Cholera (*Vibrio cholerae*)

Laboratory case definition

The Public Health Laboratory Network (PHLN) has developed standard case definitions for the diagnosis of key diseases in Australia. This document contains the laboratory case definition for *vibrio cholerae*.

Authorisation: PHLN
Consensus date: 25 September 2006

---

1 PHLN SUMMARY LABORATORY DEFINITION

1.1 Condition

Cholera

1.1.1 Definitive Criteria

Growth of *Vibrio cholerae.*

1.1.2 Suggestive Criteria

None

2 Introduction

Cholera is an epidemic diarrhoeal disease, first described as a distinct clinical entity by Thomas Sydenham in 1817. The bacterial aetiology was established in 1854 by Pacini who gave the organism its name, *Vibrio cholerae.* Pacini's findings were subsequently confirmed by Koch in 1883. John Snow recognised the route of transmission during his studies of the Broad Street pump incident in 1849; part of the second pandemic. *V. cholerae* is a curved, Gram negative bacillus with a single, polar flagellum. As in the Enterobacteriaceae, *V. cholerae* has somatic (O) and flagellar (H) antigens with the different O groups being referred to as serogroups or serovars *V. cholerae* O1 can be subdivided into three serotypes; Inaba (O antigens A & C), Ogawa (O antigens A & B) and Hikojima (O antigens A, B & C). There are two biotypes; the classical biotype, which is susceptible to polymyxin B and lysis by classical IV and FK bacteriophages; and the El Tor biotype, which lyses sheep erythrocytes, agglutinates chicken erythrocytes and shows a modified CAMP reaction.

The two *V. cholerae* O1 biotypes differ in their virulence. The classical biotype causes approximately equal numbers of symptomatic and asymptomatic infections, while the majority of El Tor biotype

infections are asymptomatic. There is no evidence that any one of the three serotypes is more virulent but serotype Hikojima is only rarely encountered. The other two serotypes are commonly seen in an epidemic setting.

The first six cholera pandemics were most probably caused by the classic biotype of *Vibrio cholerae* O1. The seventh pandemic was caused by the El Tor biotype of *V. cholerae* O1 and began in Sulawesi, Indonesia in 1961. Only *V. cholerae* O1 was thought to cause epidemic cholera until a non-O1 *V. cholerae* belonging to serogroup O139 was isolated during an epidemic in India in 1992 and subsequently spread worldwide

Cholera has an unusual ability to exhibit both epidemic and endemic epidemiology. The disease often persists in endemic form where an epidemic has occurred. Persistence of *V. cholerae* occurs in association with the plankton that forms a part of the biofilms on the surface of marine crustaceans. *V. cholerae* can also persist in the environment in a viable but non-culturable form. *V. cholerae* O1 has been endemic in Queensland river systems since the early 1970s, when it was first detected following a locally acquired case. It can usually be recovered during the summer months when the organism changes from its non-cultivatable state as a result of increasing water temperatures. Non-O1 *V. cholerae* are also present in Australian waters and may cause mild diarrhoeal disease but are not regarded as a significant public health hazard. Cholera is not endemic in Australian communities with a treated potable water supply and high standards of sanitation.

The principal clinical feature of cholera, a profuse watery diarrhoea, is caused by the action of a toxin that binds to specific receptors on cells lining the small intestine. Cholera toxin (CT) inhibits the absorption of sodium and chloride ions by intestinal villi and promotes the secretion of chloride ions and water by intestinal crypt cells. Other *V. cholerae* toxins such as Zot (zonula occludens toxin) Ace (sodium channel inhibitor), haemolysin/cytolysin, Shiga-like toxin, ST and “new cholera toxin” are thought to add to the pathogenesis of cholera and may be responsible for diarrhoea caused by non-CT producing strains (Kaper et al 1995).

The public health significance of isolating *V. cholerae* from a case of diarrhoea will vary depending on the patient’s travel history and the organism’s virulence factors and serotype. Although there are strains of O1 and O139 which do not produce CT and do not produce cholera and there are strains of non-O1/non-O139 which do produce CT and cholera, there is no evidence that non-O1/non-O139 strains are involved in epidemics. Since *V. cholerae* is endemic to water systems around Australia and locally acquired cases are occasionally detected, all isolates presumptively identified as *V. cholerae* should be referred to a laboratory, which can fully characterise the isolate. Communicable Diseases Network Australia (CDNA) has mandated that only toxigenic strains of O1 and O139 be notified to public health authorities. Cholera is an internationally notifiable disease.

The laboratory’s dependence on a history of travel-associated diarrhoea or a specific clinical request to decide whether to use The laboratory’s dependence on a history of travel-associated diarrhoea or a specific clinical request to decide whether to use *V. cholerae* selective agar means that a definitive bacteriological diagnosis may be delayed or missed altogether in sporadic cases of endemic cholera.

3 Laboratory diagnoses/tests

3.1 Presumptive laboratory diagnosis of *V. cholerae* infection

A presumptive bacteriological diagnosis of *V. cholerae* infection can be made by phase contrast microscopy of a hanging drop preparation of diarrhoeal stool. *V. cholerae* O1 demonstrates a

characteristic spiral motility that is inhibited by O1 agglutinating antisera. This test should only be used by experienced laboratory staff and when examining watery faeces strongly suggestive of cholera.

3.1.1 Suitable specimens

Faeces
This test should only be used by experienced laboratory staff and when examining watery faeces strongly suggestive of cholera

3.1.2 Test sensitivity

No quantitative data available.
Depends on the quality of the specimen, which should be fresh. O1 agglutinating antisera should not be expected to inhibit motility of *V. cholerae* O139.

3.1.3 Test specificity

Intestinal spirochaetes may be observed and mistakenly identified as presumptive *V. cholerae* by less experienced laboratory staff. These have a screw-like motility that is not inhibited by *V. cholerae* agglutinating antisera.

3.1.4 Predictive values

Absence of bacilli with characteristic motility in a watery faecal specimen does not exclude a diagnosis of cholera.

3.1.5 Suitable acceptance criteria

Micro A hanging drop preparation of watery faeces from a patient with profuse watery diarrhoea full of motile, curved bacilli with helical movement that can be inhibited by O1 antisera can be regarded as presumptive bacteriological indication of cholera.

3.1.6 Suitable internal controls

V. cholerae cultured in liquid media will demonstrate characteristic motility.

3.1.7 Suitable test validation criteria

Culture-based isolation of *V. cholerae*, its identity confirmed by a genotypic or similarly discriminating method is the best available standard. No single phenotypic method is sufficiently reliable to be regarded as a ‘gold standard’.

3.1.8 Suitable external QC programme

None available.

3.2 Culture of *V. cholerae*

V. cholerae is a non-fastidious species, able to grow on a wide variety of bacteriological media including blood, MacConkey and thiosulphate citrate bile salt (TCBS) agars.

3.2.1 Media

The most commonly used selective media for *V. cholerae* are alkaline peptone water (APW) and thiosulphate citrate bile salt sucrose (TCBS) agar. All Vibrio species require salt supplementation for optimal growth. APW is therefore used to assist the recovery of *V. cholerae* from specimens that may contain low numbers of the species e.g. formed stools from asymptomatic persons during an outbreak of cholera. TCBS was originally developed to recover *V. cholerae* and has not been fully validated for recovery of most non-cholera Vibrios from clinical specimens. Sheep blood agar is also useful for studies on *V. cholerae* due to the b –haemolysis demonstrated by the El Tor biotype and most non-O1 serotypes of *V. cholerae.* Colonies of *V. cholerae* are yellow on TCBS due to sucrose fermentation.

3.2.2 Suitable specimens

Faeces
Watery stool samples should be inoculated fresh onto TCBS agar and incubated at 35 ºC.
Formed stools from asymptomatic individuals should be inoculated into APW, and this medium subcultured onto TCBS after 6 and 18 hours.

Rectal swabs
A rectal swab is a suitable alternative specimen from patients with profuse watery diarrhoea.

Filter paper
If prolonged specimen transport is anticipated, a small piece of filter paper can be used to absorb watery stool material and placed in a conventional laboratory specimen container.

3.2.3 Test sensitivity

No quantitative data available.
V. cholerae is easily recovered from patients with the profuse watery diarrhoeal manifestations of cholera. Asymptomatic individuals are more difficult to recover *V. cholerae* from, even in the midst of an outbreak and despite the use of APW as an enrichment step. There have been reports of sucrose-non-fermenting strains of *V. cholerae* O139 which will be missed if a laboratory is completely reliant on TCBS as a bacteriological screening method.

3.2.4 Test specificity

The presence of yellow colony growth on TCBS agar does not necessarily confirm a diagnosis of cholera, since some non-cholera Vibrios such as *V. fluvialis* also ferment sucrose. Confirmatory tests such as the string test, agglutination with O1 antisera and substrate utilisation are required before a definitive identity can be allocated to suspect isolates.

3.2.5 Predictive values

A negative culture does not exclude cholera, particularly if the stool is loose or formed. A positive culture does not establish a diagnosis in the absence of clinical symptoms.

3.2.6 Suitable acceptance criteria

On TCBS after 1–2 days incubation, bacterial colonies of *V. cholerae* are usually smooth and yellow. Rough colony variants have been described. b -haemolysis on sheep blood agar is not required but is a feature of the common El Tor biotype and many non-O1 *V. cholerae* serotypes. Colonies will be salt tolerant, oxidase and string test positive. *V. cholerae* serotype O1 agglutinates with O1 antisera. The oxidase test should not be performed on colonies from agar containing fermentable sugars (e.g. TCBS agar).

3.2.7 Suitable internal controls

Batch-to-batch variation in performance of TCBS can be considerable. A properly documented, internal quality control program is required for each batch of selective (TCBS) agar. A non-cholera Vibrio such as V. furnissii (NCTC 11218) can be used as a positive control for TCBS.

3.2.8 Suitable test validation criteria

Sensitivity to the vibriostatic agent O/129 is no longer a reliable confirmatory test due to increasing resistance in *V. cholerae* isolates from south Asia and other parts of the region. Conventional substrate utilisation panels are often unreliable due to insufficient salt concentrations in the test wells. Vibrios can therefore be difficult to identify to species level. Salt supplemented substrate utilisation tests should be used to confirm the identity of presumptive *V. cholerae.* The decarboxylase and dihydrolase reactions are particularly useful.

3.2.9 Suitable external QC programme

RCPA QAP

3.3 Identification of *V. cholerae:*

There are two levels of action in the identification of *V. cholerae*:
A. Diagnostic laboratories – Most diagnostic laboratories should be capable of presumptive identification of *V. cholerae.*
B. Reference laboratories – Isolates should be forwarded to a State reference laboratory or clinical laboratory with special interest in bacterial enteric pathogens for confirmation of the identity.

3.3.1 Presumptive identification

Presumptive identification of *V. cholerae* is based on the recognition of sucrose fermenting colonies on TCBS agar that are composed of Gram negative and oxidase positive bacilli. Suspect colonies should be subcultured to nutrient agar prior to testing for oxidase production because of false positive reactions on TCBS.

3.3.2 Definitive identification

Confirmation of the identity of presumptive *V. cholerae* requires serotype O1 agglutinating antisera and salt-supplemented substrate utilisation tests. Identification to biotype level requires additional tests including detection of haemolysis, phage susceptibility with a specific phage set and polymyxin B susceptibility. These tests are not readily available in most jurisdictions.

3.3.2.1 Predictive values

A negative result in one substrate utilisation test, particularly if the test well does not contain supplementary salt does not completely exclude *V. cholerae.* Further testing may be necessary to confirm the identification.

3.3.2.2 Suitable test criteria

An isolate that exhibits biochemical characteristics consistent with documented reactions for *V. cholerae.* Expected results for *V. cholerae* are Gram negative bacilli, oxidase positive, lysine and ornithine decarboxylase positive, arginine negative, indole, ONPG and Voges-Proskauer positive, acid from sucrose but not from lactose, salicin, inositol or arabinose. The El Tor biotype produces b -haemolysis on blood agar, a positive CAMP test, is polymyxin B resistant and shows lysis with El Tor phage 5. The classical biotype is sensitive to polymyxin B, does not produce b -haemolysis on sheep blood agar, and is lysed by classical IV and FK phages but not by El Tor 5. Agglutinating antisera are available for distinguishing *V. cholerae* serotypes Inaba and Ogawa. These reagents are not be kept by most public health laboratories due to a low throughput of *V. cholerae* isolates.

3.3.2.3 Suitable internal controls

Each batch of biochemical substrate tested with positive and negative control strains.
Results of all testing recorded and the records maintained.

3.3.2.4 Suitable validation criteria

Correct biochemical reactions exhibited by a standard *V. cholerae* strain.

3.3.2.5 External QC program

3.3.3 Kits for biochemical identification

Various substrate utilisation kits exist for the identification of *V. cholerae.*
The API 20E is commonly used in Australia. It should be noted that whilst these kits will usually confirm the identity of clinical isolates, especially if normal saline is used to prepare the bacterial suspension for inoculation, they are unreliable for environmental/food isolates and traditional tests are recommended in this case.

3.3.4 Molecular Identification

Since CDNA has mandated that only toxigenic *V. cholerae* O1 and O139 be notified to public health authorities, all isolates should be serotyped and tested for their ability to produce toxin or possession of the cholera toxin gene ctxA. Several multiplex PCR assays which target rfb (O-antigen biosynthesis) and ctxA have been published (Hoshino et al 1998) and recently Singh et al, 2002, described a hexaplex PCR which targets additional virulence factors.
These assays may be used directly on faeces since the classical rice water stools contain very few inhibitors of the PCR reaction. However, they have little application in low disease prevalence areas such as Australia.

At least one Australian jurisdiction is using a multiplex PCR for *Vibrio cholerae* that confirms the serogroups O1 and O139, as well as detecting ctxA and an internal control (*hly*A).

3.4 Molecular subtyping

Subtyping for epidemiological purposes is available in only a small number of reference laboratories. Methods that have been used include restriction fragment length polymorphism analysis (RFLP), multilocus enzyme electrophoresis (MEE) and ribotyping. The small number of cases of cholera diagnosed in Australia every year means that there has been little opportunity or need to develop molecular epidemiological skills for *V. cholerae*

4 References

1. Hoshino K et al. Development and evaluation of a multiplex PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139. FEMS Immunol Med Microbiol 20:201-207.
2. Janda JM. Vibrio, Aeromonas and Plesiomonas. Ch 45. In Balows A & Duerden BI. Systematic Bacteriology. Pp 1065-1089. Collier L, Balows A, Sussman M Eds. Microbiology and Microbial Infections. 9th edn. Arnold, London. 1998.
3. MacFaddin JF. Biochemical tests for identification of medical bacteria. 3rd edn. Lippincott, Williams & Wilkins, Philadelphia, PA, 2000.
4. Singh DV et al. Development of a hexaplex PCR assay for rapid detection of virulence and regulatory genes in *Vibrio cholerae* and *Vibrio mimicus.* J Clin Microbiol. 40:4321-4324