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A Simple, Cost-Effective Undergraduate Workshop Based on Simulated Complement Fixation Test to Teach the Concept of Complement System

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Abstract: The complement system is a key component of the innate immune system, and acts as a unique connection between innate and adaptive immune responses. The multi-protein network of complement cascade plays an important role in both normal and pathologic conditions, and is an excellent example of protein activation and interaction, making it a vital study topic for various undergraduate biology disciplines. In this article, we describe a simple method using easily sourced, inexpensive materials to reliably simulate the results of a viral complement fixation test (CFT), to demonstrate the powerful complement cascade activation to undergraduate students in immunology course. The CFT workshop described in this article comprises of a disease's diagnosis scenario (influenza virus infection), a virtual protocol, and a visual learning experience to witness the outcome of complement cascade activation via red blood cell lysis. The novelty of this exercise lies in its simplicity and cost-effectiveness in the set-up, while providing safe, engaging and effective learning without the use of potentially hazardous materials and special equipment. This workshop can be used to accompany any relevant lectures on the complement system, to enhance the students' learning on this critical yet complex immunology concept.

Keywords: *Complement system, innate immunity, undergraduate immunology, simulated complement fixation test, hemolysis.*

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Introduction

The complement system is an important part and one of the most effective mechanisms of our immune system. Complement assists antibodies and phagocytic cells to clear various pathogens and damaged cells, aids in tissue regeneration, triggers inflammatory responses to fight infections, and directly attacks pathogen's cell membrane. However, inappropriate alterations in complement functions caused by genetic variants or complement deficiencies can also lead to various pathological conditions (Noris & Remuzzi, 2013; Sarma & Ward, 2011). Complement was first discovered in the 1890s as an aid or "complement" to the killing of bacteria by sensitized antibodies present in normal serum. The complement system functions as an intricate enzymatic cascade involving the sequential activation of already-existing but inactive zymogens via three different pathways: the classical pathway, the alternate pathway, and the lectin pathway. The activation of this enzymatic cascade leads to the formation of membrane attack complex (MAC), which forms pores on the cell membrane and thus causes cell lysis. The classical pathway is triggered by the presence of antibody-antigen complex, the alternative pathway is triggered directly on pathogen surfaces, and the lectin pathway is triggered by the binding of mannan-binding lectin or ficolin to pathogen surfaces. The consequences of complement activation generates active components with various effector functions such as killing of pathogens by cell lysis, the recruitment of inflammatory cells, and opsonization of pathogens (Figure 1) (Janeway, Travers, Walport, & Capra, 2001; Qu, Ricklin, & Lambris, 2009; Trouw & Daha, 2011; Wallis, 2007).

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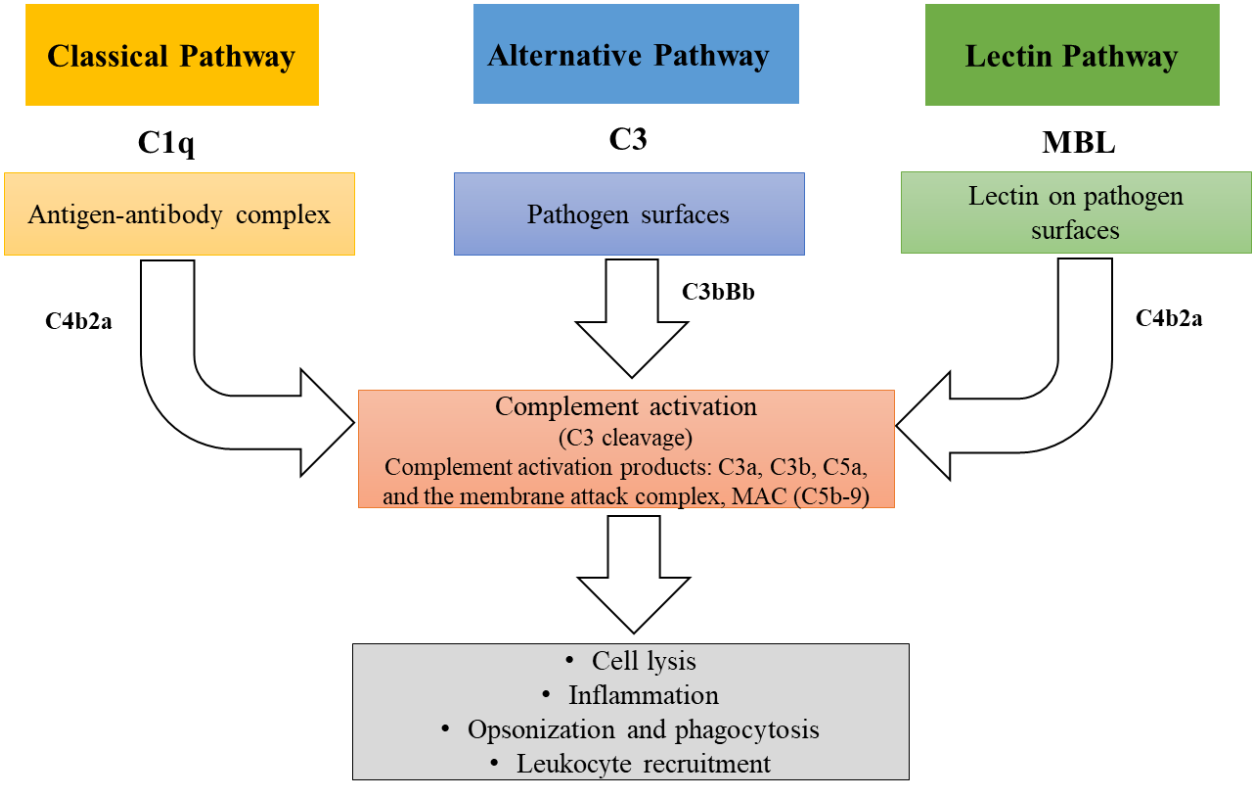

Figure 1. Simple illustration of three pathways of complement activation.

Given the significant and multitude roles of complement in our immune system, it has become an indispensable part in the curriculum of various biology-related disciplines. The specific functions and mechanisms of complement pathways are described in details in many textbooks. However, students usually lack the opportunity to appreciate the action of this fascinating system, and can hardly gain any further experience other than the somehow arid text. As the complement system consists of a tightly regulated network of more than 30 proteins, it can be difficult for educators to communicate such complex subject to undergraduate students within the laboratory. Moreover, undergraduate laboratories are often challenged by limitations in time, resources and special equipment for setting up extensive hands-on experiments. In the context of teaching complement system, published laboratory exercise such as the one from Fuller (Fuller, 2008) requires specific instrument and materials, as well as high level of student skills and safety measures due to the use of potentially hazardous biological materials. Furthermore, these exercises tend to be time-consuming in both preparation work and actual experimental procedures, making them less feasible for some undergraduate laboratories. On the other end of the spectrum, Scroferneker et al. developed a game using cardboard pieces to represent elements of the complement system to help students understand the sequential action of complement cascade (Scroferneker, 1995). This alternative method is deemed simple, effective and engaging, but is far from the actual experience of visualizing the actions of complement activation.

In this article, we describe a simplified yet compact workshop to demonstrate the action of serum complement activation via red blood cell lysis, along with the introduction of other immunology concepts such as antigen-antibody interaction and antibody titers. This workshop was originally designed as part of an immunology lab session in the final (third) year of an undergraduate biomedical science program. The method is derived from the complement fixation test (CFT), which is commonly used for the detection of either antigen or antibody in the diagnosis of many bacterial, viral and other infectious diseases. Although over time CFT has been replaced by more sophisticated detection methods such as PCR (polymerase chain reaction) or ELISA (enzyme-linked immunosorbent assay), the visible result of CFT still provides a very simple method for students to appreciate the activation of complement system in basic lab settings, and reinforces students' understanding for the concept of complement cascade system. CFT takes advantage of the initiation of the complement cascade, which requires the complement to be activated by the formation of specific antigen-antibody complexes (immune-complexes). When the antibodies to be tested are present in the assay, specific immune-complexes form and sequester the limited complement in the reaction mixture. Therefore, when an activation target, usually red blood cells (RBC) bound to anti-RBC antibodies (sensitized RBCs), is added to the reaction mixture, the remaining complement is not sufficient to lyse the cells, leaving the RBC settle as red-color aggregates. This indicates "fixation of complement" and a positive result. In a negative test, free floating complements can interact with the activation target (RBC-anti RBC antibody complexes/sensitized RBCs) when not being fully fixed on the specific immune-complexes, causing the lysis of the RBC that turns the reaction mixture pink or light red due to the release of

hemoglobin (red colored cytoplasmic respiratory pigment). Therefore, the results of this exercise can be effortlessly observed by eyes without the need of specialized equipment such as a photometry (Figure 4 under "Student's handout"). Furthermore, the intensity of the red color correlates with the amount of hemoglobin released in the solution, which provides the basis for determining the antibody titers in the test samples.

Considering the common constraints in undergraduate laboratories, such as the cost and availability of reagents and equipment, limited time frame to perform practical exercise, and the risk of exposing less-experienced students to potentially biohazard materials, the exercise described here uses a demonstration of the simulated results of a viral complement fixation test to provoke students' thinking and learning. This simple exercise comprises a medical scenario (diagnosis of influenza virus A infection), a clinical application (the complement fixation test), and a virtual assay technique (serial dilution for comparing titers and determining endpoint), alongside with the core of this exercise – an illustration of complement cascade activation. Students participating in this workshop can analyze and record the demonstrated results of complement fixation test, determine whether the serum samples from patients contain antibodies against influenza virus A antigens, and answer questions that prompt discussion about the facets of the immune system in medical conditions. The students are thus provided with an enriched learning experience that encourages active thinking and visual memory. This technically simple and informative exercise is economical for the educator to set up, and is suitable for a wide range of students of different skill levels and various biology disciplines.

Methodology

This workshop was originally designed for a class size of approximately 60 students in their final (third) year of immunology course, and was scheduled to be completed within a 15- to 20-minute period as part of a 3-hour laboratory session, while other learning activities were also included in the same class. Prior to the workshop, students would have received relevant lectures on the immune system, different components of the complement system, antigen-antibody specificity, and the application of CFT as a clinical diagnostic tool including its limitation. A brief introduction was also given before the workshop to explain the background and procedure of this CFT exercise. To gain knowledge about how the complement fixation test is carried out, students were also encouraged to watch an online virtual CFT video on the website:
(http://highered.mheducation.com/sites/0072556781/student_view0/chapter31/animation_quiz_4.html), and to answer the questions 1-5 on the website after watching the video.

The method used to simulate the result of a viral CFT is summarized in Figure 2 below. Detailed instructor notes are available in Appendix. To conduct this workshop, a laboratory bench was set-up with 4 to 6 demonstration plates (Figure 3) to display the results of simulated viral CFT for three different patient's serum samples along with relevant controls and back titration reference. Paper towels, a biohazard bin and disinfectant were also available for accidental spillage during this activity. A teaching assistant or lecturer was available to guide students' observation, recording results and to answer students' questions.

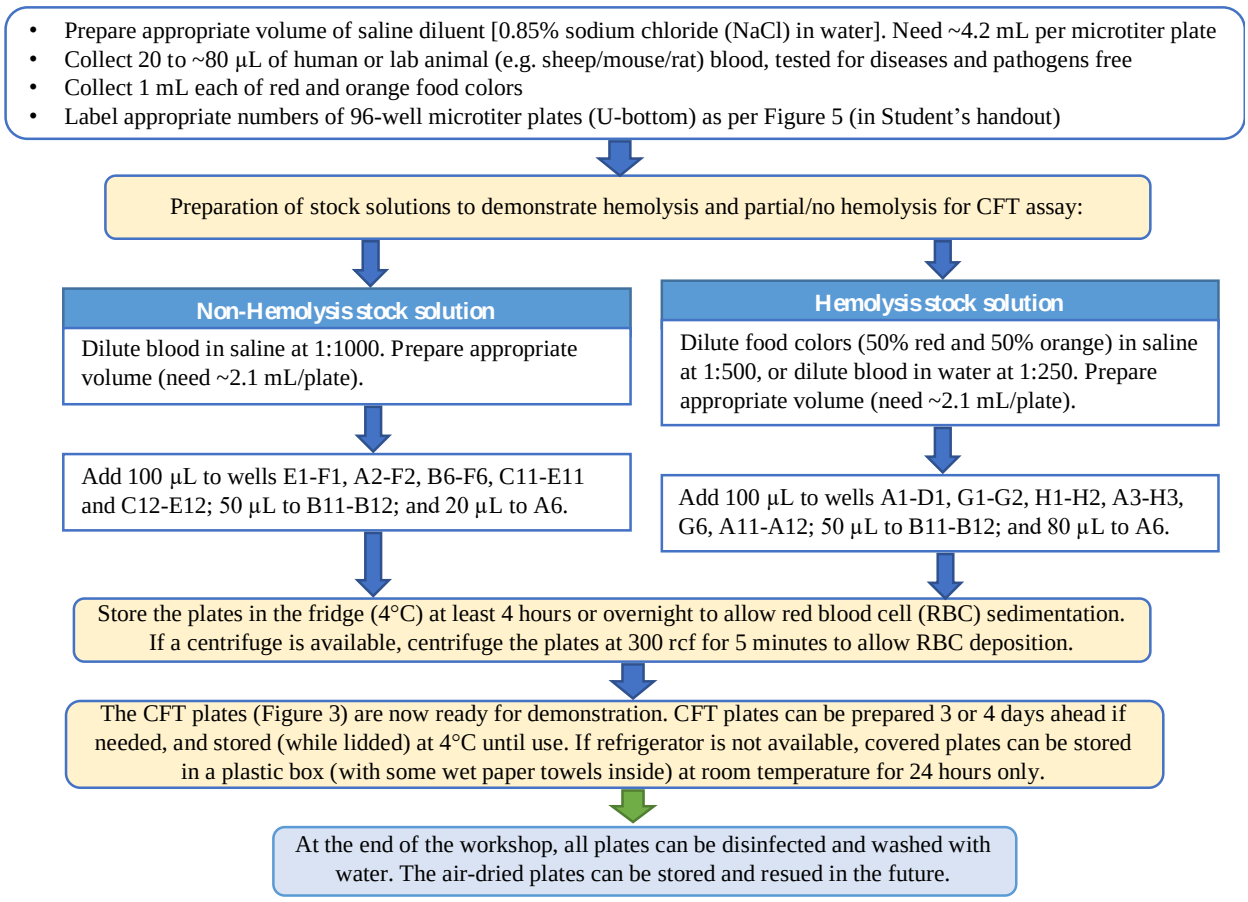

Figure 2. Schematic illustration of methods to stimulate the result of a complement fixation test (CFT).

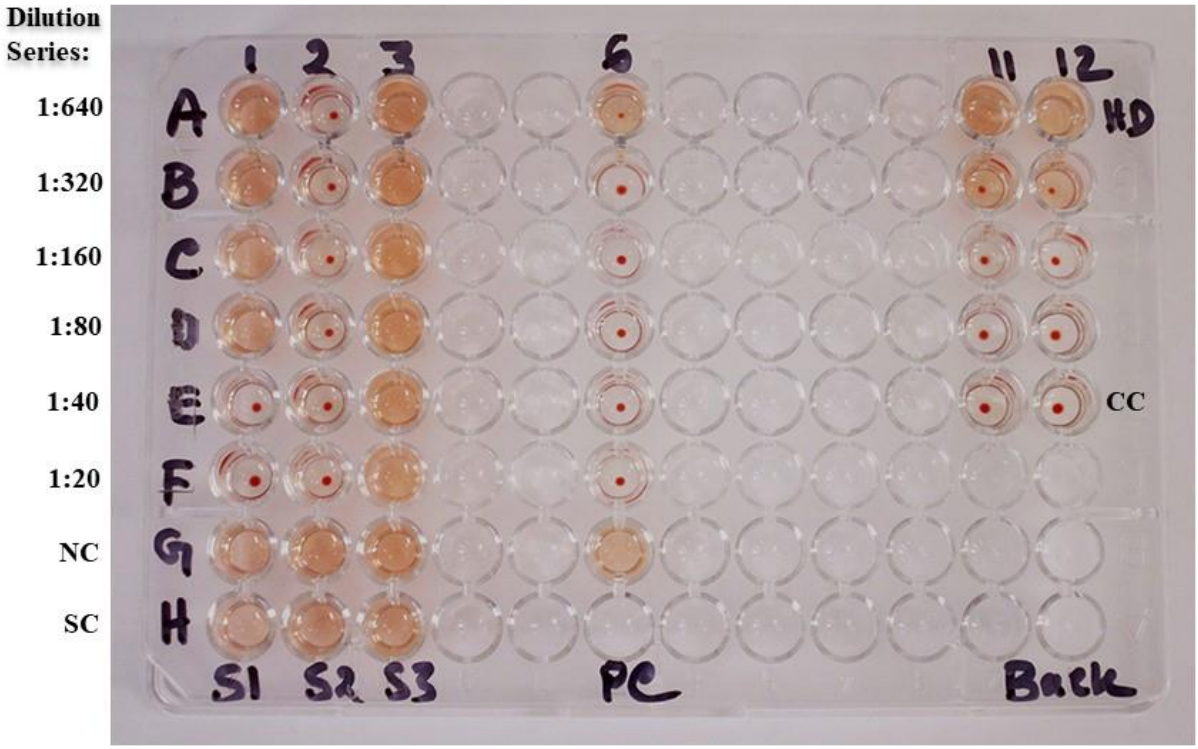

Figure 3. Photograph of the simulated viral complement fixation test result.

Patient's serum samples S1 (titer > 1:40) and S2 (titer >1:640) showed positive result, and S3 (titer <1:20) showed negative result for influenza virus A infection. Abbreviations: PC = positive control (A6-F6); NC = negative control (G1, G2, G3 and G6); SC = serum control (H1, H2 and H3); CC = cell control (E11-E12); HD = hemolysis dose (A11-A12).

During the workshop the students were given a brief introduction of the clinical scenario and were told a CFT has already been performed to determine whether serum samples from three patients have antibodies against influenza virus A antigens. Students were instructed to read the description outlined in "Student's handout" below, and understand the content of each well before presented with the CFT demonstration plate. While displaying the demonstration plate, the instructor also explained the preparation procedures of the CFT assay, and discussed the rationale of serial dilution, the inclusion of each control and back titration with the students. Students acknowledged that the sheep red blood cells (SRBC) previously coated with anti-SRBC antibody ("sensitized SRBC") were used as an indicator for this assay, and also learned that the lysis of red blood cells (hemolysis) can be detected by the release of hemoglobin (red color) (Figure 4). Students were then instructed to interpret the results of CFT (Figure 3) based on the presence or absence of hemolysis, and to determine which patient's sample contained antibodies against influenza virus A antigen. If positive results are found, the antibody levels could be quantified since the assay involved serial dilution of serum samples. In this scenario the antibody titer provides comparison of the disease severity. Students learned that the antibody titer was expressed as the dilution factor of the serum samples. The end point titer was to be determined by the highest dilution that could still cause complement fixation, i.e. the last well in which there is no hemolysis or the first well with complete hemolysis, as long as the judging criteria were consistent for all serum samples and controls. Students acknowledged the purpose of performing back titration in the assay. This was to ensure that the concentrations of complement and red blood cells being used were in an appropriate ratio for the complement to lyse red blood cells. Students also understood the negative and serum control wells should show complete hemolysis, and positive control well should show no hemolysis to validate this CFT assay as per standard laboratory-protocol. Questions at the end of "Student's handout" were used to guide and engage students in active thinking, and for the educator to assess the students' learning.

Student's handout

Viral Complement Fixation Test (CFT)

Principle:

When an antibody binds its complementary antigen, there is a conformational change in the antibody molecule. This enables its Fc portion to bind the **C1q component** of the complement system. This starts off the complement cascade. Even if the complement activation doesn't proceed all the way through to C9, components of the pathway are used up to some extent. This is called *complement fixation*. If a lot of antigen/antibody complex ("immune complex") is present, some of the complement components will be completely consumed. Sheep red blood cells (SRBCs) coated with anti-SRBC antibodies ('sensitized SRBCs') are then added to the mixture. Because the complement components have been consumed, the sensitized SRBCs will not be lysed.

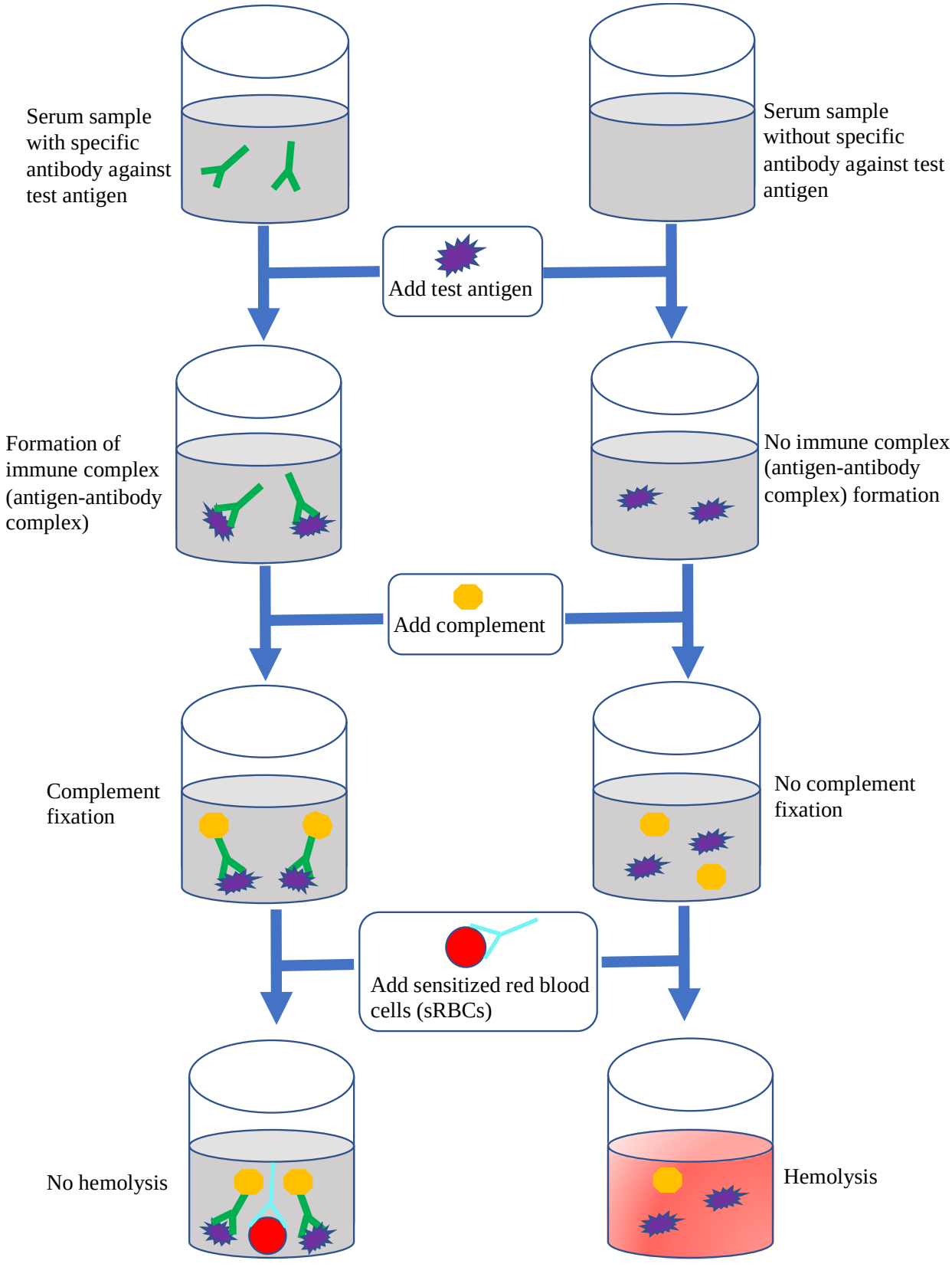

Figure 4. Procedure and result of a complement fixation test.

Procedure:

In this experiment, a complement fixation test has been used to determine whether serum samples from three patients have antibodies against influenza virus A antigens. Microtiter plates containing results of Viral Complement Fixation Test (CFT) are already prepared by the instructor using the following method, and are available as a demonstration for you to view and record the results. Please read the procedures and understand the content of each well, before looking at the demonstration plate to interpret test results.

	1	**2**	**3**	4	5	**6**	7	8	9	10	**11**	**12**	
A	640	640	640			640					2 units		**HD**
B	320	320	320			320					1 units		
C	160	160	160			160					0.5 units		
D	80	80	80			80					0.25 units		
E	40	40	40			40					0.0 CC		
F	20	20	20			20							
G	NC	NC	NC			NC							
H	SC	SC	SC										
	S1	**S2**	**S3**			**Positive Control**					**Back titration**		

Figure 5. Microtiter plate layout of the demonstrated complement fixation test.

Columns S1-S3 (from rows A-F) contain serial dilutions of the patients' serum samples; NC: negative control; SC: serum control; CC: cell control; HD: hemolysis dose. Details of the content in each well are provided in the box below.

Columns S1-S3 (from rows A-F) contain serial dilutions of serum samples from Patient 1, Patient 2, Patient 3, respectively, with veronal buffered saline (VBS), working strength influenza virus A antigen (WSA), working strength complement (WSC), sensitized sheep red blood cells (sSRBCs).

Positive control column (A6 to F6) contains serial dilution of positive control serum (which has antibodies against the influenza virus A antigen), VBS, WSA, WSC and sSRBCs.

Back titration columns (A11 to D11 and A12 to D12) contain VBS, WSC and sSRBCs.

NC (negative control) well contains VBS, negative control antigen (NCA), WSC and sSRBCs.

SC (serum-free control which has no antibodies against test antigens "influenza virus A antigen") well contains VBS, WSA, WSC and sSRBCs.

CC (cell control) well contains VBS and sSRBCs.

HD: hemolysis dose.

Step 1: Label a microtiter plate as in diagram above (Figure 5).

Step 2: Add 25 μL VBS (veronal buffered saline) to:

A1, A2, A3, A6

B1, B2, B3, B6 B11, B12

C1, C2, C3, C6 C11, C12

D1, D2, D3, D6 D11, D12

E1, E2, E3, E6 E11, E12

G1, G2, G3, G6

H1, H2, H3

Step 3: Add 50 μL of samples S1, S2, S3 and positive control serum (prepared in 1:20 dilution) to wells F1, F2, F3 and F6 respectively.

Step 4: Perform 1:2 doubling dilutions (25 μL volumes) from wells F1, F2, F3 and F6 up through to wells A1, A2, A3 and A6 respectively. Discard 25 μL from the last dilution (i.e. 25 μL remains in the well). Use a different pipette tip for each series of dilutions to avoid cross-contamination. Discard used tips into appropriate discard bin.

Step 5: Add 25 μL negative control antigen (NCA) to G1, G2, G3 and G6 (NC wells on the diagram).

Step 6: Add 25 μL working strength antigens (WSA) (test antigens: influenza virus A

antigens) to wells rows A-F (in columns 1, 2, 3 and 6,) and also to H1, H2, H3 (SC wells on the diagram).

Step 7: Add 25 μL working strength complement (WSC) to all wells in columns 1, 2, 3 and 6.

Step 8: Add 50 μL working strength complement (WSC) to wells A11 and A12.

Step 9: Perform doubling dilutions (25 μL volumes) from A11 to D11. Discard final 25 μL volume. Repeat for A12 to D12. Now wells A11 to E11 and A12 to E12 should have 25 μL in them.

Step 10: Add 50 μL VBS to wells A11 through E11 and A12 through E12.

Step 11: Put the lid on the plate and tap the side of the plate to mix. Incubate plate at 37°C for at least 30 minutes.

Step 12: After the incubation, add 50 μL of sensitized sheep red blood cells to all wells that have reagents in columns 1, 2, 3, 6, 11 and 12.

Step 13: Put the lid on the plate and tap the side of the plate to mix. Incubate plate at 37°C for 10 to 15 minutes.

The assay is complete when wells marked 2 and 1 units (hemolysis dose, HD) on the diagram demonstrate complete hemolysis. Typically, this takes between 10-15 minutes.

Result- Interpretation:

Lysis of the sensitized SRBC shows as a clear red solution, because all the SRBC released their hemoglobin. This implies that the complement was not used up, hence that the patient did not have antibodies against that virus (**negative result**).

Non-lysis is seen as a pellet of cells at the bottom of the well, and indicates fixation of complement by the immune complexes formed by patient antibodies combined with the test antigens in that well (**positive result**).

If positive results are found, the antibody levels are quantified by testing serial 2-fold dilutions of the patient's serum against the antigen to which they scored positive. The result (or antibody titer) is expressed as the highest dilution of the serum that still fixes enough complement to stop lysis of sensitized SRBC.

To better understand how the complement fixation test works, proceed to the tutorial in the link below and answer the questions 1-5 after watching the video: http://highered.mheducation.com/sites/0072556781/student_view0/chapter31/animation_quiz_4.html

WARNING!

Treat all human blood and blood products as potentially hazardous.

Review the safety procedures before embarking on this laboratory work.

Questions:

1. Among three serum samples from three patients (labelled as S1, S2, and S3) which one was “positive” for influenza virus A infection? Justify your answer.

2. Calculate the titers of anti-influenza virus A antibodies for each of the three patient samples (S1, S2 and S3) and also the positive control preparation, and explain how you worked out the titers.

3. Rank the samples in order of the titer of anti-influenza virus A antibodies. Explain the correlation of the antibody titer with disease severity among patients.

4. What is the purpose of doing positive and negative control, and also serum control in the CFT assay?

5. What is the purpose of the ‘back titration’ (done in columns 11 and 12 of the demonstrated CFT plate)? What does the level of lysis in the back titration indicate for the test?

6. What safety precautions need to be taken when handling patient's serum and human or vertebrate blood samples?

7. The sheep red blood cell (SRBC) was used as an indicator system for this CFT assay. Prior to use in this assay SRBC was first coated with anti-SRBC antibodies ("sensitized SRBC") before adding to the mixture on the well of microtiter plate, explain why.

8. What other test(s) other than CFT could be used to help diagnose patients with influenza virus infection? Compare their advantages and limitations.

Laboratory safety

Students participating in this workshop have already acquired basic laboratory skills and safety knowledge through their laboratory activities during first- and second-years' courses. Students used appropriate personal protective equipment (e.g. gloves, safety glasses, lab coat and closed-toed shoes) to observe and record the results of simulated CFT. Students were not aware that the assay didn't contain actual patient's serum/samples, and handled demonstration plate using safety guideline. Only a very small amount (less than 3 microliters) of vertebrate blood is required for each demonstration microtiter plate. Instructor can source human or lab animal blood that has been tested diseases and pathogens free, depends on the availability in different areas/countries, and handle the material in compliance with institutional regulations.

Discussion

The CFT workshop described in this article facilitates effective learning with the design of a medical scenario and detailed procedures outlined in "Student's handout", and can be completed in a very safe and economical way without specific laboratory skills or equipment. While the actual pathway of complement cascade of protein activation is rather complex, the results of complement-mediated red blood cell lysis are dramatic. The use of red blood cells as an indicator system not only allows students to visualize the activation of serum complement, but also enables qualitative interpretation of the test results without specialized instrument. Therefore, when used alongside with relevant lectures, this exercise can help students to connect theoretical knowledge and visualized experience when learning the complex concept of the complement system.

Apart from demonstrating the activation of complement, this workshop also introduces other important concepts in immunology, such as the immune complex formation via antibody-antigen specific interaction, and the correlation between antibody titer and disease severity. The students' learning journey starts from learning the disease scenario (influenza virus A infection), through understanding the test method (CFT for testing the presence of specific antibody post infection), the rationale of experimental design such as the inclusion of the appropriate controls (e.g. positive, negative, and serum control), the reason for including back titration (to verify appropriate concentration of complement and red blood cells ratio are used), the technique of serial dilution for endpoint titer determination, and finally, to the acquisition of results interpretation and data analysis skills (e.g. observation of the color change and color intensity, for diseases diagnosis and the determination of severity). By using food color to mimic the hemolysis results of CFT, the reliability and the reproducibility of this exercise are ensured, while time, skills, and cost required for setting up the lab exercise are greatly reduced. This simple method also eliminates the need of using real patient's serum which can be difficult to source and unsafe to handle. The use of very small amount of human or lab animal (e.g. sheep/mouse/rat) blood does not pose risk of contamination, nor produces significant biological wastes that require specific disposal protocols (for details see Appendix).

Student reaction and suggestions for learning assessment

Students were instructed to work as a pair to observe and record the results of the viral CFT, and used the questions outlined in "Student's handout" as a guide to discuss their observations with their lab partner and interpret the results. This discussion allowed students to have a debate among the group in exploring possible answers to the questions, which engaged students in active learning, helped to acquire new knowledge, and enhanced their critical thinking skills. Students also had the opportunities to ask questions to teaching assistants/lecturer which further allowed students to practice how to convey and receive the constructive feedback from their peers and instructors, and enhanced student's teamwork skills. Students' learning outcomes were determined through few channels: 1) Informal discussion with the instructors/teaching assistants during this workshop to determine students' ability to record and to interpret the results of CFT. 2) Students were required to submit an assignment of the results and answers to the questions individually, one week after the completion of this workshop. This contributed to their course work assessment, and reinforced the students' knowledge gained through this workshop. 3) Multiple choice questions in term-test and in final exam questions to further assist students for understanding the concept of complement system.

From our teaching experiences we noticed that, students generally found the visualized results of hemolysis (Figure 3) to be impressive and undeniable. The concept of complement fixation was usually well received by students with the help of the text and diagram (Figure 4) in the student's handout along with pre-workshop introductory lecture. Approximately 95% students (who have received relevant lecture and fully participated in the workshop) correctly answered all the questions outlined in Student's handout, and provided appropriate justification and explanation to the

satisfaction of the laboratory instructor. This showed/proved the effectiveness of this workshop in delivering new knowledge around complement activation and the CFT.

Limitations and Suggestions for Educators

The complement system, alongside with other critical concepts of the immune system, are usually missing in the undergraduate teaching laboratory due to the common perception that they are too complex, and hard to be included in laboratory activities with limited resources, time and equipment. The workshop described here offers an opportunity to introduce the often-neglected action and application of complement system, while touching on other important immunology concepts such as the antibody-antigen interaction, importance of complement system and its application to aid in clinical diagnosis. Although this workshop is still limited in providing a hands-on practical experience for the students, the simulated CFT result looks realistic and often leaves a strong positive impression. Another limitation of this workshop is that it only demonstrates the dramatic effect of the outcome of complement-mediated cell lysis, while the actual pathway cannot be visually appreciated. However, the complex cascade of protein activation can be explained in lectures more easily and effectively, especially with the help of detailed textbook chapters and multimedia resources designed for this purpose.

Due to the simplicity and cost-effectiveness to prepare, this simulated microtiter plate-based CFT exercise can easily be adapted by educators in any institutions for teaching different biology courses. For the use of this exercise in laboratories of different biological disciplines, modification to the clinical scenario or questions can be done depending on educator's teaching aims and the students' skill levels. In fact, the CFT procedures described in "Student's handout" are derived from a medical diagnostic laboratory, and have been carefully modified and optimized to suit the educational purposes. Therefore, if all materials and equipment are available in a more privileged setting, and instructor prepare and aliquot all reagents for the students, the method (which takes less than 2.5-hour to complete) can be used as the protocol for an actual CFT in a practical lab session. If time permits educators can also include other relevant controls for more advanced students to validate this CFT assay, such as testing the serum samples against a negative control antigen to rule out the possibility of false positive.

Conclusion

We have developed a simple method using easily sourced and affordable materials to reliably mimic the results of a CFT, which offers an effective teaching tool for illustrating the activation of complement cascade (through visible reaction of red blood cell lysis) to undergraduate students. This workshop serves to strengthen the links between theory and visualized experiences for a complex concept such as the complement system, that is often overlooked in undergraduate laboratories. The design of this CFT workshop also allows students to gain additional knowledge on disease diagnostic tools, and safety precautions in handling human samples. Furthermore, the simulated and demonstrated nature of this CFT workshop overcomes the difficulty of performing actual CFT exercise due to time, expense and facility constraints commonly seen in many undergraduate laboratories, and avoids the use of hazardous materials while facilitating a safe and interactive teaching environment. Finally, the protocol of this workshop can be easily modified and adapted to suit different teaching purposes for varying biology disciplines and student-levels.

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Appendix

Detailed procedure to simulate the results of viral complement fixation test demonstration

Materials:

- Sodium chloride (NaCl)
- Balance and spatula
- 96-well microtiter plates (U-shaped bottom)
- Approximately 20 to 80 µL of vertebrate blood from human or common lab animal (e.g. mouse/rat/sheep), which has been tested for diseases and pathogen free
- Approximately 1 mL each of red and orange food colors
- 10 mL and 30 mL free standing tube/bottle
- P-20, P-200 and P-5000 micropipettes
- 10-20 µL, 200 µL and 5 mL micropipette tips
- Centrifuge with rotor for 96-well microtiter plate (optional, only if available)
- Water
- Disinfectant
- Gloves, safety glasses and discard jar

Method:

A) Preparation of stock solutions ("Non-hemolysis" and "Hemolysis") to represent samples with no hemolysis and hemolysis in the CFT assay:

Note: *About 2.1 mL of each stock solution ("Non-hemolysis" or "Hemolysis") is required for each microtiter plate according to the Figure 5 in "Student's handout". Prepare appropriate volume of each of the stock solutions depending on the number of plates required for the class.*

1. Prepare "Saline diluent": 0.85% sodium chloride (NaCl) in water. Approximately 4.2 mL of saline is required for each microtiter plate. Prepare appropriate volume of saline.

2. Prepare "Non-hemolysis" stock solution for the wells showing no hemolysis: dilute whole blood with saline at a 1:1000 ratio (for example, mix 10 µL of whole blood with 10 mL of saline).

Note*: we used human blood which has been tested diseases and pathogens free, but common animal blood (e.g. mouse or sheep red blood cells) can also be used. Blood can be sourced commercially or animal blood would very likely be available from the animal facilities of universities/colleges that teach immunology or hematology. Only as little as 12 µL of fresh blood is required for preparing four CFT plates. The volume of blood sample can also be adjusted (decreased or increased) to prepare stock solution according to instructor's preference.*

3. Prepare "Hemolysis" stock solution for the wells showing full/partial hemolysis: Dilute food color (50% red and 50% orange) with saline at a 1:500 ratio (for example, mix 10 µL of red color and 10 µL of orange color with 10 mL of saline). Alternatively, if enough blood is available, instructor can prepare lysed blood sample for the wells showing hemolysis by diluting whole blood with water at a 1:250 ratio. However, please note that the use of food color (diluted with saline) works better for the wells showing partial hemolysis, as further/full lysis of red blood cells in the mixture might occur if water was used.

B) Preparation of the CFT plates for demonstration:

1. Label the appropriate number of microtiter plates as per Figure 5 in "Student's handout".

2. Add 100 µL of "Non-hemolysis" solution (diluted blood) into the E1-F1, A2-F2, B6-F6, C11-E11 and C12-E12 wells.

3. Add 100 µL of "Hemolysis" solution (diluted food color) into the A1-D1, G1-H1, G2-H2, A3-H3, G6 and A11-A12 wells.

4. Add 50 µL of "Non-hemolysis" solution into the B11-B12 and then 50 µL of "Hemolysis" solution into the B11-B12 wells to show partial hemolysis.

5. Add 20 µL of "Non-hemolysis" solution into the A6 and then 80 µL of "Hemolysis" solution into the A6 well to show partial hemolysis.

6. Centrifuge all plates at 300 rcf for 5 minutes to allow red blood cells deposition.

Note: *If centrifuge is not available, store the prepared plates at 4°C overnight (or ~ 4 hours) to allow red blood cells deposition.*

7. All plates are now ready for use. Plates can be prepared 3 or 4 days ahead, if require and stored while lidded at 4°C until use. If a refrigerator is not available, covered plates can be stored in a plastic box (with some wet paper towels inside) at room temperature for overnight only. If required and a centrifuge is available, plates can be centrifuged again for 2 min at 300 rcf immediately before the workshop.

Note: *At the end of the workshop, collect all the used microtiter plates, and soak all plates into disinfectant for appropriate time. Thoroughly rinse the wells with hot water and allow to air dry. The cleaned plates can be stored and re-used in the future.*