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Inactivating Zaire Ebolavirus in Whole-Blood Thin Smears Used for Malaria Diagnosis

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Malaria is an important mimic or coinfection in potential Ebolavirus disease patients. Here, we evaluated the efficacy of the 100% methanol-inactivating Zaire Ebolavirus Makona variant for malaria thin-smear preparation. We determined that 100% methanol completely inactivated the virus after 15 min.

The 2014-2015 West African Ebola outbreak has uncovered knowledge gaps in several areas regarding the safe handling of high-containment viruses in clinical environments, such as front-line diagnostic laboratories. A specific area of concern is the safe preparation of blood smears for malaria diagnosis. Symptoms of malaria, such as fever, headache, nausea, vomiting, and diarrhea, closely mimic those of early Ebolavirus disease (EVD) (1–3). Additionally, since the two viruses circulate in the same geographic area, coinfection is a concern (2). Malaria can be rapidly fatal if left untreated, and as such, empirical antimalarial treatments are recommended in hospitals and Ebola management centers in affected areas of Africa (3). In other areas of the world, antimalarial agents for severe malaria (e.g., artemisinin-based compounds) can be difficult to access due to limited supply or geographic distance (4). It is therefore important for laboratories to be able to provide malaria smears that are safe and concomitant with Ebola diagnostic results.

The Giemsa and Wright (Diff-Quik) staining techniques for malaria parasites in blood samples are robust diagnostic methods easily performed in most laboratory settings. Prior to staining, blood samples are prepared in either a thin- and/or thick-smear manner. Thick smears can better detect the presence of *Plasmodium* spp. than thin smears but are not methanol fixed, while the thin smears can be fixed in methanol and provide the benefit of detection and identification of the malarial species (5–8). However, it has not been definitively demonstrated that a methanol fixation step prior to a Giemsa or Wright staining procedure is sufficient to inactivate Zaire Ebolavirus (EBOV) in suspect malaria samples. Since the two agents circulate within the same areas, have similar symptoms, and can coinfect, diagnostic samples require safe handling to prevent potential EBOV infection in health care workers. In this study, we evaluated the effects of methanol and a methanol heat treatment against the EBOV/Mak variant using thin-smeared slides containing human whole blood spiked with EBOV/Mak in the absence of the *Plasmodium* parasites.

Virus stock concentrates were prepared, as previously described (9). Whole blood from a volunteer was drawn and the sample stored at 4°C. An aliquot of whole blood was spiked with concentrated EBOV/Mak virus preparation (9) to attain a final concentration of 10^8 50% tissue culture infective doses ($TCID_{50}$)/ml per milliliter of blood. Five microliters of the spiked blood was placed on a clean plastic microscope slide (catalog no. S67112A; Fisher), and smeared according to the procedure described by the CDC (5, 6). Glass was replaced by plastic slides, as per our institution's biosafety protocols within the containment level 4 laboratory.

Briefly, blood was smeared using a clean plastic slide over top of the droplet; once capillary action pulled the blood laterally, a continuous motion was used to thinly spread the blood droplet across the slide. The slides were air dried for 15 min in a biosafety cabinet at room temperature. Polypropylene Coplin jars (catalog no. S90130; Fisher) were filled with 100% methanol, and the slides were submerged in methanol for 15, 30, or 60 min, removed, and air dried for 10 min. A combination of chemical and heat inactivation was investigated by placing a second set of methanol-fixed slides at 56°C for 1 h utilizing a Boekel Scientific Slide Moat incubator (catalog no. I05-450-31; Fisher). Following each treatment, virus elution was performed by placing the treated slides within a 6-well tissue culture dish, rinsing with 1 ml of Dulbecco's modified essential medium (DMEM) plus 2% fetal calf serum (FCS) plus 10 U/ml penicillin-streptomycin (pen-strep), scraping for 1 min using a pipette tip, and titrating in Vero E6 cells (ATCC CRL 1586) using the Reed-Muench $TCID_{50}$ procedure (10). Two treatments of three biological replicates were assessed, each including three technical replicates.

Infectivity loss from the drying procedure was tested by comparing the fresh nonsmeared (5 μl) spiked blood to the dried-smear sample. This revealed a small difference, with the undried sample yielding 5.9 (± 0.12 standard deviation [SD]) log $TCID_{50}$/ml compared to 4.7 (± 0.19 SD) log $TCID_{50}$/ml for the dried sample. This loss of 1.2 logs was likely a result of the effects of drying on the virus and was considered statistically significant

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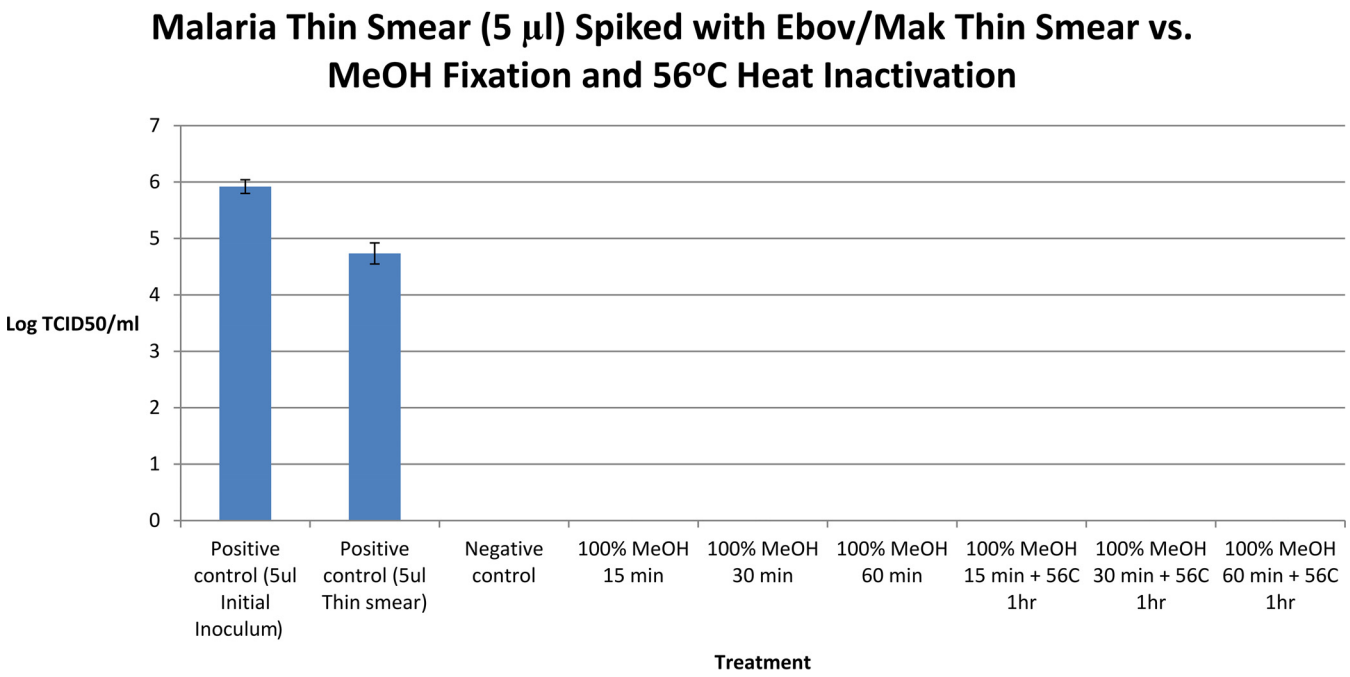

FIG 1 Effect of methanol (MeOH) fixation against whole blood spiked with 10^8 $TCID_{50}$/ml EBOV/Mak. Whole blood was spiked with 10^8 $TCID_{50}$/ml virions with 5 µl of spiked sample dried on microscope slide. Slides were submerged in 100% methanol for 15, 30, and 60 min and evaluated with an additional second set of methanol-treated slides evaluated with an additional hour of 56°C heat treatment. Infectious viral particle amounts are presented with the standard deviations.

($P = 0.00037$, Student's paired *t* test). Methanol fixation proved effective at completely inactivating the EBOV/Mak virus at all examined time points (15, 30, and 60 min), with no additional benefit from heat treatment at 56°C for 1 h following the fixation step (Fig. 1). Thus, methanol treatment alone is sufficient for EBOV inactivation and improvement of biosafety. Although not reported in this study, multiple reports have indicated that neither methanol fixation nor heat inactivation will negatively affect the quality of whole-blood thin smears or the diagnosis of malaria during the staining procedures for malaria (8, 11–14).

The CDC guidelines state that handling of suspected EBOV cases in whole blood while screening for malaria should be treated by adding small amounts of Triton X-100 (5, 6, 15). Although it is believed that the additional Triton X-100 would inactivate EBOV particles within malaria samples and that the impact on clinical biochemical and histology assays is minimal (14, 16, 17), no data have been found in peer-reviewed material to support the use of Triton X-100 in inactivating EBOV.

In conclusion, a 15-min methanol fixation step was able to inactivate a high Ebola viral load within a whole-blood thin-smear sample. Given the efficiency of methanol alone, it is unlikely that heat treatment would provide an additional benefit. While there was a statistically significant decrease in the viral titer from air-drying alone (1.2 logs), the resulting EBOV/Mak viral load (4.7 logs) is still considered to pose a high risk to individuals handling such slides prior to methanol fixation. It is our belief that that the handling of the thick smears for suspected Ebola infection poses a higher risk, as they contain larger volumes of infected blood, are not methanol fixed, and should not be used. While both the thin and thick smears allow for the detection of the *Plasmodium* parasites, the thin smears can further identify species and can accommodate the additional methanol fixation step, rendering any EBOV inert.

Studies regarding methanol fixation and its application to other important high-containment pathogens are under way. Diagnostic laboratories must therefore carefully design safe handling protocols for the preparation and handling of the malaria smear prior to the fixation step.

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