium deoxycholate, 5 g of lactose monohydrate, 4 g of dipotassium hydrogen phosphate, 1.5 g of potassium dihydrogen phosphate and 5 g of sodium chloride with 1,000 mL of water and autoclave at 121 °C for 15 minutes, then adjust the pH to 6.8 to 7.0. Cool it down below 45 °C and then add 20 mg of novobiocin sodium.

Dispense 100 mL of the solution into 200 mL of culture bottles<sup>[3]</sup>.

- 5) EMB agar medium.<sup>\*1[5]</sup> Dissolve 10 g of peptone, 10 g of lactose monohydrate, 2 g of dipotassium hydrogen phosphate, 0.4 g of eosin Y, 65 mg of methylene blue and 15 g of agar with 1,000 mL of water by heating in a boiling water bath, then adjust the pH to 6.7 to 6.9. Autoclave at 121 °C for 15 minutes.

Pour 20 mL of the medium evenly on Petri dishes and allow to solidify. Then, turn them over and slightly slide the lids and leave them at 35 to 37 °C for one hour until the surface of the agar is dry<sup>[6]</sup>.

- 6) Rainbow agar O157 medium.<sup>\*1[7]</sup> Dissolve 6 g of peptone, 35.6 g of sugar, 0.4 g of chromogenic substrate and 14 g of agar with 1,000 mL of water by heating in a boiling water bath, then adjust the pH to 6.7 to 6.9.

Pour 20 mL of the medium evenly on Petri dishes and allow to solidify. Then, turn them over and slightly slide the lids and leave them at 35 to 37 °C for one hour until the surface of the agar is dry<sup>[6]</sup>.

- 7) TSI agar medium.<sup>[8]</sup> Dissolve 15 g of peptone, 4 g of beef extract, 10 g of sucrose, 10 g of lactose



monohydrate, 1 g of glucose, 5 g of sodium chloride, 0.4 g of sodium sulfite, 80 mg of sodium thiosulfate (anhydrous), 0.2 g of ferrous sulfate heptahydrate, 20 mg of phenol red and 15 g of agar with 1,000 mL of water by heating in a boiling water bath, then adjust the pH to 7.3 to 7.5.

Dispense 4 mL portions of the medium into small test tubes and autoclave them at 121 °C for 15 minutes. Then, allow to set in a sloping position to solidify in a way of slant with butt (Slant is upper 1/3 and butt is lower 2/3 depth).<sup>[9]</sup>

- 8) SIM agar medium.<sup>[10]</sup> Dissolve 30 g of peptone, 3 g of beef extract, 0.5 g of ammonium ferric citrate, 50 mg of sodium thiosulfate (anhydrous), 0.2 g of L-cysteine hydrochloride monohydrate and 5 g of agar with 1,000 mL of water by heating in a water bath, then adjust the pH to 7.3 to 7.5.

Dispense 4 mL portions of the medium into small test tubes and autoclave them at 121 °C for 15 minutes. Then, allow to set in a vertical position to solidify in a way of butt<sup>[11]</sup>.

- 9) Simmons' citrate agar medium.<sup>\*[12]</sup> Dissolve 5 g of sodium chloride, 2 g of sodium citrate, 1 g of dipotassium hydrogen phosphate, 1 g of ammonium dihydrogen phosphate, 0.2 g of magnesium sulfate heptahydrate, 24 mg of bromothymol blue and 15 g of agar with 1,000 mL of water by heating in a boiling water bath, then adjust the pH to 6.6 to 6.8.

Dispense 4 mL portions of the medium into small test tubes and autoclave them at 121°C for 15 minutes. Then, allow to set in a sloping position to solidify in a way of slant with butt (Slant is upper 1/3 and butt is lower 2/3 depth).<sup>[13]</sup>

- 10) VP semisolid medium.<sup>\*[14]</sup> Dissolve 5 g of peptone, 7 g of pancreatic digest of casein, 1 g of yeast extract, 10 g of glucose, 5 g of sodium chloride and 3 g of agar with 1,000 mL of water by heating in a boiling water bath, then adjust the pH to 6.9 to 7.1.

Dispense 4 mL portions of the medium into small test tubes and autoclave them at 121 °C for 15 minutes. Then, allow to set in a vertical position to solidify in a way of butt.

#### B. Cultivation<sup>[15]</sup>

Pre-enrichment. Weigh<sup>[17]</sup> 25g of analysis sample<sup>[16]</sup> into buffered peptone water and shake it well. Then, incubate the solution at 35 to 37 °C for 18 to 24 hours.

Selective enrichment. Transfer a 10-mL aliquot of pre-enrichment culture solution to mEC broth with novobiocin<sup>[18]</sup>, and shake it well. Then, incubate the solution at 41 to 43 °C for 18 to 24 hours<sup>[19]</sup>.

Selective isolation. Streak a loopful of the selective enrichment culture solution on plates of the EMB agar medium and rainbow agar O157 medium, turn the plates over and incubate them at 35 to 37 °C for 18 to 24 hours.

Pure isolation.<sup>[20]</sup> Pick with needle<sup>[22]</sup> five typical or suspicious colonies<sup>[21]</sup> of *Escherichia coli*, if present, from each surface of EMB agar medium and of rainbow agar O157 medium having growth. Dilute<sup>[23]</sup> each colony with 0.3 mL of saline. Streak<sup>[24]</sup> a loopful of each diluted solution on plates of EMB agar medium, turn the plates over and incubate them at 35 to 37 °C for 18 to 24 hours.

Confirmation. Pick with needle a typical or suspicious colony of *Escherichia coli* from the surface of EMB agar medium having growth. Inoculate TSI agar medium by stabbing the butt and streaking the agar slant surface with the needle. After inoculating TSI, inoculate with the needle, SIM agar medium by stabbing. Pick with the needle the same colony again. Inoculate Simmons' citrate agar medium by

stabbing the butt and streaking the agar slant surface with the needle. After inoculating Simmons', inoculate with the needle, VP semisolid medium by stabbing. Incubate them at 35 to 37 °C for 18 to 24 hours.

### C. Identification

Identification. Identify *Escherichia coli* by judging from the following biochemical properties observed from the TSI agar medium, the SIM agar medium, the Simmons' citrate agar medium and the VP semisolid medium.

In most cases, *Escherichia coli* exhibits the following properties: lactose and sucrose fermentation (+), glucose fermentation (aerogenesis) (+), hydrogen sulfide (-), motility (+), IPA<sup>[26]</sup> (-), indole<sup>[27]</sup> (+/-), citric acid utilization (-), VP reaction<sup>[28]</sup> (-) and oxidase<sup>[29]</sup> (-).

If needed, serotyping should be performed using Pathogenic *Escherichia coli* O group mixed antisera<sup>[30]</sup>.

\* 1 For these media, commercially available dry powder media having the same compositions as those can be used. Use hydrochloric acid solution (1 mol/L) or sodium hydroxide solution (1 mol/L) to adjust the pH.

### «Summary of analysis method»

This test method is used to detect a wide range of pathogenic *Escherichia coli* (Section 1: Summary of enterohemorrhagic *Escherichia coli* O157:H7) and general *E. coli*, in addition to verotoxin-producing *E. coli*.

The method is to enrich bacteria in the sample using buffered peptone water and mEC broth with novobiocin, isolate *E. coli* using EMB agar medium and rainbow agar O157, and then test for biochemical properties. Since antisera for *Escherichia coli* except for the main pathogenic *E. coli* are not commercially available, unlike the cases of *Salmonella*, identification by serotype is usually impossible.

Target samples are formula feed and feed ingredients of animal-origin such as fish meal and meat-and-bone meal, and plant-origin such as soybean meal and maize.

Figure 18.2-5 shows the flow sheet of detection of *Escherichia coli* in feed.

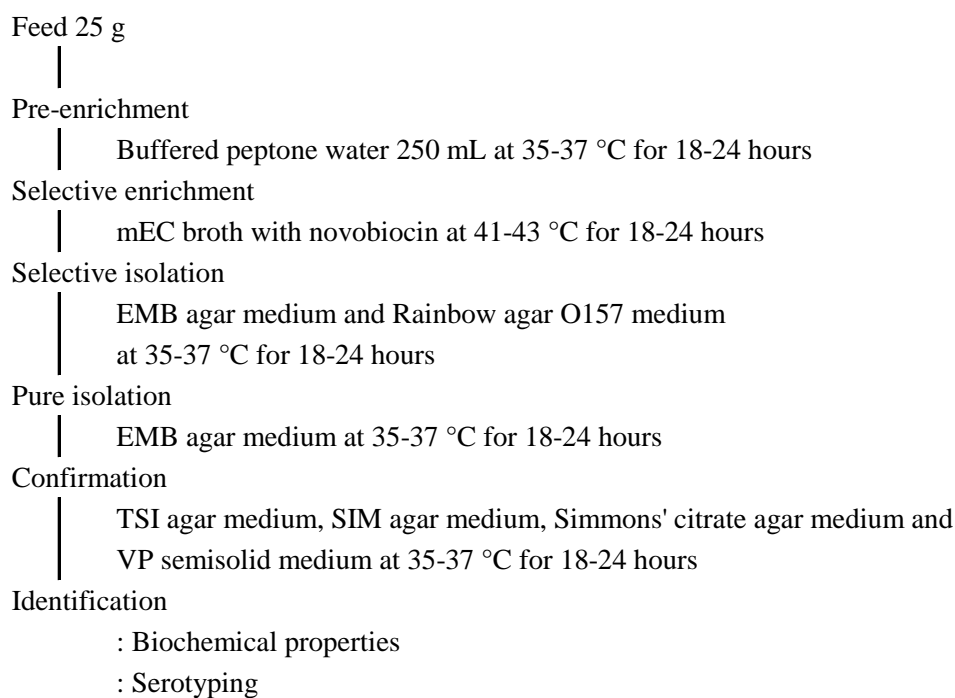


Figure 18.2-5 Flowsheet of detecting *Escherichia coli* in feed

References: Toyoko Kusama, Motomu Suginaka, Research Report of Animal Feed, 23, 128 (1998)

#### «Notes and precautions»

- [1] Added so as to disperse fatty components in the sample and obtain homogeneous culture solution. Since the surfactant (Tween 80) is hard to dissolve in low-temperature water, dissolve it by heating. The chemical name of Tween 80 is polyoxyethylene sorbitan monooleate and it is also called polysorbate 80.
- [2] Buffered peptone water (Difco, Oxoid, Eiken Chemical or Nissui pharmaceutical) or equivalent dry powder medium can be used.
- [3] Equip a glass culture bottle with an aluminum cap to keep ventilation.
- [4] mEC broth with novobiocin (Eiken Chemical, Kyokuto Pharmaceutical Industrial, Oxoid, Merck), mEC broth (Eiken Chemical, Kyokuto Pharmaceutical Industrial, Oxoid,) or equivalent dry powder medium can be used. When using the mEC broth with novobiocin, autoclave and cool it down rapidly. When using the mEC broth, autoclave and cool it down. Then, add 20 mg of novobiocin sodium (Sigma, Kanto Chemical, Wako Pure Chemical Industries or equivalent) to 1,000 mL of the broth. Before use, dissolve novobiocin sodium with water to make 2 mg/mL solution and then filter and sterilize. Novobiocin has effects to suppress enrichment of enterobacteria such as *Citrobacter* spp. and *Proteus* spp. mEC stands for modified *Escherichia coli*.
- [5] EMB agar medium (Eiken Chemical) or equivalent dry powder medium can be used. EMB stands for Eosin Methylene-Blue. Methylene blue suppresses enrichment of gram-positive bacteria. The lactose degradability of *Escherichia coli* enables it to be distinguished from other lactose non-degradable bacteria on this medium.
- [6] As shown in Figure 18.1-2, turn over the Petri dish and dry it in the incubator until the surface of the plate will be free of waterdrop. If the medium is not completely dried, bacteria spreads over

- waterdrop and grows there when it is streaked, possibly resulting in failure of generating appropriate colonies. If the medium is dried too much, the surface of the medium is likely to be cracked.
- [7] Rainbow agar O157 (Biolog) or equivalent. *Escherichia coli* O157, verotoxin-producing *E. coli*, general *E. coli* and other enterobacteria are distinguished on this medium depending on their  $\beta$ -glucuronidase activity and the  $\beta$ -galactosidase activity.
- [8] TSI agar medium (Eiken Chemical) or equivalent dry powder medium can be used. Prepare the medium by heating in a water bath for 10 minutes or in the microwave oven for several times until the agar dissolves completely. TSI stands for Triple Sugar Iron.
- [9] Dispense the medium in small test tubes (with diameter of 10 mm and length of 120 mm) up to 3 to 4 mL using a measuring pipette, Komagome pipette or a dispenser. Equip each test tube with an aluminum cap and autoclave. Then, tilt the test tube to solidify the medium in a way of slant with butt as shown in Figure 18.1-3.
- [10] SIM agar medium (Eiken Chemical) or equivalent dry powder medium can be used. Heat the medium in a water bath for about 10 minutes until the agar completely dissolves. SIM stands for Sulfide Indole Motility. This medium enables to confirm the production of hydrogen sulfide and indole, motility and IPA reactions.
- [11] Dispense the medium in small test tubes (with diameter of 10 mm and length of 120 mm) up to 3 to 4 mL using a measuring pipette, Komagome pipette or a dispenser. Equip each test tube with an aluminum cap and autoclave. Then, stand the test tube vertically to solidify the medium in a way of butt as shown in Figure 18.1-4.
- [12] Simmons' citrate agar medium (Eiken Chemical) or equivalent dry powder medium can be used. The citrate utilization of bacteria is confirmed by this medium. In cases of positive, the bacteria exhibits growth on the slant and blue color.
- [13] See [9] above.
- [14] VP semisolid agar medium (Eiken Chemical) or equivalent dry powder medium can be used. VP (Voges-Proskauer) reaction (see [28]) is confirmed by this medium.
- [15] After incubation, be sure to autoclave culture bottles, Petri dishes and others contaminated with enterobacteria and then discard or wash them.
- [16] To take a sample of feed on-site, use sampling bags and sampling shovels which have been sterilized (through radiation sterilization or gaseous sterilization) and wear sterilized gloves. If you cannot prepare these equipments, sterilize your fingers and sampling shovels with alcohol pad.
- [17] To weigh samples, use sterilized charta (with gaseous sterilization or ultraviolet sterilization) and medicine spoons sterilized with alcohol pad or dry-heat sterilized and prevent contamination through contact to other samples.
- [18] Use a Komagome pipette sterilized. After use, put the pipette in a container with antiseptic solution and autoclave it.
- [19] Compared to incubation at 35 to 37 °C, incubation at 41 to 43 °C enables to suppress enrichment of general *Escherichia coli* and enterobacteria such as *Proteus* spp. except *E. coli* O157, which shows a trend of slightly increase in the detection rate of *E. coli* O157.
- [20] This process can be omitted when a colony suspected to be *Escherichia coli* is obviously

separated from other colonies.

[21] The characteristics of colonies of *Escherichia coli* and other enterobacteria on the EMB agar medium are as described below:

*Escherichia coli*: Generate colonies with metallic green sheen due to degradability of lactose.

*Salmonella* spp., *Shigella* spp. and *Proteus* spp.: Generate translucent colonies in color of medium due to non-degradability of lactose.

For the characteristics of colonies on the rainbow agar O157, see «Notes and precautions» [18] in Section 2, “Confirmation method of verotoxin-producing *Escherichia coli*.”

[22] Pick up colony using a platinum needle or a sterilized toothpick. Most of bacteria remain alive even if they are not growing on the medium. Thus, be careful not to touch the areas other than the target colony.

[23] You may dilute the colony using a sterilized microtitration plate (about 300 µL per well).

[24] Divide the EMB agar medium into 8 sectors and streak a 2 mm loopful of diluted solution within a sector. You can substitute a medium having relatively low selectivity such as the DHL agar medium (see Article 1 “*Salmonella*”, 1-A-7)) or the rainbow agar O157 medium.

[25] The main biochemical properties of *Escherichia coli* on each confirmation medium are as described below:

· TSI agar medium

In most cases, *Escherichia coli* decompose lactose and sucrose, so that the slant of the medium becomes yellow. The butt of the medium becomes yellow due to decomposition of glucose. *E. coli* generates gas, which causes the butt to bubbles or cracks.

· SIM agar medium

In most cases, *Escherichia coli* has motility and grows throughout the medium. Those having no motility grow only along the line of needling. Since *E. coli* does not produce hydrogen sulfide, the color of the entire medium does not change. The IPA reaction (see [26] below) is negative and the indole test (see [27] below) is positive.

· Simmons' citrate agar medium

Because *Escherichia coli* do not use citric acid, growth of bacteria is not found on the slant of the medium and the medium color remains the same.

· VP semisolid medium

*Escherichia coli* is negative for the VP reaction and the reagent color remains the same by adding VP reagent.

Table 16.2-2 shows the biochemical properties of *Escherichia coli* and other enterobacteria on a part of confirmation media.

Table 18.2-2 Biochemical properties of *Escherichia coli* and other enterobacteria on confirmation media

| Type                         | Medium                             | TSI agar medium |                 |     |                  | Simmons' citrate agar medium | VP semisolid medium |
|------------------------------|------------------------------------|-----------------|-----------------|-----|------------------|------------------------------|---------------------|
|                              |                                    | Slant           | Butt            | Gas | Hydrogen sulfide |                              |                     |
| <i>Escherichia coli</i>      |                                    |                 |                 |     |                  |                              |                     |
|                              | EHEC O157                          | Yellow          | Yellow          | +   | —                | —                            | —                   |
|                              | Verotoxin-producing <i>E. coli</i> | Yellow          | Yellow          | +   | —                | —                            | —                   |
|                              | General <i>E. coli</i>             | Yellow          | Yellow          | +   | —                | —                            | —                   |
| <i>Escherichia hermannii</i> |                                    |                 |                 |     |                  |                              |                     |
|                              |                                    | Red             | Yellow          | +   | —                | —                            | —                   |
| <i>Salmonella</i> spp.       |                                    |                 |                 |     |                  |                              |                     |
|                              |                                    | Red             | Yellow or Black | +   | +                | +                            | —                   |
| <i>Citrobacter</i> spp.      |                                    |                 |                 |     |                  |                              |                     |
|                              |                                    | Yellow or Red   | Yellow or Black | +   | +                | +                            | —                   |
| <i>Proteus</i> spp.          |                                    |                 |                 |     |                  |                              |                     |
|                              |                                    | Yellow or Red   | Yellow or Black | +   | + ~ -            | + ~ -                        | —                   |
| <i>Klebsiella</i> spp.       |                                    |                 |                 |     |                  |                              |                     |
|                              |                                    | Yellow          | Yellow          | +   | —                | +                            | +                   |
| <i>Enterobacter</i> spp.     |                                    |                 |                 |     |                  |                              |                     |
|                              |                                    | Red             | Yellow          | +   | —                | +                            | +                   |

Note) + ~ - ; Positive or negative

[26] IPA (Indolepyruvic acid) reaction is a phenomenon in which indolepyruvic acid produced by deamination of tryptophan contained in peptone forms a chelate with  $Fe^{3+}$  of ammonium ferric citrate and generates a brown belt in the upper edge of the medium. This is a reaction specific to *Proteus* and related bacteria.

[27] This is a test to check for production of indole. After stratifying 1 mL of chloroform over the SIM agar medium, drop 1 mL of the following Kovač's reagent on the medium and shake it gently. If the chloroform layer becomes red, the sample is judged to be positive.

To prepare Kovač's reagent, dissolve 5 g of *p*-dimethylaminobenzaldehyde with 75 mL of isoamyl alcohol on a water bath at about 50 °C and then add 25 mL of hydrochloric acid. Store the reagent in a brown bottle.

[28] This reaction detects acetoin converted from pyruvic acid. Add 0.2 mL of  $\alpha$ -naphthol reagent and 0.4 mL of KOH-creatine solution to the VP semisolid medium and leave it at room temperature for one hour. In case the solution becomes red, it is judged to be positive. This method is used to identify *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp. To prepare  $\alpha$ -naphthol reagent, dissolve 6 g of  $\alpha$ -naphthol with ethanol and make 100 mL of solution in total. To prepare the KOH creatine solution, dissolve 40 g of potassium hydroxide and 0.3 g of creatine with water and make 100 mL of solution in total.

[29] This is a test to confirm the cytochrome oxidase activity. When streaking the culture on the CLIG agar medium onto a piece of moistened oxidase test paper (Eiken Chemical) and the paper turns deep blue within one minute, the culture is judged to be positive. This test is used to distinguish *Escherichia coli* O157 from *Aeromonas* spp. and *Plesiomonas* spp.

[30] Pathogenic *Escherichia coli* antisera (Denka Seiken) or equivalent can be used.

This set contains 8 types of O-group mixed antisera and 43 types of individual O-group antisera in order to detect main pathogenic *Escherichia coli*.

## (Reference) Eumycetes

### [Summary of Eumycetes]

Eumycetes (true fungi) are microorganisms belonging to the Eumycete division. They are classified into moulds developing through fungous filaments and yeasts growing proliferously.

The test methods introducing in this document are to detect Eumycetes in cereal, beans, formula feed and other feed ingredients by using cultivation and check the degree of contamination and presence of Eumycetes harmful to the livestock. This method mainly refers to the microbial assay described in Standard Methods of Analysis for Hygienic Chemists with Commentary (published in 2000 by Pharmaceutical Society of Japan).

There are two test methods for Eumycetes: the culture method and the direct microscopic examination. The culture method is to detect viable bacteria and is suitable for relatively low-level contaminations and detections of Eumycetes invading in grains. Eumycetes detected through cultivation can be identified finally.

The direct microscopic examination allows quick inspection of the volume of Eumycetes contained in the sample highly contaminated and even can apply to samples containing pesticide. However, this method cannot distinguish viable fungi from killed fungi.

Unlike common bacteria, it takes several days until Eumycetes colonies can be detected visually after starting cultivation. Moreover, in many cases, it takes one to two weeks until the generative organs such as spores and conidia essential for identification can be observed. The growth rate varies depending on the species of Eumycetes: Zygomycota takes three days to cover all over the Petri dish while some Eumycetes cannot be detected in 7 days. Therefore, the growing conditions should be observed every two or three days.

Since Eumycetes possibly contain Eumycetes and toxin (mycotoxin) harmful to humans, you should be very careful when handling these fungi.

Cautions on inspection of Eumycetes are as follows:

- 1) Because spores of Eumycetes are easy to scatter, control the air flow to prevent contamination in the room.
- 2) Before and after inspection, sterilize the lab bench and room interior by spraying.
- 3) Eumycetes are likely to be electrostatically charged and adhesive to fingers. Sterilize your fingers well.
- 4) Learn the knowledge on Eumycetes to be tested well, in terms of biology, physiological properties, toxicity and pathogenicity, in advance.
- 5) Store, or quickly sterilize and discard the samples used and Eumycetes detected upon completion of inspection. To sterilize them, autoclave at 121 °C for 15 minutes.

Antiseptic substances used for Eumycetes are listed below:

- 1) Ethyl alcohol (about 80 %), isopropyl alcohol (50 %); Fingers, indoor spraying
- 2) Sodium hypochlorite (available chlorine concentration; about 5 %); Equipment, fingers, sample
- 3) Phenol (equipment 3 %, fingers 1 %), cresol soap solution (equipment 3 %, fingers 1 %); Equipment, fingers, indoor spraying (phenol)

It is preferable to use Contaminon (Wako Pure Chemical Industries) to sterilize the prepared slide.

References : Edition of Pharmaceutical Society of Japan: Standard Methods of Analysis for Hygienic Chemists with Commentary 2000, Kanehara & Co. (2000)

: Edition of the Ministry of Health, Labour and Welfare: Standard methods of analysis in food safety regulation: Japan Food Hygiene Association (1992)

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: Toyoko Kusama, Isao Matsubara: Research Report of Animal Feed, 10, 88 (1985)

## [Reference]

### 1 Confirmation method of Eumycetes in unground samples

Applicable range: Cereal and beans

Use sterilized water, reagent and instrumentations, as required.

Use the sterile technique as required.

#### A. Reagent preparation<sup>\*1</sup>

##### 1) Lactophenol solution<sup>[1]</sup> (enclosed liquid)

Dissolve 10 g of phenol with 10 mL of water. Add 8 mL of lactic acid and 16 mL of glycerin to the solution and mix them.

##### 2) Potato dextrose agar medium<sup>[2]</sup>

Dissolve 200 g of potato extract, 20 g of glucose and 15 g of agar with 1,000 mL of water by heating in a water bath to adjust the pH to 5.5 to 5.7. Autoclave it at 121 °C for 15 minutes. Pour 20 mL of the medium evenly on Petri dishes<sup>[3]</sup> and leave the dish horizontally to solidify.

##### 3) Potato dextrose agar medium with chloramphenicol<sup>[4]</sup>

Dissolve all the ingredients in 2) and 100 mg of chloramphenicol (conforming with the specifications outlined in the Japanese Pharmacopoeia, hereafter called “JP”) with 1,000 mL of water. Then, do the same procedure as in 2).

##### 4) Czapek agar medium<sup>[5]</sup>

Dissolve 30 g of sucrose, 3 g of sodium nitrate, 1 g of dipotassium hydrogen phosphate, 0.5 g of magnesium sulfate, 0.5 g of potassium chloride, 0.01 g of ferrous sulfate and 15 g of agar with 1,000 mL of water by heating in a water bath, then adjust the pH to 7.2 to 7.4. Autoclave it at 121 °C for 15 minutes. Then, do the same procedure as in 2).

##### 5) Czapek agar medium with chloramphenicol<sup>[4]</sup>

Dissolve all the ingredients in 4) and 100 mg of chloramphenicol (JP) with 1,000 mL of water. Then, do the same procedure as in 4).



6) Malt salt agar medium with chloramphenicol<sup>[6]</sup>

Dissolve 25 g of malt extract, 75 g of sodium chloride, 20 g of agar and 100 mg of chloramphenicol (JP) with 1,000 mL of water by heating in a water bath, then adjust the pH to 4.9 to 5.1. Autoclave it at 121 °C for 15 minutes. Then, do the same procedure as in 2).

**B. Cultivation**

**Isolation.** Place 20 g of sample in a 200 mL stoppered Erlenmeyer flask. Add 100 mL of sodium hypochlorite solution (available chlorine concentration 10 % )<sup>[7]</sup> (9 + 1) to the flask, shake it gently and soak<sup>[8]</sup> it for 3 minutes. Then, remove its supernatant, add 200 mL of water and shake the flask gently to wash. Leave the flask for a while and then remove its supernatant. Repeat this operation twice additionally and dry the sample well. Spread the sample onto a Petri dish covered with a filter paper<sup>[9]</sup> and leave it for 10 minutes<sup>[10]</sup>.

Place 50 particles of this sample on each potato dextrose agar media with chloramphenicol, Czapek agar media with chloramphenicol and malt salt agar media with chloramphenicol<sup>\*2</sup> using a pair of tweezers<sup>[11]</sup>, and incubate them at 24 to 26 °C for 7 days.

**Pure isolation.** Pick with a hooked platinum needle a small amount of spores or fungous filaments of Eumycetes on the above three kinds of isolation media having growth<sup>[12]</sup>. Inoculate the picked sample to the center of the potato dextrose agar medium and the Czapek agar medium. Turn over them and incubate them at 24 to 26 °C for 7 to 14 days<sup>[13]</sup>.

**C. Identification**

**Rate of contaminated grain.** On the 3<sup>rd</sup>, 5<sup>th</sup> and 7<sup>th</sup> days since the cultivation of isolation starts, observe the presence of Eumycetes on the sample surface with a 10- to 40-powered stereomicroscope. Count the number of particles contaminated by Eumycetes and calculate the rate of contaminated grain<sup>[14]</sup>.

**Identification.**<sup>[15]</sup> Pick up a small part of Eumycetes colonies, well growing through the pure isolation, on a slide glass on which lactophenol solution is dropped to make a prepared slide<sup>[16]</sup> covered by a glass. Observe the prepared slide with a 40- to 400-powered light microscope to inspect the shape and color of sporangium, conidia and fungous filaments for identification.

- \* 1 Use hydrochloric acid solution (1 mol/L) or sodium hydroxide solution (1 mol/L) to adjust the pH.
- 2 If the sample is hardly contaminated with zygomycetes, this process may be omitted.

### «Summary of analysis method»

This method is to sterilize eumycetes adhered on the sample using the sodium hypochlorite solution and to detect eumycetes invading deeply into the sample by culturing on the chloramphenicol-added medium while suppressing enrichment of bacteria.

### «Notes and precautions»

- [1] This process also functions as sterilization. Store it in a brown bottle at room temperature.
- [2] A potato dextrose agar medium (Nissui Pharmaceutical, Eiken Chemical, Difco or BBL) can be used.  
This medium is used in the largest range of applications, for example for isolation, normal culturing and storage of Eumycetes.
- [3] Use a sterilized Petri dish with 90 mm of diameter and 15 mm of depth, made of glass or synthetic resin. A synthetic resin dish is likely to absorb electrostatic spores, and likely to scatter spores of *Penicillium* or other Eumycetes which produce a great amount of spores. Thus, a clear colony is hard to be obtained.
- [4] Chloramphenicol is added to suppress enrichment of bacteria in the sample. Chloramphenicol has advantages of broad antibacterial spectrum, excellent thermal stability and availability of autoclave. In place of chloramphenicol, 1 mg of penicillin and 100 mg of streptomycin can be used for 1,000 mL of the sterilized medium.
- [5] Czapek Solution Agar (Difco) or equivalent medium can be used.  
Mainly used as the medium for isolation and identification of *Aspergillus* and *Penicillium*.
- [6] You may add sodium chloride to the malt agar plate (Nissui Pharmaceutical., Ltd., Difco or BBL). Be sure to avoid over-heating this medium and re-melting down the medium once hardened.  
This medium is used to suppress enrichment of Zygomycetes which grow fast and facilitate detection of other Eumycetes.
- [7] Sodium hypochlorite solutions (for chemical applications or food additives; antiformin as alias) (Wako Pure Chemical Industries) may be used.
- [8] Sodium hypochlorite sterilizes Eumycetes adhered on the sample surface to enable detection of Eumycetes that invade deeply into the sample.
- [9] Be sure to use a sterilized filter paper.
- [10] Dry extra moistures on the sample surface.
- [11] For wheat, barley, milo and rice, place 5 grains on a Petri dish. For maize, soybeans and peanuts, place 4 grains on a Petri dish.
- [12] Using a hooked platinum needle (Figure 18.3-1) under a stereomicroscope, pick up a species of Eumycetes carefully to avoid contamination. To maintain the spore formation capability, it is required for strains forming spores to transplant spores as much as possible.

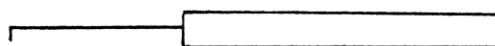
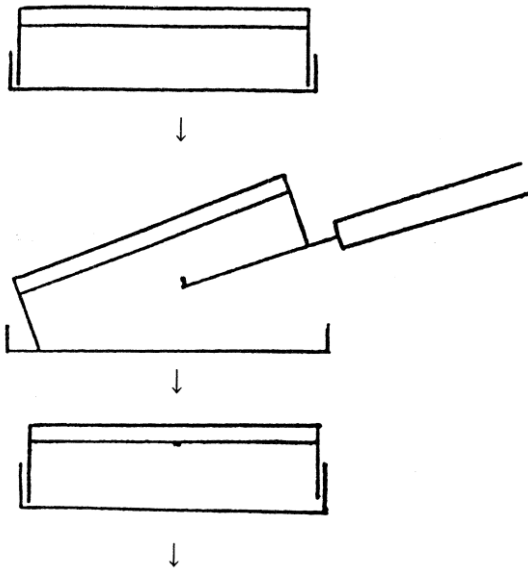


Figure 18.3-1 Hooked platinum needle

- [13] To obtain good colonies from pure isolation, handle the test equipment as shown in Figure 18.3-2. Some Eumysetes species do not show sporulation during 14 days of cultivation. In this case, continue cultivating more.



Pour 20 mL of the medium on a dish. After the medium solidified, turn over the dish.

Pick a fungus with a hooked platinum needle, fix the needle to an edge of the dish.

Inoculate it once on the center of the medium softly.

A minute amount of spores at the tip of the platinum needle will be sufficient.

Cultivate the inoculated plate tranquilly.

Use two to three plates for a Eumycetes.

Observation

Keep the plate upside-down. Lift it gently for observation.

Figure 18.3-2 Pure isolation

- [14] Many Eumycetes are often detected on a grain. In these cases, count the number of contaminated grains as one and calculate the rate of contaminated grain Eumycetes based on the following formula:

$$\text{Rate of contaminated grain (\%)} = \frac{\text{Number of contaminated grains}}{\text{Number of tested grains}} \times 100$$

As the number of tested grains increases, accuracy of the rate of contaminated grain becomes higher.

There is another method to inspect the number of detected Eumycetes per 100 tested grains by species of Eumycetes.

- [15] Perform identification by referring to tables described in references listed in [Summary of Eumycetes].

You may store the identified strains using one of the following methods:

1) Subculture method

Using the potato dextrose agar medium, transplant Eumycetes onto the center of the slant medium prepared in the test tube. Fully grow spores and then store them at room temperature and continue subculturing every 3 to 6 months.

2) Cryopreservation method

Transplant Eumycetes onto the same medium as in 1). Fully grow spores and then store them in a refrigerator with sealing to prevent the medium from drying. The survival period is 1 to 10 years.

3) Liquid paraffin superposition method

Transplant Eumycetes onto the same medium as in 1). Fully grow spores and then add sterilized liquid paraffin so that it can cover all over the medium and store them at room temperature. The survival period is in the range from 3 months to 10 years or more depending on the strain.

4) Freeze-drying method

Suspend spores in skim milk medium and transfer the suspension into an ampule. Freeze-dry the suspension using a freeze-drying device, then seal the ampule under vacuum and cryopreserve it. Long time storage is possible.

[16] The prepared slide will be easy to be observed with bubbles disappeared after passing over a flame for a couple of times.

To observe the structure of *Penicillium* or other Eumycetes containing a great amount of spores, gently wash it in advance on a slide glass on which some drops of DRIWEL K (Fujifilm) solution are placed. Then, take some of the spores onto a slide glass on which some drops of lactophenol solution are placed to make a preparation.

To store the prepared slide for a long time, seal it with nail varnish.

## 2 Confirmation method of Eumycetes in powdered samples

Applicable range: Formula feed and other powdered ingredients<sup>[1]</sup>

Use sterilized water, reagent and instrumentations, as required.

Use the sterile technique as required.

### A. Reagent preparation

1) Sodium carboxymethylcellulose solution

Autoclave sodium carboxymethylcellulose solution (1 w/v%) at 121 °C for 15 minutes.

2) Potato dextrose agar medium<sup>[2]</sup>

See 1-A-2).

3) Potato dextrose agar medium with chloramphenicol<sup>[3]</sup>

See 1-A-3).

4) Czapek agar medium<sup>[4]</sup>

See 1-A-4).

### B. Cultivation

Isolation.<sup>[5]</sup> Place 10 g of sample in a 200 mL stoppered Erlenmeyer flask. Add 100 mL of sodium carboxymethylcellulose solution<sup>[6]</sup> to the flask and shake it so that the solution become homogeneous<sup>[7]</sup> to make the sample stock solution.

Dispense a 1-mL aliquot of the sample stock solution in a test tube, add 9 mL of sodium carboxymethylcellulose solution<sup>[6]</sup> to the test tube to prepare a 10-fold diluted solution. Transfer 1 mL of the 10-fold diluted solution in a test tube, add 9 mL of sodium carboxymethylcellulose solution<sup>[6]</sup> to the test tube to prepare a 100-fold diluted solution.

Dispense each 1-mL aliquot of the sample stock solution, 10-fold diluted solution and 100-fold diluted

solution on each potato dextrose agar medium with chloramphenicol (3 Petri dishes for each solution) and spread the solution evenly<sup>[8]</sup>. Turn over the dishes and incubate them at 24 to 26 °C for 7 days.

Pure isolation. Pick with a hooked platinum needle, a small amount of spores or fungous filaments of Eumycetes on the above isolation media having growth<sup>[9]</sup>. Inoculate the picked samples to the center of the potato dextrose agar medium and the Czapek agar medium. Turn over them and incubate them at 24 to 26 °C for 7 to 14 days<sup>[10]</sup>.

### C. Identification

Count of Eumycetes.<sup>[11]</sup> On the 3<sup>rd</sup>, 5<sup>th</sup> and 7<sup>th</sup> days since the cultivation of isolation starts, inspect the number of Eumycetes colonies generated on each Petri dish of each medium and count the number of Eumycetes per 1 g of sample.

Identification. See 1-C.

### «Summary of analysis method»

This method is to detect Eumycetes while suppressing their enrichment in the sample by diffusing the powder sample evenly with the surfactant solution and then incubating the sample on the chloramphenicol-added medium.

Reference: Nakajima et. al. : Study of Animal Husbandry; 37, 11 (1983)

### «Notes and precautions»

[1] For the powdered sample, unlike the case of unground sample in section 1, surface sterilization is not needed. Inspect the degree of sample contamination with Eumycetes based on the number of Eumycetes per 1 g of sample.

[2] See «Notes and precautions» [2] in section 1.

[3] See «Notes and precautions» [4] in section 1.

[4] See «Notes and precautions» [5] in section 1.

[5] Perform isolation cultivation as shown in Figure 18.3-3. When the degree of contamination with Eumycetes is high, dilute the sample solution further.

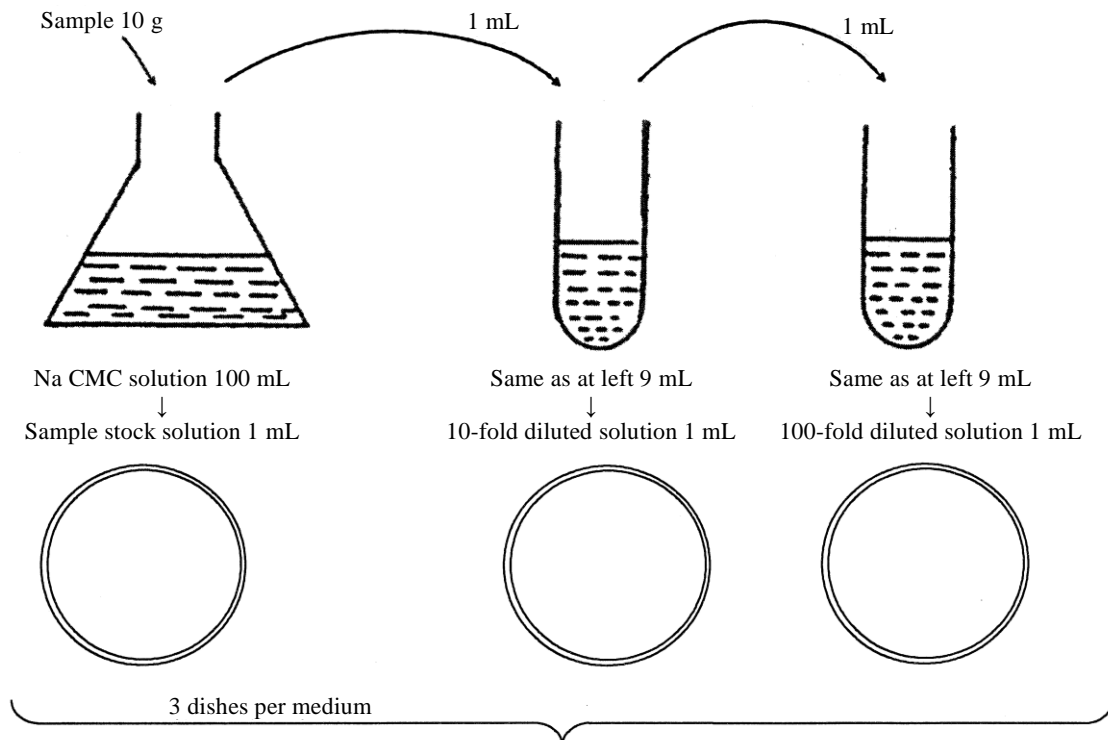


Figure 18.3-3 Isolation cultivation method to count Eumycetes

- [6] You may use sterilized agar solution (0.05 w/v%) instead.
- [7] You may put about 100 sterilized glass beads (6 mm diameter) in a Erlenmeyer flask and shake it well to homogenize the solution evenly.
- [8] Spread the sample promptly over the entire medium aseptically using a sterilized bacteria spreader.
- [9] See «Notes and precautions» [12] in section 1.
- [10] See «Notes and precautions» [13] in section 1.
- [11] The number of Eumycetes counted by this method is the sum of conidia or spores and fungus filaments (all of them are viable bacteria). Since the fungus filaments have various degrees of segmentation depending on the conditions of grinding or shaking (or homogenizing in some cases) for extraction of samples, the resultant count of Eumycetes may differentiate.

To count Eumycetes, use a plate on which 20 to 50 colonies are formed.

The number of Eumycetes in formula feed is  $2 \times 10^3 \sim 2 \times 10^4$ /g on the mush sample. However, in the pellet feed, steam heating reduces Eumycetes, which can hardly be detected (detection limit:  $10^2$ /g).

For feed ingredients, maize (ground) contains the largest number of Eumycetes:  $2 \times 10^2$  to  $2 \times 10^5$ /g. Milo, brown rice and wheat contain from  $< 10^2$  to  $2 \times 10^2$ /g. Wheat flour, soybean meal and wheat bran contain from  $< 10^2$  to  $10^5$ /g depending on the manufacturer and production lot.