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Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Shigella* spp.

Microbiologie des aliments — Méthode horizontale pour la recherche de Shigella *spp.*

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 21567 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods which are specific to these products may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this method in the case of particular products.

The harmonization of test methods cannot be immediate and, for certain groups of products, International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed they will be changed to comply with this International Standard so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Shigella* spp.

WARNING — In order to safeguard the health of laboratory personnel, it is essential that the whole of this method is only carried out by skilled personnel using good laboratory practices and preferably working in a containment facility. Relevant national Health and Safety Regulations relating to this organism shall be adhered to. Care shall be taken in the disposal of all infectious materials.

1 Scope

This International Standard specifies a horizontal method for the detection of *Shigella* species.

Subject to the limitations discussed in the Introduction, this International Standard is applicable to

— products intended for human consumption and the feeding of animals, and

— environmental samples in the area of food production and food handling.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 6887-1, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

ISO 6887-2, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 2: Specific rules for the preparation of meat and meat products*

ISO 6887-3, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products*

ISO 6887-4, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products*

ISO 7218:1996, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations*

ISO 8261, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

ISO/TS 11133-1, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 1: General guidelines on quality assurance of culture media in the laboratory*

ISO/TS 11133-2, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 2: Practical guidelines on performance testing of culture media*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
Shigella
microorganisms which form colonies fitting the description of these species on the solid selective media used, and which display the biochemical and serological characteristics described when tests are carried out in accordance with this International Standard

3.2
detection of *Shigella* spp.
determination of the presence or absence of these microorganisms in a particular mass of product, when tests are carried out in accordance with this International Standard

4 Principle

4.1 General

The detection of *Shigella* necessitates four successive stages (see Annex A).

4.2 Enrichment in selective liquid medium

A test portion is inoculated into *Shigella* broth containing 0,5 µg/ml of novobiocin, then incubated anaerobically at (41,5 ± 1) °C for 16 h to 20 h.

4.3 Plating out and identification of colonies

From the enrichment culture obtained, three selective differential media are inoculated: MacConkey agar with low selectivity; XLD agar with moderate selectivity; and Hektoen enteric agar with the greatest selectivity. All are incubated at 37 °C for 20 h to 24 h.

4.4 Biochemical and serological confirmation

Typical and suspect colonies are selected from each of the three selective agars. The colonies are purified on nutrient agar, then biochemical and serological characterizations are carried out using the tests described.

5 Culture media, reagents and antisera

For current laboratory practices, see ISO 7218, ISO/TS 11133-1 and ISO/TS 11133-2 for the preparation, production and performance testing of culture media.

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

See Annex B for descriptions of all media, reagents and antisera.

Commercially available dehydrated media should give more consistent results than media prepared from their component parts in the laboratory. Follow the manufacturer's instructions exactly, as small changes in preparation can significantly change the performance of selective media. Excessive heating of the selective agars used in this International Standard by autoclaving, storage and then re-heating for use may result in loss of selectivity.

6 Apparatus and glassware

Disposable equipment is an acceptable alternative to re-usable glassware if it has suitable specifications.

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

6.1 Apparatus for dry sterilization (oven) and **wet sterilization (autoclave)** of equipment.

See ISO 7218.

6.2 Drying cabinet or **oven**, ventilated by convection, and capable of operating at a set temperature between 37 °C and 55 °C.

6.3 Incubators, capable of operating at (37 ± 1) °C and (41,5 ± 1) °C.

6.4 Modified atmosphere jars or **anaerobic incubation cabinets**, and related apparatus to achieve anaerobic conditions, with a gas composition of < 1 % O_2 and 9 % to 13 % CO_2. See ISO 7218.

6.5 Water baths, operating at a set temperature of (47 ± 3) °C, for the cooling of molten media prior to plate pouring, and another set at (50 ± 1) °C (see B 6.3.2 and B 10.2.2).

6.6 Peristaltic homogenizer (stomacher) or **rotary blender**.

See ISO 7218.

6.7 Inoculation needles and **loops**, made of platinum/iridium or nickel/chrome, of diameter approximately 3 mm, or plastic disposable loops and needles of suitable specifications.

6.8 pH-meter, having an accuracy of calibration of ± 0,1 pH unit at 25 °C.

6.9 Flasks and **bottles**, with closures, of suitable capacities for use in the preparation of enrichment broths and agars and their storage.

6.10 Measuring cylinders.

6.11 Tubes, 18 mm in diameter and 160 mm in length (plugged or with screw caps), or **culture bottles**, of nominal capacity 30 ml and 10 ml, with non-toxic metallic caps with liners or plastic disposable caps.

6.12 Petri dishes, of diameter between 90 mm to 100 mm and diameter 140 mm.

6.13 Glass slides or **plates,** suitable for use in agglutination tests.

7 Sampling

It is important that the laboratory receive a sample that is truly representative and has not been damaged or changed during transit or storage.

Sampling is not part of the method specified in this International Standard. See the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

8 Preparation of test sample

Prepare the test sample in accordance with the appropriate part of ISO 6887 and/or ISO 8261.

Analysis of samples should begin as quickly as possible, as survival of *Shigella* is poor.

9 Procedure (see diagram in Annex A)

9.1 Test portion

See the appropriate part of ISO 6887 and/or ISO 8261 dealing with the procedures for the different types of products concerned.

9.2 Enrichment

In general add x g or x ml of test portion to $9x$ ml of *Shigella* broth containing 0,5 µg/ml of novobiocin (B.1.2) to make a 1 in 10 dilution of the test sample. Homogenize the test portion in the broth using a peristaltic homogenizer or rotary blender (6.6). Aseptically adjust the pH to 7,0 ± 0,2, if necessary.

Incubate (6.4) the *Shigella* broth under anaerobic conditions with caps and closures loose, or with equipment giving an equivalent effect, so that gas exchange can readily occur without contamination at (41,5 ± 1) °C (6.3) for 16 h to 20 h.

9.3 Plating out and colony selection

9.3.1 Using the cultures obtained in 9.2, gently mix the contents by hand and allow the larger particles to settle.

Inoculate, by means of a loop (6.7), the surface of the following selective agars to obtain well-isolated colonies: MacConkey agar (B.2.1) with low selectivity; XLD agar (B.2.2) with moderate selectivity; and Hektoen enteric agar (B.2.3) with a greater selectivity.

9.3.2 Incubate (6.3) the plates at (37 ± 1) °C for between 20 h and 24 h.

9.3.3 The appearance of different *Shigella* species can vary on the same selective agar. See Annex C for a description of *Shigella* colonies on the different selective agars used.

Shigella species can form a minority proportion of the total microbial flora when contaminating a food sample or after enrichment. In these circumstances, the direct streaking of the enrichment broth onto one plate per selective agar may fail to allow the detection of *Shigella* colonies. It may therefore be appropriate in some circumstances (e.g. the investigation of foods implicated in illness) to consider the inoculation of either two 90 mm dishes or one large (140 mm) Petri dish (6.12) to increase the possibility of detection.

The colonies of some Enterobacteriaceae strains are very similar in appearance to those of *Shigella*. Any typical or suspect colonies shall be confirmed (see 9.4) as *Shigella* species or not. Also in some circumstances (e.g. foods implicated in food poisoning), it may be appropriate to investigate more than five colonies from a plate to increase confidence in the absence of *Shigella* in the food sample tested.

Mark any typical or suspect colonies found on each plate.

If no typical colonies are seen and the growth of other microorganisms is weak (particularly on the more selective agar), re-incubate the plates for a further 24 h. Examine them again for typical *Shigella* colonies.

Carry out the confirmation procedure described in 9.4.

9.4 Confirmation of colonies

9.4.1 General

Identification kits (currently commercially available) that have been shown by the user to be reliable for the identification of the different species of *Shigella* may be used. Follow the manufacturer's instructions precisely.

For confirmation, sub-culture from each dish of each selective medium (see 9.3) five marked typical or suspect colonies.

If on one dish there are fewer than five typical or suspect colonies, take all the marked colonies for confirmation.

Use pure cultures for biochemical and serological confirmation.

9.4.2 Purification of colonies

Streak the selected colonies onto the surface of nutrient agar plates (B.3) so as to gain well-isolated colonies.

Incubate (6.3) the plates at (37 ± 1) °C for 18 h to 24 h.

If the cultures on nutrient agar are mixed, sub-culture the suspect colony onto a further plate of nutrient agar and incubate at (37 ± 1) °C for 18 h to 24 h to obtain the pure culture.

Shigella sonnei can give two colony types on the same agar plate: a smooth round domed colony (phase 1), and a flat irregular colony with a mat surface (phase 2).

NOTE It is possible to first test the most characteristic colony from each selective agar plate. If positive, it is not necessary to test other colonies. If negative, progress through the other selected colonies until either all are negative or a positive is found.

9.4.3 Biochemical confirmation

9.4.3.1 General

By means of an inoculation needle (6.7), inoculate the media specified in 9.4.3.2 to 9.4.3.9 respectively with each of the cultures selected in 9.4.1 and record all the results.

9.4.3.2 Triple sugar iron agar (TSI slopes) (B.4)

Stab the butt and streak the agar slope.

Incubate (6.3) at (37 ± 1) °C for (24 ± 3) h.

Interpret the changes in the medium as follows:

Area of slope	Appearance	Indication
Butt	Yellow	Glucose fermented: positive
	Red or unchanged	Glucose not fermented: negative
	Black	Formation of hydrogen sulfide: positive
	Bubbles or cracks	Gas formation
Slant surface	Yellow	Lactose and/or sucrose utilized: positive
	Red or unchanged	Lactose and sucrose not utilized: negative

Typical *Shigella* cultures show a yellow butt (acid formation) and no gas bubbles, there is no change in the colour of the slant (no utilization of lactose or sucrose) and no hydrogen sulfide production (see Table 1).

9.4.3.3 Semi-solid nutrient agar for motility tests (B.5)

Stab the semi-solid nutrient agar with a colony using an inoculation needle (6.7).

Incubate (6.3) tubes at (37 ± 1) °C for 18 h to 24 h.

Examine the line of inoculation for spreading growth. Non-motile microorganisms will give a discrete line; motile strains will give diffuse growth away from the inoculum line.

All *Shigella* species are non-motile.

9.4.3.4 Urea agar (B.6)

Streak the agar surface.

Incubate (6.3) at (37 ± 1) °C for (24 ± 3) h and examine at intervals.

If urea is hydrolysed, a rose-pink to deep cerise colour develops from the release of ammonia by the decomposition of the urea with a change in the colour of the pH indicator. There is no change in colour of the agar with a negative reaction.

Shigella species do not hydrolyse urea.

9.4.3.5 L-Lysine decarboxylase medium (B.7)

Inoculate below the surface of the liquid broth.

Incubate (6.3) at (37 ± 1) °C for (24 ± 3) h.

Turbidity and a purple colour after incubation indicate a positive reaction; yellow indicates a negative result.

Shigella species do not decarboxylate lysine.

NOTE The use of a paraffin overlay in the tubes can help to ensure anaerobic conditions.

9.4.3.6 L-Ornithine decarboxylase medium (B.8)

Inoculate below the surface of the liquid broth.

Incubate (6.3) at (37 ± 1) °C for (24 ± 3) h.

If a purple colour develops, the test is positive; a yellow colour means a negative result.

Shigella sonnei decarboxylates ornithine, but other *Shigella* species do not (see Table 1).

9.4.3.7 Detection of indole formation (B.9)

Inoculate a tube containing 5 ml of tryptone/tryptophan medium (B.9.1) with the pure culture.

Incubate (6.3) at (37 ± 1) °C for (24 ± 3) h.

After incubation, add 1 ml of Kovac's reagent (B.9.2).

The formation of a red ring within 10 min indicates indole formation, and a yellow/brown colour indicates a negative reaction.

Shigella sonnei is negative whilst other strains give variable reactions (see Table 1).

9.4.3.8 Detection of β-galactosidase (B.10)

Suspend a loopful of the purified culture from the nutrient agar into 0,25 ml of saline solution (B.12) in a screw cap bottle or test tube.

Add one drop of toluene and shake to mix well.

Put the tube in an incubator (6.3) set at 37 °C and leave for several minutes.

Add 0,25 ml of the complete reagent and mix.

Replace in the incubator set at 37 °C and leave for (24 ± 3) h, examining at intervals.

A yellow colour indicates the formation of β-galactosidase, which can occur in as little as 20 min.

Shigella sonnei is positive. *S. dysenteriae* and *S. boydii* give variable reactions and *S. flexneri* is negative. (see Table 1).

9.4.3.9 Utilization of carbohydrates (B.11)

Inoculate each of the prepared carbohydrate broths with a small inoculum.

Incubate (6.3) at (37 ± 1) °C for (24 ± 3) h.

A positive reaction when carbohydrate is utilized gives a change in the pH indicator from purple to yellow.

See Table 1 for the reactions of different *Shigella* species.

9.4.3.10 Interpretation of biochemical results

Strains within some *Shigella* species vary in their biochemical reactions (see Table 1), therefore interpretation based only on biochemical results is difficult and serotyping is essential to establish identity.

Shigella are Gram-negative bacilli, 2 µm to 4 µm by 0,5 µm in size, but often show a tendency to shorter cocco-bacillary forms and typically do not produce gas from glucose. They are non-motile, do not produce hydrogen sulfide or decarboxylate lysine, and are lactose negative at 24 h. The other tests described above give variable reactions or differing reactions according to the species.

Within the genus *Shigella,* mannitol discriminates *Shigella dysenteriae* (negative*)* from other species and L-ornithine decarboxylase differentiates *Shigella sonnei* (positive) from other species.

9.4.4 Additional biochemical differentiation

9.4.4.1 General

It is recommended to carry out additional biochemical differentiation tests for a better identification of the strains: some strains of *Escherichia coli* and *Shigella* species are similar.

9.4.4.2 Sodium acetate

Streak the slope of the sodium acetate medium (B.13) with the pure culture (9.4.1). Use a straight wire to minimize the amount of culture medium transferred with the inoculum, or use an inoculation needle.

Incubate under aerobic conditions for 2 days at (37 ± 1) °C.

Examine the green medium for growth: a positive result is found when the medium turns blue.

Look for the growth, a blue colour indicates a positive reaction.

If no growth occurs, incubate the culture for 2 additional days at (37 ± 1) °C. Examine the medium again.

Shigella species do not grow or grow very poorly. Strains of *E. coli* give blue colonies with the surrounding medium blue/green.

Table 1 — Biochemical differentiation and confirmation of *Shigella* species from *Escherichia coli*, *Hafnia* and *Providencia* species

Test	*Escherichia coli*	*Hafnia* species	*Providencia*	*Shigella sonnei*	*Shigella flexneri*	*Shigella dysenteriae*	*Shigella boydii*
H_2S from TSI	–	–	–	–	–	–	–
Gas from glucose (TSI)	+	V	+	–	–	– [e]	– [e]
Motility	+	V	V	–	–	–	–
Urease	–	–	V	–	–	–	–
L-Lysine decarboxylase	V	+	–	–	–	–	– [i]
L-Ornithine decarboxylase	V	+	–	+	–	–	–
Indole formation	+	–	+	–	V [d] (61 %)	V [d] (44 %)	V [d] (29 %)
β-Galactosidase	+	V	-	+ (95 %)	-	V [f] (50 %)	V [f] (11 %)
Acid from:							
Dulcitol	V	V	–	–	V [g] (9,4 %)	V [g] (4.5 %)	V [g] (6,7 %)
Glucose	+	+	–	+ (100 %)	+ (100 %)	+ (100 %)	+ (100 %)
Lactose	V	V	–	– [c]	– [a]	–	– [a]
Mannitol	+	+	V	+ (99 %)	+ [b] (94 %)	–	+ (98 %)
Melibiose	V	V	V	–	V	V	V
Raffinose	V	V	–	– [c] (2,5 %)	V (53 %)	–	–
Salicin	V	V	–	–	–	–	–
Sorbitol	+	–	–	–	V (31 %)	V (29 %)	V (42 %)
Sucrose	V	–	V	– [c] (1,5 %)	–	–	–
Xylose	+	+	–	–	–	V [h] (4,0 %)	V (57 %)

V: strains variable within or between serovars of a species and, where given, (x %) indicates percentage of positive strains [1)].

[a] Some strains of *S. flexneri* serovar 2a and *S. boydii* 9 produce acid.

[b] Some strains of *S. flexneri* serovars 4 and 6b do not produce acid.

[c] *Shigella sonnei* produces acid after several days incubation.

[d] Some serotypes of *Shigella dysenteriae* and *S. flexneri* serovar 6 and *S. boydii* are negative.

[e] Strains of *S. flexneri* and *S. boydii* serovars 13 and 14 produce acid and gas.

[f] Strains of *S. dysenteriae* serovar 1 and *S. boydii* serovar 13 are always positive.

[g] Strains of *S. dysenteriae* serovar 5 and *S. flexneri* serovar 6 are positive.

[h] Strains of *S. dysenteriae* serovars 8 and 10 are positive and 4 and 6 are variable.

[i] Only strains of *S. boydii* serovar 13 are positive.

1) From Ewing W.H. and Lindberg A.A. Serology of the *Shigella.* In: *Methods in Microbiology* (Ed. Bergan T.), Vol. 14, Academic Press, 1984.

9.4.4.3 Christensen's citrate

Inoculate the slant surface of the Christensen's citrate (B.14) using an inoculation needle with a pure culture (9.4.1). Minimize as far as possible the quantity of medium transferred with the inoculum.

Incubate aerobically for 2 days at (37 ± 1) °C.

Examine to detect a cream/pink growth. In this case, the medium changes to red.

If no growth occurs, incubate the culture for a further 2 days and examine again.

Shigella species do not grow.

9.4.4.4 Sodium mucate

Inoculate the test broth (B.15.1) and the control broth (B.15.2) with the pure culture (9.4.1).

Incubate aerobically for 2 days at (37 ± 1) °C.

Examine the medium to detect growth and colour development. A blue colour indicates a negative reaction and a yellow/straw colour indicates a positive reaction.

If no growth occurs in the test broth, incubate the culture for a further 2 days at (37 ± 1) °C. Examine the medium again.

Shigella sonnei shows variable reactions but other *Shigella* species are negative.

For further details on reactions of *Shigella* species, see Table 2.

Table 2 — Additional biochemical tests[a] to differentiate some strains of *Shigella* spp. and *Escherichia coli*

Species	Biochemical reactions (growth) for a determined period after incubation					
	Sodium acetate		Christensen's citrate		Sodium mucate	
	+ % at 2 days	+ % after 2 days	+ % at 2 days	+ % after 2 days	+ % at 2 days	+ % after 2 days
S. dysenteriae	– (0)	– (0)	– (0)	– (0)	– (0)	– (0)
S. flexneri	– (0)	– (0)	– (0)	– (0)	– (0)	– (0)
S. boydii	– (0)	– (0)	– (0)	– (0)	– (0)	– (0)
S. sonnei	– (0)	– (0)	– (0)	– (0)	V (6,4)	V (36,7)
E. coli	V (83,8)	+ (93,5)	V (> 15,3)	V (> 34,2)	+ (91,6)	+ (93,0)

\+ > 90 % strains positive.

– > 90% strains negative.

V Variable results with between 10 % to 89 % of strains positive.

% Percentage of positive strains after determined incubation.

a From *Bacteriological Analytical Manual*, 8th Edition (Revised 1997), FDA, USA.

9.5 Serological confirmation

9.5.1 Antigenic differentiation

Shigella species are non-motile and therefore do not have flagella antigens. Differentiation within and between species depends upon the analysis of distinct somatic group “O” and specific “O” type antigens (see Table 3). Typing to the serovar level is best performed by an Enteric Reference Laboratory.

Growth from a fresh culture on nutrient agar is required (see 9.4.1). Carry out agglutination tests on clean glass slides or plates of glass (6.13) of the appropriate size.

Table 3 — Antigenic differentiation within the *Shigella* species

***Shigella* species**	**Antigenic group**	**Serovars (specific antigen designation)**
S. dysenteriae	A	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13
S. flexneri	B	1a, 1b, 2a, 2b, 3a, 3b, 3c, 4a, 4b, 5a, 5b, 6, X, Y
S. boydii	C	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18
S. sonnei	D	1

NOTE 1 The group antigens (A, B, C, D) can contain minor antigens that may cross react with other group antigens; this is avoided by the use of absorbed antisera and/or its dilution to a stipulated level. Some species, particularly *Shigella dysenteriae,* have envelope antigens that will mask the group and serovar antigens which prevent agglutination with specific type antisera. The envelope antigen is removed by heating a suspension at 100 °C for 15 min to 60 min.

NOTE 2 The *Shigella sonnei* group D antigen is present in both the smooth and rough colony types and has no cross reactivity with the other *Shigella* group antigens. Unlike some other Enterobacteriaceae, the rough colony types of *S. sonnei* do not necessarily auto-agglutinate. *Shigella sonnei* has no envelope antigen.

9.5.2 Agglutination tests

Follow precisely the instructions given by the manufacturer for preparing antisera and conducting agglutination tests.

Place one drop of the group antiserum and one drop of saline solution (B.12) separately on a glass slide (6.13). Disperse part of the colony to be tested in the saline and part of the colony in the antiserum solution so as to obtain a homogeneous and turbid suspension in each.

Rock the slide gently for 30 s to 60 s.

Observe the result against a dark background, if necessary with the aid of a magnifying lens.

If the bacteria in the antiserum have clumped into more or less distinct particles and there is no agglutination in the saline, the isolate is positive for the group tested. If there is agglutination in the saline, the strain is considered to auto-agglutinate, and shall not be used further. The testing of other colonies from the same culture and the other isolates selected for examination from the original selective agar and giving biochemical reactions indicative of *Shigella* should then be tested. If all selected colonies auto-agglutinate, consult an Enteric Reference Laboratory for isolate identity.

If all tests are negative and biochemical tests are characteristic of *Shigella,* heat a suspension of the pure cultures in a water bath at 100 °C for 60 min and repeat the agglutination tests.

9.5.3 Definitive confirmation

For confirmation and/or analysis beyond the “group” factor (see Table 3), isolates should be sent to a recognized Enteric Reference Laboratory for confirmation and definitive typing.

The dispatch shall be accompanied by all possible information concerning the isolates.

10 Expression of results

In accordance with the results of the interpretation, indicate the presence or absence of confirmed *Shigella* species in the test portion of x g or x ml of product.

11 Test report

The test report shall specify:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

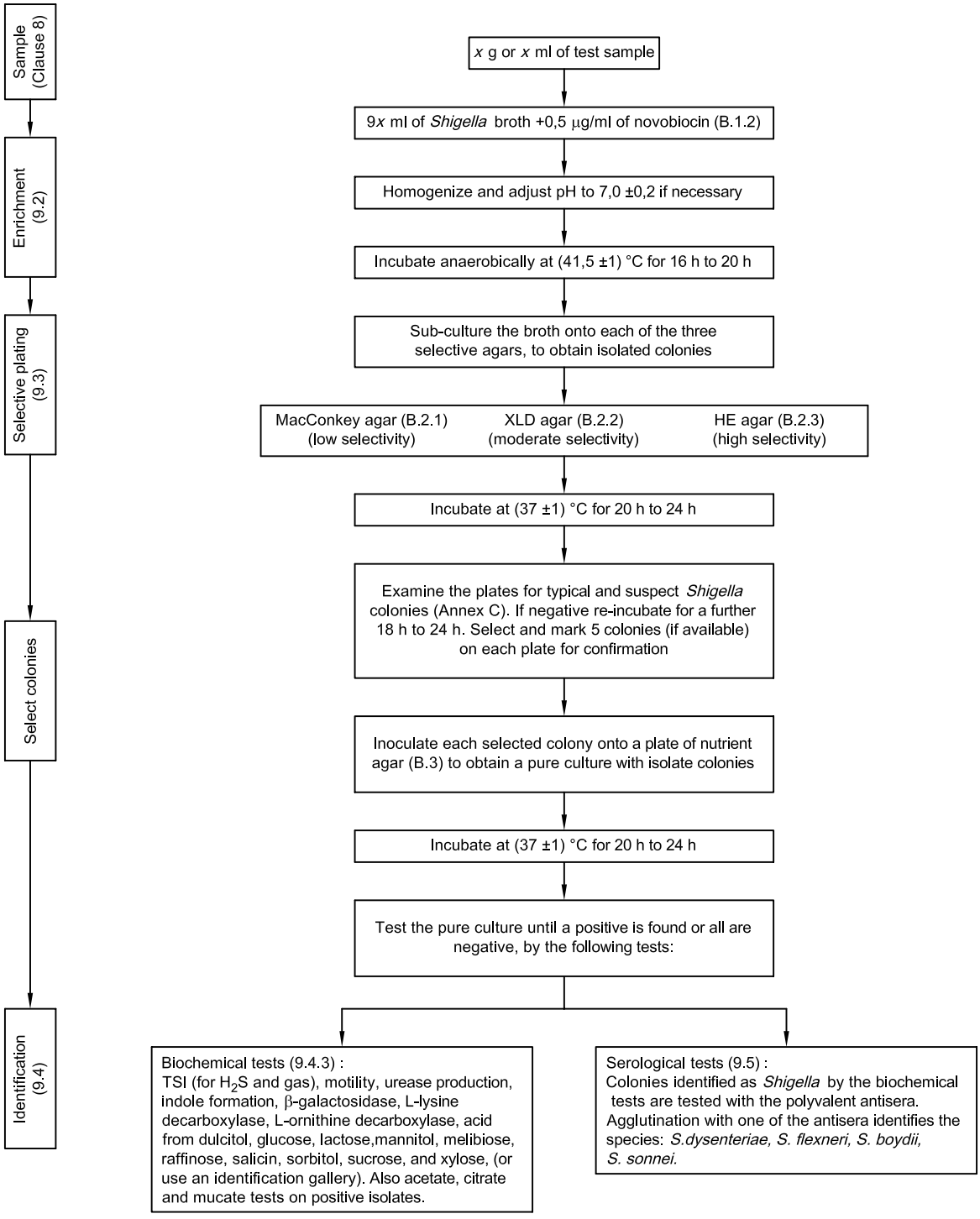
c) the test method used, with reference to this International Standard;

d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

e) the test results obtained.

Annex A
(normative)

Diagram of test procedure



Annex B
(normative)

Composition and preparation of culture media and reagents

B.1 Selective enrichment broth

B.1.1 *Shigella* broth

B.1.1.1 Composition

Enzymatic digest of casein	20,0 g
Potassium hydrogen phosphate (anhydrous)	2,0 g
Potassium dihydrogen phosphate (anhydrous)	2,0 g
Sodium chloride	5,0 g
D(+)-Glucose	1,0 g
Polyoxyethylenesorbitan monooleate (Tween 80)	1,5 ml
Water	1 000 ml

B.1.1.2 Preparation

Dissolve the components (or the dehydrated complete medium) in the water, by heating if necessary.

Adjust the pH, if necessary, using the pH meter (6.8), so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Dispense the medium in 225 ml amounts in flasks or bottles (6.9) of appropriate capacity, or in greater bulk if dispensed gravimetrically at the time of use.

Sterilize in the autoclave (6.1) set at 121 °C for 15 min.

If not used immediately, store at (5 ± 3) °C and discard if not used within 1 month.

B.1.2 Novobiocin solution

B.1.2.1 Composition

Novobiocin	25,0 mg
Distilled water	1 000 ml

B.1.2.2 Preparation

Dissolve the novobiocin in the water.

Sterilize by filtration through a 0,2 µm membrane (see ISO 7218).

Store at (5 ± 3) °C and discard if not used within 1 month.

B.1.3 Complete medium

At the time of use add either 5 ml of the novobiocin solution (B.1.2) to each 225 ml of *Shigella* broth (B.1.1) or 22 ml of novobiocin solution per litre of *Shigella* broth if prepared in larger volumes. Mix well.

This gives a final concentration of novobiocin of 0,5 µg/ml broth after 25 g or 25 ml of sample is added.

B.2 Selective differential agars

B.2.1 MacConkey agar

B.2.1.1 Composition

Enzymatic digest of casein and animal tissues	20,0 g
Lactose	10,0 g
Bile salts No. 3	1,5 g
Sodium chloride	5,0 g
Neutral red	0,03 g
Crystal violet	0,001 g
Agar	9 g to 18 g[a]
Water	1 000 ml
[a] Depending on the gel strength of the agar.	

B.2.1.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by boiling for a minimum time until dissolved.

Adjust the pH (6.8), if necessary, so that after heating it is 7,1 ± 0,2 at 25 °C.

Sterilize in the autoclave (6.1) set at 121 °C for 15 min, or otherwise as stated by the manufacturer.

Cool the molten medium in a water bath (6.5) set at 47 °C.

Pour about 15 ml into Petri dishes and allow to set in a horizontal position.

Immediately before use, dry the plates carefully until the agar surface is free of water droplets, either in a drying oven (6.2) set between 37 °C to 55 °C with their lids off and the agar surface downwards, or in a drying cabinet with the agar surface upwards.

If prepared in advance, the undried plates may be packed in plastic bags (or similar) to avoid drying out, and stored in a refrigerator at (5 ± 3) °C. Discard if not used within 2 weeks.

B.2.2 Xylose lysine desoxycholate (XLD) agar

NOTE Commercial formulations vary depending on whether the medium is autoclaved at 121 °C or only boiled.

B.2.2.1 Composition

Yeast extract	3,0 g
L-Lysine HCl	5,0 g
Xylose	3,75 g
Lactose	7,5 g
Saccharose	7,5 g
Sodium chloride	5,0 g
Sodium desoxycholate	1,0 g
Sodium thiosulfate	6,8 g
Ammonium ferric citrate	0,8 g
Phenol red	0,08 g
Agar	9 g to 18 g[a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

B.2.2.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by boiling for a minimum time until dissolved.

Adjust the pH (6.8), if necessary, so that after heating it is 7,4 ± 0,2 at 25 °C.

Cool the molten medium in a water bath (6.5) set at 47 °C.

Pour about 15 ml into Petri dishes and allow to set in a horizontal position.

Immediately before use, dry the plates carefully until the agar surface is free of water droplets, either in a drying oven (6.2) set between 37 °C to 55 °C with their lids off and the agar surface downwards, or in a drying cabinet with the agar surface upwards.

If prepared in advance, the undried plates may be packed in plastic bags (or similar) to avoid drying out, and stored in a refrigerator at (5 ± 3) °C. Discard if not used within 2 weeks.

B.2.3 Hektoen enteric (HE) agar

B.2.3.1 Composition

Enzymatic digest of meat	12,0 g
Yeast extract	3,0 g
Lactose	12,0 g
Saccharose	12,0 g
Salicin	2,0 g
Bile salts No. 3	9,0 g
Sodium chloride	5,0 g
Sodium thiosulfate	5,0 g
Ammonium ferric citrate	1,5 g
Acid fuchsin	0,1 g
Bromothymol blue	0,065 g
Agar	12 g to 18 g[a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

B.2.3.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by boiling for a minimum time to dissolve the agar, or according to the manufacturer's instructions.

Adjust the pH (6.8), if necessary, so that after heating it is 7,5 ± 0,2 at 25 °C.

Cool the molten medium in a water bath (6.5) set at 47 °C.

Pour about 15 ml into Petri dishes and allow to set in a horizontal position.

Immediately before use, dry the plates carefully until the agar surface is free of water droplets, either in a drying oven (6.2) set between 37 °C to 55 °C with their lids off and the agar surface downwards, or in a drying cabinet with the agar surface upwards.

If prepared in advance, the undried plates may be packed in plastic bags (or similar) to avoid drying out, and stored in a refrigerator at (5 ± 3) °C. Discard if not used within 2 weeks.

B.3 Nutrient agar

B.3.1 Composition

Meat extract	3,0 g
Enzymatic digest of casein	5,0 g
Agar	12 g to 18 g[a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

B.3.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by boiling if necessary.

Adjust the pH (6.8) so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Transfer the culture medium into flasks or bottles of appropriate capacity.

Sterilize in an autoclave (6.1) set at 121 °C for 15 min.

B.3.3 Preparation of nutrient agar plates

Transfer about 15 ml of molten medium, cooled to 47 °C (6.5), into Petri dishes.

Immediately before use, dry the plates carefully so that the surface is free of water droplets, preferably with the lids off and the agar surface downwards, in an oven (6.2) set between 37 °C and 55 °C, or in a drying cabinet.

If prepared in advance, store the undried plates in plastic bags in a refrigerator at (5 ± 3) °C. Discard if not used within 2 weeks.

B.4 Triple iron sugar (TSI) agar

B.4.1 Composition

Meat extract	3,0 g
Yeast extract	3,0 g
Peptone	20,0 g
Sodium chloride	5,0 g
Lactose	10,0 g
Sucrose	10,0 g
Glucose	1,0 g
Ferric citrate	0,3 g
Sodium thiosulfate	0,3 g
Phenol red	0,024 g
Agar	9 g to 18 g[a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

B.4.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by heating if necessary.

Adjust the pH (6.8), if necessary, so that after sterilization it is 7,4 ± 0,2 at 25 °C.

Dispense the medium in about 10 ml amounts into tubes or dishes (6.11) of appropriate capacity.

Sterilize in an autoclave (6.1) set at 121 °C for 15 min.

Allow to set in a sloping position to give a butt of depth 2,5 cm to about 5 cm.

If not used immediately, store at (5 ± 3) °C. Discard if not used within 1 month.

B.5 Semi-solid nutrient agar

B.5.1 Composition

Meat extract	3,0 g
Enzymatic digest of animal tissue	5,0 g
Agar	4 g to 9 g[a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

B.5.2 Preparation

Dissolve the components in the water by heating.

Adjust the pH (6.8), if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Transfer 10 ml of the molten medium to tubes or screw cap bottles (6.11) of appropriate capacity.

Sterilize in an autoclave (6.1) set at 121 °C for 15 min.

If not used immediately, store at (5 ± 3) °C. Discard if not used within 1 month.

B.6 Urea agar (Christensen's medium)

B.6.1 Base

B.6.1.1 Composition

Enzymatic digest of animal tissue	1,0 g
Glucose	1,0 g
Sodium chloride	5,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	2,0 g
Phenol red	0,012 g
Agar	9 g to 18 g[a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

B.6.1.2 Preparation

Dissolve the components or the complete dehydrated base in water by heating, if necessary.

Adjust the pH (6.8), if necessary, so that after sterilization it is 6,8 ± 0,2 at 25 °C.

Sterilize in an autoclave (6.1) set at 121 °C for 15 min.

B.6.2 Urea solution

B.6.2.1 Composition

Urea	400 g
Water to a final volume of	1 000 ml

B.6.2.2 Preparation

Dissolve the urea in the water.

Sterilize by filtration through a 0,2 µm membrane and check a portion for sterility. See ISO 7218:1996, 7.3.2.

B.6.3 Complete medium

B.6.3.1 Composition

Base medium (B.6.1)	900 ml
Urea solution (B.6.2)	50 ml

B.6.3.2 Preparation

Add, using aseptic techniques, the urea solution to the base which has been previously melted and then cooled to 47 °C (6.5).

Dispense the complete medium in quantities of 10 ml into tubes or dishes (6.11) of appropriate size.

Allow to set in a sloping position.

B.7 L-Lysine decarboxylase medium

B.7.1 Composition

L-Lysine monohydrochloride	5,0 g
Yeast extract	3,0 g
Glucose	1,0 g
Bromocresol purple	0,015 g
Water	1 000 ml

B.7.2 Preparation

Dissolve the components in the water, by heating if necessary.

Adjust the pH (6.8), if necessary, so that after sterilization it is 6,8 ± 0,2 at 25 °C.

Transfer the medium in quantities to of 2 ml to 5 ml to narrow culture tubes (6.11) with screw caps.

Sterilize in an autoclave (6.1) set at 121 °C for 15 min.

B.8 L-Ornithine decarboxylase medium

Proceed as for the whole of B.7, but substituting 5,0 g of L-ornithine monohydrochloride for the L-lysine monohydrochloride.

B.9 Reagents for indole reaction

B.9.1 Tryptone/DL-tryptophan medium

B.9.1.1 Composition

Pancreatic digest of casein	10,0 g
Sodium chloride	5,0 g
DL-Tryptophan	1,0 g
Water	1 000 ml

B.9.1.2 Preparation

Dissolve the components in the water by heating.

Adjust the pH (6.8), if necessary, so that after sterilization it is 7,5 ± 0,2 at 25 °C.

Transfer the medium in quantities of 5 ml into narrow culture tubes or screw cap bottles (6.11).

Sterilize in an autoclave (6.1) set at 121 °C for 15 min.

If not used immediately, store at (5 ± 3) °C. Discard if not used within 1 month.

B.9.2 Kovac's indole reagent

B.9.2.1 Composition

4-Dimethylaminobenzaldehyde	5,0 g
Hydrochloric acid (ρ = 1,18 g/ml to 1,19 g/ml)	25 ml
2-Methylbutan-2-ol	75 ml

B.9.2.2 Preparation

Mix the components.

Store at (5 ± 3) °C and use within 1 month.

B.10 β-Galactosidase reagent

B.10.1 Buffer solution

B.10.1.1 Composition

Sodium dihydrogen phosphate (NaH_2Po_4)	6,9 g
Sodium hydroxide (10 mol/l solution)	about 3 ml
Water to a final volume of	50 ml

B.10.1.2 Preparation

Dissolve the sodium dihydrogen phosphate in approximately 45 ml of water in a 50 ml volumetric flask.

Adjust the pH (6.8) to 7,0 ± 0,2 at 25 °C with sodium hydroxide solution.

Add water to a final volume of 50 ml.

B.10.2 ONPG solution

B.10.2.1 Composition

o-Nitrophenol-β-D-galactopyranoside (ONPG)	0,08 g
Water	15 ml

B.10.2.2 Preparation

Dissolve the ONPG in the water at 50 °C (6.5), then cool the solution.

B.10.3 Complete medium

B.10.3.1 Composition

Buffer solution (B.10.1)	5 ml
ONPG solution (B.10.2)	15 ml

B.10.3.2 Preparation

Add the buffer solution to the ONPG solution.

Store at (5 ± 3) °C. Discard if not used within 1 month.

B.11 Bromocresol purple broth

B.11.1 Composition

Enzymatic digest of animal tissue	10,0 g
Meat extract	3,0 g
Sodium chloride	5,0 g
Carbohydrate or alcohol[a]	10,0 g
Bromocresol purple	0,04 g
Water	1 000 ml

[a] The following carbohydrates and alcohols are used individually: dulcitol, glucose, lactose, mannitol, melibiose, raffinose, salicin, sorbitol, sucrose, xylose.

B.11.2 Preparation

Dissolve the components or the complete dehydrated base in the water, by heating if necessary

Adjust the pH (6.8) so that after sterilization, it is 7,0 ± 0,2 at 25 °C.

Sterilize by filtration through a 0,2 µm membrane filter into a sterile container.

Dispense 5,0 ml amounts into sterile tubes or bottles of appropriate size (6.11).

Check a few units of each complete medium for sterility by incubation at 37 °C for 24 h.

The broth may be stored for up to 4 weeks at (5 ± 3) °C

B.12 Saline solution

B.12.1 Composition

Sodium chloride	8,5 g
Water	1 000 ml

B.12.1.1 Preparation

Dissolve the sodium chloride in the water.

Adjust the pH (6.8), if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Transfer the solution into flasks to screw cap bottles in quantities of about 10 ml.

Sterilize in an autoclave (6.1) set at 121 °C for 15 min.

B.13 Sodium acetate agar

B.13.1 Composition

Sodium acetate	2,0 g
Sodium chloride	5,0 g
Magnesium sulfate (anhydrous)	0,2 g
Ammonium phosphate	1,0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	1,0 g
Bromothymol blue	0,08 g
Agar	9 g to 18 g[a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

B.13.2 Composition

Dissolve all the components except the $MgSO_4$, in the water by boiling and mixing, then add the $MgSO_4$ or otherwise as stated by the manufacturer for dehydrated complete medium.

Adjust the pH (6.8), if necessary, so that after sterilization it is 6,7 $\pm$ 0,2 at 25 °C.

Dispense about 8 ml portions into test tubes (6.11).

Sterilize in an autoclave (6.1) set at 121 °C for 15 min.

Allow to set in a sloping position to give a 5 cm slant surface.

B.14 Christensen's citrate agar

B.14.1 Composition

Sodium citrate	3,0 g
Glucose	0,2 g
Yeast extract	0,5 g
Cysteine monohydrochloride	0,1 g
Ferric ammonium citrate	0,4 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,0 g
Sodium chloride	5,0 g
Sodium thiosulfate	0,08 g
Phenol red	0,012 g
Agar	9 g to 18 g[a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

B.14.2 Preparation

Dissolve the components or the complete dehydrated medium in water by boiling and mixing.

Adjust the pH (6.8), if necessary, so that after sterilization it is 6,9 ± 0,2 at 25 °C.

Dispense into test tubes (6.11) to fill one-third of the tube, then cap.

Sterilize in an autoclave (6.1) set at 121 °C for 15 min.

Allow to set in a sloping position to give a 4 cm to 5 cm slant surface and a butt of agar 2 cm to 3 cm deep.

B.15 Mucate broth

B.15.1 Test broth

B.15.1.1 Composition

Enzymatic digest of casein[a]	10,0 g
Mucic acid	10,0 g
Bromothymol blue	0,024 g
Water	1 000 ml

[a] Alternatively, 25,0 g of nutrient broth No. 2 may be used.

B.15.1.2 Preparation

Dissolve the mucic acid by slowly adding small amounts of 5 mol/l sodium hydroxide, then mix.

Dissolve the enzymatic digest of casein in water, then add the mucic acid.

Adjust the pH (6.8) so that after sterilization it is 7,4 ± 0,2 at 25 °C.

Dispense 5 ml portions into test tubes (6.11)

Sterilize in an autoclave (6.1) set for 10 min at 121 °C.

B.15.2 Control broth

B.15.2.1 Composition

Enzymatic digest of casein	10,0 g
Bromothymol blue	0,024 g
Water	1 000 ml

B.15.2.2 Preparation

Dissolve the components in the distilled water.

Adjust the pH (6.8) so that after sterilization it is 7,4 ± 0,2 at 25 °C.

Dispense about 5 ml quantities into tubes (6.11).

Sterilize in an autoclave (6.1) set for 10 min at 121 °C.

B.16 Antisera of *Shigella* species

Group antisera (types A, B, C, and D, see Table 3) are required. These are available from either an Enteric Reference Laboratory or commercially.

Follow the instructions of the supplier concerning their preparation and use.

Annex C
(normative)

Description of *Shigella* colony morphology and colour on selective agars, for both identification and quality control purposes

All Enterobacteriaceae on all these media have round colonies with a smooth surface and entire edge. The size of well-isolated colonies is generally > 2 mm. See Table C.1 for details.

Table C.1 — Description of *Shigella* colony morphology and colour on selective agars

Bacterial species	Selective agars		
	MacConkey agar	XLD agar	Hektoen agar
Shigella sonnei [a]	Colourless to pale pink, translucent, lactose negative.	Translucent with red/cerise centre, same colour as the agar.	Green and moist raised colonies.
Shigella, other species	Colourless, translucent, lactose negative.	Translucent with red/cerise centre, same colour as the agar.	Green and moist.
Escherichia coli	Red with turbid precipitate in agar.	Yellow, opaque, surrounded by yellow precipitate in agar.	Red/salmon with zone of precipitate in agar.
Enterobacter cloacae	Red with turbid precipitate in agar.	Yellow, opaque, surrounded by yellow precipitate in agar	Red/salmon with zone of precipitation agar.
Klebsiella pneumoniae	Red with turbid precipitate in agar.	Yellow, opaque and mucoid, surrounded by yellow precipitate.	Red/salmon with zone of precipitation agar.
Salmonella	Colourless and translucent, agar yellow around colony.	Red with black centre.	Blue green, with or without black centre.
Proteus mirabilis	Colourless and translucent, agar yellow around colony.	Yellow, black centre, yellow agar with precipitate.	Blue/green, with or without black centre.
Enterococcus faecalis	Red, small round colonies.	None or poor growth, colonies yellowish.	None or poor growth, colonies yellowish.

[a] *Shigella sonnei* may ferment lactose after > 40 h incubation, resulting in a weak reaction similar to *Escherichia coli* after this time. *Shigella sonnei* colonies can also show a smooth to rough variation; this is generally accompanied with a loss of a 120 megadalton plasmid and a loss of virulence, and colonies may auto-agglutinate upon suspension in saline and antiserum.

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