भारतीय मानक
Indian Standard

IS 11061 : 2023

विब्रियो कॉलेरा का डायग्नोस्टिक सेरा तैयार करने की संहिता

(पहला पुनरीक्षण)

Code for Preparation of *Vibrio cholerae* Diagnostic Sera

(First Revision)

ICS 70.100.99

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June 2023 **Price Group 5**

Food Microbiology Sectional Committee, FAD 31

FOREWORD

This Indian Standard (First Revision) was adopted by the Bureau of Indian Standards, after the draft finalized by the Food Microbiology Sectional Committee had been approved by the Food and Agriculture Division Council.

The production of reliable diagnostic sera, by immunizing rabbits, demands close attention at all stages in the process. The sera produced should be of good quality and specific in nature. Since the production of the diagnostic sera is costly, the users must be made aware of the need to conserve this reagent.

In the preparation of this standard, considerable assistance has been derived from the Central Research Institute, Kasauli.

This standard was first published in 1984. The first revision of the standard has been brought out to incorporate following important modifications keeping in view the technological advancements in this area along with the editorial changes to align it in the latest style and format of Indian Standard:

a) Preservation and storage temperature and conditions of sera has been updated at **9**;
b) The procedure of 'Bleeding Test' has been modified in **5.3** keeping in view the guidelines issued by CPCSEA (Committee for the purpose of control and supervision of experiments on animals); and
c) *Vibrio cholerae* O139 Serum has been added in the new clause **8.5**.

The composition of the committee responsible for the formulation of this standard is listed in Annex D.

For the purpose of deciding whether a particular requirement of this standard is complied with, the final value, observed or calculated, expressing the result of a test or analysis, shall be rounded off in accordance with IS 2 : 2022 'Rules for rounding off numerical values (*second revision*)'. The number of significant places retained in the rounded off value should be the same as that of the specified value in this standard.

Indian Standard

CODE FOR PREPARATION OF *Vibrio cholerae* DIAGNOSTIC SERA

(First Revision)

1 SCOPE

This standard specifies the guidelines for raising, absorption and testing of various diagnostic antisera used for serotyping of *Vibrio cholerae*. This is the guidance document and the actual method may vary depending on the manufacturing procedures validated by individual manufacturers.

2 REFERENCES

The standards listed below contain provisions which, through reference in this text, constitute provisions of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of these standards listed below:

IS No.	*Title*
IS 6850 : 2023	Specification for agar, microbiological grade (*first revision*)

3 SELECTION OF STRAINS

3.1 In the production of diagnostic agglutinating antisera, the antigen for immunization of rabbits must be prepared from a known classical strain or any other strain standardized/validated by the individual manufacturer as per its validated procedures.

For this purpose, the recommended strains (*see* Annex A) or any other strain standardized/validated by the individual manufacturer can be obtained from the following centres:

a) National Salmonella & Escherichia Centre and National Collection of Type Cultures, Central Research Institute, Kasauli;
b) National Institute of Cholera and Enteric Diseases, Calcutta;
c) National Salmonella Centre (Veterinary), Izzatnagar, Bareilly;
d) Microbial Type Culture Collection, IMTECH, Chandigarh;
e) National Collection of Type Cultures, Salisbury, UK;
f) American Type Culture Collection, USA; and
g) Any other type culture collection centre.

3.2 It is advisable not to use wild and field strains for preparation of antigens in the production of diagnostic sera.

4 PREPARATION OF ANTIGEN

4.1 Plate out the bacterial strain (freeze dried) on a good quality nutrient agar (*see* Annex B) plate which has been well dried and tested for sterility. Incubate overnight at 37 °C. Observe the appearance of the colonies and if any rough colonies are visible, repeat the plating to obtain smooth colonies.

4.1.1 From a satisfactory plate select 5 well isolated smooth colonies and test for the absence of auto-agglutination by slide agglutination in physiological saline (0.85 percent) and 1 : 500 acriflavine aqueous solution.

4.1.2 Characterize the selected colonies biochemically and serologically

4.1.3 Subculture all 5 characterized colonies in 10 ml of nutrient broth and incubate at 37 ºC.

4.1.4 After 4 h to 6 h, inoculate a nutrient agar plate (diameter 145 mm) or a rolled whisky agar bottle with 1 ml or 5 ml of the nutrient broth cultures, respectively. Spread the culture over the surface of the plate/bottle and incubate overnight at 37 ºC.

4.1.5 Harvest the growth in 2 ml/10 ml of physiological saline (0.85 percent) in case of plate and bottle, respectively, so as to obtain a thick suspension.

4.1.6 Steam at 100 °C for 2 h 30 min and then cool.

4.1.7 Add thick steamed suspension, drop by drop, to 20 ml of buffered formal saline (*see* Annex C) until the bacterial concentration reaches about 3 000 x 10^6 organisms per ml. This antigen suspension usually remains satisfactory for six months, if stored at 4 °C.

4.2 Auto-Agglutination Test

4.2.1 Before use, the antigen suspension shall be tested for auto-agglutination.

4.2.2 Dilute the suspension to 400 x 10^6 organisms per ml using buffered formol saline and place 0.5 ml into each of 4 Dreyer's tubes. Incubate at 50 °C to 52 °C for 18 h and check for auto-agglutination.

4.2.3 The antigen suspension should be tested in tubes against a homologous serum and should give at least standard agglutination at the stated titre of the serum.

4.2.4 Standard *Vibrio cholerae* serum used for the tube agglutination tests are prepared by immunizing rabbits. These should have a sufficient titre (1/600) and should show no cross reactions with heterologous antigens.

5 IMMUNIZATION AND BLEEDING SCHEDULES

5.1 Selection of Rabbits

Disease - free rabbits weighing 1.5 kg to 2 kg are test bled for pre-immunity agglutinating titres. If any rabbit serum shows agglutination against *Vibrio cholerae,* that animal is not suitable for raising the serum and is discarded.

5.2 Immunization

Rabbits are injected (*see* **4.1.6**) according to the schedule shown with the immunizing suspension below:

Day	*Suspension in ml*
First	0.25
Third	0.5
Sixth	1.0
Ninth	1.5
Twelvth	2.0
Sixteenth	Bleeding

Different immunization schedule may be used as per the standardized/validated procedure by individual manufacturer.

5.3 Bleeding Schedule

The rabbits are test bled on the 4th day of the last injection. If the titre is found suitable, the rabbits are bled out by cardiac puncture.

NOTE — The animal procedures should be undertaken strictly as per the CPCSEA (Committee for the purpose of control and supervision of experiments on animals) guidelines.

5.3.1 Blood is collected into 150 mm × 16 mm test tubes or other glass containers, which have been washed and rinsed thoroughly. Allow the blood to clot, first placing in a 37 °C incubator for 2 h and a refrigerator (4 ºC) overnight.

5.3.2 Remove the serum on the following day. Any serum which shows haemolysis should be discarded.

5.3.3 After being tested and any necessary absorptions having been completed, the serum is seitz filtered or membrane filtered. The serum is preserved by adding 0.4 percent phenol saline/0.01 percent thiomersal/0.08 percent sodium azide.

5.3.4 The serum shall be stored in the refrigerator at 4 °C.

NOTE — Most antigens produce an adequate response after one course of injections. Repeated immunization courses often produce sera with cross reactions to heterologous antigens. Such sera require extensive absorptions to ensure specificity and therefore repeated immunization courses are not recommended.

6 TESTING OF SERUM

The serum is tested for homologous and heterologous antibody titre by tube agglutination. The results of this testing will determine the extent of absorption required.

7 ABSORPTION

The sera showing heterologous agglutination require to be absorbed. The absorbing strain(s) should be smooth. The organisms for absorption are grown in nutrient agar plates (145 mm) or whisky agar bottles. The growth is harvested using normal saline and heated at 100 °C for 2 h 30 min, centrifuged and the supernatant is discarded. The serum under absorption is added to the sediment and mixed thoroughly. It is then kept in a water bath at 52 ºC for 2 h and centrifuged. The clear supernatant serum is pipetted out and tested for agglutination. If the serum gives agglutination against a heterologous culture, second absorption is done as before. After the absorption procedure, the serum is seitz filtered or membrane filtered and agglutination titre is determined.

NOTE — Serum should be discarded if heterologous titre persists even after second absorption.

8 FINAL TESTING OF ABSORBED SERA

Absorbed serum should give the under mentioned titre:

8.1 In Case of *Vibrio cholerae* Inaba Serum

Vibrio cholerae Inaba = 160 or above (Homologous)
Vibrio cholerae Ogawa = 20 Negative (Heterologous)
Vibrio cholerae Rough = 20 Negative (Heterologous)

8.2 In Case of *Vibrio cholerae* Ogawa Serum

Vibrio cholerae Ogawa = 160 or above (Homologous)
Vibrio cholerae Inaba = 20 Negative (Heterologous)
Vibrio cholerae Rough = 20 Negative (Hetcrologous)

8.3 In Case of *Vibrio cholerae* Rough Serum

Vibrio cholerae Rough = 160 or above (Homologous)
Vibrio cholerae Inaba = 20 Negative (Heterologous)
Vibrio cholerae Ogawa = 20 Negative (Heterelogous)

8.4 In Case of *Vibrio cholerae* Non-Differential Serum

Vibrio cholerae Inaba = 160 or above
Vibrio cholerae Ogawa = 160 or above
Vibrio cholerae Rough = 20 negative

8.5 In Case of *Vibrio cholerae* O139 Serum

Vibrio cholerae O139 = 160 or above
Vibrio cholerae Rough = 20 negative

9 PRESERVATION AND STORAGE OF SERA

9.1 After testing and any necessary absorptions the serum is seitz filtered or membrane filtered. This is then preserved in 0.4 percent phenol or 0.01 percent thiomersal or 0.08 percent sodium azide.

9.2 The sera are stored in the dark at 2 °C to 8 °C and usually the titre remains steady. They must not be frozen. However, sometimes the homologous titre falls and the fall in titre is abrupt. But in general, absorbed sera show this tendency more frequently than unabsorbed sera.

9.3 Before filling, the titre of the stock serum should be determined and the serum is diluted with 0.4 percent phenol/normal saline to give a titre of about 1 in 160.

9.4 In addition to giving a satisfactory titre the diluted serum must give a rapid agglutination when tested by slide agglutination with an appropriate suspension of the homologous strain.

9.5 The diluted serum is distributed in 1 ml to 2 ml amounts into suitable sterile ampoules or vials.

10 LABELLING OF *Vibrio cholreae* SERA

10.1 Absorbed *Vibrio cholerae* Ogawa Sera

It shall be labelled as *Vibrio cholerae* Ogawa sera with its date of manufacturing, quantity and the titer. It will agglutinate strains of *Vibrio cholerae* Ogawa and will not agglutinate strains of *Vibrio cholerae* Inaba when tested by the slide agglutination test.

10.2 Absorbed *Vibrio cholerae* Inaba Sera

It shall be labelled as *Vibrio cholerae* lnaba sera with its date of manufacturing, quantity and the titer. It will agglutinate strains of *Vibrio cholerae* Inaba and will not agglutinate strains of *Vibrio cholerae* Ogawa when tested by the slide agglutination test.

10.3 Absorbed *Vibrio cholerae* Non-Differential Serum

It shall be labelled as *Vibrio cholerae* non-differential sera with its date of manufacturing, quantity and the titer. It shall agglutinate with strains of *Vibrio cholerae* Inaba and *Vibrio cholerae* Ogawa when tested by slide agglutination test.

10.4 Absorbed *Vibrio cholerae* Rough Serum

It shall be labelled as *Vibrio cholerae* Rough serum with its date of manufacturing, quantity and the titer. It shall agglutinate with *Vibrio cholerae* rough strains but not with *Vibrio cholerae* Inaba and *Vibrio cholerae* Ogawa strains.

10.5 Final Label

The bottles (or ampoule) containing *Vibrio cholerae* agglutinating serum shall be labelled to give the following information:

a) Name of the manufacturer and manufacturing licence number, if any;
b) Name of the serum shall be written as indicated above;
c) Quantity, titre of the serum, the preservative used, if any, and temperature of storage;

d) Batch (or lot) number, manufacturing and expiry date;
e) Label should have the words 'FOR LABORATORY USE ONLY'; and
f) Not to be frozen, to be stored at 2 °C to 8 °C.

11 TEST FOR STERILITY

The sera should be suitably tested for sterility.

12 RECORDS TO BE MAINTAINED BY THE MANUFACTURER

It shall be mandatory for the manufacturer to maintain all the relevant records of manufacturing and quality control processes.

ANNEX A

(*Clause* 3.1)

Vibrio cholerae STRAINS FOR IMMUNISATION AND ABSORPTION

Name of Serum	Raising Strain	Absorbing Strains
Vibrio cholerae Inaba serum	*Vibrio cholerae* Inaba No. 569/B	1) *Vibrio cholerae* Ogawa S/121/58 2) *Vibrio cholerae* Ogawa B-53-2 3) *Vibrio cholerae* Ogawa 41
Vibrio cholerae Ogawa serum	*Vibrio cholerae* Ogawa B-53-2	1) *Vibrio cholerae* Inaba 49520 2) *Vibrio cholerae* Inaba 569/B 3) *Vibrio cholerae* Inaba B-53-1
Vibrio cholerae Rough serum	*Vibrio cholerae* Rough B-53-3	1) *Vibrio cholerae* Inaba 49520 2) *Vibrio* cholera Ogawa S/121/58

ANNEX B

(*Clause* 4.1)

NUTRIENT AGAR

B-1 COMPOSITION

Ingredients	Gms/Litre
Peptone	5.0
Sodium chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5
Agar	15.0

B-2 PREPARATION

Suspend 28.0 g media in 1 000 ml of purified/distilled water. Adjust final *p*H (7.4 ± 0.2) at 25 °C. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure 103 kPa (15 lbs) for 15 min. Cool to 45 °C to 50 °C. Mix well and pour into sterile petri plates.

ANNEX C

(*Clause* 4.1.6)

PREPARATION OF BUFFERED FORMOL SALINE (BFS)

C-1 STOCK BFS, 2.5 PERCENT

Commercial formalin (40 percent) 50 ml
Physiological saline 2 000 ml
Adjust *p*H to 7.6 by addition of Na_2HPO_4 crystals

C-2 WORKING BFS, 0.25 PERCENT

Stock (2.5 percent BFS) 40 ml
Physiological saline 360 ml

ANNEX D

(*Foreword*)

COMMITTEE COMPOSITION

Food Microbiology Sectional Committee, FAD 31

Organization	*Representative(s)*
ICAR - Indian Veterinary Research Institute, Izzatnagar, Bareilly	DR KIRAN N. BHILEGAONKAR **(*Chairperson*)**
CSIR - Central Food Technological Research Institute, Mysuru	DR ALOK K. SRIVASTAVA DR ASHA MARTIN (*Alternate*)
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Member Secretary
SHRIMATI VARSHA GUPTA
SCIENTIST 'D'/JOINT DIRECTOR
(FOOD AND AGRICULTURE), BIS

Panel responsible for Review of Indian Standards related to Microbiological Media Ingredients, FAD 31 : Panel 3

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