WHO/V&B/03.03
ORIGINAL: ENGLISH

Guidelines for environmental surveillance of poliovirus circulation

Vaccines and Biologicals

World Health Organization

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The Department of Vaccines and Biologicals thanks the donors whose unspecified financial support has made the production of this publication possible.

This publication was produced by the
Vaccine Assessment and Monitoring
of the Department of Vaccines and Biologicals

Ordering code: WHO/V&B/03.03
Printed: March 2003

This publication is available on the Internet at:
www.who.int/vaccines-documents/

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Abbreviations

AFP	acute flaccid paralysis
CPE	cytopathogenic effect
EPI	Expanded Programme on Immunization (WHO)
ITD	intratypic differentiation of poliovirus isolates to determine whether wild or vaccine like
MOH	Ministry of Health
NIDs	national immunization days
NPL	national poliovirus laboratory
NSL	non-Sabin-like (wild) viruses (result reported from certain ITD tests of polioviruses)
OPV	oral polio vaccine
PEG	polyethylene glycol
PV	poliovirus
RRL	regional reference laboratory
SL	Sabin-like poliovirus. Result reported from certain
ITD	tests of polioviruses
VDPV	vaccine-derived poliovirus (isolates of poliovirus demonstrating 1 to 15% difference from parent OPV strains by full VP1 sequence homology consistent with an extensive period of virus excretion or transmission in the community)
WHO	World Health Organization

Summary

Acute flaccid paralysis (AFP) surveillance is the gold standard for surveillance in the polio eradication initiative. Under certain circumstances valuable supplementary information can be obtained by environmental surveillance. However, because of inherent limitations and additional resource requirements, environmental surveillance should be restricted to selected populations where deficiencies in AFP surveillance are suspected and where conditions exist that render the population at risk for poliovirus circulation (e.g. low polio vaccination coverage or risk of poliovirus importation). Environmental surveillance should only be implemented after careful planning of all steps in the operation and thorough assessment of the potential benefits and alternatives.

Different principles for sampling and sample processing have been used in the past for environmental surveillance for enterovirus infections. Sufficient comparative data have not been published to unequivocally prove that there is an advantage of any one principle over the others for detection of low-level poliovirus circulation in human populations. Therefore, countries already exploiting environmental surveillance might continue to use their established approach, provided that other aspects of these guidelines are followed and the procedures they use are validated as recommended.

Countries planning to start environmental surveillance should consult the WHO regional office at an early stage and incorporate the following guidelines in their planning. In particular, before starting activities there should be guaranteed availability of sufficient laboratory resources, advance training of personnel, and validation of adopted laboratory procedures. An environmental surveillance plan should be developed that clearly indicates reporting responsibilities to assure coordinated use of all epidemiological information on possible circulation of poliovirus in the population.

1. Introduction

The examination of stool samples from patients identified through acute flaccid paralysis surveillance links poliovirus isolates to specific individuals and permits a focused investigation of that individual and the immediate community at risk. AFP surveillance is the gold standard for the polio eradication initiative.

The examination of composite human faecal samples through environmental surveillance links poliovirus isolates from unknown individuals to populations served by the wastewater system. Environmental surveillance can provide valuable supplementary information, particularly in urban populations where AFP surveillance is absent or questionable, persistent virus circulation is suspected, or frequent virus re-introduction is perceived.

The rationale for environmental surveillance is based on the characteristic poliovirus excretion pattern. Infected individuals excrete poliovirus in faeces for periods up to several weeks, whether or not they are symptomatic. Large numbers of excreted poliovirus particles remain infectious in the environment for varying lengths of time depending on the immediate conditions. Virus presence may be detected by a variety of laboratory methods for concentration, separation and identification.

Environmental surveillance has been used successfully in monitoring enteric virus circulation and assessing the extent or duration of epidemic poliovirus circulation in specific populations. In several countries, wild polioviruses have been detected in the environment in the absence of reported AFP cases. Environmental surveillance is also a potential tool for monitoring circulating vaccine-derived poliovirus (VDPV) and assessing population immunity of populations vaccinated with inactivated polio virus (IPV).

Effective environmental surveillance requires researched application, special laboratory skills, and sustainable field and laboratory resources. The first step in making the decision to introduce environmental surveillance into local or national programmes is an evaluation of its role in the context of regional and national polio surveillance goals. The following guidelines provide information to assist countries in evaluating the advantages of environmental surveillance, formulating an effective plan, selecting appropriate methodologies, interpreting findings, and anticipating effective programme responses to the results.

2. Formulating a plan for environmental surveillance of poliovirus circulation

2.1 Identification of potential target populations

Properly conducted environmental surveillance has the best potential to reveal useful information on poliovirus circulation if the efficiency of AFP surveillance is suspected to be sub-optimal and the target population has one or more of the following properties:

- inadequate immunization coverage (known or suspected) through the routine immunization services and/or supplementary immunization activities, e.g. national immunization days (NIDs) conducted in the target population either recently or in the past;
- evidence of recent circulation of wild poliovirus or VDPV in the target population; and
- perceived risk of importation of wild poliovirus either by cross-border contacts or through other type of connections to another population presenting with concurrent wild poliovirus transmission.

A consultation with the local sanitary engineering authorities should be carried out to assess possibilities to collect representative environmental samples derived from the desired population. Natural routes of household wastewater should be carefully investigated. A converging sewer network serving the target population is preferable because it enables monitoring of large groups of people by analysing samples collected at a single site, from a main collector sewer. Other types of known routes of wastewater flow (such as open canals or water channels) have in some cases enabled successful demonstration of wild poliovirus circulation in the relevant population, but sampling in such situations may result in lower surveillance sensitivity (compared to converging sewer networks), due to unknown or uncontrolled factors.

Increasing size of the source population may decrease the sample sensitivity, depending on the concentration of virus in the wastewater sample. In some situations, therefore, a larger number of poliovirus-infected individuals may be needed for a given sample to reveal poliovirus. In large cities, it may be necessary to segment the population and to sample preferred subgroups within the segment. It is likely that under urban conditions, poliovirus will spread readily through the susceptible population, and therefore, monitoring subgroups within the urban population will be adequate.

2.2 Components of a national plan for environmental surveillance

After national decision to conduct environmental surveillance, a written detailed and comprehensive plan should be created and ratified by the Ministry of Health (MOH). Development of the plan should exploit different expertise including the national Expanded Programme on Immunization (EPI), national polio laboratory (NPL), local sanitary engineering and other relevant provincial and local authorities. The WHO regional office should be consulted at an early phase of the planning.

The plan should include the following elements:

- length and time schedule of sampling;
- details of the actual sampling sites (location and population sizes likely to be represented);
- responsibilities for sampling, instructions for sampling and sample logistics;
- provision of laboratory space, personnel, equipment and reagents;
- protocols of sample processing and virus identification;
- data management and reporting (contents of reports and reporting channels);
- training and quality assurance; and
- envisaged consequences of different laboratory results.

It is important to acknowledge in the planning and budget that processing and analysing of environmental specimens will cause a significant workload for the laboratory. Otherwise, surveillance and laboratory performance (for polio and/or other activities) may suffer due to "over stretched" resources.

2.3 Length and time schedule of sampling in different situations

Environmental surveillance can be instituted for different purposes. If aimed at providing supplementary evidence for elimination of wild poliovirus circulation in a population, a long term, regular sampling programme of a representative population is preferable. Sampling frequency should, preferably, be twice a month, but at least once a month. Sampling should be continued for at least one year, and preferably three years after the last wild poliovirus isolation. If environmental surveillance is prompted by known or suspected reintroduction of wild poliovirus or appearance of cases caused by circulating VDPV (cVDPV), the initial plan may cover a shorter period (not less than 12 months) and apply more frequent sampling, targeted to more selected populations. This must always be accompanied by intensified AFP surveillance (see Annex 4). Whatever the purpose, it is expected that a significant proportion of the samples will contain virus strains derived from oral polio virus (OPV) in countries immunizing with OPV. Environmental surveillance may thus be a potential approach to monitor silent circulation of VDPV in human populations. The actual sampling dates should be coordinated with the people in charge of the logistics and the NPL to avoid unnecessary storage of the collected samples before processing can be arranged.

2.4 Principles for selecting sampling sites

Recommended sampling sites are inlets to sewage treatment plants or other major collector sewers. Industrial wastes may contain compounds that may be toxic to cell cultures and/or interfere with poliovirus replication. This has to be taken into account when selecting the sampling sites. In the absence of a sewer network, representative sampling may be difficult to achieve and environmental surveillance should only be started if the major flow routes of wastewater containing human faecal material are sufficiently well known. Targeted, carefully designed stool surveys may be considered as an alternative approach to environmental surveillance in the absence of a sewer network.

Sampling sites chosen for regular monitoring should represent selected high-risk populations. The preferable size of the source population is 100 000–300 000. If smaller populations exist and the sampling sites are close to each other, one can consider generation of composite samples by mixing portions derived from different sites to reduce the laboratory workload. If the source population is larger, the consequently reduced sample sensitivity can be compensated for by collecting more frequent samples, acknowledging, however, that this would increase the laboratory workload.

2.5 Sampling principles and sample logistics

The plan should clearly indicate who is responsible for collecting the samples at each sampling site. Sampling can be organized by the local authorities or centrally, through the MOH or NPL, whichever is considered the most suitable alternative for the particular situation. Exploiting the use of an existing sewage sample collection system should be considered whenever possible. Training and written instructions for sampling should be provided to persons collecting the samples.

There are two principal modes of collecting environmental samples for virological analysis, referred to as grab and trap sampling. In the grab method an amount of raw sewage is collected at a selected sampling site, either at one point in time, or, preferably, at different predetermined times to form a time-adjusted composite sample. Many sewage treatment plants use automated equipment for collecting samples at regular intervals during a 24-hour period or over the peak hours of household sewage flow. Manual collection of composite samples is also possible but sustained adherence to the relatively tedious practice may be difficult to guarantee. If automated collectors are not available and peak hours of household sewage flow are not known, samples collected at one point in time can readily be used.

Grab sample volumes of one litre are recommended. The larger the volume of sewage analysed the higher the theoretical sensitivity to detect poliovirus circulation in the source population. In practice volumes larger than 1 litre are difficult to handle in the laboratory and may be replaced by several parallel regular samples. Larger volumes or parallel samples mean, however, increasing time and workload per site, and may limit the number of sites that can be monitored. Instructions for collecting grab samples are described in Annex 1.

Trap samples are collected by hanging a bag of non-specifically absorbing material in the sewage stream. After one or more days the bag is taken out of the sewage and shipped to the laboratory, where the absorbed material is eluted and analysed for the presence of (polio)viruses.

Grab sampling is preferred to trap sampling as it is more feasible for quantitative estimation of detection sensitivity of the system, and long-term experience suggests that programmes exploiting concentrated grab samples detect polioviruses and non-polio enteroviruses more often than those using trap sampling. If grab sampling is not feasible, the use of standard amounts of macroporous glass in permeable bags is recommended as a trap sampling method (Annex 2). Gauze pads are not recommended, as adequate standardization of the absorbent is too difficult to achieve. Whatever the sampling principle, collected samples should be immediately refrigerated and kept cool during transport to arrive at the NPL within 48 hours of collection. The laboratory should be notified in advance and the laboratory should acknowledge the receipt of the sample.

> ***Comment:*** An alternative way to collect composite samples is to grab contents of a primary sedimentation pond at a sewage treatment plant, if the known mean transit time is less than 24 hours. However, in spite of being theoretically sound, evidence for the advantage of this approach over a simple grab sample from the inlet collector sewer fluid is scarce. Hourly fluctuation of household waste flow may become significant (and the need to collect time-adjusted composite samples realistic), in situations where the target population is relatively small and the sampling site is close to the residential area.

2.6 Sample processing in the laboratory

The concentration of poliovirus in environmental specimens, even after concentration, is usually lower than that in faecal specimens of poliovirus infected humans. The processing of both grab and trap samples in the laboratory contains steps that may generate aerosols, and all precautions should be taken to avoid cross-contamination of samples. Processing and analysis of environmental specimens must not interfere with that of samples collected from AFP patients. It is recommended that separate space and personnel should be assigned for the work with environmental and AFP samples.

2.6.1 Processing fluid grab samples in the laboratory

Half (500 ml) of the collected raw sewage specimens should be concentrated before inoculation into cell cultures to improve detection sensitivity. The other half should be kept at 4°C as a backup until the concentrate from the first half has been successfully inoculated into cell cultures. The first step in any concentration procedure is clarification of the sample, i.e. pelleting of larger suspended solids by centrifugation (Annex 1). Poliovirus may be partly bound to these solids. Therefore, the pellet should be kept at 4°C, to be later combined with the concentrated supernatant.

One frequently used method for sewage sample concentration is the so-called two-phase separation method (Annex 1). A given volume of clarified sewage is mixed with defined amounts of two polymers, dextran and polyethylene glycol (PEG).

The homogenous mixture obtained by vigorous shaking is left to stand overnight at 4°C in a separation funnel. This allows the polymers to separate and form two distinct layers (phases) in the funnel. Enteroviruses accumulate in the smaller bottom layer and/or at the boundary between the layers (interphase). The bottom layer and the interphase are collected drop-wise. The pellet from the initial centrifugation is suspended in this concentrate, which is then treated with chloroform and assayed for presence of virus. The resulting nominal sample concentration is 50–100-fold.

This method is simple enough to be adopted in any NPL provided that necessary training has been given, the equipment is available and sustained provision of the reagents can be guaranteed.

> ***Comment:*** Several alternative methods have been used to successfully concentrate sewage samples. Two other principles are well documented:

- Precipitation with PEG, which requires a high-speed centrifuge with a large volume rotor. A maximum of three samples can be processed in one centrifuge during a working day, and the mean hands-on time per sample is greater than that of the two-phase separation method.
- Ultra-filtration using specific equipment.

Using either of these two methods, a more than 100-fold nominal concentration can be obtained, but this should be viewed with caution for sewage specimens because (i) toxic compounds may also be concentrated, (ii) recovery of the virus from the concentrate does not increase proportionally to the nominal concentration, and (iii) if higher nominal concentration results in inoculation of a smaller number of cell culture vials, separation of virus mixtures may become more complicated.

Whichever concentration method is used, it must be validated for the laboratory by spiking experiments (see Annex 1 for "validation of the concentration step"). A good procedure should be able to detect 10–20 $TCID_{50}$ of poliovirus in a 500 ml sample.

2.6.2 Processing trap samples in the laboratory

A specific elution procedure has been developed to release poliovirus trapped in the ground glass containing bags (Annex 2). The glass powder is first transferred to a small glass column and sequentially rinsed with defined buffer solutions. The eluates are treated with chloroform and inoculated in cell cultures.

Spiking experiments (see Annex 1) should be used to validate the use of this procedure in a laboratory.

2.7 Detection of poliovirus in environmental samples

In principle, environmental sample concentrates and trap eluates are examined for presence of poliovirus in the same way as faecal specimens. Because of the specific nature of the specimens, some modifications are, however, recommended. For possible confirmatory tests at a later stage, one quarter of the processed sample (at least 1 ml) should be frozen at -20°C. For optimal performance, most of the remainder of the processed sample should be inoculated into cultures of L20B and RD(A) cells.

At least 75 cm^2 of cell monolayer should be used per sample. This is equal to three 50 ml flasks (about 25 cm^2 each), two of which should contain L20B and the other RD(A) cells. After replacing the culture medium with 4.5 ml of maintenance medium, a maximum of 0.5 ml of processed environmental sample should be inoculated per flask. If more than three flasks are to be used per sample, it is advisable that additional L20B flasks be used rather than RD(A), since the L20B is selective for polioviruses. Environmental samples often contain abundant non-polio enteroviruses which may mask poliovirus growth in the RD(A) cells. However, isolation of non-polio enteroviruses in inoculated RD(A) cells serves as an internal control for field and laboratory procedures.

Maintenance and follow-up of the inoculated cultures are similar to those inoculated with clinical specimens (see WHO Polio Laboratory Manual), including blind passages when necessary, cross-passaging RD(A) isolates in L20B cells (passages in tube cultures) and serotyping using standardized sets of WHO-approved antisera. L20B cell isolates should be prioritized in serotyping as environmental specimens frequently contain difficult-to-sort-out mixtures of non-polio enterovirus serotypes.

> ***Comment:*** By using the above bulk culture approach there is a risk of missing minor and low-fitness components in a virus mixture. Using multiple tube cultures might partially overcome this problem but requires a lot of laboratory work. Using a plaque assay to isolate individual virus strains from environmental specimens has been shown to be a useful means to avoid this risk, especially when coupled to a second passage at high temperature to select likely wild poliovirus strains for rapid further characterization. This approach requires special training and a separate reliable high temperature incubator.

2.8 Characterization of poliovirus isolates

All poliovirus isolates from environmental specimens should be differentiated as wild or vaccine-like, ideally within 14 days of detection, and in a WHO-accredited regional reference laboratory (RRL). RRLs, however, give highest priority to characterizing polioviruses obtained from AFP cases and their contacts. Environmental surveillance is likely to generate a substantial workload for ITD tests because of the likely preponderance of Sabin polioviruses in countries where OPV is used. Therefore logistic arrangements for ITD tests must be incorporated in the planning stages. It is noteworthy that parallel poliovirus isolates derived from a given environmental specimen may not be identical even if belonging to the same serotype, and may contain a mixture of Sabin-like (SL) and non-Sabin-like (NSL) strains. A backup portion of the sample should be kept at the NPL to be made available to the RRL for repeat testing as necessary.

Poliovirus (PV) isolates and strains showing contradictory ITD results in genetic and antigenic assays may represent cVDPVs and should be sequenced for further characterization, as is done for corresponding clinical isolates. It is important to keep in mind that poliovirus mixtures are common in environmental specimens, which may cause confusion in interpreting ITD results.

2.9 Reporting laboratory results

Reporting of laboratory results from environmental surveillance to MOH and WHO should in principle follow the guidelines of reporting for clinical surveillance with respect to the need for regular reporting of activities and findings as well as immediate reporting of wild poliovirus isolation. The plan should describe the reporting procedures and indicate who is responsible for reporting the results.

2.10 Interpretation of results and consequences

The route of poliovirus from an infected individual through the environment to the cell cultures at NPL is very complex, and thus the results obtained in environmental surveillance should be interpreted with caution. A useful criterion of satisfactory overall performance of the surveillance is detection of non-polio enteroviruses in the samples. At least 30% of concentrated sewage from grab samples should reveal NPEV and at least 10% of the ground glass traps should reveal NPEV. In populations immunized with OPV, environmental surveillance should also reveal SL strains, especially during and after NIDs and other campaigns.

Abundant OPV-derived strains in the sewage may theoretically mask the presence of small amounts of wild poliovirus if the standard techniques without specific selective conditions for wild poliovirus are being used. However, there is plenty of evidence from practical experience of successful isolation of wild poliovirus during and immediately after NID, and hence there is no need to interrupt environmental surveillance because of an OPV campaign.

Isolation of wild poliovirus from an environmental specimen should raise the same question and result in similar actions as diagnosing a paralytic case caused by wild poliovirus, i.e. a determination should be made of whether the results represent recent importation of the virus or wild poliovirus circulation in the community (see Annex 4). This should result in intensified AFP surveillance in the community (see Annex 4), more frequent and possibly redesigned environmental sampling and preparation for supplementary immunization activities. Environmental findings should be assessed in the context of all other epidemiological information. The WHO regional office should be consulted about proposed programmatic actions.

Isolation of a wild poliovirus from an environmental specimen usually means that a number of individuals are excreting the virus. Negative results are more difficult to interpret and should be assessed in relation to the sampling design and efficiency of laboratory procedures. The theoretical maximum sample sensitivity can be calculated by using some assumptions (Annex 3). Repeated sampling will increase the probability of detecting low-level transmission of wild poliovirus or cVDPV in a population. If a population is monitored using the recommended methods with acceptable quality indicators, consistently negative wild poliovirus results for 12 months suggest that wild poliovirus is not circulating in the population. If this situation continues for three successive years, wild poliovirus circulation is highly unlikely in the source population. These conclusions should be drawn with caution if there is a high risk of importation of wild poliovirus.

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Annex 1:
Collection and concentration of grab samples

A. Collection of grab specimens of sewage

The National Environmental Surveillance Plan should contain unequivocal detailed instructions for the following matters.

- *Sampling sites and persons responsible for sampling*

  Sewage specimens may be collected at sewage treatment plants, preferably from the inlet collector canal or, if the source population is considered to be too large, from other major collector sewers in the network. Accessibility of the actual sampling site should be agreed upon with the local sanitary engineering authorities.

- *Details and responsibilities for provision of the sample vials to be used*

  Sturdy sample vials of either glass or plastic with a volume of 1–1.5 litre can be used. They should be cleaned but sterilization is not essential. The form of the vial is not important (i.e. bottle, can, etc.) but it should be sealable and compatible with the container to be used for cold transportation of samples. The vial should have an unequivocal identification code and should be accompanied with a form indicating the sampling site and sampling time.

- *Sampling procedure at each sampling site*

  If automated sampling equipment is not available, samples should be taken from mid-stream of a collector sewer using a bucket or other suitable means that may be available locally. Composite samples can also be generated by hand by collecting smaller volumes at intervals to cover known peak hours of household wastewater flow, or to combine samples representing smaller than optimal adjacent population sizes. A sample of one litre of raw sewage fluid should be collected in the vial, and the outside of the tightly closed vial wiped with a disinfectant before packaging in a cold transport container.

- *Transport of specimens*

  Persons responsible for arranging the transport of specimens should be mentioned by name. The parcel should be kept at 4°C before and during the transport to the laboratory. The cold transport container should be labelled with the name and address of the laboratory.

B. Concentration of sewage specimens using the two-phase separation method

Reagents for 4 x 0.5 litre specimens

1. 22%(w/w) Dextran
   - 40 g Dextran T40 (Amersham – Pharmacia; products of other manufacturers may be suitable but have not been validated); and
   - 142 ml sterile distilled water.

Use magnetic stirring for dissolving. Can be kept for 2 weeks at 4°C.

2. 29%(w/w) PEG 6000
   - 363 g PEG6000 (Fluka AG; products of other manufacturers may be suitable but have not been validated); and
   - 888 ml sterile distilled water.

Use magnetic stirring for dissolving. Can be kept for 2 weeks at 4°C. Can also be autoclaved, 15 min at 115°C.

3. About 150 ml 5N NaCl
4. 1N NaOH (1N HCl) for pH adjustment
5. pH paper with 0.5 unit (or tighter) scale

Concentration of 0.5 litre specimen

1. Centrifuge the entire sample, in several portions if necessary, for 10 min at 1000 g.[1] Pool supernatants in a 1 litre Erlenmyer flask. Keep the pellets at 4°C.
2. Adjust the pH of the supernatant to neutral (pH 7 –7.5). Usually only a few ml 1N NaOH is needed. Measure the volume of the supernatant.
3. To 500 ml of the supernatant, add 39.5 ml of 22% dextran, 287 ml 29% PEG6000, and 35 ml 5N NaCl. Mix thoroughly and keep in constant agitation for 1 hour at 4°C using a horizontal shaker or magnetic stirrer.
4. Prepare a sterile conical 1 litre separation funnel per sample being evaluated and attach the funnel to a stand. Spread grease on the gliding glass surfaces of the valves but do not obstruct the holes. (Teflon valves do not require smearing). Check water tightness with a small volume of sterile water. Pour the mixture from #3 into the funnels and leave overnight at 4°C.
5. Open the valve with caution. Collect the entire lower layer and the interphase slowly drop-wise, into a sterile tube (usually 5–10 ml per 0.5 litre of original sample).

[1] g = relative centrifugal force; to convert to RPM use the following formula:
$g = (11.7 \times 10^{-7}) RN^2$
where R = radius in mm from centrifuge spindle to extreme point on the tube, and N = speed of centrifuge spindle in RPM.

6. Re-suspend the pellet from #1 into the harvest of #5. Extract with 20% volume of chloroform by shaking vigorously for 1 min. Centrifuge as with faecal suspensions. Collect the upper water phase in a sterile tube and add antibiotics (e.g. penicillin G and streptomycin to final concentrations of 100 IU/ml and 100 mg/ml, respectively).

7. Freeze 1 ml aliquot of the extracted concentrate at -20°C (-70°C if available) for potential future use. Inoculate the remaining extracted concentrate in fresh monolayer cultures of L20B or RD(A) cells in 50 ml (25 cm^2) flasks.

C. Validation of the concentration step

The nominal concentration power of the two-phase separation method is 50–100 fold but its feasibility should be tested by spiking experiments in each laboratory using it. A known amount poliovirus type 1 Sabin is mixed into a selected sewage sample, the mixture is concentrated, and the concentrate is analysed for poliovirus.

1. Make a standard series of 10-fold dilutions of a PV1 Sabin stock with known titer. Calculate the dilution and volume containing 20 $CCID_{50}$ per 100ml – 1.0 ml. Make a standard backup titration of the virus stock on a microwell plate.

2. Divide a 1 litre sewage sample in two equal parts, add 20 $CCID_{50}$ of PV1 Sabin to one part of sample and concentrate both parts as described above.

3. After chloroform extraction, make dilutions of 1/3 (0.7 ml to 1.4 ml) and 1/10 (0.2 ml original to 1.8 ml) of the concentrate in maintenance medium, and freeze them to -20°C.

4. Inoculate 5 L20B flasks and 1 RD(A) flask with 0.5 ml of chloroform treated spiked concentrate and 2 L20B flasks and 1 RD(A) flask of the unspiked concentrate. Monitor for the development of CPE and confirm isolates by serotyping.

5. If the obtained volume of the concentrate is 5 ml, the maximal amount of virus per 0.5 ml aliquot to be inoculated per 50 ml flask is 2 $CCID_{50}$. At least 2 of the 6 flasks inoculated with the spiked sample should reveal poliovirus type 1, if the back titration shows that the amount of virus used for spiking was as designed. If no poliovirus was found in the spiked sample, repeat the experiment with 100 $CCID_{50}$. If poliovirus is also found in the unspiked sample, or if all six flasks inoculated with the concentrate of the spiked sample revealed poliovirus, go back to and analyse the frozen dilutions (3 flasks each).

Other concentration methods can be validated in principle using the same procedure.

Annex 2:
Using bags with sorbent (macroporous glass) to "trap" viruses

A. Sampling

During sampling, special precautions should be taken to prevent cross contamination.

In the field a sorbent-bag with sorbent should be fixed using fishing-line so that the bag will be in the stream of water. After exposure for 3–7 days the sorbent-bag should be placed in a separate plastic parcel or sterile flask and transported to the laboratory in a cold bag or cold box. Each sorbent-bag should be labelled (locality, point of sampling, date of beginning of the sampling, duration of exposure). Samples should be kept at +4°C for no more than 24 h, and should be kept cool during transport to the laboratory.

B. Treatment of samples

The bag with sorbent is placed in a sterile Petri dish. The edge of the sorbent-bag is cut off, the glass sorbent is washed out with sterile distilled water (about 5 ml) using a pipette in the same Petri dish. Put the glass into a column of 5–10 ml volume. Viruses are eluted stepwise with 3 sterile solutions (use 3 ml of each).

1. 0.05 M Tris-HCl pH 9.1
2. 0.05 M Tris-HCl pH 9.1 with 0.5 M NaCl
3. 3 % beef extract in 0.05 M Tris-HCl pH 9.1

Each of the 3 fractions (eluates) is collected and investigated All fractions are treated with chloroform. For that purpose, add 2 volumes of chloroform per volume of eluate, vigorously shake for 10 min and centrifuge at 2000 rpm for 10 min (to separate the phases). The water phase (upper) is transferred with a pipette into a sterile flask, and penicillin and streptomycin are added to final concentrations of 100 IU/ml and 100 mg/ml, respectively.

C. Preparation of macroporous glass

To increase sorption ability of macroporous glass, it is treated as follows:

- Prepare a mixture of 1 part of 3 % H_2O_2 and 1 part of 6 M HCl.
- Add 1 volume of glass to 1 volume of the above mixture and boil with care in hood for 1 h without cover.
- Wash the glass with an excess of distilled water to neutral pH and dry at 100°C.
- Put 3 cm^3 of prepared glass in a parcel (5 x 7 cm) of special water-permeable material.

D. Pretreatment of glass columns

To prevent unwanted adsorption of viruses to the column wall, wet the column (inner surface) with silicone fluid (Sigmacote, SL-2), pour out the fluid, keep the column at 100°C for 1 h. The silicone fluid can be reused repeatedly.

Annex 3:

Theoretical considerations on sensitivity of environmental surveillance

The theoretical maximum sample sensitivity can be calculated by using some assumptions when grab samples are collected.

1. All people in a given population are connected to a converging sewer network, and all poliovirus they excrete will end up into the sewer network.
2. Once in the sewage, poliovirus will remain detectable by cell culture for the necessary period of time.
3. The mean amount of PV excreted daily per person is 10^7 infectious units.
4. The daily flow of sewage per person varies greatly between different localities, but an assumption can be made of a flow of 100 litres per person per day.
5. The NPL will detect wild poliovirus if there are at least two infectious units in the entire assayed 2 ml fraction of a given sample (1 CCID-50/ml).
6. Possible coexistence of SL viruses does not interfere with the system.

With these assumptions, the daily output of a single person (10^7 infectious units) can be diluted to 10^7 ml = 10 000 litres (corresponding to the daily flow of 100 individuals), and the system could still detect the virus without sample concentration, assuming that the virus was evenly distributed in the sampled sewage.

If the tested sample is 100-fold concentrated, the system might detect one infected individual among 10 000 uninfected ones.

Thus, if the source population of a given specimen is 100 000 or more, it is obvious that a specimen can only accidentally be virus-positive if one or a few persons only are excreting the virus. Please, note that all above assumptions are made using perfect performance at each step. This will not happen in real life. In practice this means that individual importations of wild poliovirus are highly unlikely to be detected by this system and that repeated detection of virus in a sampling site almost guarantees that virus is circulating in the population.

Annex 4:
Responding to wild poliovirus or VDPV detection in environmental samples

The detection of wild poliovirus or VDPV in the environment demands follow-up investigations to determine the significance of the findings. Various factors influence the nature and scope of the programme response, including:

- the status of the country as polio-free, recently endemic or endemic;
- the polio immunization coverage in the population;
- the quality of AFP surveillance in the population; and
- the reliability of the laboratory results and trends in wild poliovirus or VDPV detection in the environment.

In polio-free countries wild polioviruses or VDPV detected through either AFP or environmental surveillance strategies represent a public health emergency warranting immediate further investigation to determine if there is an outbreak and to plan for an appropriate immunization response. In recent or current polio-endemic countries or areas, wild poliovirus or VDPV detected in the environment may serve as an impetus for targeting and improving surveillance and immunization performance, especially if no concomitant paralytic cases are detected through routine AFP surveillance.

A. Investigations to determine if there is an outbreak

Investigations should proceed simultaneously along several fronts to determine the significance of wild poliovirus or VDPV detected through environmental surveillance. The following programmatic actions should be taken to determine if there is an outbreak:

1. Communicate information

- Notify all reporting units within the country within 24 hours of receiving news of a suspected outbreak of poliomyelitis. Rapid communication regarding a possible polio outbreak is key to initiating appropriate action and preventing further spread.
- Request heightened active surveillance for AFP cases and strict attention to completeness and timeliness of reporting.
- Inform WHO and UNICEF within 48 hours that a suspected outbreak is being investigated.

2. *Enhanced environmental sampling*

- Review information on the population represented by the sampling site and the frequency of environmental sampling, and determine whether there are opportunities for increasing sensitivity of virus detection. Ongoing transmission may be deduced from repeated wild poliovirus detection through intensified sampling (e.g. weekly sampling).
- Investigate additional sampling sites for surveillance of sub-populations and/or neighbouring or contact populations.

3. *Search for poliovirus-infected persons*

- Review routine surveillance data to determine whether polio cases may have been missed. Include in the review the previous 12 months and focus on surveillance quality indicators (non-polio AFP detection rate, timeliness and adequacy of stool collection from cases, proportion of cases with stools tested in a WHO-accredited laboratory and the available laboratory results).
- Review retrospective records in health facilities in the immediate and surrounding areas of the suspected outbreak to determine if polio cases were not reported or were inadequately investigated.
- Initiate an active case search in the suspected community.
- Assess the value of stool surveys, taking into consideration issues related to timing, representative sampling, logistic arrangements for samples collection/handling, and assuring adequate laboratory support.

4. *Assess polio immunization coverage*

- Review routine and supplemental polio immunization coverage to assess the likelihood of susceptible populations capable of sustaining poliovirus transmission.
- Begin preliminary planning for an immunization response while immunization coverage is reviewed, focusing on logistic, operational and financial needs.

5. *Enhance virologic investigations*

- Expedite genome characterization of the wild poliovirus or VDPV isolates to assist in the investigation of their possible source and possible chains of transmission.
- Request that all virus negative faecal specimens from AFP cases and untyped or non-typable virus isolates from faecal and environmental samples be submitted to a WHO-accredited laboratory for further investigations.
- "Flag" all subsequent poliovirus isolates, environmental samples and faecal samples from the area of the suspected outbreak for high priority testing in a WHO-accredited laboratory.

B. Responding to a confirmed outbreak of wild poliovirus or VDPV

A decision should be made as soon as possible (and no later than one month after detection) as to whether a suspected outbreak has been confirmed or if there is a sufficiently high index of suspicion to warrant an immunization response. An outbreak is confirmed if any of the following conditions are met:

- multiple detection of wild polioviruses or VDPV in the environment (e.g. over several weeks or in several sites);
- a number of genetically distinguishable wild polioviruses or VDPV are detected; and
- paralytic polio cases, polio-compatible cases, wild poliovirus or VDPV-infected persons are found during follow-up investigations.

If an outbreak is confirmed, countries should notify WHO and UNICEF within 24 hours and the existing immunization services or a special steering group of experts within the Ministry of Health should advise and coordinate response activities nationwide. The response should be appropriate to the outbreak and include the following actions:

- Contact all surveillance units and major hospitals nationally to inform them of the outbreak and provide them with information and materials to assist in identifying further cases.
- Enhance national surveillance activities to determine whether poliomyelitis cases occurred in areas beyond that where the outbreak was confirmed.
- Institute systematic monitoring of surveillance reports at the national level.
- Conduct an immunization response consistent in size and nature with the findings of the outbreak investigation.

The Department of Vaccines and Biologicals was established by the World Health Organization in 1998 to operate within the Cluster of Health Technologies and Pharmaceuticals. The Department's major goal is the achievement of a world in which all people at risk are protected against vaccine-preventable diseases.

Five groups implement its strategy, which starts with the establishment and maintenance of norms and standards, focusing on major vaccine and technology issues, and ends with implementation and guidance for immunization services. The work of the groups is outlined below.

The *Quality Assurance and Safety of Biologicals team* team ensures the quality and safety of vaccines and other biological medicines through the development and establishment of global norms and standards.

The *Initiative for Vaccine Research* and its three teams involved in viral, bacterial and parasitic diseases coordinate and facilitate research and development of new vaccines and immunization-related technologies.

The *Vaccine Assessment and Monitoring team* assesses strategies and activities for reducing morbidity and mortality caused by vaccine-preventable diseases.

The *Access to Technologies team* endeavours to reduce financial and technical barriers to the introduction of new and established vaccines and immunization-related technologies.

The *Expanded Programme on Immunization* develops policies and strategies for maximizing the use of vaccines of public health importance and their delivery. It supports the WHO regions and countries in acquiring the skills, competence and infrastructure needed for implementing these policies and strategies and for achieving disease control and/or elimination and eradication objectives.

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