FIELD DETECTION OF EASTERN EQUINE ENCEPHALITIS VIRUS IN THE
AMAZON BASIN REGION OF PERU USING REVERSE
TRANSCRIPTION–POLYMERASE CHAIN REACTION ADAPTED FOR FIELD
IDENTIFICATION OF ARTHROPOD-BORNE PATHOGENS

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Abstract. In support of efforts to develop rapid diagnostic assays for use in the field, reverse transcription–polymerase chain reaction (RT-PCR) assays were developed to detect arboviruses circulating in the Amazon Basin region of Peru. Previous knowledge of arthropod/pathogen relationships allowed a focused evaluation to be conducted in November 2000 that assessed the feasibility and reliability of a mobile, rapid, field-expedient RT-PCR diagnostic system aimed at detecting eastern equine encephalitis virus (EEEV) in Culex (Melanoconion) pedroi mosquitoes. Modifications were made to a commercially available mobile molecular laboratory kit and assay procedures were tailored for use under harsh environmental conditions with field-collected and field-processed mosquitoes. From CO2 baited mosquito light traps, 3,227 Cx. (Mel.) pedroi mosquitoes were collected and sorted into 117 pools. The pools were processed and assayed in the field by RT-PCR and five of those pools were found positive for EEEV. Laboratory sequence analysis confirmed the presence of two distinct subtypes of EEEV.

INTRODUCTION
The rapid identification of relevant arthropod-transmitted pathogens and the determination of potential human disease risk, especially in field environments, is of great importance to the United States military. The development of standardized RNA extraction procedures and rapid nucleic acid–based diagnostic techniques designed to identify arthropod-borne pathogens within any given region of the world is a major preventive medicine concern to deployed military and humanitarian support organizations. Currently, the accepted techniques for virus isolation, identification, and characterization include cell culture, animal inoculation, hemagglutination inhibition assay, plaque-reduction neutralization assay, enzyme-linked immunosorbent assay (ELISA), and viral serology.1,2 Even though these methods are highly sensitive and specific for the detection of disease pathogens, modifying these techniques for field use is either not feasible (i.e., animal inoculation and cell culture based assays) or practical (i.e., the large volumes of buffers required for ELISAs). An additional technique referred to as a real-time polymerase chain reaction (PCR) could easily be adapted for field use and could be considered as a candidate for development as the next generation of nucleic acid–based field diagnostics assay. For highly pathogenic viruses such as eastern equine encephalitis virus (EEEV), Venezuelan equine encephalitis virus (VEEV), Crimean Congo hemorrhagic fever virus, and Rift Valley fever virus, culturing these viruses requires biosafety level 3 or 4 (BSL-3/4) laboratory facilities.3 For certain viruses, i.e., dengue virus, growth in cultured cells is poor, thus making isolation difficult.4 After isolation, the viruses require identification. Simple identification methods do exist, (i.e., immunochromatographic dipstick assays), but are available for a very limited number of viruses such as West Nile virus and St. Louis encephalitis virus (SLEV).5 A more involved identification procedure, namely, reverse transcription–polymerase chain reaction (RT-PCR), can be used in the laboratory to identify any number of selected arboviruses from either laboratory-grown viruses6 or from field-collected and laboratory-processed mosquitoes.7,8 Therefore, the use of RT-PCR in the field for identifying medically relevant viruses can provide timely information to health care personnel and military leaders about relative disease risks to personnel in urban, peri-urban, and field environments.

From previous studies conducted from 1995 to 2000, mosquitoes collected from the Amazon Basin region of Peru yielded more than 180 viral isolates from three different viral families: Togaviridae, Flaviviridae, and Bunyaviridae (Klein T and others, unpublished data).9,10 From the previously collected data on mosquito abundance, seasonality, and distribution coupled with knowledge of virus and vector relationships (Klein T and others, unpublished data), Culex (Melanoconion) pedroi mosquitoes and EEEV virus (family Togaviridae, genus Alphavirus) were selected as targets for the first field evaluation of an RT-PCR system developed for use in the field. Alphaviruses are known to cause disease in humans and are considered to be medically relevant arthropod-borne pathogens.11 They contain positive-sense single-stranded RNA genomes encapsidated by a single type of capsid protein. The nucleocapsid is surrounded by a lipid bilayer containing virus-encoded glycoproteins. The Alphavirus genome contains a 5’-methylguanosine cap and a 3’-polyadenosine tail.

During November 2000, a field laboratory was set up in the Amazon Basin region of Peru, near the city of Iquitos, to conduct the field evaluation of the RT-PCR field system. The system was designed to provide a rapid, highly sensitive, and deployable pathogen-detection system for augmentation into military field laboratories. Conducting molecular diagnostics in the field under non-optimal laboratory conditions (extreme temperature and humidity, generator-supplied electrical power, non-filtered air, and a minimal cold chain) required development of processes and procedures to compensate for the harsh field conditions. For this technology to work in the field, standardized, reliable, and easily performed procedures were developed and tested first under optimal laboratory conditions, then by evaluation under harsh environmental field conditions. The RT-PCR assays were designed as generic assays that could be used with any number of PCR primer sets.
to detect any number of viruses. The primer annealing temperatures were not optimized in favor of a single set of reaction conditions that would simplify use of the assays and prevent mistakes in the field. The use of RT-PCR in the field is a powerful tool for determining risk assessments in real time. Within one day, mosquitoes can be trapped, processed, and evaluated for the presence of any number of medically relevant pathogens. We have shown that RT-PCR diagnostics can be accomplished in the field under harsh environmental conditions.

**MATERIALS AND METHODS**

**Laboratory mosquito manipulations.** Laboratory-reared 7–10-day-old *Ochleratatus (Ochleratatus) taeniorhynchus* mosquitoes were inoculated intrathoracically with 0.2 μL of 10^7 plaque-forming units (PFU) of EEEV isolated from mosquito pools previous collected in the Amazon Basin region of Peru and passed in Vero cells. Separate groups of mosquitoes were inoculated with VEEV serotype IA/B, VEEV serotype IIIC, or SLEV as described earlier. Mosquitoes were held at 26°C with a 16-hour light to 8-hour dark photoperiod for seven days after inoculation. Mosquitoes were immobilized with triethylamine (TEA), which was previously demonstrated to effectively immobilize mosquitoes while having no effect on viral titers, and pooled into groups containing one inoculated mosquito and either 0, 4, 9, 14, 24, or 49 negative mosquitoes. Mosquito pools were triturated in a 1.5-mL microcentrifuge tube containing one 4.5-mm spherical copper, zinc, or glass ball (the 4.5-mm spherical copper ball is a type of bead (Amersham Biosciences, Corp., Piscataway, NJ). The mosquito homogenate was clarified by centrifugation in a microcentrifuge tube containing 0.25 mL of PBS and RNA was collected into the bottom of the tube by brief centrifugation and then stored on ice or frozen at −70°C.

To compare the efficiency of the extracting medium, mosquitoes were also triturated in 0.75 mL of Trizol®-LS (Invitrogen, Inc.) and E-gel® II (Invitrogen, Inc.) was added to each tube. After trituration, the mosquito homogenate was clarified by centrifugation in a microcentrifuge at 4°C and 12,000 rpm for five minutes. The supernatant was transferred to a clean microcentrifuge tube and stored on ice or frozen at −70°C.

**Isolation of RNA and synthesis of cDNA.** Clarified mosquito-PBS homogenate or virus infected cell culture supernatant (250 μL) was combined with 0.75 mL of Trizol®-LS in a microcentrifuge tube, vortexed briefly, and incubated at room temperature for five minutes. Chloroform (200 μL) was added to the tube, vortexed or shaken for 10 seconds, and incubated for 10 minutes at room temperature. The aqueous phase containing the viral RNA was separated from the organic phase by centrifugation at 4°C and 12,000 rpm for 10 minutes. After centrifugation, 500–550 μL of the clear aqueous solution was removed and transferred to a clean RNase-free 1.5-mL microcentrifuge tube containing 1 μL of glycogen (20 mg/mL). Isopropanol (500 μL) was added, and the tube was vortexed briefly. The RNA was pelleted on the bottom of the tube by centrifugation at 4°C and 12,000 rpm for 10 minutes. The clear supernatant was decanted, leaving a white pellet at the bottom of the tube. Ethanol (500 μL, 75%) was added to the tube containing the pellet. The tube was inverted several times and then centrifuged at 4°C and 12,000 rpm for two minutes. The clear supernatant was decanted, and the tube was briefly centrifuged to collect the residual alcohol into the bottom. The residual alcohol was removed from the RNA pellet with a pipet. The RNA pellet was dissolved in 12–25 μL of nuclease-free water at room temperature for 5–10 minutes. After vortexing, the RNA was collected into the bottom of the tube by brief centrifugation and then stored on ice or frozen at −70°C.

The presence of inhibitors co-isolated during the RNA purification step was evaluated by subjecting the RNA to a second round of purification. RNA isolated from mosquitoes triturated in PBS or in Trizol®-LS was further purified using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer’s instructions.

The RNA extracted from the mosquito homogenate was converted into cDNA as follows. RNA (10 μL) and 2 μL of random hexamer (100 nmol) were combined in a dNTP mixture in a PCR tube and placed in a thermal cycler programmed as follows: 70°C for 10 minutes, 4°C for five minutes, 25°C for 15 minutes, 42°C for 50 minutes, 70°C for 15 minutes, then 4°C for storage. At the beginning of the 25°C step, the thermal cycler program was paused and 4 μL of 5× RT buffer (Invitrogen, Inc.), 2 μL of 0.1M dithiotheitol, 1 μL of deoxyribonucleotide triphosphate mixture (10 mM each), and 1 μL of Superscript™II (Invitrogen, Inc.) was added to each tube. Random hexamer was used instead of a reverse primer specific to EEEV to allow for the reverse transcription of any viral RNA genome present in the mosquito homogenates. This procedure was designed to allow for the detection of any possible viruses circulating in the same region as EEEV (i.e., VEEV or western equine encephalitis virus) by using the same cDNA with different PCR primer sets. The tubes were inverted several times to mix the contents, centrifuged to collected the material into the bottoms of the tubes, and then placed back into the thermal cycler to complete the program. Once completed, the cDNAs were stored on ice or frozen at −20°C.

**PCR amplification and gel electrophoresis.** The sequences of the PCR primers are listed in Table 1. The PCR amplification of targeted viral sequences present in the cDNA prepared from the RNA isolated from the mosquitoes was accomplished as follows. For a 25-μL reaction, 1–2 μL of cDNA, 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), and 21×22 μL of water was added to each PCR tube containing one puRe Taq™Ready-To-Go™ PCR bead (Amersham Biosciences, Corp., Piscataway, NJ). The PCR amplification was performed as follows: 95°C for two minutes, followed by 35 cycles at 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 45 seconds, followed by 72°C for seven minutes and a final hold at 4°C.

Agarose gel electrophoresis of the PCR products was achieved by using pre-cast, bufferless agarose gels containing embedded electrodes and ethidium bromide (E-gel® gels; Invitrogen, Inc.) and E-gel® power bases. The electrophoresis was conducted according to the manufacturer’s recommendations with the following modifications. Water (15 μL) was added to the DNA molecular weight marker well and 5 μL of water was added to each of the sample wells prior to the electrophoresis. The PCR products were visualized under UV light. The identity of the PCR products was confirmed by sequencing.
addition of DNA marker or sample. Electrophoresis dye (5 µL, 0.05% bromophenol blue and 0.05% xylene cyanol in 50% glycerol) was mixed with each PCR product and 15 µL of this was loaded into each sample well. After the completion of the electrophoresis, the PCR product bands were visualized using a transilluminator and recorded using a Polaroid® camera (Polaroid, Waltham, MA) and 667 film.

**Field site location and mosquito collections.** Mosquitoes were collected from a secondary jungle site near the village of Puerto Almendras, located approximately 25 km from the city of Iquitos, in the Amazon Basin region of Peru. The field laboratory was setup in a village hut with a tin roof, dirt floors, and open windows. Electricity was supplied from a small 240-volt, gasoline-powered generator that was step-down transformed to 110 volts. Mosquito light traps baited with CO2 (American Biophysics Corp., North Kingstown, RI) were set at dusk and collected at dawn. The mosquitoes collected in the traps were immobilized with TEA, identified to species, and pooled into groups of 25–30. *Culex (Mel.) pedroi* mosquito pools were separated from all other pools, processed as outlined earlier, and tested for the presence of EEEV by RT-PCR. The remaining pools were returned to United States Army Medical Research Institute of Infectious Diseases (Fort Detrick, Frederick, MD) for other testing.

**Field equipment.** A commercially available mobile molecular laboratory kit (model MML-0150; MJ Research, Inc., Waltham, MA) designed for field use was evaluated in the field. The components of the laboratory kit consisted of a 25-well thermocycler (model PTC-150HB), a microcentrifuge (16,100 × g maximum), a compact ultraviolet transilluminator, and a Polaroid® camera (Polaroid, Waltham, MA) and 667 film.

**Laboratory sequencing and phylogenetic analysis.** The RT-PCR amplification product (15 µL) from processed mosquito pools that tested positive for EEEV was purified using the Qiaquick® PCR purification kit (Qiagen, Inc.) according to the manufacturer’s instructions. Automated sequencing was performed on an ABI 310 genetic analyzer using a Big-Dye™ sequencing kit (PE Biosystems, Inc., Foster City, CA) according to the manufacturer’s instructions. Primer, excess nucleotides, and buffer were removed from the Big-Dye™ sequencing reaction by eluting the material from a Sephadex™G-50 (Amersham Biosciences Corp.) column equilibrated with water. Sequences were aligned using the MegaAlign program (Lasergene analysis software; DNASTAR, Inc., Madison, WI) and sequence ends were trimmed to a uniform length. Phylogenetic analyses of aligned sequences were performed with the ClustalW method with a gap penalty of 15 and a gap length of 0.66. The phylogenetic tree generated by MegaAlign is a rooted tree with the number of substitution events indicated at the bottom of the tree.

**RESULTS**

**Selection and evaluation of primers for the PCR amplification of EEEV.** An appropriate primer set for the amplification of EEEV was selected by evaluating primer sets previously published by others (Table 1). The selected primer sets targeted different regions of the *Alphavirus* genome. The results of six different primers sets used in the amplifying EEEV isolated from a single infected mosquito are shown in Figure 1. The PCR cycle times and temperatures used for the amplification of EEEV were generalized for an amplification product (amplicon) of 1,000 base pairs or less with low primer annealing temperatures. The low annealing temperature would also compensate for minor changes in the primer binding sites due to genetic drift. Even though the PCR assay was not optimized for any given primer set, the E-9657/E-10004 primer set produced the most abundant amplicon with the appropriate number of basepairs. The E-9657/E-10004 primer set also targeted conserved envelope sequences where the variable bases were replaced with degenerate bases. The primer sets E1 (+)/(−),13 VEE 0091/00092 (Oberste M and
others, unpublished data), and M2W/cM3W\textsuperscript{16} produced adequate amounts of amplicon with the appropriate number of basepairs and could serve as additional primer sets for confirmational testing of presumed positive samples in the field. The primer set C (+)/(−)\textsuperscript{13} produced a small amount of amplicon with the wrong number of basepairs, while VEEV 115/116\textsuperscript{15} failed to produce an amplicon with the appropriate number of basepairs.

The specificity of the E-9657/E-10004 primer set was evaluated by comparing the amplicon generated from the RT-PCR assay of EEEV, VEEV serotype IA/B, VEEV serotype IIIC, and SLEV infected mosquitoes (Figure 2). St. Louis encephalitis virus, a flavivirus, was used as an unrelated control virus for the experiment. Eastern equine encephalitis virus was specifically amplified using the E-9657/E-10004 primer set while VEEV IA/B and SLEV were not amplified. Marginal amplification of VEEV IIIC was noted and probably resulted from sequence homology between the alphaviruses. The primer set E-9657/E-10004 was also used in the RT-PCR assay of other cell culture-passed alphaviruses, bunyaviruses, and flaviviruses isolated from Peru. For alphaviruses, Una was weakly amplified whereas Oriboca and Trocara were not amplified, for the bunyaviruses, Caraparu and Itaqui were not amplified, and the flavivirus Ilheus was also not amplified. Additional PCR assays were conducted on all samples that tested negative with the E-9657/E-10004 primer set by using other primer sets to confirm the presence of the appropriate viruses.

**Preparation of mosquito homogenate.** A comparison of copper, zinc, and glass BBs showed that the copper and zinc BBs performed similarly, while the glass BB required longer vortexing times to achieve efficient trituration of the mosquitoes. Pools of 25 mosquitoes were efficiently triturated with one metal BB and pools of 1–5 mosquitoes were more efficiently triturated with two metal BBs. Use of a small, motorized microcentrifuge pestle also efficiently triturated pools of one or 25 mosquitoes but caused splattering and potential aerosols and was discontinued for field processing of mosquitoes. Use of a BB for triturating the mosquitoes was preferred because the mosquitoes and BBs were sealed inside of microcentrifuge tubes, thereby greatly reducing the generation of aerosols and cross-contamination.

The ability to detect one virus-infected mosquito in pooled mosquitoes was investigated by triturating one EEEV-infected mosquito with increasing numbers of uninfected mosquitoes (Figure 3). Trituration of one infected mosquito with up to 49 uninfected mosquitoes yielded RNA that was efficiently converted into cDNA. The PCR amplification of the cDNA resulted in approximately the same amount of amplicon for each pool containing one infected mosquito and either 0, 4, 9, 14, 24, or 49 uninfected mosquitoes. A standard plaque assay of each of the homogenates in Vero cells produced between $5.42 \times 10^4$ PFU/mL and $1.85 \times 10^6$ PFU/mL except for the pools containing only uninfected mosquitoes and those resulted in no plaques. Even though the amount of virus present in the infected mosquitoes varied by more than one log, the amount of amplicon produced after PCR amplification was approximately the same.
To determine the optimal extraction medium, laboratory mosquitoes inoculated with EEEV were pooled (one infected plus 24 uninfected mosquitoes) and triturated in either PBS or in Trizol®-LS. The isolated RNA was then reverse transcribed into cDNA and then PCR amplified using the E-9657/E-10004 primer set. Trituration of the mosquitoes in Trizol®-LS resulted in the precipitation of a black RNA pellet that failed to produce an amplicon after RT-PCR amplification, whereas mosquitoes triturated in PBS produced a tan-white RNA pellet that produced an amplicon after RT-PCR. Removing inhibitors co-isolated with the RNA prepared from the mosquitoes triturated in Trizol®-LS required an additional RNA purification step. After removal of the inhibitors, RT-PCR amplification produced an abundant amplicon. Subsequent experiments performed with cell culture medium containing 10% fetal bovine serum as the mosquito trituration medium showed a stabilizing effect of the medium, presumably the serum proteins, on the viruses contained in the mosquito homogenates that were freeze-thawed several times. Experiments comparing buffers containing different amounts of serum proteins are in progress. We currently triturate mosquitoes in the field with cell culture medium containing 10% fetal bovine serum.

Detection of EEEV in field-processed mosquitoes. In the course of a two-week field evaluation, 3,227 Cx. (Mel.) pedroi mosquitoes were captured in CO2 baited light traps, pooled into groups of 25–30, and assayed by the RT-PCR for the presence of EEEV. All steps were completed at the field site, and the results after repeating the RNA extraction, RT assay, and after using 2 μL of cDNA in the PCR assay are shown in Figure 4. Presumptive positive and negative pools were reprocessed in the field and used to confirm the initial field results that included only 1 μL of cDNA. The EEEV amplicon generated in the field with the E-9657/E-10004 primer set co-migrated with a sample prepared in the laboratory from cell culture-derived EEEV. Pools 59, 60, 74, 110, and 153 tested positive for the presence of EEEV while the remaining 112 pools tested negative. Even though pools 59 and 60 were sequential, they were initially prepared and found to be positive by RT-PCR on two different days, thus making cross-contamination of pool 60 with pool 59 unlikely. The infection rate was calculated to be approximately 1.6 EEEV infected per 1,000 Cx. (Mel.) pedroi. This infection rate was similar to that determined by others in previous years for mosquitoes.

**Figure 3.** Effect of the number of mosquitoes in a pool on the ability to detect a single laboratory-infected mosquito by a reverse transcription-polymerase chain reaction (RT-PCR). A single eastern equine encephalitis virus-inoculated mosquito was combined with different numbers of uninfected mosquitoes, triturated in 0.75 mL of phosphate-buffered saline, and processed into cDNA. The PCR amplification of the cDNA was accomplished using the E-9657/10004 primer set. The number of infected and uninfected mosquitoes per pool is shown. Lane 1, DNA molecular weight marker. Values on the left are in basepairs.

**Figure 4.** Detection of eastern equine encephalitis virus (EEEV) in field-captured and processed mosquitoes. Culex (Melanoconion) pedroi mosquitoes collected in the field were pooled into groups of 25–30, triturated in phosphate-buffered saline, and processed into cDNA. Polymerase chain reaction (PCR) amplification of the cDNA was achieved using the E-9657/10004 primer set. Amplified product was detected in the field using pre-cast agarose gels. Lanes are marked with the pool number except lane D, DNA molecular weight marker; lane L, laboratory prepared PCR product from amplification of EEEV with E-9657/10004 primer set (bright lower band) mixed with product from amplification of EEEV with M2W/cM3W primer set (faint upper band). Values on the left are in basepairs.
collected during November and assayed by plaque assay (Klein T and others, unpublished data).

**Sequence and phylogenetic analysis of field-detected EEEV.** Samples generated in the field were returned to the laboratory for additional testing and for virus confirmation. After cell culture passage of material from the mosquito pools that tested positive for EEEV, the isolated viruses were amplified by the RT-PCR and the resulting amplicons were then sequenced using the same primers as those used in the PCR assay. Growth of all five viral isolates in cell cultures produced the expected cytopathic effect and produced plaques in standard plaque assays. Figure 5 shows the relationship between the different viral isolates and between the Brazil-Peru (lineage II) and the Argentina-Panama (lineage III) subtype of EEEV. North American EEEV is also shown for comparison. Virus from pools 59, 60, 110, and 153 were found to be phylogenetically related to the Argentina-Panama subtype and virus from pool 74 was found to be related to the Brazil-Peru subtype. We noted no difference in the amplicon sequences among viruses from pools 59, 60, 110, and 153.

**DISCUSSION**

During the field evaluation of an RT-PCR system in the Amazon Basin region of Peru, diagnostic assays and techniques were developed that allowed us to detect arboviruses circulating in jungle-dwelling mosquitoes. A field laboratory was set up in a bamboo hut with a hard packed dirt floor and covered with a corrugated tin roof. The daily internal temperature of the field laboratory exceeded 32°C with a relative humidity of 95%. The goal of this project was to assemble and field test existing equipment, simplify and refine protocols, to include addressing contamination and containment issues, and to develop specimen handling and processing methods that could be used in a field environment.

Several methods exist for detecting virus in arthropod samples. Agglutination or inhibition of agglutination-based assays, either using latex particles or red blood cells, require antisera specific for all the different viruses under investigation. The need for antisera, the temperature sensitivity of the assays, and the potential lack of specificity due to shared antigen-epitopes between viruses within the same families make this technique less desirable for use in the field. Other assay such as complement fixation, direct or indirect immunofluorescence assay (IFA) or enzyme immunoassay (EIA) analysis, dipstick assays (discussed earlier), and time-resolved fluorometry also require antisera or antibodies specific to each virus. Some procedures such as an ELISA require large amounts of buffer for washing the plates, which is not practical in the field. Direct IFA and EIA also requires the preparation and maintenance of conjugated antibodies or antisera specific for each virus. This requires either a commercial source or in-house facilities to prepare the high quality antibodies and antisera needed for the assays and may be cost prohibitive. Also, nonspecific binding of the antibodies to the different proteins present in the triturated arthropod samples encountered in the field may diminish the effectiveness of the assays for detecting virus antigen in the presence of nonspecific background staining. The most specific immunologic test for the presence of a specific virus is the neutralization test. This test also requires antibodies specific for each virus and also requires the use of cultured cells, which is not feasible in the field. Biosensor-based methods, those that can detect the binding of an antigen to an antibody adsorbed onto the surface of a transducer, may have utility in the field but are currently restricted to the laboratory due to the size or fragile state of the analysis instrument. Other techniques useful in the laboratory, but not in the field, for identifying viruses are plaque assays, host range restriction analysis by growth in cultured cells, electron microscopy for determining the size, shape, and density of the viral particles, and sequence analysis.

Non-immunologic methods that are capable of detecting viruses present in arthropods are based on the amplification and detection of the viral genomes present in the sample by a PCR. A multitude of PCR methods exist (to include but not limited to conventional PCR and RT-PCR, real-time PCR, ligation-activated transcription PCR, in situ PCR, multiplex PCR, and nested PCR) that when coupled with multiple probe amplification procedures and detections methods make the PCR a versatile diagnostic tool. The advantages and disadvantages of the different PCR methods for detecting viruses have been reviewed elsewhere. This report describes our efforts at designing protocols and procedures that would minimize the problems normally associated with the detection of viruses in arthropods in the field using conventional RT-PCR.

We simplified specimen processing by eliminating the need for wet ice or a chill table for specimen identification. Chill tables and wet ice can cause condensation build-up on specimens that degrades the quality of the arthropod. For the purpose of this study, it was important to process specimens in an efficient and timely manner to allow for virus detection. Use of TEA as an immobilizing agent eliminated the condensation build-up associated with chill tables while keeping the specimens physiologically alive for an extended period of time. This provided the taxonomist with more time to make proper identifications.

To address the issue of virus containment, we modified our method of triturating specimens from using a battery-operated tissue homogenizer, which can splatter material out-
side of a microcentrifuge tube, to vortexing specimens within a sealed microcentrifuge tube containing a copper BB. Triturating mosquitoes in a chaotropic buffer (e.g., Trizol®-LS) neutralizes any viruses present and minimizes the safety concerns surrounding processing of virus-infected mosquitoes in the field, but has several drawbacks. We showed that triturating mosquitoes directly in Trizol®-LS requires an additional purification step, and that additional step require more time and money. More importantly, triturating mosquitoes directly in chaotropic buffers results in the loss of that viral species for further characterization. The virus cannot be returned to the laboratory, grown in culture, characterized, and preserved for future research. The procedures reported here were developed to minimize safety concerns while retaining any possible viral isolates for future characterization and identity confirmation.

Incorporating temperature-stable, pre-assembled products into the RT-PCR protocols provided two benefits, in addition to temperature stability. First, use of the Ready-To-Go™PCR beads simplified the PCR protocol so that anyone with basic laboratory skills could successfully complete the assay. The product required fewer manipulations, thereby reducing the probability of cross-contamination. One goal of the research was to develop procedures that could be augmented into the United States Army’s Theater Army Medical Laboratory and could be completed by soldiers with basic laboratory skills. Second, using E-gel® agarose gels for analyzing PCR products in the field did not require a microwave to melt the agarose, did not require gel casting time (which would have been difficult in the tropical environment), and did not require liquid agarose gel electrophoresis buffer (which would have contributed to excess weight and possible transport spillage). The plastic sealed gels also facilitated gel handling and minimized exposure to ethidium bromide. In addition, the E-gel® power bases provided a light-weight electrophoresis power supply with a built-in timer.

Other commercially available products that were considered but not used in the field included RNA extraction kits and a product similar to the Ready-To-Go™ PCR beads but incorporated RT-PCR into one temperature stable bead. Since the aim of this project was to develop low-cost, simplified procedures, the addition of extraction kits, although extremely effective for RNA isolation, would have significantly increased the total cost of the process. Even though Trizol®-LS extraction uses volatile reagents, it is a very cost-effective procedure but could be replaced with an RNA isolation kit that does not use volatile reagents when the cost is comparable. The use of RT-PCR beads requires adding a virus-specific primer to initiate priming of the RT reaction. Therefore, a separate RT-PCR bead would be necessary for each and every virus under investigation and would rely on the isolated RNA (an enzymatically and chemically unstable nucleic acid) as the source of the nucleic acid used in the RT-PCR assay. Using random hexamer to primer the RT reaction produces cDNA that is more enzymatically and chemically stable, more resistant to freeze-thaw degradation, and that can be used as necessary with any primer set in the PCR assay. The costs associated with the use of the RT-PCR beads for one, two, or three or more assays is less, the same, or more, respectively, than the costs associated with our reported RT-PCR assay, yet the easy of use and temperature stability of the RT-PCR beads warrants additional testing and field evaluation. Research that is currently ongoing is focusing on developing PCR primers sets that can detect, for example, all alphaviruses present in a mosquito pool. This approach would allow for rapid screening of large numbers of mosquito pools. After detecting an alphavirus, specific primers would be used to identify the viral species.

The reported mosquito processing, RNA isolation and cDNA synthesis, and PCR amplification can detect EEEV in the field under extreme environmental conditions. Select military laboratories have the mission to identify and evaluate health hazards in the areas where deployed personnel operate. Incorporating the reported protocols and techniques into the Theater Army Medical Laboratory’s capabilities will enable personnel in the field to detect arthropod-borne viruses and to perform health-associated risk assessments in a timely manner. Currently, we are expanding the RT-PCR diagnostic protocols to include procedures for detecting malaria and other parasites found in mosquitoes and other arthropods in the field.

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