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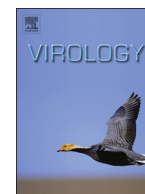
Effect of time at temperature on wild poliovirus titers in stool specimens

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ABSTRACT

Background: The effect of transport temperature on the viability of poliovirus in stool specimens from paralyzed cases has not been tested. Quality assurance of programmatic indicators will be necessary in the final phase of polio eradication.
Objective: To estimate the effect of time at elevated temperatures on wild poliovirus titers in stool specimens.
Methods: We exposed aliquots of pooled wild poliovirus type 1 specimens to elevated temperatures (27 °C, 31 °C, and 35 °C) for varying time periods up to 14 days. We determined the virus titer of these aliquots and created decay curves at each temperature to estimate the relationship between time at temperature and virus titer.
Results: We found significantly different slopes of decay at each temperature. The negative slopes increased as the temperature increased.
Conclusions: While poliovirus in stool remains relatively stable at moderately elevated temperature, transport at higher temperatures could impact sample integrity and virus isolation results.

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Introduction

The Global Polio Eradication Initiative (GPEI) is a remarkable public health and scientific venture. Success with poliovirus will make it the second human pathogen, after smallpox, to be eradicated from the natural world. To achieve the goal of eradicating all 3 wild polioviruses (WPV), the program relies on surveillance, leading to the timely investigation of infants, children and other persons with a clinical syndrome of acute flaccid paralysis (AFP). AFP surveillance entails the detection of any case of AFP in a child under 15 years and any case of any age where the physician suspects polio (WHO, 1996). AFP surveillance systems in endemic regions are expected to identify at least two non-polio AFP cases per 100,000 population aged < 15 years to demonstrate capacity for detecting poliovirus transmission should it be occurring (WHO, 1996). High quality surveillance combines timely identification, and prompt and complete investigation of suspected AFP cases. Proper investigation includes collection of two adequate stool

specimens from each AFP case at least 24 h apart, within 14 days of onset of paralysis. Alongside identification of suspect cases, the appropriate collection, handling and shipment of stool specimens to a global network of laboratories that are capable of performing virus isolation, typing and intratypic differentiation, using both classical virological and molecular techniques is vital to the eradication effort. The success of the program depends on sensitive and timely field investigations and accurate diagnostic testing by the laboratories. A fundamental attribute of the GPEI has been the standardization of laboratory and field procedures to achieve the goal virus eradication. To ensure validity of the laboratory testing that will document the successful eradication of poliovirus, stool specimens should be transported to a WHO-accredited laboratory within 72 h after collection in a "reverse cold chain", maintaining a temperature < 8 °C, and without leakage or desiccation (WHO, 1989). Laboratory activities to identify and characterize poliovirus depend on the execution of the standardized field implementation of the reverse cold chain.

WPV in solution has been shown to be temperature-sensitive in virus inactivation studies during vaccine development stability testing (Melnick, 1991), but the temperature standard specified for stools during transport is based on studies of oral poliovirus vaccine viability in solution. Oral poliovirus vaccine is the most

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heat-sensitive vaccine in use; the vaccine can be stored at 2–8 °C for a maximum of 6 months to maintain potency (WHO, 1989). Serotype 1 is the most temperature-sensitive of the three poliovirus serotypes in solution. The recommended temperature range is based on data from multiple studies defining time- and temperature-dependent titer loss, including a 0.3 $\log_{10}$ titer loss after storage at 4 °C for 18 months (WHO, 1990; Sokhey et al., 1988; Peetermans and C.G., 1980; World Health Organization Polio Laboratory Manual, 2004; Wood and Hull, 1999; Finney, 1978). Exposure to higher temperatures increased the rate of decay for stabilized vaccine; oral polio vaccine titers declined ~20% when stored at 22 °C for 14 days, and ~50% when stored at 36 °C for 14 days; Storage at 36 °C for three weeks resulted in nearly complete titer loss (Sokhey et al., 1988). No studies characterizing the heat sensitivity of wild or vaccine poliovirus in specimens have been published.

AFP cases are classified as polio cases based on the results of poliovirus isolation; characterization as "non-polio" following a negative results assumes proper collection, storage and shipment to an accredited laboratory, i.e., an intact reverse cold chain. However, little is known about how transport conditions would affect the detection of WPV in stool specimens. Anecdotally, temperatures in transit frequently vary outside the recommended range, especially in tropical and semi-arid regions where polio remains endemic. We undertook this study to estimate the effect of time at elevated temperatures on WPV titers in stool specimens.

Objective

The objective of this study was to estimate the effect of time at elevated temperatures on wild poliovirus titers in stool specimens and describe the slope of decay of type 1 poliovirus when exposed to different elevated temperatures.

Methods

RD (human rhabdomyosarcoma) and L20B (mouse L cell engineered to express the human poliovirus receptor (Wood and Hull, 1999)) were maintained in a 5% CO_2 atmosphere at 37 °C in minimal essential medium with Earle's salts (MEM; Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, or Thermo Scientific, Lafayette, CO).

A pilot experiment was performed to establish the appropriate range of temperatures and incubation times to be tested in the main study. Approximately 400 μL from each of five previously characterized stool specimens, containing wild poliovirus type 1 (WPV1) detected by the standard WHO virologic surveillance protocol (World Health Organization Polio Laboratory Manual, 2004), were pooled, mixed thoroughly, and divided into aliquots of 50 μL in 23 individual 0.65 mL polypropylene microcentrifuge tubes. Two aliquots were placed at 4 °C and seven aliquots were placed in heat blocks at each of three temperatures, 25 °C, 35 °C, and 45 °C. One aliquot was removed from each heat block at approximately the same time each day on days 4, 8, 12, 16, 20, 24, and 28, and stored at 4 °C until further analysis. Stool suspensions (10% w/v) were made from each of the aliquots using standard protocols (World Health Organization Polio Laboratory Manual, 2004). Ten-fold serial dilutions were prepared for each suspension and each dilution was inoculated in duplicate onto monolayers of RD cells in 24-well plates in MEM-2% FBS.

For the main experiment, we calculated power using the non-central F density: $1 - \beta = pr(F > F_{crit})$, where F_{crit} is the critical value of the central F distribution for 1 and υ_2 degrees of freedom and a significance of 0.05 (43). Assuming a moderate temperature effect on WPV decay in specimens (range of slope decay of -0.1 to -0.4), lower correlation among the observations (0.5) and larger standard deviation (0.75), we found that $n = 8$ different pools of homogenized stool aliquoted and exposed to elevated temperatures will result in a $1 - \beta = 0.91$ for determination of the slope of the decay curves. Ten pools of stool were created, each comprised of six different poliovirus type 1-positive stools. Stools were selected non-systematically from a library of WPV-positive specimens at CDC. Approximately 250 μL of each of six stools were pooled, mixed thoroughly and divided into 50 μL aliquots as above. The baseline titer of each stool pool was determined by titration in L20B cells (L20B cells are selective for poliovirus and are used to reduce background CPE due to non-polio enteroviruses that might be in the original stools (Wood and Hull, 1999)). One aliquot from each pool was placed immediately at 4 °C and seven aliquots were placed in each of three heat blocks at 27 °C, 31 °C, and 35 °C. An aliquot was removed from each heat block at approximately the same time each day on days 2, 4, 6, 8, 10, 12, and 14, and stored at 4 °C. Stool suspensions were made as described above. Ten-fold serial dilutions were prepared and 10 replicates of each dilution were inoculated onto L20B cells in 96-well plates. At five days post-inoculation, plates were stained with crystal violet solution (0.05% crystal violet, 0.5% Tween-20, 50% ethanol), washed three times with deionized water, and allowed to dry overnight. Inoculated cultures were maintained in a 5% CO2 atmosphere at 37 °C in both the pilot and main experiment and were observed daily for five days for development of cytopathic effect (CPE), an indicator of the presence of intact poliovirus. For each incubated aliquot, WPV1 titer (50% cell culture infectious dose ($CCID_{50}$) per gram stool) was calculated using the Spearman-Kärber formula (Finney, 1978).

For both the pilot and main experiments, reverse transcription-semi-nested polymerase chain reaction (RT-snPCR) and sequencing (Nix et al., 2006) were used to determine whether viral nucleic acid was detectable in stools that were negative in cell culture at days 4, 8, 12, 16, 20, 24 and 28 for the pilot and days 1, 2, 3, 4, 7, 10 and 14 for the main experiment.

We analyzed the WPV1 isolation and titer results to determine the difference in the loss of titer by temperature and time at temperature, examining the slope of the decay curve at each temperature using least squares regression. We did not assume that data points below the titration assay's limit of detection (10^2 $CCID_{50}$ per gram) equaled 0, and therefore excluded them from the analysis to prevent biased estimates of the slopes. WPV1 titers were analyzed using a generalized linear model with a repeated measures ANOVA design using SAS software, version 9.1 (SAS Institute Inc., Cary, NC). The measured dependent variable was the slope of the $\log_{10}$ (titer) of WPV1 remaining vs. time, calculated for each pool at each temperature. The independent variable was temperature. Non-detection of WPV1 in the aliquot by cell culture indicated the time endpoint for each temperature.

Results

In the pilot experiment, WPV1 was highly stable at 25 °C for at least 28 days, with only minimal loss from 10^6 to 10^5 $CCID_{50}$/g (Fig. 1). WPV1 was stable at 35 °C for four days but rapidly lost titer thereafter, losing 3 logs at 12 days and becoming undetectable by 16 days. After incubation at 45 °C, WPV1 was undetectable at 4 days, the earliest time point tested.

For the main experiment, the median baseline titer of the stool pools was $10^{4.66}$ $CCID_{50}$/g with a range of $10^{2.9}$–10^6 $CCID_{50}$/g. Plots of WPV1 decay curves by temperature showed an increased rate of titer loss at higher temperatures (Fig. 2). We estimated the mean

slope of WPV decay at 27 °C, 31 °C and 35 °C (Table 1). The three mean temperature decay slopes were significantly different from each other ($p < 0.01$) (Table 1). The mean time to negative cell culture results was eight days at 35 °C for high titer ($> 10^{5.5}$ $CCID_{50}/g$) specimens. Specimens with a low WPV titer at baseline ($< 10^{3.5}$ $CCID_{50}/g$) reached the point of non-detection in a mean of five days at 35 °C.

Negative aliquots from both experiments were tested using RT-snPCR to determine the detectability of WPV after heat inactivation. Poliovirus was detectable by RT-snPCR and typeable by sequencing the PCR amplicon in the pilot study samples exposed at 25 °C or 35 °C for up to 28 days, and to 45 °C for up to 8 days, even though virus was no longer detectable in culture after day 8 at 35 °C or after day 0 at 45 °C (Fig. 3A). In the main study samples, WPV was detectable and typeable up to 14 days (the longest incubation tested) at 27 °C, 31 °C, and 35 °C (Fig. 3B).

Discussion

The partners in the GPEI have developed quality control guidelines to assure vaccine effectiveness, including monitoring of the "forward" cold chain with vaccine vial monitors. Guidelines for transport of stool specimens are in place to assure stool integrity and the ability of laboratories to isolate poliovirus in the specimen
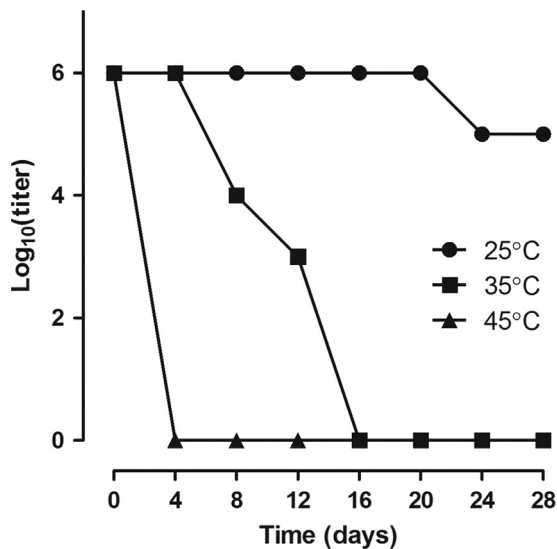
if it is present (WHO, 1989). These quality assurance guidelines of the "reverse" cold chain assume the potential negative effect of adverse specimen transport (not arriving under proper cold conditions) on the laboratory result; however, the conditions of

Table 1
Mean WPV decay slopes.

Temperature (°C)	Mean slope	Standard error	Standard deviation
27	−0.0855	0.01340	0.04591
31	−0.1411	0.03844	0.13805
35	−0.2581	0.04017	0.13446

Fig. 3. Detection of wild poliovirus type 1 (WPV1) by reverse transcription-semi-nested polymerase chain reaction in stool incubated at to elevated temperatures over time. (A). WPV1-containing stool exposed for 4–28 days at 35 °C or 45 °C, and for 28 days at 25 °C. (B). WPV1-containing stool exposed for 1–14 days at 31 °C or 35 °C, and for 14 days at 27 °C.

Fig. 1. Change in wild poliovirus type 1 titers in stool pools incubated for 4–28 days at 25 °C, 35 °C, and 45 °C.

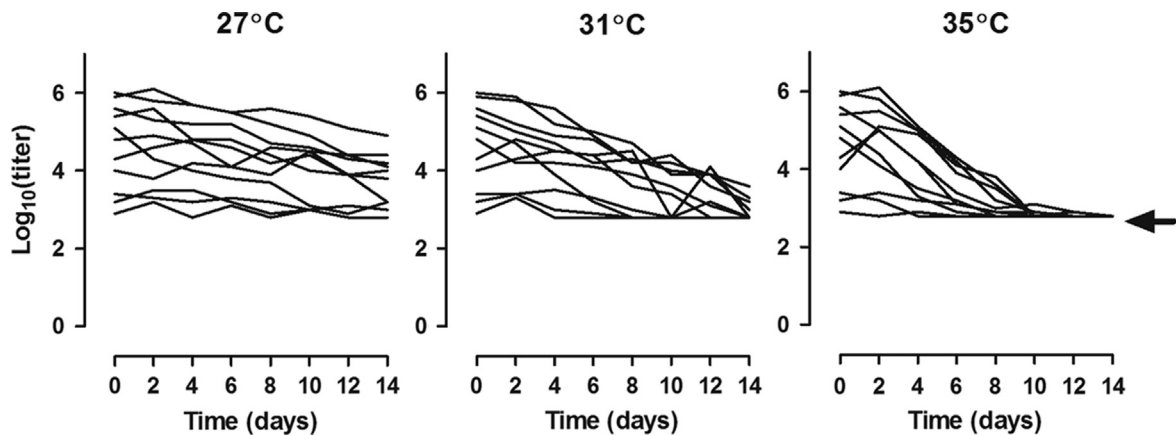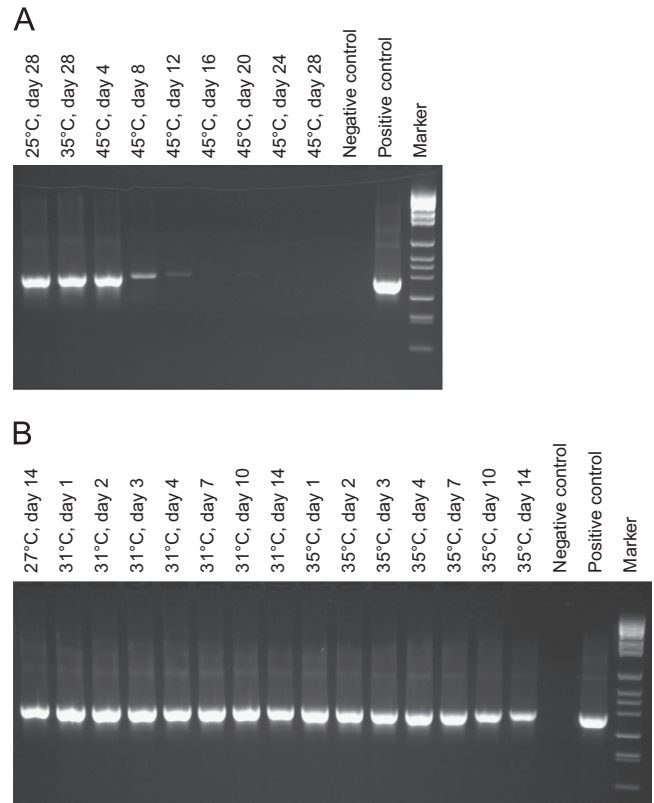**Fig. 2.** Change in wild poliovirus titers in 10 stool pools incubated for 0–14 days at 27 °C, 31 °C, and 35 °C.

specimens are not monitored during transport. In this study, estimating the slopes of decay over time for WPV type 1 at multiple temperatures common in arid and semi-arid locations where poliovirus has or continues to circulate allowed more accurate assessment of the risk of missing circulating virus (falsely negative laboratory results) due to an inadequate reverse cold chain. Increasing temperature will increase the rate of WPV titer decay as will the amount of time a specimen is exposed to a given temperature. The data presented underline the effect of high temperature on virus decay and emphasize the need to prevent specimen exposure to high ambient temperatures. However, this study shows that under moderate heat stress, poliovirus titer decreases are moderate even with extended exposure times. In extreme conditions, with limitations in timely transport of specimens, monitoring of temperatures inside specimen carriers may be necessary to ascertain possible titer loss over time and the potential impact on the quality of results reported to the GPEI.

Field observations have indicated that the emphasis placed on the forward cold chain has not been placed on the reverse cold chain. However, our data demonstrate that current guidelines for the transport of specimens within 72 h are sufficient to maintain specimen integrity even at temperatures well above the recommended 2–8 °C. While it is reassuring that poliovirus in stool remains relatively stable at moderately elevated temperature, transport at temperatures > 25 °C, especially if delivery is delayed, could impact sample integrity and virus isolation results.

Based on the decay curves, a stool specimen with an average baseline poliovirus titer of $10^{4.57}$ $CCID_{50}/g$ would arrive at the laboratory after 72 h of travel at 27 °C with a titer of $10^{4.4}$, or less than a half-log titer loss from baseline due to time and temperature. When traveling at 31 °C, an average titer specimen would arrive at the laboratory after 72 h with a titer of $10^{4.2}$ $CCID_{50}/g$. If an average-titer specimen experienced temperatures of 35 °C for the 72 h transport, the titer expected at the laboratory would be $10^{3.9}$ $CCID_{50}/g$.

Under moderate to extreme time and temperature conditions, however, falsely negative laboratory results could occur. There was no loss in titer after up to 20 days incubation at 25 °C and only minimal loss of titer due to temperature exposure at 27 °C for up to 14 days, indicating a reverse cold chain transit of up to two weeks at or below 25 °C would lead to isolation of poliovirus by the laboratory if it were present in the specimen in average titers. If the initial titer of the specimen is low, the titer will reach undetectable levels more quickly than an average-titer specimen. When low-titer specimens ($< 10^{3.80} CCID_{50}/g$) were exposed to 31 °C and 35 °C, they became undetectable by cell culture in 2 to 4 days.

Assuring that current reverse cold chain guidelines are followed provides the best means for maintaining confidence in the laboratory results; this includes specimen storage and transport below 8 °C to the extent possible and, more importantly, a transit time of ≤ 72 h from collection to the laboratory. Data to document adherence to current guidelines is necessary for use by regional certification committees when deliberating a region or nation's polio-free status. Given the public health importance of polio eradication and ensuring polio-free status, a low-cost time and temperature monitoring method may be important in some settings to assure that the extremes of time or temperature during transport are uncommon.

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