



Virology Division

Specialized Molecular Diagnostic Unit

BACKGROUND

The Specialized Molecular Diagnostic Unit (SMDU) offers a wide variety of molecular assays for viral diagnosis and disease monitoring, such as PCR, qualitative PCR, quantitative PCR, real-time PCR, multiplex PCR and sequencing, in support of the NICD surveillance activities and operational research. These NAT techniques have high sensitivity, allowing for early and accurate diagnosis of viral diseases. The laboratory's workload has increased with the growing demand for tests to detect diseases early and for more targeted therapies. Viral loads and subtyping are now part of the biological monitoring of patients chronically infected with human immunodeficiency virus (HIV), Hepatitis B virus (HBV) and Hepatitis C virus (HCV). New applications, multiplex detection and test automation to improve practicability, increase throughput, reduce turnaround times and lower costs are continually evaluated. The key areas that the SMDU has supported include the HVTN 503 trial where nucleic acid testing (NAT) plays a key role in confirming so-called "infected status" or in cases of recent exposure where an algorithm includes NAT testing. The role of acutely infected HIV and transmission has generated a great deal of interest and the Unit has applied a commercial assay as a tool in collaboration with the STIRC Unit that detects for early B-cell responses. An exciting development is the introduction of therapy for HCV-infected individuals and the usefulness of the HCV genotype and responses to therapy are being monitored. An important role from a public health perspective is the early identification of HIV infection in infants. To this end the NICD as part of its regional support of Swaziland and Lesotho performs routine DNA PCR testing. Standardization, quality control, adequate staff training, proficiency testing and error detection remain important key elements in testing result integrity. The three areas of activity include (1) HIV-1 and other viruses (EV, HSV, JC), (2) Hepatitis B and C viruses, and (3) Polioviruses.

Since 1995, the NICD has been a WHO Regional Reference laboratory for the Polio Eradication Initiative. The molecular polio section serves as a reference laboratory outside the southern African region for many of the countries that fall under the WHO African Regional Office. Major activities within the section using molecular sequence-based analysis have answered several epidemiological questions regarding the likely location of endemic poliovirus reservoirs and patterns of virus transmission. They also determine if a virus is

similar to endemic strains or has been introduced (imported), i.e. closely related to viruses circulating in another country or region. The unit contributes to training scientists and technologists from other African countries through WHO-based workshops. Unit members are involved in the following Polio Network activities:

- Global Polio LabNet
- Regional Polio LabNet
- National Polio Expert Committee (NPEC)
- National Task Force for Polio (NTF)
- Global LabNet Working Group
- Data Management for Polio LabNet

Specialized Molecular Section

ACTIVITIES, HIGHLIGHTS AND ACHIEVEMENTS

● **HIV-1 DNA PCR diagnosis in infants:** HIV-1 DNA PCR assay using DBS offers a sensitive and specific test appropriate for the diagnosis of HIV-1 in infants. There are many advantages to using HIV-1 DNA PCR with dried blood specimens over HIV-1 DNA PCR with whole-blood specimens. Whole blood can be blotted on filter paper from heel stick or finger punctures in infants. Therefore, the sample centrifugation and extraction procedures are reduced. Dried blood on filter paper is biologically stable and can be stored at room temperature. It can be transported easily and is therefore convenient to use in resource-limited settings. SMDU is collaborating with the Clinton Foundation and the Center for Disease Control (CDC) by assisting with scaling-up and establishing infant diagnosis of HIV-1 in Lesotho, Swaziland, Liberia and the Caribbean. Over 6,000 specimens were processed during 2007. The LightCycler 480 real-time PCR platform was validated for confirming all positive test results. The collaboration also includes support, training and transfer of technologies and providing sub-Saharan HIV-1 material to be used in EQA programs.

● **STI testing on the ANRS 1265 male circumcision trial:** Further STI testing for the ANRS 1265 male circumcision trial includes:

- *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) testing: 2,500 urine specimens processed for the detection of CT/NG, using the COBAS/Amplicor system.
- *Trichomonas vaginalis* PCR: 2,500 urine specimens processed using real-time, high-throughput LightCycler 480 platform.
- Human papillomavirus screening and genotyping: 1,900 swabs processed to screen, detect and genotype low-, intermediate- and high-risk strains of human papillomavirus (HPV).

● **FACSCount and Panleucogating:** SMDU introduced a second technology for CD4+ assessment in addition to the FACSCount (BD Biosciences). Panleucogating (PLG) is an efficient and cost-effective technique that has been evaluated for obtaining CD4+ T cell counts. It utilizes the Beckman Coulter EPICS XL flow cytometer. Validation and comparison of the two methods are in process.

● **HIV-1 viral load testing:** During 2007, the unit obtained the COBAS Ampliprep/COBAS Taqman instrument for real-time viral load tests for HIV (and HBV and HCV). Offering fully automated sample preparation and analysis, this platform increased laboratory productivity and test result integrity. The NASBA/EasyQ and the COBAS Ampliprep/ COBAS Amplicor semi-automated systems are currently utilized for different projects. The unit performed HIV-1 RNA testing for the HVTN 503 trial as confirmation of infection in conjunction with serology testing. The COBAS AmpliScreen HIV-1 qualitative test is used for early diagnosis of HIV-1 infection for the PlasmAcute/WAHR study during the acute infection stage and to pinpoint seroconversion. Samples were also analyzed for the Herpes Episodic Acyclovir Therapy study to identify acute HIV-1 infection in men who were HIV-1 serology negative.

● **Herpes, Enterovirus and JC PCR:** Only Herpes 1 and 2 and Enterovirus PCR specimens are currently analyzed for aseptic meningitis diagnosis, and a real-time multiplex PCR for all aetiological agents responsible for aseptic meningitis is under development, to be able to report results within 24 hours to improve treatment schedules and decrease hospitalization of patients. There was a definite increase in the number of specimens requested for JC virus PCR during the 2007 period. The unit is currently developing a real-time method for the detection of the JC virus to improve turnaround times.

● **EQA:** During 2007, the laboratory successfully completed proficiency panels referred by NEQAS, REQAS, QCMD (Quality Control for Molecular Diagnostics), the Centers for Disease Control (DBS HIV-1 DNA PCR) and the Virology Quality Assessment Program for all the test methods offered. In collaboration with QCMD, NICD is introducing an Internal Process Control program for HIV-1 RNA tests in South African laboratories. The purpose of the program is to have continuous monitoring of

laboratories that currently provide a service for the ARV program.

COLLABORATIONS

Centers for Disease Control and Prevention (CDC): HIV-1 diagnosis in infants

Clinton Foundation: HIV-1 diagnosis in infants

QCMD/CDC: Sub-Saharan HIV-1 material to be used in EQA programs and for use as internal run controls for HIV-1 RNA quantification

HVTN 503 HIV-1 Vaccine Trial: HIV-1 RNA viral load testing on participants

PHRU: CD4 testing and viral load testing for the TB mortality trial

STIRC: CD4 testing and viral load testing for the VCT1 and VCT2 studies; qualitative HIV-1 RNA PCR screening for acute HIV-1 infections (PlasmAcute study)

CAPACITY BUILDING

Training: SMDU was involved in molecular technology transfer training sessions. These include Roche PCR Academy training sessions, as well as HIV DNA DBS training and FACSCount (BD Biosciences) training sessions for CLS and IAVI staff. Registrars and medical technology students were trained on a regular basis, and introduced to the full range of molecular tests performed in the unit. In the case of the Roche Academy a total of 31 persons from various African countries were trained in a wide array of technologies including automated extraction, viral load assays and real time PCR.

HBV and HCV Section

ACTIVITIES, HIGHLIGHTS AND ACHIEVEMENTS

Although past studies in this unit have included research studies of both Hepatitis E and G, current studies in the laboratory are confined to the Hepatitis B (HBV) and C (HCV) viruses.

● **The prevalence of Hepatitis C virus genotypes in two patient groups over an eight-year period:** Previous studies have shown that Hepatitis C virus (HCV) prevalence in South Africa is low (2.6%), and genotype 5 is the dominant genotype. This laboratory-based study, the first sequence-based genotype study from this country, was a retrospective study using serum specimens stored from two time periods: a three-year period, 2000-2003, and, more recently, during the year 2007. The study determined the prevalence of genotypes in two different patient groups (haemophiliacs and patients with liver disease (LD))

during these time periods. The predominant genotype was the same: genotype 5 and genotype 1 for the haemophiliac and LD groups, respectively, over the time periods studied. Genotype 2 and a higher prevalence of genotype 3 were observed in the haemophiliac group from 2007 when compared to the earlier specimens. There was an overall decrease in the prevalence of genotype 5 over time. A cluster of genotype 5 sequences had only one adenine at nucleotide position 107 of the 5'UTR. Subtyping in the less conserved NS5B region further characterized the 2000-2002 isolates. Sequencing in the 5'UTR was adequate for determining genotypes, but sequence data from the NS5B region was more informative, as it allowed genotypes and subtypes to be differentiated. The study shows the changing prevalence and frequencies of HCV genotypes in HCV patients from Johannesburg, South Africa, over an eight-year period. The changes and trends observed could be explained by the greater sensitivity and specificity of new technologies or the effects of travel, immigration and/or emigration.

● **Pilot study to determine the usefulness of using a position-specific scoring matrix (PSSM) to characterize the subtypes of genotype A of Hepatitis B virus (HBV):** The evolutionary origins of HBV are obscure. With the discovery of widespread recombination in HBV, standard phylogenetic methods cannot be used in evolutionary studies. Pattern matching, using a position-specific scoring matrix, is an exquisitely sensitive method that ranks a query sequence against two related, but evolutionary divergent, subsets of sequences. Because of the variety of subtype A sequences now being found in Africa, it is postulated that genotype A originated in Africa. If this postulate is true, African subtype A1 must be ancestral to European subtype A2, and scoring representative sequences from African countries against a matrix formed from a subset of specimens from these two groups should give an indication of the origins and divergence of HBV in Africa. Figure 1 shows a plot of PSSM scores (using a moving window of size 10 amino acids) for a subtype A3 specimen from Cameroon in West Africa, AB194950. Of the 276 amino acids examined, there were 34 informative sites. Informative sites are primarily in the preS2 region (12) or the fourth membrane-spanning helix (8) of the surface antigen. The overall score was -8.653994233, indicating, as is to be expected, that the Cameroon subtype is of African origin. However, used with a selection of query sequences from all over southern Africa, the ranking of the respective scores will enable the origins and evolutionary patterns of these specimens to be plotted relative to one another. In addition, from an evolutionary perspective, the fact that only a few sites are required to discriminate the origins of the query virus so well suggests that the amino acid changes may have important functions that differentiate the viruses, and may not just be geographic accidents. These preliminary results are encouraging and indicate that PSSM is an imminently

useful tool for analysis of mutations in a recombining virus, with the additional constraints of overlapping reading frames, like HBV. Other questions that could be answered include an analysis of preCore and Core mutations from different disease cohorts, which would enable the establishment of cut-off values for the different stages of disease or, alternatively, be used to measure and monitor the emergence of antiviral resistance and/or vaccine mutation phenotypes.

- **Diagnostic service:** Standard operating procedures (SOP) for HCV viral load (using the COBAS Amplicor) and genotyping by sequencing the 5' UTR have been established and have now been upgraded from research protocols to routine diagnostic procedures within the Hepatitis section of the SMDU. An NHLS grant was obtained to evaluate and compare the Line Probe Assay (LiPA) with the gold standard of sequencing, and this is currently being implemented for high-throughput HCV genotyping. The COBAS AmpliPrep/COBAS Taqman HCV and HBV viral load assays were evaluated and are now used routinely to determine the progress of patients receiving therapy. Other routine diagnostic Hepatitis assays available include an in-house qualitative HBV PCR.
- **Quality control for molecular diagnostics (QCMD):** There were two panels (each of eight plasma specimens) for the QCMD HCV RNA proficiency programme for 2007. These were tested by a qualitative PCR method (Roche Amplicor Manual), and we scored 100% on both panels. There was one panel of eight plasma specimens for the QCMD HCV Genotyping proficiency programme for 2007. We used two methods for genotyping: LiPA (Bayer-Siemens) and sequencing (in-house), and scored 100% for the panel. Mixed infections in the panel were easily detected by LiPA and sequencing confirmed the LiPA results. There were also two panels of eight plasma specimens for the QCMD HBV DNA proficiency programme for 2007. These were tested using the in-house qualitative PCR, and we consistently scored 100%, thus confirming the ability of our diagnostic PCR to reliably detect at least 100 copies of HBV per ml.

COLLABORATIONS

HCV prevalence study specimens and patient demographics in collaboration with Dr Peter Barrow, Liver Clinic, Johannesburg General (LD specimens), and Dr Johnny Mahlangu, Head of both the Haemophiliac Clinic, Johannesburg General, and Medical and Scientific Advisory Council of the National Haemophilia Foundation (MASAC; haemophiliac specimens)

CAPACITY BUILDING

- Ongoing training and supervision of student Nishi Prabial-Sing who was registered for her second year as a PhD student at the University of the Witwatersrand Health Sciences in 2007.

- Registrars were trained on hepatitis diagnostics and phylogenetic methods used in the laboratory.
- Trained Polio staff in basics of Perl programming and principles of Position Specific Scoring Matrices for use in characterizing and classifying Vaccine Derived Polio viruses.
- Attended the first annual Bioinformatics Conference (28-30th January 2007) and gave a report back at the Bioinformatics Group meeting on the 26.03.08.

Registered for Degrees: Nishi Prabdial-Sing, PhD

Polio Section

WHO Regional Reference Laboratory for Polio

ACTIVITIES, HIGHLIGHTS AND ACHIEVEMENTS

ACTIVITIES

During 2007, the unit received 1,245 poliovirus isolates (Figure 1), which were characterized as vaccine or wild type using two intratypic differentiation methods, viz., PCR and ELISA. These isolates were sent to the NICD from national and regional laboratories throughout Africa, namely Angola, Benin, Burkina Faso, Burundi, Cameroon, Chad, Cote d'Ivoire, Congo, Eritrea, Ethiopia, Kenya, Madagascar, Malawi, Mozambique, Namibia, Nigeria, Niger, Democratic Republic of Congo (DRC), Rwanda, South Africa, Somalia, Sudan, Uganda, Zambia and Zimbabwe (Figure 2). Original specimens from AFP cases were received from several southern African countries and any polio isolates were

treated as described above. The total number of wild poliovirus cases with onset of paralysis in 2007 was 366. Nigeria remains the country with the highest number of endemic cases in Africa, with a total of 286 wild-type polioviruses (not all data shown) (Figure 3). PV1 wild-type isolates are distributed into three genotypes: India (SOAS), West African B (WEAF-B) and East African (EAAF) (Figure 4). The WEAF-B genotype consists of viruses from Nigeria, Niger, Chad, Benin and Cameroon. The EAAF genotype consists of the viruses from Ethiopia and Somalia, while SOAS viruses are from Angola and DRC. Of the identified PV1 wild types, 44 were from DRC. Other wild-type polioviruses identified in 2007 were from Angola, Benin, Cameroon, Chad, Ethiopia, Niger, Nigeria and Somalia. In 2007, Chad was re-infected by two polio wild-type viruses. One of the cases of wild-type 1 was near the border with Cameroon. The second case was close to the border with Darfur, Sudan, in the same area from which a polio importation into Darfur occurred in 2004. The sequence of the index case showed a 98.68% identity to the case from Nigeria. WEAF-B wild PV3 is divided into clusters and sub-clusters, and cluster D still remains the most active cluster (Figure 5). The wild-type 3 case identified in Chad was from the N'Djamena region and the first wild-type 3 since November 2006. The Chad virus has moved into the south of the country in the area that was not covered by Supplemental Immunization Activities (SIAs), putting neighboring countries, like the Central African Republic (CAR), at risk. The National Immunization Days (NIDs), covering 95% of the target population of the country, were held on 23-26 February 2008, using monovalent oral polio vaccine type 1 (mOPV1). No Vaccine-Derived Polioviruses (VDPVs) were identified in 2007.

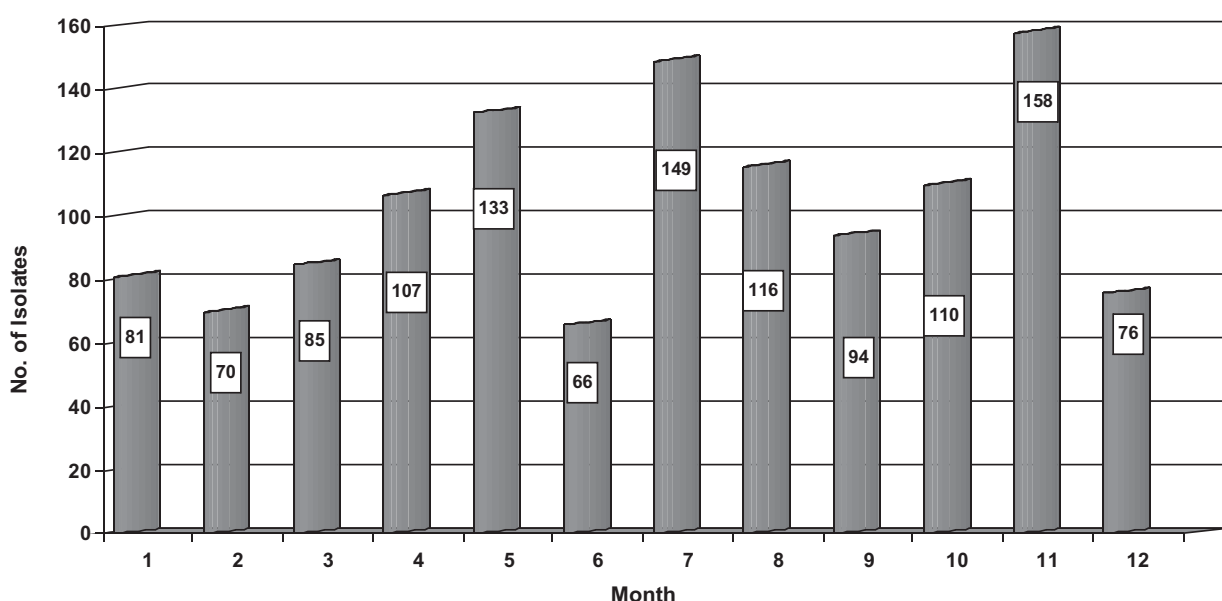


Figure 1: Number of isolates received per month

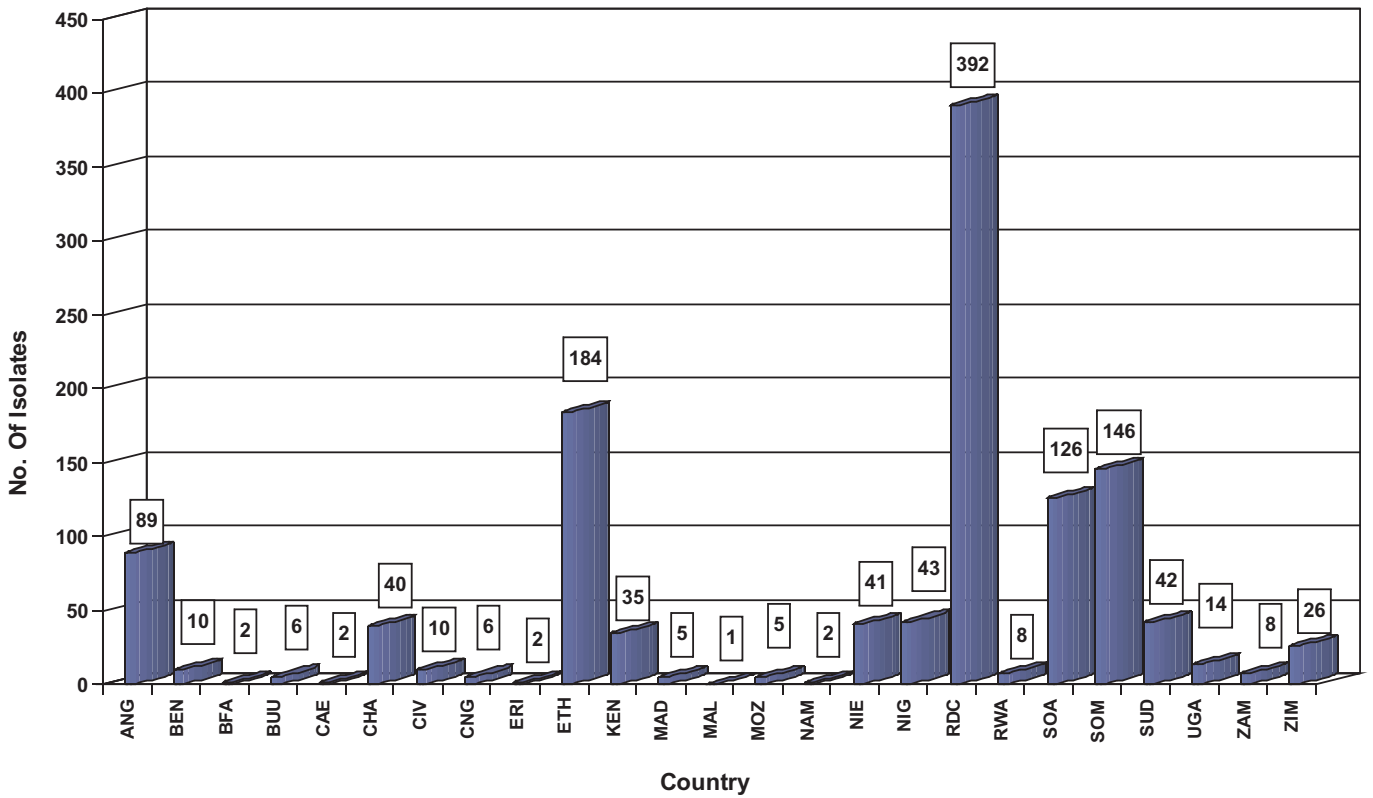


Figure 2: Poliovirus samples received from African countries

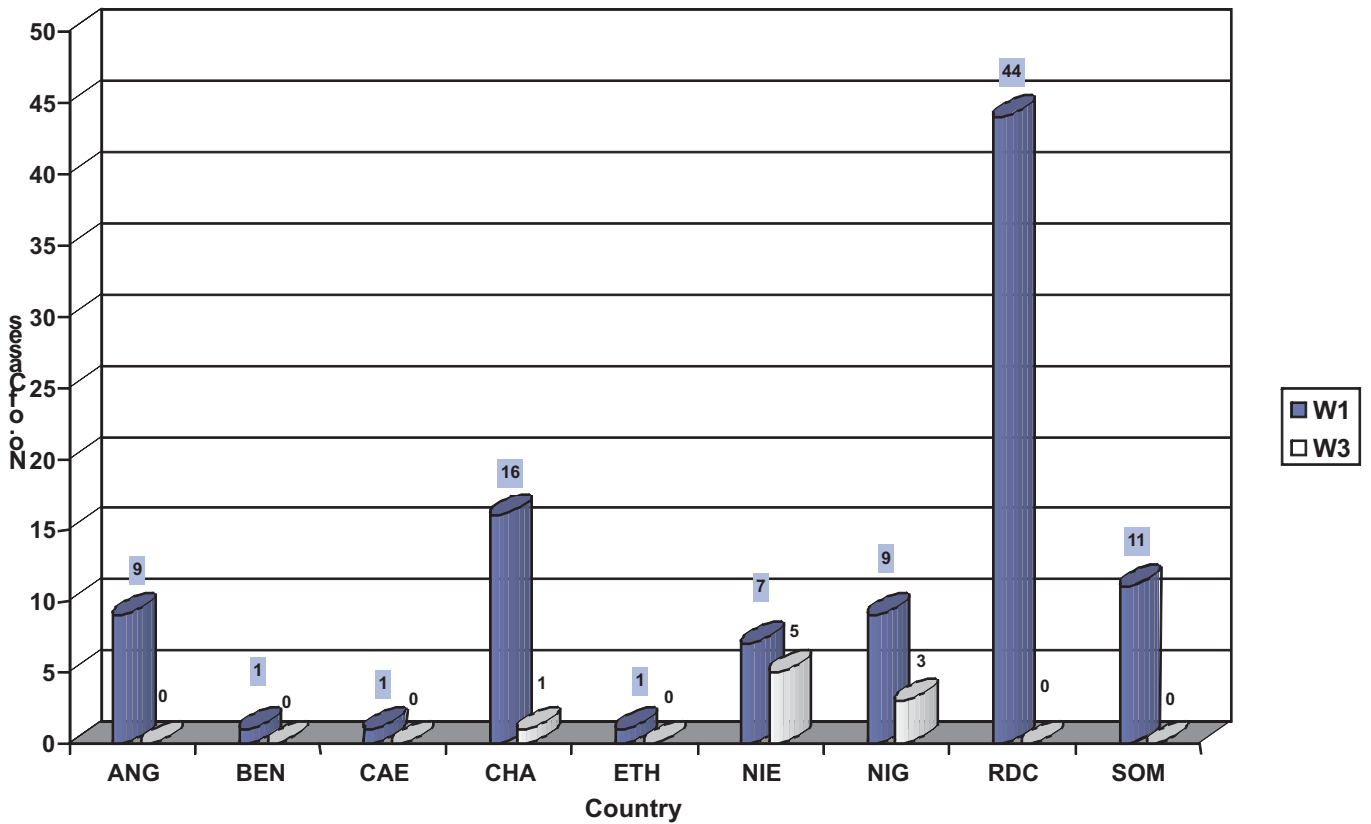


Figure 3: Wild-type isolates of type 1 and type 3 identified in 2007

HIGHLIGHTS

● **WHO Database Training for the NICD Polio Laboratories:** WHO Regional Officers conducted WHO database training for the NICD Polio Laboratories at the NICD IT training centre from 26 March 2007 to 30 March 2007. The training was based on various modules.

● **CDC Consultation Training for the NICD Polio Laboratories (Human Capacity):** In an effort to increase the skills base of staff employed in the NICD Molecular Polio Unit, as well as optimize laboratory workflow, a consultation with personnel from the Polio Unit at the Center for Disease Control (Atlanta, USA) was held from 2 December 2007 to 15 December 2007. The consultation focused on the following aspects, which are relevant to the unit:

- Real-time PCR: The current real-time PCR methodology contains a considerable advantage over conventional PCR diagnostics, in that it allows for the identification of Vaccine-Derived Polioviruses, as well as viruses that have undergone recombination in either the VP1 or 3D regions of the viral genome without the requirement for the generation of sequence data.
- Database upgrades: The database was upgraded with additional features, which included the

creation of a non-AFP database, the creation of an auto labeling and coloring utility for phylogenetic trees for WHO reports, and a streamlined interfaced and auto-backup utility for the preparation of weekly database reports.

- Implementation of new sequencing protocols.
- Phylogenetic tree construction: upgraded functionality.
- Geographic mapping.

COLLABORATIONS

- Center for Disease Control and Prevention, Georgia, USA: Polio Eradication Initiative (PEI)
- WHO/HQ, Geneva, Switzerland: Polio Eradication Initiative (PEI)
- WHO/AFRO, Harare, Zimbabwe: Polio Eradication Initiative (PEI)
- African Polio LabNet: Polio Eradication Initiative (PEI)

CAPACITY BUILDING

Registered for Degrees

- Nicksy Gumede-Moeletsi, PhD