

S1. Supplement to the WHO Polio Laboratory Manual

An alternative test algorithm for poliovirus isolation and characterization

S1.1 Rationale for the alternative test algorithm

Early confirmation of infection and transmission of polioviruses is an important first step towards implementing public health interventions to limit virus spread. Experiences in the WHO global polio laboratory network (GPLN) suggest that the following factors contribute to delays between paralysis onset in patients and laboratory confirmation of poliovirus infection:

- Three groups are involved in the investigation: surveillance personnel who detect AFP cases, collect and refer samples to laboratories; laboratory personnel who carry out virus isolation and characterization procedures; commercial shipping companies contracted for the transport of samples and virus isolates to laboratories.
- Only fifty percent of all countries have at least one laboratory that belongs to the GPLN. All of the network laboratories have capacity for virus isolation and determination of virus serotype. Some of them have additional capacity for intratypic differentiation (ITD) within the same facility. The location of technologies at different levels of the GPLN and in different countries results in a requirement for occasional intra- and inter-country shipping of faecal samples or virus isolates for testing and this can be time consuming and costly.
- Highly sensitive and specific procedures for direct and rapid (within hours) detection of poliovirus in faecal samples are currently unavailable. Lack of standardized, validated reagents, and quality assurance protocols currently limit introduction of new technologies for poliovirus detection into the GPLN.

Analysis of test performance data of the GPLN has been used to identify areas where greater efficiency is possible to improve reporting timeliness. The alternative test algorithm (i.e. step-wise stages of testing) for poliovirus isolation and characterization described in this supplement has been evaluated under field conditions in 3 different locations and has been found to provide more rapid results with greater than 95% poliovirus detection sensitivity, specificity and comparability with reference to the traditional algorithm described in Chapters 7 and 8 of the WHO polio Laboratory manual, 4th edition (WHO/IVB/04.10). When compared to the traditional algorithm, the alternative test algorithm:

- Retains inoculation of each faecal sample in duplicate in two continuous cell lines (L20B and RD).
- Has a shortened observation time for inoculated cell cultures.
- Has a different approach for passage of cell cultures.
- Requires testing of virus isolates that show cytopathogenic effects (CPE) in L20B cells by polymerase chain reaction (PCR) for simultaneous identification of virus serotype and poliovirus intratypic differentiation (ITD), followed by immediate reporting of any poliovirus with non-Sabin-like (NSL) results.
- Uses RD cell culture passages of L20B positive cultures for ITD tests.
- Restricts use of the micro neutralization test (NT) for serotype identification to only cell cultures containing mixtures of viruses (identified by PCR) in order to separate individual poliovirus strains for ELISA testing.
- Tests monotypic polioviruses (those with monotypic Sabin-like (SL) viruses identified by PCR or isolates separated from mixtures by NT) by ELISA to screen

for potential vaccine derived polioviruses (VDPV) based on antigenic characteristics.

S1.2 Isolation of viruses in continuous cell lines

Have available the following items:

- tube cultures of L20B and RD cells;
- maintenance medium;
- 1 ml and 5 ml plastic disposable pipettes;
- Specimen extract/s (see Chapter 6, section 6.2 of WHO Polio laboratory Manual for protocol for specimen preparation)

Do the following:

- Microscopically examine recently prepared monolayer cultures to be sure that the cells are healthy and at least 75% confluent. A suitable monolayer would be one formed within 2–3 days of seeding.
- Remove the growth medium and replace with 1 ml maintenance medium.
- Label two tubes of RD and two of L20B for each specimen to be inoculated (specimen number, date, passage number).
- Label one tube of each cell type as a negative control.

Note: Both cell lines must be inoculated at the same time.

- Inoculate each tube with 0.2 ml of specimen extract and incubate in the stationary sloped (5°) position at 36°C. L20B cells will not survive being rotated and it is unnecessary for either L20B or RD cell lines for poliovirus isolation.
- Examine cultures daily, using a standard or inverted microscope, for the appearance of CPE.
- Record all observations of inoculated and control cultures, recording CPE as 1+ to 4+ to indicate the percentage of cells affected (1+ represents up to 25% of cells; 2+ represent 25 to 50%; 3+ represents 50 to 75%; 4+ represents from 75 to 100%), toxicity¹, degeneration or contamination².
- If no CPE appears after at least 5 days of observation, perform a blind passage³ *in the same cell line* and continue examination for a further 5 days. (NB. Contents of duplicate negative cell cultures from an individual case should not be pooled for passage, i.e. individual negative cell culture tubes should be passaged

¹ **Toxicity:** If cell cultures show rapid degeneration within twenty-four hours of inoculation this may be due to non-specific toxicity of the specimen. These tubes should be frozen at –20°C, thawed, and 0.2 ml volumes transferred (i.e. this is procedurally similar to a passage but should not be counted as a passage) into cultures of the same cell type. If toxic appearances recur, return to the original specimen extract and dilute the extract in PBS at 1/10 and re-inoculate cultures as described above (this should be considered as inoculation of the specimen and day 0 for observation).

² **Microbial contamination:** Contamination of the medium and cell death resulting from bacterial or fungal contamination makes detection of viral CPE uncertain or impossible. Return to the original specimen extract, **re-treat with chloroform** (see Chapter 6 section 6.2 of WHO Polio Laboratory Manual) and inoculate fresh cell cultures as described above.

³ **Blind Passage:** Freeze the tubes at –20°C, thaw, and passage 0.2 ml of culture fluid to tubes containing fresh monolayers **of the same cell type** and examine daily for at least 5 days. If cultures show no CPE by this stage, the result is regarded as negative.

- separately). Cultures should be examined for a total of at least 10 days (i.e. minimum of 5 days post-inoculation and minimum of 5 days post-passage) before being judged as negative and discarded.
- If characteristic enterovirus CPE appears at any stage after inoculation, i.e. rounded, refractile cells detaching from the surface of the tube, record observations and allow CPE to develop until at least 75% of the cells are affected ($\geq 3+$ CPE). At this stage, perform a second passage⁴ in the opposite cell line (see Figure S1.1). Duplicate cultures of the same sample in the same cell line that both show $\geq 3+$ CPE can be pooled⁵ for passage into a single tube containing a monolayer of cells from the other cell line in 1 ml of maintenance medium:
 - **CPE positive L20B cultures** are passaged into RD cell cultures and incubated at 36°C and observed.
 - Characteristic enterovirus CPE will appear in the majority of RD cultures and should be allowed to develop until $\geq 3+$ CPE is observed. Tubes are then frozen at -20°C until referred to an ITD laboratory for typing and ITD.
 - A small number of samples contain viruses that show CPE in L20B cells that is not reproducible when the viruses are passaged into RD cells⁶. RD cultures should be examined for at least 5 days before being reported as negative.
 - **CPE positive RD cultures** are passaged into L20B cells and incubated at 36°C and observed daily. This passage is aimed at separating polioviruses that may be present in mixtures with other enteroviruses and amplifying the titre of any polioviruses that may be present.
 - If no CPE is obtained when L20B cultures are examined for at least 5 days, the culture is considered negative for polioviruses and is reported as NPEV (see Figure S1.1)
 - Characteristic enterovirus CPE, however, will appear in some L20B cultures and should be allowed to develop until $\geq 3+$ CPE is observed. Another passage of the virus is performed, back into RD cells⁴, with subsequent observation for CPE. This passage is aimed at amplifying virus titre. Any $\geq 3+$ CPE positive RD cultures are stored at -20°C until referred to an ITD laboratory. A small number of RD cultures may remain negative when examined for at least 5 more days⁶ and should be reported as negative.

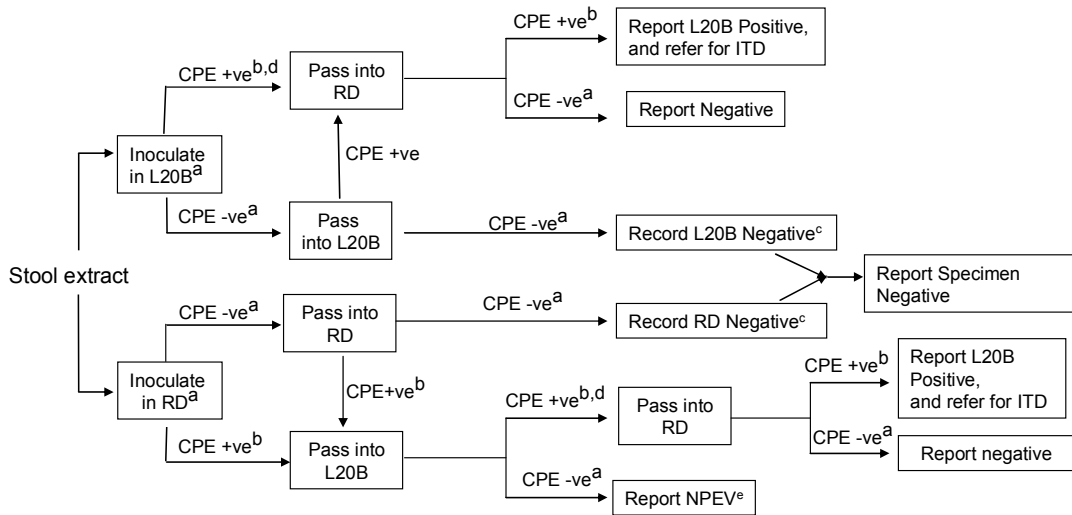
⁴ **Passage of CPE+ cultures:** Freeze the tubes at -20°C, thaw, and passage 0.2 ml of culture fluid to tubes containing fresh monolayers of a different cell type and examine daily for at least 5 days.

⁵ **Pooling of CPE positive cell cultures:** Pooling should only be done if $\geq 3+$ CPE is observed in duplicate cultures of the same cell line on the same day. In any other situation, each cell culture is passaged separately into a single new cell culture.

⁶ **Non-reproducible CPE.** Such viruses may be reoviruses, adenoviruses or other non-enteroviruses that can grow in mouse L-cells. Some of these viruses may cause CPE clearly distinctive from enterovirus-characteristic CPE.

Figure S1.1

Poliovirus Isolation New Algorithm



- a Observed for a minimum of 5 days
- b Observe until $\geq 3+$ CPE obtained (usually 1-2 days, 5 days maximum; re-inoculate when toxicity or contamination observed)
- c Total minimum observation time of 10 days (2 x 5 days)
- d Pool positive tubes (if both tubes show $> 3+$ CPE on the same day) before final RD passage
- e Isolates can be serotyped by laboratories with an interest in NPEV diagnosis or to confirm proficiency

S1.3 Reporting of virus isolation results and follow-up

- Laboratories without on-site ITD capacity must report **preliminary virus isolation results** to national authorities and to WHO at this stage.
- The result for an individual specimen is obtained by combining results for duplicate cultures in both L20B and RD cell lines.
- The result for an individual case is obtained by combining the results for all specimens from that case.
- The possible outcomes of virus isolation procedures and suggested wording of reports are provided in Table S1.1.
- **Untyped** isolates should be sent as soon as possible to an appropriate ITD laboratory (and ideally within 7 days of detection) where virus serotype and intratype will be determined. Accompanying documentation must include patient and isolate identification information (e.g. EPID and laboratory number) and isolates must be labeled with laboratory number and passage information (e.g. expressed as L+R+ or R+L+R+).
- Laboratories wishing to do so may continue to perform virus serotyping by microneutralization assay on an aliquot of the isolate in their own location. It is emphasized, however, that laboratories should not await serotyping results or separate viruses before shipping isolates to ITD laboratories.

Table S1.1: Possible outcomes and reporting of virus isolation results for an individual specimen

Outcome of virus isolation tests	Comment	Action required
Negative	No viral CPE was observed post-inoculation or post passage of L20B and RD cell cultures for any of the inoculated cultures or specimens of the case.	Report "Negative" or "No virus isolated". No further action required.
L20B positive	Viral CPE was obtained in L20B cells either post-inoculation or post-passage for at least one of the cultures or specimens of the case. CPE was reproducible when the L20B isolate was passaged into RD cells.	Report "L20B positive isolate obtained. Likely to be poliovirus. Isolate will be characterized by ITD tests". Refer the RD passage of the L20B isolate to an ITD laboratory within 7 days of detection.
Non-polio Enterovirus detected.	Viral CPE was obtained in RD cells either post-inoculation or post passage and no CPE was obtained when the RD isolate was passaged into L20B cells.	Report "NPEV positive". No further action required.
Both L20B positive and Non-polio Enterovirus detected.	One or more cultures of the specimen was identified as having an "L20B positive isolate" <u>and</u> one or more other cultures of the same specimen was also identified as being "NPEV positive"	Report "L20B and NPEV positive isolates detected".

S1.4 Determination of virus serotype and poliovirus intratype

Within the GPLN, polioviruses have traditionally been isolated using L20B and RD cells, identified and serotyped in NT using polyclonal antisera, and monotypic viruses have been subsequently subjected to ITD using 2 methods, one based on antigenic and the other on a molecular principle. All isolates giving concordant SL results in 2 ITD tests have been reported. Polioviruses with any result different from SL in either ITD test have been reported but also forwarded to a Global Specialized Laboratory for genomic sequencing. This strategy has been effective in detecting wild polioviruses and VDPVs with high sensitivity and specificity. Experience gained over several years in the GPLN, however, shows that:

- There is close to 100% agreement in the determination of serotype of isolates by NT and PCR.
- Serotype results are available within 3 to 5 days by NT and within hours by PCR.
- When PCR procedures are used as outlined in Chapter 8 of the WHO Polio Laboratory Manual 4th Edition, virus serotype and poliovirus intratype can simultaneously be determined.

- There is close to 100% agreement in the designation of non-Sabin-like viruses based on PCR results and poliovirus VP1 nucleotide sequences.
- Polioviruses present in isolates as monotypes, or, in mixtures with other enteroviruses can be accurately serotyped and intra-typed by PCR. RD-cultured isolates give better PCR results than L20B-cultured isolates.
- Poliovirus intratype can be accurately determined within twenty-four hours using ELISA tests, but the test requires use of monotypic polioviruses. Polioviruses present in mixtures must therefore be separated prior to performance of the ELISA test.

An alternative approach for determining virus serotype and intratype was developed to improve efficiency and shorten reporting timelines, taking into consideration all of the above experiences (see Figure S1.2). In the alternative approach, the highest priority is given to rapid reporting of non-Sabin-like polioviruses.

Do the following:

- ITD laboratories will receive 2 categories of isolates for testing, as follows: those that showed CPE in L20B cells at some stage of testing and CPE was reproducible when isolates were passed in RD cells (categories are L20B+RD+ and RD+L20B+RD+ isolates).
 - ITD tests are performed on all L20B+RD+ isolates using the PCR method described in Chapter 8 (section 8.3 on pages 112 to 118) of the WHO Polio Laboratory Manual 4th Edition. NB. Isolates are not passaged or serotyped prior to testing by PCR, and all primer sets listed in Table 8.2 (page 118) must be used.
 - RD+L20B+RD+ isolates are tested using the PCR method in only limited situations: if this is the only category of isolate obtained from an individual specimen or if the PCR result obtained for the corresponding L20B+RD+ isolate from the same specimen was interpreted as negative or NPEV.
- Interpret PCR results, as described in Table 8.1 (page 117) of the Polio Laboratory Manual. Based on the interpretation:
 - Report to national authorities and WHO the result of any poliovirus isolate identified as "wild poliovirus of indicated serotype(s)". The report should specify the poliovirus serotype obtained and indicate the intratype as "non-Sabin-like". Reports should be provided within 24 hours of completion of the PCR, and the isolate should be referred to a specialized laboratory for sequencing within 7 days of detection. Non-Sabin-like (preliminary) results from any isolate should be reported and isolates referred for sequencing as they become available. Final results can be issued at a later time when all virus isolation and ITD results are available for all cultures and specimens of the case.
 - Perform the ELISA test on all monotypic Sabin-like polioviruses (see Section 8.1 of the WHO Polio Laboratory Manual). NB. The ELISA test is not performed on "non-Sabin-like" viruses. There is no need to perform serotyping by NT before performing ELISA on monotypic polioviruses identified through prior PCR testing. ELISA results are interpreted and ITD results are reported according to the protocols in Chapter 8, sections 8.1.4 and Table 8.3 of the WHO Polio Laboratory Manual.
 - Perform NT (see Section 7.3 of WHO Polio Laboratory Manual) to separate viruses present in any isolate with a PCR result interpreted as

having a mixture of polioviruses of different serotypes. Perform ELISA test on separated monotypic viruses (see Section 8.1 of the WHO Polio Laboratory Manual).

- Provide final results to national authorities when virus isolation and ITD results are available for all specimens and isolates of the case. Table S1.2 provides examples of results and their interpretation.

Figure S1.2: Flow chart for performing and reporting ITD tests

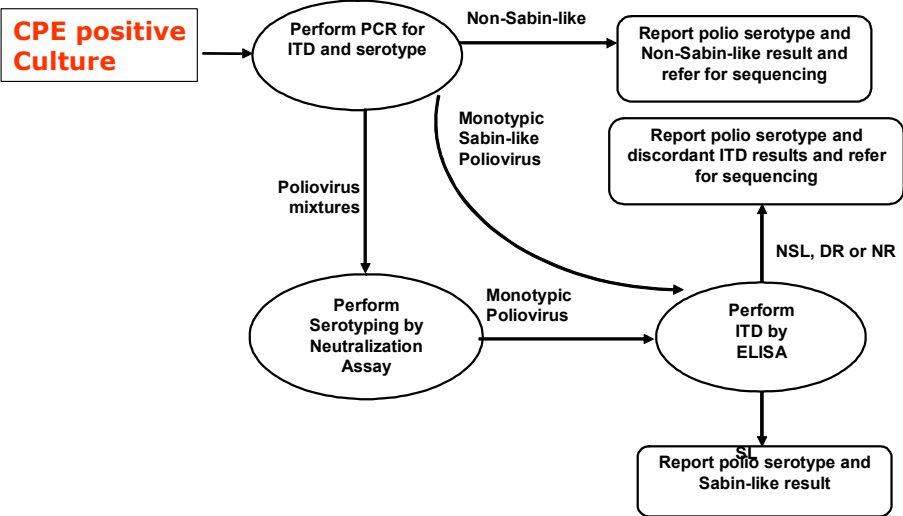


Table S1.2
Example of results and interpretation

	Stool No.	Virus isolation result		Results of PCR								PCR Result	Action required	ELISA result	Final Result Case
		Where found	Result	Pan EV	Pan PV	Sero 1	Sero 2	Sero 3	Sabin Multiplex						
									S1	S2	S3				
A	Stool 1	L20B arm RD Arm	Negative R+L-	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NPEV
	Stool 2	L20B arm RD arm	Negative R+L-	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	
B	Stool 1	L20B arm RD arm	L+R+ R+L+R+	+ NN	+ NN	- NN	- NN	+ NN	- NN	- NN	+ NN	P3SL NN	Perform ELISA NN	P3SL NN	P3SL + NPEV
	Stool 2	L20B arm RD arm	Negative R+L-	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	NN NN	
C	Stool 1	L20B arm RD arm	L+R+ R+L+R+	+ NN	+ NN	+ NN	- NN	- NN	+ NN	- NN	- NN	P1SL NN	Perform ELISA NN	P1SL NN	P1SL
	Stool 2	L20B arm RD arm	L+R+ R+L+R+	+ NN	+ NN	+ NN	- NN	- NN	+ NN	- NN	- NN	P1SL NN	Perform ELISA NN	P1SL NN	
D	Stool 1	L20B arm RD arm	L+R+ R+L+R+	+ NN	+ NN	+ NN	- NN	- NN	- NN	- NN	- NN	P1NSL NN	Report P1NSL * NN	NN	P1NSL + P3SL. P1 virus to be referred for sequencing.
	Stool 2	L20B arm RD arm	Negative R+L+R+	NN +	NN +	NN -	NN -	NN +	NN -	NN -	NN +	NN P3SL	NN Perform ELISA	NN P3SL	
E	Stool 1	L20B arm RD arm	L+R+ R+L+R+	+ NN	+ NN	- NN	+ NN	+ NN	- NN	+ NN	+ NN	P2SL + P3SL NN	Perform NT and ELISA NN	P2SL + P3SL NN	P2SL + P3SL
	Stool 2	L20B arm RD arm	L+R+ R+L+R+	+ NN	+ NN	- NN	- NN	+ NN	- NN	- NN	+ NN	P3SL NN	Perform ELISA NN	P3SL NN	
F	Stool 1	L20B arm RD arm	L+R+ R+L+R+	+ NN	+ NN	- NN	+ NN	- NN	- NN	+ NN	- NN	P2SL NN	Perform ELISA NN	P2 DR* NN	P3SL + P2 with discordant ITD. P2 virus to be referred for sequencing.
	Stool 2	L20B arm RD arm	L+R+ R+L+R+	+ NN	+ NN	- NN	- NN	+ NN	- NN	- NN	+ NN	P3SL NN	Perform ELISA NN	P3SL NN	

NN	= Testing not needed	NT	= Neutralization test
SL	= Sabin-like	NSL	= Non-Sabin-like
NEV	= Not Enterovirus	NPEV	= Non-polio Enterovirus
P1	= Poliovirus Serotype 1	P2	= Poliovirus Serotype 2
P3	= Poliovirus Serotype 3	*	= Refer virus for sequencing