Preface

The avian influenza (AI) virus, which is the type A influenza virus adapted to an avian host, has been an important pathogen for the poultry industry worldwide for many years. Although the cause was unknown at the time, one of the first recognized outbreaks of highly pathogenic AI (HPAI) virus occurred in Europe in the 1870s. Since 1955, when the AI virus was first identified, there have been about 24 outbreaks of the HPAI virus in domestic poultry worldwide.

Of those, perhaps the most consequential HPAI virus strain to emerge has been the Asian H5N1 HPAI virus, which was first seen in Southeast Asia around 1997. Until sometime in 2003, this strain had been mainly involved in sporadic outbreaks and was relatively contained. Then in 2004, reports of infection in poultry increased throughout Asia at about the same time as fatal cases of human infections in several Southeast Asian countries were confirmed. By 2006, the virus had spread further throughout Asia, into Africa and Europe, and as far west as the United Kingdom.

In addition to the rapid and distant spread of the Asian H5N1 HPAI virus, and the low number of human infections with a high fatality rate (approximately 50–60%), this virus's lineage has developed unusual pathogenic properties in birds in that it can cause disease and even mortality in some species of ducks. These factors have contributed to an increasing focus on the AI virus as a basic research area, and surveillance for the virus in domestic poultry and wild bird species has also increased considerably in the past few years.

This volume focuses on both the essential virological methods that are foundational for AI virus research and diagnostics as well as some of the newest molecular procedures used for basic and applied research. Since the AI virus has had such a long history as a veterinary pathogen, many of the classical virological procedures for the basic manipulation and characterization of the virus have been in use for many years. Therefore, they are well established and well optimized. These tests will form the foundation for either research or diagnostics. The recently developed molecular methods focus on elucidating the virus pathogenesis (reverse genetics), and some methods focus on the avian host response to the AI virus. Although some of these methods are applicable to type A influenza in general, the focus is the AI virus and the avian host system.

The aim of this book is to create a resource that includes both basic methods that are currently used and well established as well as some of the most exciting new methods for studying the virus itself, and to include methods that focus on work with avian hosts, an area that has been greatly lacking. In that context, this volume will be of interest to both diagnosticians and researchers, but it does assume a basic knowledge of virology and molecular biology.

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Chapter 2
Avian Influenza Virus Sample Types, Collection, and Handling

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Summary Successful detection of the avian influenza (AI) virus, viral antigen, nucleic acid, or antibody is dependent upon the collection of the appropriate sample type, the quality of the sample, and the proper storage and handling of the sample. The diagnostic tests to be performed should be considered prior to sample collection. Sera are acceptable samples for ELISA or agar gel precipitin tests, but not for real-time RT-PCR. Likewise, swabs and/or tissues are acceptable for real-time RT-PCR and virus isolation. The sample type will also depend on the type of birds that are being tested; oropharyngeal swabs should be collected from poultry, and cloacal swabs should be collected from waterfowl. This chapter will outline the collection of different specimen types and procedures for proper specimen handling.

Keywords diagnostics; virus replication; virus detection; specimen collection; sample processing.

1. Introduction

Sample selection and handling are important in the detection of the avian influenza (AI) virus. The type and quality of samples collected will determine the success of diagnosis. In addition, storage conditions from the time of collection until the specimen is processed in the laboratory are very important for the diagnosis of avian influenza.

Low pathogenic avian influenza (LPAI) is primarily a respiratory disease in poultry and in general replicates mainly in the respiratory tract in gallinaceous birds as well as in the gastrointestinal (GI) tracts of waterfowl (see Chapter 1). In contrast, highly pathogenic avian influenza (HPAI) is a systemic disease in poultry and can be isolated from most organ systems [1]. Most HPAI viruses replicate poorly in waterfowl and produce few clinical signs. Specimens collected within three days of the onset of clinical symptoms in poultry will yield the highest viral load for virus isolation or nucleic acid detection [2]. The collection of specimens from dead animals should be done as soon as possible after death to preserve the virus for testing.

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The National Veterinary Services Laboratories (NVSL) uses brain-heart infusion broth without antibiotics for the collection of tracheal and cloacal swabs. Other viral transport media containing protein buffer may also be used (tryptose, nutrient, or peptone broth) [3]. The presence of protein in the viral transport media will help prevent the degradation of live virus during handling and transport to the laboratory. Antibiotics may be added to the viral transport media to prevent microbial growth during transportation provided the specimens will not be processed for bacterial identification.

AI viruses are unstable in the environment and are easily inactivated by extreme heat or drying [1]. Transporting dry swabs to the laboratory decreases the likelihood of isolating the AI virus. The swabs should be placed into one of the previously mentioned viral transport media immediately following collection. Collection of specimens in a protein-containing solution followed by refrigeration increases the stability of the virus. The specimen should be maintained at refrigeration (2–8 °C) and transported to the laboratory as soon as possible. The virus titer will decrease if the specimen is frozen and thawed multiple times. This may result in a failure to detect virus that is present in low levels [4]. In order to avoid freezing and thawing, do not store specimens at 0 °C.

Polyester swabs with plastic handles should be used for the collection of swab specimens rather than cotton swabs with wooden handles. The wooden handles are frequently treated with substances that can inactivate live virus and inhibit the isolation of viral nucleic acid. In addition, swabs treated with calcium alginate have been shown to inactivate virus and can decrease the viral titer up to 1000-fold in 48 hours [4].

2. Materials

1. Sterile glass tubes, screw cap
2. Brain-heart infusion broth or other viral transport media
3. Sterile polyester or Dacron swabs with plastic handle

3. Methods

3.1. Collection of Swab Specimens

AI viruses replicate primarily in the respiratory and gastrointestinal tracts of poultry and waterfowl, respectively. Swab specimens can be used for virus isolation, nucleic acid-based assays, and antigen immunoassays. Swabs collected for flock surveillance should be taken from clinically ill or dead animals [5]. The virus is present in the highest quantity at this time [1, 5]. Oropharyngeal swabs are the preferred specimen from poultry for rRT-PCR testing; however, waterfowl shed the AI virus primarily from the GI tract. For this reason, cloacal swabs should be used for

detection from these species (see Chapter 11 for more detail). However, there are substances in these samples that can inhibit the PCR reaction (see Chapter 4). Therefore, virus isolation (see Chapter 6) is the best test method to accurately identify the presence of the AI virus from cloacal swab samples. Collecting cloacal swabs from small living birds may be harmful to the animal; therefore, in this case fresh feces may be used for testing [6].

Swab specimens from a single flock or species may be pooled for testing. Up to five oropharyngeal swabs or five cloacal swabs from a single poultry flock may be pooled in the same viral transport media. Oropharyngeal and cloacal swabs should not be pooled in the same viral transport media if the specimens will be tested by rRT-PCR. Combining oropharyngeal and cloacal swabs is acceptable if the specimens will be tested only by virus isolation. Swab specimens from wild birds or waterfowl should not be pooled in the same viral transport media. These birds may carry multiple types of the AI virus, and pooling the swabs in a single tube may inhibit the detection of one or more virus subtypes. Therefore, cloacal swabs from wild birds should be collected in individual tubes. If the samples are to be tested by rRT-PCR, a small amount of the swab material from up to five swabs from a single flock or species may be pooled into a separate tube for RNA extraction and tested as a single sample. If the rRT-PCR results are positive, the specimens should be tested individually by virus isolation to reduce the chance of mixing virus subtypes in a single sample.

1. Insert the swab into the cloaca or oropharyngeal area, swabbing the area thoroughly. Be sure to swab the tracheal opening and draw the swab through the choanal cleft on the upper palette when collecting swabs from the respiratory tract. The trachea may also be swabbed; however, the oropharyngeal area is easier to sample and can yield a higher titer. If collecting fecal swabs, insert the swab into freshly deposited wet feces in order to saturate the swab.
2. Place the swab into 3–4 mL of viral transport media and swirl vigorously to dispel the contents of the swab into the media.
3. Lift the swab out of the media; press the swab firmly against the side of the tube to remove any remaining liquid from the swab.
4. Discard the swab into a disinfectant solution.
5. Label each tube with a unique sample identifier.

3.2. Collection of Tissue Specimens

Tissues are appropriate specimens for virus isolation, nucleic acid-based assays, and immunohistochemistry. Isolation of the AI virus from internal organs (other than respiratory or gastrointestinal tissues) may indicate systemic disease, which is often associated with highly pathogenic AI virus [1]. Detection of virus in tissues other than lung and trachea from animals infected with low pathogenic AI virus is very rare and may occur from contamination with secretions from the respiratory or digestive tract.

Tissues should be collected and placed into sterile plastic bags or tubes labeled with a unique identifier. Tissues to collect may include trachea, lung, air sac, intestine, spleen, kidney, brain, liver, and heart. Tissues may be processed individually or pooled; however, tissues from one system (respiratory, digestive, cardiovascular) should not be mixed with tissues or organs from other systems. Recommended tissue pools include respiratory tissues (lung, air sac, trachea), digestive tissues (liver, pancreas, proventriculus), intestinal tissues (large and small intestine, cecum), lymphoreticular tissues (spleen and Bursa of Fabricius), lung and spleen, liver and kidney, heart, and spleen/bursa. Brain and other nervous system tissues should always be processed individually. Tissues from more than one bird should never be pooled together, as individual birds within a flock may be at various stages of infection and if antibody is present in tissues from one bird, it may neutralize the virus in tissues from a second bird.

3.3. Specimen Handling and Storage

Swab specimens should be chilled at 4 °C immediately following collection for up to 48 hours [1–3]. If testing cannot be initiated within 48 hours, samples should be frozen (−70 °C or lower). Fresh tissue specimens may be frozen immediately without viral transport media and homogenized at the time of processing. Alternatively, fresh tissues may be placed in a viral transport media, frozen and thawed up to three times to disrupt the cells, and processed without homogenization.

In order to avoid freezing and thawing multiple times, specimens should never be maintained at 0 °C. Swab specimens can be maintained for weeks to months at −20 °C or below, and for up to a week at 4 °C. Tissue specimens should be stored at either 4 °C for up to 48 hours or at −70 °C or below for long-term.

3.4. Collection of Serum Samples

A presumptive diagnosis of AI virus infection may be made by detecting antibodies in sera. Without the isolation of a virus, the detection of antibody assumes a recent infection with the AI virus. Antibodies are primarily an indication of prior exposure and may be detected long after an active infection is over. Numerous tests are available for AI virus antibody detection. Sera can be tested by enzyme-linked immunosorbant assay (ELISA), agar gel immunodiffusion (AGID) assay (Chapter 6), and hemagglutination inhibition (HI) assay (Chapter 8). ELISA and AGID assays are not type-specific because they detect antibody to either the matrix or nucleocapsid proteins; the HI assay detects antibody to specific hemagglutinin (HA) subtypes.

If possible, serum should be collected no later than 7 days after the onset of clinical signs, and convalescent phase serum should be collected 2–4 weeks later

[2]. Three to four mL of blood should be collected from a wing vein into a 5-mL syringe. Following collection, pull the plunger all the way out on the syringe to allow air into the barrel and to maximize the surface area, as this will allow for the most serum to be collected. Lay the syringe on its side so the blood pools to one side of the syringe (the surface area is maximized). Allow the syringe to sit at room temperature for 24 hours to allow the serum to separate from the clot. Alternatively, the syringe can be placed at 37 °C for 4 hours and stored at 4 °C overnight. After separation, the serum can be poured off of the clot into a clean tube. Serum should always be collected within 24 hours of blood collection. The serum should be centrifuged at a low speed to remove excess erythrocytes before testing.

3.5. Transportation of Specimens to the Laboratory

Samples should be transported to the laboratory as soon as possible following collection. The specimens should be shipped on ice pack or wet ice and delivered for testing. To prevent degradation of the virus, the specimens should not be held at refrigeration temperatures for longer than 48 hours. If the specimens must be stored before transportation, the samples should be frozen (preferably at −70 °C). The specimens should be packed for shipping with sufficient ice packs to ensure the specimens stay frozen until they are received in the laboratory for testing. Samples should not be shipped on dry ice unless they are in sealed containers or are sealed, taped, and double plastic-bagged [2], because the CO_2 gas released during dry-ice sublimation will inactivate the AI virus.

In the United States, the transportation of diagnostic specimens via public roadways or airspace must follow the regulations outlined by the International Air Transport Association (IATA) and the Department of Transportation (DOT). Complete regulations can be found at www.iata.org or in the Code of Federal Regulations (49 CFR Parts 171, 172, 173, and 178).

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