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Research Paper

West Nile Virus Infection Rates in Pooled and Individual Mosquito Samples

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ABSTRACT

The detection of West Nile virus (WNV) in mosquitoes by real-time RT-PCR provides valuable information on the epidemiology of the virus and identifies mosquito species that are potential vectors. Testing sets of pooled mosquitoes of the same species is logistically the easiest and most cost-effective approach for WNV testing; however, little information is available on how the results of small pooled sets relate to those of testing individual mosquitoes. During the 2002 outbreak, we compared pooled and individual samples of two mosquito species (*Culex pipiens* and *Culex restuans*) collected from three Health Unit regions in Ontario, Canada. Significantly more *Cx. restuans* were infected with WNV compared to *Cx. pipiens*. We show that with pool sizes of five individuals both MIR (minimum infection rates) and MLE (maximum likelihood estimation) values were acceptable in estimating infection rates. Key Words: West Nile virus—Mosquito—Infection rate. Vector-Borne Zoonotic Dis. 4, 198–208.

INTRODUCTION

West Nile virus (WNV) is a single-stranded plus sense RNA virus in the family Flaviviridae (genus *Flavivirus*) and possesses a genome of approximately 11,000 nucleotides (Peterson and Roehrig 2001). The natural transmission cycle of WNV involves mosquito vectors and birds, with mammals such as humans and horses being incidental dead-end hosts (Lanciotti et al. 2000). The first documented case of WNV infection in the Western Hemisphere was reported in New York City in 1999 (CDC 1999). In Canada, the first WNV positive bird was reported from Windsor-Essex County, Ontario, in August 2001 (OMHLTC 2001) and the first positive mosquito pool was reported in October 2001 from the Region of Peel, Ontario (Drebot et al. 2003). Approximately 1 year later, September 2002, the first confirmed human case was reported in the Region of Peel. In the 2002 season, a total of 281 birds, 101 horses, 319 humans, and 598 mosquito pools were reported positive for WNV in Ontario; Halton, Peel and Toronto Health Units had the highest number of positive mosquito pools and reported human cases in Ontario (mosquito pools 72, 132, 175; human cases 57, 40, 129, respectively) (OMHLTC 2002).

Since the introduction of WNV in the United States and Canada, intensive surveillance programs have been introduced to monitor the spread of the virus among mosquitoes and birds. In 2002 the standard protocol used in Canada for mosquito testing involved pooling mosquitoes of the same species from the same collection in groups of up to 50 individuals, homogenizing them, extracting total RNA, and

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then testing for the presence of WNV using two TaqMan® reverse transcriptase polymerase chain reaction (RT-PCR) assays using generic and envelope primer and probe sets (Lanciotti et al. 2000). From all positive samples RNA was then re-extracted and re-tested with TaqMan® RT-PCR assays using the same primer and probe sets to confirm the presence of WNV (Drebot et al. 2003). The testing of mosquito pools gives an indication of which mosquito species harbor WNV and, if sufficient numbers are tested, infection rates can be calculated; however, the actual number of individual WNV positive mosquitoes in a pool is unknown. The estimation of the proportion of infected mosquitoes in a specific area or in pooled samples can be calculated using two indices, namely, MIR (minimum infection rate) or MLE (maximum likelihood estimation). MIR calculations assume a minimum of one positive mosquito in a sample but this is not always the case. Furthermore, MIR requires that the sample size is >1000 mosquitoes tested (Bernard et al. 2001). When sample size is <1000 mosquitoes, it is recommended that MLE be used instead.

The purpose to this study was twofold: (1) to determine the actual infection rate (IR) (i.e., the number of individual WNV positive mosquitoes per 1000 mosquitoes) of *Culex pipiens* and *Culex restuans* in samples collected from Halton, Peel and Toronto during the peak of transmission to humans in 2002 and (2) to evaluate whether infection data based on pooled MIR and MLE values overestimates or underestimates WNV infection rates compared to the calculated IR value.

MATERIALS AND METHODS

Mosquito collection

Adult mosquitoes were collected in CO_2-baited CDC miniature light traps (JW Hock Co.) as part of Ontario's WNV surveillance program. Trained staff from participating Health Units set mosquito traps out in the late afternoon, and the collection cups containing one night's mosquitoes were retrieved the following morning. Traps containing live mosquitoes were transported within 24 h to Brock University in coolers on ice packs. The collection cups were then frozen at −20°C for at least 2 h to kill the mosquitoes. Mosquitoes were identified to species using the keys of Wood et al. (1979) on a BioQuip® chill plate using a Leica® dissecting microscope. Identified mosquitoes were pooled according to species, health unit, trap location, and collection date in sterile 2-mL Sarstedt® vials. Vials were then stored at −80°C until virus testing. Individual mosquitoes were separated from the pooled samples, placed into individual sterile 2-mL Sarstedt® vials, and processed separately.

Mosquito homogenization

One sterile BB (Copperhead premium grade airgun shot, steel BB cal. 4.5 mm) and 1 mL of BA-1 diluent (0.05M Tris buffer pH 7.5, 0.36% sodium bicarbonate, 1.0% bovine albumin fraction V, 100 units/mL penicillin-streptomycin, 1 × media 199) were added to each vial. Mosquitoes were homogenized at 30 Hz for 2 min using a Qiagen® Mixer Mill MM 300 and then centrifuged at 10,000 rpm for 2 min. One hundred μL of mosquito homogenate was removed and added to 250 μL of lysis buffer for total RNA extraction.

RNA extraction

All mosquitoes were processed individually. RNA extraction was performed according to the manufacturer's instructions for Qiagen RNeasy® Mini Kit except for the following modifications. RNA was eluted in 100 μL of RNase free water instead of 75 μL and total RNA was then stored at −80°C until used. Second RNA re-extractions followed the same manufacturer's instructions except that 200 μL of mosquito slurry was added to 400 μL of RLT lysis buffer instead of 100 μL mosquito slurry to 250 μL of lysis buffer; RNA was eluted in 100 μL of RNase free water.

WNV detection

Two sets of primers and probes were used for WNV identification. Generic primers and probes (GPR) were used for initial identification and envelope primers and probes (EPR) were used for the first confirmatory test. Probes were labeled at the 5′ end with 6-carboxyfluo-

rescein (FAM) reporter dye and were labeled at the 3′ end with 6-carboxytetramethylrhodamine (TAMRA) quencher dye. Nucleotide positions refer to WNV NY-99 complete genome sequence accession number AF196835. WNV Egyptian strain Eg101 (accession number AF260968) supplied by Dr. Robbin Lindsay of the National Microbiology Laboratory Winnipeg, Manitoba was used in positive WNV controls and water samples were used as negative controls.

Viral testing

Each 5-μL RNA sample contained 1 μL each of individual mosquito sample to make up a mosquito pool. The 5-μL RNA sample was combined with the primers and probes in a 50-μL reaction volume using TaqMan® One-Step RT-PCR Master Mix Reagents from Applied Biosystems [17.45 μL RNase free H_2O, 25.00 μL of TaqMan 2 × Universal PCR master mix, 0.5 μL of generic primer 1 (100 μM; genome position 10668 5′CAGACCACGCTACGGCG3′), 0.5 μL of generic primer 2 (100 μM; genome position 10770c 5′CTAGGGCCGCGTGGG3′;), 0.3 μL of generic probe (20 μM; FAM-5′CTGCGGAGAGTGCAGTCTGCGAT3′-TAMRA), 1.25 μL of ×40 Multiscribe and RNase inhibitor mix]. Samples were amplified using an iCycler iQ™ Real-Time PCR Detection System (BioRad) with the following cycles and temperatures: 1 cycle of 50°C for 30 minutes and 95°C for 10 min and 40 cycles of 90°C for 15 sec, 60°C for 1 min and hold at 4°C. The iCycler iQ™ Real-Time PCR Detection System graphically displays relative fluorescence for each sample at every cycle and generates corresponding threshold cycle (Ct) values. Ct values represent the PCR cycle number at which the reporter dye first exceeds a baseline signal. Ct values are inversely related to concentration of cDNA such that, as the concentration doubles the Ct value decreases by one. Samples with Ct values below 37 were designated as positive, and those above 37 were negative. If WNV was detected with the generic primer and probe set, another 5 μL of RNA from the same mosquito pool was tested using the above conditions with the envelope primer and probe set (envelope primer 1; genome position 1160 5′TCAGCGATCTCTCCACCAAAG3′; envelope primer 2; genome position 1229c 5′GGGTCAGCACGTTTGTCATTG3′; envelope probe sequence: FAM-5′TGCCCGACCATGGGAGAAGCTC3′-TAMRA). In addition, the individual mosquito samples from which each pool was derived were tested separately to determine the number of individual mosquitoes positive in a pool. From all positive samples RNA was then re-extracted and tested further with TaqMan® RT-PCR using the above conditions to confirm the presence of WNV. A confirmed WNV-positive sample is defined as a sample in which WNV was detected in the RNA extraction and in the re-extraction using both the generic and envelope primer and probe sets.

Mathematical equations and statistical analysis

Infection rates (IR, MIR and MLE) were calculated using the equations below (Bernard et al., 2001). Chi-squared tests were used to compare infection rates among mosquito species. All Chi-squared tests were generated with Epi Info version 3.2 CDC. Significance was tested at a level of alpha = 0.05.

$$\text{Infection rate (IR)} = (\text{no. positive individuals/no. mosquitoes tested}) \times 1000$$

$$\text{Minimum infection rate (MIR)} = (\text{no. positive pools/total no. mosquitoes tested}) \times 1000$$

$$\text{Maximum likelihood estimation (MLE)} = [1 - (n - X/n)^{1/m}] \times 1000$$

For this last equation, n is the number of pools tested, X is the number of positive pools, and m is the pool size. A requirement for using MLE is that the pool size remains constant (Chiang and Reeves 1962). MLE assumes a binominal distribution of positive mosquitoes among pools and calculates the infection rate most likely observed from the results (Gu et al. 2003).

RESULTS

A total of 310 mosquitoes (*Cx. pipiens* and *Cx. restuans*) from Halton, Peel and Toronto were tested individually for WNV. These were collected in CDC light traps between August 18

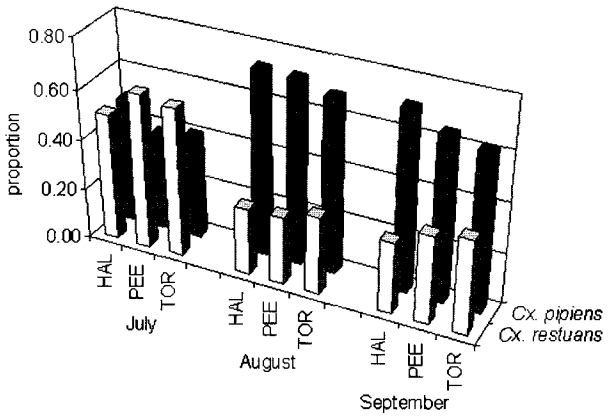

FIG. 1. 2002 monthly collection proportions for field-collected female *Cx. pipiens* and *Cx. restuans* in Halton (HAL), Peel (PEE), and Toronto (TOR) regions. *Cx. restuans* mosquitoes peak in July whereas *Cx. pipiens* mosquitoes peak in August–September. All mosquitoes used in the current study were collected between August 18 and September 14, 2002.

and September 14, 2002 when *Cx. pipiens* mosquitoes were more common than *Cx. restuans* (Fig. 1). The average pool sizes collected for *Cx. pipiens* and *Cx. restuans* were 6.25 and 5.94, respectively. Of the 310 mosquitoes tested, 18 (5.81%) were positive for WNV. The positive individual mosquitoes include six from Halton (one *Culex pipiens*, five *Culex restuans*), 10 from Peel (four *Cx. pipiens*, six *Cx. restuans*), and two from Toronto (two *Cx. restuans*) (Table 1). Combining the data for the three Health Units gave IR values of 34.48 for *Cx. pipiens* and 78.78 for *Cx. restuans*. Chi-square analysis showed a significantly lower IR value calculated for *Cx. pipiens* than for *Cx. restuans* ($\chi^2[1] = 18.99$, $p = 0.0000131$). *Cx. pipiens* average Ct values were 29.45 GPR and 30.18 EPR, and the average Ct values on re-extracted RNA samples were 21.16 GPR and 28.94 EPR. *Cx. restuans* average Ct values were 28.57 GPR and 27.44 EPR, and the re-extracted RNA samples were 24.83 GPR and 28.36 EPR.

A total of 52 pooled mosquito samples (*Cx. pipiens* and *Cx. restuans*) from Halton, Peel, and Toronto were tested for WNV, and of these, 17 of 52 (32.7%) were positive for WNV. The positive mosquito pools included five pools from Halton (one *Cx. pipiens* pool, four *Cx. restuans* pools), nine pools from Peel (four *Cx. pipiens* pools, five *Cx. restuans* pools) and three pools from Toronto (one *Cx. pipiens* pool,* two *Cx. restuans* pools) (Table 1). MIR values calculated for the *Cx. pipiens* and *Cx. restuans* mosquito pools were 41.37 and 66.67, respectively. Chi-square analysis showed significant differences between MIR values calculated for *Cx. pipiens* and *Cx. restuans* ($\chi^2[1]=6.62$, $p = 0.01$). MLE values calculated for *Cx. pipiens* and *Cx. restuans* were 45.30 and 77.89, respectively. Chi-square analysis showed significant differences between MLE values calculated for *Cx. pipiens* and *Cx. restuans* ($\chi^2[1] = 9.43$, $p = 0.002$). No significant differences were observed among *Cx. pipiens* IR, MIR and MLE values nor among *Cx. restuans* IR, MIR and MLE values. *Cx. pipiens* average Ct values for pooled samples were 27.67 GPR and 29.09 EPR and the re-extracted RNA samples were 23.86 GPR and 28.98 EPR. *Cx. restuans* average Ct values for pooled samples were 28.87 GPR and 29.46 EPR, and the re-extracted RNA samples were 26.29 GPR and 29.53 EPR.

DISCUSSION

Many intensive surveillance programs have monitored the spread of WNV and provide valuable information about which mosquito species harbour the virus and the role they may play in the transmission cycle. It is thought that *Cx. restuans* may have an important role in the initiation of WNV at the beginning of mosquito season and that *Cx. pipiens* may have a greater role in amplification of the virus later in the season (Andreadis et al. 2001). Since the mosquito samples tested here were collected when *Cx. pipiens* were more abundant than *Cx. restuans*, we had initially expected IR values to be higher in *Cx. pipiens*. However, the opposite was true. Approximately two times as many *Cx. restuans* tested positive as *Cx. pipiens*, which may be the result of seasonal environmental conditions (Dohm et al. 2002) or vector biology (Turell et

*The *Cx. pipiens* pool from Toronto was confirmed as positive, but none of the individual samples from which the pool was derived was confirmed as positive. The Ct values generated with the re-extracted samples were above 37. Degradation of WNV RNA may be occurring during freeze-thawing of the mosquito homogenates.

TABLE 1. VIRAL TESTING RESULTS OF *CX. PIPIENS* AND *CX. RESTUANS* FROM THREE HEALTH UNITS IN ONTARIO (HALTON, PEEL, AND TORONTO)

		No. pools			*Positive pools with*		
Location	*Species*	*Pos. pools*	*Neg. pools*	*Total no. pools*	*1 pos. IND/5*	*2 pos. IND/5*	*Total no. IND*
HAL	*Cx. pipiens*	1	8	9	1	0	45
	Cx. restuans	4	7	11	3	1	55
PEE	*Cx. pipiens*	4	6	10	4	0	50
	Cx. restuans	5	5	10	4	1	50
TOR	*Cx. pipiens*[a]	1	9	10	0	0	50
	Cx. restuans	2	10	12	2	0	60
Totals				52			310

[a]The *Cx. pipiens* pool from Toronto was confirmed as positive, not the individual samples that were used to make the pool.

The panels to the left are the number of positive pools, negative pools and total number of pools tested while the panels to the right are the number of positive individuals found in each positive pool of five and the total number of individual mosquitoes tested. HAL, Halton; PEE, Peel; TOR, Toronto; pos, positive; neg., negative; IND, individual; no., number.

al. 2001). We speculate that the *Cx. restuans* individuals were older females that had a greater opportunity to pick up a WNV infection than the presumably younger *Cx. pipiens* females. This is a testable hypothesis that warrants further study in subsequent WNV seasons by age-grading the females prior to testing them for WNV by RT-PCR.

True infection rates (IR) can be determined by testing individual mosquitoes but this is time consuming and expensive. Instead testing sets of pooled mosquitoes of the same species is an easier and more cost effective approach. However, there remains uncertainty concerning the best way to estimate IR using pooled data. According to Bernard et al. (2001) MIR can only be used if more than 1000 mosquitoes are tested. Thus, if fewer than 1000 are tested it is suggested that MLE be used instead. MLE values are thought to provide an improved estimation of infection rates (Walter et al. 1980), estimating the infection rate itself (Gu et al. 2003). Gu et al. (2003) demonstrated that MLE values were more accurate in calculating infection rates compared to MIR values when pool size was large. According to Walter et al. (1980) no significant differences were observed between MIR and IR when sample size is small; however, no data were presented to back up this claim. In the current study when the pool size was held at 5 individuals per pool, no significant differences were observed among IR, MIR and MLE values for either *Cx. pipiens* or *Cx. restuans*; thus, MIR and MLE are both acceptable estimates of IR, the true infection rate. The "pooled infrate program," developed by Brad Biggerstaff. PooledInfRate: a Microsoft® Excel Add-In to compute prevalence estimates from pooled samples, CDC, Fort Collins, CO), was considered for determining MIR and MLE values. However we were reluctant to use the program. First, the MIR and MLE values generated by this program did not coincide with the values obtained from the equations provided. Secondly, the disclaimer stated at the end of the pooledinfrate manual does not provide any guarantee that the results generated by the program are accurate.

WNV is widespread throughout both the United States and Canada. It is necessary to fine tune mosquito surveillance methods, so that decisions about control strategies can be made as quickly as possible. Reliable indices for estimating infection rates are particularly important in situations where a vector species is in low abundance, but is heavily infected, as was the case with *Cx. restuans* in the current study. Our evaluation of pooled and individual mosquito samples demonstrated no differences when calculating WNV infection rates with MIR or with MLE when pool sizes were kept at five individuals. Since the testing of pooled samples is likely to remain the cornerstone of arboviral surveillance, and since it is opera-

tionally difficult to have equal sized, large pools to test, we conclude that the common practice of using MIR to estimate infection rate is defensible.

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