Detecting the Presence of Nora Virus in *Drosophila* Utilizing Single Fly RT-PCR

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ABSTRACT: A single fly RT-PCR protocol has recently been developed to detect the presence of the persistent, horizontally transmitted Nora virus in *Drosophila*. Wild-caught flies from Ohio were tested for the presence of the virus, with nearly one-fifth testing positive. The investigation presented can serve as an ideal project for biology students to gain relevant laboratory experience. The study can be easily adapted to best meet the needs of instructor and student, and provide exposure to PCR, work with *Drosophila*, data analysis, and molecular biology.

Key words: single fly RT-PCR, *Drosophila*, Nora virus, wild-caught flies

INTRODUCTION

Molecular biology is at the forefront of biology. Current students must have an understanding of basic laboratory techniques and practices to show competency in their field and be informed about innovations in molecular biology. Polymerase chain reaction (PCR) is one molecular technique that is an integral tool in the laboratory today. It is commonly used in a wide array of biological disciplines, including forensics, industry, and medicine (Jordan & Lynch, 1998; Bartlett & Stirling, 2003; Yue, 2014). Developed in the early 1980s by Kary Mullis and his colleagues at Cetus Corporation in California, the technique replicates sequences of DNA. This enables small samples of DNA to produce larger quantities, which in turn can be used for further research (Mullis et al., 1986; Jordan & Lynch, 1998; Yue, 2014). As a result of the frequency of use, ease, and potential that PCR has to offer, current biology students need to become familiar with the process and its applications. Additionally, an understanding of PCR at the molecular level is crucial and personal experience will enable students to continue to explore the expanding field of molecular biology.

PCR has a multitude of uses that are diverse and innovative (Jordan & Lynch, 1998). The combination of reverse transcription with PCR allows RNA sequences to be analyzed in a process called reverse transcriptase polymerase chain reaction (RT-PCR). RNA cannot be used directly in standard PCR, so RT-PCR first produces DNA copies (cDNA) of the RNA via reverse transcription of the RNA template, followed by amplification of product (Bustin, 2000). RT-PCR is a sensitive and flexible process, allowing one to get quick results from very small sample sizes (Freeman et al., 1999; Bustin, 2000). Not only should biology students become familiar with conventional PCR, they also need to perform applications like RT-PCR to understand some of the techniques alternate uses.

A RT-PCR protocol has recently been developed utilizing single female *Drosophila.* The protocol to detect the presence of Nora virus is discussed. The study that is presented is recommended for biology students as a tool to use and understand the process of RT-PCR, and the protocol can be easily adapted to serve other applications. Completion of this laboratory exercise gives students practice at catching and identifying *Drosophila* species, data analysis, and virology. Students will gain valuable field and laboratory experience, research Nora virus, work with the model organism *Drosophila*, and expand their molecular understanding of PCR.

This exercise enables students to gain first-hand experience with *Drosophila*. *D. melanogaster* has been used as a model organism for research since the early 1900s. Studies involving *Drosophila* have examined anything from the basis of heredity to physiological systems (Kounatidis & Ligoxygakis, 2012; Rämet, 2012; Teixeira, 2012). Due to *Drosophila's* conserved immune response pathways with humans in their response to viruses, *Drosophila* is commonly used as a model for innate immunity (Reiter et al., 2001; Hoffman, 2003; Hultmark, 2003). Unlike humans, who have innate and adaptive immune response systems to combat pathogens, *Drosophila* depends solely on an innate immune response (Hoffman, 2003). As a result, *Drosophila* is a successful model organism for understanding mechanisms of the innate immune system (Kemp & Imler, 2009; Sabin et al., 2010; Kounatidis & Ligoxygakis, 2012; Teixeira, 2012). Approximately 20 groups of viruses exist in natural populations of insects from 12 viral families including *Rhabdoviridae, Dicistroviridae, Birnavirdae*, and *Reoviridae* (Kemp & Imler, 2009; Sabin et al., 2010). More than twelve human viruses have been studied in *Drosophila* (Hughes et al., 2012).

Nora virus is the ideal virus for students to investigate. It is a picorna-like virus. The virions are naked icosahedral-shaped particles approximately 28

nm in diameter and contain a genome of single-stranded positive-sense RNA (Habayeb et al., 2009). The genome consists of four open reading frames (ORFs). The first two ORFs encode non-structural proteins and ORFs three and four encode the structural proteins of the virion.

Drosophila is frequently exposed to pathogens since they eat, lay eggs, and develop on decaying fruit or media. Nora virus infects the intestine and is excreted in the feces. Therefore, when *Drosophila* feed on contaminated media, the flies ingest the virus and become infected. Transmission occurs horizontally, passing the virus to other flies through the fecal-oral route, with continuous shedding of the virus at high rates. The chance of obtaining positive RT-PCR results is high, although there is variation in viral loads of individual flies (Habayeb et al., 2009). Nora virus is unique due to its ability to persist in its host without causing any pathogenic effects or influencing longevity (Habayeb et al., 2009; Ekström et al., 2011). The virus is also non-pathogenic to humans, so work with flies is safe for students.

Basic knowledge of *Drosophila* is necessary for completion of this exercise. The four stages of the life cycle progress from egg, larva, pupa, and finally to adult. Development times are dependent on rearing temperature and larval density. Flies reared at higher temperatures and lower densities develop more rapidly. Development time also varies with species. The species caught in this study, *D. melanogaster* subgroup species and *D. virilis*, have development times of 13 days and 20 days, respectively, at 18°C. *Drosophila* become sexually mature at different times, ranging from a few days to weeks in some species, and maturity differs between males and females. While adults thrive on decaying fruit and media, eggs are typically laid on ripening fruit so that larvae can feed as the food source begins to rot (Markow & O'Grady, 2006).

Only female flies were selected in this study since they are generally larger and increase the chance of having an adequate amount of genetic material for testing. *Drosophila* can be sexed by examining the genital organs under magnification. Female genitalia is not surrounded by dark bristles found in males, the tip of the abdomen is less rounded, and contains more sternites (Markow & O'Grady, 2006). Females also lack sex combs on their front legs. While they are typically larger than males, sexing should not be based on this criterion alone.

While it may seem straightforward to follow the methodology outlined in this investigation, it is important for students to gain practice following the techniques. The exposure to field and laboratory work in this exercise provides students the opportunity to gain firsthand knowledge about *Drosophila*, Nora virus, and practice with basic PCR procedures. Students must be able to show efficiency in technical problems that often arise (Freeman et al., 1999). The methods do not include molecular explanations about the temperatures specified or what the primers or RNA are doing (Jordan & Lynch, 1998). Thus, there are many areas in which this investigation can be expanded and modified to best fit the goals of the student and instructor and emphasize learning over rote performance.

MATERIALS & METHODS

Fly stocks and husbandry

Flies were collected the week of August 3, 2014 in Sylvania, Ohio. Fruit baits were made by placing overripe bananas and apples into plastic cups, placing plastic wrap over the cup, sealing the wrap with a rubber band, and poking holes in the wrap with a toothpick. Cups were placed outside for several hours in the evening. Larger species of flies were collected with sweeps over open fruit. Additionally, mushrooms were soaked for an hour in tap water and placed in plastic covered cups. These cups were placed on the ground in a shaded flower bed in the morning. Approximately 150 flies were collected, about half of which were female.

Flies were retrieved from the cups and placed in plastic vials with instant medium (Formula 424® Instant *Drosophila* Medium, Carolina Biological Supply Company,Burlington, NC). A plastic vial was placed over a hole in the plastic wrap to remove the flies, with some gentle tapping on the cup. The plastic vials were plugged with a foam stopper. Flies were anesthetized with FlyNap (Carolina Biological Supply Company) and examined with a dissecting microscope to identify the species. *Drosophila* species can be identified with the aid of Markow and O'Grady's field guide, which contains distribution maps and identification keys (Markow & O'Grady, 2006). Additionally, FlyBase (Dos Santos et al., 2015) has colored illustrations of many species. The female flies were kept in vials of approximately 40 individuals per vial and transported to the University of Nebraska at Kearney (UNK) in Kearney, Nebraska. The flies were kept together for transport until they were separated into individual microfuge tubes when they arrived at UNK, which was no longer than 5 days.

RNA extraction and RT-PCR

RNA extraction was performed on random single female flies a week after capture. Female flies were placed in 1.5 ml microcentrifuge tubes with 20 µl of TRIzol® reagent (Invitrogen, Carlsbad, CA) to extract RNA. Flies were homogenized with disposable Kontes® plastic pestles. An additional 20 µl of TRIzol® reagent was added to the homogenate and the tubes were shaken by hand. The tubes were incubated at room temperature for 5 minutes and centrifuged at 12,000 rcf for 10 minutes at 4°C to pellet the insoluble exoskeleton and debris. The supernatant fraction was transferred to new

microcentrifuge tubes and 8 µl of Chloroform was added to each tube. The tubes were shaken by hand, incubated at room temperature for 3 minutes, and centrifuged at 10,000 rcf for 15 minutes at 4°C. The upper aqueous layer was transferred to a new RNAse-free microcentrifuge tube and 20 µl of isopropanol was added. They were shaken by hand, and incubated at room temperature for 10 minutes. The tubes were centrifuged at 12,000 rcf for 10 minutes at 4°C. The supernatant fraction was removed and the pellet was washed with 40 µl of 75% ethanol. The tubes were centrifuged at 7,500 rcf for 5 minutes at 4°C. The supernatant fraction was removed and the tubes were briefly centrifuged so the remaining supernatant fraction could be removed with a micropipette. The tubes were air dried for 3 minutes and then the pellet was resuspended in 5 µl of RNase-free water by flicking the sides of the tube. Finally, 0.5 µl of RNase Out (Invitrogen) was added to each sample.

The samples were analyzed for the presence of Nora virus by RT-PCR using *Nora ORF 1* 54-844 (Forward 5'TGGTAGTACGCAGGTTGTGGGAAA3'; Reverse 5'AAGTGGCATGCTTGGCTTCTCAAC3') primers and FideliTaq RT-PCR Master Mix (2X) (USB®, Cleveland, OH). Fifty µl reactions were prepared by combining 19 µl of water, 25 µl of master mix, 2 µl (20 picomoles) of forward primer, 2 µl (20 picomoles) of reverse primer, and 2 µl (~200 ng) of the RNA samples. The tubes were thoroughly mixed by vortexing and centrifuged. The parameters for RT-PCR are as follows: 50°C for 30 minutes, 94°C for 2 minutes, followed by the amplification loop consisting of 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 2 minutes, with a final cycle of 68°C for five minutes.

The samples were prepared for agarose gel analysis by adding 10 µl of the PCR product and 2 µl of 6X loading dye. The electrophoresis apparatus was set up and samples were loaded on a 0.8% agarose gel with the separation performed at 75 volts for approximately 1 hour. The results were analyzed with a confidence interval at 95%, indicating that one can be 95% confident that the population of *Drosophila* infected with Nora virus falls in the indicated range. This is consistent with a significance level of 0.05. The formula to calculate the confidence interval boundaries (Confidence Interval Boundaries = p_{avg} +/- Z score (1 - α) * s_{pavg}) is shown, where p_{avg} is the sample proportion, the Z score (1- α) is the standard score and was calculated by taking the inverse of the standard normal cumulative distribution when α = 0.05, and s_{pavg} is the sample standard error.

RESULTS

A positive reaction for Nora virus was observed for the control and laboratory stock flies with Nora virus, showing a product at approximately 800 bp (Figure 1; Lanes 5-10). The negative controls do not show a PCR product (Figure 1; Lanes 3-4). The positive control was Nora virus RNA. The laboratory stock flies utilized have been kept for many generations and are known to be highly infected with Nora virus. This indicates that single female fly RT-PCR was successful.

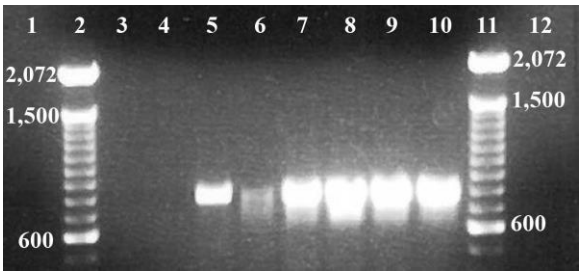

Fig. 1. Confirmation of Nora virus detection using single female flies via RT-PCR. Lane 1 = Empty; Lane 2 = 100 bp Ladder; Lanes 3 & 4 = Negative (water) control; Lanes 5 & 6 = Positive (Nora virus RNA) control; Lanes 7-10 = single female fly via RT-PCR; Lane 11 = 100 bp Ladder; Lane 12 = Empty. The product seen in Lanes 5-10 is approximately 800 bp, which is the expected size for the Nora virus product.

Female wild-caught flies tested for the presence or absence of Nora virus infection were predominantly from the *D. melanogaster* species subgroup, although *D. virilis* was also found. A total of 6 flies out of 32 tested positive for Nora virus (Figure 2a; Lanes 5, 7, 10, 13 and Figure 2b; Lanes 4, 7). Nora virus was not detected in the other 26 flies, as indicated by the absence of a PCR product (Figure 2a; Lanes 4, 6, 8-9, 11-12 and Figure 2b; Lanes 5-6, 8-13 and Figure 2c; Lanes 4-15). The results of this study show that 18.75% of wild-caught flies in Sylvania, Ohio have Nora virus. The 95% confidence interval suggests that 5.22% to 32.3% of the fruit fly population would be expected to be infected with Nora virus.

DISCUSSION

This study developed an RT-PCR protocol for single *Drosophila* and used the protocol to investigate the number of wild-caught flies infected with Nora virus. The need for single fly RT-PCR is two-fold. First, the exact number of flies with Nora virus infection can be revealed, as well as the viral titer levels. This is a more accurate approach, as opposed to conducting RT-PCR with groups of flies. Secondly, it exposes how widespread Nora virus is in different species and increases confidence in results since flies are independently examined. The single fly RT-PCR protocol provides experience for biology

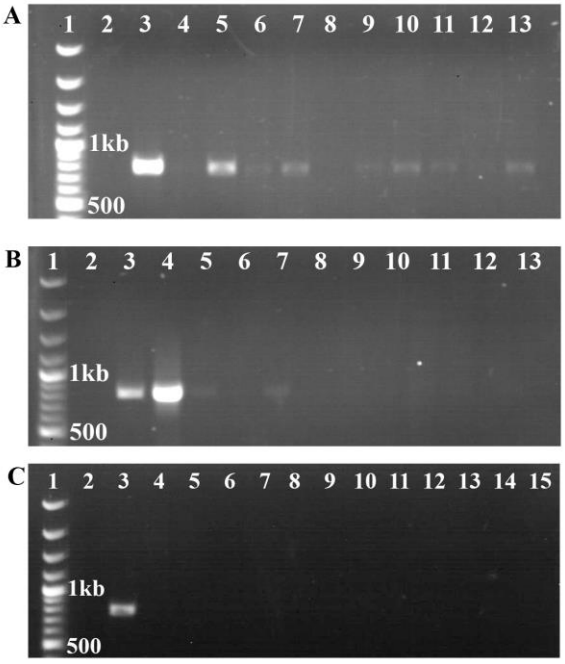

Fig. 2. Detection of Nora virus using wild-caught single female flies via RT-PCR. Panel A is samples 1-10: Lane 1 = 100 bp Ladder; Lane 2 = Negative (water) control; Lane 3 = Positive (Nora virus RNA) control; Lanes 4-13 = single female fly via RT-PCR. The product seen in Lanes 5, 7, 10, and 13 is approximately 800 bp, which is the expected size for the Nora virus product. Panel B is samples 11-20: Lane 1 = 100 bp Ladder; Lane 2 = Negative (water) control; Lane 3 = Positive (Nora virus RNA) control; Lanes 4-13 = single female fly via RT-PCR. The product in Lanes 4 and 7 is approximately 800 bp, which is the expected size for the Nora virus product. Panel C is samples 21-32: Lane 1 = 100 bp Ladder; Lane 2 = Negative (water) control; Lane 3 = Positive (Nora virus RNA) control; Lanes 4-15 = 1 female fly via RT-PCR. The product in Lane 3 is approximately 800 bp, which is the expected size for the Nora virus product.

students, even those who lack prior exposure to *Drosophila* work.

Nora virus appears to be widely distributed in laboratory stocks of *D. melanogaster*. The extent to which Nora virus infects different species of *Drosophila* in the wild is unknown. Studies have shown that organisms in the laboratory experience significant adaptation to the change in conditions within a short time period due to genetic bottlenecks and selection pressures (Gilligan & Frankham, 2003; Swindell & Bouzat, 2005; Gilchrist et al., 2012). The shift in genotype occurs even when population size is large and the environment is not stressful for the organisms (Gilligan & Frankham, 2003; Gilchrist et al., 2012). Thus, it was imperative to look for the presence of Nora virus in wild-caught flies. The results of this study indicate that Nora virus is present in *Drosophila* in their natural setting, with nearly one-fifth of flies in northwest Ohio infected.

There are several ways this study could be expanded and improved. Individual flies should be isolated immediately after capture to eliminate the potential for cross-contamination. Although the results do not seem to indicate that cross-contamination occurred since both positive and negative results were observed, contamination is possible if flies are reared together for any duration of time. Isolation can be achieved by storing wild-caught *Drosophila* in individual vials on dry ice. Additional locations and *Drosophila* species should be tested to better understand virus prevalence. Biology students can easily collect flies at different locations for testing, and will likely see a wider variety of species. Unfortunately, identification of subgroups of *D. melanogaster*, such as *D. simulans*, *D. melanogaster*, and *D. yakuba* is difficult based upon phenotype and morphology, hence the reason they were grouped in this activity. Fortunately, distinquishing between *Drosophila* species, such as *D. melanogaster*, *D. virillis*, and *D. pseudoobscura* is relatively easy, and was done in this study. By sampling a wide array of locations and *Drosophila* species, the average relative density of Nora virus RT-PCR products could then be tested to compare virus titer levels.

This study provides the opportunity for biology students to practice a new single fly RT-PCR protocol, with the potential for exposure to many other biological domains. The objectives and interests of the instructor and student should first be discussed. Then, a project can be created which introduces the student to PCR use and history, work with *Drosophila* in the laboratory and field, and data analysis, while enabling the student to learn about virology in a relevant way. Students must experience first-hand the advances that have taken place and are still taking place in molecular biology. The protocol outlined present an opportunity for students to create and complete a significant science project.

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