Service contract for the provision of EU networking and support for public health reference laboratory functions for antimicrobial resistance in *Salmonella* species and *Campylobacter* species in human samples

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Identification of Enterobacteriaceae Using API.

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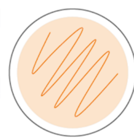
STATENS SERUM INSTITUT



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1. BACKGROUND

Efficient laboratory methods for isolation, identification and typing of Salmonella are essential elements in the control of Salmonella. There exist a number of different commercial identification kits for Salmonella that are widely acknowledged and used in public health laboraroties. In this exercise we demonstrate how Salmonella can be identified using the Enterobacteriaceae Using API ID 20E and ID 32E from Biomerieux.

2. MATERIAL AND METHODS

Kits for identification of Enterobacteriaceae

2.1. 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 may be 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. In the instruction to ID 32E, Biomerieux has recommended which strains are best used as quality control.

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 the instructions that come with the API kit.

Procedure

Day 1

Prepare the strip

Pour normaly 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.

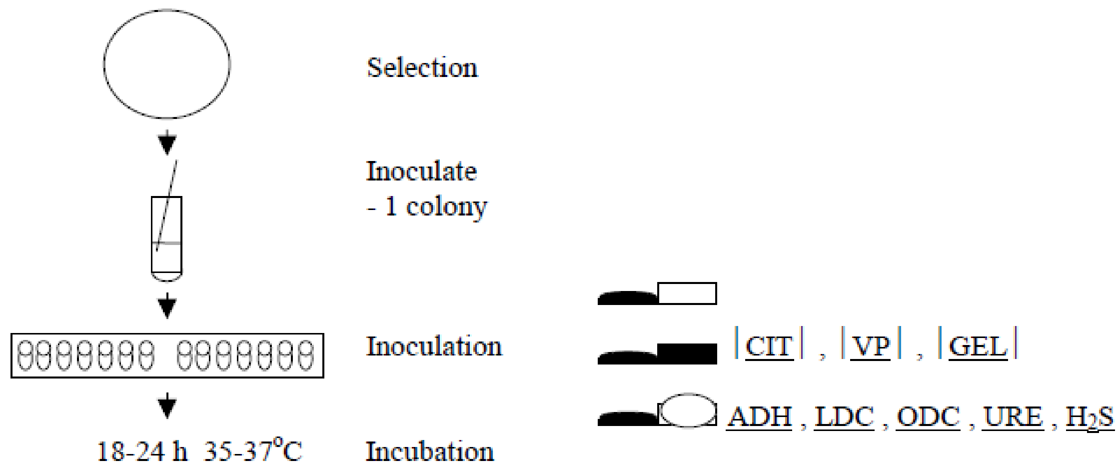
Perform the oxidase test on a similar colony according to ref. 1 or the available oxidase kit.

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 H2S by overlaying with mineral oil.

Close the incubation box and incubate at 35-37 ^{o}C for 18-24 hours.

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	Add 1 drop **OX** and read within 1-2 min	
	(e.g. use one colony on filter paper)	Colourless	Violet
NO_3-NO_2	NO_2 production	Add 1 drop **NIT 1**+**NIT 2** and read after 2-3 min	
	Use the GLU tube	Yellow	Red
N_2	Reduction to N_2 gas	If negative above add 2-3 mg **Zn** to the GLU tube and read after 5 min	
	Use the GLU tube	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).

2.2. Identification of Enterobacteriaceae and other Gram-negative rods with ID 32 E

Introduction

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

Materials

Equipment

- 37 ^{o}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 ^{o}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).

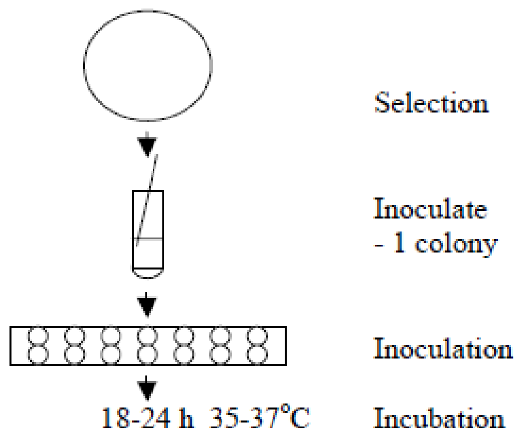
On the report sheet the tests are separated into 10 groups consisting of tests with the numbers 1, 2 and 4 and one with number 1 and 2. The numbers corresponding to the positive reactions are added in each group, and an 11-digit profile is obtained. Use this profile for identification according to the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

3. Composition and preparation of culture media and reagents

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.

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.

4. Reference

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.