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Animal Influenza Virus

Second Edition

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Preface

Influenza A viruses are among the most important pathogens for humans, food animals, and companion animals. Of the animal influenza viruses, avian, swine, and equine influenza are likely to have the greatest economic impact internationally because of their value as food animals or, with horses due to a large sport competition industry. Also, although the risk is truly unknown, as domestic animals, poultry, swine, and horses have extended contact with humans, which provides an interface for interspecies transmission, there is always the potential for these animal influenza viruses to become threats to public health.

Regardless of the possible implications for public health, influenza is highly significant to poultry, swine, and equine health. Research and diagnostics with animal influenza viruses are critical to animal health in its own right and it should be recognized that the needs and goals of animal agriculture and veterinary medicine are not always the same as those of public health. Even within these three examples of animal influenza viruses there are differences in the approach which may need to be taken, as the structure of the poultry, swine, and equine industries are different and there are some biological differences of influenza virus from each animal group as well. One of the aims of this book is to sort out those differences and to provide host, strain, and lineage specific guidance and procedures.

The reader will also recognize that in some cases the same method is described for all three of these animal viruses, for example real-time RT-PCR or hemagglutination inhibition (HI) assay. At first glance this may seem redundant; however there are often seemingly minor, but crucial differences in the assay, such as sample processing for each species (e.g., how to treat for sera the HI assay) or the specificity of reagents (e.g., primer sequences for RT-PCR; optimal laboratory host system for virus isolation). In contrast there are some methods that will be unique to an animal influenza virus group and parameters will necessarily vary. Certainly, assays may be adapted to individual study needs with proper optimization or can simply be used as they are described. The aim of this book is to provide the essential methods used in working with animal influenza viruses, and to compile more advanced information that will guide the user in designing influenza studies.

Most importantly this book would not have been possible without the contributions of the authors. The contributors are experts in their fields; therefore their input and knowledge is invaluable with the details they provide from their extensive experience. I want to gratefully acknowledge each of them for taking time from their busy schedules to contribute to this book. I would also like to thank the editorial team at Springer: John Walker, MIMB series editor, and both Patrick Marton and David Casey for all their time and help with completing this book.

Happy pipetting.

Athens, GA, USA *Erica Spackman*

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Part I

General

Chapter 1

Hemagglutination Assay for Influenza Virus

Mary Lea Killian

Abstract

The hemagglutination assay (HA) is a tool used to screen cell culture isolates or amnioallantoic fluid harvested from embryonated chicken eggs for hemagglutinating agents, such as type A influenza. The HA assay is not an identification assay, as other agents also have hemagglutinating properties. Live and inactivated viruses are detected by the HA test. Amplification by virus isolation in embryonated chicken eggs or cell culture is typically required before HA activity can be detected from a clinical sample. The test is, to some extent, quantitative as 1 hemagglutinating unit (HAU) is equal to approximately 5–6 logs of virus. It is inexpensive and relatively simple to conduct. Several factors (quality of chicken erythrocytes, laboratory temperature, laboratory equipment, technical expertise of the user) may contribute to slight differences in the interpretation of the test each time it is run. This chapter describes the methods validated and used by the US National Veterinary Services Laboratories for screening and identification of hemagglutinating viruses.

Key words Hemagglutination, Avian influenza virus, Type A influenza, Influenza detection, Virus titer

1 Introduction

The hemagglutinin protein on the surface of influenza virus particles is capable of binding to *N*-acetylneuraminic acid-containing proteins on avian and mammalian erythrocytes [1, 2]. When combined, if the influenza virus is present in a high enough concentration, there is an agglutination reaction and the erythrocytes link together to form a diffuse lattice (*see* Fig. 1). The hemagglutination assay (HA) is a classic diagnostic test used to screen cell culture supernatant or amnionic–allantoic fluid (AAF) harvested from embryonated chicken eggs.

The HA is not an identification assay. Other types of viruses (e.g., paramyxoviruses, adenovirus-127) and certain bacteria also have hemagglutinating properties [3–5]. The HA should be followed by a hemagglutination-inhibition assay (*see* Chapter 2) to determine the type and/or subtype of virus. The HA does not necessarily indicate the presence of a viable virus [1]. It is also capable

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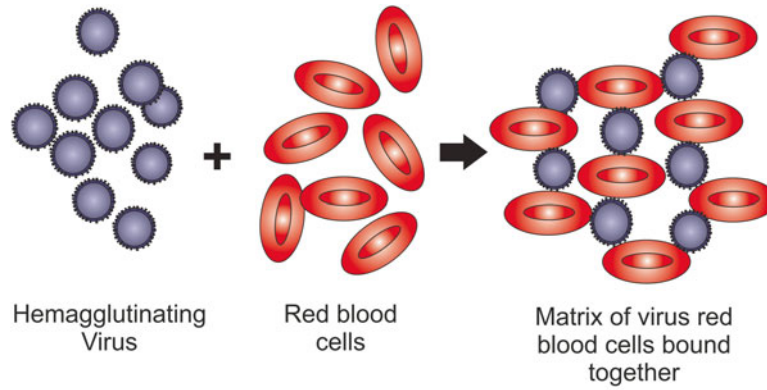

Fig. 1 Illustration of the hemagglutination process. The virus binds red bloods cells (RBCs) and forms a matrix by linking RBCs together which prevents the RBCs from settling in the diluent (often PBS)

of detecting viral particles that have been degraded or inactivated and are no longer infectious.

Generally, there is enough virus present following a single passage in culture for detection of influenza virus by HA [4, 5]. Additional passages increase the chance for cross-contamination in the laboratory. On average, a 10^5–10^6 50 % egg infectious dose (EID_{50})/ml is required for detection by HA [5]. At the time of collection, AAF that contains blood should be discarded as there may be some adsorption of the virus by the erythrocytes, decreasing the overall titer of the virus in the fluid or inhibiting the ability to detect the virus by HA assay.

The following test procedure is used at the US National Veterinary Services Laboratories (NVSL). The World Animal Health Organization (OIE) Manual of Standards for Diagnostic Tests and Vaccines outlines a slightly different procedure, however the results are equivalent. The OIE procedure utilizes different volumes of reagents and a different concentration of chicken erythrocytes, and the steps are performed in a slightly different order. For this reason, steps or reagents from the NVSL procedure and the OIE procedure should not be combined in the same test.

2 Materials

1. Sterile bottle with lid.
2. Anticoagulant, Alsever's solution or acid citrate dextrose.
   (a) Alsever's solution: Weigh out reagents into a conical flask: 0.55 g of citric acid, 0.8 g of sodium citrate, 2.05 g of D-glucose, and 0.42 g of sodium chloride. Dissolve in distilled water to a final volume of 100 ml. Dispense into sterile 10 ml

bottles, do not tighten lids and sterilize by autoclaving at 116 °C for 10 min. Use slow exhaust. Allow to cool, then tighten the lid and label the bottle. Store at 4 °C.

(b) Acid citrate dextrose (ACD): Weigh out reagents into a conical flask: 4 g of citric acid, 11.3 g of sodium citrate, and 11 g of D-glucose. Dissolve in 300 ml of distilled water then add distilled water to a final volume of 500 ml. Dispense into 100 ml bottles and do not tighten the lids. Sterilize by autoclaving at 116 °C for 10 min. Use slow exhaust. Allow to cool, then tighten the lid and label the bottle. Store at 4 °C.

3. 50 ml Conical tubes.
4. U-bottom or V-bottom 96-well plate with lid.
5. Single and multichannel pipettes and pipette tips to deliver 50 µl volumes.
6. Liquid reagent reservoirs.
7. 0.1 M phosphate-buffered saline (PBS), pH 7.2.
   (a) To prepare PBS, combine the following ingredients: 8.5 g of sodium chloride, 1.33 g of sodium phosphate dibasic, and 0.22 g of sodium phosphate monobasic. Dissolve in distilled water to a final volume of 1 l. Mix thoroughly and adjust pH to 7.2 ± 0.1.
8. Chicken erythrocytes, 0.5 % in PBS (*see* Chapter 2).
9. Positive control influenza antigen.
10. Mylar microtiter plate sealers (Thermo) or equivalent.
11. Test material: AAF harvested from eggs inoculated with AIV or an AIV suspect sample. Sufficient quantity of AAF should be harvested to provide enough material to conduct the HA assay and subsequent characterization assays. Cell culture supernatant may also be used.

3 Methods

3.1 Collection and Preparation of Rooster Red Blood Cells

1. Rooster red blood cells (RBCs) collected from specific pathogen-free (SPF) chickens are preferred for use in the HA test. Red blood cells collected from hens may contain hormones that interfere with hemagglutination. Chicken erythrocytes are typically used because the settling time is quicker and the settling patterns are typically clearer than with cells from other species [2]. Certain AI viruses may not hemagglutinate chicken erythrocytes before adaptation in embryonated chicken eggs; these viruses may be more sensitive to hemagglutination with turkey erythrocytes or guinea pig erythrocytes.

2. Fresh erythrocytes should be prepared regularly. Red blood cells will begin to hemolyze after 5–7 days, causing inaccuracies in the HA test [6]. The erythrocyte suspension should be mixed gently before use to ensure a uniform distribution of cells in each of the test wells.
3. Prepare a sterile bottle with a lid containing an anticoagulant (either ACD or Alsever's solution). Use one volume ACD to three volumes blood or one volume Alsever's solution to one volume blood.
4. Before collecting blood, draw a small amount of anticoagulant into the syringe and expel again. This will coat the syringe which will keep the blood from coagulating in the syringe before addition to the anticoagulant in the bottle.
5. Collect 3–5 ml blood from the wing vein or by cardiac puncture from an SPF rooster in accordance with the appropriate animal care and use procedures. Animals should not be vaccinated for Newcastle disease or other pathogens as this may interfere with some serological hemagglutination-inhibition tests. Add the blood to the anticoagulant. Rotate the bottle gently to mix thoroughly. Always treat the red blood cells gently to avoid hemolysis.
6. Add the suspension to a 50 ml conical tube and add a sufficient quantity of PBS to total 50 ml. Rotate gently to mix.
7. Centrifuge at approximately 800 × *g* for 10 min to pellet RBCs.
8. Aspirate supernatant and surface layer of white cells (buffy coat) from the tube without disturbing the pellet of erythrocytes.
9. Wash the erythrocytes a total of three times in PBS by repeating **steps 6–8**.
10. Add 1 ml packed red blood cells to 199 ml PBS for a final RBC concentration of 0.5 %.

3.2 Hemagglutination Assay

1. Orient a microtiter plate (U-bottom or V-bottom) so that samples will be diluted either 8 wells or 12 wells across as needed. Number the rows on each plate so that the contents of each row are uniquely identified.
2. Add 50 μl PBS to every well on the plate.
3. Add 50 μl AAF (*see* **Note 1**) or cell culture fluid to be tested ("test material") to the first well in each row (or column) to be tested. Note that this will result in a 1:2 dilution of test material. Positive control antigen and cell control (no antigen, where PBS is added instead of antigen) wells with RBCs must be included each time the test is performed. If multiple plates are being tested at the same time, one positive control antigen and one RBC only control well should be included on a minimum of every fifth plate.

4. Dilute the test material: Mix the contents of the first well by pipetting up and down slowly (avoid generating bubbles). Transfer 50 µl from the first well to the second well in the row or column as appropriate for the plat orientation. Continue to make twofold dilutions of the virus suspension across the entire row or column. Discard the excess 50 µl after the last row or column. All wells should have a final volume of 50 µl after this step.
5. Starting at the end of the plate with the highest dilution add 50 µl 0.5 % erythrocyte suspension to every well. Tap the plate gently to mix.
6. Apply adhesive plate sealer to each plate. Once the sealer is applied, the plates may be removed from the biological safety cabinet after decontaminating the surface.
7. Allow 20–30 min for the RBC to settle (*see* **Note 2**).

3.3 Interpretation of Results

1. The HA plate should be read when the erythrocytes in the cell control wells have settled to form a solid "button" in the bottom of the well (hemagglutination negative) (*see* **Note 3**) (*see* Chapter 24, Fig. 1). When the plate is tilted at approximately 45°, the RBCs will stream in a "tear-drop" fashion [2, 7]. Test fluids that are HA negative will also form solid buttons in all wells of the corresponding row. These buttons should teardrop at the same rate as the cell control. Because 10^5–10^6 EID_{50}/ml is required for hemagglutination to occur, an additional passage of negative material in embryonated chicken eggs may be optionally performed to confirm that isolations are not missed because of low levels of virus in the sample.
2. Samples showing complete hemagglutination in one or more test wells should be considered positive for a hemagglutinating agent (*see* Fig. 2). Hemagglutination positive samples may be further characterized by testing in the hemagglutination-inhibition assay (*see* Chapter 2) using monospecific antibodies or may be confirmed as influenza with another assay (e.g., rRT-PCR).
3. Incomplete hemagglutination may be observed as buttons that do not teardrop, have fuzzy margins, or form a donut-shaped ring in the bottom of the well (incomplete hemagglutination may not be observed if using V-bottom plates). Incomplete hemagglutination usually indicates an unbalanced proportion of erythrocytes and virus particles allowing partial settling of the erythrocytes. The incomplete reaction may be recorded but should be interpreted as negative.
4. The endpoint of the virus titration is the highest dilution causing complete hemagglutination (initial dilution is 1:2). The endpoint dilution is considered 1 HA unit (HAU), and the number of HAUs/50 µl is the reciprocal of the highest dilution. Example: For 6 wells of complete hemagglutination with an endpoint dilution of 1:64, there are 64 HAU/50 µl.

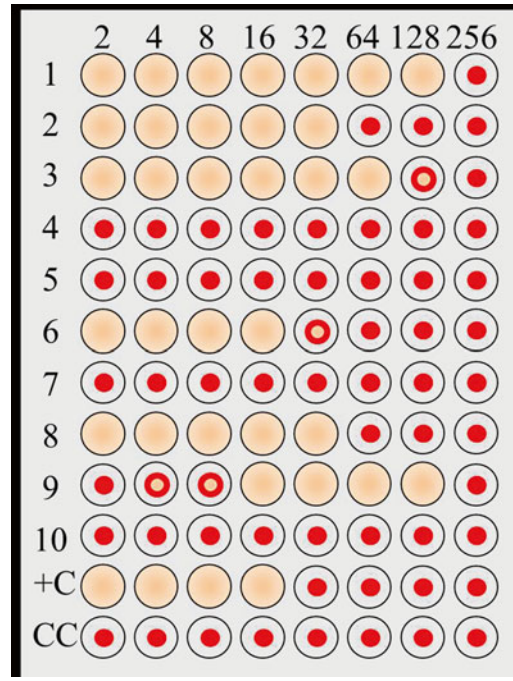

Fig. 2 Illustration of microtiter plate with positive and negative hemagglutination results. Sample ID is designated on the *left* side of the plate (+C indicates positive control antigen and CC indicates RBC control), the dilution factor is designated across the *top*. Results for each sample: (*1*) positive hemagglutination 128 HAU; (*2*) positive hemagglutination 32 HAU; (*3*) positive hemagglutination 64 HAU with incomplete hemagglutination in 128 HAU well; (*4*) negative hemagglutination; (*5*) negative hemagglutination; (*6*) positive hemagglutination 16 HAU with incomplete hemagglutination in 32 HAU well; (*7*) negative hemagglutination; (*8*) positive hemagglutination 32 HAU; (*9*) positive hemagglutination 128 HAU with prozone effect in 2, 4, and 8 HAU wells; (*10*) negative hemagglutination

4 Notes

1. All steps with infectious or potentially infectious material must be performed in a Class II biological safety cabinet. Aerosol-resistant tips and aseptic technique should be used any time an aliquot is taken from the original sample tube containing virus. Material carried on the surface of a pipette is a common source of laboratory contamination.
2. Assay plates should not be left too long before reading results [1]. The neuraminidase protein present on influenza viruses acts to break virus-cell bonds, and may eventually begin to break apart the lattice formed by the virus and erythrocytes. Some virus strains have very high neuraminidase activity and may not allow proper hemagglutination. When these viruses are encountered, the assay should be performed at 4 °C to

decrease neuraminidase activity, and the incubation period should be increased to 45–60 min before reading the plate.

3. Troubleshooting.
   (a) If the erythrocytes do not settle in cell control wells after 20–30 min possible causes are as follows: (1) The erythrocytes are not equilibrated to room temperature; (2) the erythrocyte suspension is poor quality or hemolyzed; (3) the PBS is poor quality or incorrect pH; (4) contamination of wells with viral antigen has occurred.
   (b) If the positive control is negative possible causes are as follows: (1) The positive control antigen was not added to control well; or (2) the erythrocytes have been allowed to settle for longer than 30 min.
   (c) When a sample is negative for hemagglutination in the first wells (where the highest virus concentration should be) and positive in the last wells (lower concentration wells) it is called the prozone effect. The prozone phenomenon is a high dose effect where reactions may be weak or negative in the first wells and stronger in the higher dilutions. This is caused when the virus in the high concentration wells is proportionately higher than the available receptors on the erythrocytes therefore cannot crosslink into a lattice. They may appear as either complete buttons or a partial button with fuzzy margins in the bottom of the well. Reactions may be interpreted as false negative if the dilution series is not carried out far enough (*see* Fig. 2). A dilution of 2^8 is normally sufficient to avoid a false-negative reading due to a very high virus concentration.

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Chapter 2

Hemagglutination-Inhibition Assay for Influenza Virus Subtype Identification and the Detection and Quantitation of Serum Antibodies to Influenza Virus

Janice C. Pedersen

Abstract

Hemagglutination-inhibition (HI) assay is a classical laboratory procedure for the classification or subtyping of hemagglutinating viruses. For influenza virus, HI assay is used to identify the hemagglutinin (HA) subtype of an unknown isolate or the HA subtype specificity of antibodies to influenza virus. Since the HI assay is quantitative it is frequently applied to evaluate the antigenic relationships between different influenza virus isolates of the same subtype. The basis of the HI test is inhibition of hemagglutination with subtype-specific antibodies. The HI assay is a relatively inexpensive procedure utilizing standard laboratory equipment, is less technical than molecular tests, and is easily completed within several hours. However when working with uncharacterized viruses or antibody subtypes the library of reference reagents required for identifying antigenically distinct influenza viruses and or antibody specificities from multiple lineages of a single hemagglutinin subtype requires extensive laboratory support for the production and optimization of reagents.

Key words Hemagglutination, Hemagglutination-inhibition, Avian influenza, Type A influenza, Influenza subtype identification

1 Introduction

Influenza viruses agglutinate erythrocytes through the interaction of the virus surface glycoprotein, the hemagglutinin (HA), with receptors on the surface of the erythrocyte. If viral particles are in sufficient quantity, the interaction of the HA protein with erythrocytes will form a complete network of linked erythrocytes preventing erythrocytes from settling out or precipitating as a small pellet in the bottom of a tube or microtiter plate. Agglutination of erythrocytes is the basis of the hemagglutination assay (*see* Chapter 1), and inhibition of the agglutination reaction by HA subtype-specific antisera is the basis of the hemagglutination-inhibition (HI) assay [1–3] (*see* Fig. 1).

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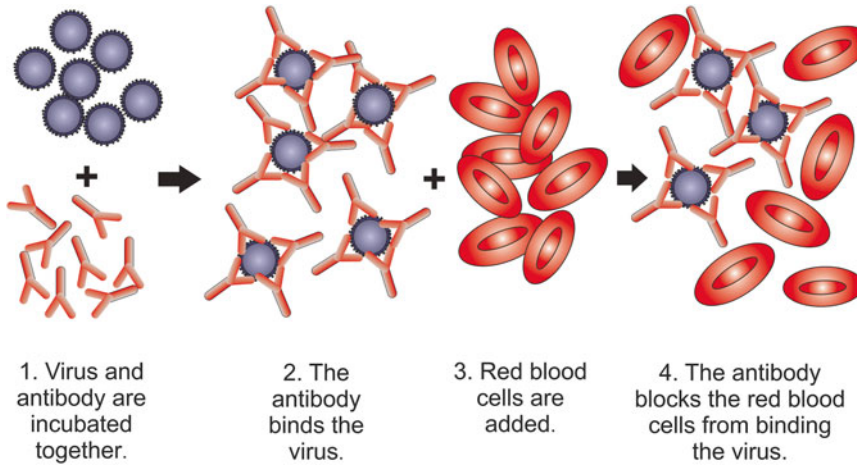

Fig. 1 Hemagglutination-inhibition assay. (*1*) Virus and antibody are mixed and incubated; (*2*) if the antibody is an antigenic match to the virus, it will bind the virus; (*3*) *red* blood cells are then added to the assay; (*4*) since the antibody is binding the virus, the virus can not bind the *red* blood cells and hemagglutination is blocked

A panel of serum prepared against the 16 distinct HA subtypes is used in the HI assay to confirm the HA subtype identity of influenza viruses and may be used to evaluate antigenic relatedness [4]. Typing is facilitated by using antisera raised against influenza isolates with neuraminidase (NA) subtypes which are heterologous with the test virus. The use of heterologous NA test virus eliminates false-positive reactions due to steric inhibition caused by the interaction of homologous neuraminidase antigen and antibodies [5]. For example a false positive could be produced by testing an H5N2 isolate with H7N2 sera, but should not cross-react with H7N3 sera.

Reference laboratories maintain a library of antigens and antisera of each HA subtype for isolate identification and testing antigenic relatedness. Production of antibodies to only the HA protein with DNA vaccines has been reported [6] and offers an alternative method for the production of sera without NA interference.

Importantly, with diagnostic and surveillance specimens an influenza virus with a novel HA subtype would not be detected or would produce a false-negative result in tests using antisera to the known HA subtypes [7]. Therefore, it is essential to confirm that a hemagglutinating agent that is negative by HI assay is not influenza by another test such as commercial antigen immunoassay, rRT-PCR, sequencing, or agar gel immunodiffusion assay.

In addition to virus subtype identification, the HI assay may be used to detect and quantitate HA subtype-specific antibodies in serum, plasma, or egg yolk following infection or vaccination. The presence of AI virus-specific antibodies may be detected as early as 7 days after infection by HI assay [4]. Antibody positive serum will inhibit the HA activity of an antigen of the same HA subtype,