Journal of the American Mosquito Control Association, 23(4):488–491, 2007

OPERATIONAL NOTE

IMPROVED HIGH-THROUGHPUT METHOD FOR MOLECULAR IDENTIFICATION OF *CULEX* MOSQUITOES

ILIA ROCHLIN,[1,3] MICHAEL P. SANTORIELLO,[1] RICHARD T. MAYER[2] AND SCOTT R. CAMPBELL[1,4]

ABSTRACT. *Culex pipiens*, *Cx. restuans*, and *Cx. salinarius* play important and most likely different roles in transmission of West Nile virus (WNV) in the northeastern United States. While *Cx. pipiens* and *Cx. restuans* are considered the main enzootic vectors of WNV, *Cx. salinarius* may be involved in epizootic cycles due to its broader host preferences. Accurate morphological identification of field-collected *Culex* specimens may be difficult, and therefore the New York State Department of Health arbovirus surveillance program allows combined *Cx. pipiens* and *Cx. restuans* pools to be tested for WNV. We have developed a modified and improved DNA isolation protocol using proteinase K digestion without traditional mosquito trituration and nucleic acid extraction to permit high-throughput screening of a large number of *Culex* specimens for species identification using polymerase chain reaction (PCR). This method utilizes a 96-well–plate format and a novel 1-step crude extraction procedure using proteinase K to obtain genomic DNA template from 1 mosquito leg in sufficient quantity for at least 2 standard 50-µl PCR reactions. Proteinase K digestion of legs from individual *Culex* mosquitoes was performed and used for PCR amplification with previously described species-specific ribosomal DNA primers. Using these rDNA primers, our modified proteinase K method successfully identified 91% to 100% of the *Culex* samples.

KEY WORDS *Culex*, mosquitoes, multiplex polymerase chain reaction, species identification, surveillance

Mosquitoes from the genus *Culex* are considered the main enzootic vectors of West Nile virus (WNV) in the northeastern United States (Andreadis et al. 2001, Kulasekera et al. 2001, Nasci et al. 2001). New data suggest that *Culex salinarius* (Coquillett) might be an important epizootic vector involved in human transmission (Andreadis et al. 2004). The respective contributions of *Cx. pipiens* (L.) and *Cx. restuans* (Theobald) to WNV epizootic cycles have not been unequivocally determined (Kilpatrick et al. 2005). In the New York State Department of Health WNV surveillance program, adult females of the 3 *Culex* species are collected and tested for WNV in pools of 10–50 specimens. Routine morphological differentiation of female *Cx. pipiens*, *Cx. restuans*, and *Cx. salinarius* to the species level can be difficult because of similar morphology and damage during the collection process (Crabtree et al. 1995, Debrunner-Vossbrinck et al. 1996, Apperson et al. 2002). As a result, *Culex* pools collected in New York are mixed *Cx. pipiens* and *Cx. restuans* (Bernard et al. 2001) and may contain *Cx. salinarius* (Bernard and Kramer 2001). The same situation may occur wherever the ranges of the 3 species overlap, namely the mid-Atlantic, northeastern, and central United States (Darsie and Ward 1981). Taking into consideration different biological roles of *Culex* species in WNV transmission, there is a need for a reliable and rapid identification procedure for surveillance.

Based on high-copy nuclear ribosomal DNA, multiplex polymerase chain reaction (PCR) primers were developed and shown to be highly sensitive and 100% species specific for *Cx. pipiens*, *Cx. restuans*, and *Cx. salinarius* (Crabtree et al. 1995). Several traditional methods for mosquito genomic DNA extraction include triturating each sample separately in liquid nitrogen or lysis buffer (Crabtree et al. 1995, Debrunner-Vossbrinck et al. 1996), using manufactured kits (Crabtree et al. 1995, Apperson et al. 2002, Toma et al. 2002, Aspen et al. 2003), using a robot with manufactured kits (Savage et al. 2006), or performing multistep purification and precipitation procedures (Crabtree et al. 1997, Walton et al. 1999, Beebe et al. 2002). However, none of these protocols are suitable for routine screening of a large number of individual specimens because of complexity, time, or prohibitive cost. An optimal method would be accurate, quick, use a small amount of easily obtainable tissue to leave sufficient material for arbovirus testing, and yield enough DNA for multiple reactions. Also, minimized conventional tissue grinding would

[1] Arthropod-Borne Disease Laboratory, Suffolk County Department of Health Services, 335 Yaphank Avenue, Yaphank, NY 11980-9744.
[2] USDA, ARS, Arthropod-Borne Animal Diseases Research Laboratory, College of Agriculture, Department 3354, 1000 East University Avenue, Laramie, WY 82071.
[3] Present address: Division of Vector Control, Suffolk County Department of Public Works, 335 Yaphank Avenue, Yaphank, NY 11980-9744.
[4] To whom correspondence should be addressed.

reduce personnel exposure to potentially infectious aerosolized material during trituration. Here we describe a simple *Culex* species-specific identification protocol adapted for a large-scale surveillance program that is consistent with the above criteria. This method utilizes a 96-well–plate format and a novel 1-step crude extraction procedure using proteinase K to obtain genomic DNA template from 1 mosquito leg in sufficient quantity for at least 2 standard 50-μl PCR reactions.

Initially, 5 crude DNA extraction methods were attempted and evaluated by PCR for efficacy and reliability. The multiplex PCR protocol used to evaluate each DNA extraction method is given below. Extraction of DNA was performed using either distilled water or a denaturing buffer (10 mM Tris-HCl, pH 8.2, 1 mM ethylenediamine tetraacetic acid (EDTA), 50 mM NaCl, 0.1% Triton-X 100; Rafferty et al. 2002). For both treatments, 40 μl of either distilled water or the denaturing buffer was pipetted into each well containing 1 mosquito leg, heated at 96°C for 10 min, and 19 μl of the sample removed for the PCR amplification. After observing zero or limited amplification (≤50%) with the denaturing buffer or distilled water method, respectively (data not shown), direct DNA extraction from the mosquito leg was attempted during the PCR reaction (Grevelding et al. 1996). Accordingly, 1 mosquito leg was placed directly into each well containing the PCR reagents and 19 μl of distilled water, but no significant improvement in sensitivity (≤50%) was noted compared to that of the aforementioned distilled water DNA extraction method (data not shown).

A Chelex resin solution, usually in conjunction with proteinase K digestion, has been widely applied for DNA extraction in forensic science when small amounts of tissue are available (Stein and Raoult 1992, Sepp et al. 1994). To adapt this for use with mosquitoes, 1 mosquito leg was put into a well with 40 μl of a 5% Chelex solution containing proteinase K (Chelex-100 [Sigma, St. Louis, MO], 100 μg/ml proteinase K [QIAGEN, Valencia, CA], 30 mM Tris-HCl, pH 8.2), incubated for 12 h at 50°C, heated at 96°C for 10 min, and 19 μl of the sample removed for PCR amplification. Compared to the previous 3 methods, this protocol produced higher efficacy and a consistently strong amplification signal (≥91%; data not shown). However, dispersing small and equal volumes of Chelex solution among the plate wells was difficult. Therefore, a 5th crude DNA extraction method was attempted using proteinase K digestion without Chelex. For DNA isolation from mosquito legs, both methods were equally efficient so the proteinase K method without Chelex was further optimized and eventually adopted for routine surveillance.

An autogenous *Cx. pipiens* laboratory colony was used for initial optimization, and the same protocol was then applied to field-collected *Culex* from the 2005 surveillance season. One leg was removed per mosquito and put into a single well of a standard full-skirted 96-well plate. Each well contained 30 μl of distilled water. Each plate held 92 legs, leaving the last 4 wells empty for positive and negative controls. These specimen-storage plates were immediately sealed with a plastic film and stored at −70°C.

Proteinase K digestion was performed in the specimen-storage plate. After thawing at room temperature, 10 μl of the digestion solution (400 μg/ml proteinase K, 120 mM Tris-HCl, pH 8.2) was added to each well using a multichannel pipette. The plate was incubated at 50°C for 12 h on an MJ Research thermal cycler (Model PTC-200; Bio-Rad Laboratories, Hercules, CA) with a heated lid to prevent excessive evaporation and subsequent condensation. Proteinase K was then inactivated by heating at 96°C for 10 min, and the plate containing the crude proteinase K DNA digests was stored at 4°C.

The multiplex PCR analysis was performed in 50 μl (total volume) containing 25 μl of GoTaq Green Master 2X Mix (Promega Corp., Madison, WI) that contains Taq polymerase and gel loading dye to reduce pipetting steps. A 10-mM stock solution of 3 previously published ribosomal 28S DNA forward primers specific for *Cx. pipiens* (PQ10; 698 base-pair [bp] product), *Cx. restuans* (R6; 506-bp product), and *Cx. salinarius* (S20; 175-bp product), and 1 mutual reverse primer (CP16) were used (Crabtree et al. 1995). The final $MgCl_2$ concentration was raised from the original 1.5 mM in the GoTaq Green Master Mix to 2.5 mM by adding 2 μl of 25-mM $MgCl_2$ per reaction to improve specificity. Consequently, the final PCR master mix contained 200 μM of each deoxynucleotide triphosphates (dNTPs), 2.5 mM $MgCl_2$, and 200 μM of each of 4 primers (1 μl each primer) for a final reaction volume of 31 μl. After loading the final PCR master mix into each well of a new 96-well plate, 19 μl of the proteinase K digest was transferred to each well using a multichannel pipette and the plate containing the remainder of the samples was returned to −70°C. For positive controls, DNA was extracted from 3 to 5 mosquitoes of each *Culex* species using the DNeasy® tissue kit (QIAGEN) per manufacturer's instructions from colony and Suffolk County field-collected *Culex* mosquitoes that were identified morphologically and by PCR analysis. Genomic DNA was diluted 1:100 or 1:1,000 to provide a species-specific signal with a relative staining intensity equal to or below that of the crude proteinase K DNA digests from the leg samples. One well per plate was left blank as a negative control by adding distilled water.

The PCR amplification was optimized and performed in the thermal cycler programmed for 1 denaturing cycle at 94°C for 5 min, 33 amplification cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and 1 72°C extension cycle for 7 min. To visualize PCR products, electrophoresis of up to 200 samples (i.e., 2 full 96-well plates, controls, and 100-bp DNA ladder II [GeneChoice Inc., Frederick, MD]) was completed using the Millipede A6 gel system (Owl Separation Systems, Portsmouth, NH). The PCR amplification products were loaded onto a 1.8% agarose gel using a multichannel pipette, electrophoresed in tris-acetate-EDTA (TAE) buffer (pH 8.3) at a constant 100 V for ~1.5 h, and stained with 0.5 μg/ml ethidium bromide in TAE buffer (pH 8.3).

Using the modified crude proteinase K digestion and PCR protocol, ribosomal DNA was amplified successfully from colony and field-collected mosquito legs. This produced species-specific bands identical to those of the positive controls (Figs. 1A, 1B). The efficacy of this method for field-collected mosquitoes varied from 91% to 100% per plate, which was comparable to the Chelex solution method, albeit with a slightly weaker signal (data not shown). Crude DNA extraction with distilled water and in the PCR reaction mix produced visible bands in up to 50% of the samples per plate, while the denaturing buffer method produced no amplification (data not shown). Previous reports indicate nearly identical sensitivity of Chelex solution and proteinase K–only treatments (Sepp et al. 1994) and a variable rate of failure with distilled water extraction (Stein and Raoult 1992, Sepp et al. 1994). Cross contamination was seen in some samples, probably due to carryover on the forceps during leg removal. The contamination was found to be eliminated by using a 95% ethanol wash and flaming of the forceps before processing each specimen.

For *Anopheles* genotyping, Rafferty et al. (2002) reported another method employing a 96-well–plate format and a 1-step crude DNA extraction from entire mosquitoes and body parts using a denaturing buffer (containing no proteinase K) and heat. However, *Culex* samples subjected to the same treatment failed to produce visible PCR products in this study (data not shown). Possible explanations for this failure may be different PCR reagents, less *Culex* tissue used for DNA extraction, different reaction volumes, or undetermined *Culex*-specific template inhibition, which was reduced or eliminated by proteinase K treatment.

In summary, the protocol reported herein permits quick identification of *Cx. pipiens*, *Cx. restuans*, and *Cx. salinarius* by combining a simple DNA extraction method in a 96-well format and a multiplex PCR analysis. If 2 plates are processed simultaneously, 184 individual specimens can be identified within a 24-h period, reducing cost, labeling, and manipulation time, while preserving high efficacy and specificity. Additional benefits include preserving mosquito bodies for further arbovirus testing and a reduced risk of exposure of personnel to aerosolized material that might occur during conventional tissue trituration. Furthermore, this protocol may be expanded to include additional *Culex* species using published primer data (Aspen 2003) or applied to other morphologically indistinguishable groups of mosquito species. Thus, this method will contribute to improved arbovirus surveillance and vector control efforts by defining

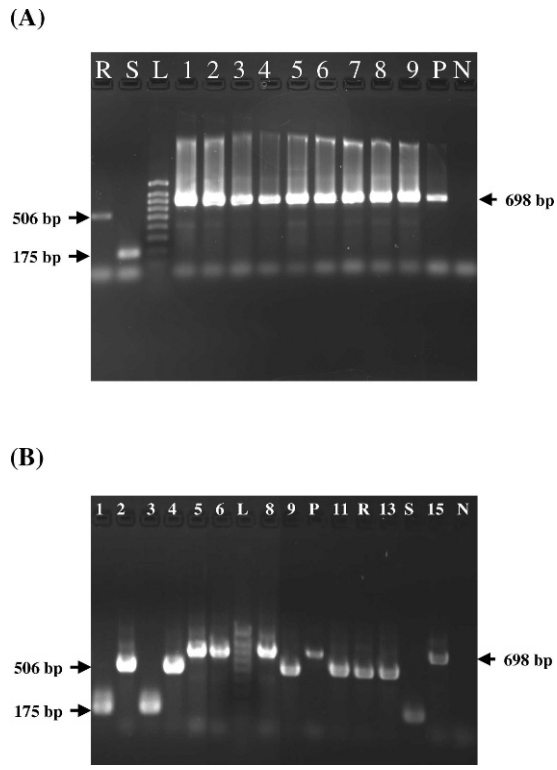

Fig. 1. Amplification products of the 4-primer polymerase chain reaction assay using proteinase K crude digest. (A) Lane R, *Culex restuans* genomic DNA control; lane S, *Cx. salinarius* genomic DNA control; lane L, 100 base-pair (bp) DNA ladder; lanes 1–9, *Cx. pipiens*, colony, single leg; lane P, *Cx. pipiens* genomic DNA control; lane N, negative control, no template. (B) Lanes 1–6, 8–9, 11, 13, and 15, field-collected female *Culex* mosquitoes, single leg; lanes 5, 6, 8, and 15, *Cx. pipiens*; lanes 2, 4, 9, 11, and 13, *Cx. restuans*; lanes 1 and 3, *Cx. salinarius*; lane L, 100-bp DNA ladder; lane P, *Cx. pipiens* genomic DNA control; lane R, *Cx. restuans* genomic DNA control; lane S, *Cx. salinarius* genomic DNA control; lane N, negative control, no template. Arrows indicate the 698-bp *Cx. pipiens* fragment, the 506-bp *Cx. restuans* fragment, and the 175-bp *Cx. salinarius* fragment.

the species composition of *Culex* populations and WNV-positive *Culex* pools.

We thank Melissa Zanini for excellent technical support and James Dantonio for assistance with the *Culex pipiens* colony. This research was partially funded by United States Department of Agriculture Specific Cooperative Agreement # 58-5410-4-338.

REFERENCES CITED

Andreadis TG, Anderson JF, Vossbrinck CR. 2001. Mosquito surveillance for West Nile virus in Connecticut, 2000: isolation from *Culex pipiens*, *Cx. restuans*, *Cx. salinarius*, and *Culiseta melanura*. *Emerg Infect Dis* 7:670–674.

Andreadis TG, Anderson JF, Vossbrinck CR, Main AJ. 2004. Epidemiology of West Nile virus in Connecticut: a five-year analysis of mosquito data 1999–2003. *Vector Borne Zoonotic Dis* 4:360–378.

Apperson CS, Harrison BA, Unnasch TR, Hassan HK, Irby WS, Savage HM, Aspen SE, Watson DW, Rueda LM, Engber BR, Nasci RS. 2002. Host-feeding habits of *Culex* and other mosquitoes (Diptera: Culicidae) in the Borough of Queens in New York City, with characters and techniques for identification of *Culex* mosquitoes. *J Med Entomol* 39:777–785.

Aspen S, Crabtree MB, Savage HM. 2003. Polymerase chain reaction assay identifies *Culex nigripalpus*: part of an assay for molecular identification of the common *Culex* (*Culex*) mosquitoes of the eastern United States. *J Am Mosq Control Assoc* 19:115–120.

Beebe NW, van den Hurk AF, Chapman HF, Frances SP, Williams CR, Cooper RD. 2002. Development and evaluation of a species diagnostic polymerase chain reaction–restriction fragment-length polymorphism procedure for cryptic members of the *Culex sitiens* (Diptera: Culicidae) subgroup in Australia and the southwest Pacific. *J Med Entomol* 39:362–369.

Bernard KA, Kramer LD. 2001. West Nile virus activity in the United States, 2001. *Viral Immunol* 14:319–338.

Bernard KA, Maffei JG, Jones SA, Kauffman EB, Ebel G, Dupuis AP, Ngo KA, Nicholas DC, Young DM, Shi PY, Kulasekera VL, Eidson M, White DJ, Stone WB, Kramer LD. 2001. West Nile virus infection in birds and mosquitoes, New York State, 2000. *Emerg Infect Dis* 7:679–685.

Crabtree MB, Savage HM, Miller BR. 1995. Development of a species-diagnostic polymerase chain reaction assay for the identification of *Culex* vectors of St. Louis encephalitis virus based on interspecies sequence variation in ribosomal DNA spacers. *Am J Trop Med Hyg* 53:105–109.

Crabtree MB, Savage HM, Miller BR. 1997. Development of a polymerase chain reaction assay for differentiation between *Culex pipiens pipiens* and *Cx. p. quinquefasciatus* (Diptera: Culicidae) in North America based on genomic differences identified by subtractive hybridization. *J Med Entomol* 34:532–537.

Darsie RF, Ward RA. 1981. Identification and geographical distribution of the mosquitoes of North America, north of Mexico. *Mosq Syst Suppl* 1:1–313.

Debrunner-Vossbrinck BA, Vossbrinck CR, Vodkin MH, Novak RJ. 1996. Restriction analysis of the ribosomal DNA internal transcribed spacer region of *Culex restuans* and mosquitoes in the *Culex pipiens* complex. *J Am Mosq Control Assoc* 12:477–482.

Grevelding CG, Kampkotter A, Hollmann M, Schafer U, Kunz W. 1996. Direct PCR on fruitflies and blood flukes without prior DNA isolation. *Nucleic Acids Res* 24:4100–4101.

Kilpatrick AM, Kramer LD, Campbell SR, Alleyne EO, Dobson AP, Daszak P. 2005. West Nile virus risk assessment and the bridge vector paradigm. *Emerg Infect Dis* 11:425–429.

Kulasekera VL, Kramer L, Nasci RS, Mostashari F, Cherry B, Trock SC, Glaser C, Miller JR. 2001. West Nile virus infection in mosquitoes, birds, horses, and humans, Staten Island, New York, 2000. *Emerg Infect Dis* 7:722–725.

Nasci RS, White DJ, Stirling H, Oliver JA, Daniels TJ, Falco RC, Campbell S, Crans WJ, Savage HM, Lanciotti RS, Moore CG, Godsey MS, Gottfried KL, Mitchell CJ. 2001. West Nile virus isolates from mosquitoes in New York and New Jersey, 1999. *Emerg Infect Dis* 7:626–630.

Rafferty CS, Campbell SR, Wirtz RA, Benedict MQ. 2002. Polymerase chain reaction–based identification and genotyping of *Anopheles* mosquitoes with a 96-pin bacterial replicator. *Am J Trop Med Hyg* 66:234–237.

Savage HM, Anderson M, Gordon E, McMillen L, Colton L, Charnetzky D, Delorey M, Aspen S, Burkhalter K, Biggerstaff BJ, Godsey M. 2006. Oviposition activity patterns and West Nile virus infection rates for members of the *Culex pipiens* complex at different habitat types within the hybrid zone, Shelby County, TN, 2002 (Diptera: Culicidae). *J Med Entomol* 43:1227–1238.

Sepp R, Szabo I, Uda H, Sakamoto H. 1994. Rapid techniques for DNA extraction from routinely processed archival tissue for use in PCR. *J Clin Pathol* 47:318–323.

Stein A, Raoult D. 1992. A simple method for amplification of DNA from paraffin-embedded tissues. *Nucleic Acids Res* 20:5237–5238.

Toma T, Miyagi I, Crabtree MB, Miller BR. 2002. Investigation of the *Aedes* (*Stegomyia*) *flavopictus* complex (Diptera: Culicidae) in Japan by sequence analysis of the internal transcribed spacers of ribosomal DNA. *J Med Entomol* 39:461–468.

Walton C, Handley JM, Kuvangkadilok C, Collins FH, Harbach RE, Baimai V, Butlin RK. 1999. Identification of five species of the *Anopheles dirus* complex from Thailand, using allele-specific polymerase chain reaction. *Med Vet Entomol* 13:24–32.