



# POLIO LAB NETWORK

## Quarterly Update



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### *Experience with cell sensitivity LQC is reviewed*

Network experience to date with establishing laboratory quality control (LQC) poliovirus standards for cell sensitivity testing reveals a wide range in QC virus titers. Some variation may be due to true loss in cell sensitivity, but much may be attributed to first experiences. To repeat the instructions provided by the RLC:

- *Make serial 10-fold dilutions of the virus preparation*
- *Use a volume of 100ul of virus dilution per well*
- *Use 4 dilutions of virus strain on the test plate; a range of  $10^4$  to  $10^7$  for the NIBSC standard reference strains; and a range of  $10^5$  to  $10^8$  or  $10^6$  to  $10^9$  for the laboratory quality control standards (LQC) prepared in your laboratory*
- *Use 20 replicate wells per dilution of virus on the test plate*
- *Calculate the virus titre using the Kärber formula*

The protocol should be amended to include an upper limit of virus titer (+0.5 log of expected titer) for the test to be valid, the need to test all three serotypes in both cell lines and the acceptance as valid of those tests where 90% CPE (instead of 100% CPE) or 10% CPE (instead of 0% CPE) are achieved. In those cases, the next lower or higher virus dilutions are to be considered 100% or 0% CPE, respectively, when applying the Karber formula to calculate the virus titer.

The dilution range for titration of LQC standards should be changed as indicated. For example, if no 0% CPE or 10% CPE point is reached, use dilutions  $^{-6}$  to  $^{-9}$  instead of  $^{-5}$  to  $^{-8}$ . Failure to repeat the tests using the optimum dilution range will cause many tests to be invalid.

To assign a titer to the laboratory quality control (LQC) poliovirus standards, THREE INDEPENDENT VALID TESTS ON DIFFERENT DAYS WITH FRESH DILUTIONS MUST BE PERFORMED IN PARALLEL WITH THE CORRESPONDING NIBSC VIRUS STANDARD. Once the results of the three tests are available for each reference strain and the virus titers of the NIBSC standards, done in parallel, are within the expected limits, a titer for the LQC standards can be es-

established. The LQC can then be used for routine testing of cell sensitivity for poliovirus infection.

The NIBSC virus standards have deliberately low titers to assist laboratories to accurately measure virus preparations with low virus content. If an NIBSC standard virus titer falls out of expected limits (+/- 0.5 log of the assigned titer), the assay should be repeated once to eliminate human error. If the results are confirmed, the procedure must be invalidated and the source of the titer discrepancy identified.

A titer higher than expected could be due to a mistake during the preparation of the virus dilutions or overspill from adjacent wells.

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**Editorial note:** At this juncture of the Polio Eradication Initiative, assurance of laboratory sensitivity for poliovirus isolation is crucial. Cell culture quality control (QC) data provide the only direct proof of cell sensitivity. WHO accreditation requires internal QC procedures to be implemented at least quarterly. Authenticated L20B and RD cells, NIBSC Sabin virus reference stocks of assigned titers, and QC protocols were distributed to all laboratories by the Regional Laboratory Coordinators (RLC) to assist in establishing laboratory quality control (LQC) standards. In the accompanying article, Javier Martin discusses general Network experience and offers some useful advice.

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# Case Study #2

**Situation:** Laboratory A is the National Polio Laboratory in a country where wild poliovirus was last isolated 5 years ago. It receives about 300 fecal samples annually and 6 months ago scored 100% on the annual proficiency test. A few weeks ago the lab reported isolation of a virus that grew on both L20B and RD cells and that could not be typed with RIVM poliovirus antiserum pools.

The Regional Reference Laboratory (RRL) identified the isolate as ECHO virus 17 and failed to confirm growth on L20B cells. As the yearly accreditation visit was due anyway, the Regional Laboratory Coordinator (RLC) visited Laboratory A. She was welcomed by the Director and staff, including a young graduate student who had joined the virology team a few months earlier.

**Investigation:** The chief technician was happy to report that the staff had maintained all lab functions during her recent absence of three months on maternity leave. She was especially proud of the cell culture activities carried out part-time by the graduate student.

The accreditation visit started in the cell culture room. The graduate student turned down the radio sound volume and described his project, which involved comparing PCR detection of rhinoviruses in clinical specimens with detection after 24 hours cultivation in three cell lines. Usually he did his work, including routine cell culture passage, in the evenings not to interfere with regular activities and to enjoy his calypso music at full volume.

The RLC discovered from the meticulous logbooks that the lab had discontinued the standard cell counting procedure. They were now routinely using a 1:5 splitting ratio for both RD and L20B cells. If tubes were needed for the next day, they used a 1:3 ratio to be sure of full monolayers. Inspection of the RD and L20B flasks seeded the day before showed nearly complete monolayers with little or no differences in cell morphology between the two.

Meanwhile, some 40 minutes after he had begun, the graduate student finished his cell passage experiments, cleaned, closed, and turned off the Biological Safety Cabinet (BSC), and turned the radio back on.

Further review revealed that only last week another isolate grew on both RD and L20B cells and was not neutralized by poliovirus serum pools. Like the previous isolate, the poliovirus-typing test showed a late breakthrough in several wells in the <sup>-3</sup> and <sup>-4</sup> dilutions and a virus titer of <sup>-4</sup> on back titration. The repeat experiment at <sup>-1</sup> and <sup>-2</sup> showed clear breakthrough in all wells after two days. Although enterovirus typing was no

longer done routinely, the RLC suggested testing the isolate undiluted against available enterovirus antisera. The next day it was identified as ECHO 7.

**Conclusions:** The RLC concluded the following, which she discussed with the lab staff:

- The current L20B cell culture is contaminated with, or is solely, RD cells, based on two observations. First, the RRL failed to confirm growth of the first NPEV isolate on L20B, and the isolation of another was highly unlikely. Second, the only NPEVs reported to grow on L20B cells are Coxsackie A viruses, which also grow in baby mice and on the mouse cell (LA) parent of the genetically modified L20B cells.
- The contamination or exchange of the L20B cells with the RD cells likely resulted from improper BSC use. The graduate student passaged three cell lines in 40 minutes, a task that should have required well over 2 hours. BSCs should be in operation 15 minutes before and after use, that is, a minimum of 30 minutes between experiments. Passaging one cell line is one experiment, after which the BSC should be completely cleaned with disinfectant. Passaging cell lines separately under strict conditions is crucial to maintain cell identity and reduce the risk of mycoplasma contamination.
- Finally, the low virus titer of the two recent NPEVs, the full cell sheets in the one-day-old flasks, and the 1:3 spitting ratio for rapid production of tubes for virus isolation all suggest low cell sensitivity. An ideal opportunity to monitor cell quality and cell yields was lost when the lab discontinued the cell counting step. Cells should be seeded so that a full sheet is obtained after 3-4 days. In this manner, trypsinized cells recover, attach to the surface and divide several times, generating a layer of freshly divided cells with optimal sensitivity for poliovirus or enterovirus isolation. The cell sensitivity test by poliovirus titration is an ideal starting point, but equally important is the quality of the cells in contact with the inoculum.

**Recommendations:** The RLC complemented the staff on overall lab performance, but noted that recent shortcuts had been taken with dramatic consequences. She recommended that the laboratory:

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## ***Warning: Chloroform Instability***

**Dr. Bassioni**, Regional Reference Laboratory, VACSERA, Cairo, has associated a bottle of chloroform used for sample processing with loss of ability to isolate poliovirus and other enteroviruses from environmental samples. Laboratories should be aware that phosgene (a hazardous corrosive gas) is a breakdown product of chloroform. Virus inactivation by phosgene is highly plausible. Pharmaceutical grade chloroform usually contains alcohol or, less frequently, amylene as stabilizers to retard chloroform breakdown. Amylene has been reported as less effective than alcohol in retarding phosgene generation over time. The chloroform bottle in question at VACSERA contained amylene.

As a precaution, all Network laboratories should examine chloroform labels to make certain the contents include ethanol (usually 1%) as a stabilizer. Laboratories should replace chloroform that contains amylene (or no stabilizer) with bottles that contain ethanol for processing all samples. Check with the manufacturer if the stabilizer is not listed. Chloroform is a time-sensitive chemical. Opened bottles should be dated and discarded after 2 years.

*Recommendations continued from page 2*

1. Check the characteristics of the L20B and RD cells in the cell bank. If ECHO 7 or ECHO 17 grow on the new L20B cells from the cell bank, request the RRL to provide authenticated L20B cells and prepare a new local cell bank. Old cells should be discarded once sensitive authenticated cells are stored in the new cell bank.
2. Determine the optimal cell culture conditions for seeding flasks and tubes. Cell counting and constant seeding conditions for each cell line provide extra assurance of cell quality and sensitivity.
3. Follow the WHO Laboratory Manual and SOPs, especially the SOPs for using BSCs and maintaining cell lines. Never passage different cells lines at the same time.
4. Maintain an atmosphere of positive criticism concerning laboratory practices. Newly appointed staff members must understand that SOPs are based on experience. Shortcuts can have dire consequences.
5. Re-test the AFP samples from the last three months. The presence of polioviruses in these samples may have been missed because of low cell sensitivity and the absence of L20B cells.

*Harrie van der Avoort, RIVM, Bilthoven*

## ***Network News***

With regret, we report the retirement of **Dr. Owale Tomori**, the pioneer Regional Laboratory Coordinator for the African Region. He began AFR LabNet in 1994 from, in his own words, “a scattering of decaying laboratories dotted all over Africa”. As he passes the reigns of today’s strong LabNet, those words are difficult to imagine. We are proud to learn he has been appointed Vice Chancellor of a new University in Nigeria.

We also extend our best wishes and congratulations to **Dr. Gene Gavrilin**, who was selected in August for the well-earned position of European Regional Laboratory Coordinator. He replaces **Dr. Galina Lipskaya**, the pioneer EUR Coordinator, who began in 1996 and met the formidable challenges of organizing the largest number of laboratories in Global Network. Galina officially retired last year, but continues as a consultant, making for a smooth transition in the Region.

We are saddened to learn of the untimely death of **Mr. Apollo Muwonge**, Senior Technician of the EPI Laboratory, Uganda. He was a major force in the laboratory for twenty years, contributing to the success of EPI in Uganda and neighboring countries and to the development of the WHO African Regional Polio and Measles Laboratory Network.

*Experience continued from page 1*

A lower than expected virus titer could similarly reflect a procedural error, but, if confirmed in a second test, it most likely reveals low cell sensitivity. The same rules apply to the LQC standards once they have been validated for use in routine cell sensitivity tests.

If low sensitivity is confirmed, all elements and actions related to cell culture and the QC process should be reviewed including SOPs, staff, management, reagents/materials, equipment, and records. If cells appear to be the most likely cause, discard those in use and replace with a batch of cells of documented sensitivity from a Regional Reference Laboratory, if necessary.

Labs should provide full details of their titration experiments and results using the forms provided and keep a chart on their historical data.

*Javier Martin, NIBSC, London*



# Poliovirus Surveillance Report, January - June 2004

REGION	Number of AFP cases with specimens	P1 only	P2 only	P3 only	Polio mix	Polio + NPEV	NPEV only	Pending culture results	% Positive for NPEV	% Results within 28 days
AFR	4948	479	47	191	68	96	679	76	15%	94%
AMR	810	4	10	6	3	0	91	0	11%	94%
EMR	2808	46	19	40	35	8	488	48	16%	99%
EUR	519	3	8	3	10	0	13	0	3%	99%
SEAR	6487	119	48	113	94	49	1560	45	24%	99.6%
WPR	2807	22	58	40	36	10	208	348	6%	94%

  

Regional Ref. Lab	Poliovirus Intratypic Differentiation Results												
	No. of cases with isolates submitted	Type 1			Type 2			Type 3			Pending		% AFP cases with ITD results within 14 days
Wild		Sabin	VDPV ##	Wild	Sabin	VDPV ##	Wild	Sabin	VDPV ##	ITD Discordant results	ITD Tests Pending*		
AFR	818	421	80	0	0	93	0	88	133	0	0	78	99%
AMR	23	0	5	0	0	12	0	9	0	0	0	0	95%
EMR	148	18	54	0	0	44	0	6	66	0	0	2	92%
EUR	24	0	8	0	0	16	0	0	10	0	0	3	100%
SEAR	426	29	200	0	0	99	0	1	214	0	0	8	88%
WPR	175	0	34	0	0	91	0	0	55	0	17	22	94%

includes some cases with onset in 2003 received for testing in 2004

#No. cases with any isolates pending completion of ITD tests

## only for isolates designated as VDPV after VP 1 sequence obtained

AFR: Between January and June 2004, wild polioviruses were isolated from AFP cases in 8 countries: Nigeria (453), Niger (19), Côte d'Ivoire (8), Chad (12), Central African Republic (2), Burkina Faso (6), Benin (5), and Botswana (1). With the exception of Botswana, all other countries also had wild viruses in 2003, as did Ghana, Cameroun, and Togo. Only Nigeria and Niger were considered poliovirus endemic at the end of 2003. Genetic characteristics of viruses from other countries indicated direct importations from northern Nigeria, or indirect importations from Nigeria via Chad or Burkina Faso. No vaccine derived polioviruses (VDPV) were detected in the region since 2002 when type 2 VDPVs were detected in Madagascar (4 cases) and Nigeria (1 case).

AMR: No wild polioviruses were detected in the region up to June 2004. No VDPVs have been detected since the type 1 outbreak in Haiti and Dominican Republic in the first half of 2001.

EMR: Wild polioviruses were detected in AFP cases from 4 countries between January and June 2004: Afghanistan (3), Egypt (1), Sudan (1) and Pakistan (19). With the exception of Sudan, all of these countries were considered as polio-endemic in 2003. Type 1 virus in Sudan was genetically linked to viruses in Chad. Virus from a single type 1 polio case in Lebanon in 2003 was linked to imported virus from India. Supplementary surveillance in Egypt revealed indigenous wild type 1 polioviruses in 4 sewage samples collected from Minya and Asyut provinces up to June 2004. Twelve sewage samples from 4 provinces in Egypt were positive in 2003.

EUR: No wild polioviruses were detected in the region in 2003. Several type 2 VDPVs have been isolated from sewage samples collected in 2 locations in Slovakia between April 2003 and June 2004. These isolates are not associated with paralytic cases and are the subject of an on-going investigation. A single type 2 VDPV was detected in sewage in Israel in April 2004.

SEAR: India remained the only country with wild polioviruses detected in the region. There were 30 wild virus AFP cases between January and June 2004, compared to 77 cases in 2003. A type 2 VDPV was isolated from an immunodeficient patient in Thailand in 2003.

WPR: No wild polioviruses were detected in the region up to June 2004. A type 1 VDPV was isolated from a single, healthy, non paralysed, child in Mongolia in 2003. A type 1 VDPV isolated from an AFP case in June 2004 is currently under investigation in China.

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