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**Meeting of the Regional Reference Laboratories (RRLs)
WHO/Europe Polio Laboratory Network
Paris, France
13-15 March 2013**

SUMMARY OF DISCUSSIONS AND RECOMMENDATIONS

A meeting of the Regional Reference Laboratories (RRLs) in the European Region of the WHO Global Polio Laboratory Network (GPLN) was held in Paris, France with representatives from 8 member laboratories, CDC and WHO laboratory scientists with responsibility for coordinating the network's activities.

The Lab Coordinator opened the meeting by reviewing the state of completion of the recommendations from last year's meeting and provided an overview of the performance by GPLN laboratories and activities in the Region. The Lab Directors provided updates on their testing, research and training activities over the past year. A representative from CDC presented an update on changes in polio diagnostic protocols with special focus on molecular ITD and sequencing procedures.

Performance of the Polio Laboratory Network in the European region in 2012 remained high. All laboratories in Europe are accredited with the exception of Uzbekistan which is pending administrative processes and other technical issues. There was also significant improvement in the communication between National Labs (NLs) and Regional Reference Labs (RRLs) and in the reporting of laboratory results to the Regional Office although some concerns remain as reports from countries vary in quantity and frequency and some countries are reporting very few or no poliovirus (PV) and/or non-polio enterovirus (NPEV) isolates. No wild PV isolations were reported during this year. Challenges to improve timeliness of detection of wild PVs and vaccine-derived polioviruses (VDPVs) continue as international shipment of materials and reagents such as virus panels, cells, clinical samples, etc., is getting increasingly more complex and expensive due to strict regulations in most countries.

All labs are now reporting through the Laboratory Data Management System (LDMS) which is proving to be a very useful tool as a platform that integrates lab-based acute flaccid paralysis (AFP), enterovirus (EV) and environmental (ENV) surveillance. New functionalities have been added to LDMS that facilitate the geo-localization of supplementary surveillance activities.

Efforts to develop improved laboratory methods and standardized quality assurance procedures continue to be a priority of the GPLN. RRLs in the European Region implemented the new dual-staged ITD rRT-PCR protocol and participated in the first official proficiency testing for sequencing of poliovirus isolates. Concerns related to labs having problems understanding the correct process of cell sensitivity testing were also discussed.

A session was included to debate the possibility of holding a laboratory meeting during 2013. The group concluded that a meeting involving all polio laboratories of the WHO European Region would be useful at this stage

of the Global Polio Eradication Initiative (GPEI). This would help scientists adapting to current and future changes in testing and to be fully aware of the significance of proficiency testing and quality assurance of laboratory procedures.

SUMMARY OF DISCUSSIONS

1. Surveillance for poliovirus in the European Region

Performance of the Polio Laboratory Network in the European Region in 2012 remained high with 96% of isolations of PVs and 84% of ITD results produced/obtained within the required timelines. A total of 8038 specimens from all surveillance activities were processed in the European region during the period January – December 2012. Of those, 4283 were faecal specimens from AFP cases. No wild PVs were detected during 2012. In the European region, all WHO polio laboratories except one (Uzbekistan) were fully accredited by WHO as of December 2012. Some countries include enterovirus diagnostic labs in different regions which do not undergo accreditation by WHO. The corresponding National Laboratory (NL) is responsible for the quality assurance of all laboratory work performed in these sub-national laboratories and for making sure that all poliovirus isolations in the country are performed using WHO-approved algorithms and procedures.

There has been significant progress in Spain and Portugal following recent major assessments and implementation of corrective measures. This was judged by results from proficiency testing and evaluation of results shown in accreditation checklists. Communication between RRLs and their associated NLs has also improved during this period. These interactions provide benefits to both sides - NL and RRL. Challenges to improve timeliness of detection of wild PVs and VDPVs still remain particularly those concerning the shipment of stool samples and PV isolates to NLs or RRLs.

2. Supplementary surveillance

The European Region of the GPLN prioritizes the isolation and characterization of polioviruses from faecal specimens from AFP cases, supplemented by other surveillance approaches such as ENV and EV surveillance in its support of the GPEI. Laboratories in this Region have a broad range of experience and laboratory techniques. ENV and EV surveillance are essential because AFP surveillance is not universally used throughout the Region. More countries are introducing these supplementary surveillance activities into their national programs.

About 40 Member States report the use of EV surveillance as the only mechanism of polio detection or in conjunction with either AFP or ENV surveillance. Guidelines covering different aspects related to EV surveillance such as recommended procedures for specimen transport, EV detection and characterization and documentation and reporting of results have been developed by the Regional Office.

ENV surveillance for PV is also relevant for the Region and needs to be maintained and expanded in the immediate future in order to supplement AFP surveillance or to substitute for it where it is not implemented. 20 Member States have some ENV surveillance activities at the moment. However, standardization and establishment of a quality assurance program for environmental surveillance is complex as virus isolation rates might vary significantly between countries/regions. Many variables can affect isolation rates from environmental samples such as differences in sewage infrastructure, population density, national immunization policies, climatic conditions, migration movements, etc.

Methods for sample collection and virus isolation algorithms might also vary between laboratories and virus isolations are often not performed in WHO-accredited labs, which makes evaluation of performance and tracing of results more complicated. Efforts are ongoing to develop updated “WHO Guidelines for ENV Surveillance of PV circulation” and detailed standard laboratory procedures to improve the efficiency of analysis of sewage samples. New functionalities have been added to LDMS to improve reporting of results from EV and ENV surveillance including the geographical locations where sampling is taking place that can now be shown in graphical maps. A new reporting form showing results from EV and ENV surveillance has been developed and contains information on the total number of samples analyzed, including specimens with negative results. The results are shown by country and geographical area within that country. These control systems should help identifying quality indicators to assess the extent and effectiveness of these supplementary surveillance activities.

3. Detection of Vaccine Derived Polio Viruses (VDPVs)

VDPVs have been isolated from various sources in the European region although none from AFP cases. A type 1 VDPV with 12 mutations in VP1 was isolated in Turkey from an immunodeficient child that had been identified as a contact of an AFP case from whom a SL type 1 PV had been isolated. The two children shared one room in a hospital, but the type 1 PV isolates were different. The isolate from the AFP case had three mutations in VP1, none of them common to the 12 mutations found in the isolate from the contact child. Two related type 3 VDPVs were also isolated in Turkey, one containing 9 mutations in the VP1 (L20B isolate) and the other 10 VP1 mutations (RD isolate).

Two related plaque isolates of type 2 aVDPVs were isolated from a sewage sample collected in Tel Aviv (Israel) in August 2012. They had 16.2% VP1 sequence divergence from Sabin 2 and differed by only one nucleotide between them. These strains belong to the cluster of highly diverged type 2 aVDPV isolates first identified in sewage samples in 1998. A type 1 aVDPV with 13.7% VP1 divergence from Sabin 1 was isolated from a sewage sample in Haifa in December 2012. This isolate was related to a previous strain isolated Haifa in 2009.

Two iVDPV2 isolates were obtained from the well-known long-term immunodeficient excreter in the UK who has now been excreting PV for an estimated 27 years.

VDPVs have been periodically found in ENV samples from European countries such as Finland, Slovakia, Estonia and Israel during the last several years. Prolonged VDPV detections remain a concern because of their potential of VDPVs to circulate in communities with low immunity. Molecular characterization of these aVDPV strains found in sewage samples indicate that they have properties similar to VDPVs excreted by immunodeficient individuals. This suggests that the prevalence of long-term polio excretion by immunodeficient individuals might be higher than expected. Few ad hoc studies have been conducted in Europe to assess the incidence of polio excretion among immunodeficient individuals which are inconclusive.

WHO/Europe and HQ are in dialogue with a non-governmental organization working with individuals with primary immunodeficiencies that have agreed to contact laboratories in different countries to request testing for poliovirus excretion.

4. LDMS reporting system

All labs in the Polio Laboratory Network in the European Region are now reporting through LDMS although some concerns remain on the consistency of reporting between countries/regions as reports vary in quantity and

frequency. As reviewed in detail during last year's RRL meeting, the online LDMS provides the WHO European Regional Office with a platform for a comprehensive evaluation of polio surveillance. It integrates data from lab-based AFP, EV and ENV surveillance and helps minimizing reporting errors between surveillance and laboratory databases enabling the efficient analysis of performance indicators. It also allows the immediate notification of wild PVs to WHO/Europe and the tracking of stool samples in near-real time. LDMS now includes several improvements such as the possibility to enter geographic information for samples from any type of surveillance. LDMS allows labs not only to report data from routine surveillance activities but also to enter results from other studies such as stools studies or analysis of samples from immunodeficient patients and other information related to the characterization of poliovirus isolates such as sequencing data. The reporting form has been updated to integrate results from AFP, EV and ENV surveillance. It includes entries for the number of specimens analyzed for each of the three surveillance activities, the number of non-polio enterovirus isolations and the number of poliovirus isolations classified as wild type, VDPV or Sabin-like.

5. Laboratory Methods

5.1 Update on methods

RRLs and particularly Global Specialized Laboratories (GSLs) in the European Region continue to contribute to the validation, implementation and improvement of methods used by the GPLN. Scientists from the CDC are continuously updating protocols for ITD rRT-PCR to help increasing the sensitivity and specificity for detection of wild PVs and VDPVs.

The dual-staged rocket rRT-PCR was implemented in RRLs in Italy, Germany and Finland (ongoing). Implementation in other GSLs might be more difficult depending on the technical specifications of the different RT-PCR platforms available at RRLs. New rRT-PCR assays for the direct detection of VDPVs (rather than by exclusion) are being developed. Assays for type 1 and 2 VDPVs are complete and that for type 3 VDPV is proving the most challenging assay to develop. Concerns were noted regarding the possibility of missing VDPVs which have new sequence changes at probe sites. Locked Nucleic Acid (LNA) are being used to increase the specificity of the current CDC kits. CDC has been using the LNA version of the PV and EV probes for over 2 years. They provide better signals and will improve data interpretation especially when serotype mixtures are present. The next batch of ITD kits will include these LNA probes. Future developments in ITD rRT-PCR testing will include a quadruplex assay to detect Sabin 1, 2 and 3 + EV in the same reaction.

The development of efficient methods for the direct detection of virus RNA in stool samples is still elusive as current methods sometimes fail to detect viruses detected by cell culture.

A transition is planned to the use of commercial buffers for rRT-PCR reactions. The CDC have performed a comparison of several commercial kits versus current CDC reagents. The use of optimal buffer reagents will help increasing the sensitivity of PV assays for use in ENV and/or direct stool testing.

Improvements in methods for virus isolation from ENV samples are also needed. The current ENV concentration/processing algorithm is slow and labour-intensive. ENV isolates in some regions are currently tested by probe hybridization, ELISA and neutralization. These assays do not detect all VDPVs and are less sensitive than the ITD rRT-PCR method used for testing of AFP specimens in more than 80 GPLN labs. Efforts to streamline the processing and testing of environmental samples are ongoing; they will include a switch to rRT-PCR assays for ITD and the use of improved methods for collection and treatment of samples.

The current state of the standardization and quality assurance of methods for sequencing polio isolates was also discussed. The first official proficiency testing (PT) exercise for sequencing was conducted in 2012. The results were very positive overall: 16 labs passed (90% or higher), 2 labs failed (>80%), one lab score is pending, one lab hasn't received the panel and one lab had equipment problems. 17 labs sequenced virus mixtures, which was optional. All European RRLs participating in the study passed the PT.

5.2 Reagents and reference standards

The availability of adequate reagents and reference standards to support polio laboratory testing is critical to ensure accurate and reliable results as most assays used by the GPLN require specific reagents that have been validated:

- Some reagents are available through the WHO catalogue.
- CDC reagents such as rRT-PCR kits can be requested for laboratory projects other than routine surveillance testing. Priority will be given to WHO-related projects such as studies that will contribute to the endgame for poliovirus eradication.
- A new batch of AG pools for EV typing will be available from March 2013, it will most likely be the last batch available and will cover the needs for 2-3 years.
- Sabin virus reference standards for cell sensitivity can be ordered online:
http://www.nibsc.ac.uk/science/vaccines/cell_substrates1.aspx
- Results from the evaluation by CDC of different kits for rRT-PCR are available to help identifying suitable reagents for lab tests.

6. Laboratory Quality Assurance Program

6.1. Annual accreditation process

The annual PT and assessment of laboratories continue to be critical for the quality assurance of the performance in polio labs. Seven different PT panels are in use for evaluating (i) accuracy of virus isolation (two versions, for the old and new virus isolation algorithms); ITD by (ii) ELISA, (iii) probe hybridization, (iv) traditional PCR, (v) real-time PCR (rRT-PCR), and, (vi) rRT-PCR for VDPV screening, (vii) nucleic acid sequencing. The PT program is coordinated by WHO in collaboration with the GSLs in the United States and the Netherlands. Laboratories that attempted ELISA PT in 2012 attained passing scores of > 90%. All laboratories that attempted the traditional PCR PT attained passing scores of > 90%. All laboratories that attempted the two rRT-PCR panels for ITD and VDPV screening attained passing scores of > 90%. Similarly, all laboratories that attempted the sequencing panel attained passing scores of \geq 90%. 13 labs did not undergo virus isolation PT of 2012 yet, since the Russian RRL is experiencing problems with importing the PTs into the Russian Federation. All other labs passed the isolation PT in 2012.

6.2 Cell lines

The quality assurance of all aspects related to the work in GPLN labs is critical to guarantee optimal conditions for the isolation of PV and NEPV from stool and ENV samples. The requirement to test regularly the cell sensitivity for poliovirus infection was introduced in the GPLN several years ago and evaluation of results sent by labs has proven

to be a very useful tool to monitor lab performance and to detect labs that were missing virus isolation in samples that were later shown to contain PVs or NPEVs. However, during the meeting, participants agreed that this test is still not used to its full potential. The concept of cell sensitivity testing appears not to be well understood by most labs and this problem needs to be addressed. The virus titre results from cell sensitivity testing should be critically evaluated during the accreditation process. It might be necessary to update the accreditation checklist to include evidence for the correct interpretation of results and any action taken following any failed tests. Efforts are ongoing to update the chapter describing cell sensitivity testing as part of a general revision of the WHO Polio Manual to be undertaken by scientists from GSLs.

An SOP for cell authentication has been developed by the UK GSL and has been shared with GSLs.

6.2. Laboratory training

Training activities during this period included a visit by two scientists from the Russian Federation to the Finland RRL to be trained in molecular detection methods for the characterization of PVs and other intestinal viruses. The new polio lab director from Sweden also had induction training at the Finland RRL. One scientist from the NL in Poland trained in Germany RRL on the molecular analysis of poliovirus strains from environmental samples.

Scientists from the RRL in Germany participated in the celebration of a world polio day in Germany to raise awareness about polio in the different sectors of society. Scientists from the RRL in the UK participated in a Polio Outbreak Simulation Exercise (POSE), designed by WHO/Europe and the HPA, UK, to thoroughly review the UK's polio plans and preparedness and then test them in a rigorous exercise. These activities are very useful to evaluate the ability and efficiency in detecting polio importation/outbreak and the public health responses available, particularly in countries where polio has been eradicated many years ago and both clinical experience and public awareness are lacking.

7. All-Regional Laboratory meeting

A session was dedicated to discuss the possibility of holding a regional laboratory meeting in the next few months. The convenience, timing and logistics related to the organization of such a meeting were assessed. The group concluded that a meeting involving all polio laboratories of the WHO European Region would be very useful at this stage of the GPEI. It has been a long time since representatives from all laboratories met in Malta in 2007. There have been significant modifications in the methods and testing algorithms used in the labs and indeed more are anticipated in view of future changes in immunization policies and biosafety requirements related to the endgame for polio eradication.

Points for the agenda will include an update on the global situation and the strategies for the endgame of the GPEI, details on the recent and future changes in methodologies and discussions on the expectations, challenges and responsibilities that the labs face, with an emphasis on the importance of proficiency testing and quality assurance of laboratory procedures. The significance of the future legacy left by participants in all aspects of the GPEI program will also be discussed. The expertise acquired by GPLN scientists will be very useful for them to contribute to current or future programs for the control of viral diseases.

It was also agreed that this would be a good opportunity to organize a practical workshop on biosafety or lab management in the program, although the selection of appropriate participants would be required.

RECOMMENDATIONS

1. RRL directors should maintain close communication with associated NLs to ensure the fast shipment of isolates and the early detection of performance concerns. To achieve this:
 - a. Communication between RRLs and their associated NLs should include all aspects of lab functionality.
 - b. Accreditation checklists of associated NLs should be made available to RRLs.
 - c. RRL directors should discuss with the Regional Office plans for accreditation visits in 2014.
2. Laboratories of the WHO/Europe polio laboratory network should report all results from surveillance activities by using LDMS. To achieve this:
 - a. Results should include those from AFP, EV and ENV surveillance.
 - b. Laboratories are also encouraged to report results from other activities such as stool studies or analysis of samples from immunodeficient patients.
 - c. Laboratories can also enter other data related to the characterization of polioviruses such as sequences from polio isolates.
 - d. RRL directors can provide technical support and assist non-WHO labs to ensure all activities involving poliovirus isolation are reported to the Regional Office.
3. The Regional Office should help to increase the quality of reporting by LDMS. To achieve this:
 - a. The Regional Office can assist if problems in implementation are encountered by labs.
 - b. The quality of reporting through LDMS will be made public.
4. There is a need to identify quality indicators and to improve standardization procedures for supplementary surveillance activities conducted in the European region as these are the only means for PV detection in many countries. To achieve this:
 - a. Guidelines for EV surveillance developed by the Regional Office should be distributed to labs.
 - b. Guidelines for ENV surveillance should be updated.
 - c. A critical review by the participating laboratories in the Member States on the quality of their supplementary surveillance activities, particularly EV surveillance, is strongly recommended.
 - d. RRL directors should identify ways to support these activities whether they are directly involved in laboratory testing or not.
5. RRL Laboratories should continue participating in the development, validation, pilot and proficiency testing of laboratory methods for the GPLN methods to contribute to the improvement, standardization and quality assurance of these procedures.
 - a. The new ITD Rocket rRT-PCR method should be pilot tested in laboratories with rRT-PCR ITD capacity that have still not done it following instructions from CDC.
 - b. Protocols for sequencing methods should be compiled by the Regional Office to be shared between sequencing laboratories with a view to harmonize procedures and reagents.
 - c. All RRLs should switch to the standard elution protocol for samples arriving on Whatman® FTA® paper.
6. The annual evaluation of the quality assurance of activities conducted in all polio laboratories in the Region should continue to be a high priority.
 - a. Laboratories should review their training procedures to ensure an SOP exists with details of how competence for the different laboratory techniques should be acquired and evaluated.

- b. Laboratories distributing cells to NLs should send samples from RD and L20B master cell stocks to the GSL in the UK to be tested for authenticity.
7. An all-region laboratory meeting in late 2013 involving all WHO polio laboratories of the WHO European Region is recommended at this stage of the GPEI.