Serious Events Supposedly Attributable to Vaccination or Immunization Associated with Receipt of Yellow Fever Vaccine During a Mass Immunization Campaign Peru, 2007

Findings and Recommendations of an Expert Panel

Washington, D.C., March 2008

Table of Contents

Expert Panel Members

Thomas P. Monath, MD (Chairman)

Kleiner Perkins Caufield & Byers Pandemic & Biodefense Fund, USA

Edward B. Hayes, MD

Surveillance and Epidemiology Activity of Arboviral Diseases, Centers for Disease Control and Prevention, USA

Martin Cetron, MD

Global Migration and Quarantine, Centers for Disease Control and Prevention, USA

Alan D. T. Barrett, MD

Center for Biodefense and Emerging Infectious Diseases, University of Texas Medical Branch, Galveston, Texas, USA

Expedito Luna, MD

Instituto Butantan, Brazil

The investigation of serious ESAVIs that occurred in October 2007 following administration of yellow fever vaccine (17DD sub-strain) manufactured by Bio-Manguinhos, Brazil, was led by staff from PAHO's Immunization Unit (Family and Community Health Area) with cooperation from PAHO's Essential Medicines, Vaccines, and Health Technologies Unit (Technology and Health Services Delivery Area) and staff from WHO's Quality, Safety and Standards Unit (Family and Community Health Cluster).

Acronyms

ARDS	Acute Respiratory Distress Syndrome
CDC	Centers for Disease Control and Prevention (USA)
DNA	Deoxyribonucleic acid
ESAVI	Event Supposedly Attributable to Vaccination or Immunization
GACVS	Global Advisory Committee on Vaccine Safety (WHO)
GI	Gastrointestinal
INS/NIH	Instituto National de Salud/National Institute of Health (Peru)
NMRCD	Naval Medical Research Center Detachment (USA)
PAHO	Pan American Health Organization
SAGE	Strategic Advisory Group of Experts (WHO)
SLE	Systemic lupus erythematosus (Lupus)
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid Interference
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
TAG	Technical Advisory Group on Vaccine-preventable Diseases (PAHO)
WHO	World Health Organization
YEL-AND	Yellow Fever Vaccine-associated Neurotropic Disease
YEL-AVD	Yellow Fever Vaccine-associated Viscerotropic Disease

Serious Events Supposedly Attributable to Vaccination or Immunization Associated with Receipt of Yellow Fever Vaccine during a Mass Immunization Campaign Peru, 2007

Introduction

Yellow fever remains a substantial public health problem in tropical regions of South America and Africa, and there is a risk of introduction and spread in non-endemic regions infested with the urban vector *Aedes aegypti.* Peru has contributed about 50% of the cases reported in the Americas. Over the past seven years, 331 cases have been reported in Peru with a case fatality rate of 50%. In 2007-2008, a resurgence of yellow fever virus activity has occurred in Brazil, with a southward extension of an epizootic wave, with associated human cases, into Paraguay and Argentina. The first occurrence in many years of what appears to be urban (*Ae. aegypti*-borne) yellow fever has occurred in the suburbs of Asunción, Paraguay. There is no specific treatment for yellow fever. Vaccination using the live, attenuated 17D vaccine remains the most effective measure for prevention and control of yellow fever. With the strong support of the Pan American Health Organization (PAHO) and the World Health Organization (WHO), most countries in the endemic region now incorporate 17D vaccines into their Expanded Program on Immunization (EPI). In addition, mass campaigns have been conducted in the following settings: i) for catch-up immunization; ii) when virus activity increases, or in response to outbreaks in the human population; and iii) in special settings such as areas bordering the endemic region fromwhere the population frequently migrates into the endemic area or where there is deemed to be a risk of introduction and spread of the virus. The 17D vaccine has been administered to hundreds of millions of people over 70 years, with a long history of confidence in its safety and effectiveness. Nevertheless, new precautions concerning yellow fever 17D vaccines have recently been recognized because of the occurrence of rare but serious Events Supposedly Attributable to Vaccination or Immunization (ESAVIs).

Yellow fever vaccine-associated viscerotropic disease (YEL-AVD) represents a rare but life-threatening complication first described in the medical literature in 2001. Up to September 2007, a total of 36 YEL-AVD cases had been reported worldwide following administration of yellow fever vaccines (17D and 17DD substrains) from 5 manufacturers. The mean age was 49 years (range 4-79 years), the male:female ratio was 2:1, and the case-fatality rate was 60%. Reported risk factors include age >60 years and thymic disease/thymectomy.

In September-October, 2007 a mass yellow fever immunization campaign was conducted in Ica Department, Peru, following a major earthquake, with approximately 63,000 doses delivered. Five persons, aged 23 to 79 years, who received yellow fever vaccine (17DD substrain) developed suspected YEL-AVD. This was the first time that multiple YEL-AVD cases had clustered within a short timeframe (patients were immunized between 23 September and 1 October 2007) and delimited space (Ica Department, Peru). Four persons died (case-fatality rate 80%). All four fatal cases occurred within a population of ~43,000 who had received a single lot of 17DD vaccine (designated 050VFA-121Z, referred to below as 121Z) manufactured by a WHO-prequalified producer, Bio-Manguinhos from Brazil. A second lot of Bio-Manguinhos vaccine (123Z) had been used in ~20,000 persons in the mass campaign in Ica Department, without any associated cases of YEL-AVD. The vaccine lot used in a 5th (non-fatal, but hospitalized) case is unknown. No ESAVIs were reported when lot 121Z was administered in Venezuela prior to its use in Peru. However, this finding should be interpreted with caution due to limited sensitivity of passive surveillance systems for ESAVIs.

A statement was issued by PAHO/WHO on 2 November 2007[1] to alert the global public health community to the occurrence and investigation of these events. It provided preliminary information on the four fatal cases and outlined a series of steps in the planned investigation for determining the etiological role of yellow fever vaccine in the ESAVIs, for reviewing the manufacturing and control of the implicated vaccine lot, for enhanced surveillance to detect ESAVIs, and for additional case-finding studies to be undertaken in Peru. Use of the 121Z lot and related lots was suspended pending investigation of a possible causal role in the ESAVIs. An Expert Panel was convened on 1 November to review preliminary data and lead the investigation. The Panel met again on 4-5 March 2008, together with representatives of the Ministry of Health of Peru, the vaccine manufacturer (Bio-Manguinhos), and PAHO/WHO staff. This report summarizes the results and conclusions of the laboratory and field investigations that were conducted between November 2007 and February 2008.

1 Fatal Adverse Events Following Receipt of Yellow Fever Vaccine Produced by Bio-Manguinhos, Brazil. Available at http://www.paho.org/English/AD/FCH/IM/PAHO_WHOStatement_YellowFever_Nov07.pdf.

Clinical and Pathological Features of the Cases

The affected patients presented with a similar clinical syndrome, summarized in Table 1. Cases 1-4 had received yellow fever vaccine for the first time; the prior history of vaccination in case 5 is unknown.

Table 1. Clinical and Pathological Features of YEL-AVD Cases, Peru, 2007

Case	Age/ Sex	Pre-existing conditions	Days between vaccination and		Symptoms/Signs	Laboratory abnormalities (furthest removed from normal range)	Outcome and Salient Autopsy Findings	Meets Case Definition
			Onset	Death				
1	23/F	Rosacea	1	9	Fever, headache, arthralgia, myalgia, malaise, nausea, vomiting, diarrhea 8 days after vaccination developed shock, ARDS, acidosis, encephalopathy, multi-organ failure	WBC: 66,400 mm^3 Platelets: 54,000/mm^3 AST: 850 IU/mL ALT: 222 U/L Creatinine: 4.1mg/dL; CPK 4055	Died Midzonal necrosis, steatosis (liver); acute tubular necrosis; Thyroid neoplasia, chronic thyroiditis	Definite YEL-AVD
2*	24/F		<1	14	Fever, headache, malaise, myalgia, nausea, vomiting, diarrhea 11 days after vaccination developed shock, encephalopathy, acidosis, GI bleeding, jaundice, ARDS, multi-organ failure	Hct 15.5% WBC: 11,470 mm^3 Platelets: 15,000/mm^3 AST: 850 IU/mL ALT: 222 U/L Bili: 6.2 mg/dL BUN: 112 mg/dL CPK: 3173	Died Steatosis, focal necrosis (liver); cerebral edema; pulmonary edema; severe Candida infection (larynx, trachea, stomach)	Definite YEL-AVD
3	79/M	Cardiac disease	3	11	Fever, malaise, dyspnea, abdominal pain, vomiting, diarrhea 9 days after vaccination developed progressive shock, ARDS, acidosis, renal failure.	WBC: 17,400 mm^3 Platelets: 249,000/mm^3 AST: 416 IU/mL ALT: 231 U/L Bili: 2.9 mg/dL Creatinine: 2.8 mg/dL	Died Midzonal necrosis, steatosis (liver); acute tubular necrosis; depletion white pulp (spleen); congestion	Definite YEL-AVD
4*§	49/F	Systemic lupus and rheumatoid arthritis	Unclear (7-18 days)	30	Headache, malaise, arthralgia. 29 days after vaccination hospitalized with generalized edema, jaundice, altered mental status, jaundice, bleeding, acidosis, cardio-respiratory distress	Hct: 31% WBC: 5,530 mm^3 Platelets: 57,000/mm^3 AST: 123 mMol/L Bili: 5.2 mg/dL Creatinine: 3,3 mg/dL	Died Midzonal necrosis; steatosis (liver); acute tubular necrosis and glomerulonephritis; pulmonary edema	Definite YEL-AVD
5	43/M		4	--	Fever, headache, malaise, diarrhea. Admitted to ICU, possible encephalopathy, scleral icterus. Defervesced and was discharged 16 days after vaccination	AST: 158 (normal 2-35), ALT: 244 (normal 9-43) Bili: 0.5 mg/dL	Survived	Probable YEL-AVD

F: Female / M: Male * Patients 2 and 4 received injections of a potentially immunosuppressive drug (dexamethasone) during the early phase of illness.
§ Patient 4 also may have taken the immunosuppressive drugs methotrexate and prednisone orally after the vaccination was administered.
Note: All patients received antibiotics.

The Panel reached the following conclusions after review of the cases:

- The syndrome in cases 1-3 was consistent with prior reports of YEL-AVD.
- The features in case 4 were atypical by being protracted, and the syndrome was likely modified by the underlying autoimmune disease (SLE) or by immunosuppressive drugs administered to the patient.
- Diarrhea was a prominent feature in this series, and was severe in several cases. Diarrhea has also been noted in previous case reports and appears to be a part of the YEL-AVD syndrome. The pathogenesis is unknown and the GI tract should be subject to investigations for yellow fever viral infection and damage.
- None of the cases had a clear contraindication to vaccination. However, case 3 had a precaution (advanced age, a known pre-disposing risk factor for YEL-AVD). In general, persons of this age should not receive the vaccine unless there is a clear risk of exposure to wild-type virus.

- Treatment with immunosuppressive drugs might have enhanced infection with the vaccine virus in cases 2 and 4. Precautions against use of such drugs in the 10 days following yellow fever vaccination (i.e., until immunity appears) may be warranted; such precautions would need to be reviewed and accepted by the relevant regulatory authority.
- The Panel noted that autoimmune disease might constitute a new risk factor for ESAVIs following yellow fever vaccination. One of the 5 cases in Peru had rheumatoid arthritis and systemic lupus and case 1 had thyroiditis noted on autopsy. At least two other previous cases of YEL-AVD (in the US and Brazil) also had lupus.
- The *Candida* infection in case 2 was likely secondary to antibiotic treatment and severe systemic illness rather than reflecting pre-existing primary immune suppression.
- With the possible exception of case 3, in whom there was serologic conversion to typhoid O and typhoid H antigens, there was no evidence for a concurrent infection with an unrelated agent that could explain the severity or outcome of illness in the patients with YEL-AVD. Moreover, there was no evidence for a common environmental or toxic exposure, and no concomitant medications were shared by all patients. However, it should be recognized that there has been no systematic investigation of the potential role of another infectious agent in these cases of YEL-AVD.
- The Panel noted that the management and treatment of patients with YEL-AVD was difficult and that little guidance was available. The last meeting on the management of patients with yellow fever (wild-type) was held by PAHO more than 20 years ago (in 1985).

Epidemiological Investigations and Search for Additional Cases

A surveillance system for ESAVIs is established in Peru. Additional retrospective case-finding investigations were conducted in November 2007 by personnel from the Ministry of Health and allied institutions in Peru and the Centers for Disease Control & Prevention (CDC), Fort Collins (USA). The data from the existing surveillance system and field investigations captured a total of 11 cases of suspected serious ESAVIs:

- Six cases were reported from Ica. One of these was subsequently determined to not meet working case definitions for either YEL-AVD or YEL-AND (yellow fever vaccine-associated neurotropic disease). Retrospective case-finding found no additional suspect cases of YEL-AVD or other serious ESAVIs.
- Five cases of serious ESAVIs after yellow fever vaccination were identified by the existing surveillance system from other regions (none had received lot 121Z), including 1 death (a 20-month old infant). This fatal case was investigated virologically and there was no detectable yellow fever virus by infectivity, RT-PCR (reverse transcriptase-polymerase chain reaction), or immunohistochemistry. The cause of death was hemolytic anemia. Other non-fatal cases were investigated but none were suspected to be YEL-AVD cases.

The Panel took note of the fact that the existing surveillance system had been sensitive, had identified all the fatal cases and one non-fatal case of YEL-AVD in Ica Region, and had led to the prompt cessation of the vaccination campaign in Ica Department. Despite an intensive review of over 28,000 hospital records in Ica Department, no additional cases were found.

The Peruvian authorities lacked an established protocol for the investigation of cases of yellow fever vaccine-associated ESAVIs, including the collection, handling and testing of serological and virological specimens. It was noted that such a protocol has been developed in Brazil and would be useful for the facilitated systematic investigation of future cases throughout regions where yellow fever vaccinations are performed.

Incidence of YEL-AVD in Ica Department

The incidence of YEL-AVD in Ica Department in this event is significantly higher than observed previously in other settings. Five YEL-AVD cases occurred among 63,174 people who received yellow fever vaccine in September-October 2007 in Ica province (for a rate of 7.9 per 100,000). If the total of 42,742 persons receiving lot 121Z is used as the denominator, the incidence is 9.4 per 100,000 if only 4 cases received that lot and 11.7 per 100,000 if all 5 received that lot. In travelers who received yellow fever vaccine in the US and Europe, the overall incidence of YEL-AVD has approximated 0.3 to 0.4 cases per 100,000. If this rate is accepted as the true rate, the probability of seeing four cases after 42,742 doses is extremely small (0.00001).

The association of all 4 fatal cases with one vaccine lot (121Z) while no such cases were detected in another lot of vaccine used at the same time in Ica was also unexpected, as no instance of more than one case of YEL-AVD caused by a single vaccine lot has been reported heretofore. About 42,742 people were given the implicated vaccine lot (121Z) in Ica. An additional 20,432 people were vaccinated in Ica with a different lot (050-VFA123Z), and no deaths were reported in this group. The incidence of fatal YEL-AVD associated with the 121Z lot is not statistically significant from that in the other lot at the $p<0.05$ level, and thus it is possible that the association with one lot happened by chance. (Even if the non-fatal case is assumed to have arisen from lot 121Z, the difference in incidence of YEL-AVD between the two lots is not significant at the $p<0.05$ level.)

A number of hypotheses were considered to explain the higher rate of YEL-AVD in Ica, including altered pathogenicity of the vaccine virus, altered susceptibility of the individuals experiencing the ESAVIs, and altered susceptibility of the population as a whole. The possibility that the 121Z lot contained a genetic change responsible for enhanced virulence was the focus of an extensive investigation, summarized below. Possible host factors (advanced age, autoimmune disease) may have contributed to susceptibility in two of the four cases.

The Panel noted that population-based factors were probably very important in this event. The cases occurred in the setting of a mass immunization campaign in a non-endemic area, where yellow fever vaccinations had not previously been used, where the population had no background immunity to yellow fever, where large numbers of adults, including elderly people were vaccinated, and where ESAVI surveillance was heightened. The Panel believed that the risk of YEL-AVD in such a setting is substantially higher than in other situations where mass campaigns are undertaken in endemic areas where a high background of immunity to yellow fever exists. To better define the risk of YEL-AVD in South America and to determine if the Ica episode is aberrant or not, a high priority should be placed on obtaining data on YEL-AVD incidence in other similar circumstances where mass campaigns have been performed outside the enzootic area in the past, such as the eastern part of São Paulo State, Brazil and in Paraguay and Argentina where mass campaigns are now underway.

Virological Analyses and Potential Causal Relationship Between ESAVIs and the 121Z Lot

The laboratory diagnosis of the cases was undertaken by the National Institute of Health (*Instituto Nacional de Salud*) of Peru, the United States Naval Medical Research Center Detachment (NMRCD), and by CDC Atlanta and Fort Collins. Examination revealed a wide tissue distribution of virus (including many vital organs), high viremia, high virus load, and high antibody titers consistent with previous reports of cases who had died following yellow fever immunization.The genomic sequences of viral RNA from 3 cases were also determined. The results are summarized in Table 2.

Table 2. Laboratory Diagnosis, Confirmed and Probable Yellow Fever Viscerotropic Disease, Ica, Peru, 2007

Case	Day of Death (after day of vaccination)	Virus Isolation	Viral RNA in Tissue (PCR)	Viral Antigen in Tissue	Antibody	Genome Sequence
1	9	Positive: S, Li, B (Negative: U, Lu, K, Sp)	Positive: S, U, Lu, Li, K, B (Negative: None)	Positive: Lu, Li, K, B (Negative: None)	IgM+ PRNT 1:160	RNA from lung: full length consensus sequence indistinguishable from Bio-Manguinhos 17DD secondary seed (102/84)
2	14	Positive: None (Negative: B, K, Li, Lu, S, U, Sp)	Positive: S, U, Lu, Li, K, B (Negative: Sp)	Positive: Lu, Li, K (Negative: B)	IgM+ PRNT 1:10,240	RNA from liver: full length consensus sequence indistinguishable from Bio-Manguinhos 17DD secondary seed (102/84) except for non coding nucleotide change at nt 4921 in the NS3 gene
3	11	Positive: None (Negative: B, K, Li, Lu, S, Sp)	Positive: S, Lu, Li, K, B, Sp (Negative: None)	Positive: Li, Sp (Negative: Lu, K, B)	IgM+ (PRNT ND)	Partial sequence (nucleotides 1550-2870 and 6235 to 8435) indistinguishable from secondary seed 102/84
4	30	Positive: None (Negative: S, Li, K, B, Sp)	Positive: S, Li, K (Negative: B)	Positive: K (Negative: Lu, Li, B)	IgM+ PRNT >1: 20,480	No sequence data available
5	Non-fatal	ND	ND	ND	IgM+ (PRNT ND)	ND

S=serum; U=urine; Lu=lung; Li=liver; K=kidney; B=brain; Sp=spleen; ND=not done; PRNT: Plaque Reduction Neutralization Test

Note: Testing was performed at the four different laboratories, INS – Peru, NMRCD – Peru, CDC – Fort Collins, and CDC – Atlanta. If a sample tested positive in one of the labs, it was recorded as being positive even if testing results from other labs were negative.

Bio-Manguinhos reported that the full genomic consensus sequence of the 121Z lot was determined and showed no changes from the secondary seed 102/84. These data had yet to be reviewed by the Expert Panel. In addition, approximately 75 clones of each of three vaccine lots manufactured by Bio-Manguinhos, including the 121Z and 123Z lots used in Ica, were partially sequenced (E gene) at CDC, Fort Collins. The clonal analysis confirmed that the vaccines contained a 'genetic swarm' of multiple virion subpopulations differing at approximately 0.15% of their amino acids. No conclusions could be reached by this analysis regarding any changes relevant to the ESAVIs.

Most important was the finding that the 121Z lot virus consensus sequences obtained from vital organs from three of the confirmed YEL-AVD cases were indistinguishable from the parental 17DD secondary seed lot used since 1984. These findings provide strong evidence that the vaccine virus is genetically stable and that no mutations occurred during replication in the affected host, or selection of a variant subpopulation from the vaccine that was responsible for enhanced virulence. If mutation or selection had occurred, and was responsible for the tissue and organ damage seen in these cases with severe infections, it would be expected that the altered virus would represent at least 10% of the total virion population and thus be detectable in a consensus sequence.

Retention samples of Lot 121Z from the manufacturer and samples of vaccine used in Ica at the end of shelf life were tested with a validated infectivity assay by a WHO-contracted laboratory. No change in potency between original release and end-of-shelf-life samples were found, confirming results from the manufacturer, INS, and CDC that indicated that there were no altered (low) dose or changes in vaccine potency in the vaccine lot.

The Panel emphasized the importance of the need for a WHO repository of samples and vaccine viruses involved in YEL-AVD cases for future studies as new scientific advances and technological approaches to elucidating the pathogenesis of YEL-AVD become available.

Inspection of Bio-Manguinhos Manufacturing Facility

A site visit by a team composed of PAHO/WHO, CDC, and Expert Panel members reviewed batch production records and the manufacturing facilities. No deviations or other issues impacting product quality relevant to the ESAVIs were found. It was recommended that during the different steps of the manufacturing process, samples be retained on intermediates (i.e., flasks of viral suspension) of each production lot be kept until the expiration date to assist in future investigation, and that representative vials of any lot implicated in a viscerotropic ESAVI be maintained indefinitely. Currently, only final container samples are maintained until the expiration date is reached, at which time they are discarded. A one-off test of the 121Z lot for human adventitious agents was recommended to exclude the remote possibility of a contaminant in this lot. Although a validated animal model to test for reversion to viscerotropism does not exist, experimental in vivo studies should be considered. A supply of the 121Z lot and selected sister lots should be retained indefinitely to allow for such future studies.

The Panel was informed that the WHO Requirements for Yellow Fever Vaccine[2] (dated 1998) is scheduled for revision, and strongly encouraged discussion to the value of genetic testing on a lot-by-lot basis during this process.

Major Conclusions of the Expert Panel

The following conclusions were reached by the Panel:

- The occurrence of multiple YEL-AVD cases clustered in time and space, and associated with one lot of yellow fever vaccine (050VFA-121Z, Bio-Manguinhos, Brazil), was unprecedented.
- The incidence of YEL-AVD, which ranged from 7.9-11.7 cases per 100,000, is more than 20 times higher than previously reported. The incidence among persons who received the 121Z lot was not statistically higher (at the $p<.05$ level) than in persons who received a different lot (123Z) during the mass campaign.
- The investigation showed clinical, virological, and pathological evidence of confirmed YEL-AVD in 4 fatal cases and prob-

2 World Health Organization. WHO Expert Committee on Biological Standardization: forty-sixth report. Annex 2: Requirements for Yellow Fever Vaccine. WHO Technical Report Series No. 872, 1998. Document available at www.who.int/biologicals/publications/trs/areas/vaccines/yellow_fever/WHO_ TRS_872_ A2.pdf.

able YEL-AVD in 1 surviving case. The cause of death was an overwhelming infection with 17DD vaccine virus, probably associated with a severe immune response syndrome.

- Consensus genome sequencing of the 121Z vaccine lot, a parental secondary seed lot, and viral RNA from patients indicated that there were no consensus genetic changes in the vaccine virus that were responsible for causing YEL-AVD.
- No virologic or production process evidence could be found to suggest that vaccine lot 121Z had anything inherently wrong with it to explain the higher frequency of YEL-AVD in persons receiving that lot.
- The adverse event surveillance system in Peru was sensitive. No additional fatal cases of YEL-AVD were identified in Ica after a retrospective review of hospital records and death registries.
- Host and unknown factors may have increased risk of severe infection with 17DD, including advanced age in one case, and autoimmune disease (lupus) in another. In two cases, potentially immunosuppressive medication was administered after vaccination. Autoimmune disease is not a recognized risk factor up to now, but further attention to this is warranted in the future. The apparent association of diarrhea with YEL-AVD cases suggests the potential involvement of yellow fever virus directly in infection of the GI tract or as a co-factor with another infectious agent.
- Population factors were likely important in this outbreak–these factors include wide use of yellow fever vaccine in adults (many of whom have risk factors such as advanced age) in a non-endemic area that has not previously been subjected to yellow fever vaccinations and therefore has no protective immunity.

Recommendations

The following recommendations were made by the Expert Panel. These recommendations cover a spectrum of activities that should be undertaken to improve knowledge of the risk of ESAVIs associated with yellow fever vaccines and steps to reduce such risk, as well as to guide the clinical management of such cases, thereby potentially improving outcomes.

a) Communicating the Information

- Risk communication related to information about these ESAVIs should be balanced with communication about risks of yellow fever and vaccine effectiveness in preventing yellow fever.
- PAHO should make this report available to international panels, such as SAGE, TAG, and GAVCS.
- PAHO should compile a full report of the investigations of these ESAVIs and publish it.

b) Further Investigation of Cases

- The Virology Working Group of the Expert Panel should develop a research agenda aimed at further elucidating the causal factors underlying these cases of viscerotropic disease. Agenda topics should include the following:
  - → Further virological evaluation of the Ica YEL-AVD cases including:
    - Considering sequencing viral isolates from case 1;
    - Considering attempts to obtain a viral isolate from cases 2-4 through blind passage;
    - Sequencing viral nucleic acid from case 4;
    - Considering comparison of virulence of lot 121Z with other lots through animal testing;
    - Further characterizing viral RNA from case tissues; and
    - Testing the 121Z vaccine lots for adventitious infectious agents.
  - → Obtaining results of host DNA testing of the four fatal cases to identify potential genetic factors responsible for increased susceptibility.
  - → Considering further host factor evaluation among YEL-AVD survivors to be able to further explore the role of the immune system (innate immunity). This would entail collection of peripheral blood mononuclear cells and could include a study of toll-like receptor activation and the induction of interferon-stimulated genes in response to 17D virus,

genetic studies of the 2',5' oligoadenylate synthetase genes, and other studies to be designed by an expert panel.

→ Considering the potential role of yellow fever vaccine virus and other infectious agents as co-factors in YEL-AVD.

- PAHO/WHO should take steps to ensure that specimens from YEL-AVD cases, virus isolates, and retained samples of lot 121Z (and sister lots) be retained indefinitely and be made available to *bona fide* researchers in the future who may develop new tools for determining causality and pathogenesis.
- WHO should develop a protocol for investigation of YEL-AVD. The protocol should include:
  - → Description of YEL-AVD characteristics (include possible rapid onset, diarrhea);
  - → Case definitions for YEL-AVD and YEL-AND;
  - → Guidelines for clinical assessment of cases and autopsy work-up if applicable;
  - → Guidance on what samples should be obtained, with appropriate handling and shipment instructions; and
  - → Specification of reference laboratories where samples should be sent for specific testing.
- WHO should work with the relevant investigators/institutions to revisit the case reports for YEL-AVD cases reported previously to document the relevance of diarrhea as a clinical feature of this condition.
- PAHO/WHO should consider revising the Guidelines for clinical case management of yellow fever and to include the management of YEL-AVD and YEL-AND, considering, for example, the use of prophylactic antibiotics, supportive care, and management of systemic inflammatory response and multi-organ failure. The status and availability of antiviral drugs, antibodies, and RNAi, among others should also be considered.

c) Improved Information on ESAVI Incidence and Risk Factors

- Countries should consider establishing real-time vaccine registries, using computer-searchable databases, when administering yellow fever vaccines either in vaccine campaigns or during routine vaccination.
- Countries should consider surveying vaccinated populations following yellow fever vaccination campaigns, including epidemiological and laboratory-based studies, in order to evaluate the vaccine coverage rate and whether recommendations for vaccination have been followed, e.g., whether higher risk populations or individuals were vaccinated during the campaign. The outcomes of such surveys should be used to elucidate clearer guidance for precautions and contraindications for yellow fever vaccine use. The incidence of vaccine-associated ESAVIs should be documented in campaigns conducted outside the endemic region where the population does not have a high background of immunity, e.g., Argentina, Brazil (eastern São Paulo State), and Paraguay. The age- and sex-specific rates should be determined, and rates calculated by other risk categories, where possible. Incidence should be expressed with common denominators, e.g., x per 100,000 doses.
- WHO should review information contained in the yellow fever vaccine product inserts (product labeling) from different manufacturers to ensure that adequate information on ESAVIs is provided and up to date. New precautions against use of immunomodulating drugs in the 10 days following yellow fever vaccination (i.e., until immunity appears) may be warranted.
- WHO and countries should consider the role of autoimmune conditions, such as systemic lupus erythematosus, ulcerative colitis, Crohn's disease, and rheumatoid arthritis, as possible risk factors for severe YEL-AVD.
  - → Obtain estimates of background prevalence of various autoimmune conditions and compare to the prevalence of these conditions among cases of YEL-AVD.
  - → Document underlying conditions in future cases.
- Countries should consider providing all health care providers and vaccinees a simple information sheet with indications and contraindications for vaccination and description of potential ESAVIs.
- PAHO/WHO should encourage collection of specific data on the incidence of YEL-AVD (and YEL-AND) in endemic versus non-endemic areas (where the background of yellow fever immunity is low), for example Argentina and the eastern region of São Paulo State, Brazil.

d) Other

- Further data on outcomes of vaccination with 121Z lot in Venezuela should be obtained, since it is believed that 70,000 persons received this lot prior to its use in Ica Department and ESAVI surveillance was of unknown sensitivity.
- The WHO Requirements for Yellow Fever Vaccine (dated 1998) should be reviewed with consideration to the value of genetic testing.

Serious Events Supposedly Attributable to Vaccination or Immunization Associated with Receipt of Yellow Fever Vaccine During a Mass Immunization Campaign

Peru, 2007

Annexes

Annex 1

Case Summary (Cases 1-4)

Case Summary

CASE 1 (RCQ)

a) Clinical Summary:

This 23 year-old female, a medical student in Ica (Peru), received yellow fever vaccination (YF) with vaccine lot number 050VFA121Z (manufactured by Bio-Manguinhos, Brazil; expiration October 2007) by subcutaneous injection on 27 September 2007. Her past medical history was remarkable only for acne rosacea, diagnosed in 2005. Interview with the patient's family revealed that this was her first dose of YF (no confirmation available by health records). History of any regular medications, allergies, any acute illnesses in the month prior to receiving YF, or recent travel were unknown.

One day post-vaccination she developed generalized malaise, fever (39.5°C), headache, athralgias, and myalgias. She treated her symptoms with paracetamol (acetaminophen), with doses as much as 3 grams per day for four to five days. She also took metamizole (a nonsteroidal anti-inflammatory drug –not available in the U.S. since 1977) in unspecified doses. Five days post-vaccination, she received an intramuscular injection of metamizole with subsequent improvement of her fever, but not the other symptoms. Over the next two days, she experienced nausea, vomiting, and diarrhea with four to five watery, yellow-colored stools per day. She presented to the Emergency Department of Ica Regional Hospital the following day (eight days post-vaccination), and was admitted with a diagnosis of gastroenteritis and dehydration. She was treated with fluids, dimenhydranate, and ciprofloxacin all administered intravenously.

Her initial white blood cell count was 14,500/mm^3 with 86% neutrophils and 10% lymphocytes. Hemoglobin and platelet count (150,000/mm^3) were normal. Her aspartate aminotransferase (AST) level was 78 U/L, alanine aminotransferase (ALT) 65 U/L, alkaline phosphatase 243 U/L, total bilirubin 0.85 mg/dL, albumin 3.8 g/dL, creatinine 1.6 mg/dL, and urea 50 mg/dL.

Her condition deteriorated throughout the first hospital day (day eight post-vaccination) as she developed hypotension, metabolic acidosis (blood pH was 7.191 and pCO_2 was 18.5 mm Hg), and acute renal failure. Subsequently, she developed psychomotor agitation and respiratory failure requiring intubation, mechanical ventilation, and placement of a central venous catheter in the shock trauma unit early in the morning of the second hospital day (day nine post-vaccination). Multiorgan system failure and encephalopathy were thought to be caused by either septic shock or a serious adverse reaction to YF. She was treated with intravenous ciprofloxacin, ceftriaxone, hydrocortisone, furosemide, dopamine, and sodium bicarbonate.

Her white blood count increased to 66,400/mm^3 with 91% neutrophils (including 28% bands) and 8% lymphocytes. Her platelets decreased to 54,000/mm^3. Her AST increased to 850 U/L, ALT to 222U/L, international normalized ratio (INR) to 3.2, and creatinine to 4.1. Serum lactate was 140 mmol/L and total creatine phosphokinase was 4,055. A typhoid O agglutination test yielded an agglutinin titer of 1/160. Additional studies included a chest radiograph which showed pulmonary congestion with prominent hilar markings, and ultrasound examination which showed hepatosplenomegaly, ascites, and bilateral pleural effusions. A stool culture was negative.

Her systolic blood pressure decreased to 60 mm Hg despite treatment with vasopressors. During the course of the second hospital day her shock became refractory to medical treatment and she died (nine days post-vaccination).

b) Serology Results:

Serological studies performed at the National Institute of Health-Peru (NIHP) demonstrated no evidence of recent infection with HIV, hantavirus, hepatitis B, leptospira, mayaro virus, oropuche virus, or Venezuelan equine encephalitis virus.

c) Post-mortem Findings: Microscopic

Post-mortem tissue samples examined at CDC and NIHP showed the following: liver tissue had scattered midzonal necrosis, diffuse microvesicular steatosis, and sparse mixed inflammatory cell infiltrates; kidney had acute tubular necrosis and focal hemorrhage; lung had intra-alveolar edema and vascular congestion; brain had edema and discrete gliosis (brain findings noted at NIHP only).

The Central Morgue of the Legal Medicine Institute found thyroid neoplasia, chronic thyroiditis.

d) Tests Specific for Yellow Fever Vaccine:

Serum obtained nine days after vaccination tested positive for anti-yellow fever virus (YFV) IgM, but negative for anti-YFV IgG by ELISA (a negative YFV-IgG ELISA test is not unusual after vaccination with YF). A plaque reduction neutralization test (PRNT) titer of anti-YFV antibody was 1:160.

RT-PCR showed the presence of 17D YFV RNA in serum, urine, liver, lung, kidney, and brain (NIHP and CDC). 17D YFV was cultured from post-mortem specimens of serum, liver, and brain (NIHP and CDC). Viral load was determined by real-time PCR at CDC. High amounts of 17D YFV were detected in lung (7.6×10^6 PFUeq/ml) and serum (3.9×10^6 PFUeq/ml), while 3.5×10^5 , 3.9×10^4 , 1.2×10^4, and 6.8×10^1 PFUeq/ml were detected in kidney, brain, liver and urine respectively. The viral load in serum was determined from a sample taken nine days after vaccination. The sequence of viral RNA obtained from lung tissue was identical to the reference sequence for YF 17-DD vaccine virus strain.

17D YFV antigen was detected by immunohistochemistry (IHC) at CDC with abundant and extensive staining in the liver, lung, kidney, and rare staining in the central nervous system (CNS). IHC performed at NIHP was positive for staining in the liver only.

Case 2 (GSAL)

a) Clinical Summary:

This 24 year-old female from Chincha Province (Ica Department) received yellow fever vaccination (YF) with vaccine lot number 050VFA121Z (manufactured by Bio-Manguinhos, Brazil; expiration October 2007) by subcutaneous injection on 27 September 2007. Her past medical history was remarkable for having fallen out of a tree at age eight years and sustaining unspecified head trauma, with the subsequent development years later of intermittent headaches which became worse during times of psychological stress. Also, she had delivered one normal term infant on 4 July 2007. Records indicate that she was allergic to egg yolk and also to unspecified medicine(s). Interview with the patient's family revealed that this was her first dose of YF (no confirmation available by health records). History of any regular medications, any acute illnesses in the month prior to receiving YF, or recent travel were unknown. She was employed as a hair stylist prior to the Ica earthquake (15 August 2007). According to information from the hospital in Lima, she also bred guinea-pigs, and, according to her husband, she only worked helping him in a street market, where she usually had her meals. She also bred hens, cats, dogs and doves.

During the night of 27 September (day of vaccination), the patient began to have symptoms of headache, malaise, myalgias, and fever (temperature undocumented), which lasted nine to ten days. Seven days after vaccination, she developed nausea, vomiting, and diarrhea with three to four yellow-colored liquid stools per day. Records indicate that she was evaluated in a physician's office at some point during day three to day eight post- vaccination, at which time she was diagnosed with urinary tract infection and intestinal disorder, and was administered amikacin 500 mg intramuscularly. She was also prescribed oral ciprofloxacin (500 mg) and dexamethasone (4 mg). It is unknown what total doses of these medications were prescribed and whether she took them. Her husband noted that the frequency of diarrhea increased to 10 to 20 stools per day on day 10 post- vaccination. On day 11 post-vaccination, a physician made a home visit and diagnosed dehydration and shock with watery diarrhea of unknown etiology and possible hyponatremia. The physician administered an unknown injection for "liver protection" (possibly a vitamin preparation) and counseled the patient to go to the hospital emergency department.

On day 11 post-vaccination, she was brought by her husband to the Chincha Hospital Emergency Department, where initial examination demonstrated a blood pressure of 60/30 mm Hg, heart rate of 108/min. respirations of 26/min. and a temperature of 36°C. Her examination was significant for a diminished level of consciousness, dry mouth and mucous membranes, slow capillary refill time, tachypnea without abnormal breath sounds, and tachycardia without arrhythmia. Her abdomen was soft and non tender. Initial laboratory tests were performed. Her white blood cell count was 16,300 with 87% neutrophils (including 2% bands) and 13% lymphocytes. Hematocrit was 44% and platelet count was not noted. Creatinine was 1.6 mg/dL, urea 188 mg/dL and glucose 52mg/dL. The clinical impression was hypovolemic shock from severe dehydration, and acute renal failure. Due to the seriousness of her condition, she was transferred later that day (day 11 post-vaccination) to a national hospital in Lima.

At the national hospital in Lima, she was noted to have a blood pressure of 80/50 mm Hg, heart rate of 100 and respiratory rate of 30. Her examination findings were similar to those noted by the referring hospital; it was also noted that she was agitated,

moaning, and had mydriatic pupils. She was immediately administered intravenous fluids which resulted in diuresis within one hour. Initial laboratory results at this hospital included hematocrit 31%, white blood count 11,470/mm^3 (with 82% neutrophils, including 18% bands), platelet count 24,000/mm^3, sodium 121mmol/L, creatinine 3.2 mg/dL, urea 154mg/dL, aspartate aminotransferase (AST) 735 U/L, alanine aminotransferase (ALT) 167 U/L, total bilirubin 6.3 mg/dL, direct bilirubin 6.2 mg/dL, albumin 1.6 g/dL, international normalized ratio (INR) 2.99, total creatine phosphokinase 3,173 U/L, and serum myoglobin 1851 ng/ml. Arterial blood gas results were: pH 7.095, pCO_2 34, pO_2 223 mmHg, and bicarbonate 11.

The revised diagnostic impression was refractory distributive shock with multiorgan system failure and severe metabolic acidosis, acute respiratory failure, encephalopathy, coagulopathy, and upper gastrointestinal bleeding. She was intubated and mechanically ventilated in the intensive care unit. Treatment included intravenous fluids, vasopressors, hydrocortisone, meropenam, vancomycin and insulin. Despite aggressive treatment, her clinical condition deteriorated over the next few days. She became jaundiced, edematous and developed a fever with maximum temperature of 40.2ºC. Her hematocrit dropped to 15.5%, platelets to 15,000/mm^3, creatinine to 1.7 mg/dL, urea to 112mg/dL and fibrinogen was 62 mg/dL. She remained acidemic with a blood pH of 7.093–7.23. Her AST increased to 850 U/L, and ALT to 222 U/L. Her stool culture grew E. coli O86. A first blood culture grew Staphylococcus, species unspecified; it was noted on the laboratory report that this was possibly a contaminant. A second blood culture grew yeast, unofficially reported later to be Candida.

On the third hospital day (day 14 post-vaccination), she had a cardiac arrest and died after unsuccessful attempts at resuscitation.

b) Serology Results:

Serological studies performed at the National Institute of Health-Peru (NIHP) demonstrated no evidence of recent infection with HIV, hantavirus, hepatitis B, leptospira, mayaro virus, oropuche virus, Venezuelan equine encephalitis virus, or rickettsia.

c) Post-mortem Findings: Microscopic

Post-mortem tissue examined at CDC and NIHP showed the following: the liver had diffuse microvesicular steatosis, rare hepatocyte necrosis, and sparse mixed inflammatory cell infiltrates; kidney had red blood cells and red cell casts in the tubules; brain had cerebral edema and discrete gliosis; lung had congestion, interstitial inflammation, and intraalveolar edema (lung findings only noted by CDC).

The Central Morgue of the Legal Medicine Institute found candida infection in larynx, epiglottis, trachea, and stomach. They also found endometritis.

d) Tests Specific for Yellow Fever Vaccine:

Serum obtained 11 days after vaccination tested positive for anti-yellow fever virus (YFV) IgM, but negative for anti-YFV IgG by ELISA. A plaque reduction neutralization test (PRNT) titer of anti-YFV antibody was 1:10240.

RT-PCR showed 17D YFV RNA in serum, urine, liver, lung, kidney, and brain (NIHP, NMRCD, and CDC). YFV was not successfully isolated by culture from any post mortem tissue. Viral load was determined by real-time PCR at CDC. 17D YFV RNA was detected in liver (1.1×10^4 PFUeq/ml), and brain (4.2×10^3), with 9.6×10^2, 4.6×10^2, 2.6×10^2, and 2.5×10^2 PFUeq/ml detected in urine, lung, serum, and kidney respectively. The viral load in serum was determined from a sample taken 11 days after vaccination. The sequence of the serum PCR fragment was consistent with glycoprotein E of YF virus (NIHP). The sequence of the brain PCR fragment was 100% identical to 17-DD YF vaccine virus strain in the E region (NMRCD). The sequencing of RNA from liver tissue was identical to the 17-DD YFV reference strain except for one silent nucleotide change (CDC).

17-D YFV antigen was detected by immunohistochemistry (IHC) with rare staining in liver, lung, kidney and brain (CDC).

Case 3 (MCP)

a) Clinical Summary:

This 79 year-old male resident of Nazca Province (Ica Department), received yellow fever vaccination (YF) with vaccine lot number 050VFA121Z (manufactured by Bio-Manguinhos, Brazil; expiration October 2007) by subcutaneous injection on 1 October

2007. His past medical history was significant for benign prostatic hypertrophy with prostatectomy (2002), gastritis (2006), and an allergy to sulfa. His family reported that during the two months prior to hospitalization the patient had onset of dyspnea on moderate exertion, which eventually progressed to dyspnea with mild exertion, as well as exertional precordial chest pain. No documentation of a diagnosis of cardiac disease was found on available medical records. Interview with the patient's family revealed that this was his first dose of YF (no confirmation available by health records). History of any regular medications or recent travel were unknown.

Three days post-vaccination, the patient developed fever (not quantified), malaise, dyspnea, and severe abdominal pain. Five days post-vaccination, he began to have vomiting and five to seven watery, bloody stools with mucous per day. Six days post-vaccination, he was brought to the hospital in Nazca complaining of fever, cough, and worsening abdominal pain. His vital signs on initial examination were blood pressure 100/60 mm Hg, heart rate 88/min., respiratory rate 22/min. and temperature 38ºC (axillary). His examination was significant for mild rales and diminished breath sounds in the right lung base. Initial laboratory test results included white blood cell (WBC) count of 7,200/mm^3, with 75% neutrophils (including 10% bands); stool with pink color and mucous, WBC >100/field, red blood cells (RBC) >400/field, and 8–16 polymorphonuclear neutrophils/field; urine with moderate bile, 1–2 WBC/field, 3–4 RBC/field, and rare bacteria. Platelet count and serum chemistry tests were not performed. The admitting diagnosis was bronchopneumonia and possible urinary tract infection. He was treated with intravenous fluids and ceftriaxone.

The patient's condition deteriorated over the next few days. On hospital day three (day nine post-vaccination), he was noted to have a blood pressure of 60/20 mm Hg, a heart rate of 144/min., and to have abdominal pain and watery diarrhea. He was diagnosed with hypovolemic shock and was transferred to the regional hospital in Ica. There, the initial examination was remarkable for tachypnea with no abnormal breath sounds, tachycardia, and hyperactive bowel sounds. Laboratory test results were remarkable for hematocrit 43%, WBC 9,400/mm^3, with 96% neutrophils (along with 13% bands); creatinine 2mg/dL; urea 37 mg/dL. The admitting diagnoses were congestive heart failure (CHF), chronic bronchitis, and acute watery diarrhea – possible dysentery. He was treated with furosemide, captopril, ceftriaxone, and amikacin.

His condition subsequently deteriorated with the development of atrial fibrillation with a rapid ventricular response as well as a distended and diffusely tender abdomen. The next day (day 10 post-vaccination), he was intubated and mechanically ventilated because he had developed pulmonary edema and had become tachypneic and acidemic (blood pH of 7.05, PCO_2 of 34). His hematocrit rose to 53%, and WBC to 17,400/mm^3 with a platelet count of 249,000/mm^3. His creatinine rose to 2.8 mg/dL. The aspartate aminotransferase was 416 U/L, alanine aminotransferase was 231 U/L, alkaline phosphatase was 605 U/L, total bilirubin was 2.9 mg/dL and direct bilirubin was 2.3 mg/dL. A PSA was 76.45 ng/ml. A typhoid O agglutination test yielded an agglutinin titer of 1/80 and typhoid H agglutination test yielded a titer of 1/320. An abdominal ultrasound showed diffuse hepatopathy, splenomegaly, bilateral nephropathy, ileus, and cholelithiasis. The diagnoses were revised to distributive shock, probably septic from an abdominal source; acute respiratory failure most likely from pulmonary edema; acute renal failure; and possible chronic coronary artery disease. His medications were changed to dopamine, adrenaline, furosemide, imipenem, hydrocortisone, synthetic colloid, as well as transfusions of platelets (10 units) and fresh frozen plasma (2 units).

The patient's condition became refractory to treatment and he died on day 11 post-vaccination.

b) Serology Results:

Serological studies performed at the National Institute of Health-Peru (NIHP) demonstrated no evidence of recent infection with leptospirosis or HIV infection.

c) Post-mortem Findings: Microscopic

Post-mortem tissue examined at CDC showed the following: liver tissue had diffuse micro- and macrovesicular steatosis with extensive multifocal hepatocyte necrosis; spleen had extensive congestion and depletion of white pulp; kidney had acute tubular necrosis with interstitial hemorrhage; CNS had congestion. NIHP report noted that adequate microscopic visualization of the tissues was limited by severe congestion and focal hemorrhage although, overall, the histopathologic findings were compatible with congestive heart failure.

The Central Morgue of the Legal Medicine Institute found chronic cardiopathy, liver damage consistent with chronic cardiopathy, mid-zonal necrosis area in liver.

d) Tests Specific for Yellow Fever Vaccine:

Serum obtained 10 days after vaccination tested positive for anti-yellow fever virus (YFV) IgM, but negative for anti-YFV IgG by ELISA. PRNT was not done due to an insufficient volume of the sample.

RT-PCR showed the presence of 17D YFV RNA in serum, liver, lung, kidney, spleen, and brain (NIH Peru and CDC). YFV was not successfully isolated by culture from any post mortem tissue specimens. Viral load was determined by real-time PCR (CDC). High amounts of 17D YFV were detected in spleen or liver[1] (3.5×10^4 PFUeq/ml), kidney (2.7×10^4 PFUeq/ml), and lung (2.1×10^3) while 2.6×10^2, and 1.9×10^2 PFUeq/ml were detected in brain, and serum respectively. The viral load in serum was determined from a sample taken 10 days after vaccination. CDC was only able to amplify a portion of the YFV genome by RT-PCR and therefore only perform partial genome sequencing. In the regions for which sequence data (2870 bases) was obtained for the YFV amplified from patient tissue, the sequence was identical to the 17-DD virus secondary seed lot.

Scant amounts of 17D YFV antigen were detected by immunohistochemistry (IHC) in only the spleen at CDC.

Case 4 (MYS)

a) Clinical Summary:

This 49 year-old female resident of Chincha Province (Ica Department) received yellow fever vaccination (YF) with vaccine lot number 050VFA121Z (manufactured by Bio-Manguinhos, Brazil; expiration October 2007) by subcutaneous injection on 24 September 2007. Her past medical history was significant for an initial rheumatoid arthritis diagnosis in 1993, systemic lupus erythematosis (SLE) diagnosed in 1996, chronic renal insufficiency, hypertension, and a cerebrovascular accident in 2003 resulting in the need for crutches to ambulate. She had been treated with prednisone twice a day (dose unknown), captopril daily (dose unknown), and ibuprofen prn. However, records indicate that she had stopped taking these medications three years prior. Interview with the patient's family revealed that this was her first dose of YF (no confirmation available by health records). History of allergies or recent travel was unknown.

Four days after vaccination, she was seen at a hospital outpatient clinic in Chincha complaining of hip pain of one week-duration, which made it hard to walk. She was given an intramuscular injection of dexamethasone and diclofenac (dose unknown), and also was prescribed oral methotrexate (16 tablets; dose unknown) and oral tenoxicam (50 tablets; dose unknown). Records do not indicate if she took any of these oral medications. She was instructed to return for re-evaluation in two weeks. Seven days after vaccination she experienced pain in her legs, several episodes (number unknown) of melena, and three episodes of vaginal bleeding. Eighteen days after vaccination she returned to the hospital outpatient clinic in Chincha as scheduled, complaining of intense headache, general malaise, and arthralgias. She was prescribed diclofenac, vitamin B_{12}, and prednisone (route, dose, and quantity for each is unknown) and was to follow up in two weeks.

Twenty nine days after vaccination, she returned to the emergency department with severe headache, diminished urine output and signs of dehydration. She was found to be afebrile, but quite morbid overall with generalized edema, mild jaundice, dehydration, and diminished consciousness. Initial laboratory tests were performed. Her aspartate aminotransferase was 91 U/L, alanine aminotransferase was 128 U/L, alkaline phosphatase was 742 U/L, total bilirubin was 5.2 mg/dL, and direct bilirubin was 4.2 mg/dL. No CBC or other chemistry tests were done. She was evaluated by a gastroenterologist who diagnosed her with encephalopathy of unknown etiology, acute cholestasis, and "post-yellow fever vaccination syndrome", and recommended transfer to a hospital offering a higher level of care.

The following day (day 30 post-vaccination), the patient was transferred to a hospital in Lima. On arrival there her temperature was 37.2ºC, and it was noted on examination that she had marked pallor of her skin and mucous membranes, signs of mucosal bleeding, scleral icterus, lower extremity ecchymoses, and tachycardia. Laboratory tests were performed. Her white blood cell count was 5,530/mm^3, hematocrit was 31%, and platelet count was 57,000/mm^3. Other laboratory results were aspartate aminotransferase of 100 U/L, total bilirubin of 5.2 mg/dL, creatinine of 3.3mg/dL, urea of 272 mg/dL, sodium of 123 mmol/L, potassium of 6.8 mmol/L, and lactate of 4.4 mmol/L. An abdominal ultrasound examination showed chronic hepatic disease, splenomegaly, and increased renal echogenicity bilaterally. Her diagnoses were metabolic encephalopathy, metabolic acidosis, possible sepsis, chronic renal insufficiency with decompensation, jaundice probably secondary to acute liver disease,

1 Uncertain if frozen tissue received and used by CDC for RT-PCR and real-time PCR was liver or spleen.

exacerbation of SLE, and "acute symptoms of yellow fever from vaccination". She was treated with intravenous ceftazidime, hydrocortisone, and fluids. She experienced a respiratory arrest with bradycardia and was intubated and mechanically ventilated. Subsequently, she had a generalized seizure and another cardiopulmonary arrest, which was refractory to resuscitation efforts. She died on day 30 post-vaccination.

b) Serology Results:

Serology tests performed at the National Institute of Health-Peru (NIHP) were negative for HIV and Leptospirosis.

c) Post-mortem Findings: Microscopic

Post-mortem tissue examination showed the following: liver had diffuse microvesicular steatosis and scattered hepatocellular necrosis (CDC and NIHP), as well as discrete areas of mixed inflammatory cell infiltrates (NIHP); kidney had interstitial mononuclear infiltrates (CDC), and membranoproliferative glomerulonephritis with acute tubular necrosis (NIHP); lung had pulmonary edema (NIHP); brain had no significant histopathologic changes (CDC).

Tissue gram staining (CDC) revealed abundant gram positive and gram negative microorganisms in lung, liver, and kidney. Because many of these organisms were present in clusters with no corresponding inflammatory reaction, they were felt by the reviewing pathologist to most likely represent postmortem polymicrobial overgrowth.

The Central Morgue of the Legal Medicine Institute found damage in different organs compatible with SLE, especially in kidneys. Liver damage consistent with yellow fever: mid-zonal necrosis areas.

d) Tests Specific for Yellow Fever Vaccine:

The patient's serum tested positive for anti-yellow fever virus (YFV) IgM, but negative for anti-YFV IgG by ELISA. The plaque reduction neutralization titer (PRNT) of anti-YFV antibody was >1:20,480 (CDC).

RT-PCR showed the presence of 17D YFV RNA in serum (NIHP), liver (CDC), and kidney (NIHP and CDC). Attempts at isolation of yellow fever virus by culture from all tissues were unsuccessful (NIHP). Viral load was determined by real-time PCR (CDC). The viral load of 17D YFV in kidney was 1×10^4 PFU eq/ml and in liver 3–5 PFU eq/ml. Insufficient quantities of RNA were obtained from tissue for sequence analysis.

17D YFV antigen was detected by immunohistochemical (IHC) staining in liver (NIHP) and kidney (CDC).

Annex 2

Possible Non-fatal Yellow Fever Vaccine-associated Viscerotropic Disease

Possible Non-fatal Yellow Fever Vaccine-associated Viscerotropic Disease

Chief complaint: JDC, a 43 year-old male was admitted to Hospital III, Felix Torrealva Gutierrez, Ica, on 4 October 2007, with a 7-day history of fever of 38-39°C, general malaise, and headache.

History of present illness: Patient is a 43 year-old male from Pachacutec District, Ica, who was in good health until 27 September, 7 days prior to admission, when he developed fever, headache and general malaise. Four days prior to admission, the patient developed loose stools and was seen at a local clinic. He had a negative urinalysis there and was started on amoxicillin. Because of continued fever, headache, and diarrhea, patient was seen in the emergency room at Hospital III on 4 October.

Immunization History: Patient was immunized on 23 September 2007 at Health Center Pachacutec, Ica, as part of an immunization campaign according to the investigation form for events supposedly attributable to vaccines or immunization (ESAVIs). According to vaccination record, "carnet," patient received a Td. Patient says he received yellow fever vaccination on that date (no documentation in hospital record of receipt of YF vaccine).

Travel History: None

Occupational History: Teacher / Professor ("Docente")

Alcohol: Drinks alcohol once a week

Past Medical History:

- Appendectomy in February 2007
- Fractured tibia at age 9
- No chronic illnesses

Vital Signs: In the Emergency Room the patient had a temperature of 39°C. On admission had a temperature of 37.5°C, Pulse – 84, respiratory rate 24, and was normotensive.

Physical Exam: Patient weighed 77 kilos. He was alert and oriented. Physical exam was unremarkable except for diaphoresis, and an injected pharynx. Abdomen was non-tender and patient had no meningismus or focal neurologic signs.

Laboratory: Hemoglobin 15.7, hematocrit 44.4, platelets 267,000, white blood count 9680 (67% neutrophils, 25% lymphocytes), creatinine 0.6, TGO 158 (2-35), TGP 244(9-43).

Initial Diagnosis: Febrile syndrome of undetermined origin (liver enzymes not immediately available). Emergency room note mentions rule out urinary tract infection, nephrolithiasis, typhoid, and post-vaccine reaction

Hospital Course: Patient was admitted to internal medicine ward with plan to culture urine, perform febrile agglutinins, hydrate with intravenous fluids, and paracetamol for fever. He was given metamizol IM.

The patient continued to have fever and headache. On 7 October, the patient was transferred to the intensive care unit for further workup and "neurologic observation." The patient at that time was felt to be mildly dehydrated and to have mild scleral icterus. Physical exam was otherwise unremarkable. Patient continued to receive intravenous fluids. He also was put on oxygen with excellent saturation, and sucralfate. The diagnosis in the ICU was "rule out" post-vaccine encephalitis. Additional laboratory studies on 8 October showed TGO 19, TGP 28, total bilirubin 0.5, total protein 8.2, and normal clotting studies. The patient defervesced on 8 October.

On 9 October, the patient was seen by an infectious disease consultant and transferred out of the intensive care unit to the infectious disease service. The infectious disease consultant felt the patient had a probable vaccine reaction to Td.

Additional laboratory results were negative urine culture, negative febrile agglutinins (H antigen 1:80), and negative stool for parasites. An echo study of the liver done on 3 October (?) showed an increase in echogenicity diffusely consistent with chronic hepatic disease (?). The patient had serum and urine obtained by epidemiology on 6 and 9 October; where these specimens were tested and for what is not clear.

The patient was discharged clinically improved from hospital on 11 October with a diagnosis of complications from Td vaccination and improving intestinal infection.

ANNEX 3

Protocol for Yellow Fever ESAVI Investigation
Ica, Peru, 2007

MINISTRY OF HEALTH
General Bureau of Epidemiology

Prepared by:

Alvaro Whittembury Vlásica
Physician/Epidemiologist, General Bureau of Epidemiology

Washington Toledo
PAHO Medical Consultant

María Ticona Zegarra
Epidemiology Nurse, General Bureau of Epidemiology

Jorge Uchuya Gómez
Physician/Epidemiologist, General Bureau of Epidemiology

Víctor Aquije Méndez
Physician/Epidemiologist, Ica Regional Bureau of Epidemiology

Reviewed by:

Ned Hayes
CDC Atlanta

Steve Waterman
CDC Atlanta

Mark Gershman
CDC Atlanta

Gladys Ramírez Prada
General Director, General Bureau of Epidemiology

José Bolarte Espinoza
Sectoral Director, Public Health Surveillance

Protocol for Yellow Fever ESAVI Investigation
Ica, Peru, 2007

1. Introduction

Epidemiological surveillance is a regular ongoing process that enables us to determine the magnitude of the problem, identify vulnerable populations, stratify risk, and assess the impact of current prevention and control measures.

Epidemiological surveillance of events supposedly attributable to vaccination or immunization (ESAVIs) has been conducted officially in Peru since 2002. The appropriate regulations are in place and technical guidelines are available in the report "How to Deal With Adverse Events Supposedly Attributable to Vaccination or Immunization". This report defines severe ESAVIs as post-vaccination disease that requires hospitalization or causes death. The system includes four lines of action: (a) communication to deal appropriately and promptly with "crises" that may occur, to prevent loss of confidence in vaccination by the population; (b) education through appropriate counseling for mothers and fathers, to enable them to detect post-vaccination warning signs (e.g., persistent crying, high fever that does not subside, convulsions) early and seek immediate medical attention; (c) epidemiological surveillance based on reporting and immediate investigation of ESAVI cases; and d) appropriate clinical management of the events that occur. Standards, research protocols, forms, and reporting flows have been established.

Between 2002 and 2006, ESAVI epidemiological surveillance performed by the General Bureau of Epidemiology of the Ministry of Health identified 13 vaccine-related ESAVIs.[1] See Table 1.

Table 1. General Bureau of Epidemiology
Number of Severe ESAVIs Recorded in the ESAVI Epidemiological Surveillance System, Peru 2001-2006

YEAR	VACCINES AND SEVERE ESAVIs											
	BCG	HVB	OPV	DTP	Pentavalent	Hib	Yellow Fever	DT Pediatric	DT Adult	Measles	MMR	MR	Subtotal
2001	0	0	0	0	n/a	n/a	0	0	0	0	n/a	n/a	0
2002	0	0	0	(2) Anaphylactic Shock (2) Febrile Convulsive Syndrome (1) Vaccine Reaction (Persistent)	n/a	n/a	0	0	0	0	n/a	n/a	5
2003	0	0	0	0	(1) Anaphylactic Shock (1) Febrile Convulsive Syndrome	0	0	0	0	0	0		2
2004	0	0	0	0	0	0	0	0	0	0	0	(2) Thrombocytopenic Purpura	2
2005	0	0	0	0	0	0	(1) Encephalitis	0	0	0	0	(2) Thrombocytopenic Purpura	3
2006	0	0	0	0	0	0	0	0	0	0	0	(1) Anaphylactic Shock	1
Total	0	0	0	5	2	0	1	0	0	0	0	5	13

n/a: no applicable

1.1 Proposed Investigation:

From 23 September to 6 October 2007, the National Health Strategy for Immunization conducted yellow fever vaccination in the population aged 15-59 years in the Ica region. A total of 63,174 persons were vaccinated: 42,742 persons were vaccinated with lot 050VFA121Z and 23,172 persons were vaccinated with lot 050VFA123Z. From 4 to 6 November 2007, the ESAVI surveillance system reported the death of four persons with ages ranging from 23 to 79 years. All of these deaths occurred after yellow fever vaccination with lot 050VFA121Z (substrain 17DD manufactured by Bio-Manguinhos, Brazil). In addition, on 31 October 2007 another ESAVI case associated with the yellow fever vaccine with a fatal outcome was reported. This patient was vaccinated in Lima with lot 064VFA035Z.

The first four cases had similar clinical symptoms --fever, headache, malaise, and watery diarrhea-- which rapidly progressed to distributive shock and irreversible multiple organ failure. Onset of symptoms occurred between 24 hours and 1 week after vaccination. The fifth case was diagnosed as autoimmune hemolytic anemia. Based on the clinical and laboratory data available to date, four of the five cases have been classified as acute yellow fever vaccine-associated viscerotropic disease in accordance with WHO[2] classification criteria. The fourth and fifth cases are still pending laboratory tests.[3]

Acute viscerotropic disease following yellow fever vaccination was first recognized in 2001. This condition is rarely reported. Thirty-seven suspect or confirmed cases have been reported to date worldwide following vaccination with vaccine substrains

17DD and 17D204. This condition usually develops as a yellow fever-like condition with multiple organ failure. Onset of symptoms occurs 2 to 5 days after the administration of yellow fever vaccine. The risk of viscerotropic disease following yellow fever vaccination is 0.1 to 0.3 per 100,000 persons vaccinated. Higher risk has been recorded for persons aged >60 years. At present, little is known about host factors or vaccine factors that potentially contribute to the risk of contracting viscerotropic disease.

The cases reported are the first cluster of reported cases of viscerotropic disease associated with a single lot of vaccine. Based on the number of doses of the vaccine lot administered in Ica from 23 September to 6 October, the reported case rate is approximately 10 per 10,000 doses. This is significantly higher than previously reported rates.[4,5,6]

In view of the high rate of reported cases of viscerotropic disease associated with vaccine lot 05OVFA121Z in Peru, PAHO/WHO recommended immediate discontinuation of the use of Bio-Manguinhos yellow fever vaccine lot 05OVFA121Z and lots related during the production process, specifically lots 05OVFA118Z, 05OVFA119Z, 05OVFA120Z, 05OVFA122Z, 05OVFA123Z, 05OVFA124Z, 05OVFA125Z, 050VFA126Z, and 064VFA035Z.

In response to this situation, the decision was made to conduct an epidemiological investigation of cases or deaths that met the definition of viscerotropic and neurotropic disease that were not detected by regular ESAVI surveillance. Two strategies were proposed for this purpose:

- Retrospective case-finding, searching for cases that meet the definition of viscerotropic disease in hospital records of the Ica region, including inpatient and emergency care: Look for an association with yellow fever vaccination; describe clinical and epidemiological characteristics; identify risk factors.
- Retrospective search for deaths in the municipal records offices: Identify cases that meet the definition of viscerotropic disease based on a review of medical histories and/or interviews with health workers and family members; look for an association with yellow fever vaccination for clinical, epidemiological, and laboratory description; identify risk factors (Annex 1).

A third strategy proposed identification of incident cases in selected hospitals in the country that meet the case definition for viscerotropic disease: Look for an association with yellow fever vaccination for clinical, epidemiological, and laboratory description; identify risk factors. However, this activity could not be carried out.

2. Objectives

2.1 General Objective:

- Identify and describe probable cases of yellow fever vaccine-associated viscerotropic and neurotropic disease.
- Determine which risk factors are related to yellow fever vaccine-associated viscerotropic disease.

2.2 Specific Objectives:

- Evaluate the case definition for yellow fever vaccine-associated viscerotropic and neurotropic disease.
- Identify probable cases of yellow fever vaccine-associated viscerotropic and neurotropic disease in Ministry of Health hospitals and other institutions selected by the Ica Regional Bureau of Health (DIRESA).
- Identify probable cases of yellow fever vaccine-associated viscerotropic disease in the death records of DIRESA.
- Describe the clinical, epidemiological, and laboratory profile of probable cases of viscerotropic disease in the population vaccinated against yellow fever in 2007 in Ministry of Health hospitals and other institutions selected by DIRESA.
- Identify risk factors related to yellow fever vaccine-associated viscerotropic disease

3. Population and Sample

3.1 Type of Study:

The study consisted in conducting retrospective case-finding in hospitalized patients that met the selection criteria (See 3.5.1). After selection was completed, medical histories were reviewed to determine whether they met the definition of probable or confirmed cases of yellow fever vaccine-associated viscerotropic or neurotropic disease.

3.2 Population:

The study population was made up of users of health services in Ministry of Health, EsSalud, Armed Forces, and Police hospitals of the Ica Regional Bureau of Health who were treated between 23 September and 6 November 2007.

3.3 Inclusion and Exclusion Criteria:

3.3.1 Inclusion Criteria:

- *Case* refers to all patients that meet the selection criteria (See 3.5.1).
- *Control* refers to all patients of the same sex and age (±2 years) who received treatment on the same day in the same hospital, and do not meet the selection criteria (See 3.5.1). The patients selected will preferably have been treated immediately before or after a case by the same physician.

3.3.2 Exclusion Criteria:

All patients that met the selection criteria were included.

3.4 Sample:

3.4.1 Calculating Sample Size:

All patients who met the *selection criteria* (3.5.1) and were treated in regional hospitals between 23 September and 6 November 2007 were included.

3.4.2 Sample Selection:

Sampling was not done. Work was conducted with the entire target population.

3.5 Operational Definition of Variables:

3.5.1 Selection Criteria:

All patients with fever over 38ºC (or who felt feverish) for more than 24 hours, and **one or more** of the following signs and symptoms:

1. Severe headache
2. Altered senses
3. Tonic-clonic seizures
4. Nausea/vomiting
5. Watery stools
6. Myalgia lasting more than 24 hours
7. Joint pain lasting more than 24 hours
8. Increased respiration rate (>20 breaths per minute)

3.5.2 Definition of Probable Case of Yellow Fever Vaccine-associated Viscerotropic Disease:

All cases vaccinated within 15 days prior to onset of symptoms with no evidence of other etiologies that clinical symptoms could be attributed to; with fever and one or more of the following symptoms: nausea, vomiting, malaise, watery stools, myalgia, joint pain, dyspnea; and one or more of the following:

- Serum transaminase at least 3 times higher than normal;

- Total serum bilirubin at least 1.5 times higher than normal;
- Serum creatinine at least 1.5 times higher than normal;
- Total creatine phosphokinase (CPK) more than 5 times higher than normal;
- Thrombocytopenia (blood platelets <100,000/mL);
- Myocarditis (including compatible abnormalities: electrocardiogram, echocardiogram, cardiac enzyme changes, or biopsy-related inflammation of cardiac tissue);
- Elevation of prothrombin time (INR) or partial activated thromboplastin time; and
- Histopathology compatible with yellow fever (e.g., medial liver necrosis, Councilman's bodies).

3.5.3 Definition of Confirmed Case of Yellow-Fever Vaccine-associated Viscerotropic Disease:

All probable cases with one or more of the following:

- 17D* yellow fever virus isolated in blood more than 7 days after vaccination and/or by PCR more than 11 days after vaccination;
- Viral load of 17D* yellow fever virus in serum greater than 10^3 PFU/mL recorded on any day;
- YF specific antigen in visceral tissue detected by immunohistochemistry (IHC);
- 17D* yellow fever virus isolated in visceral tissue; and
- "Amplification" of 17D* yellow fever virus in visceral tissue.

3.5.4 Definition of Probable Case of Yellow Fever Vaccine-associated Neurotropic Disease:

All cases vaccinated within 30 days prior to onset of symptoms with no evidence of other etiologies that clinical symptoms could be attributed to; with fever, severe headache, focalized or generalized neurological symptoms, or any of the following:

- Cerebrospinal fluid (CSF) with signs of viral infection (pleocytosis, primarily mononuclear);
- Magnetic resonance with signs of multifocal demyelinization;
- Abnormal electroencephalogram compatible with encephalopathy; and
- Electromyography with signs of demyelinization

3.5.5 Definition of Confirmed Case of Yellow Fever Vaccine-associated Neurotropic Disease:

All probable cases with the following:

- 17D* yellow fever virus isolated in blood more than 7 days after vaccination and/or by PCR more than 11 days after vaccination;
- Viral load of 17D* yellow fever virus in serum greater than 10^3 PFU/mL recorded on any day;
- Yellow fever specific antigen in nerve tissue or CSF detected by IHC;
- 17D* yellow fever virus isolated in nerve tissue or CSF;
- PCR positive for 17D* yellow fever virus in nerve tissue or CSF; and
- Yellow fever IgM detected in CSF.

4. Procedures

Case-finding in the study was conducted by searching active institutional records of hospital discharges (emergency, hospitalization, referrals) and death records in the municipalities (See Flow Chart 1-Conceptual Diagram, page 39).

* Virus confirmed as 17D by monoclonal antibody analysis or nucleotide sequencing in cases with possible wild-type YF virus, including all vaccines in 17D lineage.

4.1 Active Institutional Case Finding:

1. Active case-finding for viscerotropic and neurotropic disease was conducted in selected hospitals by reviewing the records for health care provided between the date the yellow fever vaccination campaign began and 30 days after the end of the campaign. Two activities were conducted:
   - The list of patients treated was obtained from the database (statistics) of discharges between 23 September and 6 November 2007, and from the health care records in these departments.
   - Medical histories were located for all patients who received emergency care or were hospitalized during this period, as well as patients transferred to more complex facilities.
2. Patients with clinical symptoms who met the selection criteria (See 3.5.1) were selected. The medical history of each patient was reviewed and the case selection card filled out.
3. If the patient met the selection criteria, the investigation card was filled out and a photocopy was made of the complete medical history of each case. In addition, for each case selected, two controls were identified and copies were made of the medical history of these patients (See 3.3.1).
4. All investigation cards and medical histories of patients who met the selection criteria were sent to the General Bureau of Epidemiology in Lima for review to determine whether they met the criteria for probable or confirmed cases of viscerotropic and neurotropic disease (See 3.5.2–3.5.5).
5. The history of yellow fever vaccination was then verified. The National Health Strategy for Immunization was in charge of preparing a database with information on the persons vaccinated against yellow fever in Ica during the campaign.

4.2 Case Finding in Health Records:

1. This was conducted by reviewing the death records in the municipalities. Patients who died between 23 September and 6 November 2007 were included in the investigation.
2. The register of death certificates and death records in the records office of the provincial municipalities was reviewed.
3. If the patient died in a hospital or health care facility, a search for his/her medical history was conducted. Otherwise, the verbal autopsy of the patient was obtained. This entailed visiting the physician who filled out the death certificate and the family of the deceased. The date of yellow fever vaccination was also verified.
4. Finally, it was confirmed whether the patient met the case definitions defined for this study.

5. Results

On 13 November 2007, a meeting was held with the Ica regional health authorities and the directors of the health care facilities selected for this study. Five working groups were formed, one for each province in the Ica region: Chincha, Ica, Nazca, Palpa, and Pisco. Staff from the General Bureau of Epidemiology, regional and local epidemiology offices, EsSalud, and NMRCD participated, with the support of a representative from PAHO and another from the U.S. CDC. Four of the teams worked on information gathering from 13 to 16 November. Only the team for the Pisco province worked from 20 to 23 November.

The case-finding results are shown in Tables 2 and 3.

Table 2. Records Reviewed by Province and Hospital, Ica, Peru, 2007

Province/Hospital		Hospitalization	Emergency	Transfers	Total
Chincha	Hospital San José	130	3330	18	3478
	Hospital EsSalud RTG	347	5864	214	6425
	Policlínico Policía	-	329	-	329
	Deaths				55
	Total	477	9523	232	10287
Ica	Hospital Regional	227	5200	26	5453
	Hospital Socorro	40	3234	3	3277
	Hospital EsSalud FT	433	556	14	1003
	Deaths				103
	Total	700	8990	43	9836
Nazca	Hospital de Apoyo Nazca	1164	3686	125	4975
	Deaths				8
	Total	1164	3686	-	4983
Palpa	Hospital de Apoyo Palpa	24	455	-	479
	Deaths				1
	Total	24	455	-	480
Pisco	Hospital San Juan de Dios	389	2435	42	2866
	Policlínico EsSalud	123	-	84	207
	Hospital Cubano 1	155	-	-	155
	Hospital Cubano 2	118	-	-	118
	Deaths				66
	Total	785	2435	126	3412

Table 3. Hospital Records Reviewed and Cases Identified, Ica, Peru, 2007

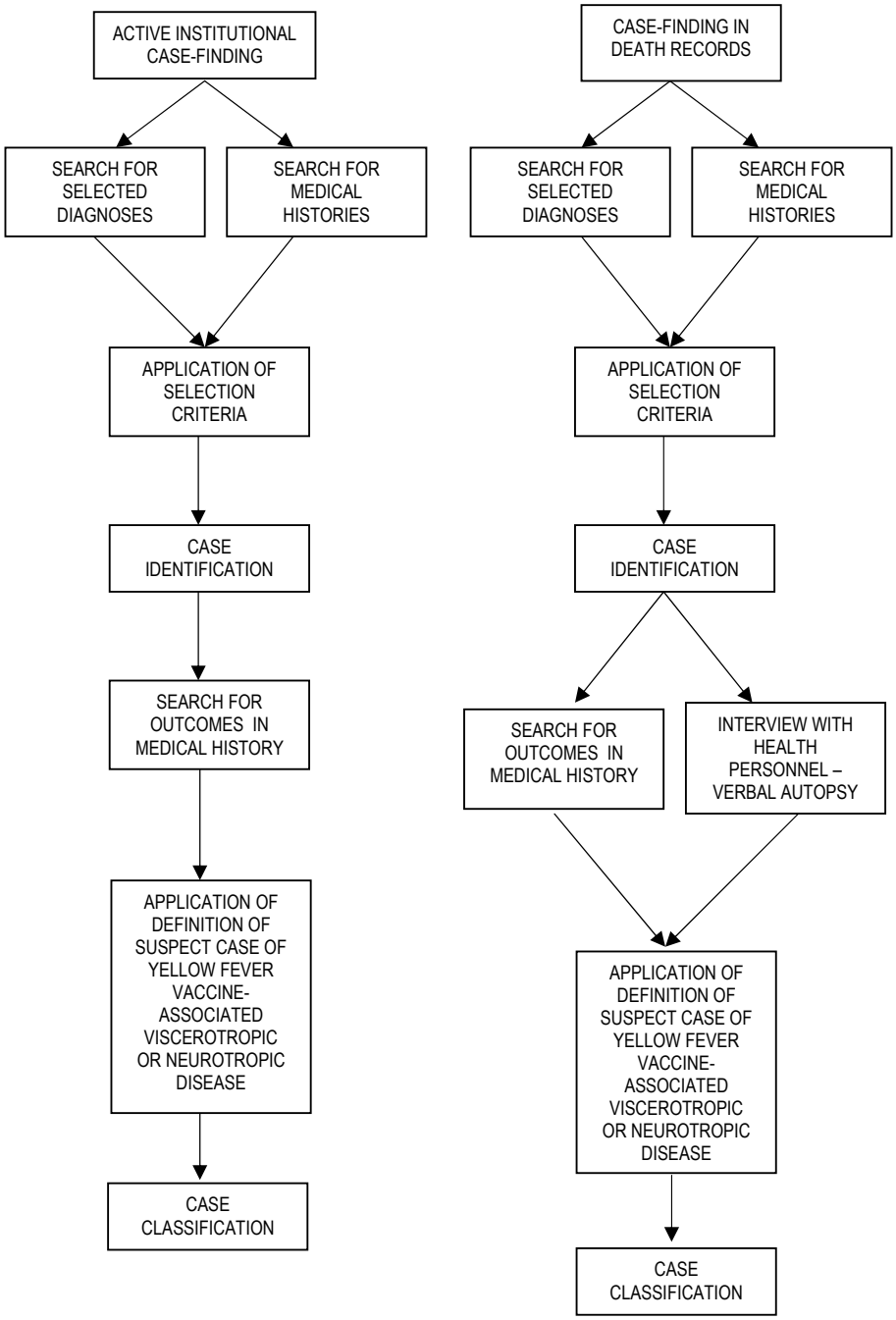
Province	Chincha	Ica	Nazca	Palpa	Pisco
Total	10232	9710	5020	480	3346
Suspected	180	33	45	3	50
Probable	3	2	0	0	0
Confirmed	2	2	0	0	0

A total of 28,788 medical records were reviewed from hospitals in the 5 provinces of Ica. A total of 311 cases that met selection criteria were identified. Five of these cases met the criteria for probable cases. Four out of 5 of these cases were identified by ESAVI surveillance. All of these cases were finally associated with yellow fever vaccine. The fifth case was not found in the list of vaccinated persons. It was a 10-year-old girl from the province of Chincha who visited Hospital San José de Chincha for symptoms of generalized jaundice, dry cough, high temperature, and vomiting. She was admitted with probable diagnosis of viral hepatitis and pharyngitis. The laboratory tests showed elevated bilirubin (total bilirubin: 5.14 mg/dL, direct bilirubin: 0.59 mg/dL, indirect bilirubin: 4.55 mg/dL), particularly indirect bilirubin, and anemia (9.8 g/dL). Transaminase profile and coagulation profile were within normal range. Hepatitis A, B and C test results were negative.

6. Conclusion

Case-finding identified only cases previously detected by the ESAVI epidemiological surveillance system. Other probable or confirmed cases of yellow fever vaccine-associated viscerotropic or neurotropic disease in the population in the Ica region who received medical treatment at hospitals in this region have not been identified.

FLOW CHART 1: STUDY CONCEPTUAL DIAGRAM



References

1. Ministerio de Salud, Dirección General de Epidemiología, Dirección Ejecutiva de Vigilancia Epidemiológica. NOTI 99 [Electronic database updated weekly]. Lima: Ministerio de Salud, 2007.
2. Case definitions. Yellow fever vaccine-associated adverse events. 2004.
3. Ministerio de Salud, Dirección General de Epidemiología, Instituto Nacional de Salud, Dirección General de Salud de las Personas, Dirección General de Medicamentos Insumos y Drogas.
4. CDC. Yellow fever vaccine recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR* 2002;51(RR17):1-10.
5. Vellozzi C, Mitchell T, Miller E, Casey C, Eidex R, Hayes E, Yellow fever vaccine safety working group. Yellow fever vaccine-associated viscerotropic disease (YEL-AVD) and corticosteroid therapy: eleven United States cases, 1996-2004. *Am J Trop Med Hyg* 2006;75(2):333-6.
6. Ministério da Saúde. Fundação Nacional de Saúde. Eventos Adversos sérios Associados com a vacina 17D Contra Febre Amarela.

Annex 4

Report of the Virology Subcommittee Investigating ESAVIs in Ica Department, Peru, September-October 2007

Alan D.T. Barrett,
University of Texas Medical Branch at Galveston, USA

Gwong-Jen J. Chang,
Centers for Disease Control and Prevention, Fort Collins, USA

Ricardo Galler,
Fio Cruz, Brazil

Robert S. Lanciotti,
Centers for Disease Control and Prevention, Fort Collins, USA

Thomas P. Monath,
Kleiner Perkins Caufield & Byers, Harvard, USA

Report of the Virology Subcommittee Investigating ESAVIs in Ica Department, Peru, September-October 2007

Summary

Four individuals died in Ica Department, Peru, in October 2007 following administration of 17DD vaccine lot 050-VFA-121Z manufactured by Bio-Manguinhos. Although the virus in the vaccine lot 05-VFA-121Z was not sequenced, genomic sequences of viruses isolated from cases #1 and #2 were indistinguishable from the 102/84 secondary seed sequence published by Galler et al. (Phenotypic and molecular analyses of yellow fever 17DD vaccine viruses associated with serious adverse events in Brazil. Virology. 2001 Nov 25;290(2):309-19). Taken together with partial genomic sequence data for case #3, there is no evidence to suggest that a variant virus in lot 05-VFA-121Z or enriched in the patients was responsible for the deaths following immunization. Examination revealed a wide tissue distribution of virus (including many vital organs) in the vaccinees who died, high viremia, and a high virus load (in lung, Case #1), consistent with previous reports of cases who had died following yellow fever immunization. Studies on stability of lot 05-VFA-121Z indicate that the virus had not lost any significant viability even though the virus was close to its expiry date (October 2007) and had been transported multiple times between countries. Evidence was obtained to suggest that there is heterogeneity of the vaccine virus RNA population in each of three 17DD vaccine lots. The significance of these data is difficult to evaluate but previous studies have shown heterogeneity in yellow fever 17D-204 and 17DD vaccine virus populations, and there was no evidence that any particular virus in the vaccine population has been enriched in vaccinees. Overall, the results of studies to date suggest that the cold chain was maintained for lot 05-VFA-121Z and the same lot contained 17DD vaccine indistinguishable from 17DD vaccine in other lots used since 1984. Finally, there was no apparent selection of a variant in the vaccinees who died following immunization with this lot. In summary, within the limits of the testing performed, there is no evidence for a change in the vaccine virus that could explain the ESAVIs.

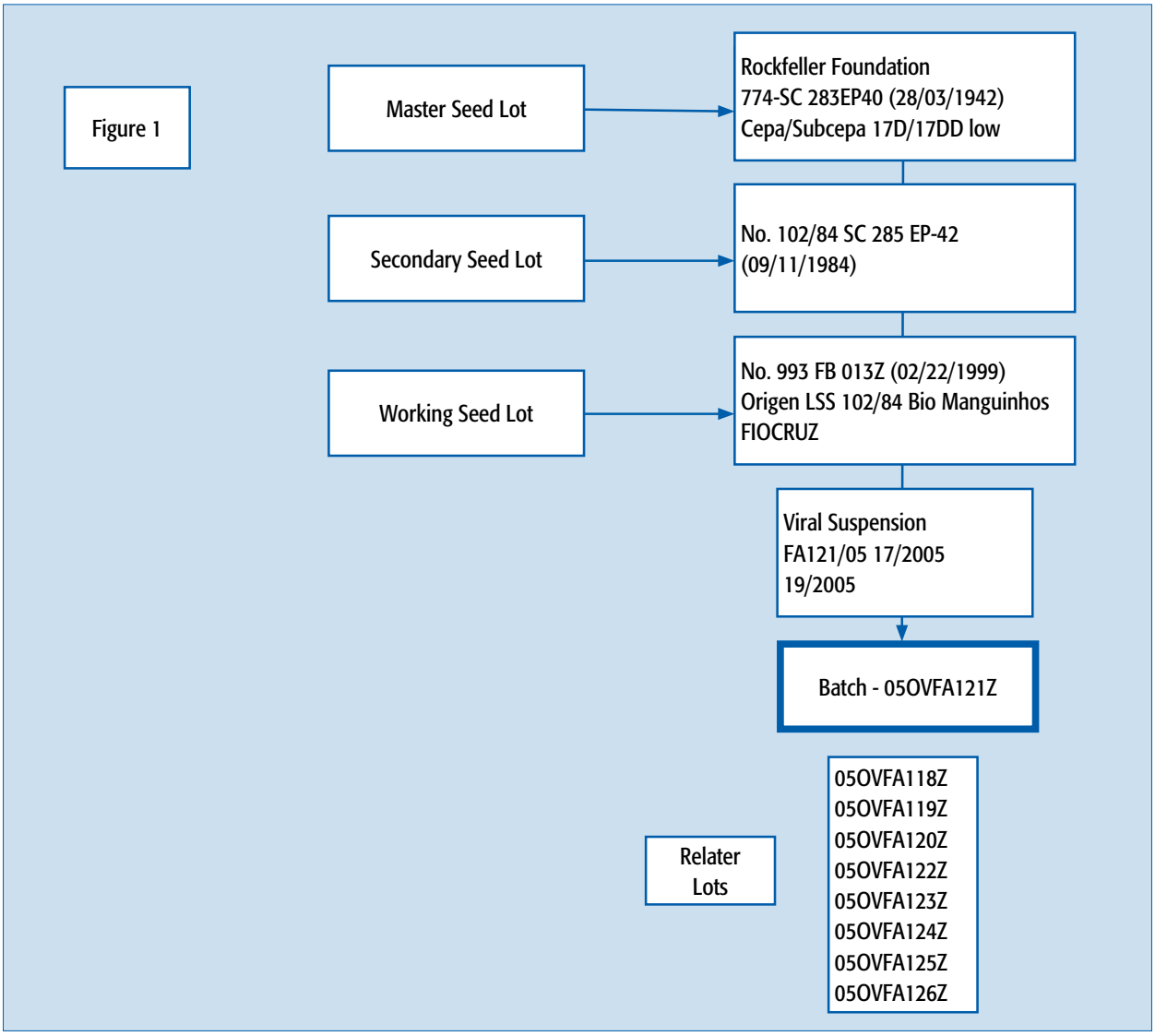
Nevertheless, it is acknowledged that the association of all 4 fatal cases with one lot of vaccine (05-VFA-121Z) while no such cases were detected in another lot of vaccine used at the same time in Ica is unexplained. About 42,742 people were given the implicated (050VFA121Z) lot of vaccine in Ica. An additional 20,432 people were vaccinated in Ica with a different lot (050VFA123Z), and no deaths were reported in this group. The incidence of YEL-AVD associated with the 121Z lot is not statistically significant from that in the other lot (p=0.2095, Fisher's exact test, one-way), and it is therefore likely that the association with one lot was by chance.

However, the incidence of YEL-AVD in Ica is significantly higher than observed in previous episodes, where the overall rate has approximated 0.3 per 100,000. The probability of seeing four cases after 43,000 doses is 0.00001. Since there was no statistical difference in incidence across lots, and the genomic sequence had not changed, the most likely explanation of the events in Peru would appear to be one or more of the following as potential contributors in the fatal cases: host factors that increase susceptibility to YF 17DD virus, some unidentified adventitious agent, concomitant infections, or previous circulation of other unknown agents in the earthquake-affected area. Clearly, these remain uncertain, However, it should be noted that the vaccine lots underwent QC/QA by the manufacturer in which assays for bacteria, fungi, mycoplasma, and a number of known avian virus adventitious agents (but not mammalian viruses) in the vaccine preparations were checked and were found to be negative.

In conclusion, the virologic studies undertaken following the deaths do not identify a role for the vaccine virus in the deaths, based on the studies undertaken to date. There are a number of additional studies that should be considered to continue investigation of these ESAVIs following yellow fever immunization to generate hypotheses to explain the cluster and this subcommittee would like to contribute to such studies. In addition, there needs to be an organized collection and storage of samples from all cases of yellow fever ESAVIs in order that systematic and consistent research studies can be applied to all cases so that data are comparable between cases. It is only in this way that we will understand the mechanism of the severe ESAVIs.

Introduction

This report describes yellow fever virologic studies undertaken as part of the investigation of four deaths in October 2007 following immunization with the 17DD vaccine manufactured by Bio-Manguinhos. It should be emphasized that the studies were limited to investigation of the 17DD vaccine virus to determine whether or not there were any differences in the vaccine virus administered to the vaccinees who died or in the vaccine virus that multiplied in the vaccinees. Other than standard tests by the manufacturer during production of the vaccine (which revealed nothing out of the normal), no studies were undertaken to look for adventitious agents or other infectious agents as potential co-factors in the fatal cases. All four deaths occurred in Ica Department in Peru following immunization with one particular lot of vaccine: 050-VFA-121Z. Note: a different lot, 050-VFA-123Z, was also used in the same Department with no serious ESAVIs associated with this lot. The pedigree of lot # 050-VFA-121Z is shown in Figure 1.



The four individuals who died after receiving the vaccine are as follows:

Case #1: 23 year-old woman

Case #2: 24 year-old woman

Case #3: 79 year-old man

Case #4: 49 year-old woman

Laboratories Involved in the Virologic Studies

Initial studies were undertaken in Peru by the Instituto Nacional de Salud (INS) and the United States Naval Medical Research Center Detachment (NMRCD). Subsequently, samples were sent to the United States Centers for Disease Control and Prevention in Atlanta (CDC-ATL) and some were forwarded to the CDC Division of Vector Borne Infectious Diseases at Fort Collins (CDC-FC). In addition five lots of vaccine were sent from INS to CDC-FC (Table 1).

Table 1: Yellow Fever Vaccine Received at the CDC from INS

Number	Product	Lot Number
1	Lyophilized Vaccine	050-VFA-119Z
	Diluent	057-DFA-053Z
2	Lyophilized Vaccine	050-VFA-121Z
	Diluent	058-DFA-056Z
3	Lyophilized Vaccine	050-VFA-123Z
	Diluent	057-DFA-053Z
4	Lyophilized Vaccine	050-VFA-124Z
	Diluent	057-DFA-053Z
5	Lyophilized Vaccine	050-VFA-125Z
	Diluent	057-DFA-053Z

Nucleotide Sequencing Studies of Samples from Patients

The first virologic studies were undertaken in Peru where INS and NMRCD utilized RT-PCR and began to isolate virus from clinical samples. Both laboratories used RT-PCR based on the envelope protein gene and obtained consensus sequence data indicating the presence of 17DD vaccine virus RNA in samples from cases # 1 and #2.

Subsequent studies at CDC-FC resulted in the determination of the consensus nucleotide sequences of the full-length genome for some samples (including complete genomic sequences for cases # 1 [from lung] and #2 [from liver]) (Table 2).

Table 2: Comparison of Consensus Genomic Sequences of Cases #1 and #2 with Published Genomic Sequences of 17DD Vaccine in Genbank and the Bio-Manguinhos Secondary Seed 102/84						
Nucleotide	Original 17DD GenBank U17066	102/84 Secondary seed from Table 1 Galler et al. Virology 2001	CASE 1 25% mixed analysis	Comment	CASE 2 25% mixed analysis	Comment
1003	T	T/C	T	C visible at 10%	T/C	
2110	A	G	G		G	
2356	T	C	C		C	
2677	C/T	T	T		T	
4523	C/T	C/T	C/T (1:1)		C/T	
4921	G	G			A	SILENT
4948	C	C	C		C	T visible at 20% in 2 of 6 reads. SILENT
5362	A	C	C		C	
6673	T	T*	C/T (1:1)	silent	C/T	
9988	C	C/T	C	C visible at 10%	C/T	
10,174	A	A/G	A	G visible at 10%	R	
10,243	A/G	G	G		G	
10,291	C	C	C		Y	
10,367	C/T	T	T		T	
10,675	A	A/G	A/G		A/G	

* Marchevsky reports C/T at this position for the secondary seed.

The results are summarized as follows:

Case # 1: The full-length consensus genetic sequence of virus RNA in the lung from the first case is indistinguishable from the 102/84 secondary seed sequence published by Galler et al. Note: there is an isolate from case #1 but this has not been sequenced.

Case #2: The full-length consensus genetic sequence of virus RNA sequenced directly from liver tissue is indistinguishable from the 102/84 secondary seed sequence published by Galler et al. with the exception of one silent mutation at nucleotide position 4921 (GA; NS3).

Case #3: It was only possible to RT-PCR a portion of the genome; many attempts were made from all available tissues. Only partial sequence data for case #3; 2870 bases (nucleotide positions 1550 to 2870 and 6235 to 8435) were generated. In these regions the sequence is indistinguishable from the 17DD secondary seed lot.

Case #4: No samples available for characterization.

It should be noted that the term "indistinguishable" is used as the nucleotide sequence appears identical but potential variation in a minority of the RNAs in the population (probably less than 10%) of viruses in the vaccine cannot be excluded.

Extensive studies were undertaken characterizing virologic aspects of the four cases and these are summarized in Tables 3-6.

PFUeq/ml was determined by parallel testing dilutions of YF 17D virus by plaque assay and real-time RT-PCR. The data are utilized to construct a standard curve which can estimate the number of pfu present in the original samples. This approach assumes that there is a fairly constant ratio of pfu to viral copy number between YF 17D preparations. However we have observed that this ratio can vary by as much as a factor of 10; therefore the PFUeq/ml could be off by a factor of 10.

Table 3: Virologic Aspects of Case 1

Case 1 - 23yo F

Date of Vaccination: 27 September 2007
Date of Illness Onset: 28 September 2007
Date of Death: 6 October 2007

Yellow Fever Testing			Test Result by Location			
Laboratory Test	**Sample**	**Sample Date**	**NIH - Peru**	**NMRCD - Peru**	**CDC**	**CDC**
ELISA IgM	Serum	6-Oct-07	Positive	Negative	Positive	
ELISA IgG	Serum	6-Oct-07	Negative	Negative	Negative	
PRNT	Serum	6-Oct-07	NA	NA	160	
						Quantitative PFUeq/ml
RT-PCR	Serum	6-Oct-07	Positive	NA	Positive	3924000
	Urine	6-Oct-07	Positive	NA	Positive	68
	Lung	6-Oct-07	Positive	NA	Positive*	7600000
	Liver	6-Oct-07	Positive	NA	Positive	11600
	Kidney	6-Oct-07	Positive	NA	Positive	350000
	Brain	6-Oct-07	Positive	NA	Positive	39320
Culture	Serum	6-Oct-07	Positive	NA	Positive	
	Urine	6-Oct-07	NA	NA	NG - Final	
	Lung	6-Oct-07	NA	NA	NG - Final	
	Liver	6-Oct-07	Positive	NA	NG - Final	
	Kidney	6-Oct-07	NG-Final	NA	NG - Final	
	Brain	6-Oct-07	Positive	NA	Positive	
	Spleen	6-Oct-07	NG-Final	NA	NA	
Histopathology - IHC staining	Lung	6-Oct-07	NA	NA	Positive - abundant	
	Liver	6-Oct-07	Positive	NA	Positive - abundant	
	Kidney	6-Oct-07	NA	NA	Positive - abundant	
	Brain	6-Oct-07	NA	NA	Positive - rare	

NA = Not applicable (did not test) NG - final = No growth final

* Sequence of RNA from lung tissue is identical to the reference sequence for 17-DD.

Table 4: Virologic Aspects of Case 2

Case 2 - 24yo F

Date of Vaccination: 27 September 2007
Date of Illness Onset: 27 September 2007
Date of Death: 11 October 2007

Yellow Fever Testing			Test Result by Location			
Laboratory Test	**Sample**	**Sample Date**	**NIH - Peru**	**NMRCD - Peru**	**CDC**	**CDC**
ELISA IgM	Serum	8-Oct-07	NA	Positive	Positive	
	Serum1	5-Oct-07	Positive	NA	NA	
	Urine	5-Oct-07	Positive	NA	NA	
ELISA IgG	Serum	8-Oct-07	NA	Negative	Negative	
	Serum1	5-Oct-07	Negative	NA	NA	
	Urine	5-Oct-07	Negative	NA	NA	
PRNT	Serum	8-Oct-07	NA	NA	10240	
						Quantitative PFUeq/ml
RT-PCR	Serum	5 or 8-Oct-07	Positive*	Negative	Positive	262
	Urine	5-Oct-07	Positive	NA	Positive	956
	Lung	11-Oct-07	Positive	Negative	Positive	464
	Liver	11-Oct-07	Positive	Positive	Positive***	10960
	Kidney	11-Oct-07	Positive	Negative	Positive	247
	Brain	11-Oct-07	Positive	Positive**	Positive	4160
	Spleen	11-Oct-07	NA	Negative	NA	NA
	Blood	11-Oct-07	NA	Negative	NA	NA
Culture	Serum	5 or 8-Oct-07	NG - Final	NG - Final	NG - Final	
	Urine	5-Oct-07	NA	NA	NG - Final	
	Lung	11-Oct-07	NG - Final	NG - Final	NG - Final	
	Liver	11-Oct-07	NG - Final	NG - Final	NG - Final	
	Kidney	11-Oct-07	NG - Final	NG - Final	NG - Final	
	Brain	11-Oct-07	NG - Final	NG - Final	NG - Final	
	Spleen	11-Oct-07	NA	NG - Final	NA	
Histopathology - IHC staining	Lung	11-Oct-07	NA	NA	Positive - rare	
	Liver	11-Oct-07	Negative	NA	Positive - rare	
	Kidney	11-Oct-07	NA	NA	Positive - rare	
	Brain	11-Oct-07	NA	NA	Negative	

NA = Not applicable (did not test) NG - final = No growth final

* Sequence of serum PCR fragment consistent with glycoprotein E of yellow fever virus.
** Sequence of brain PCR fragment 100% identical to 17-DD in the E region.
*** Sequencing of RNA from tissue is identical to 17-DD reference strain except for one silent nucleotide change.

Table 5: Virologic Aspects of Case 3

Case 3 - 79yo M

Date of Vaccination: 1 October 2007
Date of Illness Onset: 4 October 2007
Date of Death: 12 October 2007

Yellow Fever Testing			Test Result by Location			
Laboratory Test	**Sample**	**Sample Date**	**NIH - Peru**	**NMRCD - Peru**	**CDC**	**CDC**
ELISA IgM	Serum	8-Oct-07	Negative	Positive	Positive	
ELISA IgG	Serum	8-Oct-07	Negative	Negative	Negative	
PRNT	Serum	8-Oct-07	NA	NA	QNS	
						Quantitative PFUeq/ml
RT-PCR	Serum	8-Oct-07	Positive	Negative	Positive	192
	Lung	12-Oct-07	Positive	Negative	Positive	2084
	Liver	12-Oct-07	Positive	Negative	Positive*	35360*
	Kidney	12-Oct-07	Positive	Negative	Positive**	27160
	Brain	12-Oct-07	NA	Negative	Positive	264
	Spleen	12-Oct-07	Positive	Negative	NA*	NA*
Culture	Serum	11-Oct-07	NG - Final	NG - Final	NG - Final	
	Lung	12-Oct-07	NA	NG - Final	NG - Final	
	Liver	12-Oct-07	NG - Final	NG - Final	NG - Final*	
	Kidney	12-Oct-07	NG - Final	NG - Final	NG - Final	
	Brain	12-Oct-07	NG - Final	NG - Final	NG - Final	
	Spleen	12-Oct-07	NG - Final	NG - Final	NA*	
Histopathology - IHC staining	Lung	12-Oct-07	NA	NA	Negative	
	Liver	12-Oct-07	NA	NA	Positive	
	Kidney	12-Oct-07	NA	NA	Negative	
	Brain	12-Oct-07	NA	NA	Negative	
	Spleen	12-Oct-07	NA	NA	Positive - rare	

NA = Not applicable (did not test) NG - final = No growth final

* Note: Fixed tissue received by the CDC labeled "liver" was spleen. Therefore, it is uncertain if the frozen tissue used in the RT-PCR testing and culture at the CDC was liver versus spleen.

** CDC only able to RT-PCR a portion of the genome; many attempts were made from all available tissues, with results coming from the kidney. Partial sequence data included 2870 bases (nucleotide positions 1550 to 2870 and 6235 to 8435). The sequence in these regions was identical to the 17DD secondary seed lot.

Table 6: Virologic Aspects of Cases 4

Case 4 - 49yo F

Date of Vaccination: 24 September 2007
Date of Illness Onset: 1 October 2007
Date of Death: 24 October 2007

Yellow Fever Testing			Test Result by Location			
Laboratory Test	**Sample**	**Sample Date***	**NIH - Peru**	**NMRCD - Peru**	**CDC**	**CDC**
ELISA IgM	Serum	24-Oct-07	Positive	Positive	Positive	
ELISA IgG	Serum	24-Oct-07	Negative	Negative	**	
PRNT	Serum	24-Oct-07	NA	NA	>20480	
						Quantitative PFUeq/ml
RT-PCR	Serum	24-Oct-07	Positive	Negative	Negative	Negative
	Lung	24-Oct-07	NA	NA	NA	NA
	Liver	24-Oct-07	Positive	Negative	Very weak positive	3.3/4.8
	Kidney	24-Oct-07	NA	Negative	Positive	10464
	Brain	24-Oct-07	NA	Negative	Equivocal	NA
Culture	Serum	24-Oct-07	NG - Final	NG - Final	NA***	
	Lung	24-Oct-07	NA	NA	NA	
	Liver	24-Oct-07	NG - Final	NG - Final	NA***	
	Kidney	24-Oct-07	NG - Final	NG - Final	NA	
	Brain	24-Oct-07	NG - Final	NG - Final	NA	
	Spleen	24-Oct-07	NG - Final	NA	NA	
Histopathology - IHC staining	Lung	24-Oct-07	NA	NA	Negative	
	Liver	24-Oct-07	Positive	NA	Negative	
	Kidney	24-Oct-07	NA	NA	Positive - abundant	
	Brain	24-Oct-07	NA	NA	Negative	

NA = Not applicable (did not test) NG - final = No growth final

* Samples were retrieved on 25 October 2007; however, the patient died on 24 October 2007.
** Uninterpretable due to high background.
*** Due to the very low copy number or lack of virus detected by quantitative RT-PCR, cultures from the specimens were not attempted.

Note: CDC unable to obtain enough RNA from tissues for sequencing.

Stability of Virus in Lot # 050-VFA-121Z

Studies were also undertaken by Bio-Manguinhos (using current S.O.P required for pre-qualification by WHO) and CDC-FC to look at stability of virus in lot # 050-VFA-121Z.

Bio-Manguinhos found that there was no significant loss in infectivity (shown as log10 pfu) of samples of the lot held by the manufacturer.

	Date for 11-8-05	Date for 12-4-07
2-8ºC	5.16/dose	4.90/dose
37° for 14 days	4.75/dose	4.70/dose

CDC-FC took unopened vials of vaccine provided by INS in November 2007, reconstituted one vial and assayed infectivity in Vero cells. The results were as follows (log10 pfu/ml):

Lot # 050-VFA-119Z: 4.96 pfu/ml

Lot # 050-VFA-121Z: 5.33 pfu/ml

Lot # 050-VFA-123Z: 4.87 pfu/ml

Lot # 050-VFA-124Z: 5.08 pfu/ml

Lot # 050-VFA-125Z: 4.97 pfu/ml

While the results obtained in the two laboratories cannot be directly compared, the results indicate that the lots of vaccine had the titers within the potency specifications, even though the vaccine had an expiry date of October 2007. In addition, the results indicate that the cold chain had been maintained for lot # 050-VFA-121Z, as it had been moved to multiple locations in Venezuela, Bolivia, and Peru prior to being used in Ica Department.

Heterogeneity in Viral RNAs Within the Vaccine Population

Clonal sequencing was performed at CDC-FC from viral RNAs extracted directly from three vaccine lots (050-VFA-119Z, -121 and -123). Extracted RNAs were amplified by RT-PCR using a high-fidelity commercial kit and cloned into Invitrogen's TA vectors. Ninety-six white colonies from each transformation were randomly picked; and recombinant plasmids, amplified directly from E. coli colonies, were used as templates for sequencing by an extremely high-fidelity rolling circle enzyme (phi-29 polymerase).

Clonal sequencing focused on the E protein gene (with some of the NS1 gene) only, between genomic nucleotides 1249 and 2646. Between 75 and 78 randomly selected clones were sequenced from each lot. Nucleotide changes were counted for all clones and categorized as leading to an amino acid substitution or not (silent mutation). A summary of the clonal sequencing results is listed in Table 7.

Table 7: Results of Clonal Sequencing for Three Yellow Fever Vaccine Lots

Lot	# of clones sequenced	# (and %) of variant clones/lot	Nucleotide substitutions			
			Total #	# of AA substitution	# of silent mutations	% of AA substitution/ total #
119	75	43 (57)	119	50	69	42
121	78	57 (73)	121	54	67	48
123	76	37 (49)	123	35	88	28

This study found that the percentage of variable sites identified by clonal sequencing for any one vaccine lot was between 8.5 and 8.9%. However, if the total number of nucleotides sequenced are taken into account [# of nucleotide changes/(# of clones sequenced*1397 base pair)], 0.11% of nucleotides for lots 119 and 121, and 0.12% nucleotides for lot 123 had a nucleotide change. The overall percentage of amino acid substitutions [# of amino acid substitutions/(# of clones sequenced*466 amino acids)] was 0.14% for lot 119, 0.15% for lot 121, and 0.10% for lot 123.

The raw data for every clone sequenced can be found in the appendix. The maximum number of nucleotide changes observed in any one clone was 7 (0.5% nucleotides changed) seen in Lot 119 Clone 11E. The maximum number of amino acid substitutions per clone was 4 (0.86% of AA changed) seen with four clones in two lots (Lot 119 clones 6F and 11E and Lot 121 clones 3D and 4B). The most commonly mutated site was at position A2093 which was mutated in six clones (all in lot 119). There were 16 nucleotide positions where the same mutation was noted in more than one vaccine lot.

Interestingly, one clone in Lot 121, clone 3D, had two amino acid (AA) substitutions in regions which have been noted to be important B-cell epitopes: E-AA position 104 – B cell epitope recognized by flavivirus group-reactive monoclonal antibodies (such as 4G2, 6B6C-1) and E-AA position 153 – an residue identified by the neutralizing escape variant study using human monoclonal antibodies derived from 17D vaccinees. An amino acid substitution in E-AA 153 was also previously noted in another viscerotropic case (Martin et al. Lancet 2001 358: 98-104). Neither of two AA substitutions was identified in lot 119 or 123.

While the population of variants within the implicated Lot 121 differs from the other lots sequenced, the fact remains that the consensus sequence of isolates from vital organs of two fatal cases failed to reveal that the subpopulations representing the mutations at E104 or E153 dominated, indicating that these mutants had not become dominant in vivo and were therefore unlikely to be responsible for the pathological events. It is generally accepted that consensus sequencing is sensitive enough to reveal the presence of a mutant virus at 10% of the total virion population. Thus, at present there is no evidence that the apparent mutants identified in vitro exist in vivo, including the tissues from patients. It is unknown whether a minority virus population, even if virulent, could cause disease expression.

Limitations of Clonal Sequencing

It is recognized that there are limitations to clonal sequencing, including the following:

- Process of clonal sequencing could lead to the introduction of nucleotide changes;
- Inability to address whether changes noted could lead to phenotypic changes or all the viral RNAs are found in infectious virus; and
- The sequence is just of the E protein and did not explore other areas of the gene which could influence the virulence of virus

Summary of Clonal Sequencing

Data from clonal sequencing of the E protein gene from three vaccine lots indicate that variant clones are present in each lot (49-73% of clones) and roughly 9% of the sequenced region contains variant sequences. Heterogeneity has been identified previously in yellow fever vaccine viruses. The genomic sequence data obtained from patients' samples suggests that none of the apparent variants in the vaccine population was enriched in the patients to a significant level that was identifiable by direct sequencing.

Potential for a Different Population of Viruses to Segregate into One Vaccine Batch

The Head of yellow fever vaccine production at Bio-Manguinhos summarized the situation as follows: assume a routine production round in which about 2,400 embryonated eggs are inoculated with a suspension of the seed lot. After incubation embryos are collected in groups of 40 and homogenized. Every two groups of 40 are pooled and centrifuged. The supernatant is aspirated into a flask, which, after addition of stabilizer, will contain approximately 450mL of suspension, the equivalent to 80 embryos. Therefore, the inoculation of 2,400 eggs will yield at the end of the day 30 such flasks, each of which is tested for potency and sterility. This group of 30 flasks is coded and constitutes the intermediate viral suspension and only after QC releases the potency/sterility data they can be used for further processing. The minimum size of a vaccine batch will use 6 to 7 such flasks, which are pooled and formulated before filling and freeze-drying. The processing of 6-7 flasks of the intermediate viral suspension will provide over 40,000 5-dose vials. Therefore, a single fill lot will contain virus from 420-560 embryos.

It is evident that virus from a single "bad" egg could wind up segregated to one fill lot. Consequently the amount of "bad" virus

(a population from one egg with a significant nucleotide/amino acid change with regard to virulence) per dose would be ~50 PFU, assuming that the virus from one egg corresponds to roughly 0.17% of the total virus in a lot made up with 560 embryos and a human dose of 4.5 log10 PFU.

Discussion

Lot 050-VFA-121Z was given to 42,742 individuals in Ica Department in Peru and, unfortunately, four vaccinees died. The four were not located in one specific area of the Department and resided in three of the five provinces in the Department. In addition, 20,432 individuals in Ica Department received lot 050-VFA-123Z and none had reported severe ESAVIs. This is the first time a cluster of deaths has been reported following administration of one particular lot of yellow fever vaccine. Previous studies of ESAVIs suggest that the incidence of a death following administration of yellow fever vaccine is 0.3 per 100,000 while this cluster represents a ratio of 10 in 100,000. The probability of this taking place is 0.00001 suggesting that there are factors unique to this situation in Peru. The hypothesis currently used to explain the deaths is a role of the host (genetics or immune response) but this is not consistent with the cluster seen in Peru. The virologic studies undertaken following the deaths do not identify a role for the vaccine virus in the deaths based on the studies undertaken to date. While there are many discussion points that could potentially generate hypotheses to explain the cluster, the major point is the limited research undertaken to investigate the yellow fever ESAVIs and there is a clear need to develop a research agenda to investigate such cases, including further studies on the Peru cases. This subcommittee would like to contribute to such studies. In particular, there needs to be an organized collection and storage of samples from all cases of yellow fever ESAVIs in order that systematic and consistent research studies can be applied to all cases so that data are comparable between cases. It is only in this way that we will understand the ESAVI mechanism.