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Summary: Surveillance of wild bird populations for avian influenza viruses (AIV) contributes to our understanding of AIV evolution and ecology. Both real-time reverse transcriptase polymerase chain reaction (RRT-PCR) and virus isolation in embryonating chicken eggs (ECE) are standard methods for detecting AIV in swab samples from wild birds, but AIV detection rates are higher with RRT-PCR than isolation in ECE. In this study we tested duck embryos, turkey embryos, and multiple cell lines for AIV growth as compared to ECE for improved isolation and propagation of AIV for isolation representing all 16 hemagglutinin subtypes. There were no differences in LPAIV propagation titers in duck or turkey embryos compared to ECE. The replication efficiency of LPAIV was lower in each of the cell lines tested compared to ECE. LPAIV titers were 1-3 log mean tissue culture infective doses (TCID₅₀) lower in Madin-Darby chicken kidney (MDCK), primary chicken embryo kidney (CEK), and primary chicken embryo fibroblast (CEF) cell cultures and 3-5 log TCID₅₀ lower in chicken bone marrow macrophage (HD11), chicken fibroblast (DF-1), and mink lung epithelial (Mv1Lu) cells than the corresponding mean embryo infective doses (EID₅₀) in ECE. The quail fibroblast (QT-35) and baby hamster kidney (BHK-21) cell lines produced titers 5-7 log TCID₅₀ less than EID₅₀ in ECE. Overall, ECEs were the most efficient system for growth of LPAIVs. However, the savings in time and resources incurred, with the use of the MDCK, CEK, and CEF cultures, would allow a higher volume of samples to be processed with the same fiscal and financial resources, thus being potentially advantageous despite the lower replication efficiency and lower isolation rates.

Introduction

Surveillance of wild bird populations for avian influenza viruses (AIV) is essential to our understanding of AIV evolution and ecology. Subsequently, efficient detection and isolation of AIV from surveillance samples continues to be a high priority. Currently, the use of molecular techniques such as real-time reverse transcriptase polymerase chain reaction (RRT-PCR) for the detection of viral nucleic acid has emerged as the preferred test for identification of AIV in wild bird samples [7]. However the recovery and propagation of viable AIV still depends on the use of embryonating chicken eggs (ECE). This the "gold" standard method of isolation of AIV, ECEs are able to support growth of a broad spectrum of AIV. However, some RRT-PCR positive surveillance samples have failed to yield AIV on isolation attempts in ECEs [3]. This fact has prompted investigation of other species of avian embryos and potential cell culture systems for higher isolation rates for AIV from surveillance samples. Virus isolation using ECE tends to be costly and requires much forethought concerning scheduling due to the fact that embryos must be incubated 9-11 days prior use [7]. In addition, the persistent propagation of AIV in ECEs has been shown to lead to the emergence of mutations in the hemagglutinin glycoprotein [5]. Finally, the ability of diagnostic laboratories to maintain a large volume of high quality avian embryos can be a limiting factor in virus isolation and propagation [3]. Other methods of AIV isolation and propagation have been explored, such as the use of cell culture. Overall the MDCK cell line has been the most widely used cell culture system since 1975 when Tobita et al. reported that MDCK cells were useful for the propagation of influenza viruses [1]. Other cell lines have been tested for permissiveness to AIV such as embryonic swine kidney (ESK), African green monkey kidney (Vero), swine kidney (SK), hamster lung (HmLu-1), and monkey kidney (JTC-12) [6, 8]. While these cell lines are permissive to AIV, the sensitivity is lower than ECE. Of all the cell lines tested, the MDCK was identified as the best for propagation of influenza viruses, but the number of hemagglutinins subtypes of influenza A tested has been limited [6]. In this study, we evaluated different species of avian embryos for the highest yield when inoculated with a diverse selection of LPAIVs and determined which avian embryos would produce the highest isolation rate when inoculated with wild bird surveillance samples. In addition, we evaluated which cell lines or cell cultures with and without supplemental trypsin would produce the highest yield when inoculated with a diverse selection of LPAIVs.

Methods and Materials

Viruses. The viruses isolated used in this study are listed in Table 1. The viruses were obtained from the repository of the Southeast Poultry Research Laboratory (SEPR) with original viruses provided by Dennis Senne (NVSL, Ames, IA), Robert Webster (St. Jude's, Memphis TN), David Stallknecht (University of Georgia, Athens, GA), and Richard Slemmons (Ohio State University, Columbus, OH). Individual virus strains were selected to cover all 16 hemagglutinin subtypes and eight of nine neuraminidase subtypes (N1-6, N8 and N9). The viruses were originally isolated from a variety of wild bird species or domestic waterfowl.

Cell Cultures/Cell Lines. The cell cultures used in this study are listed in Table 2. The impact of passage on replication titer was determined for one virus, A/CanadaGoose/WI/902/75 (H5N2), through passages 1-10 with and without supplemental L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-trypsin in the MDCK cell line. For determination of HI through H16 virus growth in different cell lines, cells were seeded in 48-well cell-culture plates and allowed to monolayer for 24 hours. When the monolayers were between 90 to 95% confluent, five replicates were inoculated with a ten fold serial dilution of a specific virus strain. Each virus was inoculated onto two plates per cell culture: one plate received TPCK-trypsin supplemented media, and the other received non supplemented media. After four days of incubation, the cells were examined microscopically for presence of cytopathic effect (CPE). Plates were frozen at -70°C, allowed to thaw and the supernatant was harvested for hemagglutination (HA) testing. The median tissue culture infectious dose (TCID₅₀/ml was calculated by the Reed and Muench method using the HA pattern [4]. Ten day of age specific pathogen free (SPF) embryonating chicken eggs (ECE) were inoculated via the chorioallantoic sac route (CAS) according to standard methods with the same ten fold serial dilution [9]. Chorioallantoic fluid was harvested on a 4 days post inoculation (DPI) to test for HA activity. Using the HA pattern the embryo infectious dose (EID₅₀/ml was calculated by the Reed and Muench method [4].

Avian Embryos. The embryos selected for this study were specific pathogen free embryonating chicken (SEPR), Athens, GA), and turkey (SEPR, Athens, GA), and conventional embryonating duck eggs (Maple Leaf Farms, Milford, IN) eggs. All of the embryos were tested with the same HI through H16 LPAIVs listed in Table 1. The duck and turkey embryos were used on thirteen days of incubation, while the chicken embryos were used on ten days of incubation. Embryos were inoculated via the CAS route according to standard methods [9]. On 4DPI, chorioallantoic fluid was harvested for HA testing. The EID₅₀ was calculated by the Reed and Muench method using the HA pattern [4]. In addition, the efficiency of the embryos for primary isolation of AIV from wild bird surveillance samples was compared. Initially, the surveillance samples were determined to be positive for AIV matrix protein by RRT-PCR and had cycle threshold (CT) values of greater than or equal to 32. The samples were inoculated into the CAS according to the standard methods. On 4DPI, embryos were candled for mortality and chorioallantoic fluid was harvested for HA testing. Samples with a positive HA were tested for AIV by antigen capture test (BinaxNow® test, Inverness Medical, Scarborough, ME). If samples were determined to be negative for a hemagglutinating agent, they were re-inoculated into embryos for a second and third isolation attempt.

Table 1. Virus strains and subtypes

Virus Strain	Subtype	Figure 2 Graph
A/Duck/NewYork/15024-21/96	H1N1	A
A/HerringGull/Delaware/677/88	H2N8	B
A/Duck/NewYork/6874/78	H3N2	C
A/Mallard/Ohio/338/86	H4N8	D
A/CanadaGoose/Wisconsin/802/75	H5N2	E
A/Duck/Pennsylvania/69	H6N1	F
A/Goose/NewYork/8600-3/98	H7N2	G
A/Duck/Louisiana/B174/86	H8N4	H
A/RuddyTurnstone/NewJersey/650658/02	H9N9	I
A/GreenWingTeal/Louisiana/272W/87	H10N2	J
A/RuddyTurnstone/NewJersey/650678/02	H11N4	K
A/Duck/Louisiana/188D/87	H12N5	L
A/Gull/Maryland/1824/78	H13N9	M
A/Mallard/Gurjev(Russia)/263/82	H14N5	N
A/Shearwater/WesternAustralia/2576/79	H15N6	O
A/Black-HeadedGull/Mongolia/1756/06	H16N3	P

Table 2. Selected cell cultures and cell lines

Name	Origin	Tissue	Morphology
CEK	Chicken	Kidney	Epithelial
CEF	Chicken	Embryo	Fibroblast
BHK-21	Hamster	Kidney	Fibroblast
DF-1	Chicken	Embryo	Fibroblast
HD11	Chicken	Bone Marrow	Macrophage
Mv1Lu	Mink	Lung	Epithelial
QT-35	Quail	Fibrosarcoma	Fibroblast
MDCK	Canine	Kidney	Epithelial

Tables and Figures

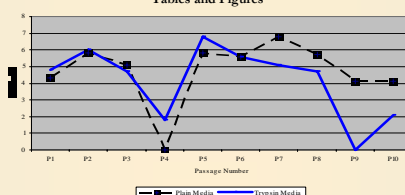


Figure 1. TCID₅₀/ml produced by the MDCK cell line inoculated with A/CanadaGoose/Wisconsin/902/75 (H5N2). The vertical axis is the TCID₅₀/ml. The horizontal axis is the passage number. The virus was passed in the MDCK cells ten times. The solid line represents trypsin supplemented media (Trypsin Media) and the dashed line with squares represents non supplemented media (Plain Media).

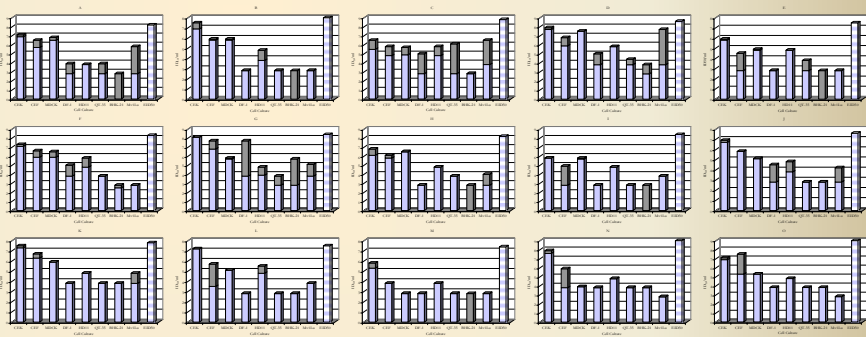


Figure 2. Graphs A through P represent the selected cell lines and cell cultures inoculated with the H1 through H16 virus subtypes listed in Table 1. The vertical axis represents TCID₅₀/ml. The horizontal axis represents all cell lines and cell cultures. The far right bar in each graph represents the EID₅₀/ml produced by ECEs, placed for comparison to the TCID₅₀/ml. As for the cell lines and cell culture TCID₅₀/ml, the light colored bars represent the TCID₅₀/ml achieved without the use of supplemental trypsin (Plain). The dark bar stacked on top represents the increase in the TCID₅₀/ml with the addition of supplemental trypsin (Trypsin).

Results and Discussion

The BHK-21, QT-35, and DF-1 cell lines had greater susceptibility to trypsin toxicity; these lines received 0.14µg/ml or less to the Mv1Lu, CEF, CEK, and HD11 cultures showed moderate resistance to trypsin; these lines received 0.15µg/ml, 0.2µg/ml, 0.2µg/ml, and 0.25 µg/ml, respectively. The MDCK cell line received 0.45µg/ml and was the most resistance to trypsin by trypsin. To determine impact of cell passage on virus replication titers, ten serial passages of the H5N2 virus were conducted in the MDCK cell line with and without supplemental trypsin (Figure 1). The titers ranged from undetectable by HA testing on passages four and nine, to as high as 10^{6.8} TCID₅₀/ml on passages five and seven. Also noted was lack of differences in titer between passage one and passage ten. Based on this information, one passage of each virus in cell culture and calculation of the TCID₅₀ would be a sufficient indicator of the cell culture's ability to support LPAIV replication. The TCID₅₀ results for all cell cultures and lines tested with the H1 through H16 LPAIVs allowed categorization of the cell cultures into three groups. First, the MDCK, CEK, and CEF with supplemental trypsin were placed into the group that produced TCID₅₀ s on average between 10^{3.5} less than the corresponding EID₅₀ in ECE. The second group consisted of the DF-1, HD11, Mv1Lu, QT-35 with supplemental trypsin, and the CEF without supplemental trypsin. These cultures replicated LPAIV on average between 10^{3.5} TCID₅₀ less than the EID₅₀ in ECE. The third group represents the cultures that replicated LPAIV on average between 10^{7.7} TCID₅₀ less than the EID₅₀ in ECE; this group consisted of the BHK-21 and the QT-35 without supplemental trypsin. The addition of supplemental trypsin did allow for an increase in titer for certain cell lines. The CEF, DF-1, QT-35 and the Mv1Lu cultures experienced an increase ranging from 10^{3.5} TCID₅₀ /ml to as much as 10³ TCID₅₀ /ml, whereas the kidney epithelial cultures, e.g. MDCK and CEK, experienced on average less than 10³ TCID₅₀/ml increase in titer with the addition of supplemental trypsin. The passage of the H5N2 virus ten times in the MDCK cell lines produced fluctuations in titer that were interpreted as adaptation cycles to growth in the cell culture system. Most influenza A and B isolates undergo periods of adjustment or adaptation to host cells but can be converted to high growth viruses after several passages through MDCK cells [1]. Two factors seem to play a role in the efficient propagation of LPAIV in cell culture: (1) the cell culture selected for use and (2) the particular virus strain used. The MDCK cell line has been the most consistently used for culturing and propagating AIV. The data here indicates that the primary cultures, CEK and CEF, may also be added as efficient cultures for AIV propagation, although the production of these primary cultures is moderately labor intensive compared to the perpetual MDCK line. The majority of the fibroblast cell lines required the addition of supplemental trypsin to facilitate LPAIV growth. LPAIV require trypsin-like enzyme cleavage of the hemagglutinin precursor protein to enable multiple replication cycles. Epithelial cell cultures contain endogenous trypsin-like enzymes and are permissive to LPAIV growth, but fibroblastic and other mesenchymal cells lack trypsin-like enzymes and require addition of trypsin to cultures for LPAIV replication [2, 7]. In our study, MDCK and CEK cell cultures exhibited small increases in LPAIV growth titers with the addition of supplemental trypsin. The addition of supplemental trypsin may not be necessary with the kidney epithelial cells, which are known to contain endogenous trypsin-like proteases. The most inefficient cultures for LPAIV growth were the BHK-21 and QT-35, whereas the remaining cell lines supported moderate levels of LPAIV replication. Some of the cell lines produced titers in a more consistent fashion than others; the HD11 cell line consistently produced TCID₅₀ s between 10^{4.8} TCID₅₀/ml and 10^{5.8} TCID₅₀/ml, whereas the Mv1Lu cell line showed varying results from a low of 10^{2.8} TCID₅₀/ml to the high of 10^{7.7} TCID₅₀/ml.

Avian Embryos. As a whole, the propagation titers on first passage for embryonating chicken, turkey, and duck eggs inoculated with H1 through H16 LPAIVs, were similar. Minor differences in titer were noted between different embryo types for individual virus strains; however the range of these differences was as low as 10^{1.1} EID₅₀/ml to the high of 10^{1.2} EID₅₀/ml. To determine if different embryos could affect AIV isolation from surveillance swabs, two sample sets totaling fifty six wild bird surveillance samples with RRT-PCR Ct values of 32 or greater for the matrix protein were inoculated into all three types of embryos. The first group, consisted of twelve samples taken from gulls within the United States, was processed using embryonating chicken and turkey embryos. No viable isolates were recovered from this sample set. The second group, consisted of forty four samples collected from various avian species in Mongolia, was processed with embryonating duck and chicken embryos. One AIV isolate was recovered in both the duck and chicken embryos from this sample set. While the data did not identify a species of embryos which was better for isolation of AIV from wild bird surveillance samples, the results suggest the three types of embryonating avian eggs may be used interchangeably. However, the use of a larger sample set for isolation attempts may result in a higher number of positive isolations for one or more of the three types of avian embryos.

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