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Methods for Microbiological and Immunological Studies of Space Flight Crews

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ACRONYMS AND ABBREVIATIONS

ASTP	Apollo-Soyuz Test Project
AU	agglutination unit
BA	blood agar
BBL	Baltimore Biological Laboratories
BTB	bromthymol blue
CMMY	cornmeal/malt-extract/yeast-extract agar
HIA	heart infusion agar
MR	methyl red
OF	oxidation-fermentation
PHA	phytohemagglutinin
RTD	routine test dilution
SAB	Sabouraud's dextrose agar
TGY	tryptose glucose yeast
TSA	trypticase soy agar
TSB	trypticase soy broth
TSI	triple-sugar iron
TTC	2,3,5-triphenyltetrazode chloride
VP	Voges-Proskauer
YEME	yeast-extract/malt-extract agar
YPSS	yeast phosphate soluble starch agar

1. INTRODUCTION AND GENERAL IDENTIFICATION SCHEMA

By Gerald R. Taylor* and S. N. Zaloguev†

Microbiological evaluations of American space-flight crewmen were initiated at the beginning of the Apollo mission series and were designed to characterize the microbial load of astronauts preparing for lunar surface exploration. This procedure was considered necessary to facilitate the identification of any possible terrestrial contaminants in the returned lunar material. Studies conducted during the final missions in the Apollo series were designed to identify and prepare for possible microbial problems associated with the lengthy Skylab missions. Microbiological evaluations conducted as part of the U.S. Skylab Program identified the response of autoflora to long-duration space flight.

Preflight and postflight microbiological analyses designed to detect potential pathogens, with some additional emphasis on selected species, have been conducted on crewmembers of the Russian Vostok, Voskhod, and Soyuz space exploration missions. On the longer duration missions, such as the 425-hour flight of Soyuz 9 and the 570.5-hour flight of Soyuz 11, detailed analyses of the fungal and bacterial autoflora were performed. Although the objectives of the U.S. and U.S.S.R. space microbiology studies were often similar, the methods employed were different.

Prior to the joint Apollo-Soyuz Test Project (ASTP) flight in the summer of 1975, scientists of both countries had not attempted to formally standardize the methods for collecting samples, conducting analyses, and interpreting data. With the advent of ASTP, the U.S. National Aeronautics and Space Administration and the U.S.S.R. Academy of Sciences, which were involved in space research, concluded an agreement on conducting a joint biological experiment (Microbial Exchange Experiment) during which the scientific-research laboratories of both countries, employing jointly developed research methods, would study samples collected from cosmonauts, astronauts, and various areas of the Apollo and Soyuz spacecraft. To expand the potential scope for this type of experiment, the specialists of both countries developed a joint method which made it possible to achieve maximum correlation between ASTP research and the studies performed by both countries in the past. This set of laboratory guidelines consists of a collection of joint methods agreed upon by scientific personnel from the United States and the U.S.S.R. In some cases,

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certain methods not considered optimal by all laboratory personnel were nevertheless incorporated into this collection so that data obtained during previous studies could be more easily correlated. In addition, this manual does not replace other manuals in use currently; rather, the objective of the manual is its use in the following capacities.

1. As a reference consisting of methods jointly acceptable to both U.S. and U.S.S.R. scientists

2. As a source of information on research performed by both countries in the area of space microbiology

3. As a reference aiding in the planning and implementation of future unilateral or joint microbiological research in space

Although the same areas were not always sampled and the same techniques were not always used throughout all U.S. and U.S.S.R. space flights, it is possible to present the protocol used for the ASTP mission as an example of the general methodology involved. During each preflight and postflight sampling period, 10 microbial specimens were collected from each crewmember. Calcium alginate swabs, wetted in 0.3-millimolar phosphate buffer, were used to sample each of seven body surface areas. This swab technique was the only collection method compatible with the flight program. Although it is recognized that subsurface microbes may be overlooked when this method is used, procedures for sampling subsurface areas were not acceptable. Dry calcium alginate swabs were used to sample the throat before collection of the mouthwash specimen. For the latter sample, the subject gargled with 0.3-millimolar phosphate buffer followed by a repeated rinse of the teeth with the same solution. Swabs were placed in 5 cubic centimeters of 0.3-millimolar phosphate buffer for transport to the laboratory. Analysis of all samples was initiated within 1 hour of specimen collection.

The contents of each swab and mouthwash specimen were serially diluted under aseptic conditions and subsequently inoculated onto the surface of nutrient media as outlined in table 1-I. The variety of media, the number of plates inoculated, and the dilution range were selected on the basis of requirements for isolating and quantitating the aerobic autoflora components present in each sampled area. The Sabouraud dextrose agar plates were incubated at 298 K (25° C) for 7 days. All other plates were incubated at 310 K (37° C) for 48 hours. Following incubation under the appropriate conditions, all resulting colonies on every plate were categorized and counted. Subsequently, one sample of each morphologically different colony type was transferred from each dilution series to the appropriate nutrient media (figs. 1-1 to 1-7) and Gram stained. Specific studies of selected U.S. (refs. 1-1 to 1-10) and U.S.S.R. (refs. 1-7 and 1-10 to 1-16) crews have previously been reported.

Citations to foreign literature sources generally are printed as submitted by the investigators, and the information has not been verified in all cases.

As an aid to the reader, where necessary the original units of measure have been converted to the equivalent value in the Système International d'Unités (SI). The SI units are written first, and the original units are written parenthetically thereafter.

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TABLE 1-I.- SAMPLE SITES AND PRIMARY ISOLATION MEDIA EMPLOYED

IN THE JOINT U.S.-U.S.S.R. SPACE FLIGHT

Sample designation	Media	Number of plates	Dilution range, $\log_{10}$ units
Back of neck, Hair, Hands, Axillae, Ears	Blood[a]	2	0 to 4
	Mannitol[b]	3	0 to 1
	CMMY[c]	4	0
	SAB[d]	5	0
Mouthwash (natural)	Blood	2	0 to 5
	Mannitol	3	0 to 1
	CMMY	4	0 to 2
	Rogosa[e]	3	0 to 3
	Choc[f]	3	0 to 5
Mouthwash (centrifugate)	CMMY	4	0
	SAB	5	0
Nose	Blood	2	0 to 4
	Mannitol	3	0 to 3
	CMMY	4	0
	SAB	5	0
	Choc	3	0 to 1
Toes and groin	Blood	2	0 to 5
	Mannitol	3	0 to 1
	CMMY	4	0
	SAB	5	0
Throat swab	Blood	2	0 to 5
	Mannitol	3	0 to 1
	CMMY	4	0 to 2
	SAB	5	0
	Choc	3	0 to 5
	Rogosa	3	0 to 3

[a]Blood agar.
[b]Mannitol salts agar.
[c]Cornmeal/malt-extract/yeast-extract agar.
[d]Sabouraud's dextrose agar.
[e]Rogosa agar.
[f]Chocolate bacitracin agar.

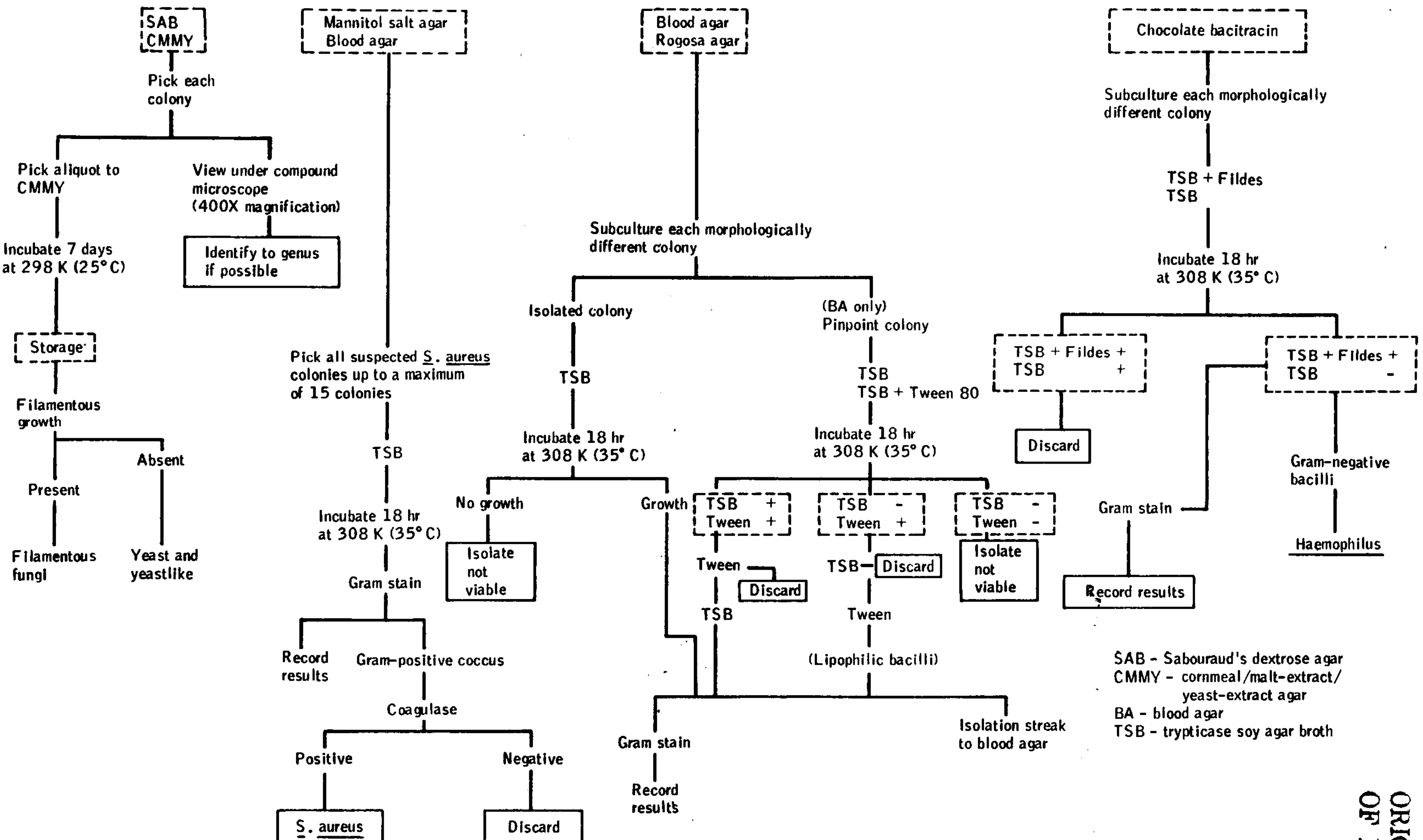

Figure 1-1. Schematic diagram for analysis of initial quantitation/isolation media.

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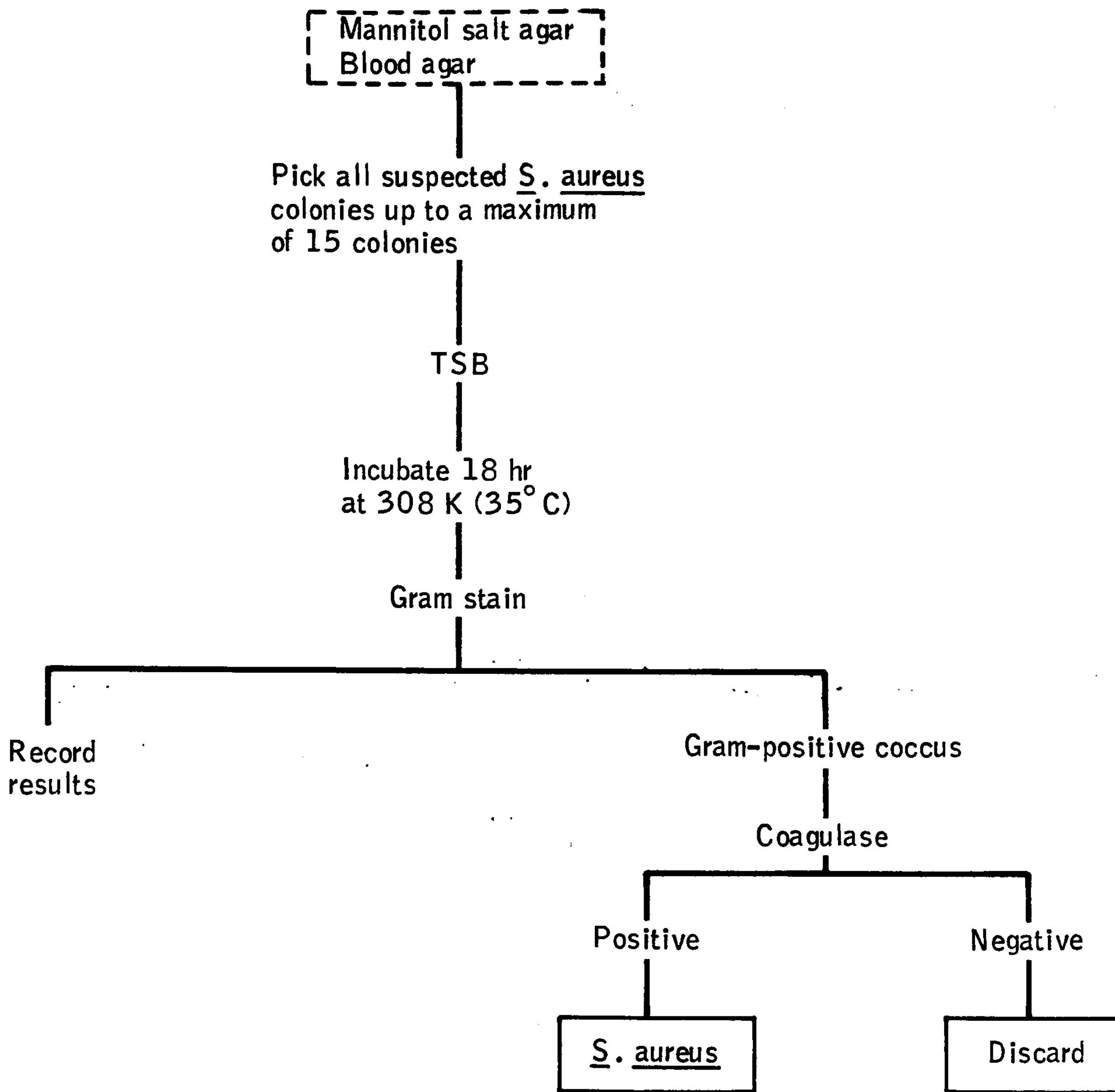

Figure 1-2.- Schematic diagram for selection of *Staphylococcus aureus* cultures for special studies.

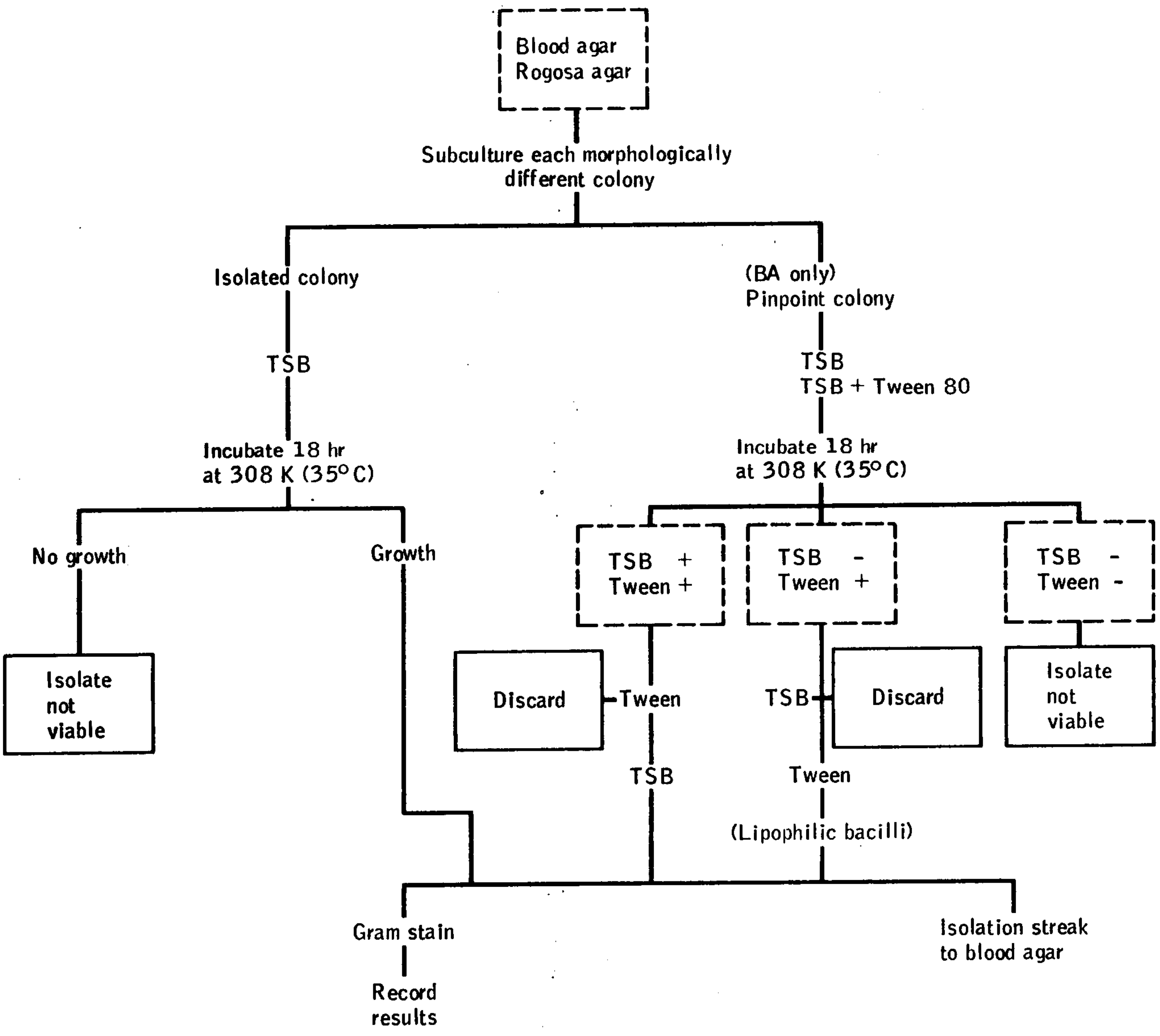

Figure 1-3.- Schematic diagram for selection of nonfastidious bacteria.

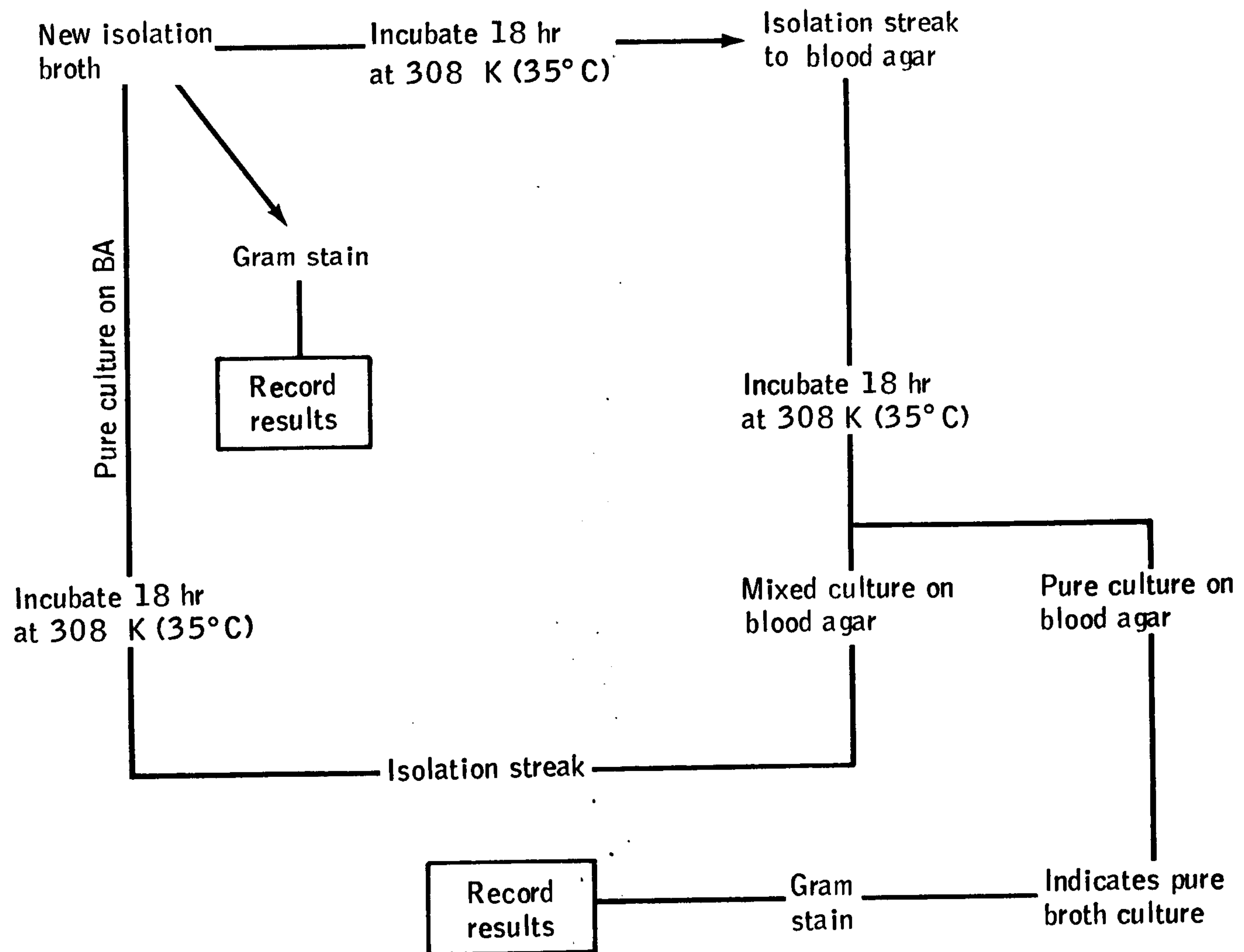

Figure 1-4.- Schematic diagram for purity check of nonfastidious bacteria.

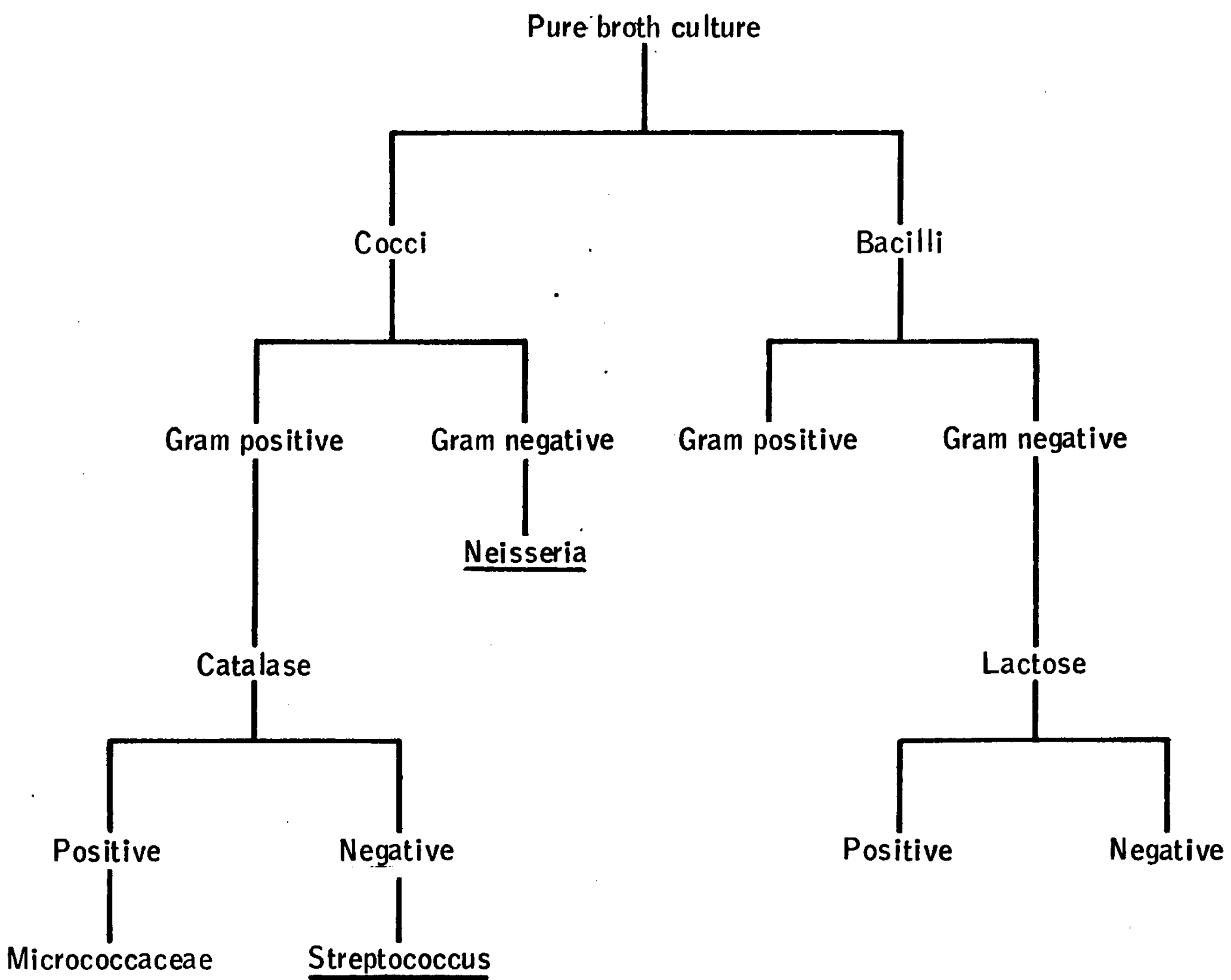

Figure 1-5.- Schematic diagram for division of nonfastidious bacteria.

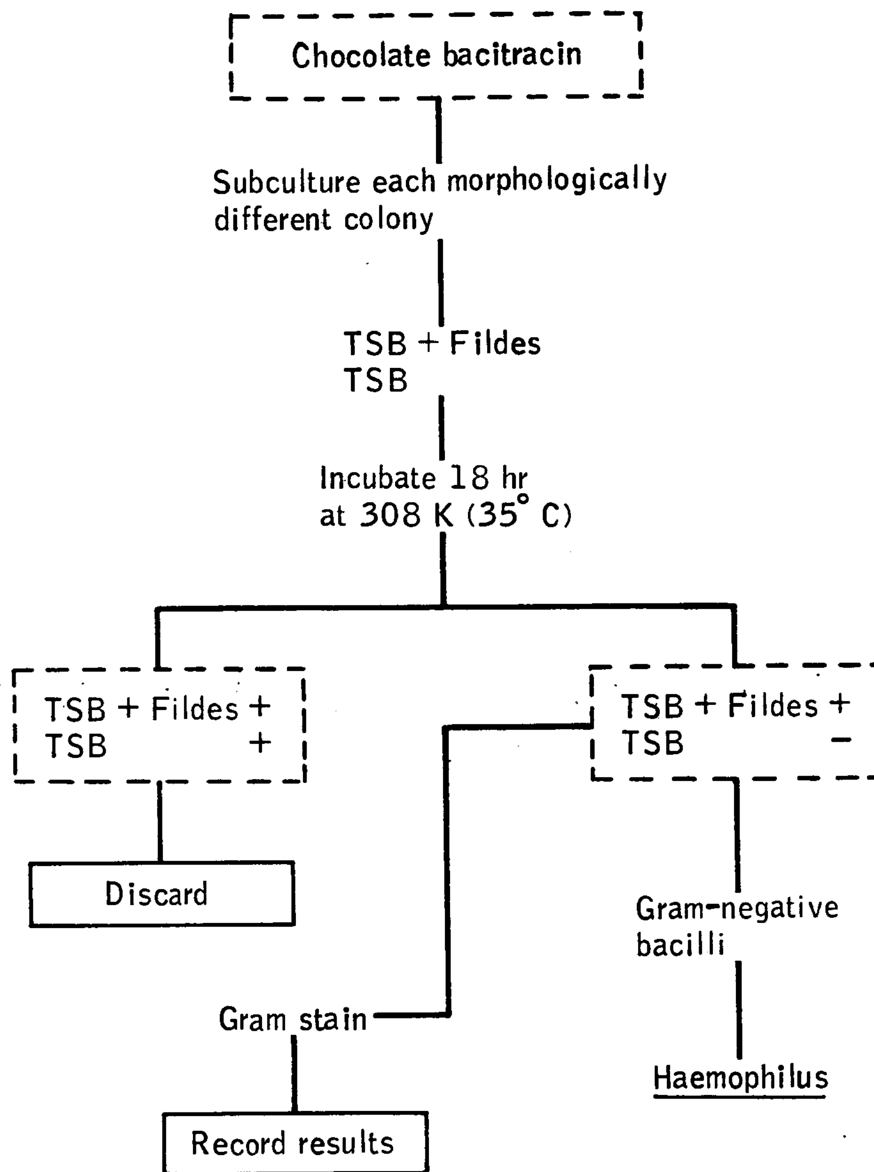

Figure 1-6.- Schematic diagram for selection of _Haemophilus_ species.

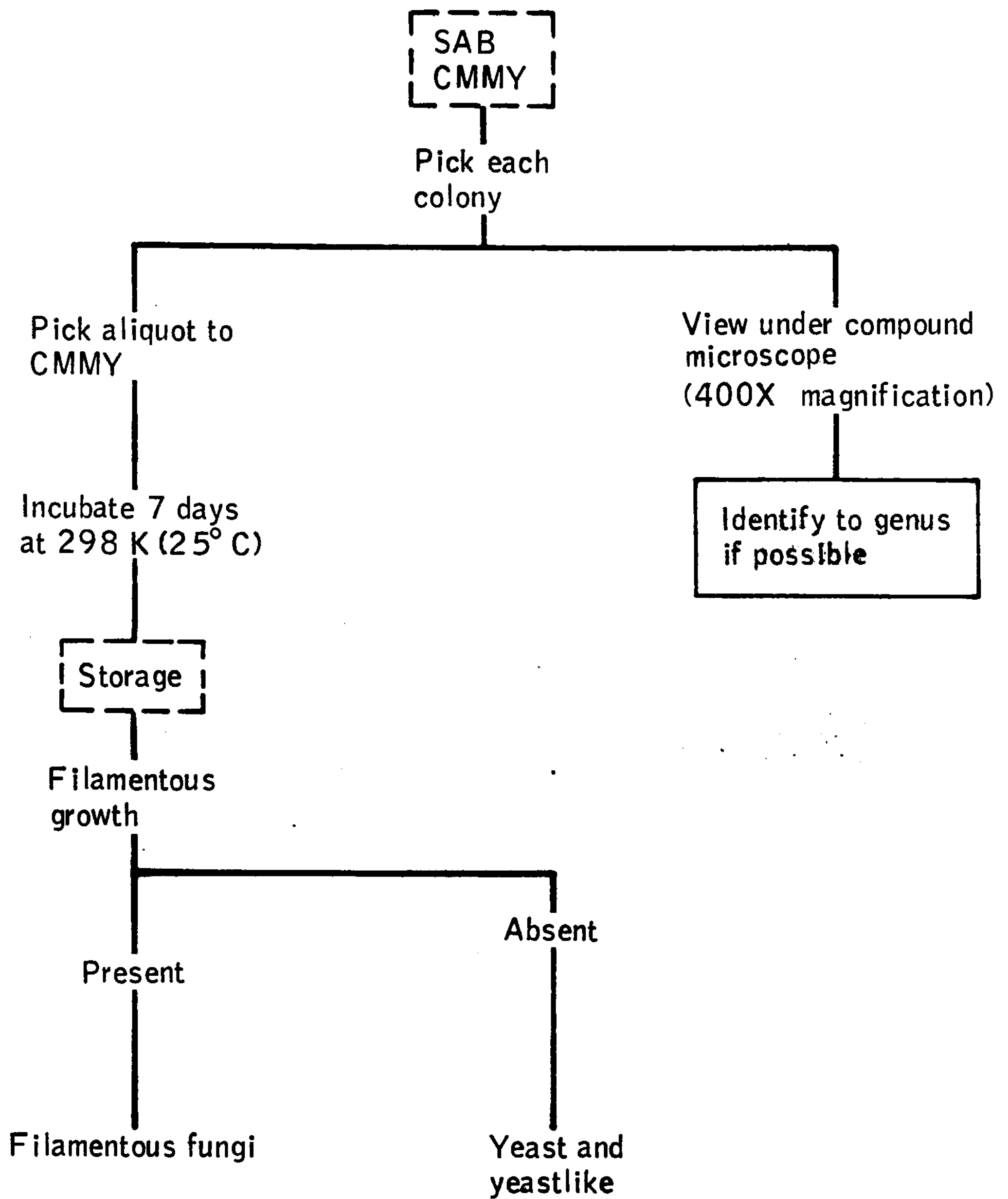

Figure 1-7.- Schematic diagram for selection of yeasts and filamentous fungi.

2. IDENTIFICATION OF GRAM-POSITIVE COCCI

By J. Glee Decelle,* A. N. Victorov,† and V. M. Shilov‡

2.1 PURPOSE

The purpose of this chapter is to describe methods for identifying Gram-positive cocci.

2.2 MATERIALS

The following materials are used in identifying Gram-positive cocci.

1. Trypticase soy broth (TSB) (medium 1)
2. Heart infusion agar (HIA) (medium 29)
3. Hydrogen peroxide (H_2O_2)
4. Micrococcus glucose medium (medium 7)
5. Liquid paraffin (reagent 16)
6. Rabbit plasma (medium 53)
7. Phosphatase medium (medium 8)
8. Ammonia (0.880 specific gravity) (reagent 17)
9. Acetoin medium (medium 27)
10. 5-percent solution of alpha naphthol in absolute ethyl alcohol (reagent 3a)

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11. 40-percent aqueous potassium hydroxide (KOH) solution (reagent 3b)

12. Ammonium phosphate base medium for carbohydrate decomposition (medium 11)

13. 1-percent glucose medium (medium 12)

14. 10-percent bile broth (medium 13)

15. Blood agar with 40-percent bile (medium 14)

16. Kalina's enterococci medium (medium 15)

17. 0.2-percent inulin medium (medium 16)

18. 1-percent raffinose medium (medium 16)

2.3 METHODS

2.3.1 DETERMINATION OF CATALASE FORMATION

The following procedure is used in determining catalase formation.

1. An HIA slant (medium 29) is inoculated with two drops of the 24-hour TSB culture of Gram-positive cocci.

2. The slant culture is allowed to incubate at 308 K (35° C) for 18 hours.

3. Three drops of a 3-percent aqueous solution of H_2O_2 are added to the culture growing on the HIA (medium 29).

4. The immediate formation of gas bubbles in the H_2O_2 indicates the presence of catalase.

5. Cultures that produce catalase (catalase positive) are further identified as shown in section 2.3.2.

6. Cultures that do not produce catalase (catalase negative) are further identified as shown in section 2.3.3.

7. All Gram-positive cocci are identified according to figure 2-1.

2.3.2 IDENTIFICATION OF GRAM-POSITIVE CATALASE-POSITIVE COCCI

2.3.2.1 Determination of Glucose Decomposition

2.3.2.1.1 Anaerobic decomposition of glucose.- Anaerobic decomposition of glucose is determined by inoculating the 24-hour TSB culture being studied into deep tubes of *Micrococcus* glucose medium (medium 7) and by layering with liquid paraffin (reagent 16). The following procedure is used.

1. Before inoculation with the culture, the medium is heated in a boiling water bath for 15 minutes and then cooled in an ice bath (at ≈277 K (4° C)).

2. A Pasteur pipette is used to inoculate the medium with culture throughout the entire length of the tube. A column of liquid paraffin at least 15 millimeters high, previously autoclaved at 394 K (121° C) for 30 minutes, is layered on the surface of the medium to create conditions of reduced oxygen.

3. The culture is grown on the medium for 5 days at 308 K (35° C).

4. A complete color change from purple to yellow throughout the sealed tube is an indication of anaerobic fermentation of glucose.

5. Cultures that decompose glucose under these conditions of reduced oxygen (anaerobic) are considered to be staphylococci according to the recommendations of Baird-Parker (ref. 2-1).

2.3.2.1.2 Aerobic decomposition of glucose.- Aerobic decomposition of glucose is determined by inoculating the 24-hour TSB culture being studied into *Micrococcus* glucose medium (medium 7) without layering with liquid paraffin. The following procedure is used.

1. Before inoculating with a culture, the medium is heated in a boiling water bath for 15 minutes and then cooled in an ice bath (at ≈277 K (4° C)).

2. A Pasteur pipette is used to inoculate the medium with culture throughout the length of the tube.

3. The culture is grown in the medium for 5 days at 308 K (35° C).

4. Any change in color from purple to yellow in the aerobic tube is an indication of aerobic production of acid from glucose.

5. Cultures that decompose glucose under aerobic conditions and do not decompose glucose under anaerobic conditions are considered to be micrococci according to the recommendations of Baird-Parker (ref. 2-1). Also, some micrococci do not decompose glucose under any conditions.

2.3.2.2 Determination of Coagulase

The following procedure is used in determining coagulase.

1. Two drops of the 24-hour TSB culture are added to 0.5 cubic centimeter of rabbit plasma (medium 53 rehydrated with distilled water in a ratio of 1:3) in sterile test tubes.

2. The tubes are incubated at 308 K (35° C) and checked at 3 hours and at 24 hours for the presence of a clot.

3. The presence of a clot indicates that the culture under study has the capability of producing the enzyme coagulase.

4. Control tests are conducted with each set of samples.

   a. Control 1 - Rabbit plasma (0.5 cubic centimeter) plus two drops of sterilized broth (medium 1). No clot should occur.

   b. Control 2 - Rabbit plasma (0.5 cubic centimeter). No clot should occur.

   c. Control 3 - Rabbit plasma (0.5 cubic centimeter) plus two drops of broth (medium 1) containing *Staphylococcus aureus*. A clot should occur.

2.3.2.3 Determination of Phosphatase

The following procedure is used in determining phosphatase.

1. The 24-hour TSB culture is seeded on a nutrient medium containing 1-percent phenolphthalein phosphate (medium 8) using one of the following methods.

   a. Patch method - The bottom of a Petri dish containing phosphatase medium (medium 8) is marked into 10 to 15 sections. A small amount (less than one drop) of the 24-hour TSB culture is placed onto one section. Thus, 10 to 15 cultures may be tested for phosphatase production on one Petri dish of medium.

b. Alternate method - An alternate method consists of pipetting 1 cubic centimeter of medium into a sterile 3.7-milliliter (1 dram) vial. The vial containing the phosphatase medium is inoculated with one drop of the 24-hour TSB culture.

2. The Petri dishes or vials with inoculated cultures are incubated for 3 days at 303 K (30° C).

3. The presence of free phenolphthalein is determined by placing ammonia (0.88 specific gravity) (reagent 17) on the inner surface of an inverted Petri dish cover or on the inside of the vial cap.

4. The Petri dish bottom, with culture, is inverted and placed into the lid, with the lid underneath the bottom.

5. The vial is inverted in a test-tube rack so that the ammonia does not flow onto the surface of the medium.

6. Phosphatase-producing colonies turn dark pink when exposed to ammonia vapors.

2.3.2.4 Determination of Acetoin

The following procedure is used in determining acetoin.

1. The 24-hour TSB culture is inoculated into acetoin medium (medium 27).

2. The inoculated tubes are incubated for 7 days at 303 K (30° C) according to Baird-Parker (ref. 2-1).

3. To 1 cubic centimeter of incubated culture are added 0.6 cubic centimeter of a 5-percent solution of alpha naphthol in absolute ethyl alcohol (reagent 3a) and 0.2 cubic centimeter of a 40-percent aqueous solution of KOH (reagent 3b).

4. The contents of the tube are mixed thoroughly on a vortex mixer and allowed to stand at room temperature (≈293 K (20° C)) for 2 hours with the cap loosened.

5. A pink color indicates the presence of acetoin.

6. If a negative reaction occurs, the acetoin culture is reincubated for another 7 days at 303 K (30° C) and retested.

2.3.2.5 Determination of Carbohydrate Decomposition

The decomposition of arabinose, lactose, maltose, and mannitol is determined as follows.

1. The 24-hour TSB culture is inoculated onto the surface of ammonium phosphate base medium (medium 11) with corresponding carbohydrate.

2. The inoculated media in screwcap test tubes are incubated 7 days at 303 K (30° C) and are observed daily.

3. A change in color from purple to yellow is considered a positive indication of carbohydrate decomposition.

2.3.2.6 Identification of Cultures

Cultures are identified as follows.

1. Cultures that produce catalase and decompose glucose under anaerobic conditions are identified as staphylococci.

2. Catalase-positive cultures that do not decompose glucose under anaerobic conditions are identified as micrococci.

3. The two recognized species, Staphylococcus aureus and Staphylococcus epidermidis, are separated by their ability or inability to produce coagulase.

   a. Strains of Staphylococcus aureus produce coagulase.

   b. Strains of Staphylococcus epidermidis do not produce coagulase.

4. Gram-positive catalase-positive cocci are grouped according to the biochemical reactions outlined in table 2-I.

2.3.3 IDENTIFICATION OF GRAM-POSITIVE CATALASE-NEGATIVE COCCI

2.3.3.1 Alpha-Hemolytic Streptococci

Alpha-hemolytic streptococci are identified as follows.

1. Gram-positive catalase-negative cocci that produce alpha hemolysis on sheep blood agar are tested for bile resistance.

2. For these tests, 1 cubic centimeter of the 18-hour TSB culture of the strain being tested is inoculated into 5 cubic centimeters of 10-percent bile broth (medium 13) previously distributed into test tubes.

3. If, after incubation for 24 hours at 310 K (37° C), the broth becomes clear because of lysis of the microorganisms, the culture is classified as a pneumococcus (Streptococcus pneumoniae).

4. The alpha-hemolytic streptococci that are resistant to 10-percent bile broth are tested for resistance in a 40-percent bile medium.

   a. For this test, a 24-hour streptococci culture, grown in TSB broth, is inoculated onto the surface of sheep blood agar with 40-percent bovine bile (medium 14) using the "streak" method and a loop.

   b. If there is growth of colonies after 48 hours of incubation at 310 K (37° C), the microorganism is classified as enterococcus.

5. Enterococci grown on sheep blood agar with 40-percent bovine bile are seeded onto Kalina's medium for enterococci (medium 15).

   a. The cultures are incubated for 24 hours at 310 K (37° C).

   b. Enterococci colonies that produce dark-red coloring in this medium are classified as Streptococcus faecalis, whereas those that produce lilac color are Streptococcus faecium.

6. The alpha-hemolytic streptococci that are not classified as pneumococci or enterococci are implanted in test tubes with broth (medium 16) to which a 0.2-percent inulin solution has been added. These cultures are also inoculated into test tubes with broth (medium 16) to which a 1-percent solution of raffinose has been added.

   a. The cultures are incubated at 310 K (37° C).

   b. Streptococci may be separated into four biochemical groups on the basis of raffinose and inulin assimilation as shown in table 2-II.

2.3.3.2 Beta-Hemolytic Streptococci

Beta-hemolytic streptococci are identified as follows.

1. Gram-positive catalase-negative cocci that produce beta hemolysis on sheep blood agar and form chains are inoculated into broth with 1-percent glucose (medium 12).

2. These organisms are grouped serologically using the Lancefield method to establish group A characteristics.

2.3.3.2.1 Preparation of antigen extracts.- Antigen extracts are prepared in the following manner.

1. A 4-hour culture of streptococci is incubated on a compound scarlatinal broth (medium 65).

2. The prepared culture of streptococci is inoculated into 100-millimeter bottles filled with scarlatinal broth. The bottles with the culture are incubated at 310 K (37° C) for 24 hours.

3. After incubation, the microbial suspension is centrifuged, and the centrifugate is thrice washed with a 0.85-percent solution of sodium chloride (NaCl) and suspended in 2 cubic centimeters of a 1/20-normal solution of hydrochloric acid in a 0.85-percent solution of NaCl.

4. The bacterial suspension is placed into a boiling water bath for 15 minutes and periodically agitated.

5. The suspension is centrifuged once more, the supernatant fluid is decanted, and an indicator (bromthymol blue) is added to the precipitate.

6. The extract is neutralized with a 1-normal solution of sodium hydroxide. During the process of neutralization, the yellow color of the extract changes to green. The obtained extract contains the group-identifying substance of the hemolytic streptococcus and can be used in the ring precipitation test.

2.3.3.2.2 Ring precipitation test.- The ring precipitation test is performed in 30-millimeter-high tubes having a diameter of 2 to 2.5 millimeters, as follows.

1. The undiluted group serum is placed on the bottom of a vial; then, an undiluted antigen extract of the tested culture of the hemolytic streptococcus is added slowly with a capillary pipette as a layer above the serum.

2. The reaction is positive if within 15 to 20 minutes, at room temperature, a visible opalescent ring of precipitate develops at the interface of the two layers.

2.3.3.3 Gamma-Hemolytic Streptococci

Those Gram-positive catalase-negative microorganisms not showing alpha or beta hemolysis are classified as gamma hemolytic.

2.4 REFERENCE

2-1. Baird-Parker, A. C.: A Classification of Micrococci and Staphylococci Based on Physiological and Biochemical Tests. J. Gen. Microbiol., vol. 30, 1963, pp. 409-427.

TABLE 2-I.- BAIRD-PARKER DIAGNOSTIC SCHEME FOR CLASSIFYING

STAPHYLOCOCCI AND MICROCOCCI

Subgroups	Staphylococcus (a, b)						Micrococcus (b)							
	I	II	III	IV	V	VI	1	2	3	4	5	6	7	8
Pink pigment	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Acid from glucose														
Aerobic	+	+	+	+	+	+	+	+	+	+	+	+	±	±
Anaerobic	+	+	+	+	+	+	–	–	–	–	–	–	–	–
Coagulase	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Phosphatase	+	+	+	–	–	–	–	–	–	–	–	+	–	–
Acetoin	+	+	–	+	+	+	+	+	+	+	–	–	–	–
Acid from –														
Arabinose	–	–	–	–	–	–	–	–	–	+	v	+	–	–
Lactose	+[c]	v	v	–	+	v	–	+	v	+	+[c]	+	–	–
Maltose	+	+	–	v	+	v	v	+	+[c]	+	+[c]	+	–	±
Mannitol	+[c]	–	–	–	–	+	–	–	+	+	+[c]	+	–	–

[a]Staphylococcus subgroup I corresponds to Staphylococcus aureus, and subgroups II to VI correspond to Staphylococcus epidermidis.

[b]+, positive reaction; –, negative reaction; ±, negative or very weakly positive; v, variable reactions.

[c]Usually positive.

TABLE 2-II.- BIOCHEMICAL TYPES OF ALPHA-HEMOLYTIC STREPTC

Biochemical type	Designation	Decomposition
		Raffinose
1	*Streptococcus salivarius*	+
2	*Streptococcus mitis*	+
3	Group G streptococci	–
4	Streptococci grouped by specific sera	–

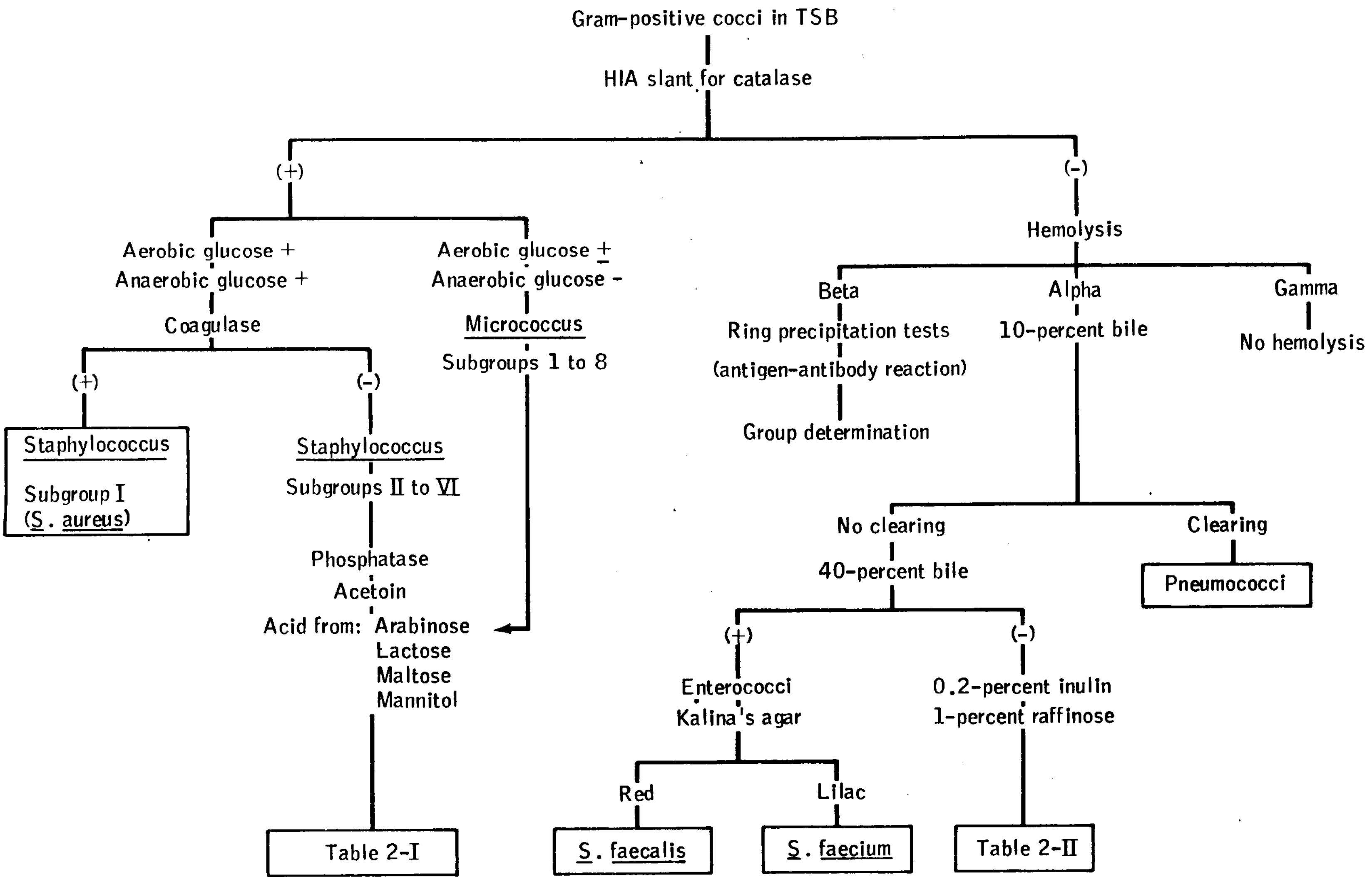

Figure 2-1.- Schematic diagram for identification of Gram-positive cocci.

3. IDENTIFICATION OF GRAM-NEGATIVE COCCI

By K. V. Zarubina*

3.1 PURPOSE

The purpose of this chapter is to describe the methods for sample collection and identification of Gram-negative cocci.

3.2 MATERIALS

The following materials are used.

1. Hottinger's agar containing serum (medium 66)
2. Hottinger's agar containing serum and ristomycin (medium 67)
3. Hottinger's agar containing serum and carbohydrates (medium 68)
4. One-percent aqueous solution of dimethyl-paraphenylenediamine hydrochloride
5. Phenol red indicator (reagent 28)

3.3 METHODS FOR IDENTIFYING GRAM-NEGATIVE COCCI

The following methods are used for identifying Gram-negative cocci.

3.3.1 MATERIALS USED FOR IDENTIFICATION

All varieties of colonies grown on agar which contains ristomycin and only those colonies of microbes grown on agar containing serum (medium 66) which have a smooth or rough, shiny or dull

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surface and are circular or oval shaped, transparent, opalescing in transmitted light, or nontransparent due to the presence of yellow or whitish pigment are used as materials for identification.

3.3.2 METHODS FOR ISOLATING A PURE CULTURE

The following procedure is used for isolating a pure culture.

1. Colonies possessing morphological properties given in section 3.3.1 are reinoculated with the use of a bacteriological loop from the surface of ristomycin- or serum-containing agar onto the surface of medium 66 distributed into Petri dishes, by the patch method or the streak method, onto a section of agar (six to nine sections on each Petri dish). The inoculum is incubated at 310 K (37° C) for 24 hours.

2. After the culture has been incubated, the nature of its growth is determined and Gram staining is performed.

3.3.3 OXIDASE REACTION

To determine whether the Gram-negative cocci belong to the genus *Neisseria*, the oxidase reaction is conducted as follows. A drop of freshly prepared solution of dimethyl-paraphenylenediamine hydrochloride is deposited onto the surface of the tested cultures grown on serum agar. A color change to pink indicates that the microorganisms belong to the genus *Neisseria*.

3.3.4 IDENTIFICATION OF MICROORGANISMS OF THE GENUS *NEISSERIA*

Microorganisms of the genus *Neisseria* are identified as follows.

1. With the use of a bacteriological loop, the colonies of microorganisms which produce a positive oxidase reaction are transferred onto Hottinger's agar (medium 64), onto Hottinger's agar containing horse serum (medium 66), and onto media containing carbohydrates (glucose, maltose, saccharose, levulose) (medium 68). In the case of a positive reaction to carbohydrates, the media change color from red to yellow.

2. After the culture has been grown for 24 hours at 310 K (37° C) on the previously mentioned media, *Neisseria* microorganisms are identified on the basis of the cultural, morphological, and biochemical properties given in table 3-I.

TABLE 3-I.- MORPHOLOGICAL, CULTURAL, AND BIOCHEMICAL PROPERTIES OF NEISSERIA

SPECIES MOST OFTEN FOUND IN THE NASOPHARYNX

Species of *Neisseria*	Formation of yellow pigment	Reaction to oxidase	Reaction to the cultivating conditions			Reaction to carbohydrates			
			Growth on serum agar at 310 K (37° C)	Growth on agar without serum at 310 K (37° C)	Growth on serum agar at 293 to 295 K (20° to 22° C)	Glucose	Maltose	Levulose	Saccharose
N. meningitidis	–[a]	+[b]	+	–	–	+	+	–	–
N. catarrhalis	–	+	+	+	±	–	–	–	–
N. sicca	–	+	+	±	±	+	+	+	+
N. perflava	+	+	+	–	–	+	+	+	+
N. flava	+	+	+	–[c]	–[c]	+	+	–	–
N. subflava	+	+	+	+[d]	+[d]	+	+	–	–
N. flavescens	+	+	+	±	±	–	–	–	–

[a]– indicates absence of a given property.
[b]+ indicates presence of a given property.
[c]Rarely +.
[d]Rarely –.

4. IDENTIFICATION OF SPORE-FORMING GRAM-POSITIVE RODS

By Theron O. Groves*

4.1 PURPOSE

The purpose of this chapter is to describe methods of identifying spore-forming rods.

4.2 MATERIALS

The following materials are used in identifying spore-forming rods.

1. Simmon's citrate agar (medium 38)
2. Gelatin (medium 46)
3. Nitrate broth (medium 39)
4. Methyl red/Voges-Proskauer (MR-VP) medium (medium 37)
5. Litmus milk (medium 48)
6. Heart infusion agar (HIA) slants (medium 29)
7. 7-percent sodium chloride (NaCl) broth (medium 30)
8. Tryptose glucose yeast (TGY) spore medium (medium 31)
9. Starch agar (medium 32)
10. Trypticase soy broth (TSB) (medium 1)
11. Ammonium phosphate base (medium 11)
12. 10-percent glucose, lactose, sucrose, mannitol, or xylose medium (medium 41)
13. Motility medium (medium 45)

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14. Nitrate reagent I (reagent 4a)

15. Nitrate reagent II (reagent 4b)

16. Zinc dust (reagent 14)

17. Voges-Proskauer (VP) reagents (reagent 3)

18. Gram's iodide solution (reagent 6b)

4.3 METHODS

4.3.1 DETERMINATION OF SPORE FORMATION

The spore formation is determined as follows.

1. The 24-hour TSB culture is streaked onto TGY spore medium (medium 31).

2. The cultures are incubated at 308 K (35° C), and Gram stains are made from each culture. Negative cultures are restained daily until spores are observed. If spores are not observed after 7 days, the microorganism is considered to be not a spore former.

4.3.2 IDENTIFICATION OF SPORE-FORMING GRAM-POSITIVE RODS

Identification is based on the biochemical reactions obtained from the following series of media.

4.3.2.1 Simmon's Citrate Agar (Medium 38)

Simmon's citrate agar (medium 38) is used to detect the ability of the microorganism to utilize citrate as the sole source of carbon. The following procedure is used.

1. The surface of the slant is inoculated (using a Pasteur pipette) with a very small amount of the TSB culture.

2. A positive test is indicated by a color change from green to blue after incubation for 24 hours at 308 K (35° C) or by visible growth after 48 hours.

4.3.2.2 Gelatin (Medium 46)

Gelatin (medium 46) is used for the detection of the enzyme gelatinase by means of the following procedure.

1. The medium is inoculated with two drops of the TSB culture and incubated at room temperature (≈293 K (20° C)) for 7 days.

2. After incubation, the cultures are refrigerated at ≈277 K (4° C) for 30 minutes.

3. A positive test is indicated by failure of the gelatin to solidify after refrigeration. A negative test is indicated by a solidified gelatin.

4.3.2.3 Nitrate Broth (Medium 39)

Nitrate broth (medium 39) is used to test the ability of the microorganism to reduce nitrate to nitrite or to free nitrogen gas. The following procedure is used.

1. The medium is inoculated with two drops of the TSB culture and incubated at 308 K (35° C).

2. After 48 hours of incubation, three drops of nitrate reagent I (reagent 4a) and three drops of nitrate reagent II (reagent 4b) are added.

3. A pink-red color indicates the presence of nitrite, produced by microbial reduction of the nitrate. If no pink or red color appears, nitrite is absent.

4. If nitrite is absent, a few grains of zinc dust (reagent 14) are added to the culture tube. The appearance of a red color indicates the presence of nitrate and the test is considered negative. If no red color appears, nitrate is absent and the microorganism is assumed to have reduced the nitrate to free nitrogen. This is recorded as a positive test for nitrate reduction.

4.3.2.4 MR-VP Medium (Medium 37)

The MR-VP medium (medium 37) is used to test the ability of the microorganism to produce acetyl-methyl-carbinol from glucose as follows.

1. The medium is inoculated with two drops of the TSB culture and incubated at 308 K (35° C) for 48 hours.

2. To 1 cubic centimeter of culture, add 0.6 cubic centimeter of a 5-percent solution of alpha naphthol in absolute alcohol (reagent 3a) and 0.2 cubic centimeter of 40-percent potassium hydroxide (KOH) solution (reagent 3b). The contents of the tube are mixed on a vortex mixer and allowed to stand at room temperature (≈293 K (20° C)) for 2 hours with the cap loosened.

3. A positive VP test (acetyl-methyl-carbinol from glucose) is indicated by a red color. A negative VP reaction is indicated by a yellow color.

4.3.2.5 Litmus Milk (Medium 48)

Litmus milk (medium 48) is used to determine the action of the microorganism on milk in the following manner.

1. The medium is inoculated with two drops of the TSB culture and incubated at 308 K (35° C).

2. The tube is read daily for 7 days.

3. The reactions that may occur include clot formation, peptonization (digestion), and/or acid production.

   a. Presence of a clot is recorded as a "C."

   b. An area of clearing at the top of the medium is indicative of peptonization and is recorded as a "P."

   c. Acid production is indicated by a change in the indicator from blue to pink and is recorded as an "A."

4.3.2.6 Ammonium Phosphate Base Medium (Medium 11)

Ammonium phosphate base medium (medium 11) is used to determine carbohydrate decomposition as follows.

1. The following carbohydrates (0.5 percent in ammonium phosphate base (medium 11)) are each inoculated with two drops of the TSB culture: glucose, lactose, sucrose, mannitol, and xylose.

2. The media are incubated at 308 K (35° C) and observed daily for 7 days.

3. Acid production resulting from carbohydrate decomposition is indicated by a color change from purple to yellow and is recorded as a positive reaction. A negative test is indicated by no change in color.

4.3.2.7 Seven-Percent NaCl Broth (Medium 30)

The 7-percent NaCl broth (medium 30) is used to determine which microorganism will grow in the presence of a 7-percent salt concentration as follows.

1. One drop of the TSB culture is inoculated into the medium and incubated for 7 days at 308 K (35° C).

2. A positive reaction is indicated by a turbid culture after incubation. Care must be taken so that the inoculum will not be confused with growth.

4.3.2.8 Starch Agar (Medium 32)

By means of the following procedure, starch agar plates are used to determine whether the microorganism hydrolyzes starch.

1. The TSB culture is streaked onto the plate and incubated for 7 days at 308 K (35° C).

2. After incubation, two drops of Gram's iodine solution (reagent 6b) are placed on the growth.

3. A positive reaction is indicated by a yellow coloration of the medium. A purple color indicates a negative reaction.

4.3.2.9 Trypticase Soy Broth (Medium 1)

Trypticase soy broth (medium 1) is used as follows.

1. The TSB is inoculated with one drop of the TSB culture and incubated at 328 K (55° C) for 7 days.

2. The tube is read daily for 7 days for the presence or absence of growth at 328 K (55° C).

4.3.2.10 Motility Medium (Medium 45)

The motility medium (medium 45) is used to determine whether the Bacillus is motile or nonmotile as follows.

1. The medium is inoculated with two drops of the TSB culture by stabbing to two-thirds the depth of the medium using a Pasteur pipette.

2. After incubation at 308 K (35° C) for 24 hours, motile cultures show diffuse growth or turbidity away from the line of inoculation.

3. Microorganisms that produce hydrogen sulfide darken the medium.

4.4 IDENTIFICATION OF BACILLUS SPECIES

Biochemical reactions for the identification of *Bacillus* species are shown in table 4-I.

TABLE 4-I.- BIOCHEMICAL REACTIONS FOR THE IDENTIFICATION OF BACILLUS SPECIES

Species	Test[a]														
	Catalase	Gelatin	Starch	Nitrate	VP	Citrate	7% NaCl	Milk	Glucose	Lactose	Xylose	Sucrose	Mannitol	Growth 55C	Motility
B. alvei	+	+	+	-	+	-	-	ACP	+	-	-	v	-	-	+
B. anthracis	+	+	+	+	+	v	+	CP	+	-	-	+	-	-	-
B. badius	+	+	-	+	-	-	-	v	-	-	-	-	-	-	+
B. brevis	+	+	-	v	-	v	-	Alk	v	-	-	+	v	+	+
B. cereus	+	+	+	v	+	v	+	P	+	v	-	v	-	-	+
B. cereus var. mycoides	+	+	+	v	+	v	v	v	+	v	-	v	-	-	-
B. circulans	+	v	+	v	-	v	v	A	+	0	+	+	+	-	+
B. coagulans	+	v	+	v	+	v	-	A	v	v	v	v	v	+	+
B. firmus	+	+	+	+	-	-	+	v	+	0	v	0	+	-	+
B. laterosporus	+	+	-	+	-	-	-	P	+	0	-	-	+	-	+
B. larvae	-	+	-	+	-	-	-	v	+	-	-	-	v	-	v
B. lentimorbus	-	-	-	-	-	-	0	v	+	0	-	0	-	-	-
B. lentus	+	-	+	-	-	+	-	NC	+	0	+	0	+	-	+
B. licheniformis	+	+	+	+	+	v	+	P	+	v	+	+	+	+	+
B. macerans	+	v	+	+	-	-	-	AG	+	+	+	+	+	-	+
B. megaterium	+	+	+	v	-	+	v	P	+	v	v	+	+	-	v
B. pantothenticus	+	+	+	v	-	v	+	v	+	0	-	+	-	-	+
B. pasteurii	+	v	-	v	-	-	+	0	-	-	-	-	-	-	+
B. polymyxa	+	+	+	+	+	v	-	AC	+	+	+	0	+	-	+
B. popilliae	-	-	-	-	-	-	0	v	+	0	-	0	-	-	v
B. pulvifaciens	-	+	-	+	-	-	-	v	+	0	-	0	+	-	+
B. pumilus	+	+	-	-	+	+	+	P	+	-	+	+	+	-	+
B. sphaericus	+	v	-	-	-	v	v	v	-	-	-	-	-	-	+
B. stearothermophilus	+	v	+	v	-	v	-	v	v	0	v	0	v	+	+
B. subtilis	+	+	+	+	+	v	+	v	+	-	+	+	+	v	v

[a]+, positive; -, negative; v, variable; A, acid; NC, no change; AG, acid-gas; C, clot; P, peptonization; Alk, alkaline; and 0, results not recorded.

5. IDENTIFICATION OF NON-SPORE-FORMING GRAM-POSITIVE RODS

By V. P. Gorshkov*

5.1 PURPOSE

The purpose of this chapter is to describe methods for id
ing non-spore-forming Gram-positive rods.

5.2 MATERIALS

The following materials are used.

1. Nitrate broth (medium 39)
2. Medium containing sugars (medium 41)
3. Broth containing urea (medium 54)
4. Volutin granule stain (reagent 13)

5.3 METHODS

The following methods are used.

5.3.1 DETECTION OF VOLUTIN GRANULES

Volutin granules are detected as follows.

1. A smear of the microorganism, grown on a solid nutrie
dium (medium 2 or 3), is prepared by emulsifying the
microorganism in a drop of water on a microscope slid
2. The preparation is dried and fixed by passing the sli
through the flame of a burner.

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3. The slide is flooded with volutin granule stain (reagent 13) and heated to the steaming point.

4. After the preparation has been washed and dried, the bacteria appear to be light red in color; the volutin granules, dark blue.

5.3.2 CLASSIFICATION OF DIPHTHEROIDS

Further identification is based on biochemical reactions shown in table 5-I (ref. 5-1).

5.3.2.1 Reduction of Nitrates

The ability of the cultures to reduce nitrates into nitrites can be determined with the help of procedures described in section 4.3.2.3 of chapter 4.

5.3.2.2 Fermentation of Carbohydrates

The ability to ferment carbohydrates is determined for glucose, saccharose, and maltose as follows.

1. Several drops of 1-day-old broth culture are pipetted into each tube of medium 41 containing one of the carbohydrates.

2. The cultures are incubated at 310 K (37° C) for 24 hours. A change of color of the medium to yellow indicates carbohydrate fermentation.

5.3.2.3 Production of Urease

Urease production is determined in the following manner.

1. The 24-hour broth culture being studied is inoculated into medium 54.

2. The inoculated medium is incubated at 310 K (37° C) for 24 hours.

3. A change of color in the medium to bright red indicates the presence of the enzyme urease.

5.4 REFERENCE

5-1. Evans, N. M.: The Classification of Aerobic Diphtheroids From Human Skin. British J. Dermatol., vol. 80, Feb. 1968, pp. 81-83.

TABLE 5-I.- CLASSIFICATION OF DIPHTHEROIDS ACCORDING TO N. M. EVANS

[From ref. 5-1]

Group	Nitrate reduction	Carbohydrate fermentation			Urease production	Species of microorganism
		Glucose	Saccharose	Maltose		
A	+	+	+	+/-	-	Corynebacterium xerosis
B	+	-	-	-	+/-	Corynebacterium pseudodiphtheriticum
C	+	+	-	+	-	--
D	-	+	+	+		Corynebacterium striatum
		+	+	-	-	
		+	-	-		
E	-	+	+/-	+	+	Corynebacterium ovis or ulcerans
F	-	+	-	+	-	--
G	-	-	-	-	-	--

6. IDENTIFICATION OF LACTOSE-POSITIVE GRAM-NEGATIVE RODS

By G. P. Kalina* and N. D. Startseva*

6.1 PURPOSE

The purpose of this chapter is to describe methods for identifying lactose-positive Gram-negative rods.

6.2 MATERIALS

The following materials are used.

1. Bromthymol blue (BTB) medium (medium 6)
2. Giss medium with 1-percent lactose (medium 19)
3. Two-layer complex medium for detecting mannitol and glucose fermentation and the production of gas from glucose (medium 22)
4. Liquid complex medium for determining glucose oxidation, acetoin production, and urease activity (medium 24)
5. Two-layer complex medium for detecting motility, lysine decarboxylase, and indole production (medium 21)
6. Three-layer complex medium for detecting dulcite fermentation, hydrogen sulfide (H_2S) production, and sodium citrate decomposition (medium 23)
7. Two-layer complex medium for determining gelatinous activity, culture purity, and pigment production (medium 25)
8. Cytochrome oxidase reagent (medium 20)
9. Kovac's reagent for determination of indole production (reagent 1)

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10. Endo medium (medium 28)

6.3 METHODS

6.3.1 DETERMINATION OF LACTOSE DECOMPOSITION

Lactose decomposition is determined as follows.

1. Each isolate from the BTB plate is transferred to a tube of Giss medium with 1-percent lactose (medium 19) and streaked on an Endo plate (medium 28).

2. After 24 hours incubation at 310 K (37° C), the Giss medium is examined for lactose fermentation.

3. A change in the color of Giss medium from blue to yellow indicates a positive test.

6.3.2 IDENTIFICATION OF LACTOSE-POSITIVE GRAM-NEGATIVE RODS

Identification is based on the biochemical reactions obtained from the following series of media.

6.3.2.1 Medium 28

Endo medium (medium 28) is used in the cytochrome oxidase test as follows.

1. A colony from Endo medium is placed on filter paper and impregnated with reagent (medium 20) using a platinum loop.

2. A rapid development of blue color (within 10 seconds) indicates a positive reaction.

6.3.2.2 Medium 21

Medium 21 is a two-layer medium for detecting motility, lysine decarboxylase, and indole production as follows.

1. The medium is inoculated by stabbing through the upper layer into the semisolid layer.

2. After 24 hours incubation at 310 K (37° C), growth throughout the entire surface indicates motility. Lysine decarboxylation is indicated by a blue-green color in the upper layer.

Indole production is indicated by the development of a dark-red color after several drops of Kovac's reagent are deposited on the surface of the agar following a second 24-hour incubation period.

6.3.2.3 Medium 22

Medium 22 is a two-layer complex medium for determining mannitol and glucose decomposition and formation of gas from glucose as follows.

1. The medium is inoculated by stabbing the butt and streaking the surface of the slant.

2. After 24 hours incubation at 310 K (37° C), glucose decomposition is indicated by a yellow butt and gas production is indicated by the formation of gas bubbles in the butt. Mannitol decomposition is indicated by a yellow slant.

6.3.2.4 Medium 23

Medium 23 is a three-layer complex medium for determining dulcite fermentation, hydrogen sulfide production, and sodium citrate utilization as follows.

1. The medium is inoculated by stabbing the butt and streaking the surface of the slant.

2. After 24 hours incubation at 310 K (37° C), dulcite fermentation is indicated by yellowing of the lower column. Hydrogen sulfide production is indicated by the formation of a black ring in the middle layer. Sodium citrate utilization is indicated by a blue slant.

6.3.2.5 Medium 24

Medium 24 is a complex liquid medium for determining glucose oxidation, acetoin production, and urease activity as follows.

1. The medium is inoculated with a 1-day-old agar culture of Gram-negative bacteria.

2. After 16 to 18 hours incubation at 310 K (37° C), glucose oxidation is indicated by a color change in the medium from blue to yellow. After 0.2 cubic centimeter of 50-percent urea solution is added to the medium, the tube is placed in a 310-K (37° C) water bath for 3 to 4 hours.

a. Urease activity is indicated by the development of a dark-green, green-blue, or blue color. If the test is negative, the tube is reincubated for 24 hours.

b. After the urease test is completed, 0.4 cubic centimeter of a 40-percent aqueous potassium hydroxide (KOH) solution (reagent 3b) and 0.6 cubic centimeter of a 5-percent solution of alpha naphthol in alcohol (reagent 3a) are added. The appearance of a lilac-colored ring in 10 to 30 minutes indicates a positive acetoin reaction.

6.3.2.6 Medium 25

Medium 25 is a two-layer complex medium for determining gelatinase activity and pigment production in the following manner.

1. The medium is inoculated by streaking the surface of the plate.

2. After 24 hours incubation at 310 K (37° C), gelatinase activity is determined by the formation of zones of turbidity around the streak. Pigment production is indicated by development of color of the growth on the plate.

6.4 DIFFERENTIATION OF ENTEROBACTERIACEAE

On completion of the studies, the colors resulting from the tests that yield a positive reaction are summed up. Table 6-I (G. P. Kalina) gives the basic features of the representatives of the family of lactose-positive enterobacteria. Each feature is coded with a number from 2 to 2048 as follows: mannitol, 2; gas in glucose, 4; motility, 8; lysine, 16; citrate, 32; dulcite, 64; urease, 128; H_2S, 256; indole, 512; gelatin, 1024; and acetoin, 2048. The codes for each combination (from 2 to 3830) are presented in table 6-II and for convenience are included in table 6-I, where the basic codes characterizing the typical set of features are underlined.

TABLE 6-I.- DIFFERENTIATION OF THE BASIC GENERA AND SPECIES OF THE ENTEROBACTERIACEAE FAMILY

Genera and species	Reactions (a)											Codes (b)
	2	4	8	16	32	64	128	256	512	1024	2048	
Arizona	+	+	+	+	+	-	-	±	∓	±	-	62, 318, 830, 1086, 1342, 1598, 1854
Citrobacter	+	+	+	-	+	∓	±	±	∓	-	-	46, 174, 238, 302, 366, 430, 494, 686, 814, 942
Enterobacter cloacae	+	+	+	-	+	-	±	-	-	±	+	2094, 2222, 3118, 3246
Enterobacter aerogenes	+	+	+	+	+	-	∓	-	-	±	+	2110, 2238, 3134, 3262
Enterobacter liquefaciens	+	+	+	±	+	-	∓	-	-	+	∓	1070, 1086, 1198, 1214, 3118, 3134, 3262
Enterobacter hafnia	+	+	+	+	∓	-	-	-	-	-	∓	30, 62, 2078, 2110
Escherichia coli	+	±	+	±	-	±	-	-	±	-	-	30, 78, 94, 526, 538, 542, 586, 590, 602, 606
Klebsiella aerogenes	+	+	-	+	+	∓	+	-	-	-	+	2230, 2294
Klebsiella edwardsii	+	-	-	+	±	-	+	-	-	-	+	2194, 2226
Klebsiella oxytoca	+	+	-	+	+	±	+	-	+	+	±	1718, 1782, 3766, 3830
Klebsiella pneumoniae	+	+	-	+	+	+	+	-	-	-	-	246
Klebsiella ozaenae	+	±	-	∓	±	-	∓	-	-	-	-	2, 6, 22, 34, 38, 50, 54, 134, 162, 166, 182
Klebsiella rhinoscleromatis	-	-	-	-	-	-	-	-	-	-	-	2
Serratia	+	∓	+	+	+	-	∓	∓	-	+	±	1082, 1086, 1210, 1338, 3130, 3134, 3258, 3262, 3386, 3390, 3514

[a]+, positive indication; -, negative indication; ±, more frequently positive; ∓, more frequently negative.
[b]The codes are given in accordance with table 6-II. Underscore indicates the basic code characterizing the typical set of features.

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TABLE 6-II.- CODING OF GENERA AND SPECIES OF ENTEROBACTERIACEAE FAMILY AND CLASS I AND II VARIANTS ON THE BASIS OF 11 SELECTED REACTIONS

Genera and species	Variant (a)	Reactions (b)											Code
		2	4	8	16	32	64	128	256	512	1024	2048	
Klebsiella ozaenae	II	+	(-)	-	-	(-)	-	-	-	-	-	-	2
Klebsiella rhinoscleromatis	T	-	-	-	-	-	-	-	-	-	-	-	2
Klebsiella ozaenae	I	+	+	-	-	(-)	-	-	-	-	-	-	6
Klebsiella ozaenae	II	+	+	-	(+)	(-)	-	-	-	-	-	-	22
Enterobacter hafnia	T	+	+	+	+	-	-	-	-	-	-	-	30
Escherichia coli	II	+	+	+	+	-	(-)	-	-	(-)	-	-	30
Klebsiella ozaenae	I	+	(-)	-	-	+	-	-	-	-	-	-	34
Klebsiella ozaenae	T	+	+	-	-	+	-	-	-	-	-	-	38
Citrobacter	II	+	+	+	-	+	-	(-)	(-)	-	-	-	46
Klebsiella ozaenae	II	+	(-)	-	(+)	+	-	-	-	-	-	-	50
Klebsiella ozaenae	I	+	+	-	(+)	+	-	-	-	-	-	-	54
Enterobacter hafnia	I	+	+	+	+	(+)	-	-	-	-	-	-	62
Arizona	II	+	+	+	+	+	-	-	(-)	-	(-)	-	62
Escherichia coli	II	+	+	+	(-)	-	+	-	-	(-)	-	-	78
Escherichia coli	I	+	+	+	+	-	+	-	-	(-)	-	-	94
Klebsiella ozaenae	II	+	+	-	-	(-)	-	(+)	-	-	-	-	134
Klebsiella ozaenae	II	+	(-)	-	-	+	-	(+)	-	-	-	-	162
Klebsiella ozaenae	I	+	+	-	-	+	-	(+)	-	-	-	-	166
Citrobacter	I	+	+	+	-	+	-	+	(-)	-	-	-	174
Klebsiella ozaenae	II	+	+	-	(+)	+	-	(+)	-	-	-	-	182
Citrobacter	II	+	+	+	-	+	(+)	+	(-)	-	-	-	238
Klebsiella pneumoniae	T	+	+	-	+	+	+	+	-	-	-	-	246
Citrobacter	I	+	+	+	-	+	-	(-)	+	-	-	-	302
Arizona	I	+	+	+	+	+	-	-	+	-	(-)	-	318
Citrobacter	II	+	+	+	-	+	(+)	(-)	+	-	-	-	366

[a]T, typical set of reactions; I, variant with deviation in one reaction; II, variant with deviation in two reactions.
[b]Parentheses indicate reactions that have changed for the given variant.

TABLE 6-II.- Continued

Genera and species	Variant (a)	Reactions (b)											Code
		2	4	8	16	32	64	128	256	512	1024	2048	
Citrobacter	T	+	+	+	-	+	-	+	+	-	-	-	430
Citrobacter	I	+	+	+	-	+	(+)	+	+	-	-	-	494
Escherichia coli	II	+	+	+	(-)	-	(-)	-	-	+	-	-	526
Escherichia coli	II	+	(-)	+	+	-	(-)	-	-	+	-	-	538
Escherichia coli	I	+	+	+	+	-	(-)	-	-	+	-	-	542
Escherichia coli	II	+	(-)	+	(-)	-	+	-	-	+	-	-	586
Escherichia coli	I	+	+	+	(-)	-	+	-	-	+	-	-	590
Escherichia coli	I	+	(-)	+	+	-	+	-	-	+	-	-	602
Escherichia coli	T	+	+	+	+	-	+	-	-	+	-	-	606
Citrobacter	II	+	+	+	-	+	-	+	(-)	(+)	-	-	686
Citrobacter	II	+	+	+	-	+	-	(-)	+	(+)	-	-	814
Arizona	II	+	+	+	+	+	-	-	+	(+)	(-)	-	830
Citrobacter	I	+	+	+	-	+	-	+	+	(+)	-	-	942
Enterobacter liquefaciens	I	+	+	+	(-)	+	-	-	-	-	+	-	1070
Serratia	I	+	-	+	+	+	-	-	-	-	+	(-)	1082
Enterobacter liquefaciens	T	+	+	+	+	+	-	-	-	-	+	-	1086
Arizona	I	+	+	+	+	+	-	-	(-)	-	+	-	1086
Serratia	II	+	(+)	+	+	+	-	-	-	-	+	(-)	1086
Enterobacter liquefaciens	II	+	+	+	(-)	+	-	(+)	-	-	+	-	1198
Serratia	II	+	-	+	+	+	-	(+)	-	-	+	(-)	1210
Enterobacter liquefaciens	I	+	+	+	+	+	[illegible]	(+)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

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TABLE 6-II.- Concluded

Genera and species	Variant (a)	Reactions (b)											Code
		2	4	8	16	32	64	128	256	512	1024	2048	
Arizona	I	+	+	+	+	+	-	-	+	(+)	+	-	1854
Enterobacter hafnia	I	+	+	+	+	+	-	-	-	-	-	(+)	2078
Enterobacter cloacae	II	+	+	+	-	+	-	(-)	-	-	(-)	+	2094
Enterobacter aerogenes	I	+	+	+	+	+	-	-	-	-	(-)	+	2110
Enterobacter hafnia	II	+	+	+	+	(+)	-	-	-	-	-	(+)	2110
Klebsiella edwardsii	I	+	-	-	+	(-)	-	+	-	-	-	+	2194
Enterobacter cloacae	I	+	+	+	-	+	-	+	-	-	(-)	+	2222
Klebsiella edwardsii	T	+	-	-	+	+	-	+	-	-	-	+	2226
Klebsiella aerogenes	T	+	+	-	+	+	-	+	-	-	-	+	2230
Enterobacter aerogenes	II	+	+	+	+	+	-	(+)	-	-	(-)	+	2238
Klebsiella aerogenes	I	+	+	-	+	+	(+)	+	-	-	-	+	2294
Enterobacter cloacae	I	+	+	+	-	+	-	(-)	-	-	+	+	3118
Enterobacter liquefaciens	II	+	+	+	(-)	+	-	-	-	-	+	(+)	3118
Serratia	T	+	-	+	+	+	-	-	-	-	+	+	3130
Enterobacter aerogenes	T	+	+	+	+	+	-	-	-	-	+	+	3134
Enterobacter liquefaciens	I	+	+	+	+	+	-	-	-	-	+	(+)	3134
Serratia	I	+	(+)	+	+	+	-	-	-	-	+	+	3134
Enterobacter cloacae	T	+	+	+	-	+	-	+	-	-	+	+	3246
Serratia	I	+	-	+	+	+	-	(+)	-	-	+	+	3258
Enterobacter aerogenes	I	+	+	+	+	+	-	(+)	-	-	+	+	3262
Enterobacter liquefaciens	II	+	+	+	+	+	-	(+)	-	-	+	(+)	3262
Serratia	II	+	(+)	+	+	+	-	(+)	-	-	+	+	3262
Serratia	I	+	-	+	+	+	-	-	(+)	-	+	+	3386
Serratia	II	+	(+)	+	+	+	-	-	(+)	-	+	+	3390
Serratia	II	+	-	+	+	+	-	(+)	(+)	-	+	+	3514
Klebsiella oxytoca	I	+	+	-	+	+	(-)	+	-	+	+	+	3766
Klebsiella oxytoca	T	+	+	-	+	+	+	+	-	+	+	+	3830

[a]T, typical set of reactions; I, variant with deviation in one reaction; II, variant with deviation in two reactions.
[b]Parentheses indicate reactions that have changed for the given variant.

7. IDENTIFICATION OF LACTOSE-NEGATIVE GRAM-NEGATIVE RODS

By Kathryn D. Kropp* and Theron O. Groves*

7.1 PURPOSE

The purpose of this chapter is to describe methods for identifying lactose-negative Gram-negative rods.

7.2 MATERIALS

The following materials are used.

1. Triple-sugar iron agar (TSI) (medium 35)
2. 1-percent tryptone medium (medium 36)
3. Methyl red/Voges-Proskauer (MR-VP) medium (medium 37)
4. Simmon's citrate agar (medium 38)
5. Nitrate broth (medium 39)
6. Urea agar slant (medium 44)
7. Purple broth base (medium 40)
8. Sugars, 1 percent, in purple broth base (medium 41)
9. Decarboxylase medium base (medium 47)
10. Decarboxylase medium with arginine, ornithine, or lysine (medium 47)
11. Oxidation-fermentation (OF) medium base (medium 34)
12. Gelatin (medium 46)
13. Motility medium (medium 45)

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14. 10-percent glucose slant (medium 42)

15. 10-percent lactose slant (medium 43)

16. OF medium with glucose (medium 41)

17. Litmus milk (medium 48)

18. Nitrate test reagents (reagent 4)

19. Methyl red (MR) test reagent (reagent 2)

20. Voges-Proskauer (VP) test reagents (reagent 3)

21. Indole test reagent (reagent 1)

22. Oxidase test reagent (reagent 7)

23. Heart infusion agar (HIA) slant (medium 29)

24. Zinc dust (reagent 14)

25. MacConkey agar (medium 33)

26. Chocolate bacitracin agar (medium 51)

27. Rabbit blood agar (medium 52)

28. Trypticase soy broth (TSB) (medium 1)

29. Trypticase soy agar (TSA) (medium 2)

30. TSB with Fildes enrichment (medium 71)

31. Growth-factor strips (reagent 15)

32. Liquid paraffin (reagent 16)

7.3 METHODS

7.3.1 DETERMINATION OF LACTOSE DECOMPOSITION

Lactose decomposition is determined as follows.

1. The 24-hour TSB culture of a Gram-negative rod is streaked for isolation onto MacConkey agar (medium 33) and inoculated into 1-percent lactose broth (medium 41).

2. The seeded media are incubated at 308 K (35° C) for 48 hours.

3. After incubation, the media are examined for lactose fermentation.

   a. Rods forming pink to red colonies on MacConkey agar and acid production in 1-percent lactose broth are considered positive for lactose decomposition and are discarded.

   b. Rods forming colorless colonies on MacConkey agar and having no acid production in 1-percent lactose broth are considered negative for lactose decomposition.

7.3.2 IDENTIFICATION OF LACTOSE-NEGATIVE GRAM-NEGATIVE RODS

The major division in lactose-negative Gram-negative rods is based on the method of glucose decomposition, on growth or no growth on MacConkey agar, and on the oxidase reaction. Species identification is based on the biochemical reactions obtained from the following series of media.

7.3.2.1 Oxidation-Fermentation Medium Containing Glucose (Medium 41)

The OF medium with glucose (medium 41) illustrates the method of glucose decomposition as follows.

1. Two tubes of media are inoculated with two drops each of the TSB culture by stabbing the medium (to a depth of 1.5 centimeters) with a sterile Pasteur pipette.

2. One tube is overlayed with sterile liquid paraffin to a depth of 1.5 centimeters. Both tubes are incubated at 308 K (35° C) for 4 days.

3. The production of acid from glucose will change the color of the medium from blue to yellow.

   a. The fermentation of glucose is indicated by acid production in both the overlay tube and the open tube.

   b. The aerobic reduction of glucose (oxidation) is indicated by acid production in the open tube only.

   c. A nonoxidative reaction is indicated by no utilization of glucose in either tube.

7.3.2.2 MacConkey Agar (Medium 33)

Medium 33 is used to test the ability of the microorganism to grow on MacConkey agar as follows.

1. A loopful of the TSB culture is streaked onto MacConkey agar for isolation using a sterile inoculating loop.

2. The plate is incubated at 308 K (35° C) for 48 hours and then examined for growth.

3. If growth is observed, the test is considered to be positive. If no growth is observed, the test is considered negative.

7.3.2.3 Heart Infusion Agar (Medium 29)

Heart infusion agar (medium 29) is used to determine the production of cytochrome oxidase as follows.

1. The HIA slant is inoculated with two drops of the TSB culture and incubated for 24 hours at 308 K (35° C).

2. A colony is transferred to a paper disk impregnated with para-aminodimethylaniline reagent (reagent 7) by using a sterile loop or Pasteur pipette. A rapid development of a dark-purple color indicates a positive test.

7.3.2.4 Triple-Sugar Iron Agar Slant (Medium 35)

Medium 35 indicates the ability of a microorganism to produce acid from the decomposition of lactose, sucrose, and/or glucose, and to produce gas and/or hydrogen sulfide. The following procedure is used.

1. The medium is inoculated with the TSB culture by stabbing the butt and streaking the surface of the slant with a sterile Pasteur pipette.

2. After 24-hour incubation at 308 K (35° C), acid from glucose is indicated by an alkaline slant and an acid butt. Acid from lactose and sucrose is indicated by an acid slant. If acid is produced from all three sugars, both butt and slant are acid. An acid reaction is indicated by a color change from red to yellow. Hydrogen sulfide production is indicated by blackening of the medium.

7.3.2.5 One-Percent Tryptone Medium (Medium 36)

Medium 36 is used to detect the production of indole as follows.

1. The medium is inoculated with two drops of the TSB culture and incubated at 308 K (35° C).

2. After a 48-hour incubation, three drops of Kovac's reagent (reagent 1) are added to the culture. A dark-red (eosin) color in the surface layer indicates a positive test; yellow indicates a negative test.

7.3.2.6 MR-VP Medium (Medium 37)

Medium 37 is used to test the ability of the microorganism to produce acid and/or acetyl-methyl-carbinol from glucose. The MR test evaluates acid production from glucose, and the VP test evaluates acetyl-methyl-carbinol production from glucose. The following procedure is used.

1. Two tubes of medium are inoculated with two drops each of the TSB culture and incubated at 308 K (35° C) for 48 hours.

2. For the MR test (acid from glucose), three drops of methyl red solution (reagent 2) are added to 1 cubic centimeter of the culture.

3. A positive reaction is indicated by a distinct red color (acid); a negative reaction is indicated by a yellow color.

4. For the VP test (acetyl-methyl-carbinol from glucose), 0.6 cubic centimeter of a 5-percent solution of alpha naphthol in absolute alcohol (reagent 3a) and 0.2 cubic centimeter of a 40-percent aqueous potassium hydroxide (KOH) solution (reagent 3b) are added to 1 cubic centimeter of culture. The contents of the tube are mixed on a vortex mixer and allowed to stand at room temperature (≈293 K (20° C)) for 2 hours with the cap loosened.

5. A positive test is indicated by a red color; a negative reaction is indicated by a yellow color.

7.3.2.7 Simmon's Citrate Agar (Medium 38)

Simmon's citrate agar (medium 38) is used to detect the ability of the microorganism to utilize citrate as the sole source of carbon. The following procedure is used.

1. The surface of the slant is inoculated with a very small amount of TSB culture using a Pasteur pipette.

2. A positive test is indicated by a color change from green to blue after a 24-hour incubation at 308 K (35° C) or by visible growth after 48 hours.

7.3.2.8 Nitrate Broth (Medium 39)

Medium 39 is used to test the ability of the microorganism to reduce nitrate to nitrite or to free nitrogen gas. The following procedure is used.

1. The medium is inoculated with two drops of the TSB culture and incubated at 308 K (35° C).

2. After 48 hours of incubation, three drops of nitrate reagent I (reagent 4a) and three drops of nitrate reagent II (reagent 4b) are added.

3. A pink or red color indicates the presence of nitrite, produced by microbial reduction of the nitrate. If no pink or red color appears, nitrite is absent.

4. If nitrite is absent, a few grains of zinc dust (reagent 14) are added to the culture tube. The appearance of a red color indicates the presence of nitrate and the test is considered negative. If no red color appears, nitrate is absent and the microorganism is assumed to have reduced the nitrate to free nitrogen. This is recorded as a positive test for nitrate reduction.

7.3.2.9 Urea Agar (Medium 44)

Urea agar (medium 44) is used to detect production of urease as follows.

1. The surface of the slant is inoculated with two drops of the TSB culture and incubated at 308 K (35° C) for 24 hours.

2. A positive reaction is indicated by a deep-pink color in both the butt and the slant, indicating complete hydrolysis of urea. Partial hydrolysis is indicated by a deep-pink slant and a yellow butt. A negative test is indicated by a yellow slant and butt or by no change.

7.3.2.10 Ten-Percent Sugar Solutions (Medium 41)

Medium 41 is used to determine carbohydrate decomposition as follows.

1. The following carbohydrates in purple broth base (medium 41) are each inoculated with two drops of the TSB culture: glucose, lactose, sucrose, maltose, mannitol, and xylose.

2. The media are incubated at 308 K (35° C) and observed daily for 7 days.

3. Acid production resulting from carbohydrate decomposition is indicated by a color change from purple to yellow and is recorded as a positive test. A negative test is indicated by no color change.

4. If the results are positive for less than three of the six carbohydrates tested, the test is repeated using the carbohydrates in OF medium base (medium 41).

   a. The OF medium tubes are inoculated with two drops of the TSB culture.

   b. The media are incubated at 308 K (35° C) and observed daily for 7 days.

   c. Acid production resulting from carbohydrate decomposition is indicated by a color change from blue to yellow and is recorded as a positive test. A negative test is indicated by no change in color.

7.3.2.11 Decarboxylase Medium Base (Medium 47)

Decarboxylase medium base (medium 47) is used to detect the ability of the microorganism to utilize lysine, arginine, and ornithine as follows.

1. One tube each of lysine, arginine, and ornithine decarboxylase medium is inoculated with two drops of TSB culture. A control tube without amino acid is also inoculated. The inoculated tubes are overlayed with sterile liquid paraffin and incubated for 4 days at 308 K (35° C).

2. A positive reaction is indicated by a purple color in the tube. A positive reaction in either the lysine or the ornithine tube indicates the activity of a decarboxylase enzyme system. A positive reaction in the arginine tube indicates the activity of either a dihydrolase enzyme system or a decarboxylase enzyme system.

3. A negative reaction is indicated by a yellow color in the tubes and indicates the absence of these enzyme systems.

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4. The control tube may show two types of reactions. A yellow color indicates glucose utilization by the microorganism. A purple color indicates no growth of the microorganism in this medium, and the reactions in the lysine, arginine, and ornithine tubes should be disregarded.

7.3.2.12 Gelatin (Medium 46)

Gelatin (medium 46) is used for the detection of the enzyme gelatinase as follows.

1. The medium is inoculated with 2 drops of TSB culture and incubated at room temperature (≈293 K (20° C)) for 7 days.

2. After incubation, the tube is refrigerated (≈277 K (4° C)) for 30 minutes.

3. A positive test is indicated by failure of the gelatin to solidify after refrigeration. A negative test is indicated by a solidified gelatin.

7.3.2.13 Motility Medium (Medium 45)

Motility medium (medium 45) is used to determine whether the microorganism is motile or nonmotile in the following manner.

1. The medium is inoculated with two drops of TSB culture by stabbing to two-thirds the depth of the medium using a Pasteur pipette.

2. After incubation at 308 K (35° C) for 24 hours, motile cultures show diffuse growth or turbidity away from the line of inoculation.

3. Microorganisms producing hydrogen sulfide will darken the medium.

7.3.2.14 Ten-Percent Glucose Agar (Medium 42)

Medium 42 is used to test the ability of the microorganism to decompose a 10-percent glucose concentration as follows.

1. The surface of the slant is inoculated with two drops of TSB culture and incubated at 308 K (35° C).

2. After 48 hours incubation, the tube is observed for decomposition of glucose.

3. Three types of reactions may be demonstrated. An acid reaction is indicated by a yellow slant, an alkaline reaction is indicated by a deep purple slant, and no change in the color of the slant is recorded as no change.

7.3.2.15 Ten-Percent Lactose Agar (Medium 43)

Medium 43 is used to test the ability of the microorganism to decompose a 10-percent lactose concentration as follows.

1. The surface of the slant is inoculated with two drops of TSB culture and incubated at 308 K (35° C).
2. After 48 hours incubation, the tube is observed for decomposition of lactose.
3. Three types of reactions may be demonstrated. An acid reaction is indicated by a yellow slant, an alkaline reaction is indicated by a deep-purple slant, and no change in the color of the slant is recorded as no change.

7.3.2.16 Litmus Milk (Medium 48)

Litmus milk (medium 48) is used to determine the action of the microorganism on milk as follows.

1. A tube of litmus milk is inoculated with two drops of TSB culture and incubated at 308 K (35° C).
2. The tube is read daily for 7 days.
3. The reactions that may occur include clot formation, peptonization (digestion), and/or acid production.
   a. Presence of a clot is recorded as "C."
   b. An area of clearing at the top of the medium is indicative of peptonization and is recorded as a "P."
   c. Acid production is indicated by a change in the indicator from blue to pink and is recorded as an "A."

7.3.2.17 Identification

Gram-negative rods are identified as shown in table 7-I.

7.3.2.18 Biochemical Reactions

Biochemical reactions of non-lactose-fermenting Gram-negative rods are shown in table 7-II.

7.3.3 IDENTIFICATION OF FASTIDIOUS GRAM-NEGATIVE RODS (HAEMOPHILUS)

7.3.3.1 Hemolysis Determination

Hemolysis is determined as follows.

1. The TSB culture (enriched with Fildes) is streaked to rabbit blood agar (medium 52) for isolation, using a sterile inoculating loop. The beginning of the streak is stabbed with the loop.

2. The plate is incubated at 308 K (35° C) in a carbon dioxide (CO_2) (candle) jar for 24 hours and examined for beta hemolysis.

3. If a clear zone is observed around the colonies or the area stabbed, the organism is beta hemolytic and is so recorded.

7.3.3.2 Growth-Factor Requirements

Growth-factor-requirements tests are performed as follows.

1. The entire surface of a TSA plate (medium 2) is inoculated with the TSB culture (enriched with Fildes) using a sterile loop or swab.

2. Using sterile forceps, X-factor, V-factor, and combination X- and V-factor strips are added to each plate as illustrated in figure 7-1.

3. The TSA plates are incubated at 308 K (35° C) in a CO_2 (candle) jar for 24 hours.

4. After incubation, the plates are observed for growth around the strips.

5. If growth occurs around a specific strip, this growth factor is needed for growth of this organism and is recorded as positive. If no growth occurs around a specific strip, the growth factor is not necessary for growth and is recorded as negative.

6. Accessory growth-factor requirements of various species of the genus *Haemophilus* are shown in table 7-III, and identification of members of the genus *Haemophilus* is shown in figure 7-2.

TABLE 7-I.- KEY TO THE IDENTIFICATION OF NON-LACTOSE-FERMENTING GRAM-NEGATIVE RODS

MacConkey positive		MacConkey negative	
Oxidase negative	Oxidase positive	Oxidase negative	Oxidase positive
	Gram-negative fermenters		
Yersinia pestis Yersinia pseudotuberculosis Yersinia enterocolitica Chromobacterium violaceum Enterobacteriaceae	Providence species Pasteurella haemolytica Aeromonas hydrophila Aeromonas (Plesiomonas) shigelloides Chromobacterium violaceum Actinobacillus lignieresi (late lactose) Actinobacillus equuli Actinobacillus suis	Actinobacillus actinomycetemcomitans	Pasteurella multocida Pasteurella pneumotropica Pasteurella ureae Pasteurella gallinarum Cardiobacterium hominis
	Gram-negative questionable fermenters[a]		
None	IIK Flavobacterium meningosepticum Flavobacterium species, IIb	IIKl	IIK Flavobacterium species, IIb Flavobacterium meningosepticum IIc IIe IIh IIi
	Gram-negative glucose oxidizers		
IIK Pseudomonas cepacia Pseudomonas mallei Ve-1 Ve-2 Herellea species	Pseudomonas pseudomallei Pseudomonas aeruginosa Pseudomonas fluorescens Pseudomonas putida Pseudomonas stutzeri Pseudomonas stutzeri complex Vb-2 Pseudomonas stutzeri complex Vb-3 Pseudomonas cepacia (late lactose) Pseudomonas vesiculare Vd Va	Pseudomonas mallei	Moraxella kingii
	Gram-negative glucose nonoxidizers		
Acinetobacter (Mima) polymorpha Pseudomonas maltophilia	Moraxella osloensis Moraxella phenylpyruvica Moraxella nonliquefaciens Acinetobacter (Mima) polymorpha var. oxidans Comamonas terrigena Alcaligenes faecalis Alcaligenes odorans Alcaligenes denitrificans Pseudomonas alcaligenes Pseudomonas pseudoalcaligenes Pseudomonas denitrificans Pseudomonas diminuta Pseudomonas putrefaciens IIIa IIIb IVc-2 IVe	Acinetobacter (Mima) polymorpha	Moraxella nonliquefaciens Moraxella lacunata Moraxella bovis Moraxella osloensis Moraxella phenylpyruvica IIf IIj Pasteurella anatipestifer

[a]Early reaction that of an oxidizer.

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TABLE 7-II.- BIOCHEMICAL REACTIONS OF NON-LACTOSE-FERMENTING GRAM-NEGATIVE RODS

(a) Serratia, Proteus, and Providencia

Test	Serratia	Proteus				Providencia	
		P. vulgaris	P. mirabilis	P. morganii	P. rettgeri	P. alcalifaciens	P. stuartii
Gas from glucose	-(+)	V	+	V	V	+,-	-
Carbohydrates	F	F	F	F	F	F	F
Base used:							
Glucose	A	A	A	A	A	A	A
Xylose	-(A,L)	A(L)	A	-	V	-	V
Mannitol	A	-	-	-	V	-	V
Lactose	-(A)	-	-	-	-	-	-
Sucrose	A	A	V	-	V	V	V
Maltose	A	A	-	-	-	-	-
10% glucose slants	0	0	0	0	0	0	0
10% lactose slants	0	0	0	0	0	0	0
Oxidase	-(w)	-	-	-	-	-(w)	-(w)
MacConkey agar (growth)	G	G	G	G	G	G	G
Simmon's citrate	+	V	+(L)	-	+	+	+
Urea agar	-(+)	+	+	+	+	-	-
Nitrate reduction	+	+	+	+	+	+	+
TSI							
Slant	Alk(A)	A	Alk	Alk	Alk	Alk	Alk
Butt	A/AG	AG	AG/A	AG	A	AG(A)	A
H_2S-butt/paper	-/+	+/+	+/+	-/+	-/-(+)	-/-	-/-
Gelatin hydrolysis	+	+(L)	+	-	-	-	-
Litmus milk	A,P	Alk,P	Alk,P	A,w/Alk	A,w/Alk	A,w/Alk	A,w/Alk
Pigment	Pink,-	-	-	-	-	-	-
Decarboxylase							
Lysine	+	-	-	-	-	-	-
Arginine	-	-	-	-	-	-	-
Ornithine	+	-	+	+	-	-	-
Indole	-	+	-	+	+	+	+
MR	V	+	+	+	+	+	+
VP	+	-	V	-	-	-	-
Motility	+	+	+	+	+	+	+

TABLE 7-II.- Continued

(b) *Yersinia*, *Chromobacterium*, *Acinetobacter*, and *Mima*

Test	*Yersinia*			*Chromobacterium violaceum*	*Acinetobacter calcoaceticus* (*Herellea*)	*Mima*	
	Y. pestis	*Y. pseudotuberculosis*	*Y. enterocolitica*			*M. polymorpha* var. *oxidans*	*M. polymorpha* (*Acinetobacter*)
Gas from glucose	-	-	-	-	-	-	-
Carbohydrates	F	F	F	F	OF	OF	OF
Base used:							
Glucose	A	A	A	A	A	Alk(NC)	Alk
Xylose	A	A	A(L)	-	A	Alk(NC)	Alk
Mannitol	A	A	A	-	Alk	Alk(NC)	Alk
Lactose	-	-	-(A,L)	-	A(L)	Alk(NC)	Alk
Sucrose	-	-	A	A	Alk	Alk(NC)	Alk
Maltose	A	A	A(L)	-	Alk/AL	Alk(NC)	Alk
10% glucose slants	A	A	A	0	A	Alk(NC)	Alk
10% lactose slants	Alk	Alk	Alk(A)	0	A	Alk(NC)	Alk
Oxidase	-	-	-	-,w	-	+	-
MacConkey agar (growth)	G	G	G	G	G	G(-)	G(-)
Simmon's citrate	-	-	-	+	+(-)	+	-(+)
Urea agar	-	+	+	-(L)	V	-	-(+)
Nitrate reduction	+	+	+(-)	+	-	-	-
TSI							
Slant	Alk(A)	Alk(A)	A	Alk(A)	Alk	Alk	Alk
Butt	A	A	A	A	-(Alk)	-(Alk)	-(Alk)
H_2S-butt/paper	-/+	-/Tr,l+	-/+	-/-,l+	-/-(+)	-/Tr	-/-(l+)
Gelatin hydrolysis	-	-	-	+	V	-	-(+)
Litmus milk	-	-	-	P	V	-	-(Alk)
Pigment	-	-	-	Violet	-	-	-(brown)
Decarboxylase							
Lysine	-	-	-	-	0	0	0
Arginine	-	-	-	+	0	0	0
Ornithine	-	-	+	-	0	0	0
Indole	-	-	V	-	-	-	-
MR	+	+	+	-	-(+)	-	-
VP	-	-	-(+)	-	-	-	-
Motility	-	-	-	+	-	-	-

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TABLE 7-II.- Continued

(c) *Moraxella*, *Cardiobacterium*, and *Actinobacillus*

Test	*Moraxella*						*Cardiobacterium hominis* (Group II-d)	*Actinobacillus actinomycetemcomitans*
	M. kingii (n. sp. I)	*M. osloensis*	*M. non-liquefaciens*	*M. lacunata* (*liquefaciens*)	*M. bovis*	*M. phenylpyruvica* (n. sp. II)		
Gas from glucose	-	-	-	-	-	-	-	+
Carbohydrates	F	OF	OF	OF	F	OF	F	F
Base used:								
Glucose	A	Alk	N	N	-	Alk/N	A	A A A A
Xylose	-	Alk	N	N	-	Alk/N	-	- - A A
Mannitol	-	Alk	N	N	-	Alk/N	A	A - A -
Lactose	-	Alk	N	N	-	Alk/N	-	- - - -
Sucrose	-	Alk	N	N	-	Alk/N	A	- - - -
Maltose	A	Alk	N	N	-	Alk/N	A	A A A A
10% glucose slants	NG	Alk	Alk	NG	?	?	NG	NG
10% lactose slants	NG	Alk	Alk	Alk	sl Alk	?	NG	NG
Oxidase	+	+	+	0	+	+	+	-/w
MacConkey agar (growth)	-	V	V	-	-	G(-)	-	-
Simmon's citrate	-	-	-	-	-	-	-	-
Urea agar	-	-(+)	-	-	-	+(-)	-	-
Nitrate reduction	-	±	+	+	-	+(-)	-	+
TSI								
Slant	NG/N	Alk	Alk	Alk	Alk	N/Alk	A	A
Butt	Alk/N	-(Alk)	N/Alk	Alk	N/Alk	N/Alk	A/NG	A
H_2S-butt/paper	-/Tr	-/+	-/+	-/Tr	-/1+	-/Tr	-/1+	-/1+
Gelatin hydrolysis	V	-	-	+	+	-	-	-
Litmus milk	P	-	-	P	P	Alk	-	-
Pigment	-	sl yellow	-	-	-	-	-	-
Decarboxylase								
Lysine	0	0	0	0	0	0	0	0
Arginine	0	0	0	0	0	0	0	0
Ornithine	0	0	0	0	0	0	0	0
Indole	-	-	-	-	-	-	+(w)	-
MR	NG	0	-	NG	-	NG	NG	NG
VP	NG	0	-	NG	-	NG	NG	NG
Motility	-	-	-	-	-	-	-	-

TABLE 7-II.- Continued

(d) *Pasteurella*

Test	*Pasteurella multocida*	*Pasteurella* n. sp. I or "gas" (*multocida*?)	*Pasteurella ureae*	*Pasteurella pneumotropica*	EF-4	*Pasteurella hemolytica*	*Pasteurella gallinarum*	*Pasteurella anatipestifer*
Gas from glucose	-	- or +	-	-	-	-	-	-
Carbohydrates	F	F	F	F	F	F	F	F
Base used:								
Glucose	A AA AA	A	A	A	A	A	A	-
Xylose	A -- AA	-	-	A	-	A	-	-
Mannitol	A -A AA	-	A	-	-	A	-	-
Lactose	- -- A-	-	-	A(L)	-	A(L)	-	-
Sucrose	A AA AA	A	A	A	-	A	A	-
Maltose	- -- -A	A	A	A	-	A	A	-
10% glucose slants	vlg	NG(A)	vlg	vlg	V	vlg	vlg	NG
10% lactose slants	vlg	NG(A)	vlg	vlg	Alk	vlg	vlg	vlg
Oxidase	+	+	+	+	+	+	+	+
MacConkey agar (growth)	-	-	-	-	V	+	-	-
Simmon's citrate	-	-	-	-	-	-	-	-
Urea agar	-	+	+	+	-	-	-	+
Nitrate reduction	+	+	+	+	+ or +,gas	+	+	-
TSI								
Slant	A	A	A	A	Alk or A,w	A	A	Alk
Butt	A	A	A	A	A or N	A	A	N
H_2S-butt/paper	-/- or Tr	-/+	-/1 to 2+	-/1 to 2+	-/+(3)	-/1 to 2+	-/2 to 4+	-/Tr
Gelatin hydrolysis	-	V	-	-	V	-	-	+
Litmus milk	-(A,Alk)	-	-(Alk)	-(A)	-(Alk)	-(A)	-(A)	-
Pigment	-	-	-	-	- or sl yellow	-	-	sl browning
Decarboxylase								
Lysine	-	-	0	V	-	0	0	0
Arginine	-	-	0	-	+	0	0	0
Ornithine	V	-	0	+	-	0	0	0
Indole	+	+	-	+	-	-	-	-
MR	NG	-	NG	NG	V	NG	NG	NG
VP	NG	-	NG	NG	-	NG	NG	NG
Motility	-	-	-	-	-	-	-	-

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TABLE 7-II.- Continued

(e) *Pseudomonas*, first group

Test	*Pseudomonas aeruginosa*	*Pseudomonas fluorescens*	*P. fluorescens* group (not *fluorescens*; possibly *P. putida*)	*Pseudomonas cepacia*	Group V-A	*Pseudomonas stutzeri* complex		
						P. stutzeri Vb-1	*P. stutzeri* Vb-2	*P. stutzeri* Vb-3
Gas from glucose	-	-	-	0	0	-	-	-
Carbohydrates	OF	OF	OF	OF	OF	OF	OF	OF
Base used:								
Glucose	A	A	A	A	A	A	A	A
Xylose	A(-)	A	A	A,L(N)	A	A	A	A
Mannitol	A or -	V	V	A(wk)	-	A,w	A,w	A,w
Lactose	-	V	V	A(L)	A	-	-	-
Sucrose	-	V	V	A(Alk)	-	-	-	-
Maltose	-	V	V	A(wk)	A	A	-	A
10% glucose slants	A	A	A	A	0	A	A	A
10% lactose slants	Alk	Alk(A)	Alk(A)	A	0	Alk(A)	Alk(A)	Alk(A)
Oxidase	+	+	+	-/w	+	+	+	+
MacConkey agar (growth)	+	+	+	+	+	+	+	+
Simmon's citrate	+	+	+	+	+	+	+	+
Urea agar	+(-)	V	V	-(+)	+	V	V	V
Nitrate reduction	NO_2^+,gas	- or +(gas)	-(+)	V,no gas	+,gas	NO_2^+,gas	NO_2^+,gas	NO_2^+,gas
TSI								
Slant	Alk	Alk	Alk	Alk	Alk	Alk	Alk	Alk
Butt	Alk	Alk	Alk	Alk	Alk	N to Alk	N to Alk	N to Alk
H_2S-butt/paper	-/-	-/-	-/-	-/-(Tr)	-/-(Tr)	-/-(Tr)	-/-(Tr)	-/-(Tr)
Gelatin hydrolysis	+(-)	+	-	-(+)	-	-	-	-
Litmus milk	P(-)	P	Alk	P(-)	Alk	Alk	Alk	Alk
Pigment	0	0	0	V	0	sl yellow, wrinkled	sl yellow	sl yellow, wrinkled
Decarboxylase								
Lysine	-	-	-	+(V)	-	-	-	-
Arginine	+	+	+	-	-	-	+	+
Ornithine	-	-	-	V	-	-	-	-
Indole	-	-	-	-	0	-	-	-
MR	-	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+

TABLE 7-II.- Continued

(f) Pseudomonas, second group

Test	Pseudomonas pseudomallei (all recent isolates)	Pseudomonas (Actinobacillus) mallei	Pseudomonas diminuta	Pseudomonas vesiculare	Ib-1 (Pseudomonas putrefaciens)	Ib-2 (possibly P. putrefaciens)	Pseudomonas maltophilia
Gas from glucose	-	-	-	-	-	-	-
Carbohydrates	OF	OF	OF	OF	OF	OF	OF
Base used:							
Glucose	A	A	Alk,(A,w)	A	Alk(A)	NG,Alk	Alk,Aw
Xylose	A	Alk(N)	Alk	A(Alk)	Alk	NG,Alk	Alk,Aw
Mannitol	A	Alk	Alk	Alk	Alk	NG,Alk	Alk
Lactose	A	Alk	Alk	Alk	Alk	NG,Alk	Alk,Aw
Sucrose	A(Alk)	Alk	Alk	Alk	A(Alk)	NG,Alk	Alk,Aw
Maltose	A	Alk	Alk	A	A(Alk)	NG,Alk	A
10% glucose slants	A	-	Alk,(A,w)	A	Alk(A)	Alk	Alk
10% lactose slants	A	0	Alk	Alk	Alk	Alk	Alk
Oxidase	+	-	+	+	+(-)	+(w)	-
MacConkey agar (growth)	+	NG	+	+	+	+	+
Simmon's citrate	+(-)	-	-	-	-	-(+)	-(+L)
Urea agar	-(pink, +)	-	-(+)	-(+)	-(+)	-(+)	-(+)
Nitrate reduction	+, gas	+	-	-	+	+	+,-
TSI							
Slant	A(Alk)	N,Alk	Alk	Alk	Alk	Alk	Alk
Butt	N(Alk)	N,Alk	N	N	IR	IR	N,Alk
H_2S-butt/paper	-/-(Tr)	-/Tr, 3+	-/-	-/-	+/+	+/+	-/1 to 3+
Gelatin hydrolysis	+,1 to 4 days	0	+	+	+(-)	+	+
Litmus milk	P	-	P	-	P,IR	P,IR	P
Pigment	White, yellow, some wrinkled	-	-	-(orange)	-, apricot	-, apricot	-
Decarboxylase							
Lysine	-	-	-	-	0	0	+
Arginine	+	+	-	-	0	0	-
Ornithine	-	-	-	-	0	0	-
Indole	-	-	-	-	-	-	-
MR	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	-
Motility	+	-	+	+	+	+	+

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TABLE 7-II.- Continued

(g) *Pseudomonas* and *Alcaligenes*

Test	*Comamonas terrigena*		*Pseudomonas alcaligenes*	*Pseudomonas pseudoalcaligenes*	*Pseudomonas denitrificans*	*Alcaligenes denitrificans* (Group V-C)	*Alcaligenes faecalis* (Group VI)	*Alcaligenes odorans*
	Pseudomonas acidovorans	*Pseudomonas testosteroni*						
Gas from glucose	-	-	-	-	-	-	-	-
Carbohydrates	OF	OF	OF	OF	OF	OF	OF	OF
Base used:								
Glucose	Alk	Alk	Alk	Alk	Alk	Alk	Alk	Alk
Xylose	Alk	Alk	Alk	Alk	Alk	Alk	Alk	Alk
Mannitol	N to Aw	Alk	Alk	Alk	Alk	Alk	Alk	Alk
Lactose	Alk	Alk	Alk	Alk	Alk	Alk	Alk	Alk
Sucrose	Alk	Alk	Alk	Alk	Alk	Alk	Alk	Alk
Maltose	Alk	Alk	Alk	Alk	Alk	Alk	Alk	Alk
10% glucose slants	Alk	Alk	Alk	Alk	Alk	Alk	Alk	Alk
10% lactose slants	Alk	Alk	Alk	Alk	Alk	Alk	Alk	Alk
Oxidase	+	+	+	+	+	+	+	+
MacConkey agar (growth)	+	+(L)	+	+	+	+	+	+
Simmon's citrate	+(L or -)	-(L)	V	V	V	+	+	+
Urea agar	-(L)	V	-(+)	-(+)	-(+)	-(pink)	-(pink)	-(pink)
Nitrate reduction	+(-)	+(-)	V	+	NO_2^+	NO_2^+, gas	V, no gas	±
TSI								
Slant	Alk	Alk	Alk	Alk	Alk	Alk	Alk	Alk
Butt	N,Alk	N,Alk	N,Alk	N,Alk	N,Alk	Alk	Alk	Alk
H_2S-butt/paper	-/-, Tr	-/Tr, 2+	-/Tr, 1+(-)	-/Tr, 3+(-)	-/Tr, 2+	-/-, 2+	-/-(Tr)	-/-(Tr)
Gelatin hydrolysis	-	-	-	-	-	-	-	-
Litmus milk	Alk	Alk	Alk	Alk	-(Alk)	Alk	Alk	Alk
Pigment	-	-	-	0	0	-	-	-
Decarboxylase								
Lysine	-	-	-	-	0	0	0	0
Arginine	-(L?)	-	-(L)	+(-)	0	0	0	0
Ornithine	-	-	-	-	0	0	0	0
Indole	-	-	-	-	-	-	-	-
MR	-	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+

TABLE 7-II.- Continued

(h) Actinobacillus and Flavobacterium

Test	Actinobacillus lignieresi	Actinobacillus equuli	Actinobacillus suis	Flavobacterium meningosepticum (Group II-A)	Flavobacterium sp. (Group II-B)
Gas from glucose	-	-	-	-	-
Carbohydrates	F	F	F	OF	OF
Base used:					
Glucose	A	A	A	A,L A	-(A,L) A(-)
Xylose	A	A	A(L)	- -	- -(Aw)
Mannitol	A	A	-,A	A,L A(-)	- -(A)
Lactose	A(L)	A	A	A,L -(A)	- -
Sucrose	A	A	A	- -	-(A,L) -(A)
Maltose	A	A(1-)	A	A,L(-) A(L)	-(A,L) A(-)
10% glucose slants	vlg	vlg	0	A	(A)Alk
10% lactose slants	vlg	vlg	0	Alk(A)	Alk
Oxidase	+	+	+	+	+
MacConkey agar (growth)	+	+,L	+	+(-)	+(-)
Simmon's citrate	-	-	-	-(+,L)	-
Urea agar	+	+	+	-(+,L)	-,+,L
Nitrate reduction	+	+	+	-	$-(NO_2^+)$
TSI					
Slant	A	A	A	Alk	Alk
Butt	A	A	A	N,Alk(A)	N,Alk(A)
H_2S-butt/paper	-/1 to 2+	-/1 to 2+	-/2+	-/+	-/+
Gelatin hydrolysis	-	+	-	+	+
Litmus milk	-	A	A	P	P
Pigment	-	-	-	sl yellow	Very yellow
Decarboxylase					
Lysine	0	0	0	0	0
Arginine	0	0	0	0	0
Ornithine	0	0	0	0	0
Indole	-	-	-	+	+
MR	NG	NG	-,NG	-	-
VP	NG	NG	-,NG	-	-
Motility	-	-	-	-	-

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TABLE 7-II.- Continued

(i) National Center for Disease Control groups II-C to II-K

Test	Group II-C		Group II-E		Group II-F		Group II-H		Group II-I		Group II-J		Group II-K, type 1		Group II-K, type 2	
Gas from glucose	-		-		-		-		-		-		-		-	
Carbohydrates	F	OF	F	OF	F	OF	F	OF	F	OF	F	OF	F	OF	F	OF
Base used:																
Glucose	A	A	A	A	-	Alk	A	A	-(A,L)	A	-	NG,Alk	-	A(w)	A,L(-)	A
Xylose	-	Alk	-	Alk	-	Alk	-	Alk(N)	-	A	-	NG,Alk	-(LA)	A	A,L(-)	A
Mannitol	-	Alk	-	Alk	-	Alk	-	Alk(N)	-	Alk	-	NG,Alk	-	Alk	-	Alk
Lactose	-	Alk	-	Alk	-	Alk	-	Alk(N)	-	A	-	NG,Alk	-	A	A,L(-)	A
Sucrose	A	A	-	Alk	-	Alk	-	Alk(N)	-	A	-	NG,Alk	-	A	A,L(-)	A
Maltose	A	A	A	A	-	Alk	A	A	-	A	-	NG,Alk	-	A	A,L(-)	A
10% glucose slants	A		A(N)		Alk		A		A		NG(Alk)		N(A)		A(L)	
10% lactose slants	-		Alk(N)		Alk		-		A		NG		Alk(A)		A(L)	
Oxidase	+		+		+		+		+		+		V		+	
MacConkey agar (growth)	-		-		-(+)		-		-		-		-(L)		+(L)	
Simmon's citrate	-		-		-		-		-		-		-(few +)		-	
Urea agar	-		-		-		-		-		+		-(few +)		V	
Nitrate reduction	NO_2^+,+(-)		-		-		-		-		-		-(rare +)		V	
TSI																
Slant	A(Alk)		Alk		Alk		Alk		Alk		N,Alk		N,Alk,A		N,Alk,A	
Butt	N,A		N		N,Alk		N		N,Alk		N,Alk		N,A		N,A	
H_2S-butt/paper	-/Tr		-/3+		-/+(-)		-/Tr		-/2+		-/+		-/+		-/+	
Gelatin hydrolysis	-		-		+(-rare)		-		-		+		-		-	
Litmus milk	-		-		V,P		-		-		V		-		-	
Pigment	-		-		0		-		-,tan,sol		0		Yellow or -		- to sl yellow or yellow	
Decarboxylase																
Lysine	0		0		-		0		0		0		-		-	
Arginine	0		0		-		0		0		0		-		-	
Ornithine	0		0		-		0		0		0		-		-	
Indole	+		+		+,+w		+		+(w)		+,+w		-		-	
MR	NG		-		-		-		-		-		-		-	
VP	NG		-		-		-		-		-		-		-	
Motility	-		-		-		-		-		-		+		-	

TABLE 7-II.- Continued

(j) National Center for Disease Control groups III-A to VE-2

Test	Group III-A	Group III-B (prob. var. III-A)	Group IV-C(2)	Group IV-E	Group V-D	Group VE-1	Group VE-2
Gas from glucose	-	-	-	-	-	-	-
Carbohydrates	OF	OF	OF	OF	OF	OF	OF
Base used:							
Glucose	N,A,w	N,A,w	Alk	Alk,NG	A	A	A
Xylose	A	A	Alk	Alk,NG	A	A	A
Mannitol	Alk	Alk	Alk	Alk,NG	A(-)	A	A
Lactose	Alk	Alk	Alk	Alk,NG	-	-	-
Sucrose	Alk	Alk	Alk	Alk,NG	A(-)	-(rare A)	-(A)
Maltose	Alk	Alk	Alk	Alk,NG	-(A)	A	A
10% glucose slants	Alk	Alk	Alk	NG,Alk	A(Alk)	A	A
10% lactose slants	Alk	Alk	Alk	NG,Alk	N	Alk(A,L)	Alk
Oxidase	+	+	+	+	+	-	-
MacConkey agar (growth)	+	+	+	+	+	+	+
Simmon's citrate	+	+	+	-	+	+	+
Urea agar	-	-	Part, +24 hr	+S	+, 18 hr	+	+
Nitrate reduction	+	NO_2^+,gas	-(+)	NO_2^+,gas (+,gas)	NO_2^+,gas (NO_2,+)	+(-)	-
TSI							
Slant	Alk	Alk	Alk	Alk	Alk	Alk	Alk
Butt	N,Alk	N,Alk	N,Alk	N	Alk	N,Alk	N,Alk
H_2S-butt/paper	-/-	-/-	-/-(Tr)	-/Tr	±2+/3 to 4+	-/Tr	-/Tr
Gelatin hydrolysis	-	-	-	-	-	V	-
Litmus milk	Alk	Alk	Alk	Alk	Alk	Alk(P)	Alk
Pigment	-	-	-	-	0	Yellow	Yellow
Decarboxylase							
Lysine	0	0	0	0	0	-	-
Arginine	0	0	0	0	0	+	-
Ornithine	0	0	0	0	0	-	-
Indole	-	-	-	-	-	-	-
MR	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+

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TABLE 7-II.- Concluded

(k) Key to symbols

Symbol	Meaning	Symbol	Meaning
-	Negative	1+, 2+, etc.	Degree of reaction
+	Positive	N	Neutral
V	Variable	sl	Slight or slightly
F	Fermentation	?	Results questionable
A	Acid	vlg	Very light growth
L	Late	NG	No growth
O	Results not recorded	wk	Weak or weakly
w	Weak	A,w	Acid or weakly acid
G	Growth	IR	Intermediate reaction
AG	Acid and gas	NO_2^+	Nitrate to nitrite
Alk	Alkaline	sol	Soluble
P	Peptonization	^+S	Positive slant
NC	No change	H_2S	Hydrogen sulfide
OF	Oxidation-fermentation	()	Less common reaction
Tr	Trace		

TABLE 7-III.- ACCESSORY GROWTH-FACTOR REQUIREMENTS OF VARIOUS SPECIES OF THE GENUS *HAEMOPHILUS*

Species	Growth factors required (a)			Hemolysis
	X only	V only	X and V	
H. influenzae	-	-	+	Absent
H. parainfluenzae	-	+	+	Absent
H. aegyptius	-	-	+	Absent
H. haemolyticus	-	-	+	Present
H. parahaemolyticus	-	+	+	Present
H. aphrophilus	+/-[b]	-	+	Absent
H. ducreyi	+	-	+	Slight

[a] +, growth; -, no growth.
[b] Requires X factor only when grown under CO_2.

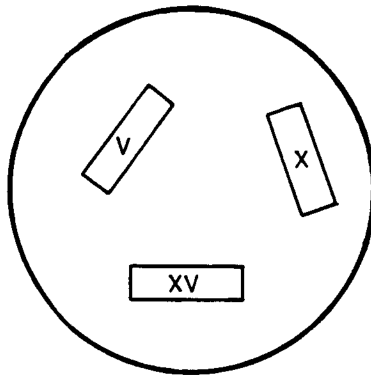

Figure 7-1.- Location of growth-factor strips on inoculated TSA plate.

Chocolate bacitracin agar
→ TSB with Fildes
→ Incubate for 18 hr at 308 K (35°C) → Gram stain → Record results
→ Streak to rabbit blood agar

- Hemolytic (beta)
  - Growth-factor requirements
    - X and V +; X -; V + → *H. parahaemolyticus*
    - X and V +; X -; V - → *H. haemolyticus*
- Nonhemolytic
  - Growth-factor requirements
    - X and V +; X -; V - → *H. influenzae*, *H. aegyptius*
    - X and V +; X +; V - → *H. aphrophilus*, *H. ducreyi**
    - X and V +; X -; V + → *H. parainfluenzae*

Figure 7-2.- Schematic diagram for identifying members of the genus Haemophilus. Growth is indicated by + and no growth by -; an asterisk indicates that the species may be slightly hemolytic.

8. IDENTIFICATION OF YEAST AND YEASTLIKE ORGANISMS

By P. N. Kashkin* and V. M. Knyazen*

8.1 PURPOSE

The purpose of this chapter is to describe methods for identifying yeast and yeastlike microorganisms. A schematic diagram of identification of medicinally important representatives of the yeast flora is given in figure 8-1.

8.2 MATERIALS

Yeast colonies of varied morphology, consistency, and color are used as materials for identification. The following nutrient media are used for isolation and identification of yeast and yeastlike microorganisms.

1. Sabouraud's medium containing glucose and antibiotics (penicillin plus streptomycin, 50 to 100 units per cubic centimeter of medium) (medium 5)
2. Gorodkova's medium (medium 18)
3. Potato agar containing glucose (medium 58)
4. Peptone water containing carbohydrates (medium 81)
5. Yelinov's medium (medium 82)
6. Christensen's medium (medium 83)
7. Medium for carbohydrate auxanogram (medium 84)
8. Medium for nitrous materials auxanogram (medium 85)
9. Medium containing arbutin (medium 86)

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10. Potato/carrot medium containing thiamine and glucose (medium 87)

11. Sterile human blood serum or horse serum

12. Fermentation test medium with gas tubes (medium 88)

13. Carbohydrate solutions (medium 89)

14. 5-percent aqueous solution of malachite green (reagent 29)

15. 0.25-percent solution of basic fuchsin (reagent 30)

16. Ferric chloride ($FeCl_3$) (reagent 31)

17. Glycerin (reagent 32)

18. India ink (reagent 33)

8.3 METHODS

8.3.1 ISOLATION OF A PURE CULTURE

Petri dishes with inoculated Sabouraud's medium (medium 5) are allowed to incubate at 298 K (25° C) for 5 days. After the 5 days, the grown colonies are counted. Five colonies of each morphological type are transferred onto slants with Sabouraud's medium containing glucose and incubated for 3 days. The purity of the culture is determined by examining a squashed drop preparation at 430× magnification. (The culture is placed into a mixture of glycerin (reagent 32) and ethyl alcohol and Gram stained.)

8.3.2 METHODS OF IDENTIFICATION

8.3.2.1 Initial

The isolated cultures are inoculated into a sporulation medium (medium 18) and maintained at 298 K (25° C) for 7 to 25 days.

8.3.2.1.1 Ascospore detection.- For ascospore detection, the preparation is stained as follows (Peshkov's method, modified by Trujillo (ref. 8-1)). A smear is prepared. The preparation is fixed above a flame, flooded with a 5-percent aqueous solution of malachite green (reagent 29), and heated for 3 minutes until it starts steaming. Then, the stain is washed off with running water, and the preparation is placed into a 0.25-percent solution

of basic fuchsin (reagent 30) for 1 minute, rinsed with water, and dried. When examined microscopically, the spores appear to be green; the cells, red. The presence of spores indicates that the culture is a true yeast. When spores are absent, the cultures are subjected to further identification according to section 8.3.2.1.2.

8.3.2.1.2 Mycelium or pseudomycelium identification.- For mycelium or pseudomycelium identification, a suspension of yeastlike fungus culture is deposited with a bacteriological loop or a bacteriological spatula in parallel streaks (at one-third of the agar depth) onto Petri dishes containing a medium (medium 58) marked into four sections. The dishes with their contents are maintained at 298 K (25° C). After 48 to 72 hours of incubation, the nature of growth and the type of filaments formed are determined. (See table 8-I.)

8.3.2.2 Subsequent

The further identification of the cultures consists of a microscopic examination of a squashed drop preparation. (A suspension of the culture is placed into a drop of glycerin/ethyl alcohol mixture; the drop is deposited onto a sterile microscope slide and covered with a coverslip.)

8.3.2.2.1 Genus Geotrichum.- The presence of mycelium which easily gives rise to square or elongated arthrospores that form chains (1.6 to 2.5 micrometers by 4 to 6 micrometers) and, when aging, assume a spherical shape indicates that the yeastlike fungi are a member of the genus Geotrichum.

8.3.2.2.2 Genus Trichosporon.- The presence of square or elongated arthrospores and blastospores (round, single, budding cells, 1.2 by 2 to 3.2 micrometers in size) indicates that the yeastlike fungi are a member of the genus Trichosporon.

8.3.2.2.3 Genus Candida.- Cultures which form pseudomycelium on potato agar and look like orbicular, budding cells in a squashed drop preparation belong to the genus Candida.

8.3.3 IDENTIFICATION OF YEASTLIKE FUNGI BELONGING TO THE GENUS CANDIDA

For identification of species belonging to the genus Candida, the following properties are studied: the presence of chlamydospores and buddings; the ability to ferment carbohydrates, to assimilate carbohydrates and nitrous materials (method of auxanogram), and to decompose arbutin; and the nature and type of growth on potato agar containing glucose (medium 58). (See section 8.3.2.1.2.)

8.3.3.1 Detection of Chlamydospores

For early detection of chlamydospores, Yelinov's medium (medium 82) is employed (N. P. Yelinov, 1964 (ref. 8-2)). A suspension of a 1-day-old culture of yeastlike fungi in a saline solution is inoculated onto a block of rice medium (medium 82) prepared as follows: melted medium, in a quantity sufficient to form a thin layer (1 to 1.5 millimeters thick), is poured into a polished Petri dish; when solidified, the medium is cut with a sterile scalpel into squares, the size of which remains within 0.5 to 0.8 square centimeter. With the aid of the same scalpel, one square is deposited onto a sterile microscope slide placed into a sterile Petri dish. A suspension of a yeastlike fungus is deposited on the surface of the block, using a bacteriological loop. Conditions are optimal if an examination at 400× magnification reveals in the field of vision 5 to 7 cells of the tested microorganism. After a coverslip has been put on the block (with care taken not to leave air bubbles under the coverslip), the edges of the block are lined with paraffin (reagent 16).

The culture is maintained at room temperature (291 to 293 K (18° to 20° C)) for 2 to 5 days. If chlamydospores are formed, they can be easily detected by a microscopic examination at 100× or 400× magnification (large, round, granular cells of 10 to 20 micrometers diameter and having a clearly defined double-contoured membrane).

8.3.3.2 Identification of Buddings

For the identification of buddings, a suspension of a 1-day-old culture of a yeastlike fungus is placed, with a bacteriological loop, into a sterile vial containing 0.5 cubic centimeter of human blood serum (or horse serum). After 3 hours of incubation at 310 K (37° C), a squashed drop preparation of the culture is examined microscopically.

The presence of chlamydospores and buddings permits the culture being studied to be classified as Candida albicans.

8.3.3.3 Classification

Yeastlike fungi belonging to the genus Candida and not forming chlamydospores or buds are tested for the presence of the following properties. (See tables 8-II and 8-III.)

8.3.3.3.1 Determination of arbutin decomposition.- A 24-hour culture of the microorganism under study is placed in agar containing arbutin (medium 86) and incubated at 298 K (25° C) for 6 days. In the case of a positive reaction, the medium becomes brown.

8.3.3.3.2 Determination of carbohydrate fermentation.- Two methods for determining the ability to ferment carbohydrates are described.

8.3.3.3.2.1 Method used in U.S.S.R. laboratories: In U.S.S.R. laboratories, a suspension of each microorganism under investigation is placed into a vial containing 0.5 cubic centimeter of peptone water and one of the carbohydrates in a 2-percent concentration (medium 81). The vials are incubated at 310 K (37° C) for 20 days. If carbohydrate fermentation takes place, the blue coloration of the indicating liquid turns yellow (at pH 6.0 or lower).

8.3.3.3.2.2 Method used in U.S. laboratories: In U.S. laboratories, two yeast-extract/malt-extract agar (YEME) (medium 90) slants (number 3 and number 4) are incubated for 24 hours at 298 K (25° C). After incubation, 5.0 cubic centimeters of sterile distilled water are added to the first YEME slant (number 3) to suspend the cells. A single drop of the resulting cell suspension is added to each of the tubes of fermentation test media (medium 88) containing one of the carbohydrates of medium 89.

The tubes are incubated at 298 K (25° C) and observed after 14 and 28 days of incubation. Presence of a yellow color indicates the production of acid and is recorded as an "A" but is considered to be negative. Presence of gas in the gas-collection tube is recorded as "G." The presence of acid and gas is recorded as positive. If no acid and no gas are produced, the reaction is recorded as negative.

8.3.3.4 Auxanogram Method

The carbohydrates used in the test to determine the ability to assimilate carbohydrates and nitrate materials (method of auxanogram) are 20-percent solutions of glucose, galactose, maltose, lactose, and saccharose. For auxanograms of carbohydrates and nitrous materials, media 84 and 85, respectively, are used.

The test following the auxanogram method (Beyerink method, modified by N. P. Yelinov, 1964 (ref. 8-2)) is performed as follows. One cubic centimeter of a suspension of a 1-day-old culture of the microorganism under study (100×10^6 cells), prepared in a saline solution, is placed onto the flat bottom of a Petri dish. Twenty cubic centimeters of liquid agar medium at a temperature of 333 K (60° C) of an appropriate composition are then poured into the Petri dish and uniformly mixed with its contents. After thickening, the medium is dried at 310 K (37° C).

The surface of the agar is topped with sterile, 5-millimeter-diameter disks of filter paper saturated with solutions of the materials being studied and dried at 310 K (37° C) for 30 minutes. The number of disks for each Petri dish must not exceed three. The Petri dishes are incubated at 310 K (37° C) for 5

days. The development of yeastlike microorganisms in places where the material being studied was deposited indicates that the material has been assimilated. The absence of growth is an indication that the material is unsuitable for nutrition.

8.3.4 IDENTIFICATION OF YEASTLIKE FUNGI BELONGING TO GENUS OTHER THAN CANDIDA

8.3.4.1 Aliphatic Acid Test

If the yeastlike microorganisms lack the ability to produce mycelium or pseudomycelium, they are tested to determine whether they require aliphatic acid (olive oil and oleic acid) for growth. The culture is inoculated with a bacteriological loop onto sections of Sabouraud agar (medium 5) in Petri dishes or into vials with the same medium. One drop of a sterile solution of aliphatic acid is deposited on each inoculation site in Petri dishes; if vials are used, the cultures are covered with a thin layer of aliphatic acid. The dishes with the inocula are incubated at 298 K (25° C). The presence of growth of the yeastlike fungus indicates that the culture belongs to the genus *Pityrosporum*.

8.3.4.2 Presence of Carotenoid Pigment

The presence of carotenoid pigment in cultures that do not require aliphatic acid for their growth indicates that the organisms belong to the genus *Rhodotorula*.

8.3.4.3 Determination of Urease Activity

Yeastlike fungi that are free of carotenoid pigment are tested on Christensen medium for the determination of urease activity (medium 83).

A suspension of the culture under study is placed into vials containing Christensen's medium. The vials are closed with rubber stoppers and incubated at 310 K (37° C) for 5 days. The urea hydrolysis is indicated by the change of color in the indicator from orange-yellow to pink-red.

8.3.4.4 Presence of Capsules

Simultaneously with urease activity determination, the presence of capsules in the culture is investigated. A drop of suspension of the microorganism being studied is deposited on a microscope slide and, next to it, a drop of India ink (reagent 33).

The two drops are mixed, covered with a coverslip, and viewed against a dark background with a microscope having a lowered condenser. Cells surrounded with light rings are seen if capsules are present in the culture. The detection of urease activity and capsules in a culture indicates that the microorganism is a member of the genus *Cryptococcus*.

Cryptococcus neoformans is differentiated from nonpathogenic cryptococci in the following manner. *Cryptococcus neoformans* microorganisms differ from other species of the same genus by the ability to grow at 310 K (37° C) and to produce a brown pigment on a potato/carrot medium (medium 87). To investigate pigment formation, the medium is inoculated with a culture of yeastlike organism and incubated at two temperatures (at 298 K (25° C) and at 310 K (37° C)) for 5 days.

Cultures which are urease-negative and have no capsules belong to the genus *Torulopsis*.

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TABLE 8-I.- TYPES OF FILAMENTS

Type	Description
1. Spherical type (Mycotorula)	Round-shaped verticillia comprised, as a rule, of blastospores (buds) of a uniform size.
2. Aliform type (Mycotoruloides)	The basic cells which comprise verticillia accumulate on both sides of constrictions. When overgrown with secondary and tertiary blastospores, verticillia become wing-shaped. Blastospores vary in size.
3. Chain type (Candida)	The lateral and apical segments of pseudomycelium carry long chains; under constrictions, chains of buds develop that form spherical and aliform verticillia.
4. Vestigial type (Mycocandida)	Segments of pseudomycelium are, as a rule, of a uniform size. The vegetative body of the fungus looks like a tiny tree.

TABLE 8-II.- MORPHOLOGICAL AND BIOCHEMICAL PROPERTIES OF SOME MEMBERS OF THE GENUS CANDIDA

No.	Microorganism	Type of filament (a)	Chlamydo-spores (b)	Buds (b)	Fermentation (c)					Assimilation (c)					Arbutin decompo-sition (d)
					Glucose	Galac-tose	Maltose	Lactose	Saccha-rose	Glucose	Galac-tose	Maltose	Lactose	Saccha-rose	
1	C. albicans	Mt,Mts	+	+	+	+	+	-	-	+	+	+	-	+	-
2	Var. stellatoidea	Mt,Mts	+	-	±	+	+	-	-	+	+	+	-	+	-
3	C. tropicalis	Mts,C	-	-	+	+	+	-	+	+	+	+	-	+	-
4	C. krusei	Mt,Mts,Mc	-	-	+	-	-	-	-	+	-	-	-	-	-
5	C. pseudotropi-calis	Mc	-	-	+	+	-	+	+	+	+	-	+	+	+
6	C. guilliermondii	Mc	-	-	+	+	-	-	+	+	+	+	-	+	+
7	C. zeylanoides	Mc	-	-	-	-	-	-	-	+	-	-	-	-	-
8	C. pelliculosa	Mc	-	-	+	+	+	-	+	+	+	+	-	+	+
9	C. parapsilosis	Mc	-	-	+	+	-	-	±	+	-	+	-	+	-
10	C. brumpti	Mts	-	-	+	-	-	-	-	+	+	+	-	-	-
11	C. intermedia	Mc	-	-	+	+	+	-	+	+	+	+	+	+	+

[a]Mt, Mycotorula; Mts, Mycotoruloides; Mc, Mycocandida; and C, Candida.
[b]+, present; -, absent.
[c]+, fermentation and assimilation of carbohydrates present; -, fermentation and assimilation of carbohydrates absent; and ±, irregular carbohydrate fermentation.
[d]+, positive; -, negative.

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TABLE 8-III.- BIOCHEMICAL PROPERTIES OF THE REMAINING MEMBERS OF THE GENUS CANDIDA

No.	Microorganism	Chlamydospores (a)	Fermentation (b)					Assimilation (b)					Potassium nitrate (c)	Arbutin decomposition (c)
			Glucose	Galactose	Maltose	Lactose	Saccharose	Glucose	Galactose	Maltose	Lactose	Saccharose		
1	C. mycoderma	-	+	-	-	-	-	+	-	-	-	-	-	-
2	C. pulcherima	-	+	+	-	-	-	+	+	+	-	+	-	+
3	C. mesenterica	-	+	-	+	-	+	+	-	+	-	+	-	+
4	C. humicola	-	-	-	-	-	-	+	+	+	+	+	-	+
5	C. rugosa	-	-	-	-	-	-	+	+	-	-	-	-	-
6	C. rekaufii	-	+	-	-	-	-	+	+	+	-	+	-	±
7	C. utilis	-	+	-	-	-	+	+	-	+	-	+	+	+
8	C. lipolytica	-	-	-	-	-	-	+	-	-	-	-	-	±
9	C. catenulata	-	+	±	-	-	-	+	+	-	-	-	-	±
10	C. japonica	-	-	-	-	-	-	+	-	+	-	+	-	+
11	C. melinii	-	+	-	-	-	-	+	-	+	-	+	+	+
12	C. robusta	-	+	+	+	-	+	+	+	+	-	+	-	-
13	C. scottii	-	-	-	-	-	-	+	+	+	-	+	+	±
14	C. tenuis	-	+	+	-	-	-	+	+	+	±	+	-	+
15	C. curvata	-	-	-	-	-	-	+	+	+	+	+	-	±
16	C. claussenii	-	+	+	+	-	-	+	+	+	-	+	-	-
17	C. solani	-	+	-	-	-	±	+	±	+	-	+	-	+
18	C. melibiosi	-	+	±	±	-	+	+	+	+	-	+	-	+
19	C. melibiosi var. membranaefaciens	-	+	±	±	-	+	+	+	+	-	+	-	+

[a]-, absent.

[b]+, fermentation and assimilation of carbohydrates present; -, fermentation and assimilation of carbohydrates absent; and ±, irregular carbohydrate fermentation and assimilation.

[c]+, positive reaction; -, negative reaction; and ±, variable reaction.

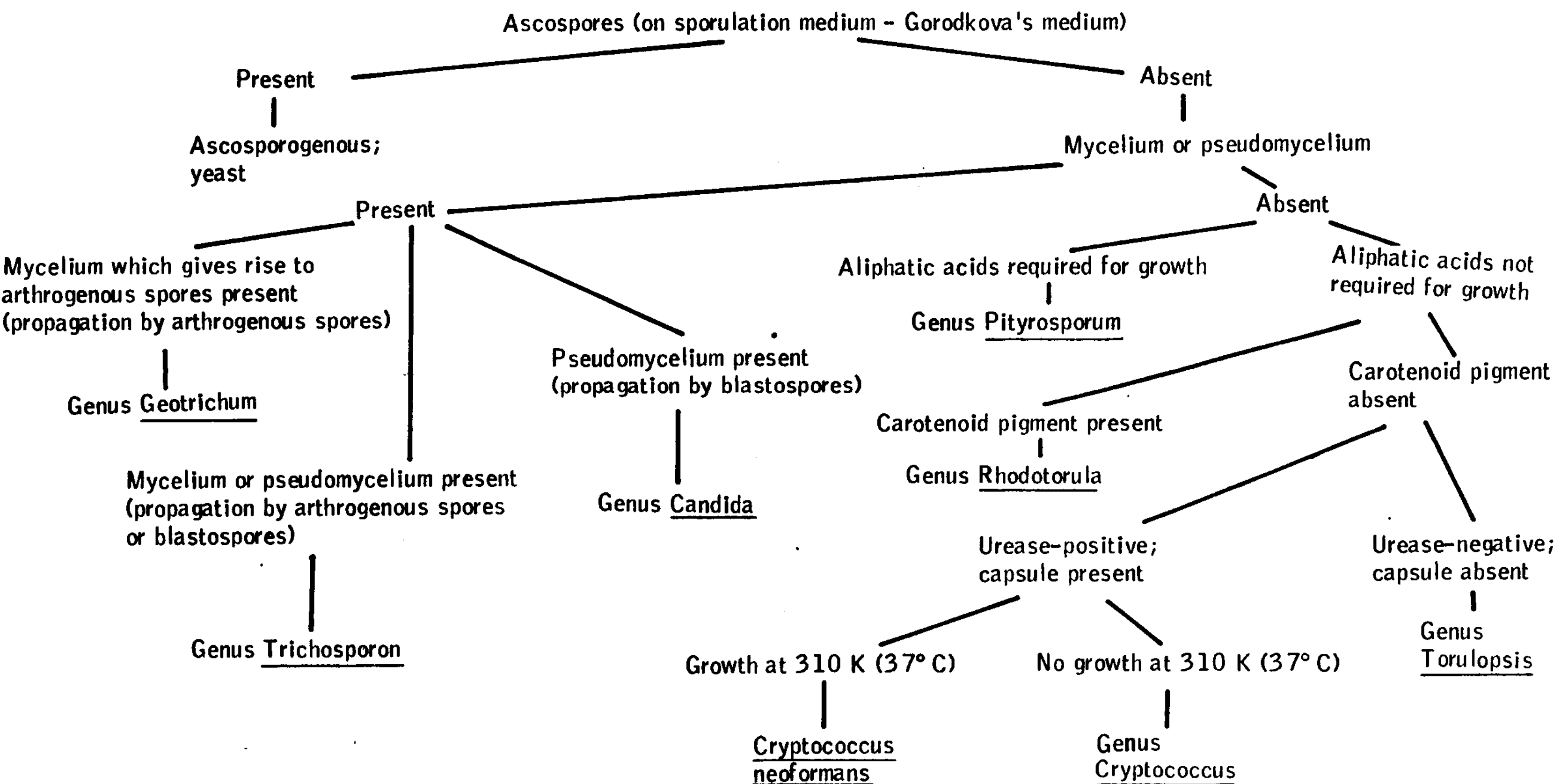

Figure 8-1.- Schematic diagram for identification of yeast and yeastlike microorganisms.

9. IDENTIFICATION OF FILAMENTOUS FUNGI

By Mary R. Henney* and Thomas C. Molina*

9.1 PURPOSE

The purpose of this chapter is to describe methods for identifying filamentous fungi.

9.2 MATERIALS

The following materials are used.

1. Czapek solution agar (medium 57)
2. Potato dextrose agar (medium 58)
3. Cornmeal/malt-extract/yeast-extract (CMMY) agar (medium 56)
4. Malt-extract agar (medium 59)
5. Sabouraud's agar (medium 5)
6. Emerson's yeast phosphate soluble starch agar (medium 60)
7. Cornmeal agar (medium 61)
8. One-half-strength cornmeal agar (medium 62)
9. Lactose yeast agar (medium 79)
10. V-8 juice agar (medium 72)
11. Hay infusion agar (medium 73)
12. Dung decoction agar (medium 74)
13. Plain agar (medium 75)
14. Potato slices (medium 76)

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15. Carrot wedges (medium 77)

16. Corn tips (medium 78)

17. Glass Petri dishes, 100 by 20 millimeters

18. Coverslips, 24 by 50 millimeters, number 1

19. Bent glass rods

20. Lacto-phenol cotton blue (reagent 11)

21. Clear nail polish (reagent 12)

9.3 METHODS

9.3.1 LABORATORY PROCEDURES

The classification of the filamentous fungi is based primarily on the morphological characteristics of the microorganisms. The following procedures are followed to make the appropriate morphological observations.

1. A single colony of each type of filamentous fungus is inoculated onto Czapek solution agar (medium 57), potato dextrose agar (medium 58), CMMY agar (medium 56), malt-extract agar (medium 59), Sabouraud's agar (medium 5), Emerson's yeast phosphate soluble starch agar (medium 60), cornmeal agar (medium 61), and one-half-strength cornmeal agar (medium 62). Plates are incubated for 7 to 28 days.

2. The inoculations are accomplished as follows. A straight wire inoculating needle is heated until the wire glows red. The wire is allowed to cool, and a small portion of mycelium is removed from the filamentous colony under investigation and transferred to the center of the plate to be inoculated.

3. The plates are examined to determine whether the appropriate morphological structures necessary for identification have developed (conidia, ascospores, basidiospores, zygospores, sporangiospores, etc.).

   a. Isolates failing to produce the necessary structures are inoculated onto lactose yeast agar (medium 79), V-8 juice agar (medium 72), hay infusion agar (medium 73), dung decoction agar (medium 74), plain agar (medium 75), potato slices (medium 76), carrot wedges (medium 77), and corn tips (medium 78).

b. Cultures are incubated for 7 to 28 days and examined for appropriate morphological structures.

4. Each type of filamentous fungus is inoculated onto a slide culture as follows.

   a. A piece of filter paper is placed on the bottom of a 100- by 20-millimeter glass Petri dish.

   b. A clean glass microscope slide (25 by 75 millimeters) and coverslip (24 by 50 millimeters, number 1) are placed on a bent glass rod in the bottom of the Petri dish, which is then covered.

   c. This apparatus is sterilized in steam at 394 K (121° C) for 15 minutes.

   d. The CMMY agar plates (medium 56) are prepared with 10 cubic centimeters of agar for each 100- by 20-millimeter plate and allowed to solidify. These plates are used to obtain agar blocks that are 10 millimeters square and 2 millimeters deep (10 by 10 by 2 millimeters).

   e. The block of agar is placed on the sterile slide in the sterile Petri dish using sterile technique.

   f. The center of each of the four sides of the block of agar is inoculated with the organism under investigation.

   g. The inoculated block is covered with the sterile coverslip.

   h. With sterile technique, 8 cubic centimeters of sterile water are added to the bottom of the Petri dish.

   i. The cultures are incubated at 298 K (25° C) until sporulation occurs (from 7 to 14 days).

   j. When spores appear, the coverslip is carefully lifted and laid aside with the mycelial surface upward.

   k. The agar square is lifted from the slide and discarded.

   l. A drop of lacto-phenol cotton blue (reagent 11) is placed on this slide and covered with a clean coverslip.

   m. A clean slide is obtained and a drop of lacto-phenol cotton blue placed near one end.

   n. This slide is covered with the original coverslip with the mycelial surface downward.

o. Excess mounting fluid is blotted away from the coverslips of the two preparations.

p. When dry, the edges are sealed with clear nail polish (reagent 12).

5. A wet mount of each type of filamentous fungus is made as follows.

   a. A drop of lacto-phenol cotton blue is placed in the center of a clean glass 25- by 75-millimeter microscope slide.

   b. A small portion of the mycelium of the microorganism under investigation is removed from the colony.

   c. The material is placed in a drop of lacto-phenol cotton blue.

   d. The mycelium is teased apart using two sterile steel needles.

   e. A 22-millimeter-square coverslip is placed over the mycelium and lacto-phenol cotton blue.

   f. The wet mount is then examined under the compound microscope.

9.3.2 OBSERVATIONS

9.3.2.1 Gross Morphology

Gross morphology is determined as follows.

1. Rate of growth of colony (in millimeters diameter per week)

2. General topography

3. Texture of colony

4. Pigmentation (both surface and reverse)

9.3.2.2 Microscopic Morphology

Microscopic morphology of the filamentous fungi is observed from slide cultures as described in section 9.3.1, item 4, and from teased preparations as described in section 9.3.1, item 5. The following characteristics are considered.

1. Type of mycelium

2. Ontogeny and type of sexual reproductive spores and sporocarps

3. Ontogeny and type of asexual reproductive spores

4. Ontogeny and type of specialized structures including

   a. Pycnidia

   b. Acervuli

   c. Spordochia

   d. Chlamydospores

9.4 IDENTIFICATION

The major groups of filamentous fungi are identified according to the following dichotomous key.

1. Hyphae aseptate, or if septate, then sexual reproduction resulting in the formation of a resting spore . . . . class Zygomycete

11. Hyphae septate; sexual reproduction not resulting in the formation of a resting spore 2, 22

2. Sexual reproduction resulting in the formation of ascospores or basidiospores 3, 33

22. Sexual reproduction lacking 6, 66

3. Spores resulting from karyogamy and meiosis borne in asci 4, 44

33. Spores resulting from karyogamy and meiosis borne on basidia . . . . class Basidiomycetes

4. Asci arising naked; no ascogenous hyphae or ascocarp produced . . . . subclass Hemiascomycetiahe (See ch. 8 for identification of this group.)

44. Ascospores produced in ascocarps, mostly from ascogenous hyphae 5, 55

5. Asci typically unitunicate; if bitunicate, then borne in an apothecium . . . . Euascomycetidae

55. Asci bitunicate; ascocarp and ascostroma . . . . Loculoascomycetidae

6. Reproduction by means of conidia, by oidia, or by budding 7, 77

66. No reproduction structures known . . . Mycelia Sterilia

7. Reproduction by means of conidia borne in pycnidia . . . . Sphaeropsidales

77. Conidia not in pycnidia 8, 88

8. Reproduction by means of conidia borne in acervuli . . . . Melanconiales

88. Reproduction by means of conidia borne otherwise 9, 99

9. Conidia phialospores, produced basipetally in chains or balls from a phialide that does not increase in length with successive spore production; a succession of mouths may be produced by sympodial proliferation to form a polyphialide . . . . Phialosporae

99. Conidia not as in 9 . . . . 10, 1010

10. Conidia aleuriospores, solitary or in botryose clusters, produced by a blowing out of the terminal portion of a hypha or sporogenous cell or conidiophore, seceding with difficulty and then by rupture of the parental hypha, which remains as a fringe around the base of the spore . . . . Aleuriosporae

1010. Conidia not as in 10 . . . . 11, 1111

11. Conidia sympodulospores, never in chains, produced on denticles from a sympodially extending sporogenous cell that elongates to form a rachis or enlarges to form a vesicle with successive spore production . . . . Sympodulosporae

1111. Conidia not as in 11 . . . . 12, 1212

12. Conidia blastospores, produced apically or laterally by budding from a hypha, a sporogenous cell, a conidiophore, or a previously formed conidium; conidia solitary or in acropetal chains, seceding readily . . . . Blastosporae

1212. Conidia not as in 12 . . . . 13, 1313

13. Conidia botryoblastospores, developing simultaneously from the swollen apex (ampulla) of a sporogenous cell, on short denticles, born singly or in acropetal chains . . . . Botryoblastosporae

1313. Conidia not as in 13 . . . . 14, 1414

14. Conidia porospores, produced through minute pores in the wall of the conidiophore or sporogenous cell or previously formed conidium, septate, pigmented solitary or in acropetal chains . . . . Porosporae

1414. Conidia not as in 14 . . . . 15, 1515

15. Conidia annellospores, produced in basipetal succession balls or chains from the apex of a flask-shaped or cylindrical sporogenous cell that increases in length with successive spores to form an elongate, annellated apex; conidia frequently truncate at the base . . . . Annellosporae

1515. Conidia not as in 15 . . . . 16, 1616

16. Conidia arthrospores, produced by basipetal septation and fragmentation of vegetative or sporogenous hyphae, nonseptate, in simple or branching chains . . . . Arthrosporae

1616. Conidia not as in 16 . . . . 17, 1717

17. Conidia meristem arthrospores, produced in basipetal chains from a conidiophore that increases in length by meristematic growth from the base . . . . Meristem Arthrosporae

1717. Conidia meristem blastospores, lens-shaped with conspicuous hyaline rim, darkly pigmented . . . . Meristem Blastosporae

Identification to generic and specific levels will be accomplished according to the classification given in references 9-1 to 9-11.

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10. BACTERIOPHAGE TYPING OF *STAPHYLOCOCCUS AUREUS*

By Suzanne Simpson Ekblad*

10.1 PURPOSE

The purpose of this chapter is to describe methods for typing *Staphylococcus aureus* using bacteriophages.

10.2 MATERIALS

10.2.1 BACTERIOPHAGES

The 22 basic phages grouped as follows are used as recommended by the International Committee on Nomenclature (ref. 10-1).

Group	Phages
I	29, 52, 52A, 79, 80
II	3A, 3C, 55, 71
III	6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85
IV	42D
Miscellaneous	81, 187

10.2.2 KNOWN PROPAGATING BACTERIAL STRAINS

The specific propagating strains of *S. aureus* are classified by the same identifying number used for the phages. A homologous type of *S. aureus* is used as a positive control for each phage type.

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10.2.3 MEDIA

10.2.3.1 Trypticase Soy Broth (Medium 1)

10.2.3.2 Trypticase Soy Agar (Medium 2)

The trypticase soy agar (TSA) plates are prepared with standard 10-centimeter Petri dishes and dried at 309 K (36° C) for 2 hours before use.

10.2.3.3 Heart Infusion Agar (Medium 29)

10.3 METHODS

10.3.1 DETERMINATION OF ROUTINE TEST DILUTION OF PHAGE STOCKS

The following procedure is used.

1. Tenfold dilutions of each phage type are made in trypticase soy broth (TSB) to a final dilution of $1:10^6$. Diluted phages are refrigerated at 277 K (4° C) when not in use.

2. To prepare each *S. aureus* inoculum, one TSB tube is inoculated from a fresh agar slant of the appropriate known propagating strain.

The following procedure is used for plating the phage and *S. aureus*.

1. The known propagating strains inoculated into TSB in step 2 of the preceding procedure are incubated for 4 hours at 309 K (36° C), at which time they will exhibit a moderate but well-defined turbidity.

2. The incubated culture is poured onto the surface of a TSA plate, and the excess is decanted.

3. The inverted rim of the Petri dish base is blotted on sterile absorbent material.

4. The opened, upright plate is allowed to dry for 10 minutes.

5. One drop of each phage dilution is then placed on the appropriate area of the dried agar plate using the template shown in figure 10-1(a) as a guide. A 1-cubic-centimeter syringe with a 0.025-cm^3/drop (26 gage) needle is used for the phage inoculation. The syringe is held perpendicular to the agar surface.

6. After the drops are dry, the Petri plate is inverted into its lid and incubated at 303 K (30° C) for 18 hours.

7. Plates are read by means of transmitted light against a dark background.

8. Reactions are recorded as follows.

Score	Number of plaques
±	1 to 20 plaques
+	21 to 50 plaques
++	More than 50 discrete plaques
+++	Confluent lysis

9. The routine test dilution (RTD) to be used for typing is the highest dilution that produces confluent lysis of the propagating strain on agar (as defined in ref. 10-2).

10.3.2 DETERMINATION OF THE LYTIC SPECTRUM

The lytic spectrum of each phage remains quite constant and provides a measure of the purity of each new lot of phage. The spectrum is determined as follows.

1. The lytic spectrum of each phage is determined by reacting the phage against the homologous propagating strain and all 21 other *S. aureus* strains as detailed in reference 10-3.

2. Each phage must show the same reaction pattern as outlined in table 10-I.

10.3.3 DETERMINATION OF PHAGE TYPES OF UNKNOWN *S. AUREUS* ISOLATES

The following procedure is used.

1. Untyped S. aureus cultures are received on heart infusion agar slants for phage typing.

2. Each TSA slant is subcultured into three TSB tubes.

3. Following incubation at 309 K (36° C) for 4 hours, each TSB broth will exhibit a moderate but well-defined turbidity. The incubated culture is poured onto two TSA Petri plates, and the excess is decanted.

4. Analysis continues as outlined in section 10.3.1, steps 3 and 4.

5. Half of the inoculated plates are spotted with a drop of each phage at its RTD. The remaining plates are spotted with a drop of phage that is 1000 times more concentrated than the RTD (1000x).

6. The phage drops are placed on the appropriate area of the dried agar plate using the template shown in figure 10-1(b) as a guide.

7. Analysis continues as outlined in section 10.3.1, items 6 through 8.

10.4 METHOD OF REPORTING RESULTS

All phage reactions at RTD and 1000x will be reported for each S. aureus isolate on the data sheet illustrated in figure 10-2.

10.5 REFERENCES

10-1. Blair, J. E.; and Parker, M. T.: Report of the Subcommittee on Phage Typing of Staphylococci to the International Committee on Nomenclature of Bacteria. Int. J. Syst. Bacteriol., vol. 17, no. 2, 1967, pp. 113-125.

10-2. Blair, John E.; and Carr, Miriam: The Techniques and Interpretation of Phage Typing of Staphylococci. J. Lab. Clin. Med., vol. 55, 1960, pp. 650-662.

10-3. Blair, J. E.; and Williams, R. E. O.: Phage Typing of the Staphylococci. Bull. World Health Org., vol. 24, 1961, pp. 771-784.

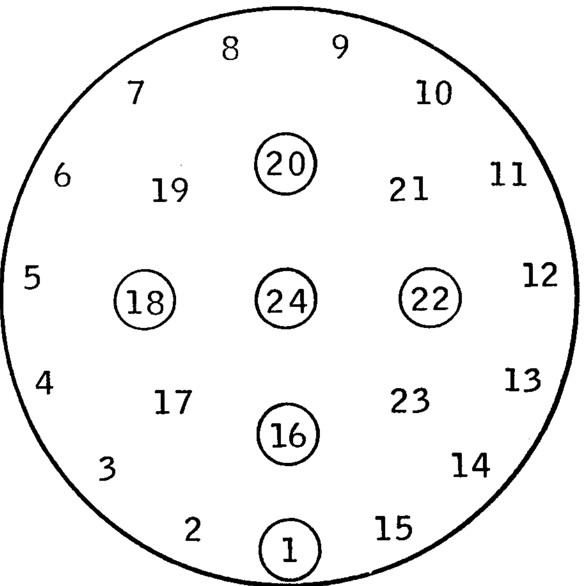
TABLE 10-I.- PHAGE REACTION PATTERNS OF THE REFERENCE *STAPHYLOCOCCUS AUREUS* PROPAGATING AND TEST STRAINS

Propagating strain	Routine test dilution (RTD) (a)	100× RTD (a, b)
29	29+++	29+++ 52°
52	52+++ 52A+ 80++	52+++ 52A+++ 80+++
52A/79	52A+++ 79+++	52± 52A+++ 79+++ 80±
80	80+++ 81+++	80+++ 81+++
3A	3A+++ 3C± 55± 71±	3A+++ 3C++ 55++ 71++
3C	3A+ 3C+++ 55++ 71+++	3A+++ 3C+++ 55+++ 71+++
55	3C++ 55+++ 71+++	3A+ 3C+++ 55+++ 71+++
71	3C++ 55++ 71+++	3C+++ 55+++ 71+++
6	6+++ 42E+ 47+++ 53+++ 54+++ 75+++ 81± 83A++ 85++ [77, 84]	6+++ 42E++ 47+++ 53+++ 54+++ 75+++ 77+++ 81++
42E	42E+++ (81)	6+ 42E+++ 74+ 53+ 54++ 81+++
47	47+++ 53++ 75+++ 77++ 84++ 85++ (29)	6+ [29] 47+++ 53+++ [54] 75+++ 77+++
53	53+++ 54+ 75++ 77++ 84++ 85+++	53+++ 54+++ 75+++ 77+++
54	47+++ 53+++ 54+++ 75+++ 77++ 84++ 85+++ (42E, 81)	42E++ 47+++ 53+++ 54+++ 75+++ 77+++ 81+
75	75+++ 77++ 84++ 85+++	47° 53++ 54° 75+++ 77+++
77	53++ 77+++ [85]	47++/^{o} 53+++ 77+++
c83A	6++ 47++ 53+++ 83A+++ 85++ [84]	6+++ [42E] 47+++ (52) (52A) 53+++ 54+/^{o} 75± 77+++/^{o} 79++ 80++ 81++
c84	84+++ 85++	47° 54° 75°
c85	84++ 85+++	6° 47° 54° 77°
42D	42D+++	42D+++
81	80+++ 81+++	80+++ 81+++
187	187+++	187+++
42C	71+ (3A, 3C)	3A++ 3C++ 71++ 81±
2009	52+++ (29)	29++ 52+++
8719	71+++	71+++

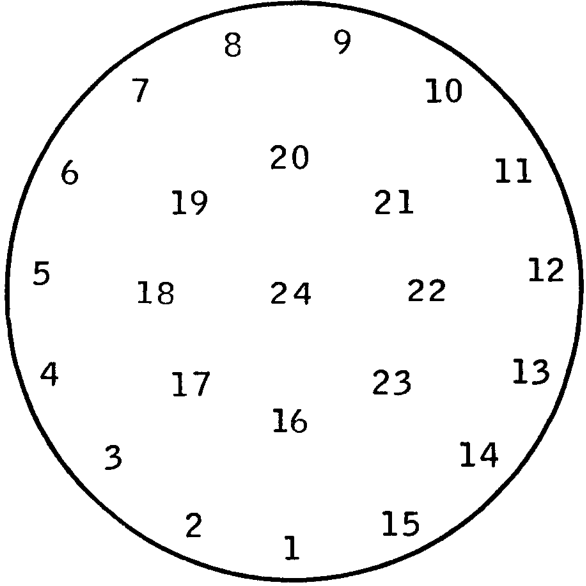
[a] +++, confluent lysis; ++, more then 50 discrete plaques; +, 21 to 50 plaques; ±, fewer than 21 plaques; (), weak reactions that sometimes occur; [], reactions that vary in occurrence and strength.

[b] o, inhibition; ±/o, +/o, ++/o, +++/o, lysis and/or inhibition. Some reactions may appear as inhibition at one time, lysis at another, or a combination of lysis and inhibition.

[c] Phages 83A, 84, and 85 may be used at RTD only.



(a) Template for phage titration.



(b) Template for phage typing.

Figure 10-1.- Templates for bacteriophage drops.

PHAGE TYPES OF Staphylococcus aureus ISOLATED FROM ASTP CREWMEMBERS

DATE RECEIVED________ ASTRONAUT________

RECEIVED FROM________ DATE CULTURED________

DATE REPORTED________ SAMPLE PERIOD________

SAMPLE SITE________

ISOLATE NUMBER________

ISOLATE NUMBERS

Position on Template	Phage nos.							Phase nos.	Position on Template
		RTD	1000X	RTD	1000X	RTD	1000X		
1	3A							3A	1
2	3C							3C	2
3	6							6	3
4	29							29	4
5	42D							42D	5
6	42E							42E	6
7	47							47	7
8	52							52	8
9	52A							52A	9
10	53							53	10
11	54							54	11
12	55							55	12
13	71							71	13
14	75							75	14
15	77							77	15
16	79							79	16
17	80							80	17
18	81							81	18
19	83A							83A	19
20	84							84	20
21	85							85	21
22	187							187	22

ISOLATE READINGS

Figure 10-2.- Data sheet for recording Staphylococcus aureus lysis patterns.

11. STAPHYLOCOCCUS SENSITIVITY TO ANTIBIOTICS

By Ye. A. Ved'mina* and M. M. Shinkareva*

11.1 PURPOSE

The purpose of this chapter is to describe the methods of studying isolated staphylococcus strains in terms of their sensitivity to antibiotics.

11.2 MATERIALS

The following materials are used.

1. Tetracycline
2. Erythromycin
3. Lincomycin
4. Ampicillin
5. Rifamycin
6. Gentamycin
7. Levomycetin
8. Oleandomycin
9. Oxacillin
10. Ceporin
11. Kanamycin
12. Hottinger's agar (medium 64)
13. Hottinger's broth (medium 63)

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11.3 METHODS

11.3.1 ANTIBIOTIC DILUTION

11.3.1.1 Preparation of Basic Antibiotic Solution

Antibiotics in powder form, stored at 277 K (4° C), are used to prepare antibiotic solutions as follows.

1. All the antibiotics are dissolved in distilled water to obtain a particular concentration of the antibiotic in micrograms per cubic centimeter.

2. The basic solution should have a potency of 1280 $\mu g/cm^3$.

3. Each test tube is filled with 2 cubic centimeters of sterile distilled water. A series of antibiotic dilutions is prepared in accordance with table 11-I.

11.3.1.2 Preparation of Antibiotic Dilutions in Nutrient Medium

One part of antibiotic solution is added to nine parts of Hottinger's agar (medium 64). The concentration of the antibiotic solution should be 10 times as high as that of the nutrient medium to be obtained.

11.3.2 DETERMINATION OF SENSITIVITY TO ANTIBIOTICS

Sensitivity to antibiotics is determined as follows.

1. An 18-hour agar (medium 64) culture is standardized and diluted to a concentration of 10^7 microbial cells/cm^3.

2. A culture of the strain being studied (0.001 to 0.002 cubic centimeter, or 20 000 microbial cells) is placed onto the surface of slightly dried Hottinger's agar (medium 64) with the use of a bacteriological loop or a special replicating inoculator. Petri dishes containing Hottinger's agar with no antibiotics added are used for control.

3. The results are analyzed after 18 to 20 hours of incubation at 310 K (37° C). The minimum effective concentration or the maximum growth-retarding concentration is determined by the antibiotic dilution used for the Petri dish with a complete absence of growth, given that the control dish is covered with growth.

TABLE 11-I.- ANTIBIOTIC DILUTION PREPARATION CHART

No.	Directions for preparing antibiotic dilutions	Concentration in the obtained solution, μg/cm^3	Final concentration in a mixture of 1 part antibiotic solution and 9 parts agar, μg/cm^3
1	6.4 parts 2000 μg/cm^3 and 3.6 parts sterile distilled water	1280	128
2	2 parts 1280 μg/cm^3 and 2 parts sterile distilled water	640	64
3	1 part 1280 μg/cm^3 and 3 parts sterile distilled water	320	32
4	1 part 1280 μg/cm^3 and 7 parts sterile distilled water	160	16
5	2 parts 160 μg/cm^3 and 2 parts sterile distilled water	80	8
6	1 part 160 μg/cm^3 and 3 parts sterile distilled water	40	4
7	1 part 160 μg/cm^3 and 7 parts sterile distilled water	20	2
8	2 parts 20 μg/cm^3 and 2 parts sterile distilled water	10	1
9	1 part 20 μg/cm^3 and 3 parts sterile distilled water	5	.5
10	1 part 20 μg/cm^3 and 7 parts sterile distilled water	2.5	.25
11	2 parts 2.5 μg/cm^3 and 2 parts sterile distilled water	1.25	.125
12	1 part 2.5 μg/cm^3 and 3 parts sterile distilled water	.63	.063
13	1 part 2.5 μg/cm^3 and 7 parts sterile distilled water	.31	.031

12. IMMUNOLOGICAL STUDIES WITH BLOOD

By Noel Funderburk,* V. M. Shilov,† L. L. Fadeyeva,‡
and I. V. Konstantinova‡

12.1 PURPOSE

The purpose of this chapter is to describe techniques used to evaluate immunological aspects of crewmember blood.

12.2 ANALYTICAL METHOD FOR MEASUREMENT OF SERUM AGGLUTINATION TITERS

12.2.1 MATERIALS

The following microtiter equipment (ref. 12-1) is used.

1. Disposable "V" plates
2. 50-cubic-millimeter loops
3. 50-cubic-millimeter droppers
4. Plate sealing tape
5. Plate viewer
6. Go/no-go delivery testers

Other material needed is as follows.

1. Isotonic saline solution (8.5 grams sodium chloride (NaCl) per cubic decimeter of distilled water (H_2O))
2. Isotonic saline with safranin (reagent 23)

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3. Bacterial antigen prepared from selected organisms isolated from each crewmember's preflight cultures or from stock cultures

4. Agglutination antigens for selected <u>Salmonella</u> and <u>Brucella</u> microorganisms (reagent 24)

5. Control sera (reagent 25)

6. Incubator, 310 K (37° C)

12.2.2 METHODS

The following methods and procedures are used.

1. Isotonic saline solution (50 cubic millimeters) is placed into all wells of microtiter "V" plates, excluding the first two wells of each row. Antigens prepared from components of the astronaut's and cosmonaut's autoflora are tested using saline diluent with safranin.

2. Test and control sera are diluted initially by adding one part isotonic saline solution to three parts serum.

3. Test serum diluted 1:4 (50 cubic millimeters) is placed into the first well of a row.

4. Control serum diluted 1:4 (50 cubic millimeters) is added to the first well of another row in the same manner as the test serum.

5. Twofold serial dilutions of the diluted sera and control serum will be prepared using 50-cubic-millimeter microtiter loops.

6. Suitably prepared antigen (50 cubic millimeters) is added to all wells.

7. The plate is sealed with transparent sealing tape, and the contents of each well are mixed by gently tapping the plate.

8. The plate is incubated at 310 K (37° C) overnight, followed by 2 hours at room temperature (between 293 and 298 K (20° and 25° C)).

9. The highest dilution of serum that inhibits formation of a compact button of antigen in the bottom of the well is read as the end point. With <u>Haemophilus</u> and <u>Brucella</u> antigens, the highest dilution that causes precipitation of the antigen is recorded.

10. The serum titer is reported as the reciprocal of the highest dilution of serum that gives a positive reaction.

11. The dilutions prepared in the described manner are 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048, 1:4096, and 1:8192.

12. Serum is not transferred into the last well of each row, which serves as an antigen control.

12.2.3 PREPARATION OF MICROBIAL ANTIGENS FOR AGGLUTINATION TESTING

12.2.3.1 Materials

The following materials are used.

1. Culture of microorganisms selected from crewmember's autoflora
2. Erlenmeyer flask, 250-cubic-centimeter
3. Mueller-Hinton broth
4. Fluid thioglycollate medium
5. Formalin-saline solution (reagent 26)
6. Saline-thimerosal solution (reagent 27)
7. Sterile glass beads, 3-millimeter-diameter
8. Sterile screwcapped tubes, 16 by 125 millimeters
9. Sterile Pasteur pipettes
10. McFarland turbidity standards (ref. 12-2)

12.2.3.2 Methods

The following methods and procedures are used.

1. Cultures of microorganisms selected from crewmember autoflora are prepared by inoculating 100 cubic centimeters of Mueller-Hinton broth.

2. These cultures are incubated at 310 K (37° C) for 18 to 24 hours.

3. After incubation, the cultures are centrifuged and decanted, and the growth is suspended in formalin-saline solution.

4. The suspension is transferred to a sterile 16- by 125-millimeter screwcapped tube containing several glass beads.

5. The antigen suspension is cultured in fluid thioglycollate medium or other suitable medium to determine sterility.

6. If no growth is obtained on cultures, the antigen is stored at 277 K (4° C).

7. Before use, the antigen suspension is further diluted with sterile isotonic saline to a turbidity equal to a number 3 McFarland standard.

8. This antigen suspension is visually inspected to see that it does not contain clumps or large particles. A suitable suspension is opalescent in appearance.

12.2.4 QUALITY CONTROL

The following quality control measures are observed.

1. To minimize variation in test results due to technique, reagents, or other variables, all serum samples are frozen immediately after collection. Preflight and postflight sera are tested simultaneously.

2. Control sera are tested concurrently with the test sera to ensure that the test is operating properly.

3. A saline control is tested with each series of tests to determine spontaneous agglutination of the antigen suspension.

12.2.5 DATA ANALYSIS

The serum antibody titers are compared between individuals and between astronaut/cosmonaut groupings before and after flight. Correlations among autoflora changes, blood leukocyte counts, and muramidase levels are noted.

12.3 ANALYTICAL METHODS FOR LEUKOCYTE DIFFERENTIAL COUNT

12.3.1 MATERIALS

The following materials are used.

1. Whole anticoagulated venous blood
2. 25- by 75-millimeter microscope slides
3. Staining tray
4. Wright's stain solutions (ref. 12-3)
5. Microscope

12.3.2 METHODS

To obtain the leukocyte count, methods outlined in the following paragraphs will be used.

12.3.2.1 Preparation of Blood Smears

Blood smears will be prepared as follows.

1. Place a 2-millimeter-diameter drop of blood onto a microscope slide, 2 centimeters from one end.
2. Place slide on table and immediately place the end of another slide (spreader slide) against the surface of the first slide, holding the spreader slide at a 45° angle.
3. Draw spreader slide against the drop of blood, spreading blood across the surface between the two slides.
4. Push the spreader slide slowly and steadily across the first slide causing the blood to follow in a thin film.
5. Dry the film rapidly by waving the slide in air; do not dry with heat.

12.3.2.2 Staining of Smears

Smears are stained in the following manner.

1. Cover the dried blood smear with Wright's stain (ref. 12-3) and let stand approximately 2 minutes. (Times are determined empirically for each lot of stain.)

2. Add an equal amount of Wright's buffer (ref. 12-3), distributing it over the entire slide.

3. Mix the stain and the buffer by blowing gently on the surface. The mixture should show a metallic sheen.

4. Let this mixture stand approximately 5 minutes. (Time is empirically determined for each batch of stain and buffer.)

5. Wash the stain from the slide with tapwater.

6. Wipe the stain from the back of the slide with a tissue.

7. Stand the slide on end and air dry.

12.3.2.3 Making the Differential Count

The differential count is made as follows.

1. Microscopically examine 500 consecutive leukocytes on the stained blood film using an oil immersion objective of approximately 1000× magnification. The nomenclature and the cell types are identified as outlined in reference 12-4. Classify and record the number of each cell type.

2. Divide the quantity of each cell type by 5 to determine the relative percentage.

12.3.3 QUALITY CONTROL

Three basic guidelines are followed to ensure quality control of the leukocyte count.

1. The leukocytes must be counted on areas of the smears where the erythrocytes are just touching each other, without overlapping. Do not count cells at the edges or ends of the smear.

2. The stain must be of sufficient quality to permit accurate identification of the cell types.

3. 500 cells are examined.

12.3.4 DATA ANALYSIS

The data are correlated with changes in the autoflora, serum muramidase levels, results of the nitroblue tetrazolium dye test, and serum antibody titers. These comparisons are made between individuals and astronaut/cosmonaut groups during inflight, preflight, and postflight sample periods, and between real-time and baseline values.

12.4 DETERMINATION OF BLOOD SERUM BACTERICIDAL PROPERTIES

12.4.1 MATERIALS

The following materials are used.

1. *Escherichia coli* culture (sec. 12.4.4, item 1)
2. Petri dishes with Endo medium (medium 28) (sec. 12.4.4, item 2)
3. Incubation chamber, 310 K (37° C)
4. Sterile 0.85-percent saline solution

12.4.2 METHODS

The following methods and procedures are used.

1. A 0.25-cubic-centimeter portion of the study serum is mixed with 0.01 cubic centimeter of *E. coli* suspension.
2. The mixture, in 0.1-cubic-centimeter amounts, is inoculated onto Endo medium both immediately after preparation and following incubation at 310 K (37° C) for 1 hour.
3. The bactericidal property of the serum is evaluated by the percentage of cells killed within 1 hour of incubation, calculated from the difference in number between the colonies grown in two harvests.

12.4.3 DATA ANALYSIS

Values of serum bactericidal activity are compared between individual astronauts and astronaut groups before and after flight.

12.4.4 SPECIFICATION

The following specification applies.

1. Suspension of a 1-day-old _E. coli_ agar culture (strain 675), containing 5000 cells per 1.0 cubic centimeter, is used.

2. Endo medium is prepared from a dry nutrient medium using 7.0 grams per 100 cubic centimeters of distilled water. The medium is contained in Petri dishes.

12.5 DETERMINATION OF PHAGOCYTIC ACTIVITY OF BLOOD NEUTROPHILS

12.5.1 MATERIALS

The following materials are used.

1. 3.8-percent solution of sodium citrate
2. Cultures of _Staphylococcus_ (sec. 12.5.4, item 1)
3. Small agglutination tubes
4. Slides
5. Azure-eosin dye (sec. 12.5.4, item 2)
6. Incubation chamber, 310 K (37° C)
7. Methanol
8. Sterile 0.85-percent saline solution

12.5.2 METHODS

The following methods and procedures are used.

1. 0.1 cubic centimeter of blood collected from a finger or vein is mixed with 0.03 cubic centimeter of 3.8-percent solution of sodium citrate.

2. 0.03 cubic centimeter of _Staphylococcus_ suspension is added to the mixture.

3. The mixture is incubated at 310 K (37° C) for 30 minutes.

4. Smears similar to blood smears required for clinical analysis are prepared. The smears are fixed in methanol for 5 to 10 minutes, dried, and stained with azure-eosin dye for 20 minutes.

5. The phagocytic activity of neutrophils is evaluated according to the percentage of active phagocytes and the phagocytic number (the mean number of cocci trapped by one active neutrophil).

12.5.3 DATA ANALYSIS

Values of phagocytic activity of blood neutrophils are compared between individual astronauts and astronaut groups before and after flight.

12.5.4 SPECIFICATION

The following specification applies.

1. A suspension of a 1-day-old agar culture of Staphylococcus (Lepin strain), containing 1×10^9 bacterial cells in a cubic centimeter, is used in the reaction.

2. Water-soluble azure-eosin dye is prepared by mixing 10 cubic centimeters of azure and 8 cubic centimeters of eosin with one drop of a 2-percent solution of sodium bicarbonate.

12.6 DETERMINATION OF ANTIBODY TITERS FOR INFLUENZA A_2 AND B VIRUSES

12.6.1 HEMAGGLUTINATION INHIBITION RESPONSE

12.6.1.1 Materials

The following materials are used.

1. Hardware

   a. Standard plexiglass plates with wells for serological reactions

   b. Graduated 1.0- and 2.0-cubic-centimeter micropipettes, 0.2-cubic-centimeter micropipettes, and Pasteur pipettes

   c. Automatic syringe for serial dilutions of sera

2. Isotonic saline solution (8.5 grams NaCl per cubic decimeter distilled H_2O)

3. Kaolin powder

4. Viral antibodies (Commercially available; see sec. 12.6.3.1, item 5.)

5. Cockerel red blood cells (sec. 12.6.3.2, item 1)

6. Control sera (sec. 12.6.3.3)

7. Device for serum inactivation

8. Incubator, 310 K (37° C)

12.6.1.2 Methods

The following methods and procedures are used.

1. 0.25 cubic centimeter of isotonic saline is dispensed into each well except the first of each row. The first wells are filled with 0.5 cubic centimeter of the test serum diluted to 1:10, then twofold serial dilutions in 0.25-cubic-centimeter volumes will be performed.

2. Specific control serum with the known titer is placed and diluted in a similar way in another row of wells. In addition, some individual wells are filled with 0.25 cubic centimeter of isotonic saline solution, 0.25 cubic centimeter of normal donor blood serum free of specific antibodies, 0.25 cubic centimeter of isotonic saline solution, and so forth.

3. 0.25 cubic centimeter of working viral dilution containing 4 agglutination units (sec. 12.6.3.2, item 6) is added to each well except those containing 0.25 cubic centimeter of isotonic saline solution, gently shaken, and incubated at 310 K (37° C) for 1 hour.

4. 0.5 cubic centimeter of 1-percent suspension of cockerel red blood cells is added to each well, mixed by gently rocking the plexiglass plate, and incubated at room temperature (293 to 298 K (20° to 25° C)) for 1 hour.

5. The highest serum dilution that completely inhibits red blood cell agglutination is considered the end point of the reaction.

6. The highest serum dilution that provides a positive reaction is considered the titer of the serum.

7. Serum dilutions obtained by the described method are in the range of 1:10 to 1:1280. In the case of serum reactivity at each level of dilution, a retitration is performed using higher dilutions.

12.6.2 TREATMENT OF THE TEST SERUM

The test serum is treated as follows.

1. The whole serum is inactivated at 329 K (56° C) for 30 minutes.

2. To one volume of the inactivated serum is added an equal volume of 25-percent kaolin suspension in isotonic saline. The mixture is shaken in a schuttel apparatus for 30 minutes and twice centrifuged at 209 rad/sec (2000 rpm) for 5 minutes, and the sediment is discarded. The supernatant fluid is diluted to 1:10 (with account of a twofold serum dilution after the addition of kaolin).

12.6.3 PREPARATION OF RED BLOOD CELLS AND TITRATION OF THE VIRAL ANTIGEN WORKING DOSE

12.6.3.1 Materials

The following materials are required.

1. Sterile flasks containing glass beads

2. Empty sterile flasks

3. Sterile gauze

4. Donor cockerels (White Leghorn)

5. Viral antigens (international diagnostic-standard viruses) of influenza A_2 (Port Chalmers/I/73) and B (Yamagata/I/73) (available from the Institute of Virusology, U.S.S.R. Academy of Sciences, Moscow)

6. The remaining materials indicated in section 12.6.1.1

12.6.3.2 Methods

The following methods and procedures are used.

1. The cockerel heart blood obtained by catheterization is dispensed into a flask with beads, shaken for 5 to 10 minutes to remove fibrin, and filtered through a double gauze layer into another flask without beads.

2. The red blood cells are rinsed three times with isotonic saline during centrifugation at 157 rad/sec (1500 rpm) for 15 minutes. The supernatant fluid is discarded. The sediment is used to obtain a 1-percent suspension of red blood cells in isotonic saline solution.

3. 0.5 cubic centimeter of isotonic saline is placed into each but the first well of the plexiglass plate; 0.9 cubic centimeter of isotonic saline is dispensed into the first well.

4. After 0.1 cubic centimeter of corresponding viral antigen is added into the first well, the viral dilution is successively transferred from well to well of each row in 0.5-cubic-centimeter volumes. The last viral dilution will be kept as a reserve.

5. 0.5 cubic centimeter of a 1-percent suspension of cockerel red blood cells is added into each well, mixed with the well content by gentle agitation of the plate, and incubated at room temperature (293 to 298 K (20° to 25° C)) for 45 minutes.

6. The highest viral dilution that provides a distinct agglutination of the red blood cells at confluent lysis is taken as 1 agglutination unit (AU).

7. The control mixture consists of 0.5 cubic centimeter of isotonic saline solution and 0.5 cubic centimeter of a 1-percent red blood cell suspension.

8. The viral dilutions in one row of the plate are from 1:10 to 1:1280. If the positive reaction exceeds the limits of these dilutions, the spare well is used for further serial dilution. The titration is conducted in a similar way.

12.6.3.3 Sera

The control sera containing antibodies for influenza viruses A_2 (Port Chalmers/I/73) and B (Yamagata/I/73) (sec. 12.6.1.2, item 2) are available from the Institute of Virusology, U.S.S.R. Academy of Sciences, Moscow, and the Institute of Vaccines and Sera, U.S.S.R. Ministry of Health, Moscow; the donor sera, free

of antibodies (sec. 12.6.1.2, item 2), are available from the Institute of Viral Preparations, U.S.S.R. Ministry of Health, Moscow.

12.7 IDENTIFICATION OF ANTIBODIES FOR THE MEASLES VIRUSES

12.7.1 HEMAGGLUTINATION INHIBITION RESPONSE

12.7.1.1 Materials

The following materials are required.

1. Those listed in sections 12.6.1.1 and 12.6.3.1
2. The red blood cells of green monkeys or macaques instead of those of cockerels
3. Viral antigens (Commercially available; see sec. 12.6.3.1, item 5.)
4. Control sera (sec. 12.6.3.3)
5. Incubator, 277 K (4° C)

12.7.1.2 Methods

The following methods and procedures are used.

1. Generally the same as in sections 12.6.1.2 and 12.6.3.2.
2. Instead of 0.25 and 0.5 cubic centimeter, 0.2 and 0.4 cubic centimeter of ingredients, respectively, will be added into the wells.
3. Instead of 0.5 cubic centimeter of a 1-percent suspension of cockerel red blood cells, 0.4 cubic centimeter of a 0.5-percent suspension of monkey red blood cells is used.

12.7.2 TREATMENT OF THE TEST SERUM

The test serum is treated as follows.

1. After the inactivation, the serum is not treated with kaolin. An equal amount of 10-percent suspension of rinsed monkey's red blood cells is added, mixed, and incubated overnight at 277 K (4° C).

2. The mixture is centrifuged once (sec. 12.6.3.2, item 2), and the supernatant fluid is studied with account of the dilution already performed (1:2).

12.7.3 PREPARATION OF RED BLOOD CELLS AND TITRATION OF VIRAL ANTIGEN WORKING DOSE

Preparation and titration are performed in the same way as described in section 12.6.3, with corrections for monkey red blood cells, their concentration (0.5 percent), measles antigens, and corresponding volume changes (0.4-cubic-centimeter amounts of red blood cells, 0.2-cubic-centimeter amounts of antigens in saline solution).

The control sera containing antibodies against measles viruses are available from the U.S.S.R. Institute of Vaccines and Sera, Moscow, and the donor sera, free of antibodies, from the U.S.S.R. Institute of Viral Preparations, Moscow. Viral antigens (commercial diagnostic-standard viruses of the measles) are available from the U.S.S.R. Institute of Viral Preparations, Moscow.

12.7.4 QUALITY CONTROL

The following quality control measures will be observed.

1. To minimize variation in test results, all serum samples from astronauts are frozen immediately after collection and stored at 263 K (-10° C). Preflight and postflight sera are tested simultaneously.
2. All tests are performed in duplicate or in triplicate depending on the quantity of serum.
3. All necessary kinds of control are provided to avoid possible technical errors.
4. The serum antibody titers are compared between individual astronauts and groups of astronauts before and after flight.

12.8 ANALYTICAL METHOD FOR MEASUREMENT OF SERUM MURAMIDASE

12.8.1 MATERIALS

The following materials and facilities are required.

1. Buffer solution, phosphate, 0.15-molar, pH 6.4 (reagent 18)
2. Muramidase substrate plates (reagent 19)
3. Control serum (reagent 20)
4. Muramidase standard solutions (reagent 21)
5. Photographic printing paper (reagent 22)
6. Photographic developer, stop bath, and fixative solutions
7. Photographic darkroom facilities
8. Semilogarithmic graph paper

12.8.2 METHODS

The following methods and procedures are used.

1. Muramidase standard solutions are placed into the first three wells of a substrate plate.
2. Another well is filled with control serum.
3. Remaining wells are filled with test sera.
4. The plates are allowed to stand at room temperature (293 to 298 K (20° to 25° C)) for 16 to 18 hours.
5. Three cubic centimeters tapwater are added to each plate, and the plate is placed into a photographic enlarger as a photographic negative.
6. The enlarger is set so that the projected image will be approximately 10 centimeters in diameter.
7. The photographic print paper is exposed and processed using empirically determined times.
8. The diameter of clear zones around each well is measured from the photographic print using a millimeter scale.
9. A standard curve is prepared by plotting the muramidase activity in each standard solution as a function of the zone diameter, using semilogarithmic paper. (The points should fall in a straight line.)
10. The values for each of the test sera and the control serum are determined from the standard curve.

12.8.3 QUALITY CONTROL

The following quality control measures are observed.

1. Each test serum is tested three times (assuming the quantity of serum is sufficient).

2. Pooled human serum is run with each substrate plate.

3. The precision of the test is determined by replicate testing of pooled serum. From these data, the mean value of the serum pool together with the one- and two-standard-deviation range are calculated.

4. Test sera are stored frozen at a temperature no higher than 253 K (-20° C) and tested simultaneously to minimize the effects of technique, reagents, and other variables.

12.8.4 DATA ANALYSIS

The values of serum muramidase are compared between individuals and groups, before and after flight. In addition, values are correlated with changes in the autoflora, blood leukocyte counts, and serum antibody titers.

12.9 DETERMINATION OF THYMUS-DEPENDENT LYMPHOCYTE (T-CELL) CONTENT IN BLOOD

12.9.1 MATERIALS

The following materials are required.

1. Hank's solution

2. Phycoll-hypate (density, 1.080 g/cm^3)

3. Heparin, free of preservative

4. Sheep red blood cells

5. Glutaraldehyde

6. Pyronine

7. Methyl green

8. 3-percent solution of acetic acid

9. Hemocytometer

12.9.2 METHODS

The following methods and procedures are used.

1. Venous blood is collected into test tubes which contain heparin (25 units per cubic centimeter of blood).

2. To obtain the leukocyte count, 9.9 cubic centimeters of 3-percent acetic acid are added to 0.1 cubic centimeter of heparinized blood, then the content of nucleated cells in blood is counted in a hemocytometer.

3. A blood smear is prepared and stained, and a differential count is made.

4. A suspension of mononuclear cells is obtained by separating a leukocyte suspension on phycoll-hypate, as follows.

   a. Heparinized blood is diluted (1:3) with Hank's solution and layered above the phycoll-hypate solution (density, 1.080 g/cm^3).

   b. The mixture is centrifuged at 400g for 20 minutes at 291 K (18° C).

   c. Mononuclear cells are collected from the interface layer.

   d. The obtained suspension of lymphocytes is diluted 1:5 with Hank's solution and centrifuged at 400g for 10 minutes.

   e. Following the removal of the supernatant fluid, the cells are twice washed with Hank's solution at 200g for 5 minutes.

   f. Using Hank's solution, the concentration of the washed suspension is brought to 2×10^6 $cells/cm^3$.

5. The reaction of spontaneous rosette formation (method for obtaining E-rosettes) includes the following procedures.

   a. Three-times-washed sheep red blood cells are diluted with Hank's solution to obtain a 0.4-percent cell suspension.

b. To 0.1 cubic centimeter of 0.4-percent suspension of sheep red blood cells in a siliconized 3-cubic-centimeter test tube, 0.1 cubic centimeter of lymphocyte suspension is added.

c. After incubating the suspension at 310 K (37° C) for 5 minutes, the test tube is centrifuged at 400g for 5 minutes.

d. The suspension is maintained at 277 to 285 K (4° to 12° C) for 60 minutes, without agitation.

e. Following the removal of the supernatant fluid, glutaraldehyde (0.05 cubic centimeter of a 0.6-percent solution) is added to the cell precipitate for 20 minutes.

f. After the cells have been washed, smears are prepared on microscope slides, fixed in methanol, and stained with methyl green-pyronine.

g. For each blood sample, two smears are examined, 200 to 300 lymphocytes on each slide. Simultaneously, the percentage of lymphocytes with three or more sheep erythrocytes agglutinated to their surfaces (percentage of rosette-forming cells or rosettes) is calculated.

12.9.3 QUALITY CONTROL

The following quality control measures are observed.

1. The analysis of the samples should be performed within 24 hours after blood collection.

2. After the fraction of mononuclear cells has been isolated, a test is conducted to determine the percentage of nonviable cells. For this test, 0.1 cubic centimeter of a 1-percent solution of trypan blue is added to 0.1 cubic centimeter of cell suspension and, after a 30-minute exposure in a hemocytometer at room temperature, the percentage of stained cells is determined. The lymphocyte suspension must contain no more than 5 percent of nonviable cells.

12.9.4 DATA ANALYSIS

Data analysis is performed as follows.

1. Based on the number of nucleated cells (cells per cubic millimeter) and the percentage of all lymphocytes and T-lymphocytes among nucleated cells, the absolute number of thymus-independent lymphocytes is counted for each sample.
2. The results of each individual postflight test are compared with the preflight results as well as with a statistical average obtained from 30 to 50 healthy individuals, using the same method.
3. Preflight and postflight crew averages are compared.

12.10 DETERMINATION OF THYMUS-INDEPENDENT LYMPHOCYTE (B-CELL) CONTENT IN BLOOD BY THE PRESENCE OF C-RECEPTORS ON THEIR SURFACES

12.10.1 MATERIALS

The following materials are required.

1. Hank's solution
2. Bull's erythrocytes
3. Specific rabbit antiserum against bull's erythrocytes
4. Glutaraldehyde
5. Methyl green-pyronine
6. Hemocytometer

12.10.2 METHODS

The following methods and procedures are used.

1. Bull's erythrocytes are twice washed in Hank's solution and centrifuged at 400g for 5 minutes.
2. A 2.5-percent suspension of washed bull's erythrocytes is prepared, using Hank's solution.

3. Two cubic centimeters of rabbit antiserum against bull's erythrocytes in a subagglutinating dilution are added to 2 cubic centimeters of the 2.5-percent suspension of bull's erythrocytes.

4. The mixture is incubated at 310 K (37° C) for 30 minutes. During incubation, the mixture is agitated every 5 to 7 minutes.

5. Following the incubation, the erythrocytes are twice washed with Hank's solution; the cells are centrifuged at 400g for 5 minutes; then, a 0.5-percent suspension is prepared.

6. To 0.1 cubic centimeter of the 0.5-percent suspension of bull's erythrocytes, placed into a siliconized vial, 0.1 cubic centimeter of suspension (containing 2×10^6 cells/cm^3) of lymphocytes obtained from the test subject is added. For suspension preparation, see section 12.9.2, items 4a to 4f.

7. The mixture is incubated at 310 K (37° C) for 5 minutes, centrifuged at 400g for 5 minutes, and incubated at 285 K (12° C) for 60 minutes.

8. After centrifugation, 0.05 cubic centimeter of a 0.6-percent solution of glutaraldehyde is added to the cell precipitate.

9. The fixed cells are smeared onto microscope slides.

10. The smears are fixed in methanol and stained with methyl green-pyronine.

11. The preparations are viewed at 1000x, 200 to 300 lymphocytes on each slide are examined, and the percentage of indirect B-rosettes (lymphocytes with 3 or more erythrocytes agglutinated to their surfaces) is determined.

12.10.3 QUALITY CONTROL

For quality control measures, see section 12.9.3.

12.10.4 DATA ANALYSIS

For data analysis procedures, see section 12.9.4.

12.11 DETERMINATION OF THYMUS-INDEPENDENT LYMPHOCYTES (B-CELLS) BY THE PRESENCE OF IMMUNOGLOBULIN RECEPTORS ON THEIR SURFACES

12.11.1 MATERIALS

The following materials are required.

1. Rabbit antiserum against human gamma globulin, labeled with fluoresceinisothiocyanate
2. Phosphate buffer solution (pH 7.2 and pH 7.4)
3. Rabbit antiserum against human IgA, labeled with fluoresceinisothiocyanate
4. Rabbit antiserum against human IgG, labeled with fluoresceinisothiocyanate
5. Rabbit antiserum against human IgM, labeled with fluoresceinisothiocyanate
6. Phycoll-hypate
7. Fluorescent microscope
8. Glycerin-phosphate mixture, pH 7.2
9. Microscope slides with Formvar coating
10. Paraffin

12.11.2 METHODS

The following methods and procedures are used.

1. The fraction of pure lymphocytes is isolated from the peripheral blood in density gradient, using phycoll-hypate. (See sec. 12.9.2, items 4a to 4f.)
2. Formvar-coated slides are each smeared with 0.5 cubic centimeter of lymphocyte suspension, containing 2×10^3 cells/cm^3, and placed into a wet chamber at 310 K (37° C) for 15 minutes.
3. The cells that do not stick to the glass are washed off with Hank's solution.

4. The layer of cells is slightly dried and a drop of antiserum (polyvalent or against one of the three types of immunoglobulins) is deposited onto it. The preparations with the stain are placed into a moisture chamber at 291 K (18° C) for 20 minutes.

5. The stained preparations are washed in six changes of phosphate buffer, pH 7.2, dried with filter paper, and placed into glycerin buffer, pH 7.4.

6. The coverslip is edged with melted paraffin.

7. The preparations are examined in a fluorescent microscope, 200 to 300 cells are counted, and the percentage of lymphocytes with a luminescent surface is determined.

12.11.3 QUALITY CONTROL

The following quality control measures are taken.

1. Before the analysis, the specificity of the antiserum that is conjugated with fluorochrome is checked by the agglutination reaction of those erythrocytes which are loaded with immunoglobulin of homologous type.

2. The analysis of content of B-lymphocytes which carry on their surfaces immunoglobulin receptors is performed within 24 hours after blood collection.

12.11.4 DATA ANALYSIS

The results are compared between individuals and between astronaut/cosmonaut groupings before and after flight.

12.12 DETERMINATION OF THE FUNCTIONAL TYPE OF THYMUS-DEPENDENT LYMPHOCYTES (T-CELLS) BY THEIR ABILITY FOR PHYTOHEMAGGLUTININ BLAST TRANSFORMATION

12.12.1 MATERIALS

The following materials are required.

1. Heparin, free of preservative

2. Hank's medium

3. Phytohemagglutinin (PHA)

4. Glacial acetic acid

5. 3-percent acetic acid

6. Uridine-H^3 (specific activity: 37×10^{12} to 185×10^{12} dis/(sec•mol) (1 to 5 Ci/mmol))

7. Thymidine-H^3 (specific activity: 37×10^{12} dis/(sec.mol) (1 Ci/mmol))

8. Sensitive nuclear emulsion (NIKFI[1] or Kodak)

9. Methyl green-pyronine

10. Hemocytometer

12.12.2 METHODS

The following methods and procedures are used.

1. Three cubic centimeters of heparinized venous blood are placed into an incubator for 60 minutes at 310 K (37° C).

2. The supernatant leukocyte-containing plasma is collected with the use of a pipette.

3. Using Hank's medium, the concentration of leukocytes in the plasma is brought to 1.5×10^6 to 2×10^6 cells/cm^3.

4. The cell suspension is dispensed in quantities of 0.1 to 0.5 cubic centimeter into flasks.

5. To the flasks with the test material, PHA Difco P (10 to 50 μg/cm^3) is added. To the flasks with the control material, an equal volume of Hank's medium is added.

6. The flasks are placed into an incubator at 310 K (37° C).

7. After 23 hours of incubation, uridine-H^3 is added to half of the test and control flasks. Thymidine-H^3 (37×10^6 dis/(sec•cm^3) (1 μCi/cm^3)) is added to the remaining flasks after 44 hours of incubation.

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[1]Motion Picture and Photography Scientific Research Institute.

8. The cultures with the isotope are incubated in a water bath at 310 K (37° C) for 4 hours.

9. To remove the isotope from the cell suspension, the latter is thrice washed with Hank's medium. Then, the suspension is smeared onto microscope slides.

10. After the smears have been fixed, they are covered with emulsion in the dark and exposed in the dark for 3 to 5 days. Then, the smears are stained with methyl green-pyronine.

11. Not less than three slides are viewed (with oil immersion) for each sample.

12. In smears with uridine-H^3, 300 cells of the lymphoid series are examined and the number of lymphoid cells, each containing more than 50 granules, as well as the average number of granules per lymphocyte are determined.

13. In smears with thymidine-H^3, not less than 1000 lymphocytes are examined and the percentage of labeled cells is determined.

12.12.3 QUALITY CONTROL

The following quality control measures are taken.

1. The lymphocyte reactivity analysis must be performed within 2 days after blood collection, the blood is preserved at 274 to 277 K (1° to 4° C).

2. The period of time between blood collection and beginning of the analysis must be equal for preflight and postflight tests.

3. To ensure that the test is operating properly, a full analysis of a blood sample collected from a healthy individual with known characteristics of T-lymphocyte reactivity is conducted concurrently with the analyses of blood samples collected from crewmembers.

4. Control and test samples are examined simultaneously.

12.12.4 DATA ANALYSIS

The results of preflight and postflight analyses are compared between individuals and between crews.

12.13 DETERMINATION OF THE FUNCTIONAL TYPE OF THYMUS-INDEPENDENT LYMPHOCYTES (B-CELLS) BY THEIR ABILITY FOR BLAST TRANSFORMATION AFTER CONTACT WITH POKEWEED MITOGEN

12.13.1 MATERIALS

The following materials are required.

1. Heparin, free of preservative
2. Hank's medium
3. Pokeweed mitogen
4. Glacial acetic acid
5. 3-percent acetic acid
6. Thymidine-H^3
7. Sensitive nuclear emulsion (NIKFI or Kodak)
8. Methyl green-pyronine
9. Hemocytometer

12.13.2 METHODS

The following methods and procedures are used.

1. Three cubic centimeters of heparinized venous blood is allowed to settle in an incubator at 310 K (37° C) for 60 minutes.

2. The supernatant leukocyte-containing plasma is collected with a pipette.

3. By adding Hank's medium, the leukocyte concentration is brought to 1.5×10^6 to 2.0×10^6 cells/cm^3.

4. The cell suspension is dispensed in quantities of 0.1 to 0.5 cubic centimeter into several flasks.

5. To the flasks with the test material, an equal amount of pokeweed mitogen (5 cubic centimeters of basic solution diluted 1:5 and 1:10) is added. To the flasks with control material, Hank's medium is added.

6. The flasks are placed into an incubator at 310 K (37° C) for 5 days.

7. For the last 4 hours of incubation, thymidine-H^3 (37×10^6 dis/(sec•cm^3) (1 $\mu Ci/cm^3$)) is added to all flasks.

8. Following the incubation, the cell suspension is thrice washed with Hank's medium, then smeared onto glass slides.

9. After the slides have been fixed, they are covered with emulsion, exposed for 3 to 5 days, developed, and then stained with methyl green-pyronine.

10. Two or three preparations of each culture are examined at 1000× magnification. To determine the percentage of labeled cells, 1000 lymphocytes are examined.

12.13.3 QUALITY CONTROL

The quality control measures listed in section 12.12.3 are observed.

12.13.4 DATA ANALYSIS

The results of preflight and postflight analyses are compared between individuals and between crews.

12.14 DETERMINATION OF THE QUANTITATIVE CONTENT OF IMMUNOGLOBULINS (IgG, IgA, IgM) IN BLOOD SERUM

12.14.1 MATERIALS

The following materials are required.

1. Immunodiffusion plates (Tri-Partigen-IgA, IgG, IgM), manufactured by Behringwerke, West Germany

2. Standard blood serums, Behringwerke, West Germany

3. Veronal/barbital buffer solution, pH 8.6

4. Slide rule, manufactured by Behringwerke, West Germany

5. 10-microliter Hamilton syringe

12.14.2 METHODS

The following methods and procedures are used.

1. Place 0.2 cubic centimeter of venous blood into a vial that contains no heparin, and draw off the serum.

2. Prepare a veronal/barbital buffer solution. For 1000 cubic centimeters of solution with pH 8.6, 10.32 grams of barbital sodium and 1.84 grams of veronal are used.

3. Dilute a sample of serum with the veronal/barbital buffer 1:9 for IgG and 1:2 for IgA.

4. Open the cover of the plate. Using the microsyringe, fill up three of the wells prepared for the reaction on each plate with the appropriate standard serum in three dilution rates; fill up the remaining wells with the sera under study. Pour 0.005 cubic centimeter of serum into each well. For IgM determination, use undiluted samples; for IgG determination, use 1:10 diluted samples; for IgA determination, use 1:3 diluted samples.

5. Close the cover. Maintain the plates with IgA and IgG at room temperature for 24 hours; the plates with IgM, for 48 hours.

6. Using a special scale, determine the square of the precipitation ring diameter.

7. Using the three known solutions, plot a calibration curve and use it for determining the level of immunoglobulins in milligrams per 100 cubic centimeters.

12.14.3 QUALITY CONTROL

The following quality control measures are observed.

1. The serum samples are frozen before and after flight. All sera are tested simultaneously.

2. Three doubled samples are taken from each specimen.

3. Test serum with a known range of deviations for this method is placed onto each dish in order to determine the fitness of the plates.

4. Control sera are initiated simultaneously with the test sera.

12.14.4 DATA ANALYSIS

The level of immunoglobulins is compared between individuals and between astronaut/cosmonaut groupings before and after flight.

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13. IMMUNOLOGICAL AND BIOCHEMICAL STUDIES WITH SALIVARY PAROTID FLUID

By Ira L. Shannon,* V. M. Shilov,† and V. M. Zemskov‡

13.1 ANTIMICROBIAL ACTIVITY

13.1.1 MATERIALS

The following materials are required.

1. 0.1-molar potassium biphthalate buffer solution, pH 6.2 (reagent 34)
2. Plates with lysozyme substrate (reagent 35)
3. Standard lysozyme solutions (reagent 36)
4. Substrate, dry acetone powder *Micrococcus lysodeikticus*
5. Punch, 8-millimeter-diameter
6. Semilog paper

13.1.2 METHODS

The following methods and procedures are used.

1. The first three wells in each plate with the substrate are filled up with standard lysozyme solutions.
2. Two other wells are filled with the test fluid.
3. The cups are incubated in an incubator at 308 to 330 K (35° to 57° C) for 18 to 20 hours.

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4. The diameter of the clear zone around each well is measured with a millimeter scale.

5. A standard curve is prepared using semilog paper. Lysozyme activity for each standard solution is plotted against the zone diameter.

6. The level of lysozyme activity for each test fluid can be determined using the standard curve.

13.1.3 QUALITY CONTROL

Each fluid is tested several times if sufficient amount is available.

13.1.4 DATA ANALYSIS

The lysozyme level in the test fluids is compared between individuals and between astronaut/cosmonaut groupings before and after flight.

13.2 LYSOZYME (MURAMIDASE) CONCENTRATION IN HUMAN PAROTID SALIVA

13.2.1 MATERIALS

The following materials and equipment are required.

1. Phosphate buffer, 0.067-molar, pH 6.2 (1.75966 grams of disodium acid phosphate (Na_2HPO_4) and 7.43119 grams of potassium dihydrophosphate (KH_2PO_4) q.s. with distilled water to 1000 cubic centimeters)

2. Stock standard (100 milligrams of lysozyme per 100 cubic centimeters of phosphate buffer)

3. Working standards (Prepare from stock standard solutions containing 0.5, 1.0, 2.0, 3.0, and 4.0 mg/100 cm^3 lysozyme in phosphate buffer.)

4. Substrate (10.0 milligrams dried Micrococcus lysodeikticus suspended in 100 cubic centimeters of the phosphate buffer)

5. Assorted volumetric and serological pipettes

6. 10- by 10-millimeter absorption cells (Beckman)

7. DB-G spectrophotometer (Beckman)
8. Linear log recorder (Beckman)
9. Assorted volumetric glassware

13.2.2 METHODS

The following methods and procedures are used.

1. Place 2.5 cubic centimeters of the substrate into a 10- by 10-millimeter absorption cell.
2. Add 0.05 cubic decimeter of sample or working standard.
3. Immediately place absorption cell into the DB-G spectrophotometer (450 nanometers).
4. Start recorder.
5. Allow reaction to proceed for 5 minutes.
6. Calculate the change in optical density per minute.
7. Prepare a curve from the readings of the standards and calculate concentration of samples.

13.3 DETERMINATION OF SECRETORY 11-S IgA LEVELS IN HUMAN SALIVA

13.3.1 MATERIALS

The following materials and equipment are required.

1. Laurell barbital buffer (15.85 grams of barbital sodium; 230 cubic centimeters of 0.1-normal hydrochloric acid (HCl); to 2 cubic decimeters distilled water for 0.05 molar)
2. One-percent agar (Heat 1 gram agarose and 50 cubic centimeters distilled water to dissolve agarose. then add 50 cubic centimeters barbital buffer.)
3. Glass slides, 7.62 by 5.08 centimeters (3 by 2 inches)
4. IgA standard, 1 to 80 mg/100 cm^3 IgA (made from human colostrum)

5. Agar slide frames (two glass slides clamped together. separated by a plastic U-shaped frame)

6. Goat antiserum for human IgA (Meloy Laboratories)

7. 5-microliter pipette

8. Gel punch

9. Microporous wicks (Gelman)

10. Paper wicks (Beckman)

11. Stain (3 grams amido black 10B; 100 cubic centimeters glacial acetic acid; 700 cubic centimeters methanol; 200 cubic centimeters distilled water)

12. Destaining solution (100 cubic centimeters glacial acetic acid; 700 cubic centimeters methanol; 200 cubic centimeters distilled water)

13. Deluxe electrophoresis chamber by Gelman

14. 4100 pulsed constant power supply by Ortec

13.3.2 METHODS

The following methods and procedures are used.

1. Pour Laurell barbital buffer into electrophoresis chamber supplied with circulating cold water to keep chamber cool so that the agar on the slides will not shrink.

2. Soak four microporous wicks for each slide in the buffer.

3. Add goat antiserum for human IgA to melted 1-percent agar for desired concentration of antiserum (0.2 to 0.5 percent).

4. Mix well.

5. Pipette melted agar and antiserum solution (328 to 333 K (55° to 60° C)) into prepared slide frames.

6. Allow agar to gel.

7. Remove one glass slide and plastic frame from agar slide. and punch seven circular wells in the agar at one end of the slide on a line along the short axis of the slide.

8. Remove agar plugs with gentle suction.

9. Place 0.005 cubic decimeter of sample and/or standard in each well.

10. Place agar slides in buffer-filled electrophoresis chamber, and attach moistened microporous wicks to the ends of the agar slides from the buffer.

11. Perform electrophoresis at 200 volts direct current for 4 hours.

12. Following electrophoresis, dry the agar slides under lintless paper wicks that have been moistened with distilled water.

13. Stain the slides for at least 10 minutes and then destain.

14. Measure length of precipitate by placing a thin. transparent ruler under the slide of a contrasting background. Measure from the edge of the well to the leading edge of the precipitate.

15. Plot the length of each standard precipitate as a function of its immunoglobulin concentration on semilogarithmic graph paper using a standard curve. Unknown concentrations can be identified by the length of their precipitate.

14. LIST AND FORMULAS FOR NUTRIENT MEDIA AND REAGENTS

14.1 NUTRIENT MEDIA USED IN MICROBIAL EXCHANGE EXPERIMENT

The following nutrient media were used in the Microbial Exchange Experiment.

1. Trypticase soy broth (TSB)

Trypticase peptone, g	17.00
Phytone-peptone, g	3.00
Sodium chloride (NaCl), g	5.00
Potassium hydrophosphate (K_2HPO_4), g . . .	2.50
Dextrose, g	2.50
Distilled water, cm^3	1000.00
Ultimate pH	7.3

Mix, dissolve, pour into test tubes, and sterilize (autoclave) at 383 K (110° C) for 15 minutes.

2. Trypticase soy agar (TSA)

Trypticase peptone, g	15.00
Phytone-peptone, g	5.00
NaCl, g	5.00
Agar, g	15.00
Distilled water, cm^3	1000.00
Ultimate pH	7.3

Mix and sterilize for 15 minutes at 383 K (110° C).

3. Blood agar - Trypticase soy agar plus 5 percent blood. Melt agar, cool to 318 K (45° C), and add 5 cubic centimeters of blood to 100 cubic centimeters of desired medium.

4. Mannitol salt agar

Beef extract, g	1.00
Polypeptone peptone, g	10.00
NaCl, g	75.00
D-mannitol, g	10.00
Agar-agar, g	15.00
Phenol red, g	0.025
Distilled water, cm^3	1000.00
Ultimate pH	7.4

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Mix, dissolve, and sterilize at 383 K (110° C) for 15 minutes.

5. Sabouraud's dextrose medium with antibiotics

Glucose (maltose), g	4.0
Peptone, g	1.0
Agar-agar, g	1.8
Distilled water, cm^3	100.0
Ultimate pH	6.0 to 6.5

Mix, dissolve, and sterilize at 373 K (100° C) for 15 minutes.

6. Medium with bromthymol blue (BTB) indicator

Agar-agar, g	2.0
Trypticase soy broth, g	100.0
1.6-percent BTB alcohol solution, cm^3	6.25
Ultimate pH	7.0

Mix, dissolve, and sterilize at 383 K (110° C) for 15 minutes.

7. _Micrococcus_ glucose medium for studying glucose decomposition under anaerobic conditions

Agar-agar, g	0.2
Tryptone, g	1.0
Yeast extract, g	0.1
Glucose, g	1.0
Bromocresol purple, g	0.004
Distilled water, cm^3	100.0
Ultimate pH	7.2

Mix, dissolve, and sterilize at 383 K (110° C) for 15 minutes.

8. Medium used to determine phosphatase-forming capability of staphylococci (medium with 1-percent phenolphthalein phosphate)

Evans peptone, g	0.5
NaCl, g	0.5
Agar-agar, g	1.5
Lab-Lemco (beef extract), g	0.5
Distilled water, cm^3	100.0

Mix, dissolve, and sterilize at 373 K (100° C) for 15 minutes. Add 1 cubic centimeter of 1-percent phenolphthalein phosphate solution to 100 cubic centimeters of melted medium (318 K (45° C)).

9. Clark medium used to determine the presence of acetoin

Peptone, g	5.0
Dextrose, g	5.0
K_2HPO_4, g	5.0

Distilled water, cm^3	1000.0
Ultimate pH	6.8

Dissolve and sterilize at 383 K (110° C) for 15 minutes.

10. Mannitol agar

Beef extract, g	1.0
Polypeptone peptone, g	10.0
D-mannitol, g	10.0
Agar, g	15.0
Distilled water, cm^3	1000.0
Ultimate pH	7.4

Dissolve and sterilize at 383 K (110° C) for 15 minutes. Add 0.025 gram of phenol red agar to melted (318 K (45° C)) agar (1000 cubic centimeters).

11. Ammonium phosphate base medium (to determine the capability of staphylococci and micrococci to form acid from maltose, lactose, arabinose, and mannitol)

Ammonium dihydrophosphate ($NH_4H_2PO_4$), g	0.1
Potassium chloride (KCl), g	0.02
Hydrated magnesium sulfate ($MgSO_4 \cdot 7H_2O$), g	0.02
Yeast extract, g	0.1
Bromocresol purple, g	0.004
Agar, cm^3	1.5
Carbohydrate (lactose, arabinose, maltose, mannitol), g	0.5
Distilled water, cm^3	100.0

Mix and heat to the point of dissolving and melting. Sterilize at 383 K (110° C) for 15 minutes.

12. Broth with 1-percent glucose for streptococci

TSB, cm^3	100.0
Glucose, g	1.0

Mix, dissolve, and sterilize at 383 K (110° C) for 15 minutes.

13. 10-percent bile solution

TSB, cm^3	90.0
Concentrated solution of oxgall, cm^3	10.0

(To prepare a concentrated solution of oxgall, dissolve 7.0 grams of dry oxgall in 100.0 cubic centimeters of water and heat until completely dissolved.)

Mix, dissolve, and sterilize at 373 K (100° C) for 15 minutes.

14. Blood agar with 40-percent bile

TSB, cm^3	100.0
Agar-agar, g	3.0

Mix and melt. Add 40 cubic centimeters concentrated oxgall to 100 cubic centimeters of 3-percent agar solution. Pour into flasks. Sterilize at 373 K (100° C) for 15 minutes. Add 5 percent of blood to agar (318 K (45° C)).

15. Kalina medium for enterococci

2-percent agar-agar solution (previously sterilized at 383 K (110° C) for 15 min)	
Amount, cm^3	100
pH	6.0
Yeast dialysate, cm^3	2.0
Glucose, g	1.0
Crystal violet, 0.01 percent, cm^3	1.25
Polymyxin M, units/cm^3	200
2,3,5-triphenyltetrazode chloride (TTC), in 0.01-percent solution, mg	10.0
Baking yeast, kg	1
Water, distilled, dm^3	3 to 5

Heat water to 328 K (55° C), add yeast, and mix. Incubate in 328-K (55° C) water bath for 3 days, filter, and sterilize at 393 K (120° C) for 15 minutes. Sterilize TTC separately with flowing steam in a small amount of water (2.0 cubic centimeters) for 30 minutes and add it to the medium just before pouring. Mix, dissolve, and sterilize at 373 K (100° C) for 15 minutes. Add 10 cubic centimeters of sterile (fatless) milk to agar (318 K (45° C)).

16. Broth with inulin or raffinose

TSB, cm^3	100.0
Medium (choose one)	
Raffinose, g	1.0
Inulin, g	0.2
1.6-percent solution of alkaline BTB, cm^3	0.2

Mix, dissolve, and sterilize at 383 K (110° C) for 15 minutes.

17. Potato agar

Potatoes, g	500.0
Peptone, g	10.0
Agar-agar, g	10.0
Distilled water, cm^3	1000.0

Mix, boil, filter, and sterilize at 383 K (110° C) for 15 minutes.

18. Gorodkova medium

Peptone, g	10.0
Agar-agar, g	20.0
Glucose, g	0.025
Beef broth, cm^3	1000.0
Ultimate pH	7.3

Mix, dissolve, and sterilize at 383 K (110° C) for 15 minutes.

19. Giss medium with carbohydrates

Peptone, g	1.0
Sodium chloride, g	0.5
Distilled water, cm^3	100
Bromthymol blue (1.5-percent alcohol solution), cm^3	1.0
Final pH	7.0

Mix and dissolve. To the specified medium, add in an amount equal to 1.0 percent of the total volume any of the carbohydrates glucose, sucrose, or maltose. Sterilize at 383 K (110° C) for 15 minutes.

20. Reagent for cytochrome oxidase test

Distilled water, cm^3	10.0
Trilon B ($C_{10}H_{14}O_8N_2 \cdot 2H_2O$), g	0.1
Sodium octyl sulfate ($Na_2S_8O_3$), g	0.005
Tetramethyl-paraphenylenediamine dihydrochloride, g	0.02

Mix.

21. Two-layered medium for determining motility, decarboxylase, and indole production

Lower layer

0.2-percent Hottinger agar solution in a 4- to 5-cm column (sterilize)

Upper layer

Ingredient	Amount
Hottinger broth, cm^3	100.0
Sodium sulfite (Na_2SO_3), g	0.05
Lysine, g	0.5
K_2HPO_4, g	0.1
Potassium dihydrophosphate (KH_2PO_4), g	0.04
Bromthymol blue (6-percent alkaline solution), cm^3	0.3

Mix, dissolve, and sterilize at 383 K (110° C) for 15 minutes. Dispense 2-cubic-centimeter volume over the lower layer.

22. Two-layered complex medium for determining mannitol and glucose fermentation and formation of gas from glucose

Lower layer

Ingredient	Amount
Distilled water, cm^3	100.0
Agar-agar, g	1.5
Peptone, g	0.2
NaCl, g	0.5
Glucose, g	0.2
K_2HPO_4, g	0.3
BTB (1.6-percent alkaline solution), cm^3	0.5

Dissolve and dispense 4- to 5-cubic-centimeter volume into tubes. Sterilize at 383 K (110° C) for 15 minutes. Allow to cool in form of a column.

Upper layer

Ingredient	Amount
2-percent Hottinger agar solution, cm^3	100.0
Mannitol, g	1.0
KH_2PO_4, g	0.1
BTB (1.6-percent alkaline solution), cm^3	0.5

Dissolve and sterilize. Dispense 4- to 5-cubic-centimeter volume onto surface of lower layer using aseptic conditions. Allow to cool in slanted position.

23. Three-layer complex medium for determining dulcite fermentation, hydrogen sulfide production, and sodium citrate disintegration

Lower layer

1-percent Hottinger agar solution, cm^3	100
Dulcite, g	0.3
BTB (1.6-percent alkaline solution), cm^3	0.3
K_2HPO_4, g	0.1

Mix and dissolve. Dispense into tube in 4- to 5-cubic-centimeter volume. Sterilize for 15 minutes at 383 K (110° C). Cool in upright position.

Middle layer

1.8-percent agar solution, cm^3	100
K_2HPO_4, g	0.1
KH_2PO_4, g	0.04
Iron ammonium citrate, g	0.05
Na_2SO_3, g	0.05

Boil to dissolve. Dispense 0.5 cubic centimeter onto surface of lower layer and cool.

Upper layer

Distilled water, cm^3	100
Agar, g	2.5
$MgSO_4$, g	0.02
K_2HPO_4, g	0.1
$NH_4H_2PO_4$, g	0.1
Sodium citrate, g	0.2
BTB (1.6-percent alkaline solution), cm^3	0.5

Sterilize at 383 K (110° C) for 15 minutes, dispense in 4- to 5-cubic-centimeter volume onto the middle layer, and cool in slanted position (column 1 to 2 centimeters in height).

24. Liquid complex medium for determining glucose oxidation, acetoin production, and urease activity

Distilled water, cm^3	100
Peptone, g	0.5
Glucose, g	0.5
K_2HPO_4, g	0.5
BTB (1.6-percent alkaline solution), cm^3	0.1

Dissolve and pour 2 cubic centimeters into test tubes. Sterilize at 383 K (110° C) for 15 minutes.

25. Two-layer complex medium for determining gelatinase activity, culture purity, and pigment production

Lower layer

Distilled water, cm^3	100.0
Peptone, g	0.5
Na_2SO_3, g	0.5
K_2HPO_4, g	0.03
Agar-agar, g	1.5
Gelatin, g	5
Crystal violet (0.01-percent aqueous solution), cm^3	2.0

Dissolve agar and gelatin, filter the solution while hot through a cheesecloth filter, boil 30 minutes in a water bath, and dispense in quantities of 12 to 15 cubic centimeters into Petri dishes.

Upper layer

Distilled water, cm^3	100.0
Agar-agar, g	2.0
20-percent solution of secondary sodium alkyl sulfate, cm^3	1.0

Sterilize aqueous agar at 383 K (110° C) for 20 minutes. When cooled to 333 to 343 K (60° to 70° C), add sodium alkyl sulfate, then dispense onto cooled lower layer (no more than 10 cubic centimeters).

26. Cornmeal agar plus Tween 80

Bacto cornmeal agar, g	17.0
Tween 80, cm^3	1.0
Distilled water, cm^3	1000.0

To 17 grams bacto cornmeal agar (infusion from 50 grams cornmeal plus 15 grams agar), add 1000 cubic centimeters distilled water and 1 cubic centimeter Tween 80. Heat to boil. Autoclave for 15 minutes at 103.4 kN/m^2 (15 psi) pressure (394 K (121° C)). Cool to 328 K (55° C) and dispense into Petri dishes. Allow to solidify.

27. U.S. medium for acetoin production

Tryptone, g	1.0
Beef extract (Lab-Lemco), g	0.3

Yeast extract, g	0.1
Glucose, g	2.0
Distilled water, cm^3	100.0
Final pH	7.2

Mix and dissolve by heating. Dispense in 3-cubic-centimeter volumes into 16- by 125-millimeter screwcap tubes. Cap and sterilize at 388 K (115° C) for 15 minutes.

28. Endo medium

Hottinger agar, cm^3	100.0
Lactose, g	1.0
Fuchsin, base (alcohol-saturated solution), cm^3	2.0
Na_2SO_3, 10-percent aqueous solution, cm^3	10.0
Final pH	7.0

Mix, dissolve, and sterilize at 383 K (110° C) for 15 minutes.

29. Heart infusion agar (HIA)

Beef heart (infusion from), g	500.0
Tryptose, g	10.0
NaCl, g	5.0
Agar, g	15.0

To rehydrate, suspend 40 grams HIA in 1000 cubic centimeters distilled water. Heat to boiling to dissolve. Distribute 5 cubic centimeters to 13- by 100-millimeter tubes, and autoclave at 394 K (121° C) for 15 minutes. Slant medium while cooling.

30. 7-percent NaCl broth

Nutrient broth (Difco), g	8.0
Bacto beef extract, g	(3.0)
Bacto peptone, g	(5.0)
NaCl, g	70.0
Distilled water, cm^3	1000.0

Distribute (after boiling) 3 cubic centimeters into 13- by 100-millimeter screwcap tubes. Autoclave at 394 K (121° C) for 15 minutes.

31. Tryptose glucose yeast (TGY) spore medium

Plate count agar (Difco), g	0.1175
Tryptone, g	(5.0)
Yeast extract, g	(2.5)
Dextrose, g	(1.0)
Agar, g	(15.0)

Agar, g	7.5
Yeast extract, g	0.025
Distilled water, cm^3	500.0

Mix ingredients and dissolve by boiling. Autoclave for 15 minutes at 394 K (121° C) and distribute into sterile Petri dishes.

32. Starch agar

Nutrient agar (Difco), g	23.0
Beef extract, g	(3.0)
Peptone, g	(5.0)
Agar, g	(23.0)
Starch, g	2.0
Distilled water, cm^3	1000.0

Mix ingredients and dissolve by boiling. Autoclave at 394 K (121° C) for 15 minutes. Distribute into sterile Petri dishes.

33. MacConkey agar (Baltimore Biological Laboratories (BBL))

Gelysate peptone, g	17.0
Polypeptone peptone, g	3.0
Lactose, g	10.0
Bile salts mixture, g	1.5
NaCl, g	5.0
Agar, g	13.5
Neutral red, g	0.03
Crystal violet, g	0.001
Distilled water, cm^3	1000.0
Final pH	7.1

Dissolve 50 grams of dehydrated medium in 1000 cubic centimeters of distilled water by heat together with agitation. Sterilize by autoclaving at 394 K (121° C) for 15 minutes. Cool to 318 K (45° C) and pour into Petri dishes.

34. Oxidation-fermentation (OF) medium base (BBL)

Trypticase peptone, g	2.0
NaCl, g	5.0
Dipotassium phosphate, g	0.3
Agar, g	2.5
Bromthymol blue, g	0.03
Distilled water, cm^3	1000.0
Final pH	7.1

Dissolve the components in 1000 cubic centimeters of distilled water by heat together with agitation. Dispense aseptically into sterile tubes (13 by 100 millimeters) in 5-cubic-centimeter volumes. Sterilize by autoclaving at 394 K (121° C) for 15 minutes. A 10-percent sugar solution (medium 41) may be used with this base.

35. Triple-sugar iron (TSI) agar slants (Difco)

Bacto beef extract, g	3.0
Bacto yeast extract, g	3.0
Bacto peptone, g	15.0
Proteose peptone, g	5.0
Bacto lactose, g	10.0
Saccharose, Difco, g	10.0
Bacto glucose, g	1.0
Ferrous sulfate, g	0.2
NaCl, g	5.0
Sodium thiosulfate, g	0.3
Bacto agar, g	12.0
Bacto phenol red, g	0.024
Distilled water, cm^3	1000.0
Final pH	7.4

To rehydrate, suspend 65 grams of bacto TSI agar in 1000 cubic centimeters of distilled water and heat to boiling to dissolve. Dispense in 4.5-cubic-centimeter volumes into 13- by 100-millimeter screwcap tubes. Sterilize by autoclaving at 394 K (121° C) for 15 minutes. Slant to allow a generous butt.

36. 1-percent tryptone solution (indole medium) (Difco)

Bacto tryptone, g	10.0
Distilled water, cm^3	1000.0

Dissolve by heat together with agitation. Dispense in 5-cubic-centimeter volumes into 13- by 100-millimeter screwcap tubes. Sterilize by autoclaving at 394 K (121° C) for 15 minutes.

37. Methyl red/Voges-Proskauer (MR-VP) medium (Difco)

Buffered peptone, g	7.0
Bacto glucose, g	5.0
Dipotassium phosphate, g	5.0
Distilled water, cm^3	1000.0
Final pH	6.9

Dissolve 17 grams of bacto MR-VP medium in 1000 cubic centimeters of distilled water by heat together with agitation. Dispense in 2-cubic-centimeter volumes into 13- by 100-millimeter screwcap tubes. Sterilize by autoclaving at 394 K (121° C) for 15 minutes.

38. Simmon's citrate agar slants (Difco)

Magnesium sulfate, g	0.2
Monoammonium phosphate, g	1.0
Dipotassium phosphate, g	1.0
Sodium citrate, g	2.0
Sodium chloride, g	5.0

Bacto agar, g	15.0
Bacto bromthymol blue, g	0.08
Distilled water, cm^3	1000.0
Final pH	6.8

Dissolve 24.2 grams of bacto Simmon's citrate agar in 1000 cubic centimeters of distilled water by heat together with agitation. Dispense in 5-cubic-centimeter volumes into 13- by 100-millimeter screwcap tubes. Sterilize by autoclaving at 394 K (121° C) for 15 minutes. Cool in slanted position.

39. Nitrate broth (Difco)

Bacto beef extract, g	3.0
Bacto peptone, g	5.0
Potassium nitrate, g	1.0
Distilled water, cm^3	1000.0
Final pH	7.0

Dissolve 9 grams of bacto nitrate broth in 1000 cubic centimeters of distilled water by heat together with agitation. Dispense in 4-cubic-centimeter volumes into 13- by 100-millimeter screwcap tubes. Sterilize by autoclaving at 394 K (121° C) for 15 minutes.

40. Purple broth base (BBL) with and without gas tubes

Gelysate peptone, g	10.0
NaCl, g	5.0
Bromocresol purple, g	0.02
Distilled water, cm^3	1000.0
Final pH	6.8

Dissolve 15 grams of the base in 1000 cubic centimeters of distilled water. Dispense in 5-cubic-centimeter volumes into 13- by 100-millimeter screwcap tubes. For gas detection, add inverted gas tube to some tubes. Cap and sterilize by autoclaving at 394 K (121° C) for 15 minutes. A 10-percent sugar solution (medium 41) may be used with this base.

41. Sugar solutions, 10-percent (to be used with mediums 34 and 40)

Glucose	Rhamnose
Lactose	Amygdalin
Sucrose	Cellobiose
Maltose	Melezitose
Mannitol	Melibiose
Xylose	Salicin (2 percent)
Galactose	Sorbitol
Fructose	Trehalose
Arabinose	Inositol
Raffinose	Glycerol

Dissolve 10 grams of each sugar in separate volumes of distilled water (100 cubic centimeters). Filter, sterilize, and store at 277 K (4° C) in sterile containers.

To use, add 0.5 cubic centimeter of desired sugars to separate tubes of purple broth base or OF medium base.

42. Glucose agar slants (10 percent)

Purple broth base, g	1.6
Bacto agar, g	1.5
Glucose, g	10.0
Distilled water, cm^3	100.0
Final pH	6.8

Dissolve the purple broth base and agar in 100 cubic centimeters of distilled water by heat together with agitation. Add glucose and dissolve without heat. Dispense in 5-cubic-centimeter volumes into 13- by 100-millimeter screwcap tubes. Sterilize by autoclaving at 394 K (121° C) for 15 minutes. Cool in a slanted position.

43. Lactose agar slants (10 percent)

Purple broth base, g	1.6
Bacto agar, g	1.5
Lactose, g	10.0
Distilled water, cm^3	100.0
Final pH	6.8

Dissolve the purple broth base and agar in 100 cubic centimeters distilled water by heat together with agitation. Add lactose and dissolve without heat. Dispense in 5-cubic-centimeter volumes into 13- by 100-millimeter screwcap tubes. Sterilize by autoclaving at 394 K (121° C) for 15 minutes. Cool in a slanted position.

44. Urea agar (Difco)

Component A (agar base)

Bacto agar, g	1.5
Distilled water, cm^3	90.0

Component B - Urea agar base concentrate purchased from Difco Laboratories as a filter sterilized solution

Dissolve the agar in 90 cubic centimeters of distilled water by heat together with agitation. Sterilize by autoclaving at 394 K (121° C) for 15 minutes. Cool to 323 to 328 K (50° to 55° C). Add 10 cubic centimeters of component B under aseptic conditions. Mix and dispense into sterile 13- by 100-millimeter screwcap tubes. Cool in a slanted position to yield a butt of 1 centimeter and a slant of 1.5 centimeters.

45. Motility medium (BBL SIM)

Trypticase peptone, g	20.0
Thiotone peptone, g	6.1
Ferrous ammonium sulfate, g	0.2
Sodium thiosulfate, g	0.2
Agar, g	3.5
Distilled water, cm^3	1000.0
Final pH	7.3

Dissolve 30 grams of dehydrated medium in 1000 cubic centimeters of distilled water by heat together with agitation. Dispense in 5-cubic-centimeter volumes into 13- by 100-millimeter screwcap tubes. Sterilize by autoclaving at 394 K (121° C) for 15 minutes.

46. Gelatin nutrient (Difco)

Bacto beef extract, g	3.0
Bacto peptone, g	5.0
Bacto gelatin, g	120.0
Distilled water, cm^3	1000.0
Final pH	6.8

Dissolve 128 grams of dehydrated medium in 1000 cubic centimeters of distilled water by heat together with agitation. Dispense in 5-cubic-centimeter volumes into 13- by 100-millimeter screwcap tubes. Sterilize by autoclaving at 394 K (121° C) for 15 minutes.

47. Decarboxylase medium base (Difco)

Bacto peptone, g	5.0
Bacto yeast extract, g	3.0
Bacto glucose, g	1.0
Bacto bromocresol purple, g	0.02
Distilled water, cm^3	1000.0
Final pH	6.5

Dissolve 9 grams of base in 1000 cubic centimeters of distilled water by heat together with agitation. To 250 cubic centimeters of base, add 1.25 grams of L-lysine, L-ornithine, or L-arginine as desired, and dissolve. No further pH adjustment is required when lysine or arginine is used. Because ornithine hydrochloride is highly acid, it requires adjustment of pH with sodium hydroxide before sterilization. Dispense in 5-cubic-centimeter volumes into 13- by 100-millimeter screwcap tubes. Sterilize by autoclaving at 394 K (121° C) for 15 minutes.

48. Litmus milk (Difco)

Bacto skim milk, g	100.0
Bacto litmus, g	5.0

Distilled water, cm^3	1000.0
Final pH	6.8

Dissolve 105 grams of dehydrated medium in 1000 cubic centimeters of distilled water. Dispense in 5-cubic-centimeter volumes into 13- by 100-millimeter screwcap tubes. Sterilize by autoclaving at 394 K (121° C) for 15 minutes.

49. Vaspar - Composed of paraffin wax and white petrolatum in equal volumes by weight. Melt paraffin wax and petrolatum in a water bath. Dispense in 100-cubic-centimeter volumes, and sterilize by autoclaving at 394 K (121° C) for 45 minutes.

50. Casman medium base (BBL)

Polypeptone peptone, g	10.0
Biosate peptone, g	10.0
Beef extract, g	3.0
Nicotinamide, g	0.05
Para-aminobenzoic acid, g	0.05
Glucose, g	0.5
Cornstarch, g	1.0
NaCl, g	5.0
Agar, g	13.5
Distilled water, cm^3	1000.0
Final pH	7.3

Suspend 43 grams of base in 1000 cubic centimeters of distilled water. Mix, heat, and boil for 1 minute. Autoclave at 394 K (121° C) for 15 minutes.

51. Chocolate bacitracin agar - Prepare 500 cubic centimeters Casman base medium (medium 50). Remove base medium from the autoclave and add 25 cubic centimeters horse blood. Mix until lysed. Cool to 318 K (45° C), add 300 $\mu g/cm^3$ bacitracin (0.15 milligram bacitracin dissolved in 10 cubic centimeters of water, filtered, and sterilized), and pour into Petri dishes.

52. Rabbit blood agar - To 500 cubic centimeters of sterile, cooled Casman's medium base, add 25 cubic centimeters of rabbit blood, mix, and pour into Petri dishes.

53. Rabbit plasma (BBL) - To a 5-cubic-centimeter vial of dehydrated rabbit plasma, add 15 cubic centimeters of sterile distilled water. Distribute 0.5 cubic centimeter each into sterile 13- by 100-millimeter test tubes.

54. Urea broth (Difco) - Add 10 cubic centimeters of bacto-urea broth concentrate to 90 cubic centimeters of sterile, cold distilled water under aseptic conditions. The distilled water should be sterilized by autoclaving at 394 K (121° C) for 15 minutes. The water must be cooled to below 328 K (55° C) before the concentrated

urea is added. This completed medium is distributed in 3-cubic-centimeter volumes into sterile 13- by 100-millimeter test tubes.

55. Phenylalanine agar (Difco)

Bacto yeast extract, g	3.0
DL-phenylalanine, g	2.0
Dipotassium phosphate, g	1.0
NaCl, g	5.0
Bacto agar, g	12.0
Distilled water, cm^3	1000.0
Final pH	7.3

Suspend 23 grams of dehydrated medium in 1000 cubic centimeters of distilled water and heat to boiling to dissolve. Dispense in 5-cubic-centimeter volumes into 13- by 100-millimeter screwcap tubes. Sterilize by autoclaving at 394 K (121° C) for 15 minutes. Cool in a slanted position.

56. Cornmeal/malt-extract/yeast-extract agar (CMMY)

Cornmeal, infusion from, g	50.0
Yeast extract, g	1.0
Malt extract, g	1.0
Agar, g	20.0
Distilled water, cm^3	1000.0

Dissolve 17 grams of bacto cornmeal agar and 5 grams of bacto agar in 1000 cubic centimeters of distilled water by heat together with agitation. Add yeast extract and malt extract. Sterilize by autoclaving at 394 K (121° C) for 15 minutes.

57. Czapek solution agar

Sucrose, g	30.0
Sodium nitrate, g	2.0
Dipotassium phosphate, g	1.0
Magnesium sulfate, g	0.5
Potassium chloride, g	0.5
Ferrous sulfate, g	0.01
Agar, g	20.0
Distilled water, cm^3	1000.0
Final pH	7.3

Dissolve 49 grams of bacto Czapek solution agar and 5 grams of bacto agar in 1000 cubic centimeters of distilled water by heat together with agitation. Sterilize by autoclaving at 394 K (121° C) for 15 minutes.

58. Potato dextrose agar

Potatoes, infusion from, g	200.0

Dextrose, g	20.0
Agar, g	20.0
Distilled water, cm^3	1000.0

Dissolve 39 grams of bacto potato dextrose agar and 5 grams of bacto agar in 1000 cubic centimeters of distilled water by heat together with agitation. Sterilize by autoclaving at 394 K (121° C) for 15 minutes.

59. Malt-extract agar

Malt extract, g	20.0
Glucose, g	20.0
Peptone, g	1.0
Agar, g	25.0
Distilled water, cm^3	1000.0

Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 394 K (121° C) for 15 minutes.

60. Emerson's yeast phosphate soluble starch agar (YPSS)

Yeast extract, g	4.0
Soluble starch, g	15.0
Dipotassium phosphate, g	1.0
Magnesium sulfate, g	0.5
Agar, g	20.0
Distilled water, cm^3	1000.0

Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 394 K (121° C) for 15 minutes.

61. Cornmeal agar

Cornmeal, infusion from, g	50.0
Agar, g	20.0
Distilled water, cm^3	1000.0

Dissolve 17 grams of bacto cornmeal agar and 5 grams of bacto agar in 1000 cubic centimeters of distilled water by heat together with agitation. Sterilize by autoclaving at 394 K (121° C) for 15 minutes.

62. Half-strength cornmeal agar

Cornmeal, infusion from, g	50.0
Agar, g	20.0
Distilled water, cm^3	1000.0

Dissolve 8.5 grams of bacto cornmeal agar and 12.5 grams of bacto agar in 1000 cubic centimeters of distilled water by heat together with agitation. Sterilize by autoclaving at 394 K (121° C) for 15 minutes.

63. Hottinger's broth

Trypticase meat digest, prepared using the Hottinger method, cm^3 . . .	500.0
Distilled water, cm^3	500.0
NaCl, g	5.0
Final pH	7.2 to 7.4

Dissolve and sterilize at 393 K (120° C) for 20 minutes.

64. Hottinger's agar

Hottinger's broth, cm^3	1000
Agar, g	20
Final pH	7.2 to 7.4

Mix, dissolve, and sterilize at 393 K (120° C) for 20 minutes.

65. Compound scarlatinal broth

Hottinger's broth, cm^3	100.0
Human blood, cm^3	5.0

Using sterile conditions, add the blood to medium 63 and dispense the mixture in 2-cubic-centimeter volumes into test tubes.

66. Hottinger's agar containing serum

Hottinger's agar, cm^3	80.0
Horse serum, cm^3	20.0

To the melted and cooled (to 323 K (50° C)), sterile Hottinger's agar (medium 64), aseptically add horse serum. Distribute the mixture into Petri dishes.

67. Hottinger agar containing serum and ristomycin

Medium 66, cm^3	100.0
Ristomycin solution containing 20 000 units/cm^3, cm^3	0.75

Add ristomycin solution to medium 66. Distribute the mixture into Petri dishes.

68. Hottinger's agar containing serum and carbohydrates (glucose, maltose, levulose, saccharose)

Medium 64, cm^3	75.0
One of the listed carbohydrates, g	0.9
Indicator, phenol red (reagent 28), cm^3	3.9
Horse serum, cm^3	20.0

Mix the first three components, sterilize at 50.7 kN/m^2 (0.5 atmosphere) for 30 minutes, and cool to 323 K (50° C). Add horse serum, and distribute into Petri dishes.

69. Medium for urease determination

Urea, g	1.0
Hottinger broth, cm^3	100.0
Cresol red (1.6-percent alcohol solution), cm^3	0.2

Add 2.0 cubic centimeters of a 50-percent aqueous solution of urea to Hottinger broth (sterile). Urea and indicator are added before dispensing the medium. The medium is dispensed into test tubes in 3.0-cubic-centimeter volumes.

70. U.S.S.R. medium for urease determination

Urea, g	1.0
Hottinger broth, cm^3	100.0
Cresol red (1.6-percent alcohol solution), cm^3	0.2

Add 2.0 cubic centimeters of a 50-percent aqueous solution of urea to Hottinger broth, pH 7.0. (The urea is self-sterilized in the specified concentration when maintained as a 50-percent solution for 2 days.) The Hottinger broth is preliminarily sterilized at 393 K (120° C) for 20 minutes. Urea and indicator are added before dispensing the medium. The medium is dispensed into test tubes in quantities of 3.0 cubic centimeters.

71. TSB and Fildes - To 5 cubic centimeters of sterile TSB in screwcap tubes (medium 1), add two drops of Fildes enrichment (reagent 10).

72. V-8 juice agar

V-8 juice, cm^3	200
Calcium carbonate ($CaCO_3$), g	3
Agar, g	20
Water, cm^3	1000

Mix, dissolve, and sterilize at 383 K (110° C) for 15 minutes. The $CaCO_3$ should be in powdered form; it should be maintained in suspension while plates are being filled.

73. Hay infusion agar

Tapwater, cm^3	1000
Decomposing hay, g	50

Autoclave for 20 minutes at 393 K (120° C) and filter.

Infusion filtrate, cm^3	1000
K_2HPO_4, g	2
Agar, g	20

Adjust pH to 6.0 to 6.5; sterilize at 393 K (120° C) for 20 minutes.

74. Dung decoction agar

Horse dung, g	200
Tapwater, cm^3	1000
Agar, g	20

Thoroughly mix dung in water and boil for 15 minutes, then filter through cheesecloth to remove coarse debris. The filtrate, made up to 1000 cubic centimeters with 20 grams of agar added, is autoclaved at 383 K (110° C) for 15 minutes.

75. Plain agar

Agar, g	20
Distilled water, cm^3	1000

Mix, dissolve, and sterilize at 383 K (110° C) for 15 minutes.

76. Potato slices - A slice of potato approximately 1 by 1 by 6 centimeters is placed in a screwcap tube and sterilized at 383 K (110° C) for 15 minutes.

77. Carrot wedges - A slice of carrot approximately 1 by 1 by 6 centimeters is placed in a screwcap tube and sterilized at 383 K (110° C) for 15 minutes.

78. Corn tips - A tip of a growing corn plant approximately 0.5 centimeter in diameter by 6 centimeters in length is placed in a screwcap tube. After 2 cubic centimeters of a 3-percent solution of glucose in distilled water are added, the mixture is sterilized at 383 K (110° C) for 15 minutes.

79. Lactose yeast agar

Lactose, g	1.0
Yeast extract, g	0.5
Agar, g	20.0
Distilled water, cm^3	1000

Mix, dissolve, and sterilize at 383 K (110° C) for 15 minutes.

80. Rogosa SL agar

Bacto tryptose, g	10.0
Bacto yeast extract, g	5.0
Bacto glucose, g	10.0
Bacto arabinose, g	5.0
Bacto saccharose, g	5.0
Sodium acetate, g	15.0
Ammonium citrate, g	2.0
Monopotassium phosphate, g	2.0
Magnesium sulfate, g	0.57
Manganese sulfate, g	0.12
Ferrous sulfate, g	0.03
Sorbitan monooleate, g	1.0
Bacto agar, g	15.0
Distilled water, cm^3	1000.0
Final pH	5.4

Suspend 75 grams of Rogosa SL agar base in 1000 cubic centimeters of distilled water and dissolve by heat together with agitation. Add 1.32 cubic centimeters of glacial acetic acid, mix thoroughly, and heat to 368 K (95° C) for 3 minutes. Do not autoclave. Pour into Petri dishes.

81. Peptone water with carbohydrates

Peptone, g	10.0
NaCl, g	5.0
Carbohydrate (sugar), g	20.0
0.05-percent solution of BTB, cm^3	1.0
Distilled water, cm^3	1000.0
pH	7.6

Sterilize at 383 K (110° C) for 15 minutes. The following sugars are used for the fermentation reaction: glucose, galactose, maltose, lactose, and saccharose.

82. Yelinov's medium

Hulled rice, g	2.0
Mannitol, g	2.0
Serine, g	1.0
Sodium sulfite, g	0.5
Agar-agar, g	2.0
Distilled water, cm^3	100

Boil 2.0 grams of hulled rice in 100 cubic centimeters of distilled water for 20 to 30 minutes, filter with paper filter, and make up to the initial volume with distilled water. Add mannitol, serine, sodium sulfite, and agar-agar and make the mixture boil

(boil until agar melts), then clarify the mixture with horse serum (7 cubic centimeters serum per 100 cubic centimeters of medium) according to general rules. Sterilize the clarified medium by autoclaving for 20 minutes at 383 K (110° C).

83. Christensen's medium (W. B. Christensen, 1946)

Peptone, g	1.0
NaCl, g	5.0
Glucose, g	1.0
Potassium dihydrophosphate, g	2.0
Agar-agar, g	15.0
Distilled water, cm^3	1000.0
Phenol red, g	0.012

Dissolve the ingredients in distilled water by heat, bring pH to 6.8, and dispense the medium in 4.5-cubic-centimeter volumes into vials. Sterilize at 393 K (120° C) for 10 minutes. Then, before the medium has solidified, pour into each vial 0.5 cubic centimeter of a 20-percent solution of urea sterilized through asbestos filters. Quickly mix the contents of the vials, and make long slants with a high column of the medium.

84. Agar used for auxanogram of carbohydrates (according to Lanjeron)

Potassium monophosphate, g	1.0
Ammonium sulfate, g	1.0
Magnesium sulfate, g	0.5
Agar-agar, g	20.0
Distilled water, cm^3	1000.0

Sterilize at 393 K (120° C) for 15 minutes.

85. Agar used for auxanogram of nitrous materials

Glucose, g	20.0
Potassium monophosphate, g	1.0
Magnesium sulfate, g	0.5
Agar-agar, g	20.0
Distilled water, cm^3	1000.0

Sterilize at 388 K (115° C) for 15 minutes.

86. Agar containing arbutin

Yeast extract, cm^3	100.0
Arbutin, g	0.5
Agar-agar, g	20.0

Sterilize at 393 K (120° C) for 15 minutes. Distribute the medium into Petri dishes, each containing a drop of ferric chloride ($FeCl_3$).

87. Potato/carrot medium (Yu. A. Lyubimov, 1971)

Potato peelings, g	200.0
Carrot peelings, g	200.0
Glucose, g	5.0
Agar-agar, g	15.0
6-percent solution of vitamin B_1 (thiamine), cm^3	1.0
Streptomycin sulfate, g	0.5
Distilled water, cm^3	1000.0

Thoroughly wash the potatoes and carrots with a brush but without soap. Thinly peel the potatoes and carrots, put 200 grams of peelings of each kind into a retort, flood with distilled water, and boil for 30 minutes. Remove the peelings by filtering through cheesecloth, and add 15.0 grams of agar-agar and 5.0 grams of glucose; make up the filtrate to a volume of 1000 cubic centimeters with distilled water. Sterilize the medium at 383 K (110° C) for 15 minutes. Before using, melt the medium in a water bath, and, when the medium starts cooling, add 1 cubic centimeter of a 6-percent solution of thiamine (vitamin B_1) and 0.5 gram of streptomycin sulfate. Distribute the medium into Petri dishes or vials.

88. Fermentation test medium with gas tubes

Component A (base)

Yeast extract, g	4.5
Peptone, g	7.5
Bromthymol blue, g	0.02
Distilled water, cm^3	1000.0

Dispense 2-cubic-centimeter quantities in 150- by 12-millimeter test tubes containing 50- by 6-millimeter inserts. Cap and sterilize by autoclaving at 394 K (121° C) for 15 minutes.

89. Carbohydrate solutions (6 percent)

Component B (for fermentation test medium)

Glucose	Lactose	Inulin
D-galactose	Cellobiose	Starch
Sucrose	Trehalose	Melezitose
Maltose	Melibiose	Raffinose (12 percent)

Solutions are sterilized by filtration and kept in the refrigerator. Add 1-cubic-centimeter quantities of the various solutions to the tubes of fermentation test medium (medium 88).

90. Yeast-extract/malt-extract agar (YEME) - Fresh young carrots are washed and cut longitudinally in quarters. Each wedge is placed

in a large (25 millimeter) test tube with 3 cubic centimeters of distilled water. The wedges are sterilized for 20 minutes with steam at 394 K (121° C).

14.2 REAGENTS USED IN MICROBIAL EXCHANGE EXPERIMENT

The following reagents were used in the Microbial Exchange Experiment.

1. Kovac's reagent (indole test)

P-dimethylaminobenzaldehyde, g	5.0
Amyl alcohol, cm^3	75.0
Concentrated hydrochloric acid (HCl), cm^3	25.0

Dissolve p-dimethylaminobenzaldehyde in the alcohol, then add acid.

2. Methyl red solution

Bacto methyl red, g	0.1
95-percent alcohol, cm^3	300.0
Distilled water, cm^3	$\approx$200

Dissolve methyl red in alcohol. Add distilled water to bring the amount up to 500 cubic centimeters.

3. Voges-Proskauer reagents

   a. 5-percent solution of alpha naphthol in absolute ethyl alcohol

Alpha naphthol, g	5.0
Absolute ethyl alcohol, cm^3	100.0

   b. 40-percent aqueous potassium hydroxide solution

Potassium hydroxide, g	40.0
Distilled water, cm^3	100.0

4. Nitrate reagents

   a. Solution I

Sulfonilic acid, g	8.0
5 normal acetic acid, cm^3	1000.0

   b. Solution II

Di-methyl-alpha naphthylamine, cm^3	12.0
5-normal acetic acid, cm^3	1000.0

c. 5-normal acetic acid

Glacial acetic acid, cm^3	56.8
Distilled water, cm^3	143.0

5. Catalase reagent - 3-percent hydrogen peroxide solution

6. Gram-stain solutions

a. Gram's solution 1

Crystal (gentian) violet, g	1.0
Distilled water, cm^3	100.0

b. Gram's solution 2

Iodine, g	1.0
Potassium iodide, g	2.0
Distilled water, cm^3	100.0

c. Gram's solution 3 - 95-percent ethyl alcohol

d. Gram's solution 4

Safranin O, g	1.0
Distilled water, cm^3	100.0

7. Oxidase test reagent

Reagent (choose one)

P-aminodimethylaniline monohydrochloride, g	0.1
Tetramethyl-p-phenylenediamine, g	0.1
Distilled water, cm^3	10.0

Prepare fresh daily or refrigerate not longer than 7 days.

8. Phenylalanine test reagent

Ammonium sulfate, g	2.0
10-percent sulfuric acid, cm^3	1.0
Ferric ammonium sulfate (half-saturated solution), cm^3	5.0

Dissolve ammonium sulfate in 10-percent sulfuric acid and half-saturated solution of ferric ammonium sulfate. Store in refrigerator.

9. 1-percent Tween solution

Polysorbate 80 (Tween), cm^3	1.0
Distilled water, cm^3	100.0

Filter and sterilize.

10. Bacto-Fildes enrichment - Sterile digest of sheep blood recommended for use in liquid mediums for culturing Haemophilus. It is rich in hematin and coenzyme required by Haemophilus. Optimal results are obtained when used in 1- to 5-percent concentration in liquid medium.

11. Lacto-phenol cotton blue mounting medium (used for preparing mounts for the study of fungus cultures)

Phenol crystals (melt in water bath before weighing), g	20.0
Lactic acid, g	20.0
Glycerin, g	40.0
Cotton blue (Poirrier's blue), g	0.05
Distilled water, cm^3	20.0

12. Clear nail polish

13. Dye for volutin granules

Ice-cold acetic acid, cm^3	5.0
96-percent rectified alcohol, cm^3	95.0
Saturated methylene blue solution, cm^3	92.0

14. Zinc dust - Elemental zinc powder

15. Growth-factor strips

   a. X-factor strips - Commercially prepared sterile paper strips impregnated with hemin

   b. V-factor strips - Commercially prepared sterile paper strips impregnated with diphosphopyridine nucleotide or coenzyme

   c. X- and V-factor strips - Commercially prepared sterile paper strips impregnated with hemin and diphosphopyridine nucleotide

16. Liquid paraffin (paraffin oil) - Liquid paraffin is dispensed in 100-cubic-centimeter volumes in screwcap bottles, capped, and autoclaved for 45 minutes at 394 K (121° C). It is used as an overlay.

17. Ammonia (0.880 specific gravity)

18. Buffer solution, phosphate, 0.15-molar, pH 6.4

a. Solution A

KH_2PO_4 (anhydrous), g	9.08
Distilled water, cm^3	1.0

b. Solution B

Disodium acid phosphate (Na_2HPO_4) (anhydrous), g	9.47
Distilled water, cm^3	1.0

c. Final solution - Mix 73.5 cubic centimeters of solution A with 26.5 cubic centimeters of solution B.

19. Muramidase substrate plates - Prepare a 1-percent solution of agarose (Sigma Chemical Co., St. Louis, Mo.) in pH 6.4 phosphate buffer with 50-mg/100-cm^3 Micrococcus lysodeikticus cells (Sigma Chemical Co.). Place 5 cubic centimeters of the buffered cell suspension into a 55-millimeter disposable Petri dish. After the agarose/cell suspension has solidified, punch 2.0-millimeter-diameter wells into the agarose, six wells per plate.

20. Broad-spectrum control serum - Pooled human serum is used.

21. Muramidase standard solutions - These solutions are obtained from Kallestad Laboratories (Chaska, Minn.). Use one each, low, medium, and high concentrations.

22. Photographic printing paper - Kodabromide F-5 is obtainable from Eastman Kodak Inc. (Rochester, Minn.). Any other black-and-white, high-contrast paper of similar characteristics may be used.

23. Isotonic saline with safranin

NaCl, g	8.5
Safranin dye, mg	0.50
Distilled water, cm^3	1000

24. Agglutination antigens for Salmonella, Brucella, and Proteus - These antigens may be obtained from Lederle Laboratories (Pearl River, N.Y.).

25. Specific control serum - Obtain antisera for Salmonella, Brucella, and Proteus antigens from Lederle Laboratories (Pearl River, N.Y.).

26. Formalin-saline solution

NaCl, g	8.5
Formalin solution, 35-percent, cm^3	9.0

Distilled water, dm^3	1.0

27. Saline-thimerosal solution

NaCl, g	8.5
Thimerosal (Sigma Chemical Co.), mg	100
Distilled water, dm^3	1.0

28. Indicator, phenol red

Phenol red, g	0.4
0.1-normal solution of sodium hydroxide (NaOH), cm^3	16.0

Mix, dissolve, keep in water bath until completely dissolved, and shake frequently. Make up to 200 cubic centimeters with distilled water. Sterilize at 101 kN/m^2 (1 atmosphere) for 30 minutes.

29. 5-percent aqueous solution of malachite green

30. 0.25-percent solution of basic fuchsin

31. Ferric chloride ($FeCl_3$)

32. Glycerin

33. India ink

34. Buffer solution, potassium biphthalate, 0.1 molar, pH 6.2

Potassium biphthalate ($C_8H_5O_4K$), g	20.42
Distilled water, cm^3	1000

Bring solution to pH 6.2 using (1) 10-normal solution of NaOH and (2) 1-normal solution of NaOH.

35. Lysozyme substrate plates - Prepare a 1-percent solution of Difco agar (Difco Laboratories, Detroit, Mich.) in pH 6.2 phthalate buffer with 150-mg/100-cm^3 *Micrococcus lysodeikticus* dry culture cells. Place 15 cubic centimeters of buffer cell suspension in agar onto 90-millimeter-diameter Petri dishes. After the suspension has solidified, punch five 8-millimeter-diameter wells in the agar of each dish.

36. Standard solutions of lysozyme - Low (4 to 8 $\mu g/cm^3$), medium (10 to 25 $\mu g/cm^3$), and high (50 to 100 $\mu g/cm^3$) concentrations are used.

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16. Abstract

Systematic laboratory procedures compiled as an outgrowth of a joint U.S./U.S.S.R. microbiological-immunological experiment performed during the Apollo-Soyuz Test Project space flight are presented. Included are mutually compatible methods for the identification of aerobic and microaerophilic bacteria, yeast and yeastlike microorganisms, and filamentous fungi; methods for the bacteriophage typing of *Staphylococcus aureus*; and methods for determining the sensitivity of *S. aureus* to antibiotics. Immunological methods using blood and immunological and biochemical methods using salivary parotid fluid are also described. Formulas for media and laboratory reagents used are listed.

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