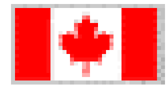

Global Salm-Surv

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

Laboratory Protocols

Level 1 Training Course

Kits for identification of *Enterobacteriaceae* Using API.

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Foreword

Despite the controls that have already been put into place, *Salmonella* infection arising from contaminated food continues to be an immense problem with millions of cases occurring annually throughout the world. In addition to the misery caused, financial loss is enormous.

Detection of *Salmonella* before contaminated foods can be consumed is therefore an essential feature of safeguarding public health and incidentally preserving the reputations and fortunes of food manufacturers and processors.

Surveillance of *Salmonella* in all the different stages of feed-food chain constitutes an important element in the exploration of epidemiology of foodborne salmonellosis, and in the development and implementation of efficient *Salmonella* control strategies.

Efficient laboratory methods for isolation, identification and typing of *Salmonella* are essential elements in *Salmonella* monitoring and control programmes. This protocol describes methods that have been extensively documented in the scientific literature, are accepted by international standardisation bodies and can be applied under most conditions and circumstances in laboratories worldwide.

It is, however, important to note the following:
There are many different procedures for isolation of *Salmonella*. The ideal method has a high sensitivity and specificity, and at the same time is simple, rapid and inexpensive. No single method fulfils all these criteria, and no single method is optimal for all purposes. Therefore, it is advisable to consult the literature before choosing a method for a particular purpose. Frequently comparing your existing methods to newer methods is also highly advisable.

In this manual we present the methods for isolation of *Salmonella* according to the ISO-6579 standard but other methods may be used. The criteria for biochemical identification of *Salmonella* are relatively standard, however the format varies. Molecular methods are increasingly being introduced as an alternative. These methods often lead to a faster diagnosis and may be simpler to conduct, but they have the disadvantage that they may be expensive.

The *Salmonella* serotyping system is probably the best phenotypic bacterial typing system ever developed. It has a high discriminatory power and provides information that has great epidemiological significance. Molecular typing methods such as PFGE can yield supplementary information, but are so far not a substitute for serotyping or micro tray agglutination, etc. Availability and cost of high quality antisera can be a problem in some countries and regions.

In a survey of national *Salmonella* reference laboratories, conducted before the launch of Global Salm-Surv, laboratories were asked about which guidelines/standards they used for antimicrobial susceptibility testing. Nearly 3 out of 4 laboratories used the NCCLS guidelines. In this light, GSS has decided to promote the use of the NCCLS guidelines, but GSS laboratories are not obligated to follow these guidelines.

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1. Kits for identification of *Enterobacteriaceae*

1.1 Identification of *Enterobacteriaceae* and other Gram-negative rods with API 20E

Introduction
API 20E is produced for identification of *Enterobacteriaceae* and other Gram-negative rods based on 23 miniaturised biochemical tests. This method is quick and simple to use, but expensive compared to ordinary biochemical tests.

Materials

Equipment
- 37°C incubator
- Refrigerator
- Loop (1 µl)
- Bunsen burner
- Sterile Pasteur pipettes

Chemicals and reagents
- Sterile normal saline, 4 ml in tubes.
- The API 20E kit consisting of strips (store at 2-8°C upon arrival) and incubation boxes.
- Reagent kit (#2012 or the individual reagents #7040 - #7046 and #7054)
- Mineral oil (#7010)
- API 20E Analytical Profile Index.
- **TDA** reagent (#7040) for detection of tryptophane deaminase.
- Kovacs reagent / **JAMES** reagent (#7054) or IND reagent for detection of indole.
- Voges Proskauer reagents **VP 1** (#7042) and **VP 2** (#7043) for detection of acetoin.
- Griess reagent **NIT 1** (#7044) and **NIT 2** (#7045) for detection of nitrites.
- Zn reagent (#7038)
- **Ox** reagent (#7046 or others) for detection of oxidase.

Bacterial strains
Strains for identification on non-selective agar

Safety
Nearly all the reagents irritate the skin, so wash with soap and water if you get some of a reagent on your skin. The reagents **IND**, **VP2**, **Ox** and **Zn** are flammable. For other safety instructions, refer to ref. 1.

Procedure

Theory / comments

Day 1

Prepare the strip

Pour water in the bottom of the incubator box. Pour off surplus water so the wells are full (approx. 5 ml) and place the strip on top of the wells in the incubator box.

Prepare the inoculum

Remove a single, well-isolated colony from a plate with a 1 µl loop or Pasteur pipette and emulsify it carefully in a tube containing 4 ml sterile normal saline.

Perform the oxidase test on a similar colony according to ref. 1 or the available oxidase kit.

Inoculation

You may use the same pipette to fill the tube and neck of tests |CIT| , |VP| , |GEL| with the bacterial suspension (avoid air-bubbles in the tubes). Fill only the tubes of these other tests.

Obtain anaerobic conditions in the tests ADH, LDC , ODC , URE and H_2S by overlaying with mineral oil.

Close the incubation box and incubate at 35-37°C for 18-24 hours.

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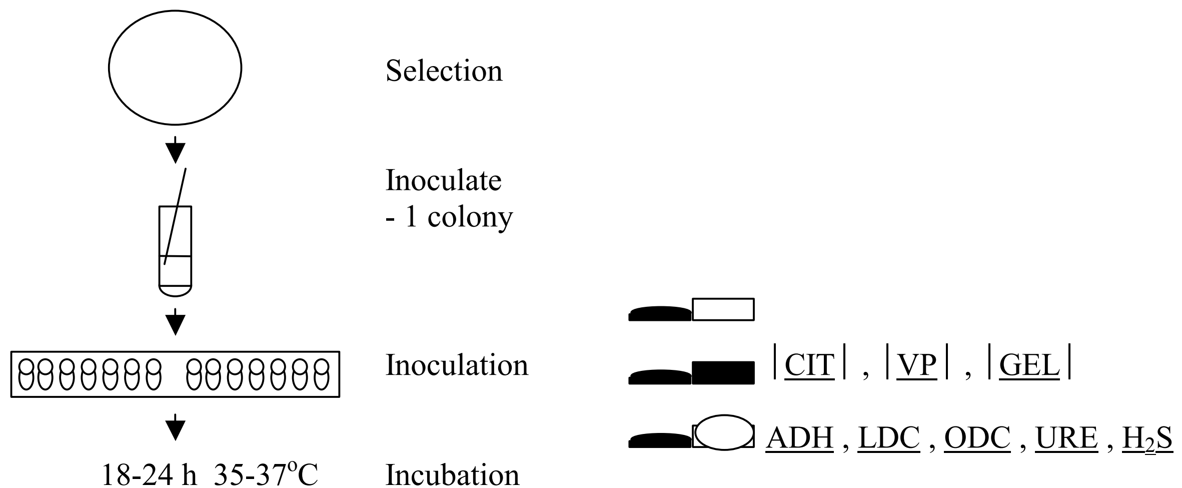
Figure 3. Overview of the API 20E procedure.



Procedure

Day 2: Reading the strip

Read the strip after 18-24 hours at 35-37°C.

All of the spontaneous reactions (except VP, TDA and IND) should be read first according to the interpretation table given on next page and the results recorded in the record sheet.

If glucose is positive and/or 3 or more tests are positive, add the following reagents to the wells:
VP: 1 drop **VP1** and 1 drop **VP2** reagents
TDA: 1 drop **TDA** reagent
IND: 1 drop Kovacs reagent **/James** reagent, and read the reactions according to the interpretation table (table 5).

On the record sheet the tests are separated into 7 groups consisting of tests with the numbers 1, 2 and 4. The numbers corresponding to the positive reactions are added in each group, and a 7-digit profile is obtained. Use this profile for identification according to the table in ref. 1.

Theory / comments

If glucose is negative and 0 or 1 test is positive, do not add reagents. Instead, reincubate the API strip for an additional 24 hours, and perform the supplementary tests by ordinary biochemical tests or refer to ref. 1:
Perform OF test.
Streak a MacConkey agar plate (MAC).
Check motility (MOB).

After incubation read all spontanous reactions, add the reagents and then perform the following tests:
NO_2: Add 1 drop **Nit1** and 1 drop **Nit2** reagents to the GLU well. If the reaction is negative (yellow) it may be due to a reduction of nitrogen, so perform the N_2 test by adding 2 to 3 mg of Zn to the GLU tube.
Read all of the reactions and the results of the supplementary tests according to table 5. In this case a 9-digit profile is obtained and is used for identification.

If the 7-digit profile is not discriminatory enough, you could also perform the NO_2, N_2, MOB, MAC, oxidation of glucose (OF-O) and fermentation of glucose (OF-F) tests as described above to get a 9-digit profile for identification according to the table in ref. 1.

Table 5. Interpretation table

Tests	Reactions/enzymes	Results	
		Negative	**Positive**
ONPG	Beta-galactosidase	Colourless	Pale yellow - yellow
ADH	Arginine dihydrolase	Yellow (orange after 24 h)	Red/orange
LDC	Lysine decarboxylase	Yellow	Orange
ODC	Ornithine decarboxylase	Yellow-(orange after 24 h)	Red/orange
|CIT|	Citrate utilisation	Pale green/yellow	Some blue-green/green
H_2S	H_2S production	Colourless/greyish	Black deposit/thin line
URE	Urease	Yellow	Red/orange
TDA	Tryptophane deaminase	Add 1 drop **TDA** reagent and read immediately:	
		Yellow	Red - dark brown
IND	Indole production	Add 1 drop Kovacs/**JAMES** and read immediately or add 1 drop **IND** and read after 2 min	
		Kovacs /**JAMES** Pale green-yellow **IND** Yellow ring	Kovacs / **JAMES** Pink **IND** Red ring
|VP|	Acetoin production	Add 1 drop **VP 1** + **VP 2** and read after 10 min	
		Colourless	Some pink/red
|GEL|	Gelatinase	No diffusion of black pigment	Diffusion of Black pigment
GLU	Glucose fermentation/oxidation	Blue/blue-green	Yellow
MAN	Mannitol fermentation/oxidation	Blue/blue-green	Some yellow
INO	Inositol fermentation/oxidation	Blue/blue-green	Some yellow
SOR	Sorbitol fermentation/oxidation	Blue/blue-green	Some yellow
RHA	Rhamnose fermentation/oxidation	Blue/blue-green	Some yellow
SAC	Sucrose fermentation/oxidation	Blue/blue-green	Some yellow
MEL	Melibiose fermentation/oxidation	Blue/blue-green	Some yellow
AMY	Amygdalin fermentation/oxidation	Blue/blue-green	Some yellow
ARA	Arabinose fermentation/oxidation	Blue/blue-green	Yellow
OX	Cytochrome oxidase (e.g. use one colony on filter paper)	Add 1 drop **OX** and read within 1-2 min	
		Colourless	Violet
NO_3-NO_2	NO_2 production Use the GLU tube	Add 1 drop **NIT 1**+**NIT 2** and read after 2-3 min	
		Yellow	Red
N_2	Reduction to N_2 gas Use the GLU tube	If negative above add 2-3 mg **Zn** to the GLU tube and read after 5 min	
		Red	Yellow
MOB	Motility	Non motile	Motile
MAC	Growth	Absence	Presence
OF	Fermentation of glucose: Closed Oxidation of glucose: Open	Green Green	Yellow Yellow

References

1. **API 20E** Identification system for Enterobacteriaceae and other Gram-negative rods. Instruction Manual version E (#2012).

1.2 Identification of *Enterobacteriaceae* and other Gram-negative rods with ID 32 E

Introduction

ID 32 E is produced for identification of *Enterobacteriaceae* and other Gram-negative rods based on 32 miniaturised biochemical tests. This method is quick and simple to use, but expensive compared to ordinary biochemical tests.

Materials

Equipment

- 37°C incubator.
- Refrigerator
- Loop (1 µl)
- Bunsen burner
- Pipettes (an automated dispenser is easiest to use)
- WhirlyWhirly mixer (if available)

Chemicals and reagents

- The ID 32 E kit consisting of strips (store at 2-8°C upon arrival) and lids.
- Sterile normal saline, 5 ml in tubes.
- Mineral oil (ref. 70 100)
- Kovacs reagent/**JAMES** reagent (ref. 70 540) for detection of indole.
- **Ox** reagent (ref. 70 460 or other kits) for detection of oxidase.

Kovacs/**JAMES** and **Ox** reagents are light sensitive, so wrap the bottles in aluminium foil and store the reagents in a refrigerator, but allow them to reach room temperature before use.

Bacterial strains

- Strains for identification on non-selective agar.

Safety

With Kovacs /**JAMES** and **Ox** reagents avoid contact with skin and eyes. In case of contact with skin, wash with soap and plenty of water. **Ox** reagent contains isoamyl alcohol which is flammable.

Procedure

Day 1

Remove a single, well-isolated colony from a plate with a 1 µl loop and emulsify it carefully in a tube containing 3 ml sterile normal saline.

Perform the oxidase test on a similar colony in advance according to ref. 1 or the available oxidase kit.

Fill each well with 55 µl of the suspension using a pipette.

Cover the tests ODC , ADH , LDC , URE , LARL , GAT and 5KG by overlaying with **2 drops** of mineral oil. Put the lid on.

Put the ID 32 E in a plastic bag with a slightly wet paper towel and incubate at 35-37°C for 18-24 hours.

Day 2: Reading the strip

Add one drop of Kovacs reagent **/JAMES** reagent to the IND well and read the tests according to the interpretation table (table 1).

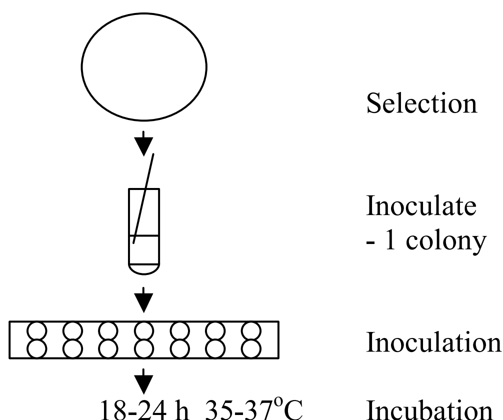
On the report sheet the tests are separated into 10 groups consisting of tests with the numbers 1, 2 and 4 and one with number 1 and 2. The numbers corresponding to the positive reactions are added in each group, and an 11-digit profile is obtained. Use this profile for identification according to the table in ref. 1.

Theory / comments

If available use a Whirly mixer to obtain a solution equivalent to 0.5 McFarland.

If available use an automated pipette for dispension

To obtain anaerobic conditions



With 2 drops of oil: ODC , ADH , LDC , URE , LARL , GAT and 5KG

Figure 4.
Overview of the ID 32 E procedure

Table 6. Interpretation table

No	Test	Reaction	Results	
			Negative	**Positive**
1.0	ODC	Ornithine decarboxylase	Yellow / yellow-orange	Red / orange
1.1	ADH	Arginine dihydrolase	Yellow / yellow-orange	Red / orange
1.2	LDC	Lysine decarboxylase	Yellow-green	Blue-violet
1.3	URE	Urease	Yellow/ yellow-orange	Pink-violet
1.4	LARL	L-Arabitol (Acidification)	Blue / blue-green	Yellow / Green-yellow
1.5	GAT	Galacturonate (Acidification)		
1.6	5KG	5 Ketoglutarate (Acidifacation		
1.7	LIP	Lipase	Colorless	Blue
1.8	RP	Phenol red (Acidification)	Red / orange	Yellow
1.9	βGLU	β-Glucosidase	Colorless	Yellow
1.A	MAN	Mannitol (Acidification)	Blue /blue-green	Yellow / Green-yellow
1.B	MAL	Maltose (Acidification)		
1.C	ADO	Adonitol (Acidification)		
1.D	PLE	Palatinose (Acidification)		
1.E	βGUR	β-Glucuronidase	Colorless	Yellow
1.F	MNT	Malonate	Yellow / pale green	Blue-green / blue
0.0	IND [1]	Indole production	Add 1 drop Kovacs/**JAMES** and read immediately	
			Colorless /-yellow / beige	Pink / red
0.1	βNAG	N-acetyl-β-glucosaminidase	Colorless	Blue
0.2	βGAL	β-Galactosidase	Colorless	Yellow
0.3	GLU	Glucose (Acidification)	Blue / blue-green	Yellow / Green-yellow
0.4	SAC	Saccharose/sucrose (Acidification)		
0.5	LARA	L-Arabinose (Acidification)		
0.6	DARL	D-Arabitol (Acidification)		
0.7	αGLU	α-Glucosidase	Colourless	Yellow
0.8	αGAL	α-Galactosidase		
0.9	TRE	Trehalose (Acidification)	Blue / blue-green	Yellow / Green-yellow
0.A	RHA	Rhamnose (Acidification)		
0.B	INO	Inositol (Acidification)		
0.C	CEL	Cellobiose (Acidification)		
0.D	SOR	Sorbitol (Acidification)		
0.E	αMAL	α-Maltosidase	Colorless / Very pale yellow	Yellow
0.F	AspA	L-Aspartic acid arylamidase	Colorless / Very pale yellow	Yellow

References

ID 32 E System for identification of *Enterobacteriaceae* and other Gram-negative rods.

[1] A beige-brown colour must be observed in the cupule before adding the reagent.

2. Composition and preparation of culture media and reagents

If no reference is given, it is the procedure used at DVL.
The media and reagents are available from several companies including Oxoid, Merck and Difco. The composition of the dehydrated media given below is an example and may vary a little among the different manufacturers. Also, the media should be prepared according to the manufacturers description if it differs from the description given here. Refer to Appendix 2 for a colour presentation of growth of *Salmonella* on selective agar media and positive and negative reactions of biochemical tests.

Kovacs reagent for indole reaction (ref. 2)

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

Preparation:
Mix the components.

1-Naphthol, ethanolic solution for VP test (ref. 2)

1-Naphthol	6 g
Ethanol, 96 % (V/V)	100 ml

Dissolve the 1-naphtol in the ethanol.

Potassium hydroxide solution for VP test (ref. 2)

Potassium hydroxide	40 g
Water	100 ml

Dissolve the potassium hydroxide in the water.

References

1. Post D. E. (1997) Food-borne pathogens monograph number I *Salmonella*. Oxoid limited, Hampshire, England.
2. ISO 6579 :1993(E) 3rd ed. Microbiology - General guidance on methods for the detection of *Salmonella*.
3. NMKL method no. 71, 2nd ed., 1999: *Salmonella*. Detection in food. Nordic committee on food analysis.