An authentic, hands-on experience in the laboratory is an important part of any undergraduate biology course. However, there are a limited number of mammalian virus systems that students can work with safely in an undergraduate teaching laboratory. For many systems, the risk to the students is too high. The influenza A virus M2 protein trans-complementation system bridges this gap. This system consists of a virus with mutations that prevent the expression of the essential M2 protein; therefore this virus can only replicate in a cell line that provides M2 in trans. Here, we describe the use of this system to carry out hemagglutination, real-time reverse transcriptase PCR, 50% tissue culture infectious dose, and plaque assays in an undergraduate lab setting.

INTRODUCTION

In the undergraduate virology laboratory, there are few systems available for students to work directly with animal viruses. This is largely due to the safety issues involved with allowing students to work with an infectious virus. While bacteriophage systems are relatively safe, and many of the lessons of virology can be taught using bacteriophage (plaque counting, host restriction, growth curves, etc.), the mechanics of working in phage systems are very similar to those learned in a bacteriology lab. They are dissimilar to those that are required to work in mammalian cell culture systems.

In this report, we describe the use of an influenza A virus trans-complementation system that is safe to use in an undergraduate laboratory setting in BSL2 conditions. The virus has been engineered to contain two consecutive stop codons in the open reading frame for the essential protein M2 (1). Viruses that contain this mutation have defects in virus entry and in the assembly of infectious virus (2–4). Because of the profound defects in replication of the M2-deficient virus, it can only be grown on a cell line that provides the M2 protein in trans. However, virus growth on an M2-complementing cell line is similar to growth of the parental virus on a wild-type cell line (5, 6). This system has been used for hands-on demonstration of plaque formation, quantification by real-time reverse transcriptase (RT)-PCR, determination of 50% tissue culture infectious dose (TCID₅₀), and hemagglutination (HA) by viruses, and students interested in human/animal diseases may find this system more relevant than systems based on bacteriophage or plant viruses. These experiments can be combined into a suite of exercises that highlight important concepts in virology, such as total:infectious virus ratios and the difference in sensitivity between different methods of virus quantification. Students were able to carry out experiments to quantify the amount of total and infectious virus in samples that they were provided, although it should be noted that the term “infectious” refers only to the activity of the virus in the M2-complementing cell line.

PROCEDURE

The corresponding author will make the virus and the complementing cell line available to others on request. This system has been used in two semesters of an undergraduate virology course with an enrollment of up to 10 students. With this class size, students were able to work individually and complete the exercises in the three-hour lab period. Larger enrollments would likely require students to work in small groups for some of the activities due to limited tissue culture hood space.
Safety issues

This system was described in 2006, and has been used in many studies of M2 function (1, 6–9). No instances of reversion of the mutated virus to wild-type have been documented. However, since reversion is theoretically possible, students should work under BSL2 conditions. Guidelines for the BSL2 teaching laboratory can be found at the following link: https://www.asm.org/images/asm_biosafety_guidelines-FINAL.pdf. Work with a potentially infectious virus should be done in a class II biological safety cabinet. Students should wear gloves, safety glasses, and lab coats. Spent media that may contain the virus should be decontaminated with 10% bleach, and solids contaminated with the virus should be autoclaved prior to their disposal.

Total virus quantification

Hemagglutination assays and real-time RT-PCR were used to quantify the total amount of virus in the samples. Depending on the desired learning objectives, it may be useful to quantify the same samples using both assays to demonstrate differences in sensitivities and to compare results from these experiments with the results from the infectious virus experiments described below. Students are generally able to set up the HA assay and begin to see agglutination by the end of a three-hour lab period. However, better results will be seen if the assays are incubated overnight at 4°C and viewed the next day. Complete protocols for HA assays can be found in Appendix 1, and example data are shown in Figure 1A.

For real-time RT-PCR experiments, RNA can be extracted using commercial viral nucleic acid extraction kits or using Trizol. RT-PCR can then be set up using extremely reliable primers that bind to segment 7 of the viral RNA (10). Although the original assay was designed to use a Taqman probe with this primer set, we have found that SYBRGreen chemistry also works well. Complete protocols for real-time RT-PCR can be found in Appendix 2, and example data are shown in Figure 1B.

Infectious virus quantification

The amount of infectious virus in samples was quantified using two methods as well, TCID$_{50}$ and plaque assays, which allowed for discussion of the benefits and drawbacks of the two protocols and for comparison with HA and real time RT-PCR assays. For TCID$_{50}$ assays, each student required approximately 30 minutes to make a 10-fold dilution series, wash the plates, and add virus to the wells. Students calculated the 50% tissue culture infectious dose from fixed and stained plates using the method of Reed and Muench (11). Complete protocols for the TCID$_{50}$ assay can be found in Appendix 3, and example data are shown in Figure 1C.

Plaque assays can also be done using this system; however, for unknown reasons, the trans-complementing cell line survives poorly when overlayed with agarose. This effect can be partially overcome by maintaining the cells at confluence for several days prior to infection and overlay. Students were able to make a dilution series, infect the cells, and overlay with agarose within the three-hour period. On day 3 post-infection, plaques can be seen as opaque areas under the agar overlay or the cells can be fixed and stained with naphthol blue-black. Complete protocols for plaque assays can be found in Appendix 4, and example data are shown in Figure 1D.
Extensions

This system is quite flexible and can be used to demonstrate a number of other important virology concepts with some modification. For example, receptor specificity can be investigated if red blood cells with different sialic acid linkages are used instead of chicken red blood cells (12), plaques could be isolated and used to make virus stocks, cells could be infected to produce single-step or multi-step growth curves, or the system could be used as a platform to demonstrate immunology concepts such as virus neutralization.

CONCLUSIONS

The use of the influenza M2 trans-complementation system is a viable, relatively safe alternative to phage systems for undergraduate virology laboratory courses. This system gives students experience with calculation of infectious titers, HA titers, genome copies, and plaque assays and can be extended to estimate total:infectious virus ratios, to test potential inhibitors of virus replication, to plaque purify virus clones, etc. Most importantly, students can gain hands-on experience working with a virus in a mammalian cell culture system.

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