

INNOVATIONS

Nora Virus Transmission in *Drosophila melanogaster*: An Investigation to Teach Viral Infection and Prophylaxis to Biology Students

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Abstract: Proper hand hygiene accompanied with environmental surface disinfection provides a comprehensive approach to control and prevent respiratory and gastrointestinal illness in schools, hospitals, work environments, and the home. The persistent non-pathogenic Nora virus common in *Drosophila melanogaster* provides a horizontally transmitted virus that students can research and design an experiment testing prophylaxis techniques for viral infection and pathogenic diseases. Students use inquiry-based methods to perform an experiment, analyze data, and draw conclusions on viral inactivation from disinfectant use on Nora virus in *D. melanogaster*.

Key words: viral infection, prophylaxis, transmission, *Drosophila*, disinfection, Nora virus

INTRODUCTION

Inquiry based investigations are necessary to stimulate learning, understanding, and excitement of the scientific process in laboratory based courses. To engage students, selecting topics that are current and relevant is essential. One topic that is seemingly timeless and relevant to the student of any age is missing work or school due to preventable illness. Absenteeism from school and work associated with respiratory and gastrointestinal illness is costly and imposes a burden on education and family financial resources. Respiratory and gastrointestinal illness results from direct or indirect contact transmission of pathogenic viruses, bacteria, and fungi. Examples of these infectious respiratory viruses are respiratory syncytial virus (RSV), rhinovirus, and influenza, including the much-publicized H1N1 strain, avian, and swine influenzas (Goldmann, 2000; Centers for Disease Control and Prevention [CDC], 2012a). Viruses causing gastroenteritis include norovirus, rotavirus, and adenovirus, with norovirus and rotavirus comprising the majority of United States infections responsible for up to 70,000 hospitalizations each year (CDC, 2012b; CDC, 2012c).

Hand hygiene in the form of hand washing with soap and water or hand rubbing with hand sanitizer is a well-documented prophylaxis for limiting indirect contact transmission of bacterial and viral infections, including both respiratory and gastrointestinal infections (Curtis & Cairncross, 2003; Rabie & Curtis, 2006; Sandora et al., 2008). One study found that 37.8% of rhinovirus remained viable after 1 hour on contaminated finger pads, and almost 16% after 3 hours (Ansari et al., 1991). Rabie & Curtis (2006) found that hand washing reduces respiratory infection

by 16%, and Ryan et al. (2001) reported a 45% reduction in respiratory illness in outpatient visits as a result of proper hand washing implementation. Furthermore, the risk of diarrheal disease is reduced by almost 50% by washing hands with soap and water (Curtis & Cairncross, 2003).

A less publicized way to control infection is fomite disinfection, as environmental surfaces become contaminated with pathogens by contact with nasopharyngeal secretions and contaminated hands. Fomites are inanimate objects, such as toilet seats or toys that can become contaminated. Rotavirus dried from fecal suspension remains viable for several days on fomites, and is readily transferrable from inanimate surfaces to hands and hands to surfaces (Ansari et al., 1988; Sattar et al., 1994). Rhinovirus and RSV are transmissible from contaminated environmental surfaces between multiple people (Gwaltney & Hendley, 1982; Sattar et al., 1993). In light of this, measures taken in preventing and controlling the spread of respiratory and gastrointestinal illness should include both proper hand hygiene and disinfection of environmental surfaces.

Using the topic of preventable viral disease, this inquiry-based investigation introduces biology classrooms to virology, molecular genetics, and the importance of hand hygiene and surface disinfection in the control and prevention of pathological disease. The students investigate pathogens, pathogen transmission, disinfectants and disinfection methods, and modern genetic laboratory techniques. The research question that the students are being asked to investigate is “What types of surface decontaminants are effective in preventing viral transmission and spread?” To test this question, we describe a novel

laboratory investigation using *Drosophila melanogaster* infected with non-pathogenic Nora virus. Nora virus is a picorna-like virus, which is seemingly endemic in both natural and laboratory populations (Habayeb et al., 2006). Because most laboratory and wild-caught stocks, as well as some commercially available stocks, are infected with Nora virus, any *D. melanogaster* stock can be tested for Nora virus infection utilizing the RT-PCR methodology outlined in this experiment. In addition, once stocks have been identified as Nora virus positive, they can be maintained indefinitely in the laboratory by transferring the stocks on a regular basis. These features negate the need for a collaborator with an infected stock to carry out experiments. *D. melanogaster* infected with Nora virus is ideal for student use because the virus is nonpathogenic to humans and *D. melanogaster* is a well-established laboratory model. To begin the inquiry process, the students first conduct a literature review to learn virus characteristics, pathogen transmission, viral disinfection, and disease control and prevention. They determine the surface decontaminant they want to test, and develop hypotheses on the efficacy of the experimental versus control surface decontaminant. The students design and perform the experiment, which includes handling Nora virus infected *D. melanogaster*, decontamination with the surface decontaminant they have selected, RNA extraction, RT-PCR for presence or reduction of Nora virus, quantitation of results, and data analysis including statistical analysis. They critically analyze their results and present them in the

structure of a peer-reviewed journal article or constructed as a conference poster to be displayed in the school to promote hand hygiene and surface disinfection in the prevention and control of respiratory and gastrointestinal diseases. Once infected fly stocks are on hand, the experiment can be conducted in a relatively short timeframe, requiring approximately 3 weeks to achieve results. Furthermore, non-infected fly stocks could be used to demonstrate the absence of Nora virus infection as an additional control, but were not used in this study.

MATERIALS & METHODS

Drosophila melanogaster rearing and culture

Nora virus (NV) infected flies (a gift from Dr. Dan Hultmark, Umeå, Sweden) were reared in 8 oz plastic bottles (Fisher Scientific, catalog #AS-117) at 25°C with diurnal light on standard molasses, torula yeast, and cornmeal media. For the experiment, the bottom of the bottles were cut off, filled with media, allowed to set, bottoms taped to the tops, and plugged with BuzzPlugs (Fisher, catalog #AS-277). In each of six bottles for the parental (P) generation, 50 NV infected males and 50 NV infected females were added. Bottles 1-3 served as the control group with either no rinsing (P flies) or eggs rinsed with *Drosophila* Ringer's solution (3mM CaCl₂•2H₂O; 182mM KCl; 46mM NaCl; 10mM Tris base; and pH adjusted to 7.2 with 1N HCl) and bottles 4-6 served as the experimental group that would later be rinsed with household bleach. The flies mated for 96 hours, with eggs removed after 24 hours for sampling and washing (Figure 1). Twenty eggs from bottles 1-3 were collected, briefly rinsed in *Drosophila* Ringer's

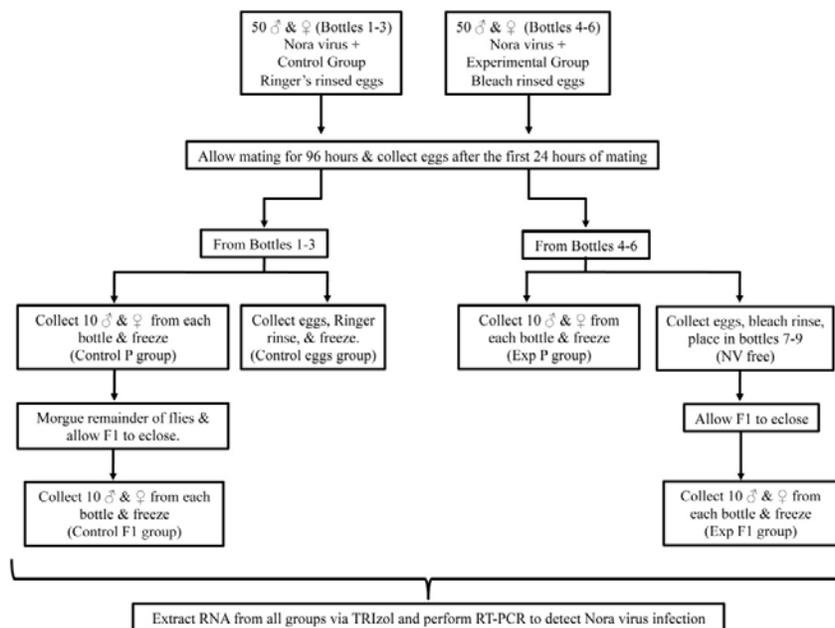


Fig. 1. Flow chart demonstrating the experiment set-up. The Ringer's wash serves as the control group, whereas the bleach wash serves as the experimental group. All groups collected including Control eggs, Control P, Control F1, Experimental (Exp) P, and Exp F1, had the RNA extracted by TRIzol and used for RT-PCR analysis.

solution in a cell strainer (Fisher, catalog #08-771-2), transferred to separate 1.5 mL sterile microcentrifuge tubes (Fisher, catalog #02-681-5) with *Drosophila* Ringer's solution, and frozen for RNA extraction. This procedure was repeated with the experimental bottles, except 15 eggs from bottles 4-6 were briefly rinsed, but not dechorionated, in a 10% sodium hypochlorite solution (household bleach) and placed on clean food in 3 new bottles (7-9), becoming the experimental NV free egg populations. At the end of the 96-hour mating period, 10 males and 10 females from Bottles 1-6 were placed in a separate 1.5 mL sterile microcentrifuge tubes and frozen for RNA extraction. These flies represented the control (1-3) or experimental (4-6) P groups. The remaining eggs in bottles 1-3 were allowed to emerge as adults and represented the control F1 flies. Ten adult males and ten adult females were collected from each control F1 flies bottle (1-3) after emergence and frozen for RNA extraction. A flowchart depicting the experiment setup is provided (Figure 1). The F1 experimental flies are explained in the next section.

D. melanogaster egg washing with bleach

The adult flies in the 3 experimental P bottles (4-6) were anesthetized using FlyNap (Carolina Biological Supply, catalog #173010). The tape was removed from the bottles, the bottom of the bottle placed on the stage of a dissecting microscope, and tweezers used to collect eggs from the surface of the media. A total of 15 eggs from each bottle were collected and placed in a cell strainer in a Petri dish containing *Drosophila* Ringer's solution. The eggs were washed 3 times. The first wash was with household bleach diluted with distilled water to a 10% bleach solution, lasting approximately 10 seconds. This wash is not long enough to remove the chorion of the egg, but long enough to penetrate the pits in the chorion where the virus may reside. The second and third washes were with fresh *Drosophila* Ringer's solution, lasting approximately 10 seconds each, to remove residual bleach. The washed eggs were placed on the surface of the clean food bottles (7-9) with tweezers and the tops of the bottles were taped back on. The washed egg bottles (7-9) were referred to as the experimental F1 flies. They were allowed to mature to adulthood and frozen for RNA extraction. The experiment setup is once again depicted in Figure 1.

RNA extraction and RT-PCR

P generation flies (bottles 1-6), control (bottles 1-3) rinsed with *Drosophila* Ringer's, control F1 flies (bottles 1-3), and experimental F1 flies (bottles 7-9), were frozen for RNA extraction and tested for the presence of Nora virus. RNA extraction was performed using TRIzol[®] per manufacturer's instructions (Invitrogen, catalog #15596-026) and concentration determined using the Nanodrop spectrophotometer. One hundred nanograms of RNA from each sample collected was analyzed for the

presence of Nora virus via RT-PCR using Nora virus specific primers ordered from Invitrogen (Forward 5'-TGGTAGTACGCAGGTTGTGGGAAA-3'; Reverse 5'-AAGTGGCATGCTTGGCTTCTCAAC-3') and a Promega Access Quick RT-PCR kit (catalog #A1250) according to manufacturer's instructions. Reactions were carried out under the following conditions: 45°C for 45 min, 94°C for 2 min, (94°C for 30 s, 55°C for 30 s, 68°C for 1 min)_{30 cycles}, 68°C for 10 min, and hold at 4°C. Five microliters of the RT-PCR products were mixed with 3 ul of 6X loading dye (Promega, catalog #G1881) and electrophoresed on a 0.8% agarose gel at 50V for 2 hours. A positive reaction yielded a product at approximately 800 bp. The relative density of the bands was quantitated by using the free program, ImageJ (<http://rsb.info.nih.gov/ij/index.html>; Abramoff et al., 2004). The results were tested for significance using a two-tailed Students *t*-test with unequal variance and $\alpha=0.05$.

RESULTS

All samples collected were tested for the presence or absence of Nora virus infection. The control (bottles 1-3) and experimental (bottles 4-6) P generation produced a positive reaction for Nora virus with a product at approximately 800 bp (Figure 2; Lanes 4-9). In addition, the control eggs removed from bottles 1-3 that were washed only in *Drosophila* Ringer's solution also tested positive for Nora virus (Figure 2; Lanes 10-12), as did the control F1 flies (Figure 2; Lanes 13-15). The intensity of the Nora virus product appeared less in the control eggs and F1 compared to the Control P flies (Figure 2; Lanes 7-12 versus Lanes 4-6), but upon statistical analysis, there is no statistical significance (Figure 3). The only statistically significant change in Nora virus expression was in the experimental F1 flies that were washed for 10 seconds in 10% bleach. These flies did not produce an 800 bp product (Figure 2; Lanes 16-18) and were found to be significantly reduced compared to all other samples tested (Figure 3).

DISCUSSION

The hypothesis for the study presented was if Nora virus is transmitted on the surface of the *D. melanogaster* eggs, it can be removed by a surface decontamination solution, such as 10% bleach, but not by a control solution (*Drosophila* Ringer's). Based on our results, it appears that washing *D. melanogaster* eggs in 10% bleach successfully disinfects the eggs, inactivating or removing Nora virus from the chorion, and eliminating viral infection in the resulting adults (Figures 2 & 3). Our procedure appears to be a successful treatment to eliminate Nora virus infection in *D. melanogaster* populations. Furthermore, these results support that Nora virus is horizontally transferred and the result of fecal-oral transmission from contaminated food



Fig. 2. Confirmation of efficacy of rinsing with 10% bleach on transmission of Nora virus via RT-PCR. Lane 1 = 100 bp Ladder; Lane 2 = Negative (water) control; Lane 3 = Positive (Nora virus RNA) control; Lane 4-6 = Control P; Lanes 7-9 = Experimental P; Lanes 10-12 = Control eggs; Lanes 13-15 = Control F1; Lanes 16-18 = Experimental F1. The product seen in Lanes 3-15 is approximately 800 bp, which is the expected size for the Nora virus product.

(Habayeb et al., 2009). This was deduced due to the fact that eggs rinsed in bleach gave rise to F1 progeny that did not test positive for Nora virus. If vertical transmission was demonstrated, the F1 progeny would have tested positive for Nora virus because the virus would have been transmitted from mother to offspring inside the egg, not on its surface. Interestingly, the control eggs tested positive for Nora virus after washing with *Drosophila* Ringer's solution, suggesting while the flies are laying eggs they are also shedding virus. This would provide an efficient way to transmit the virus to newly hatched larva as they would likely start eating the contaminated food in close proximity to where their egg was laid. Also, the intensity of the *Drosophila* Ringer's washed control eggs and adult control F1 flies Nora virus product appears to be less than that of the P flies (Figure 2, Lanes 10-15 versus Lanes 1-6), even though there is no significant difference when quantitated (Figure 3). This suggests that

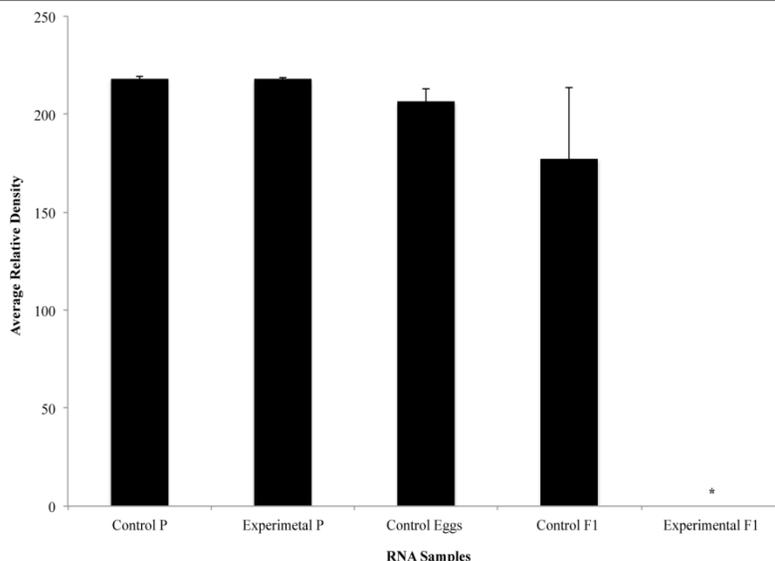


Fig. 3. Average relative density of the Nora virus RT-PCR products as analyzed by the ImageJ software. Nora virus infection was significantly diminished when infected eggs were briefly washed with 10% bleach and allowed to mature (Experimental F1) as compared to all other groups tested ($p \leq 0.05$; this comparison is represented by *). $n = 3$ for each sample, error bars are standard deviation, and the test statistic used was a two-way Student's t-test for unequal variances with $\alpha = 0.05$.

washing the eggs with *Drosophila* Ringer's may reduce viral titer levels, but does not inactivate or eliminate viral infection. These results correlate with research suggesting that water alone is not sufficient for proper hand hygiene or a reduction in viral infectivity (Sattar et al., 1994). The fact that the *Drosophila* Ringer's washed eggs contain enough virus to register a positive product using RT-PCR and the viral infection in the resultant adults should demonstrate to the students the concept of viral infection and the small amount of virus needed to induce infection. This extreme infectivity should also reinforce the idea that proper hand washing is necessary to prevent viral infections.

The results of the experimental F1 generation demonstrate that a 10% bleach solution is an effective disinfectant for Nora virus (Figures 2 & 3). Inhibiting Nora virus infection was as simple as rinsing the egg surfaces with disinfectant. Students should easily correlate this to the importance of disinfecting environmental surfaces to reduce pathogen spread.

This experiment could be modified to include washing *D. melanogaster* eggs with different disinfectant solutions, in different concentrations, and utilizing various exposure times to gauge the efficacy of different products and procedures. Students could be given different disinfectants, for example ethanol, citric acid, ammonia, or phenol based commercial products, and predicated on literature review, propose and test hypotheses for expected efficacy with regard to disinfectant type, exposure time and virus characteristics. Furthermore, there are many other persistent non-human pathogenic *D. melanogaster* viruses that could be tested. In addition to Nora virus, persistent viruses found in natural, laboratory, and commercial fruit fly populations include Sigma virus, and *Drosophila* viruses A, C, P and X, each with their own genome structure, family and transmission mode. This modification would provide

students with additional exposure to viral infection, possible virulence, and viral characteristics, which could tie into educational units on infectious disease, genetics and molecular biotechnology.

The described experiment exposes students to concepts of disease agents, disease transmission, prophylaxis measures, and molecular genetics. It could also be used to introduce students to the use of *D. melanogaster* as a model organism. Furthermore, based on their research, students could develop, promote, and implement plans to help reduce the spread of respiratory and gastrointestinal illness in their school through proper hand hygiene and environmental surface disinfection. This experiment provides students a practical investigative opportunity to apply the scientific method, reinforcing concepts introduced in lecture, and providing an opportunity for scientific research, which could ignite a spark of interest in scientific investigation unknown to them previously.

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