ABSTRACT

Progeny of broiler breeder parent flocks routinely vaccinated with a bivalent inactivated vaccine containing fowl adenovirus serotypes 8 and 11 were challenged with a new strain of fowl adenovirus serotype 9. The new strain is known to cause inclusion body hepatitis in chickens. Results indicated that the current vaccine provided protection from infection to the progeny of the vaccinated parent flocks when challenged with the non-vaccine serotype 9 fowl adenovirus.

Twelve fowl adenoviruses were characterized by molecular techniques and virus neutralization assays. The viruses were found to belong to four different fowl adenovirus serotypes: European serotypes 6, 7, 8, and 10.

INDEX WORDS: Fowl adenovirus, Virus neutralization, Vaccine, Aviadenovirus, Virus isolation, Poultry, Inclusion Body Hepatitis, Maternal antibodies, Molecular characterization
PROTECTION OF BROILER BREEDERS AGAINST A STRAIN OF FOWL ADENO VIRUS AND CHARACTERIZATION OF SEVERAL FOWL ADENO VIRUS SEROTYPES

by

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DEDICATION

To my fiancé, James, who has given me the courage to accomplish things I never thought I could and to my mother, Denise, whose unconditional love and support has always lifted me up.
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INTRODUCTION

The classification of family *Adenoviridae* has been quite dynamic over the past several decades. Most recently, the International Committee on the Taxonomy of Viruses (ICTV) has restructured its classification dividing the family into four genera: *Mastadenovirus, Atadenovirus, Siadenovirus, and Aviadenovirus* [12].

*Aviadenovirus* encompasses the twelve established fowl adenovirus serotypes (FAdv) as well as some members of the goose (GAdv), duck (DAdv), pigeon (PAdv), and turkey (TAdv) adenoviruses [12]. This study focuses upon the twelve fowl adenovirus serotypes, which are known to infect poultry.

A common challenge for researchers working with the fowl adenoviruses is the inconsistency in nomenclature among the serotypes. The twelve serotypes have been numbered differently by many countries presenting a lack of consensus due to regional differences. The United States, Europe, Northern Ireland, and Japan have all instituted their own numbering systems [59]. Many records maintained regarding the fowl adenoviruses list simply a number without regard to which system of classification the number refers. Scientists have been presented with difficulties in establishing a uniform classification system and a reliable method of differentiating one serotype from another. Classically, the virus neutralization assay has been employed in determining the serotype of an adenovirus. However, some strains exhibit broad antigenicity making cross-neutralization common in serological assays [21, 64].

Advancements in genomic analysis have allowed for improved differentiation between the serotypes by separating them into five distinct species (FAdv-A through E)
based upon their restriction fragment length polymorphism (RFLP) patterns combined with results of cross-neutralization studies [8]. While this has allowed for a more reliable method of determining the molecular identity of the fowl adenoviruses, there still exists no unifying system to classify the serotypes with an individual name to be used throughout the world in a standard fashion. One objective of this study was to characterize several fowl adenoviruses and antisera using the virus neutralization assay and confirm these results with molecular analysis.

Fowl adenoviruses are characteristically ubiquitous in nature, often proving to be non-pathogenic or causing secondary disease conditions. However, particular strains have been recognized as the causative agents in diseases such as hydropericardium syndrome (HPS) and inclusion body hepatitis (IBH). IBH has been seen sporadically in the United States in the past decade. Researchers at the Poultry Diagnostic and Research Center (PDRC) at the University of Georgia have isolated and molecularly characterized some of these pathogenic fowl adenoviruses and demonstrated their ability to cause disease. Two such viruses were isolated from a parent flock at a primary breeding company and identified as strains 1047 and 8565. They belong to the European serotypes 11 and 8, respectively. FAV 11 (strain 1047) belongs to the molecular group D and FAV 8 (strain 8565) belongs to the molecular group E [103]. The grandparent flocks at the company are routinely vaccinated with an oil emulsion vaccine containing these two strains of fowl adenoviruses. Another fowl adenovirus isolate was recently implicated in causing IBH in parent flocks at the same primary breeding company. It, too, was isolated and characterized at the PDRC and was named Stanford strain. This strain was molecularly identified as belonging to European
serotype 9 and the molecular group E [103]. Challenge studies have shown that this strain is, in fact, a pathogenic fowl adenovirus, raising the question of whether there is a need to include this strain in the company’s vaccination program. The second objective of this study explores that necessity by determining if vaccination with two fowl adenoviruses, serotype 8 (8565 strain) and serotype 11 (1047 strain), of the grandparent flocks would convey protection from disease to their progeny when challenged with the recently isolated serotype 9 (Stanford strain) fowl adenovirus.
LITERATURE REVIEW

History

The first adenovirus was identified by Cowdry and Scott, who predicted that the causative agent of infectious hepatitis in canines was a filterable agent [20]. In 1947 it was determined that this filterable agent was, in fact, an adenovirus [81]. Ironically, the first isolation of an avian adenovirus reportedly occurred when isolates of lumpy skin disease of cattle were inoculated into embryonated chicken eggs [33]. The first avian adenovirus identification in diseased birds involved isolates from quail suffering from respiratory disease. It was named quail bronchitis virus [70]. The virus that became known as chicken embryo lethal orphan (CELO) was unintentionally isolated from fowl embryonated eggs [102]. A relationship between CELO and the quail bronchitis virus was soon established [31].

While investigating an acute respiratory infection in humans, researchers observed spontaneous deterioration of primary cell cultures from human adenoids [80]. Agents isolated from human respiratory secretions were shown to induce cytopathic changes in human cell culture [46]. The agents recovered from these diseases were found to be related and were called acute respiratory disease (ARD) agents. It was not until 1956 that these agents were given the name “adenovirus” reflecting the fact that the first human adenovirus strain was isolated from human adenoid tissue [34]. “Adeno-“ is from the Greek “aden” or “adenos” meaning gland. Adenoviridae was
granted family status by the International Committee on Taxonomy of Viruses (ICTV) in 1975 [69]. Since their discovery the adenoviruses have been shown to infect a wide range of vertebrate host species from snakes and fish to birds, deer, and humans [27].

**Classification**

Members of the family are classified as such primarily on the basis of their structural morphology [99]. The family *Adenoviridae* was originally divided into two genera, *Mastadenovirus*, which included the viruses that infected mammalian hosts, and *Aviadenovirus*, which included the viruses that infected avian hosts. This separation was due to a lack of common structural proteins by cross-reaction analysis of viruses from the two genera [50]. Recently, ICTV added to the family *Adenoviridae* two proposed genera: genus *Atadenovirus* and genus *Siadenovirus*. The two proposed genera infect a wider range of vertebrate species than the two existing genera. Within a genus, the viruses have been grouped into species named for the host and assigned a letter of the alphabet [9].

According to the most recent classifications, the genus *Mastadenovirus* includes all species of the human adenoviruses (HAdv), some species of ovine adenovirus (OAdv), porcine adenoviruses (PAdv), some species of bovine adenovirus (BAdv), equine adenovirus (EAdv), canine adenovirus (CAdv), murine adenovirus (MAdv), and tree shrew adenovirus (TSAdv). Known chimpanzee adenoviruses are grouped within the human adenovirus species.

Proposed genus *Atadenovirus* was named such based on the fact that their genomes have a bias toward a high A + T content [8, 10]. Officially the genus contains only one species, which is ovine adenovirus D (OAdv-D), but it tentatively includes
some members of duck adenovirus (DAdv), possum adenovirus (PoAdv), and some species of bovine adenovirus (BAdv). Species that have been isolated but are not officially recognized as members of Atadenovirus include corvine adenovirus and snake adenovirus.

Proposed genus Siadenovirus includes turkey adenovirus A (TAdv-A) and frog adenovirus (FrAdv). An adenovirus has been isolated from the sturgeon, but it is believed that this virus belongs in a genus outside of the existing and proposed genera [27].

Historically, genus Aviadenovirus included three separate groups of viruses known as groups I, II, and III. Group I contained viruses that shared a common group antigen. They also become known as the conventional adenoviruses [58]. Group II was composed of the adenoviruses causing hemorrhagic enteritis of turkeys, marble spleen disease of pheasants, and avian splenomegaly. Group III included the egg drop syndrome virus. Members of group II and the egg drop syndrome virus of group III have been removed from genus Aviadenovirus, leaving only the viruses formerly known as group I avian adenoviruses as officially recognized species in the genus [12]. Most of the early classifications of these adenoviruses were based upon serum neutralization assays, grouping them into ten or eleven serotypes [13, 62]. Further classification divided the group I adenoviruses into at least twelve different serotypes [64]. These twelve serotypes make up the current genus Aviadenovirus.

Many problems have arisen over the classification of the twelve serotypes of these adenoviruses. First, there exist prime strains and those of broad antigenicity, which show partial cross-neutralization in serological assays [21, 64]. Additionally,
there are regional differences in the numbering of the serotypes. The numbering among the serotypes in the European classification overlaps only partially with that of the American system, and there are, still, other classification systems such as those in Northern Ireland and Japan [59]. A contributor to this disarray is the fact that there has been no thorough cross-neutralization study performed [66]. Genomic analysis has generally proven faster and more efficacious in differentiating fowl adenovirus strains [43]. The combination of using the polymerase chain reaction to detect adenovirus DNA [45, 65, 74] combined with restriction enzyme analysis is useful for the typing of fowl adenoviruses [65, 74]. This molecular classification has, until recently, been based solely upon restriction enzyme analysis of total viral DNA using *Bam*HI and *Hind*III restriction endonucleases [103]. The disadvantages of classifying the serotypes based on whole genome restriction fragment length polymorphism (RFLP) analysis are that this often conveys little about biological properties of the virus [66] and there is evidence of variation between field isolates and reference strains [45]. According to RFLP of the full genome and results of cross-neutralization assays, the ICTV has placed the twelve European serotypes in five different species: fowl adenovirus A to fowl adenovirus E [9]. FAdv-A, B, C, D, and E are currently the only recognized species that belong to the genus *Aviadenovirus*. Unassigned viruses include some of the duck adenoviruses, pigeon adenovirus, and turkey adenovirus [12].

**Virion Structure** Members of the family *Adenoviridae* are non-enveloped viruses with a diameter of 70 to 90 nm. Among viruses, they are considered medium in size. Genomic DNA comprises 11 to 13% of the virion. Accessory proteins serve to stabilize the viral capsid and connect it to the core.
The virion possesses a characteristic icosahedral symmetry [57]. The capsid is composed of a single layer containing 252 capsomers, or subunits. It has 240 nonvertex capsomers or hexons which are each 8 to 10 nm in diameter. These make up the major soluble component of viruses harvested from cell culture and contain at least two antigens. One of these is common to mammalian adenoviruses and the other gives type specific neutralizing activity [61]. The hexons are formed by three identical polypeptides [9]. They have a hexagonal base and a central cavity [12]. The virion also has twelve vertex capsomers known as pentons, each composed of a penton base tightly associated with one or two glycosylated fibers protruding from the virion surface and ranging in length from 9 to 77.5 nm [12]. The fiber proteins interact with one another forming the shaft of the fiber and a distal knob [12]. The penton contains an antigen related to group specificity [61]. The positions of the hexons, penton bases, and fibers are well established [9].

**Genome Organization**  
The genome is composed of a non-segmented, single linear double-stranded DNA molecule 43 kbp in length. It encodes for 40 different polypeptides. One third of these are for structural proteins [9]. The guanine + cytosine content is 54 to 55% in *Aviadenoviruses*. When compared to *Mastadenoviruses*, the *Aviadenovirus* genes for major structural proteins and integral functional proteins are well conserved. These include IIIa, hexon, penton base, DNA polymerase, and protease proteins. The *Mastadenovirus* early protein homologues responsible for host cell interaction and immune modulation, E1A, E1B, E3, and E4, are not present, however. The genome has terminally redundant sequences with inverted terminal repetitions (ITR). The ITRs are between 50 and 200 bp in all viruses analyzed thus far.
The genome has a virus coded terminal protein which is covalently linked to the 5'-end of each DNA strand. Sheppard and Trist identified two open reading frames (ORFs) 3' to the penton base gene. The two ORFs encode highly basic polypeptides which are especially arginine rich. They also identified the supposed poly A recognition sequence signaling the end of the L2 transcription unit [92]. The chicken embryo lethal orphan (CELO) virus, commonly referred to as fowl adenovirus 1, contains three core polypeptides. Its core is similar to that of human adenoviruses. The CELO virus core was not found to contain a nucleosome subunit or repeat pattern, suggesting that it is unlike chromatin [54]. The hexon gene is 1219 bp in length. It is the major surface protein of the virus. The hexon gene contains one conserved P region and four variable regions. L1-L2 exhibit seven hypervariable regions (HVRs) and neutralizing epitopes.

**Replication of the Virus**

There is substantial evidence for homology in the transcriptional events between *Aviadenoviruses* and *Mastadenoviruses* [72], therefore, much knowledge of *Aviadenovirus* replication has been obtained from that of *Mastadenovirus* replication.

The synthesis of viral DNA begins about ten hours post-infection after the virus attaches to host cellular receptors via at least one of the fiber proteins [32, 36, 44]. While it has been reported that internalization of some human adenoviruses involves the RGD motif of the penton base with the integrin complex on the host cell surface, this is not so for other human adenoviruses and the CELO virus [28, 55]. In these, no RGD motif exists, but rather it is speculated that the two fibers of the CELO virus are necessary for viral attachment and internalization [44, 91]. The viral particle is carried into the nucleus in an endosome.
Replication occurs in the nucleus of the host cell, producing eosinophilic and basophilic intranuclear inclusion bodies [82, 94]. Eosinophilic inclusion bodies appear medium in size, surrounded by a clear halo, while basophilic inclusions are large, filling the entire nucleus.

DNA synthesis begins at either of the 5’ ends where there is a terminal protein attached. This portion of the cycle is termed Type I replication. Here one of the two strands of DNA serve as the replication template, thus the replication products are a duplex of a daughter strand and a parental strand plus a single displaced strand of DNA [90]. Type II replication occurs when this strand forms a circular shape called a panhandle structure enabling replication machinery to recognize the terminal protein. A new viral genome is made again from one parental strand and one daughter strand [53].

The complete CELO virus genome sequence was published in 1996 by Chiocca and colleagues [16], providing transcriptional and replication data. Both early and late expression of proteins has been detected. Early proteins block the cytotoxic T lymphocyte (CTL) response in the host, modulate the cell cycle, block apoptosis of the cell, shut down host mRNA transport, and assist in DNA replication. Late proteins are involved in virion assembly and cell lysis. Also reported is a RNA bipartite leader sequence most likely involved in transcriptional activity of many of the structural proteins [72].

**Physiochemical Properties** While resistance to heat inactivation varies by strain, avian adenoviruses can generally be inactivated by heating at 60°C for 30 minutes. They are stable when stored frozen at -20°C [9]. No change in infectivity or decrease in titer is known to occur when kept between pH 5 and pH 6 [12, 58, 59]. Due to their lack
of a lipid envelope, adenoviruses are not susceptible to treatment with lipid solvents, including 50% alcohol, chloroform, 2% phenol, and protease [59]. However, inactivation may be achieved by treating for one hour in formalin, aldehydes, or iodophors [38]. The virion has a molecular mass (Mr) of 150 to 180 x 10^6 and a buoyant density of 1.32 to 1.35 gcm^{-3} in CsCl [12].

**Pathogenicity**

**Hosts**  As previously mentioned, adenoviruses infect a wide range of vertebrate hosts. The *Aviadenoviruses*, as a genus, are known to infect chickens, geese, ducks, pigeons, and turkeys. Generally a virus is specific to a given host species, but some chicken *Aviadenoviruses* have been isolated from other avian hosts [59]. The viruses can be readily isolated from healthy birds between three and fourteen weeks of age, but it is possible to isolate the virus in birds of all ages. Most adult birds have been infected by more than one serotype [58]. Hosts concomitantly suffering from immunosuppression from pathogens such as infectious bursal disease virus (IBDV) and chicken anemia virus (CAV) are often at greater risk for showing symptoms of adenovirus infection [59].

**Transmission**  Both horizontal and vertical transmission are known to occur commonly with adenoviruses, though some are transmitted only horizontally [30, 100]. After ingestion, the virus replicates within the intestinal tract and viral particles are shed in the feces or respiratory mucosa [18, 49]. Latently infected birds can sporadically shed viral particles. Horizontal transmission occurs when the virus is excreted after the decline of maternal antibody levels. This usually takes place between two and four weeks of age in birds, but excretion may occur earlier. Given that most commercial
flocks contain birds from several different parent flocks, it is not uncommon to have
more than one adenovirus serotype circulating at a time. One or two serotypes may be
isolated from an individual bird, and a broiler flock may contain four or more serotypes
[57]. Highest titers of adenovirus are normally found in fecal material. Contact with this
material is the chief facilitator of horizontal spread within a poultry flock [19]. As with
other viruses, fomites, transmission by personnel, and that by transport must also be
considered in horizontal spread [59]. Spread by aerosol has not proven a considerable
factor in dissemination of the virus when compared to the importance of fecal shedding.
Environmental persistence of adenoviruses is significant, due to their relative durability
outside a host [78]. They can remain infectious for long periods of time in water, food,
and litter. Lacking a lipid envelope, they are resistant to organic solvents.

Vertical transmission can occur, but not nearly as frequently as horizontal
transmission. Breeders that become infected by horizontal means during egg
production may transmit the virus via the egg. In a study by Saifuddin and Wilks [85]
adenovirus was detected by antigen-capture ELISA in the yolk sac and albumen of eggs
produced by infected broiler chickens showing the possibility of vertical spread. The
infected parents showed a wide range of neutralizing antibody titers.

**Immunity** Neutralizing antibodies can be detected in chickens that have been
experimentally infected for one to two weeks post-infection [60]. Adenoviruses are
routinely recovered from young, asymptomatic poultry. Surveys for antibodies in adult
poultry indicate that most adult birds have been exposed to a number of adenovirus
strains [58]. Low levels of virus shedding are associated with high concentrations of
antibodies. Levels of shedding appear to increase with a decrease in antibody
concentrations. This represents the cyclic nature of activation of latent adenoviruses. When infected experimentally, viral particles could be isolated from the gastrointestinal tract and feces of chickens up to twenty weeks post-infection, even in the presence of neutralizing antibodies [85]. Maternal antibodies may be transmitted to young through the yolk sac during embryonic development [38]. Maternal antibodies from hens possessing antibodies increase the progeny’s protection from severe disease for up to three weeks after hatching, but provide little or no protection after four weeks [37]. Infection can still occur in chicks which have maternal antibodies, but the incidence is reduced compared to those without [41, 60].

**Epizootology**

**Role in Poultry Disease**  
An important consideration when investigating the role of adenoviruses in poultry disease is their ubiquitous nature. Isolation of an adenovirus from a flock, even a diseased flock, may not necessarily mean that the virus is the primary causative agent of the problem at hand [58]. *Aviadenoviruses* are associated with inclusion body hepatitis (IBH), hydropericardium syndrome (HPS), respiratory disease, necrotizing pancreatitis, drops in production or feed conversion, and adenoviral gizzard erosion. They have also been connected with anemia and severe lymphoid depletion of the bursa of Fabricius and spleen [82]. The strains acting as primary agents in hydropericardium and inclusion body hepatitis in chickens, within serotypes 4 and 8, have been well-characterized. It is the omnipresence of the adenoviruses that often presents obstacles in identifying them as primary agents of disease.

The association of adenoviruses with drops in production and feed conversion is questionable. Adenoviruses have been related with falls in egg production and egg
shell quality [67, 101]. However, it is highly unlikely in most management systems that birds will reach their sexual maturity without infection by several adenoviral strains. Birds experimentally infected with four adenovirus strains showed no changes in egg production and minimal changes in egg quality [22]. A study reported poor feed conversion and growth retardation in birds experimentally inoculated with a serotype 8 isolate associated with inclusion body hepatitis [86], but overall there is little evidence to support the idea that naturally occurring adenoviral infections cause decreased feed conversion or growth [59].

Adenoviruses are known to act as primary agents in some respiratory disease. They have been isolated from the airsacs, lungs, and tracheas of affected birds, and researchers have been able to reproduce mild respiratory disease from such isolates [2, 18]. Lesions similar to those experimentally reproduced have been reported in field outbreaks as well [63]. Adenoviruses were more recently isolated a number of times from the tracheas of birds from Georgia flocks suffering from infectious laryngotracheitis [97].

**Infectious Hydropericardium Syndrome**  
Hydropericardium syndrome (HPS) is a contagious disease [1]. Infectious hydropericardium syndrome was first reported in Angara Goth, Pakistan in 1987 [6]. Other names for the disease include Angara Disease, after the first reported case, and Hepatitis/Hydropericardium Syndrome [45]. In Pakistan alone the disease became quite destructive causing the death of over 100 million broilers in less than two years [26, 48]. The disease spread into India, Iraq, and Kuwait, Mexico, Peru, Ecuador and Chile [89]. Examinations of electron microscopic data concluded that an adenovirus was the causative agent of HPS [14, 15], and an
adenovirus was isolated from field cases of the disease [98]. Hydropericardium syndrome was successfully reproduced in specific pathogen free chickens of varying ages using fowl adenovirus serotype 4 isolates that originated in Pakistan and Ecuador [56]. Serotype 8 has also been implicated as a primary agent of disease in HPS. The disease is transmitted horizontally among broiler poultry, usually by mechanical means [4] or via ingestion of excreted viral particles in fecal material [87]. This was debated for some time, as researchers were unable to reproduce the disease by experimental oral inoculation or by contact transmission with infected birds [5]. However, it was shown that the oral-fecal route is a possible mechanism of disease spread under field conditions [25]. It was also revealed that, when kept on infected litter, broiler chicks had a higher mortality than layers [87]. The higher growth rate of broilers relative to layers is hypothesized to be responsible for this discrepancy. Vertical transmission of the disease has been associated with concomitant infection of fowl adenoviruses with chicken anemia virus [93]. It is characterized by sudden onset and is usually observed in birds between three and six weeks of age. Cases often report high mortality of 20 to 70%. Macroscopic lesions include hydropericardium, pulmonary edema, and enlarged, mottled and friable livers. Microscopic lesions consist of intranuclear inclusion bodies in hepatocytes and necrotic foci in the liver.

The most apparent gross lesion consistent with HPS is, as expected, hydropericardium. This is seen as the accumulation of a colorless or amber-green-colored fluid in the pericardial sac. The consistency may be watery or gelatinous and may be present in volumes up to 20 ml in an affected bird [15, 51]. The apex of the heart can be seen floating in the pericardial sac [51] and the organ appears misshapen
Additionally, petechial hemorrhages and yellowish discoloration may be present in the fat of the pericardium [7]. In addition to hydropericardium there may be other lesions present. Pale, friable, swollen livers that are discolored and mottled in appearance are often seen displaying focal necrotic patches and petechial or ecchymotic hemorrhages. Edema and congestion of the lungs and pale, friable kidneys with deposits of urates in the ureters and tubules are also possible [1, 6, 15, 68]. Discoloration of subcutaneous and abdominal fat has been reported in some experimentally infected birds, as has bursal and thymic atrophy [7].

Microscopic lesions are most commonly seen in the liver. These are apparent as multifocal areas of coagulative necrosis with mononuclear cell infiltration and basophilic intranuclear inclusion bodies in the hepatocytes [1, 15, 68]. In the bursa of Fabricius, thymus, and spleen, lymphocytolysis and cyst formation have been observed leading to the depletion of lymphocytes of the bursa [1, 51].

**Inclusion Body Hepatitis (IBH)** Inclusion body hepatitis was first reported in 1963 in the US in seven week old chicks exhibiting basophilic intranuclear inclusion bodies of the hepatocytes [42]. The condition has been shown to be caused by practically all serotypes of fowl adenoviruses [59]. IBH primarily affects broiler chickens between seven and fourteen days of age. It has been reported in chicks as young as four days old and birds as old as twenty weeks [73, 75]. Clinical signs include anorexia, depression, white pasty droppings, and prostration prior to death. Mortality among infected birds can vary from 10 to 30% in less than one week’s time. Highest mortality rates are seen in birds experiencing simultaneous infections from other agents. When infected experimentally, chick mortality peaked at day three post-infection, remaining
high through the fifth day [82]. Macroscopically, lesions appear as pale, friable, and swollen livers. Petechial and ecchymotic hemorrhages may be present on the liver and/or the skeletal muscles [59]. Microscopic histological lesions include intranuclear inclusion bodies in hepatocytes and necrotic foci in the liver [11, 17, 42].

In the 1970s it was thought that adenoviral IBH resulted only when birds were also infected with IBDV, CAV, or other immunocompromising agents [37, 79]. The report of outbreaks of IBH where no immunosuppressive pathogens were indicated have since been reported, showing evidence contradictory to the first assumptions [39, 59, 73]. At that time researchers were able to reproduce the disease using isolates from infected birds showing lesions in the lymphoid tissue [41, 47]. Australia and New Zealand have experienced epidemic outbreaks of IBH in the absence of IBDV [83]. Acute IBH is defined as IBH associated with a separate immunosuppressive infection, while peracute describes IBH infection in their absence. Both of these played a substantial role in the outbreaks in Australia and New Zealand, causing losses for the industry there.

Strains of serotype 8 dominated the isolates causing the outbreaks in these two countries. The pathogenic strains isolated were later placed in molecular group E (and later species FAdv-E) according to the Zsak and Kisary method [35, 103]. Mortality was as high as 50% in affected flocks younger than three weeks of age [84]. These epidemics were found to be caused by both horizontal and vertical transmission. Breeder flocks testing serologically positive to serotype 8 transmitted the virus to progeny, who showed signs of IBH. Horizontal spread occurred when healthy chicks came into contact with infected chicks, contracting peracute IBH [40]. As indicated by
the term “peracute”, these infections were found to be unrelated to immunosuppressive agents, including IBDV and CAV [76, 77].

Most recent North American cases of IBH have been seen sporadically. They present with variable serotypes, including serotypes other than 4 and 8, which were seen in outbreaks in other countries. Previous cases occurred in older birds and were associated with IBDV. More recent cases had mortality ranging from 2 to 40% in birds younger than three weeks old, and no immunosuppressive agents were detected. The viruses isolated from these cases were also classified by the Zsak and Kisary method as belonging to molecular group E. These outbreaks have been associated with the lack of parental exposure of the parent flock to causative adenovirus before egg production leading to vertical transmission.

**Diagnosis**

**Virus Isolation** Samples for virus isolation should be taken from the alimentary tract and upper respiratory system, as they are the main sites of viral replication in fowl adenovirus infections. Feces or large intestine samples containing feces should be included in alimentary samples, as well as portions of the affected organs. For example, liver samples should be included when IBH is suspected [58]. A 10% suspension of macerated sample tissue should be made in cell culture media or bacteriologic broth containing antibiotics and antifungal. The mixture should be frozen and thawed a minimum of two times [95] to promote disruption of the tissue [58]. After centrifugation at 3000 rpms for ten minutes, supernatant should be collected and stored at -80°C until needed for inoculation. Isolation can be achieved by inoculating cell cultures or embryos [23, 57]. However, isolation in cell culture is preferred [58].
Primary chicken embryo liver cells and chicken embryo kidney cells support the replication of avian adenoviruses [58]. These cell cultures are prepared according to standard procedure [95]. Continuous cell lines may also be used for virus propagation. These include LMH (ATCC—CRL-2117) and QT6 (ATCC—CRL-1708). All twelve fowl adenovirus serotypes can be cultivated in these primary cell cultures and cell lines.

When inoculating chicken embryos the yolk sac inoculation technique is preferable for propagation of all fowl adenovirus serotypes except serotype 1, the causative agent of CELO [23]. CELO is best propagated when inoculated via the chorioallantoic sac [23, 52]. Up to five blind passages in embryos or cell culture may be necessary to ensure a positive or negative isolation from a given sample [60].

**Identification**  Fowl adenoviruses elicit a unique cytopathogenic effect (CPE) in cell culture, which is indicative of virus replication. Cells begin to round and become refractile, causing detachment of the monolayer [58]. Confirmation of adenovirus infection in cell culture can be accomplished by staining with hematoxylin and eosin (H & E stain), indirect immunofluorescence assay, or electron microscopy. H & E stain reveals basophilic intranuclear inclusions characteristic of adenoviral infection. Electron microscopy can be performed on negatively stained cell culture material. It is a fast and simple means of identification, as the adenovirus morphology is easily recognized [58]. Lesions in embryos include stunting, hemorrhage, hepatitis, visceral congestion, and mortality [23].

**Serology**  Identification and typing of isolated adenoviruses can be accomplished through the use of serology. The double-immunodiffusion (DID), or gel precipitin, test is an inexpensive and quick method of detecting group specific antigen. A disadvantage
to DID is its lack of sensitivity, however, making it difficult to detect antigen in birds undergoing primary infection by natural routes [58]. Sensitivity may be increased by pooling three antigens together for use in the test [24]. Results can be hard to interpret due to the ubiquitous nature of fowl adenoviruses.

Virus neutralization has been a standard serological technique for adenovirus typing for many years. Isolates can be typed by testing them against “known” antisera. The test has some disadvantages, however. A number of serotypes of antisera are required to perform a thorough neutralization study against all twelve types of fowl adenovirus, which can be both time-consuming and costly. This can be averted somewhat by performing the microtiter test. Another downside to neutralization tests is the affinity of adenoviruses for cross-neutralization, which can make the results difficult to interpret [58].

The indirect fluorescence antibody (FA) test is quite sensitive in detecting group-specific antibodies to the viruses and can be performed on a number of isolate [cultures]. The enzyme-linked immunosorbent assay (ELISA) is a method of detecting both group-specific and type-specific antibody to adenoviruses, and is also very sensitive [58].

**Genomic Analysis**  In 1984 Zsak and Kisary extracted DNA from the existing twelve serotypes of fowl adenoviruses and placed them in five molecular groups according to the RFLP patterns produced using restriction enzymes *BamHI* and *HindIII* separately [103]. The molecular groups, A through E, have now become known as the five species of *Aviadenoviruses*, FAdv-A through FAdv-E [9]. Since that time Pallister, Erny, and colleagues have identified distinct RFLP patterns among the members of molecular
group E marking isolates of higher virulence whose differences could not be distinguished by serological means [35, 71].

Raue and Hess have introduced a PCR/RE assay based on the hexon portion of the genome that is able to differentiate between the serotypes [74]. Samples for this technique can include phenolized tissue or infected fluids and formalin fixed tissue. This technique uses a 1.3 kb PCR amplicon of the viral DNA and the restriction endonuclease HaeII of that amplicon, and it is able to distinguish between reference strains for all serotypes except 4 and 5.

**Prevention and Control**

**Biosecurity**  Appropriate biosecurity measures are important in the control of horizontal spread of adenoviruses within a poultry house or farm. This is especially true in the prevention of IBH and HPS [3, 88]. Vertical transmission to progeny can be best prevented by practicing effective biosecurity to control horizontal spread in commercial parent flocks and limit the introduction of additional pathogens [29].

**Vaccination**  Vaccination programs involving the fowl adenoviruses were largely initiated in response to the IBH outbreak in Australia and HPS in Pakistan and Latin American countries. The live vaccine manufactured from highly virulent strains of serotypes 8 was effective in controlling the outbreak situation in these countries. It was proven safe and effective broiler breeder progeny were vaccinated at eight to eleven weeks of age. An inactivated oil emulsion vaccine is available that protects against IBH and HPS in chickens. It is recommended for use in chicks older than eight weeks and is
injected subcutaneously. It is recommended that grandparent flocks of breeders be vaccinated during growout at eight to twelve weeks of age. Broilers should be vaccinated early in life.
MATERIALS AND METHODS

This research consisted of two experiments. The first was composed of virus neutralization assays and molecular characterization of twelve fowl adenoviruses, while the second involved a broiler breeder progeny challenge.

Experimental Design: Characterization of Several Fowl Adenovirus Serotypes

Cell Cultures  Viruses were propagated and titrated, and virus neutralization assays were conducted in chicken embryo liver cell (CELiC) cultures. Livers were collected by sterile technique from thirteen to fifteen day old specific pathogen free (SPF) chicken embryos. The livers were washed several times in phosphate buffered saline (PBS) and trypsinized at 37°C in a 10% trypsin solution [95]. The cells were then centrifuged at 1500 rpm for 10 minutes at 4°C and the pelleted liver cells were resuspended at a concentration of approximately 2.5 x 10^6 cells/ml in cell culture growth media (F10-M199 with 10% fetal bovine serum) [95] containing gentamicin at a concentration of 0.1 ml/100 ml of media and Fungizone (Invitrogen Corp., Carlsbad, CA) at a concentration of 1 ml/100 ml of media. For growth of CELiC monolayers in 35 mm cell culture plates, 2 ml of cell suspension was added to each plate. For growth in microtiter plates 100 μl of cell suspension was added to each well of the microtiter plate [95]. Cell cultures were maintained at 37°C in a 5% CO₂ incubator for 48 hours to allow for monolayer formation.

Viruses  Several viruses were obtained from a stock maintained at the Poultry Diagnostic and Research Center (PDRC). These viruses were labeled as fowl adenovirus (FAV) serotypes 1, 2, 4, 5, 6, and 9. To complete the set of twelve FAV
serotypes, the remaining viruses, serotypes 3, 7, 8, 10, 11, and 12, were purchased from Charles River, SPAFAS (Storrs, CT). Viruses that were purchased were stored at -20˚C before reconstitution per the manufacturer's recommendations. To reconstitute the viruses, 0.1 ml of virus was added to 0.4 ml of sterile double distilled water. The reconstituted virus was diluted 1:100 in cell culture media. A stock of each of the twelve viruses was propagated by inoculating prepared CELiC monolayers in 35 mm cell culture plates with 0.1 ml/plate of virus stock from the PDRC or reconstituted purchased virus. Cell cultures were incubated at 37˚C for 45 minutes, then 2 ml of maintenance medium (cell culture medium with 2% fetal bovine serum) was added to each cell culture. All inoculated cell cultures were maintained at 37˚C in a 5% CO₂ incubator. Each monolayer was microscopically observed daily for the presence of cytopathic effect (CPE) and placed at -80˚C when 65 to 75% of the monolayer exhibited CPE, such as rounding of cells and detachment of the monolayer. Cell cultures were frozen for 24 hours and then placed at room temperature in a sterile laminar flow hood to thaw completely. Cells were harvested from the cell culture plates by washing the monolayer from the plate surface using a sterile pipettor and then centrifuged at 3000 rpm for ten minutes. The supernatant was collected and stored in labeled cryovials at -80˚C. All prepared viruses were stored at -80˚C. Viruses were passaged up to three times in CELiC in order to obtain the highest possible titer.

Titration Each virus was titrated in triplicate to determine its concentration. Titrations were performed in microtiter cell cultures of CELiC in ten fold dilutions with a virus control and a cell control for comparison [95]. In a dilution plate the first eleven wells of each row were filled with 180 µl of maintenance medium. Twenty µl of the virus to be
titrated was placed in the first well of each row and in the eleventh well of each row, which served as a virus control. Using a multichannel micropipettor, the contents of the first well of each row were mixed three times and 20 µl of diluted virus were transferred to the next well in the row. This procedure resulted in a series of ten-fold dilutions across the plate ending at the tenth well. Then, 180 µl of maintenance medium was added to the twelfth well in each row to serve as a cell control. The growth medium was removed from microtiter cell cultures containing prepared CELiC and 180 µl from each well of the dilution plate was added to the corresponding wells of the cell culture. Contents were transferred with a multichannel micropipettor starting with the cell control, proceeding to the diluted virus beginning with the most diluted virus, and finishing with the virus control. Inoculated cell cultures were maintained at 37˚C in a 5% CO₂ incubator and observed daily for the presence of CPE. Cell cultures were fixed and stained when 65 to 75% of the monolayer in the virus control well exhibited CPE. This generally occurred 72 to 96 hours post-inoculation. The titer of the virus was defined as the reciprocal of the dilution of virus in the last well of each row maintaining the appearance of the virus control. After determining the titer of each virus, they were all diluted in cell culture medium to a common titer of $10^2$ TCID$_{50}$/ml for use in virus neutralization assays.

**Fixing and Staining Cells** Cells were fixed by removing media from the cell cultures and adding 50 µl of ethanol to each well. After five minutes the ethanol was removed from the cell cultures and they were allowed to dry in the sterile laminar flow hood for ten minutes. To stain the cells, 50 µl of a 2% solution of crystal violet stain was added
to each well and allowed to stain for five minutes. The cell cultures were then washed gently under running water to remove excess stain [95].

**Antisera**  
Antisera to FAV serotypes 1, 2, 4, 6, 7, 8, 9, 10, 11, and 12 were obtained from the PDRC. These antisera were prepared previously at the PDRC by inoculating specific pathogen free (SPF) chickens with viruses of the same serotype, bleeding the inoculated chickens, and collecting serum from the blood samples. These were maintained at the PDRC at -80°C. Each of these antisera was used undiluted in virus neutralization assays. Antisera to FAV serotypes 3 and 5 were purchased from SPAFAS (Storrs, CT). Antisera purchased from SPAFAS were diluted 1:5 in double distilled water for use in virus neutralization assays. All prepared antisera were stored at -80°C.

**Virus Neutralizations Assays**  
Each of the six FAV serotypes obtained from the PDRC (FAV 1, 2, 4, 5, 6, and 9) and the six FAV serotypes purchased from SPAFAS (FAV 3, 7, 8, 10, 11, and 12) were tested in virus neutralization assays against each antiserum. The beta microneutralization procedure was used [95]. Viruses were kept at a constant dilution of $10^2$ TCID$_{50}$/ml and antisera were diluted serially across the microtiter cell culture. A virus control and a cell control were included in each replicate. Each test was run in duplicate. The same procedure was used for each neutralization test.

In a dilution plate, 50 µl of virus was added to the first eleven wells of each row. Next, 50 µl of antiserum was added to the first well of each row. Using a multichannel micropipettor the contents of the first well were mixed three times and 50 µl of the mixture was transferred to the next well. This was continued across the dilution plate.
ending at the tenth well, producing two-fold serial dilutions of antiserum across the wells containing constant amounts of virus. No antiserum was added to the eleventh well of each row so that it may be used as a virus control. No virus or antiserum was added to the twelfth well in each row so that it may be used as a cell control [95].

The growth medium was removed from microtiter cell cultures containing prepared CELiC and 50 µl from each well of the dilution plate were transferred to the corresponding wells of the cell cultures. Contents were transferred with a multichannel micropipettor starting with the virus control, proceeding to the wells containing the diluted antiserum beginning with the most diluted and finishing with the least diluted antiserum. The cell cultures were incubated at 37˚C in a 5% CO₂ incubator for 45 minutes, then 200 µl of maintenance medium was added to all wells. The inoculated cell cultures were kept at 37˚C for 72 hours before being fixed and stained, as described previously. The recorded results of each neutralization test reflect the reciprocal of the highest dilution of antisera in the well that still maintained the appearance of the cell control with no signs of CPE when examined microscopically.

**Viral DNA Extraction** Attempts were made to molecularly characterize each of the twelve viruses. Viral DNA was extracted from the thawed virus stock using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to instructions provided by the manufacturer. Briefly, 200 µl of cell cultured virus, 20 µl of proteinase K, and 200 µl of Buffer AL were mixed and incubated at 56˚C for ten minutes. Then, 200 µl of ethanol was added to each sample and the mixture was applied to a QIAamp spin column. After centrifugation, the DNA attached to the membrane in the column was washed with
500 µl each of buffers AW1 and AW2, and then eluted with 60 µl of Buffer EB. The purified DNA was stored at -20˚ C.

**Polymerase Chain Reaction**  The available H1 (TGGACATGGGGGC GACCTA) and H2 (AAGGGATTGACGTTGTCCA) primers (Qiagen, Valencia, CA) were used to amplify a 1219 bp segment of the hexon gene, which contains the P1, L1, and L2 regions of the genome. This segment was later used for restriction enzyme analysis [74] and sequencing. The FAV H0 (AGGTKARGCCTCCCGT) primer was obtained from the PDµC (Athens, GA) and was used to amplify the hexon region containing the ATG start codon. It was also used in conjunction with the H2 primer for sequencing purposes. The polymerase chain reaction (PCR) was executed using the FailSafe PCR System and PCR 2X premix C (Epicentre, Madison, WI) per the manufacturer's instructions. Amplification of the DNA was conducted in a total volume of 50 µl containing 100 ng DNA and 50 mM of each primer. The reaction mixtures were thermocycled 35 times with an initial denaturation step of four minutes at 94˚C. Each cycle included a denaturation at 94˚C for one minute, annealing at 55˚C for one minute, and extension at 72˚C for one and a half minutes. Next, 10 µl of each PCR product (H0-H2 and H1-H2) was separated by electrophoresis in a 1.5% agarose gel containing ethidium bromide and visualized by ultraviolet (UV) transillumination.

**DNA Purification and Sequencing**  Amplified segments of the correct size were extracted from the gel. Each segment was purified using the QIAquick Spin Kit (Qiagen, Valencia, CA) per manufacturer's instructions and used as the template for sequencing reactions. The sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according
to the manufacturer’s instructions. In each reaction 40 ng of template was used.

Sequencing reactions were run in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The deduced amino acid sequences were analyzed using the Clustal W method of DNASTar, and sequences of the first 440 amino acids of the hexon gene were compared to several published adenovirus sequences available in the GenBank database.

**Restriction Fragment Length Polymorphism (RFLP)** A total volume of 15 µl was used for conducting RFLP analysis, including 7.5 µl of PCR product obtained as described earlier and ten units of *Hae*II enzyme. Restricted products were separated by electrophoresis in a polyacrilamide Ready Gel (Bio-Rad Laboratories, Hercules, CA). The RFLP band patterns were visualized using the PlusOne™ DNA Silver Staining Kit (Amersham Biosciences, Uppsala, Sweden). The resulting electrophoretic band patterns were compared to those published for the fowl adenovirus serotypes [74].

**Correlation of Virus Neutralization and Molecular Characterization Results** The molecular identities of the twelve fowl adenoviruses used in this experiment were elucidated by the RFLP patterns and amino acid sequences obtained during their molecular characterization. Any discrepancies from the original labeling of the virus stock had to be accounted for in evaluating results of the virus neutralization assays. This allowed for true virus neutralization results to be obtained in this study.

**Electron Microscopy** No adenoviral DNA could be amplified from the virus labeled FAV 5, making it difficult to molecularly characterize. To visualize the morphology of the virus labeled FAV 5, electron microscopy was performed. A virus concentration procedure was utilized in order to prepare the virus for electron microscopy [95]. The
virus was inoculated into CELiC monolayers in 35 mm cell culture plates as described previously. The cell cultures were microscopically observed daily, and the cell cultures were placed at -80°C when 65 to 75% of the monolayer exhibited CPE. The cell cultures were then allowed to thaw completely at room temperature in a sterile laminar flow hood. Freezing and thawing was repeated twice. The cell cultures were then harvested as previously described, and centrifuged at 1500 rpm for 30 minutes at 4°C. The supernatant was then collected into sterile centrifuge tubes and centrifuged at 7000 rpm for 45 minutes at 4°C. The supernatant was collected once more, placed in sterile centrifuge tubes, and centrifuged at 30,000 x g for 120 minutes at 4°C. The supernatant was then discarded and the virus pellet was resuspended in 0.1 mL PBS [95]. Electron microscopy was conducted by the Department of Veterinary Pathology at the University of Georgia College of Veterinary Medicine (Athens, GA). The viral morphology visualized was compared to the known adenoviral morphology [12].

**Experimental Design: Protection of Broiler Breeders against a Strain of Fowl Adenovirus**

**Viruses** Two fowl adenoviruses, serotype 8 (strain 8565) and serotype 11 (strain 1047), were initially isolated by researchers at the PDRC from chickens at a primary breeding company. The grandparent flock at this facility is routinely vaccinated with an oil emulsion vaccine containing FAV 8 (strain 8565) and FAV 11 (strain 1047) at ten weeks and seventeen weeks of age. These viruses were isolated when parent flocks began exhibiting signs of peracute inclusion body hepatitis in the presence of maternal antibodies. The affected birds were between two and four weeks of age when clinical signs became apparent.
Researchers at the PDRC were able to isolate and molecularly identify the two vaccine strain viruses from samples collected from affected chickens (Fig. 1). These two viruses have been incorporated into a bivalent inactivated vaccine that is now being used in the parent flock at the facility. More recently another fowl adenovirus has been isolated in the parent flock from the same company. The virus was isolated from affected chickens and was molecularly characterized as the Stanford strain belonging to fowl adenovirus serotype 9 (Fig. 1).

In order to produce a stock of each of these three viruses, they were propagated and titrated as described previously [95, 96] in monolayers of CELiC. Passages of the viruses were kept at a minimum in order to maintain the specificity of the viruses for their natural host, the chicken. Prepared virus stocks were stored at -80°C.

**Progeny Challenge**  It was necessary to evaluate the necessity of including the non-vaccine strain of fowl adenovirus (FAV 9 Stanford strain) in the vaccine program at the primary breeding company at which it was isolated. The Stanford strain was used to challenge progeny from the parent flock at the facility. Additionally, it was necessary to challenge these birds with the two viruses currently included in the facility’s vaccination program, FAV 11 (strain 1047) and FAV 8 (strain 8565).

Each of the treatment groups included in this progeny challenge are described in Table 1. One day old broiler breeder birds were obtained from the facility. The grandparent flocks at the facility, which are the parents of these progeny, were vaccinated with the bivalent inactivated vaccine. The parents were of two different ages, in order to evaluate the possible difference in maternal antibody levels conveyed to their progeny. Birds from 55 week old parents were referred to as progeny A, and
birds from 30 week old parents were referred to as progeny B. A third group, referred to as progeny C, was used to represent commercial broiler chicks and was obtained from a separate facility. The parents of this progeny group would have had natural exposure to fowl adenoviruses rather than exposure through a vaccine. Additionally, one day old SPF chickens were obtained (Merial Select, Gainesville, GA) in order to assure that the virus challenge dose would cause infection in chickens with no significant maternal antibodies to adenoviruses.

Birds of progenies A and B were divided into seven treatment groups of 50 birds each, and progeny C and SPF birds were divided into three treatment groups of 50 birds each. The birds were housed in a climate-controlled unit at the Veterinary Farm at the University of Georgia (Athens, GA). Each group was placed in separate experimental pens with at least one unoccupied pen between groups to keep contact between birds minimal. One group of 50 birds of each progeny remained unchallenged to serve as a control group.

As detailed in Table 1, each of the remaining progeny A and B groups was inoculated subcutaneously at either one or seven days of age with $10^{4.5} \text{TCID}_{50}$ (0.1 ml inoculum per bird) of either FAV 8 (strain 8565), FAV 9 (Stanford strain), or FAV 11 (strain 1047). The groups containing progeny C and SPF birds were challenged only with FAV 9 (Stanford strain) at either one or seven days of age. Eleven birds in each treatment group containing progenies A, B, and C were weighed at one day of age to obtain an initial weight for the treatment group. These birds were weighed again at three weeks of age to obtain a final weight for each treatment group.
The birds were kept in the experimental pens for three weeks, at which point the experiment was concluded and all birds were sacrificed. Necropsy was performed on all mortality throughout the experiment and all sacrificed birds at the conclusion of the experiment in order to collect samples and record macroscopic observations.

**Serum Antibody Level Determination**  Blood samples were individually collected from 21 birds of each of the three progeny groups (A, B, and C) at one day of age. These extra birds were supplied specifically for this purpose and were sacrificed after bleeding. Blood samples were individually collected once more at three weeks of age from ten birds in each of the treatment groups prior to sacrifice. Samples were collected in sterile tubes and placed at a 45° slant to clot. Serum was collected from the clotted blood samples and stored at 4°C. Serum samples were individually tested for adenovirus antibody levels using the enzyme-linked immunosorbent assay (ELISA) manufactured by Roveko, Ltd. (Gaithersburg, MD) and evaluated using software manufactured by IDEXX Laboratories, Inc. (Westbrook, ME).

**Tissue Sample Collection**  Two liver samples were collected from each deceased bird throughout the experiment and from select sacrificed birds at the conclusion of the experiment. One of these was placed in 10% buffered formalin for histopathologic examination and the other was frozen in a sterile Whirl-Pak bag for virus isolation.

**Virus Isolation**  It was necessary to evaluate each liver sample for the presence or absence of fowl adenoviruses by attempting to isolate the viruses in cell culture. Liver samples were frozen and thawed once. Each was then minced in 4 ml PBS containing gentamicin at a concentration of 0.1 ml/100 ml of PBS and Fungizone (Invitrogen Corp., Carlsbad, CA) at a concentration of 1 ml/100 ml of PBS and frozen overnight at -80°C.
Each sample was thawed and centrifuged at 3000 rpm for ten minutes. Supernatant was collected and filtered through a sterile 0.22 µm filter into a sterile cryovial. The growth medium was removed from prepared CELiC monolayers in 35 mm cell culture plates and 700 µl of the liver homogenate supernatant was inoculated into each cell culture. Inoculated cell cultures were incubated at 37°C for 45 minutes in a 5% CO₂ incubator, then the inoculum was poured from the cell culture and 2 ml of maintenance medium was added to each cell culture. They were held at 37°C in a 5% CO₂ incubator a maximum of five days to establish the presence or absence of CPE in cells.

Each cell culture was microscopically examined daily and placed at -80°C when 65 to 75% of the monolayer displayed CPE or at five days post-inoculation if no CPE was observed. The cell culture was placed at -80°C for 24 hours and allowed to thaw completely at room temperature. Cells were harvested from the cell culture plates by washing the monolayer from the plate surface using a sterile pipettor and centrifuged at 3000 rpm for ten minutes. The supernatant was collected and stored in labeled cryovials at -80°C. Passages were performed according to the same inoculation procedure described previously in this section using the supernatant collected from the previous passage as inoculum. Samples exhibiting CPE were not passaged any additional times. If no CPE was exhibited after three passages in CELiC cultures, the sample was considered negative for virus isolation.

**Molecular Characterization** All samples exhibiting CPE in CELiC monolayers within three passages were subjected to molecular characterization, using procedures previously described. Electrophoretic DNA patterns from the samples were compared
to known positive control patterns to determine the presence or absence of adenoviral DNA.

**Histopathology**  Histopathologic examination was performed on the liver samples previously collected and stored in formalin. A section of each sample was sent to the Veterinary Diagnostic Lab at the University of Georgia (Athens, GA). The samples were stained with hematoxylin and eosin, embedded in paraffin, and mounted on glass slides. Each slide was examined microscopically and any remarkable abnormalities were recorded.

**Statistical Analysis**  Statistical analysis was performed on the body weight data and ELISA antibody titer data collected at the beginning and conclusion of the experiment. This analysis was conducted using the Dunnett’s t test (SAS Insitute, Inc., Cary, NC).
Fig. 1. RFLP patterns exhibited by three fowl adenoviruses (FAV 9, 8, and 11) isolated from the parent flock at the broiler breeder facility
Table 1. Evaluation of the effect of challenge with FAV 8, FAV 9, or FAV 11 on progeny of different parent flocks and SPF chickens

<table>
<thead>
<tr>
<th>Challenge Virus</th>
<th>Progeny A (55 Week Old Parents)*</th>
<th>Progeny B (30 Week Old Parents)*</th>
<th>Progeny C (Commercial Broilers)**</th>
<th>Specific Pathogen Free (SPF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Challenge Age</td>
<td>Day 1</td>
<td>Day 7</td>
<td>Day 1</td>
<td>Day 7</td>
</tr>
<tr>
<td>FAV 8 (strain 8565)</td>
<td>50***</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>FAV 11 (strain 1047)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>FAV 9 (Stanford strain)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Unchallenged Control</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

*Parent flock vaccinated at 10 and 17 weeks of age with FAV 8 and FAV 11
**Parent flock not vaccinated against fowl adenoviruses
***Number of birds per treatment group
RESULTS

Characterization of Several Fowl Adenovirus Serotypes

Virus Neutralization Assays  The results of the neutralization assays conducted before each fowl adenovirus was molecularly characterized are detailed in Table 2. FAV 1, 6, 9, and 12 were neutralized by their homologous antisera at titers ranging from 8 to 32. FAV 3, 4, and 5 were neutralized by their homologous antisera at titers ranging from 64 to 256. There was no logical correlation between virus and antiserum among the remainder of the virus neutralization results.

Molecular Characterization  The results of molecular characterization of the twelve adenoviruses are shown in Table 3. Eleven of the viruses (all except the virus labeled as FAV5) were positively identified as fowl adenoviruses as seen in Fig. 2. The RFLP patterns exhibited by these viruses are shown in Fig. 3. The viruses originally labeled as FAV 7 and 10 exhibited RFLP patterns corresponding to those serotypes. Upon sequencing FAV 7 exhibited 99% homology in sequence with FAV 7 (strain B3A). FAV 10 exhibited 98% homology in sequence with FAV 10 (strain VR835). Though adenoviral DNA was isolated from the virus originally labeled as FAV 8, and it exhibited RFLP patterns corresponding to serotype 8, the virus could not be sequenced. Therefore, a specific strain was not identified for this virus.

The molecular classification of the remaining viruses did not correspond to the serotypes on their labels. The viruses originally labeled as FAV 1, 2, and 12 were identified by RFLP as serotype 10 and exhibited a 98% homology in sequence with FAV 10 (strain VR835). The viruses labeled FAV 3, 4, and 9 were identified by RFLP as
serotype 7 and exhibited a 99%, 97%, and 99% homology in sequence respectively with FAV 7 (strain VR832). The virus labeled FAV 6 was identified by RFLP as serotype 8 and exhibited 99% homology in sequence with FAV 8 (strain S8). The virus labeled FAV 11 was identified by RFLP as serotype 6 and exhibited 99% homology in sequence with FAV 6 (strain CR119). The virus labeled FAV 5 could not be molecularly characterized. No adenoviral DNA could be isolated from this virus.

**Correlation of Virus Neutralization and Molecular Characterization Results**

A correlated set of virus neutralization results was constructed using the identities of the viruses as determined by molecular characterization. These are detailed in Table 4.

Three viruses were identified as FAV 7 (strain VR832). These are referred to in Table 4 as 7\textsuperscript{a}, 7\textsuperscript{b}, and 7\textsuperscript{c}. All three of these viruses were neutralized by their homologous antisera at titers ranging from 32 to 256. FAV 7 (strain B3A) was neutralized by antiserum to FAV 7 (strain VR832) at a titer of 16. FAV 8 (strain S8) was neutralized by its homologous antiserum at a titer of 8. This virus was also neutralized by antiserum to FAV 8 (strain not determined) at a titer of 1024. Four viruses were identified as FAV 10 (strain VR835). These are referred to in Table 4 as 10\textsuperscript{a}, 10\textsuperscript{b}, 10\textsuperscript{c}, and 10\textsuperscript{d}. Of these four viruses only one of them, FAV 10\textsuperscript{a}, was neutralized by its homologous antiserum. It was neutralized at a titer of 8.

FAV 7 (strain B3A), 7\textsuperscript{a} (strain VR832), 7\textsuperscript{c} (strain VR832), 10\textsuperscript{a} (strain VR835), and 10\textsuperscript{b} (strain VR835) were neutralized by antiserum to FAV 3 at titers ranging from 16 to 256. FAV 7\textsuperscript{a} (strain VR832), 7\textsuperscript{c} (strain VR832), and 10\textsuperscript{a} (strain VR835) were neutralized by antiserum to FAV 5 at titers ranging from 8 to 64. FAV 8 (strain S8) and FAV 8 (strain not determined) were neutralized by antiserum to FAV 10 (strain VR835) at titers
of 1024 and 32, respectively. Among the remaining virus neutralization results, no logical correlations could be made between virus and antiserum.

**Electron Microscopy**  The morphology of the virus labeled FAV 5, seen in Fig. 4 as visualized by the electron microscopy technique, was compared to the known morphology of the fowl adenovirus and was determined to be positive for fowl adenovirus identification.

**Protection of Broiler Breeders against a Strain of Fowl Adenovirus**

**Serum Antibody Levels**  The level of antibodies to fowl adenoviruses, as detected by ELISA, present in serum collected at one day of age from each progeny group is shown in Fig. 5. Progeny C, representing the commercial broilers, had the highest geometric mean titers (GMT) [96] at 6356. The serum samples collected from progeny A, the broiler breeder progeny with 55 week old parents, had the next highest antibody levels with a GMT of 4452. The serum samples from the progeny B group had the lowest antibody levels with a GMT of 1484. A statistical difference (P≤0.05) was seen between these antibody titers.

The antibody levels among the progeny A birds at three weeks of age were generally low, as seen in Fig. 6. The highest antibody levels were observed in the birds inoculated at seven days of age with FAV 8 (strain 8565), which had a titer of 438. Birds inoculated at seven days of age with FAV 9 (Stanford strain) had a serum antibody titer of 165. All of the progeny A treatment groups were considered negative for the presence of adenoviral antibodies.

The antibody levels among the progeny B treatment groups at three weeks of age are shown in Fig. 7. They were also all considered negative for the presence of
adenoviral antibodies. The highest titer of 592 was observed in the birds inoculated at one day of age with FAV 8 (strain 8565) and a titer of 399 was seen in birds inoculated at seven days of age with FAV 9 (Stanford strain).

As observed in the treatment groups of progenies A and B, the antibody levels in the treatment groups of progeny C at three weeks of age were also considered negative for antibody presence as detailed in Fig. 8. The highest titer of 10 was seen in the birds inoculated at seven days of age with FAV 9 (Stanford strain), and a titer of 4 was observed in the birds inoculated at one day of age with the same virus.

There was no significant difference (P≤0.05) found between the ELISA antibody titers of the seventeen treatment groups in the experiment at three weeks of age.

**Body Weight** The body weights observed in the birds at one day of age indicated that the birds of progeny A had the highest weight (45 g), with the birds of progenies B and C weighing 38 g. This is shown in Fig. 9.

The body weights of the birds in the progeny A treatment groups at the conclusion of the experiment are shown in Fig. 10. Only the treatment group inoculated at one day of age with FAV 8 (strain 8565), with a mean final body weight of 670 g, showed a significant difference (P≤0.05) when compared to the unchallenged group in progeny A, which had a mean final body weight of 764 g.

The mean final body weights of the treatment groups containing progeny B birds can be seen in Fig. 11. Among the treatment groups, a significant difference (P≤0.05) was found between the group inoculated with FAV 8 (strain 8565) at one day of age having a mean body weight of 745 g and the group inoculated with FAV 9 (Stanford strain) at one day of age having a mean body weight of 706 g when compared with the
unchallenged group of the progeny, which had a mean body weight of 632 g. A significant difference (P≤0.05) was also seen between all of the groups inoculated at seven days of age with the three challenge viruses and the unchallenged group of progeny B.

The mean final body weights of the birds in the progeny C treatment groups are detailed in Fig. 12. The mean body weight for birds in the unchallenged group was 588 g. The mean body weights of the birds in the group inoculated with FAV 9 (Stanford strain) at one day of age and at seven days of age are 705 g and 720 g, respectively. No significant difference (P≤0.05) was seen between the body weights of any of the treatment groups of progeny C birds.

**Mortality**  The percent mortality among the treatment groups throughout the experiment is summarized in Table 5. All of the SPF birds inoculated at one day of age died within 5 days post-inoculation. The SPF birds inoculated at 7 days of age had 20% mortality. The group of progeny A birds inoculated at one day of age with FAV 11 (strain 1047) had 8% mortality, as did the group of progeny B birds inoculated at seven days of age with the same virus. The progeny B group inoculated at one day of age with this virus showed 4% mortality. The unchallenged progeny A group also showed 4% mortality. The remaining treatment groups had less than 2% mortality.

**Macroscopic Observations, Virus Isolation, Histopathology, and Molecular Identification**  All of the necropsied mortality from the SPF birds challenged at one day of age and seven days of age with FAV 9 (Stanford strain) showed friable and pale livers with yellow discoloration, as seen in Fig. 13. The birds appeared dehydrated and kidney urates were evident. When liver samples from these birds were passaged in
CELiC cultures, they all exhibited typical adenovirus CPE (Fig. 14). Viral DNA was successfully extracted and positively identified as adenoviral DNA at a molecular level from these samples. Histopathologic examination of liver samples from the challenged SPF birds revealed intranuclear inclusion bodies in hepatocytes and multifocal necrosis of the liver tissue.

All except two of the liver samples collected from the mortality in progenies A, B, and C throughout the experiment and the necropsied birds at the conclusion of the experiment did not exhibit CPE (Fig. 14) when passaged three times in CELiC cell cultures. However, CPE, as shown in Fig. 14, was observed in the second passage in CELiC cell culture of the liver sample collected from mortality in progeny B that was inoculated at one day of age with FAV 8 (strain 8565) and in the third passage in CELiC cell culture of the liver sample collected from mortality in progeny A that was inoculated at one day of age with FAV 11 (strain 1047). The birds died at eleven days and seventeen days post-inoculation, respectively. No macroscopic lesions were seen during necropsy and no histopathologic abnormalities were noted under microscopic examination. Viral DNA extracted from the passage exhibiting CPE did not show an electrophoretic band pattern characteristic of fowl adenoviruses. An additional passage of the samples in cell culture was performed, and viral DNA extracted from this passage exhibited an electrophoretic band pattern characteristic of fowl adenoviruses.
Fig. 2. Electrophoresis results obtained using DNA extracted from twelve fowl adenoviruses and amplified by PCR

Fig. 3. RFLP patterns obtained from digestion of eleven fowl adenoviruses with HaeII

Fig. 4. Electron micrograph of the virus labeled FAV 5 (212,000x)
Fig. 5. Maternal adenoviral antibody titers in chicks from different parent flocks at one day of age

Fig. 6. Adenoviral antibody levels in chickens from progeny A three weeks after challenge with three fowl adenoviruses
Fig. 7. Adenoviral antibody levels in chickens from progeny B three weeks after challenge with three fowl adenoviruses.

Fig. 8. Adenoviral antibody levels in chickens from progeny C three weeks after challenge with FAV 9.
Fig. 9. Mean body weight (g) of chicks from different parent flocks at one day of age

Fig. 10. Mean body weight (g) of chickens from progeny A three weeks after challenge with three fowl adenoviruses
Fig. 11. Mean body weight (g) of chickens from progeny B three weeks after challenge with three fowl adenoviruses

Fig. 12. Mean body weight (g) of chickens from progeny C three weeks after challenge with FAV 9
Fig. 13. Macroscopic comparison of SPF birds unchallenged and subcutaneously challenged with FAV 9

Fig. 14. Chicken embryo liver cells: uninoculated (left) and infected with FAV 9 (right)
Table 2. Results of virus neutralization of twelve fowl adenoviruses*

<table>
<thead>
<tr>
<th>VIRUS SEROTYPE (AS LABELED)</th>
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<td>16</td>
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*Neutralization titers reflect the most common titer obtained after repeating each virus-antiserum cross at least three times.
**Neutralization titers <2 are represented by "x".

Table 3. Results of molecular characterization of twelve fowl adenoviruses

<table>
<thead>
<tr>
<th>Virus Serotype (As Labeled*)</th>
<th>Molecular Characterization (European Serotype)</th>
<th>Strain</th>
<th>% Homology (Hexon gene)</th>
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<td>VR835</td>
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</tr>
<tr>
<td>FAV 2</td>
<td>FAV 10</td>
<td>VR835</td>
<td>98%</td>
</tr>
<tr>
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<td>FAV 7</td>
<td>VR832</td>
<td>99%</td>
</tr>
<tr>
<td>FAV 4</td>
<td>FAV 7</td>
<td>VR832</td>
<td>97%</td>
</tr>
<tr>
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<td>**</td>
<td>**</td>
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<tr>
<td>FAV 6</td>
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</tr>
<tr>
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<td>FAV 7</td>
<td>B3A</td>
<td>99%</td>
</tr>
<tr>
<td>FAV 8</td>
<td>FAV 8</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>FAV 9</td>
<td>FAV 7</td>
<td>VR832</td>
<td>99%</td>
</tr>
<tr>
<td>FAV 10</td>
<td>FAV 10</td>
<td>VR835</td>
<td>98%</td>
</tr>
<tr>
<td>FAV 11</td>
<td>FAV 6</td>
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</tr>
<tr>
<td>FAV 12</td>
<td>FAV 10</td>
<td>VR835</td>
<td>99%</td>
</tr>
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*FAV 1, 2, 4, 5, 6 and 9 were labeled by a previous researcher at the Poultry Diagnostic and Research Center (University of Georgia, Athens, GA) and FAV 3, 7, 8, 10, 11, and 12 were labeled by SPAFAS (Storrs, CT).
**Virus could not be characterized
***Virus was characterized by electrophoretic band patterns resulting from RFLP, but sequencing was inconclusive
Table 4. Results of virus neutralization showing molecularly determined identity of fowl adenoviruses and their homologous antisera*

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<tr>
<th>ANTISERUM (SEROTYPE AND STRAIN)</th>
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<th>**</th>
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<th>VR832</th>
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*Neutralization titers reflect the most common titer obtained after repeating each virus-antiserum cross at least three times.
**No specific strains were determined for these viruses and antisera.
***Neutralization titers ≤2 are represented by "x".
Table 5. Percent mortality among broiler breeder progeny challenged with three fowl adenoviruses

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<tr>
<th>VIRUS</th>
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<td>7</td>
<td>8%</td>
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DISCUSSION

Characterization of Several Fowl Adenovirus Serotypes

This research began with the belief that the twelve viruses being studied represented the twelve serotypes of the fowl adenoviruses. The molecular characterization of the twelve viruses elucidated the true identity of each of the viruses. Unfortunately, these identities did not correspond with those indicated on the original labeling. Molecular characterization revealed that the viruses represented only four different serotypes: FAV 6, FAV 7, FAV 8, and FAV 10. This finding was somewhat surprising and obviously compromised the productivity of this research. Most surprising, by far, was the discovery that three of the six viruses obtained from the commercial source (SPAFAS, Storrs, CT) were of a different serotype than that specified by the company. For example, the virus ordered to represent serotype 9 was molecularly identified as serotype 7. It showed a very high homology in sequence (99%) with strain VR832 of FAV 7. Additionally, the stock viruses obtained from the PDRC at the University of Georgia (Athens, GA) were labeled with the incorrect serotype. It was disconcerting that all six of these viruses belonged to a different serotype than expected. It is not clear exactly why these viruses were labeled with a serotype number different from that found by molecular characterization. Several possibilities could account for this discrepancy. First, it is likely that these viruses were initially obtained from another source. Perhaps they were mislabeled from the time they were obtained. The stocks have been maintained at the PDRC for a number of years, and it is likely that the modern techniques of molecular characterization for fowl
adenoviruses were not available to researchers at the facility when the viruses were first obtained. Secondly, the possibility of cross-contamination between serotypes cannot be discarded. This is not uncommon when working with many fowl adenoviruses at one time. The viruses were most likely passed many times in the virology lab at the PDRC. If proper techniques were not strictly adhered to at all times, there is a chance that cross-contamination occurred.

The inconsistencies in virus labeling called into question the identities of the antisera as well. It is known that the antisera maintained at the PDRC were prepared using the viruses maintained at the same facility. Therefore, the names of these antisera (those to FAV 1, 2, 4, 6, 7, 8, 9, 10, 11, and 12) were altered to reflect the molecular findings for their corresponding virus. What cannot be determined at this point, however, is whether or not the antisera ordered from SPAFAS (those to FAV 3 and 5) are correctly identified. The virus neutralization results support the idea that these, too, may be incorrectly labeled. For example, antiserum against FAV 3 showed neutralizing activity (titers of 16-256) when tested against FAV 7 (strain B3A), FAV 7 (strain VR832), and FAV 10 (strain VR835). Antisera against FAV 5 showed neutralizing activity (titer of 64) against FAV 7 (strain VR832).

Even after correlation of the virus neutralization results with the confirmed identities of the viruses, many of the viruses still did not appear to be neutralized by their homologous antisera. For example, the virus identified as FAV 6 (strain CR119) did not appear to be neutralized in any way by its homologous antiserum. On the other hand, some of the viruses did appear to be neutralized by their homologous antisera: the viruses classified as FAV 7 were neutralized (titers of 32 to 256) by antisera
prepared against FAV 7. There appears to exist some cross-neutralizing activity between the antisera prepared against FAV 10 (VR835) and the virus identified as FAV 8 (strain S8). Low neutralizing titers ranging from 8 to 16 were observed when antiserum to FAV 10 (strain VR832) was combined with the virus. In fact, this virus was neutralized to some degree by antisera against all of the included viruses except that to FAV 6 (strain CR119). This can be considered an example of a strain exhibiting broad antigenicity [21].

Some obstacles were encountered with the molecular characterization of the viruses labeled as FAV 5 and FAV 8. After several attempts to isolate and amplify adenoviral DNA from FAV 5, none could be detected. The virus behaved normally in virus neutralization studies, showing clear CPE characteristic of adenoviruses in virus controls. The virus was neutralized (titer of 64) by its homologous antiserum and neutralized (titer of 8) by antiserum to FAV 2, 9, and 12. Additionally, electron micrographs of the virus revealed characteristic adenovirus morphology. Since the virus exhibited CPE characteristic of adenoviruses, was neutralized by several known adenovirus antisera, and exhibited characteristic adenoviral morphology, it is most likely that the virus is an adenovirus. It is believed that the virus might have a slightly altered nucleotide sequence making the available primers incapable of specific binding. These alterations could be due to mutations in the sequence. Since no DNA could be amplified, the virus could not be analyzed by RFLP or sequencing. Additionally, the virus obtained from SPAFAS (Storrs, CT) as FAV 8 could not be sequenced. Its DNA did show amplification in the PCR reaction and it produced a faint electrophoretic band that corresponded to the positive control when electrophoresis was performed. RFLP
patterns were also analyzed for this virus and were characteristic of FAV 8. However, results of sequencing were inconclusive and no specific strain could be ascertained.

One of the initial objectives of this portion of the research was to characterize twelve serotypes of fowl adenoviruses and confirm these results by molecularly characterizing each virus. Unfortunately, this objective was compromised by the finding that the twelve viruses only represented four serotypes.

The results obtained in this study are limited to four serotypes, but they are still of some merit. The disarray of the fowl adenovirus classification system has been exposed. The fact that viruses purchased from a reputable supplier were incorrectly identified is quite poignant. Any number of other researchers may have used these as reference viruses without the knowledge that they were not what they seemed. In a scientific arena this poses a formidable dilemma.

**Protection of Broiler Breeders against a Strain of Fowl Adenovirus**

The objective of this portion of the research was to evaluate the effect that challenge with FAV 9 (Stanford strain) would have on the progeny of broiler breeders vaccinated with serotype 11 (strain 1047) and serotype 8 (strain 8565). Serum antibody levels, mortality, body weight, virus isolation from samples, and histopathology were evaluated in three progeny groups. The groups represented progeny of an older vaccinated parent flock, a younger vaccinated parent flock, and a commercial flock whose parents obtained natural exposure to adenoviruses. SPF birds were challenged with FAV 9 (Stanford strain) to assure the pathogenicity of the challenge dose. Every bird in the challenged group received $10^{4.5}$ TCID$_{50}$ subcutaneously, and those in the unchallenged groups did not receive any virus.
It was initially found that maternal antibody titers were highest in the progeny of the commercial birds naturally exposed to adenoviruses. It was second highest in the progeny of the older vaccinated parent flock, and it was lowest in the progeny of the younger vaccinated parent flock. These results were expected. It was confirmed that the difference in age of the parent flocks was significant to the titer of maternal antibodies found in their progeny. At the end of the trial, none of the treatment groups showed a detectable titer of antibodies. The experiment lasted three weeks, at which time the titer of antibodies was determined in the chickens. At that time, maternal antibody levels had diminished. It is likely that if the experiment had been extended to four or five weeks higher antibody titers would have been obtained at that time in the challenged chickens. It is probable that at that time the chickens would have mounted their own antibody response to the challenge viruses.

The highest percentage of mortality was seen in the SPF birds challenged with FAV 9 (Stanford strain). This confirmed that the challenge dose of $10^{4.5}$ TCID$_{50}$ was sufficient to cause 100% mortality in birds with no detectable serum antibody titers against fowl adenoviruses. Among the remaining treatment groups, those challenged with FAV 11 (strain 1047) exhibited the highest mortality levels. This appears to be the most pathogenic strain among those in the study. The groups of progenies A, B, and C challenged with FAV 9 (Stanford strain) experienced lower mortality than those in the groups challenged with FAV 11 (strain 1047) and equal or lower mortality to that seen in groups challenged with FAV 8 (strain 8565) or not challenged at all. Therefore, challenge with FAV 9 (Stanford strain) does not appear to increase mortality in any of the progeny groups.
Challenge with FAV 9 (Stanford strain) was not shown to affect the body weight of the challenged birds when compared to the unchallenged birds in progeny groups A and C. The body weights of progeny B birds inoculated with FAV 9 (Stanford strain) at one day of age was actually significantly higher (P=0.05) than the body weights of the unchallenged birds of this progeny. The challenge did not compromise the growth of the birds, even though this strain was not included in the vaccine administered to the parent flock.

Virus was isolated from all of the liver samples collected from the SPF birds inoculated with the challenge dose of FAV 9 (Stanford strain). After three passages in CELiC cell cultures no virus was isolated from any of the liver samples collected from the birds of progenies A, B, and C inoculated with FAV 9 (Stanford strain). These samples included both mortality and samples taken after sacrifice of birds at the end of the trial. Adenovirus was isolated from one bird challenged with FAV 8 (strain 8565) and one bird challenged with FAV 11 (strain 1047). Additionally, no macroscopic or microscopic lesions were observed in any of the birds of progenies A, B, and C challenged with FAV 9 (Stanford strain). These findings indicate that challenge with this virus has not caused disease in birds of any of the three progeny groups.

Based upon the parameters evaluated, this study shows that progeny from parent flocks vaccinated with FAV 8 (strain 8565) and FAV 11 (strain 1047) show no signs of vulnerability to disease when challenged with the more recently isolated FAV 9 (Stanford strain). It does not appear that there is a significant necessity for the primary breeding company to include this strain in their vaccination program. It is possible that
vaccination with the FAV 8 (strain 8565) and FAV 11 (strain 1047) viruses induces an antibody response that provides a type of cross-neutralization against FAV 9 (Stanford strain) protecting chickens from disease.
REFERENCES


International Committee on Taxonomy of Viruses.  M. H. V. van Regenmortel, ed.


