Quantifying Bluetongue Virus in Adult *Culicoides* Biting Midges (Diptera: Ceratopogonidae)

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ABSTRACT A TissueLyser system (QIAGEN) was used to rapidly and accurately estimate bluetongue virus "loads" in individual adult *Culicoides sonorensis* Wirth & Jones (Diptera: Ceratopogonidae). The optimized homogenization program that was developed, involved shaking insects for 1 min at 25 Hz with 2- or 3-mm stainless steel ball bearings. This program was used to measure the quantities of bluetongue virus present in insects that had either been inoculated or had ingested a viremic bloodmeal through an artificial membrane. The virus titers obtained using either infection technique and the optimized program did not differ significantly from those obtained using a polypropylene motor-driven pestle, a method that is currently in common use for studies of vector competence). The advantages of the new method, as a rapid means of detecting fully disseminated infections in individual field-caught flies, are discussed. Its use is compared with the processing of pools of *Culicoides* by conventional methods, where the extent of dissemination of the virus is unknown and could wrongly implicate species that are of low importance in transmission.

KEY WORDS *Culicoides*, Bluetongue virus, vector competence, oral susceptibility, TissueLyser

Bluetongue virus (family *Reoviridae*, genus *Orbivirus*, BTV) is transmitted between ruminants primarily via the bite of certain species of *Culicoides* (Diptera: Ceratopogonidae) biting midges. The detection of BTV in field-caught adults can be used to implicate certain species as vectors, information that is vital to our understanding of bluetongue disease epidemiology. Studies of vector competence in the northern Palaearctic and Mediterranean regions have relied on virus isolation, or reverse transcription-polymerase chain reaction (RT-PCR) detection of viral RNA. These methods have been used to analyze pools of parous, nonengorged female *Culicoides*, caught "at light" on farms where recent transmission has occurred (e.g., Mellor and Pitzolis 1979, Caracappa et al. 2003, De Liberato et al. 2005, Ferrari et al. 2005, Savini et al. 2005, Mehlhorn et al. 2007). However, these techniques take no account of the proportion of *Culicoides* that have fed on viremic animals, but, due to the presence of internal barriers to virus dissemination within the insect, subsequently develop only a nontransmissible although persistent infection (Jennings and Mellor 1987, Fu et al. 1999). The proportion of adult *Culicoides* from European species that becomes infected in this way is currently unknown, although a laboratory-based study suggests that it may be high (Carpenter et al. 2006).

Recently, a high-throughput TissueLyser/RT-PCR system was used to amplify RNA from individuals and pools of *Culicoides sonorensis* Wirth & Jones that were either intrathoracic (i.t.) inoculated or orally infected with BTV (Kato and Mayer 2007). Similar methods are in common use as part of other arboviral surveillance programs (Shi et al. 2001, Nasci et al. 2002). The length of time taken by "noncompetent" adult *Culicoides* to clear virus from their gut after they have fed on a viremic host (to give a negative RT-PCR result) is currently unknown. This issue is further complicated by the very high sensitivity of RT-PCR techniques and their ability to detect both infectious virus, and inactivated particles that could survive for sometime within the insect gut. However, it has been reported that individual adult *C. sonorensis* containing >3 $\log_{10}$ 50% tissue culture infected doses ($TCID_{50}$) of BTV per insect, will have a fully disseminated infection (Fu et al. 1999). A method that can be used to rapidly and accurately estimate viral load in terms of the amount of infectious virus per insect would, therefore, be very valuable for vector competence studies. In this study, we have optimized a TissueLyser method for the homogenization, isolation, and quantification of BTV from individual adult female *Culicoides*. The efficiency of the TissueLyser was also compared with that using the standard grinding method (polypropylene motor-driven pestles).

Materials and Methods

Virus and *Culicoides*. All infection experiments were carried out using a Kosovo strain of BTV-9 (KOS2001/03) that had been passaged twice through eggs and four times through BHK-21 cell culture (for

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further details on the virus, see http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/btv-9). The infectivity of the initial virus preparation (tissue culture supernatant) was measured in BHK-21 cells, as 6.75 $\log_{10}$ $TCID_{50}$ per ml. Groups of 2–3-d-old colonized *C. sonorensis*, a North American BTV vector, from the PIRB-s-3 strain (Wellby et al. 1996) were infected by using semiautomatic i.t. inoculation (Boorman 1975), or by membrane-feeding on a 1:1 volume mixture with sheep blood (Mellor 1971). Inoculated midges were processed immediately after inoculation, whereas membrane-fed adults were immobilized using CO_2 and the fully engorged female collected, placed in a pillbox (Watkins & Doncaster, Cranbrook, Kent, United Kingdom), and incubated at 25 ± 1°C at 80% RH. A cotton pad moistened with a 5% sugar solution was provided daily. After 10-d incubation, surviving females were selected for use.

Homogenization of Samples. The TissueLyser (QIAGEN, Crawley, United Kingdom) is capable of simultaneously homogenizing 48 samples by using high-frequency shaking of each sample with a ball bearing (Dejay Distribution Ltd., Crowborough, United Kingdom). Initially, seven programs of varying duration and frequency of homogenization (based around the manufacturer's recommendations), were tested with 10 i.t. inoculated *C. sonorensis* each (Table 1). Before homogenization, the adult insects were transferred into a 1.5-ml Eppendorf tubes containing 100 μl of Glasgow minimal essential medium (MEM; with 0.6% field antibiotics containing 2.0 μg/ ml Fungizone, 1,000 IU/ml penicillin, 50 mg/ ml neomycin, and 1,000 IU/ml polymyxin). After grinding with 3-mm-diameter stainless steel ball bearings, each ball was removed from the tubes using a magnet, 900 μl of Glasgow MEM was added, and the tubes were centrifuged at 13,000 × *g* for 5 min. Virus titrations of the supernatant were carried out on BHK-21 cell monolayers in 96-well microtiter plates, looking for cytopathic effect at three and 5 d postinoculation (as described in Carpenter et al. 2006). The results of titer calculations were expressed as $\log_{10}$ $TCID_{50}$ per ml (Finney 1964). The presence of BTV was confirmed in all positive samples using a real-time RT-PCR assay (Shaw et al. 2007) and enzyme-linked immunosorbent assay (ELISA) (Thevasagayam et al. 1996).

An additional 10 i.t.-inoculated *C. sonorensis* also were ground using the earlier conventional method. In each case, power-driven polypropylene pestles were used in Eppendorf tubes for 30 s, and virus quantity was then determined by titration in an identical manner. After Levene's test for inequality of variance among the treatments used, the Kruskal–Wallis analysis of variance (ANOVA) followed by Tukey honestly significant difference (HSD) was used to differentiate median titers among the eight treatments (all statistical testing performed using MINITAB version 14, Minitab, Inc., State College, PA).

After this initial experiment, the effect of ball bearing size and construction material were investigated using stainless steel balls measuring 1, 2, 3, 4, and 5 mm in diameter and stainless steel versus polyethylene balls at 3-mm diameter only (Dejay Distribution Ltd.). For the former experiment, 10 i.t.-inoculated individuals were ground with each ball bearing size for 1 min at 25-Hz frequency. In the later experiment 10 i.t.-inoculated individuals were ground using 3-mm stainless steel or polyethylene balls at a variety of frequencies (Table 1). Virus presence and quantification was carried out as described previously, and statistical analyses were carried out in the same way for the ball diameter treatments using Mann–Whitney *U* test for stainless steel versus polyethylene treatments.

Table 1. Mean virus titer of intrathoracically inoculated *C. sonorensis* ground with stainless steel and polyethylene balls for different periods of time (minutes) and at different frequencies (Hz)

Treatment time/ frequency (Hz)	Mean virus titer ($\log_{10}$ $TCID_{50}$) ± 95% CI (n = 10 for each treatment): stainless steel balls	Mean virus titer ($\log_{10}$ $TCID_{50}$) ± 95% CI (n = 10 for each treatment): polyethylene balls
1 min/25	2.58 ± 0.26^a	1.90 ± 0.33^c
30 s/20: 30 s/30	2.48 ± 0.20^a	1.88 ± 0.21^c
2 min/25	2.15 ± 0.33^a	1.35 ± 0.35^c
1 min/25: 1 min/30	2.03 ± 0.29^a	1.32 ± 0.25^c
2 min/25: 1 min/30	1.08 ± 0.26^b	
2 min/25: 2 min/30	0.97 ± 0.28^b	
4 min/30	0.53 ± 0.41^b	
Power pestle	2.45 ± 0.40^a	

[a, b] Pairwise significant difference in groups recorded using Tukey HSD at $P < 0.05$.

[a, c] Pairwise significant difference in values recorded for each processing treatment using Mann–Whitney U at $P < 0.05$.

Validation of Optimized Technique and Sensitivity with Pooled Samples. To validate the method, 40 i.t.-inoculated midges were individually ground with 2-mm stainless steel balls for 1 min at 25 Hz and compared with 40 insects ground via the power-driven pestle method. Additionally, two groups of 80 *C. sonorensis* that had been orally fed virus/blood suspension and incubated for 10 d (as described previously) were ground individually with the TissueLyser, or by using the motorized pestle method, and their virus titer measured in an identical manner. Differences in titer were again analyzed using Mann–Whitney *U* test.

Virus detection also was carried out using pools of midges with different [virus positive:virus negative] ratios (1:1, 1:5, 1:10, 1:25, 1:50, and 1:100). Intrathoracic-inoculated midges were incubated for 7 d at 25°C and 80% RH to allow full dissemination of the virus infection. The pools of insects were ground in a 1.5-ml Eppendorf tube, as described previously using the 2-mm stainless steel balls for 1 min at 25 Hz and then 2 min at 30 Hz. Supernatants were titrated on BHK-21 cells (four replicates per pool). In all cases, ELISA and RT-PCR assays were used to confirm the presence of the virus.

Results

A significant difference in the amount of virus obtained was observed between different grinding programs (H = 57.73, df = 7, $P < 0.001$), although no significant difference was noted in variance (W =

Table 2. Mean virus titer of intrathoracically inoculated *Culicoides sonorensis* ground with stainless steel balls of different diameters (mm) for 1 min at 25-Hz frequency

Ball bearing diam. (mm)	Mean virus titer ($\log_{10}$ $TCID_{50}$) ± 95% CI (n = 10 for each treatment)
1	0.70 ± 0.45[a]
2	1.85 ± 0.21[b]
3	1.54 ± 0.35[b]
4	1.42 ± 0.35[b]
5	0.74 ± 0.36[a]
Power pestle	1.55 ± 0.34[b]

[a, b] Pairwise significant difference in groups recorded using Tukey HSD at $P < 0.05$.

1.18, $P = 0.325$). Optimal grinding was achieved using relatively short runs in the TissueLyser (Table 1). Four of the programs used showed no significant difference when compared with the standard power pestle method, and treatment of 1 min at 25 Hz gave the highest mean virus titer.

The use of different diameter stainless steel ballbearings resulted in the recovery of significantly different quantities of virus ($H = 22.79$, df = 5, $P < 0.001$). Of those ball-bearing sizes tested, 1- and 5-mm diameter were significantly less effective than 2, 3, and 4 mm, which gave titers that did not differ significantly from those obtained using the pestle method (Table 2). Stainless steel balls also performed significantly better than the polyethylene balls ($P < 0.01$ in all cases; Table 1).

Homogenizing a further 40 i.t.-inoculated *C. sonorensis* by using the optimized combination of 2-mm ball bearings with a 1-min, 25-Hz grinding program gave titers that were not significantly different from those obtained for 40 individuals ground by using the powered pestle method (TissueLyser, 1.48 ± 0.18 [mean ± 95% CI]; power pestle, 1.60 ± 0.19; $W = 1526$, $P = 0.368$). Thirty-five of the 80 *C. sonorensis* that were infected by membrane feeding contained virus at 10 d postinfection, of which 25 contained >3 $\log_{10}$ $TCID_{50}$ per insect. Thirty-eight of the 80 *C. sonorensis* homogenized using motorized pestles contained virus, of which 23 contained >3 $\log_{10}$ $TCID_{50}$ per insect. The maximum titer achieved using both methods was 5 $\log_{10}$ $TCID_{50}$ per insect, and no significant difference was found between the two methods in the mean titer of those midges containing >3 $\log_{10}$ $TCID_{50}$ per insect ($W = 620.5$, df = 44, $P = 0.876$). Virus was detected in all the pools of midges with titers ranging from 4 $\log_{10}$ $TCID_{50}$ per pool to 6.75 $\log_{10}$ $TCID_{50}$ per pool. All the above-mentioned results were confirmed as positive by real-time RT-PCR and indirect sandwich ELISA.

Discussion

The identification of vector(s) involved in arboviral outbreaks is essential for the design and application of appropriate control programs. Conventional methods of identifying vector species, involving detection of virus and virus replication in pools of insects, have many drawbacks, and the labor involved in the large-scale screening of individuals for transmissible infections has restricted their use. We have demonstrated the recovery of infectious BTV from both i.t.-inoculated and membrane-fed *C. sonorensis* by using 2- or 3-mm steel ball bearings and an optimized TissueLyser grinding program of 1 min at 25 Hz. The titers of the recovered virus were statistically indistinguishable from those obtained using the current standard technique of grinding with polypropylene pestles (as described by Venter et al. 2005 and Carpenter et al. 2006). Although the preparation time of individual samples for grinding is marginally increased by adding stainless steel balls to Eppendorf tubes containing the samples, the homogenization step itself is ≈24 times faster (the TissueLyser can process 48 samples simultaneously).

The method not only can identify potential vectors more rapid and accurately but also uses stainless steel balls that are inexpensive and disposable, removing any possibility of contamination via the reusable pestles. By using a standardized homogenization program, variations caused by different operators by using different conditions to grind individual insects also are removed, potentially increasing the comparability of data between laboratories.

Due to difficulties in successfully feeding northern Palaearctic species of *Culicoides*, this study used adults of *C. sonorensis* as a model species of vector (Venter et al. 2005). However, it seems likely that the technique will be applicable to other *Culicoides* species, and potentially for other orbiviruses, including African horse sickness virus and epizootic hemorrhagic disease virus. The use of real-time RT-PCR is currently limited to a simple confirmation that test or diagnostic samples contain BTV. However, once a better understanding of the relationships between threshold cycle values, virus titer and transmissibility of infection are established, it may be possible to replace virus isolation/titration with real-time RT-PCR assays (which are more rapid and have the potential for a much higher throughput). This would increase the speed of processing substantially. Pairing of the technique with PCR assays for species identification (e.g., for the northwestern Palaearctic species; Nolan et al. 2007, Mathieu et al. 2007) also should prove to be a straightforward task, given the sensitivity already achieved with these methods and the fact that identification of DNA from different insect species would not require quantification.

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