An antigenic comparison of avian group A and group D rotaviruses

by

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GENERAL INTRODUCTION

Diarrhea and enteritis associated with rotavirus infections have been reported in both avian and mammalian species, including humans. Avian rotaviruses, in association with viral enteritis, have been documented in many countries including the USA, United Kingdom, Japan, France, Belgium, Italy, and Israel (Reynolds, 1992). It has been reported that avian rotaviruses may be distributed world-wide like the mammalian rotaviruses (Reynolds, 1992).

Based on serologic antigenic relationships and electropherotyping of RNA, rotaviruses have been divided into seven groups: A, B, C, D, E, F and G (Pedley et al., 1983; Pedley et al., 1986). Of these seven groups, only groups A, D, E and F have been identified in avian species (Saif, 1990).

Both group A and group D rotaviruses have been isolated from chickens, turkeys and pheasants (Reynolds et al., 1987b). The avian group D rotaviruses have been reported to be the most frequently identified viruses in diarrheic turkey poults (Saif et al., 1985; Reynolds et al., 1987a). In addition, several reports have documented the group D rotaviruses in association with debilitating enteric disease in pheasant chicks (Reynolds et al., 1987b). It seems that avian group D rotaviruses are more prevalent than avian group A rotaviruses and are more frequently found in association with enteritis. These facts make the group D rotaviruses of primary interest in avian rotaviral disease research.

Research involving group D rotaviruses and rotaviral vaccine development has been hampered because group D rotaviruses are not readily propagated in vitro. In contrast, group A rotaviruses are propagated in vitro and may offer a potential for protection against group D rotavirus.

The purpose of this study was to determine the antigenic relatedness between group A and group D avian rotaviruses. Our hypothesis was that although avian group A and D rotaviruses have been established as distinct rotaviral groups, some antigenic relatedness exists between the two which could be utilized to provide cross protection. The information presented should prove to be useful in future studies investigating rotaviral disease prevention and control.

Thesis Organization

This thesis is written in an alternate format and includes a paper that will be submitted for publication. The thesis begins with a literature review and is followed by one manuscript that is presented as a separate chapter. This manuscript presents information about the antigenic relatedness of avian group A and group D rotaviruses. A chapter of general conclusions, including a list of references cited in the general introduction and general conclusions, follows the manuscript.

Literature Review

Rotaviruses have been identified as the major etiologic agent of viral enteritis in most mammalian species. These infections are frequently associated with outbreaks of diarrhea and are usually seen in neonates and young animals (Estes et al, 1983). In addition to mammals, rotaviruses are known to cause diarrhea in members of the avian species. These viruses can act alone or play a role in enteric disease syndromes (Reynolds, 1992).

Rotaviral-induced diarrhea was first reported, in 1969, in calves inoculated with a virus isolated from a field outbreak (Mebus et al., 1969; Mebus et al., 1971). Rotaviruses were first described in association with human disease in 1973 (Bishop et al. 1973; Flewett et al., 1973). This discovery was made when virus particles that morphologically resembled

the bovine rotavirus isolate were detected, using electron microscopy, in human feces from diarrheic infants (Bishop et al., 1974). Human rotaviruses, now thought to be one of the most important causes of infectious diarrhea and death in infants and young children, have been isolated worldwide (Offit, 1994). Rotaviruses have subsequently been identified as the cause of viral gastroenteritis in all the major species of livestock (Flewett and Woode, 1978).

In 1977, the first avian rotavirus infection was reported when rotaviruses were observed in the feces of diarrheic turkey poults (Bergeland et al., 1977). Although avian rotaviruses were first isolated from turkeys, they have now been identified in many additional avian species including chickens, pheasants, ducks, pigeons and some psittacines (Reynolds, 1992). A recent paper reports the identification and characterization of an avian rotavirus from a healthy migratory wild bird (Takehara et al., 1991).

Avian rotaviruses, like their mammalian relatives, may have a worldwide distribution. They have been reported in many countries including the USA, United Kingdom, Japan, France, Belgium, Italy and Israel (Reynolds, 1992). Group A, D and F rotaviruses have been isolated from both chickens and turkeys. The group G rotaviruses have been isolated only from chickens. Both group A and group D rotaviruses have also been isolated from pheasants (Reynolds et al., 1987b). Of the four groups of avian rotaviruses, research involving groups A and D has been most frequently noted in the literature.

The adaptation of several avian rotaviral isolates to serial propagation in primary cell cultures expedited the early work with these viruses. In 1985, serial propagation was reported in continuous cell lines as turkey rotaviruses were adapted to a fetal rhesus monkey kidney cell line (MA104 cells) (Theil et al., 1986). Until this time, only mammalian rotaviruses, including porcine, feline, lapine and human had been adapted to the MA104 cell

line (Reynolds, 1992). To date, only group A rotaviruses have been propagated in vitro. The inability to propagate other avian groups in vitro has hampered efforts to obtain purified virus and has limited research done with these rotavirus groups. Recent attempts to propagate group D rotaviruses in primary cells, embryonating eggs and continuous cell lines have proved unsuccessful. (Devitt and Reynolds, 1993)

In contrast to mammalian species, group A rotaviruses do not appear to be the most prevalent rotavirus in avian species. In turkeys, group D rotaviruses are identified more frequently than group A, from diarrheic flocks (Saif et al., 1985), and are more prevalent in diarrheic poults than in healthy poults (Reynolds et al., 1987a). In contrast, the group A rotaviruses seem to be as prevalent in healthy flocks as in diseased flocks (Reynolds et al., 1987a). Reports also show that broiler breeder chickens have a greater incidence of serologic titers to group D rotaviruses than to group A. (McNulty et al., 1984).

In 1987, group A rotaviruses and rotavirus like-viruses (RVLVs) were isolated from pheasant chicks. The RVLV isolated had many similarities to the turkey group D rotavirus (Reynolds et al., 1987b). The pheasant RVLV was recently characterized as an avian group D rotavirus on the basis of IEM, agar gel immunodiffusion (AGID) and polyacrylamide gel electrophoresis techniques (Devitt and Reynolds, 1993). Both pheasant and turkey group D viruses formed viral antibody complexes or precipitate bands with turkey and pheasant group D antisera. Based on these findings it was concluded that the pheasant RVLV was a group D rotavirus and was related to the turkey group D rotavirus (Devitt and Reynolds, 1993).

Morphology

Rotaviruses were originally called reovirus-like or orbivirus-like due to the fact that they somewhat resemble reoviruses (Kapikian et al., 1974). Rotaviruses are classified as a

genus in the virus family Reoviridae (Mathews, 1979). They are icosahedral, nonenveloped and possess a ds RNA genome consisting of 11 segments (McNulty, 1993).

There are two different forms of rotaviruses. Double-shelled particles, having both an inner and outer capsid, are termed intact or complete and are 65-80 nm in diameter. Single-shelled particles, lacking an outer capsid, are called incomplete or rough and are 55-70 nm in diameter (Devitt and Reynolds, 1993). Until recently rotaviral particles were reported as being 65-75 nm and 55-60 nm in diameter, for double and single-shelled, respectively (Theil, 1990). In 1993, a group D rotavirus was identified that measured 80 nm for double-shelled particles and 70 nm for single shelled particles (Devitt and Reynolds, 1993). The inner capsid surrounds a hexagonal core that is 40-45 nm in diameter (Bridger and Woode, 1976). Rotaviral particles that lack their nucleic acid, as seen by negative staining electron microscopy, are also referred to as incomplete.

The combination of the inner and outer capsids gives rotaviruses a distinct morphological appearance. The capsomere arrangement of the inner capsid gives rotaviruses a spoke-like internal structure. The spokes create a wheel appearance and the Latin word for wheel, "rota", is included in their name.

Their distinctive morphology allows rotaviruses to be easily distinguished from other enteric viruses. Because they can be shed in high numbers in the feces (Yason and Schat, 1987), rotaviruses can be detected in many fecal specimens by electron microscopy (EM). Due to their characteristic morphology and fecal shedding, direct EM was initially used as the primary tool for rotavirus identification (McNulty et al., 1979a). The fact that all rotaviruses have a similar electron microscopic appearance resulted in rotaviral particles being reported only as "rotaviruses". The application of immune electron microscopy (IEM) to the study of rotaviruses contributed to the classification of isolates (Reynolds and Pomeroy, 1989).

Physical properties

Although rotaviruses have been identified from a wide variety of hosts, most isolates have had similar physical properties. Separation of rotaviruses by ultracentrifugation in an isopynic cesium chloride density gradient yields two opalescent bands, one containing mainly double-shelled particles and the other single-shelled particles (Devitt and Reynolds, 1993). Mammalian rotaviruses separated in a cesium chloride gradient have had densities of 1.36 g/cm^3 and 1.38 g/cm^3, for double-shelled and single-shelled particles respectively. The naked core particles have a density of 1.44 g/cm^3 (Bridger and Woode, 1976). Avian rotaviruses separated in this same medium have buoyant densities of 1.34 g/cm^3 for double shelled virions and 1.36 g/cm^3 for single-shelled particles (Devitt and Reynolds, 1993). Rotaviral particles can also be separated by sedimentation in sucrose. Double-shelled particles sediment at 520S-530S and single-shelled particles sediment at 380-400S. Core particles have a sedimentation coefficient of 280S (Estes, 1990).

Single and double-shelled rotaviral particles have different biological properties. The outer capsid is required for normal infectivity. Treatment of double shelled particles with calcium chelating agents removes this outer capsid and results in non-infective single shelled particles. Rotaviruses retain their infectivity and particle integrity with fluorocarbon extraction and exposure to ether, chloroform and deoxycholate. It has been reported that rotaviral infectivity is slightly reduced with exposure to chloroform and greatly decreased with sodium dodecyl sulfate treatment. In addition, nonionic detergents have been noted to enhance infectivity by disrupting the rotaviral aggregates (Estes, 1990).

Rotaviruses have been described as environmentally stable viruses. A turkey rotaviral isolate was shown to be stable at pH3 and not completely inactivated by 56^{0}C temperatures for 8 hours (Kang et al., 1988). Work with rotaviruses from other species has

shown that 3% hypochlorite does not affect their virulence but virulence is decreased by 4% FAM, an iodophor disinfectant (Snodgrass and Herring, 1977). Other disinfectants are known to inactivate rotaviral infectivity including phenols, formalin, chlorine, ethanol (95%) and beta-propiolactone. Ethanol (95%) is reported to inactivate rotaviruses by removing their outer capsid (Estes,1990).

Classification

By the early 1980's, rotaviruses had been isolated from many different species and all of these isolates had been shown to share the same basic morphology and similar biochemical characteristics (Theil, 1990). When serologic studies were performed, many of the identified rotaviruses shared a common group antigen but others lacked this common antigen (Gary et al., 1982; Hoshino et al., 1984). This discovery led to a division of "conventional" or group A rotaviruses and "atypical" rotaviruses. The group A rotaviruses being the most prevalent in mammalian species. The atypical rotaviruses have also been called rotavirus-like viruses (RVLV), rotavirus-like agents (RVLA), novel rotaviruses, pararotaviruses and antigenically distinct rotaviruses (ADRV) (Eiden et al., 1986).

Group A rotaviruses possess the group A antigen and the atypical rotaviruses do not have this antigen. Serologic and nucleic acid comparison of two "atypical" rotaviruses with a "conventional" rotavirus led to the establishment of three distinct groups including A, B and C (Pedley et al., 1983). Further work using these same methods defined two additional groups, D and E (Pedley et al., 1986). Rotaviruses are now divided into seven serogroups, A, B, C, D, E, F, and G (Reynolds, 1992).

The nucleic acid comparison, utilizing electrophoretic migration of their ds RNA genome, contributed to the classification of rotaviruses. The RNA migrates into four distinct molecular mass groups. These groups have been designated I-IV (Laurenco et al.,

1981; Estes et al., 1984). The mammalian group A rotavirus was found to have segments 1-4 in group I; segments 5 and 6 in group II; segments 7-9 in group III; and segments 10 and 11 in group IV. This migration pattern is designated a "4-2-3-2" pattern (Hancock et al., 1983). In contrast, avian group A rotaviruses form a "5-1-3-2" pattern because segment 5 migrates to group I rather than group II (Kang et al., 1986). Avian group D rotaviruses migrate with a "5-2-2-2" pattern; group F with a "5-2-2-2"; and group G with a "4-2-2-3". Groups D and F, although they have the same overall migration, are differentiated by the different placement of bands within the I-IV groups (Saif, 1990).

The use of serologic immunofluorescent testing first showed that avian isolates are antigenically related to the mammalian rotaviruses. The cross-reaction was initially assumed to be with the group A antigen (McNulty et al., 1979b). This assumption resulted in this group of avian viruses being called avian group A, but later studies using monoclonal antibodies showed that this antigenic relationship is most likely due to a determinant other than the group A antigen (Gary et al., 1982; Hoshino et al., 1984). Of the seven groups now identified, only groups A, B, C and E rotaviruses have been identified from mammals. Groups D, F and G rotaviruses have been found only in avian species (Saif, 1990; McNulty, 1991).

In addition to these seven groups or serogroups, rotaviruses within each group are classified into serotypes. A single serogroup includes rotaviruses that share cross-reacting antigens which are detectable by serologic tests like immunofluorescence, enzyme-linked immunosorbent assay and immune electron microscopy. Serotypes are defined by reactivity of rotaviruses in plaque reduction neutralization assays. Because neutralization assays predominantly measure the reactivity of antibody to the glycosylated polypeptide VP7, different serotypes may be referred to as having different types of VP7 (Estes,1990). Another term, subgroup, was developed when it was discovered that some animal

rotaviruses appeared to belong to the same antigenic group as a human rotavirus. Rotaviral subgroups were used in a system for the antigenic classification of both human and animal rotaviruses that share a common group antigen. Later it was discovered that all of these subgroups belong to the group A rotaviruses (Kapikian and Chanock, 1990). Now four subgroup specificities are recognized: subgroup I, subgroup II, subgroup I and II, and neither subgroup I nor II (Hoshino and Kapikian, 1994).

In the 1980's much work was done to characterize the viral polypeptides of mammalian rotaviruses. This research resulted in different information as to the number and size of virus-specific polypeptides in mammalian rotaviruses. In addition, there is dispute over which polypeptides are structural proteins and which are non-structural. The most recent summary states that a mammalian group A rotavirus (SA11) has twelve polypeptides, six structural and six nonstructural (Both et al., 1994).

In 1987, the viral polypeptides of avian rotaviruses were characterized (Kang et al., 1987). This research, utilized sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoprecipitation analysis. These rotaviral polypeptides were detected in MA104 cells infected with a turkey group A rotavirus (AvRV-1). At ten hours post-infection ten major polypeptides were identified (VP1, VP2, VP3, VP5, VP6, VP7, VP8, VP9, NCVP1. and NCVP 2). They were named based on their decreasing molecular mass and a prefix, VP or NCVP, to represent structural or nonstructural proteins.

Seven structural proteins were identified from purified single-shelled, double-shelled and core particles of AvRV-1 (Kang et al.,1987). These structural proteins were designated VP1, VP2, VP3, VP4, VP5s, VP6 and VP7 with molecular mass of 125kD, 100kD, 90kD, 88kD, 54/55kD, 45kD and 37kD respectively. Of these seven structural proteins VP1, VP2 and VP6 are associated with the inner rotaviral capsid with VP1 and VP2 tightly associated with core particles. The other four, VP3, VP4, VP5s and VP7, form part of the outer

rotaviral capsid. VP7 (37kD) was identified as a glycoprotein and using tunicamycin treatment it was discovered that the precursor of VP7 is a 32kD polypeptide (Kang et.al, 1987).

Four polypeptides, that were not immunoprecipitated and were not detected in purified virus preparations, were considered to be nonstructural polypeptides or the precursors of structural polypeptides. These four proteins had molecular masses of 59kD, 30kD, 28kD, and 26kD. The 30kD and 28kD nonstructural polypeptides were identified as glycoproteins (Kang et al., 1987).

In mammalian group A rotaviruses VP6, the major inner capsid protein, and VP7 and VP4, two of the outer capsid proteins have been selected for most antigenic comparison studies. These proteins have been chosen because they can be easily and rapidly detected and have been well characterized. It has been reported that VP4 and VP7 are the neutralizing antigens and VP7 represents the major serotype antigen. VP6 represents the group and subgroup antigens. In addition, VP4 is the protein noted to be responsible for hemagglutination (Hoshino and Kapikian, 1994).

Pathogenesis and replication

The primary route that rotaviruses gain entry into the body is by ingestion. Rotaviruses infect enterocytes that line the villi of the small intestine. They tend to infect only the most mature villous epithelium cells which are located on the distal 1/2 to 2/3 of the villus (Mason, 1978). Virus particles utilize a glycoprotein in the outer shell to bind to the host cell. After cleavage of another outer shell protein, with a trypsin-like protease, the virus passes directly through the cytoplasmic membrane and into the cell. Replication then takes place in the cytoplasm. The viral RNA-dependent RNA polymerase (transcriptase), associated with the inner shell, is now turned-on and the messenger RNA's are translated to

viral proteins (Patton, 1994). With experimental infections, the first viral protein synthesis has been detected at 6 hours post infection (Kang et al., 1987).

The process continues with the synthesis of minus-strand RNA and the formation of dsRNA (Patton, 1994). Some single-shelled particles bud through the membrane of the endoplasmic reticulum, are transiently enveloped, and gain their outer shell. Virus particles are released by lysis of the host cell (McNulty, 1979b).

It has been published that rotaviruses in chickens and turkeys infect only the small intestine, but small numbers of infected enterocytes can be found in the colon and cecum. There is no evidence to show that rotaviruses replicate in other visceral organs (McNulty, 1993).

Avian rotaviruses are transmitted horizontally between birds through both direct and indirect contact. There has been no report of egg transmission of rotaviruses although other genera in the family Reoviridae can be transmitted via the egg. Also, there has been no documentation of a carrier state (McNulty, 1991).

Clinical disease

Most rotaviral infections in chickens, turkeys and pheasants occur in birds less than six weeks old (Yason and Schat, 1987). Experimental infections, with cell-culture propagated avian group A rotavirus, produced loose droppings in turkeys 2-5 days post-inoculation (Yason and Schat, 1986). Chickens experimentally infected with group A rotavirus showed mild or no clinical signs (McNulty, 1991). Although increased mortality has been associated with rotaviral infections in pheasants (Reynolds et al., 1987b), experimentally infected chickens and turkeys show no mortality (Yason and Schat, 1987). Pheasant chicks, experimentally infected at one-day of age, showed severe depression, heat seeking, diarrhea and spraddled wing feathers. In addition, by 7-days post inoculation, they

showed lower body weights, decreased D-xylose absorption and lower intestinal disaccharidase activity when compared with control groups (Reynolds, 1992). Field outbreaks of rotaviral infection in chickens and turkeys usually results in diarrhea which leads to dehydration, poor weight gains, high morbidity and can result in mild to moderate mortality (McNulty, 1991).

Studies involving chickens experimentally infected with group A rotaviruses have shown that older birds (greater than six weeks of age) are more susceptible to infection and show more severe clinical signs (Yason et al., 1987). In contrast, pheasant chicks infected with group D rotaviruses exhibit an age susceptibility with birds exposed over 6 weeks of age becoming infected but not developing clinical signs (Reynolds, 1992). This documented age susceptibility in pheasant chicks represents an important area to explore in the development of strategies to prevent rotaviral infections.

It is believed that the pathogenic mechanism by which rotaviruses cause diarrhea in birds is similar to what has been described in mammals. Rotaviruses infect the small intestine, replicate within the enterocytes and lyse these cells. These mature intestinal epithelial cells are then replaced by immature cells that migrate distally from the intestinal crypts (McNulty, 1993). These new cells are less differentiated than the cells they replaced and this leads to a deficiency in enzyme production and electrolyte transport. The result is malabsorption with a component of maldigestion. Due to the lack of these normal processes, materials remain in the lumen of the intestine. These undigested and unabsorbed materials exert an osmotic effect that retains water in the intestinal lumen. These materials also undergo fermentation which contributes to gas production and increases the number of osmotically active particles. The malabsorption, maldigestion, fermentation and osmotic effect all contribute to the watery diarrhea and gas production that can be seen with rotaviral infections (Moon, 1978).

The most commonly reported gross change with rotaviral infection is dilatation of the small intestine which may include the ceca. The intestinal contents are usually watery or frothy yellow fluid with gas and some solid ingesta (McNulty et al., 1983). Other gross findings can include dehydration, inflammation of the vent, litter in the gizzard, fecal material crusted on the plantar surface of the feet and signs of vent pecking (McNulty, 1993).

Histologic changes noted with group D rotaviral infection of pheasants include shortening of the intestinal villi and crypt hyperplasia. This results in a decreased villus to crypt ratio (Haynes et al., 1994). There is also leukocyte infiltration into the intestinal lamina propria, basal vacuolation of the epithelium at the tips of the villi, fusion of villi, and scalloping of the villus surface (Yason et al., 1987). In chickens and turkeys infected with group A rotavirus immunofluorescence studies showed the primary site of infection to be the enterocytes in the distal third of the small intestinal villi. The majority of fluorescence was seen in the cytoplasm of these cells (McNulty, 1991). It was noted that group A rotaviruses tend to primarily infect the duodenum. Other studies have shown that group D rotaviruses show preference for the jejunum and ileum (McNulty et al., 1983). Also it has been reported that there seems to be a difference in pathogenicity between rotaviral isolates, serogroups and the species infected.

Diagnosis and control

Detection of rotaviral particles in feces or intestinal contents, using direct electron microscopy, is still the most widely used method of diagnosis. Direct EM is relatively sensitive and provides the advantage of detecting all rotaviral serogroups but can not differentiate the particles that are detected (McNulty, 1991). IEM can be used to help determine the serogroup of the rotaviral particles and increase the sensitivity of this

technique. Rotaviral RNA can be detected in intestinal contents or feces through RNA extraction and polyacrylamide electrophoresis. This technique is also relatively sensitive and offers the ability to classify the isolate but is more time consuming (Reynolds, 1992). Isolation of rotavirus in cell cultures is only possible with group A avian rotaviruses and leaves other rotaviral groups undetected.

There are a number of commercial kits available that are designed to detect human group A rotaviruses. Some of these tests are in-office tests that give results in less than five minutes. Many of these commercial kits utilize latex agglutination assays and fecal specimens collected with a swab. These tests, because they are designed for human rotavirus detection, may not be accurate in the identification of other rotaviruses. The antibodies incorporated in the tests may not cross react with group A rotaviruses from other species and most likely will not detect other rotaviral groups. These tests use fecal swabs to detect rotaviruses and animals may not be shedding virus particles when the sample is taken.

Since rotaviruses, especially group A, seem to be ubiquitous it may be unrealistic to try and keep commercial poultry totally free from rotavirus infection. However, good management practices such as all in-all out housing and single age farms may help prevent rotaviral outbreaks. In addition, many producers suggest house rest between flocks with cleaning, fumigation and litter removal. Other programs have suggested symptomatic treatment such as raising the brooding temperature or treating with antibiotics to control secondary bacterial invasion (Reynolds, 1992; McNulty, 1991). The effect of the diarrhea on litter condition can be decreased by increasing the temperature and ventilation and by adding fresh litter (McNulty, 1993)..

Due to the high prevalence of rotaviral antibody in some flocks, serologic diagnosis of infection may not be recommended (McNulty et al., 1984). It must be remembered though that group A rotaviruses in contrast to group D, at least in turkey poults, have not

been as highly associated with playing a role in disease. It may be that serological detection of group D rotaviruses may more accurately reflect the true prevalence of rotaviral enteric disease.

Immune response and vaccination

It is after the virus has entered the intestinal epithelial cell that virus or viral proteins cross the basement membrane to the lamina propria or attach to specialized antigen presenting cells. The antigen can then enter the gut associated lymphoid tissue (GALT). Next, the rotaviral antigen is processed by specialized B lymphocytes and presented to helper T lymphocytes (Th), cytotoxic T lymphocytes (CTLs) and other B lymphocytes (Offit, 1994). It has been shown using a mouse model that rotaviruses, unlike most viral infections, induce a primary CTL response. This was shown when lymphocytes taken directly from the host were able to lyse virus-infected target cells. Also, the route of inoculation with rotaviruses appears to determine the frequency of CTL precursors at a given site. It was shown that oral inoculation resulted in 25-30 times more CTL precursors than after subcutaneous inoculation. These rotavirus CTLs are not serotype specific and tend to recognize VP7 antigens better that VP4 or VP6 (Offit, 1994).

With rotaviral infections in humans, it has been noted that infants and young children that have been previously infected are protected against severe disease with reinfection (Offit, 1994). In rotaviral infections of chickens and turkeys, it has been reported that they develop detectable serum antibodies within 4-6 days of experimental oral infection . In chicks, maternal antibodies have been found to persist for as long as 3 weeks and seem to be transferred to the chick via the yolk (McNulty, 1993).

In mammals, rotavirus-specific IgM and IgA appear in the serum 4-6 days post infection (PI). It has been noted that within one month of infection, virus specific B and T

cell precursors can be found distributed throughout the intestinal and non-intestinal lymphocyte population (Offit, 1994). In chickens the intestinal antibody response appears to be predominantly IgA. In the serum, rotavirus specific IgM, IgG and IgA can be detected (Myers and Schat, 1990). Work done with turkey poults shows that circulating maternally derived IgG plays a role in protection of the intestinal mucosa against rotaviral infection. This protection seems to be the greatest during the first week of life and is titer dependent. It was found that this IgG passive immunity reduced the severity of the rotavirus-associated small intestine mucosal lesions and increased the ability of the poults to absorb D-xylose (Shawky et al., 1993).

Vaccines for avian rotaviruses have not yet been developed. Vaccine development has been hindered by the number of rotaviral serogroups, the inability to propagate some of these groups of avian rotaviruses in vitro and the lack of a detailed understanding of the epidemiology of the disease and the extent of viral antigenic diversity (McNulty, 1991). Live attenuated oral vaccines developed for calves and piglets have not been successful (Conner et al., 1994). Vaccines can fail for many reasons including interference of passive maternal antibody, failure to induce protective immunity against heterotypic rotaviruses and exposure to field strains of rotavirus before the vaccine has induced a protective immune response (Conner et al., 1994).

A strategy for protection against rotaviral infection is to provide the young with protection from clinical disease and/or delay the onset of disease to an age when the disease is less severe. Most adult animals, including poultry, have preexisting naturally acquired antibodies to rotavirus (Conner et al., 1994). This immunity is almost certainly passed from the mother, in some degree, to offspring. This provides the potential for a vaccination strategy of providing passive protection. Maternally derived protection is important because some animals have an age susceptibility to rotaviral infection and providing

maternal antibody for the first few weeks of life may be a strategy to decrease the severity of rotaviral disease.

Other methods of vaccine development include the creation of subunit vaccines. This potentially could be achieved by expressing rotaviral proteins in a prokaryotic or eukaryotic system. Subunit vaccines can also be developed that allow rotaviral proteins to be synthesized using live viral vectors (Conner et al., 1994). With any vaccine strategy it is important to know the antigenic determinants that will convey protection. It may also be important to utilize rotaviral antigens that will react with many different serogroups of rotaviruses (Eiden et al., 1986). In order to develop these types of vaccines for poultry more must be learned about the antigenicity of rotaviruses and the antigenic relatedness of different rotaviral serogroups.

AN ANTIGENIC COMPARISON OF AVIAN GROUP A AND GROUP D ROTAVIRUSES

A paper to be submitted to *Avian Diseases*

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SUMMARY

The purpose of this study was to explore the antigenic relatedness of a turkey group A rotavirus and a pheasant group D rotavirus. In previous studies these two rotavirus groups had been shown to be distinguished from each other on the basis of serologic assays and electropherotyping. The present study, using ELISA, virus neutralization and western blot techniques, revealed an antigenic cross reaction between these two groups of avian rotavirus. In addition, the pheasant group D rotaviral polypeptides were characterized. SDS-PAGE revealed 10 group D rotaviral proteins that ranged in molecular mass from 16kD to 97kD.

INTRODUCTION

Enteritis and diarrhea of young birds is an enteric disease syndrome which is problematic for poultry producers and has been associated with various enteric viruses (12). Rotaviruses are considered a major cause of enteritis and diarrhea in many mammalian and avian species (3, 6,13,15). Rotaviruses have been incriminated as playing a role in this enteric disease syndrome. Although group A rotaviruses have been identified in turkeys,

chickens and other avian species, the group D rotaviruses were found to be the most prevalent viruses occurring in turkey flocks experiencing enteritis and diarrhea (14,15). Group D rotaviruses have also been reported to cause enteritis in pheasants resulting in increased morbidity and mortality. It is felt that group D rotaviruses may play an important role in the viral component of viral enteritis of turkeys, pheasants and potentially other avian species.

Rotaviruses are nonenveloped enteropathogenic viruses (7) classified as a genus in the family Reoviridae (16). They possess a ds RNA genome consisting of 11 segments contained within a double shelled capsid (18). Rotaviruses have the morphologic appearance of a wheel and get their name from the Latin rota (wheel). Rotaviruses are classified into 7 groups: A, B, C, D, E, F, and G. This group classification is based on serologic and electrophoretic techniques (10).

Limited information exists about group D rotaviruses because, unlike avian group A rotaviruses, the group D rotaviruses have not been readily propagated in vitro (7). This study utilized a group D rotavirus isolated from the intestinal contents of diarrheic pheasant chicks. Preliminary studies revealed that this virus was a rotavirus-like virus, but future work identified it as a group D rotavirus and characterized its properties (2). This avian group D rotavirus is morphologically indistinguishable from the group A rotaviruses and has a "5-2-2-2" nucleic acid electrophorectic pattern. This study reports the separation of the polypeptides of this group D rotavirus, using SDS-PAGE, and the identification of 10 proteins ranging in molecular mass from 16kD to 97kD.

Studies which have employed agar gel immunodiffusion (AGID), immune electron microscopy (IEM) (2) and immunofluorescence (IF) techniques have reported that group A and group D rotaviruses do not cross react (10,11). However, preliminary work done in our laboratory using an ELISA indicated some cross reactivity between these two

serogroups. The present study uses ELISA, serum virus neutralization and western blot techniques to provide evidence that these avian group A and group D rotaviruses are antigenically related.

MATERIALS AND METHODS

Propagation and purification of viruses. The turkey group A rotavirus used in this study was isolated from a diarrheic flock in Iowa. It was propagated in MA-104 cells using previously described techniques (17,19). The virus was purified by methods previously described with some modifications (2). Briefly, the rotavirus infected MA-104 cells were first freeze-thawed and then sonicated. This was followed by pelleting the cell debris by low speed centrifugation (1000xg for 20 min.), removing the supernatant and pelleting the virus through a sucrose gradient by ultracentrifugation (85,000xg for 2 hours). The pellet was then resuspended and ultracentrifuged (200,000xg for 24 hours at 4C) in an isopycnic cesium chloride (CsCl) gradient. Thin walled centrifuge tubes were used (Beckman, Fullerton, California) and the two opalescent bands, representing the purified single and double-shelled particles, were visualized and then collected by puncturing the thin walled tube with a 25G 5/8 inch needle and removing the bands using a 1cc syringe. The presence of purified group A virus was confirmed by direct and immune electron microscopy (IEM). The EM grids were prepared as previously described (2).

The group D rotavirus used in this study has been previously described (2). This group D rotavirus was propagated in day-old pheasant chicks. The chicks were placed in sterile containment isolaters and inoculated *per os* with purified group D rotavirus. The infected intestines were harvested from the pheasant chicks at seven to ten days post-inoculation. The group D rotaviruses were then purified from the intestines by methods previously described (2) with the modifications described above for the group A rotaviral

purification. IEM was used, as previously described (2) to confirm the presence of group D rotavirus.

Antisera. Turkeys, 3-4 months of age, were tested by AGID and found to be serologically negative to groups A and D rotavirus. Pre-immunization blood was collected and sera was prepared, pooled, and used as group A and group D negative turkey sera. Group A and group D rotavirus antisera was made by immunizing these turkeys with 15-20ug of viral protein per bird as determined by a microtiter plate assay (Bio-Rad, Hercules, CA). The group A or group D rotavirus was emulsified in Freund's incomplete adjuvant. The injection was made intramuscularly (IM) into the gastrocnemius muscle. All birds were boostered once with the respective group A or group D rotavirus that was prepared in the above described manner. All sera used was prepared with blood collected from these birds. The sera was heat inactivated (56C for 30 minutes), serially filtered (0.8 to 0.22um filters: Millipore Corp., Bedford, Mass), aliquoted and stored at -20C until needed. Before use, all sera was clarified by ulracentrifugation (85,000xg for 40 minutes).

Agar-gel immunodiffusion (AGID). AGID gels were prepared using methods previously described with the noted modifications (2). Clean microscope slides, that had been warmed to 37C, were covered with 2.5-3 ml of 1.0% noble agar (Difco Laboratories, Detroit, Mich.) at pH 8.5 with 8.5% NaCl, 1.0% polyethylene glycol, and 0.5% sodium azide. The slides were allowed to cool at 4C for approximately 12 hours. Two sets of wells per slide were cut using a blue-tongue virus AGID template (Veterinary Diagnostics Technologies Inc., Wheat Ridge, Colo.) which places the wells 2 mm apart. Gentle vacuum suction was used to remove the agar plugs. Each well held approximately 15ul of fluid.

The AGID was performed by incubating purified group A or Group D rotavirus with group A or group D rotaviral antisera in a humidified chamber at room temperature for 24 hours. Antisera negative for group A and group D rotavirus (negative control) and

antisera positive for either group A or group D rotavirus (positive control) was included with each AGID test.

Immune electron microscopy (IEM). Methods previously described (2) were used, with purified group A or group D rotavirus and hyperimmune sera.

Virus neutralization (VN) assay. One-way serum virus neutralization assays were performed using MA-104 cells, CsCl purified group A rotavirus, group A and group D rotaviral antisera, and fluorescein conjugated rabbit anti-chicken IgG (Sigma, St. Louis, MO). The procedure used was a modification of previously described techniques (4,8). MA-104 cells were grown to confluency in 96-well tissue culture plates using Dulbecco's Modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and antibiotics (penicilllin/streptomycin/fungizone, Biowhittaker, Walkersville, MD). The group A rotavirus was pre-treated with 10ug/ml trypsin (Type IX, Sigma, St. Louis, MO) and incubated for 45 minutes at 37C. The virus stock was then diluted to a predetermined concentration of 170 fluorescent forming units (FFU) per 100ul. Serial two fold dilutions, from 1:10 to 1:5,120, of anti-group A, anti-group D or group A and D negative sera, were made. The trypsin activated avian group A virus stock was then added to the serum dilutions and incubated at 37C for 60 minutes. Subsequently, the cell monolayers were washed twice with serum-free DMEM and each well was inoculated with 100ul of the serum-virus mixture. Column 2 of each 96 well plate was used as the positive (virus only) control. Row "G" of each plate was used as the negative (media only) control. A separate plate was used for each different serum treatment.

The plates were incubated with the virus-medium mixture for 90 minutes at 37C, to allow virus adsorption onto the cells. The inoculum was then removed by washing (2x) the monolayers with serum free DMEM with antibiotics. At 24 hours following the inoculation, the media was removed. The wells were then gently rinsed with phosphate

buffered saline (PBS), and the cells were fixed for ten minutes with cold methyl alcohol that had been diluted 4:1 with deionized distilled water. The monolayers were then gently rinsed once with PBS (25C).

The fluorescent antibody staining was then performed. Each well, excluding the primary antibody negative control wells, was inoculated with 100ul of anti-group A rotavirus serum. The cells were incubated with the anti-serum for 60 minutes at 37C. The monolayers were then washed three times with PBS, allowing the third wash to remain on the cells for 5 minutes. 50ul of fluorescein conjugated rabbit anti-chicken IgG was then added to each well at a dilution of 1:30. This secondary antibody was incubated for 30 minutes at 37C. The cells were washed twice with PBS and then once with deionized, distilled water. A drop (20ul) of a 1:1 glycerol-PBS mixture was then added to each well. The excess glycerol was removed by draining the plate and the plate was examined using a fluorescent (FA) microscope with a 546nm wavelength (green) filter. The entire well was scanned using the 20X objective (250X total magnification) to count the total of FFU's reported in each well.

ELISA. A DOT/DAB immunoassay was performed, with purified avian group A and group D rotavirus as antigen, using a previously described technique (1). Briefly, 0.12 inch nitrocellulose membrane disks (Trans-Blot Transfer Medium, Bio-Rad Laboratories, Hercules, CA) were cut and placed onto the bottom of the wells of a 96-well, flat-bottom tissue culture plate (Becton Dickinson and Company, Lincoln Park, New Jersey). A micropipetter was used to deliver 0.50 ul avian group A rotavirus to the top portion (12 o'clock) of each nitrocellulose disc and an equal amount of avian group D rotavirus to the bottom portion (6 o'clock) of each nitrocellulose disc. A 1:200 dilution of antisera was added to wells in column 2. Two-fold serial dilutions were then made across the plate to achieve a dilution range of 1:100 to 1:102,400. The wells in column 1 did not contain

antisera and were used as a negative control wells. Following a series of incubations, with primary antibody and secondary biotinylated antibody (biotinylated anti-chicken IgG, Vector Laboratories, Burlingame, CA), the plates were incubated with streptavidin-HRP and the chromogen. The plates were developed, allowed to dry and visually observed for results. A dark purple dot against the white nitrocellulose background was considered a positive reaction. It is important to note that this procedure allowed the testing of both antigens simultaneously in each well.

Western Blot. Polyacrylamide gel electrophoresis (PAGE) of avian group A and group D viral proteins was carried out using the PhastSystem^{R} (Pharmacia, Piscataway, New Jersey). The purified virus preparations were incubated at 100C for 5 minutes in a buffer containing 5% sodium dodecyl sulphate (SDS). The viral proteins, dissociated into their polypeptide subunits, were then placed on a homogeneous 12.5% polyacrylamide gel with 2% crosslinking (PhastGel^{R}, Pharmacia, Piscataway, New Jersey). The proteins were separated at the following parameters, 250 V, 10.0 mA, 3.0 W, 15C, 85 Vh. The subsequent viral proteins and protein markers were then transferred from the PhastGel^{R} separation media to an immobilizing nitrocellulose membrane using PhastTransfer^{R} (Pharmacia, Piscataway, New Jersey) at 1.0 mA/cm^{2} to obtain high transfer recovery within 15 minutes for each gel. The nitrocellulose membrane, containing the separated proteins, was cut into 8 strips corresponding to the separation lanes. Each strip was incubated with group A rotaviral antisera, group D rotaviral antisera or sera negative for both group A and group D rotavirus. The rest of the procedure (development of the blots) was performed as described above for the ELISA.

Experimental Design. Each of the preceding techniques (AGID, VN, ELISA, and Western Blot) were repeated a minimum of three times. The hyperimmune sera for the group A and group D were pooled sera from turkeys vaccinated with the respective virus.

The sera negative for group A and group D rotavirus was collected from these turkeys prior to the time that they were immunized. The serum virus neutralization results were reported as neutralization titers. The neutralization titer was calculated as the reciprocal of the highest dilution of serum causing 80% reduction in fluorescent foci compared to the virus control (9). The data from the different trials was averaged to arrive at a single neutralization titer for each treatment.

RESULTS

Agar-gel immunodiffusion. Immunoprecipitation bands were observed between wells containing group A rotavirus and anti-group A serum and wells containing group D rotavirus and anti-group D serum (Fig. 1). Results are summarized in Table 1.

Immune Electron Microscopy. Viral-antibody complexes were formed when rotavirus was incubated with antisera to the respective serogroup (Fig. 2).

Viral neutralization (VN) assay. The data from three different trials was averaged to arrive at a single neutralization titer for each antiserum (Fig. 3). There was evidence of cross neutralization between group A rotavirus and anti-group D serum. Results displayed in Table 2.

ELISA. ELISA revealed a cross reaction between group A and group D rotaviruses. All controls were negative except for some background discoloration in the wells treated with group A and group D negative serum (Fig. 4). Results are summarized in Table 3.

Group D rotaviral proteins. This separation revealed ten identifiable protein bands (Fig. 5). The proteins ranged from 97kD to 16kD in molecular mass.

Western blot. Western blot techniques revealed a cross reaction between group A and group D rotaviruses. The group A anti-serum reacted with group A rotaviral proteins

at approximately 37kD, 45kD, 54/55kD, and 89kD. These proteins represent the avian group A rotaviral proteins VP7, VP6, VP5, and VP4 or VP3 respectively. All of these proteins, except VP6, are located in the outer capsid of group A rotaviruses. Also, a protein at approximately 115kD reacted with the group A anti-serum. This molecular mass does not correspond with a particular rotaviral protein. The group D anti-serum reacted with group A proteins at 37kD and 45kD (Fig. 6). The group D anti-serum reacted with group D rotaviral proteins in the range of approximately 43-60kD and 100-180kD. The group A anti-serum reacted with group D rotaviral proteins ranging from 44-62kD and with a protein at approximately 100 kD (Fig. 7).

Table 1. Results of agar-gel immunodiffusion testing of group A rotaviral antisera, group D rotaviral antisera and group A and group D negative serum using turkey group A rotavirus and pheasant group D rotavirus as antigens.

	Antisera		
Virus	anti-group A	anti-group D	group A and group D negative
group A rotavirus	+ [a]	-	-
group D rotavirus	- [b]	+	-

[a] (+) represents precipitation line formed.
[b] (-) represents no precipitation line formed.

Table 2. Results of one-way serum virus neutralization comparing the capability of anti-group A serum, anti-group D serum and group A and D negative serum to neutralize group A rotavirus infection in MA-104 tissue culture cells.

	Antisera		
Virus	**anti-group A serum**	**anti-group D serum**	**group A and group D negative serum**
group A rotavirus	640[a]	30	<10

[a] The neutralization titer is the reciprocal of the last dilution of antisera which neutralized 80% of the fluorescent foci present in the controls.

Table 3. Results of enzyme-linked immunosorbent assay using group A and D rotavirus antigen and antisera and Newcastle Disease Virus (NDV B1) antisera.

	Antigen	
Antisera	group A rotavirus	group D rotavirus
anti-group A	12,800[a]	800
anti-group D	400	51,200
group A and group D negative	NR[b]	NR
anti-NDV (B1)	NR	NR

[a] Reciprocal of the last serum dilution that reacted with the antigen.
[b] NR designates no reaction between this antigen and this antisera at dilutions of 1:100 to 1:102,400.

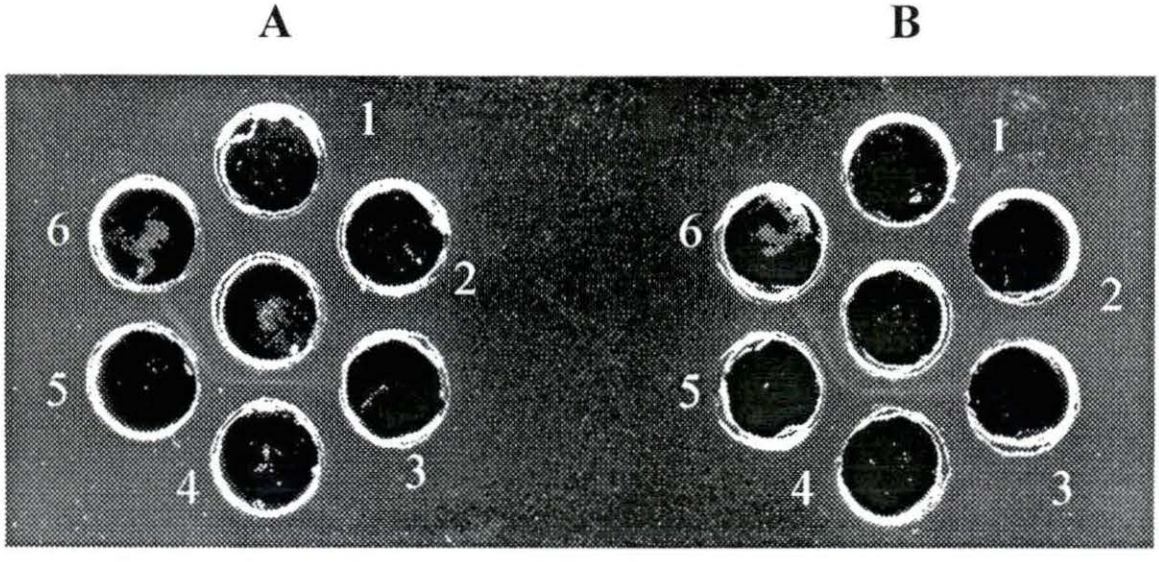

Fig. 1. Agar-gel immunodiffusion showing precipitate bands between group A rotavirus and group A antiserum and between group D rotavirus and group D antiserum. The rotaviral antigen is in the center wells; A: antigen is group D rotavirus, B: antigen is group A rotavirus. Antiserum is in the outer wells; A1: anti-group D (1:2), A2: anti-group D (1:10), A3: group A and group D negative (1:10), A4: anti-group A (1:2), A5: anti-group A (1:10), A6: group A and group D negative (1:2), B1: anti-group A (1:2), B2: anti-group A (1:10), B3: groupA and group D negative (1:10), B4: anti-group D (1:2), B5: anti-group D (1:10), and B6: group A and group D negative (1:2).

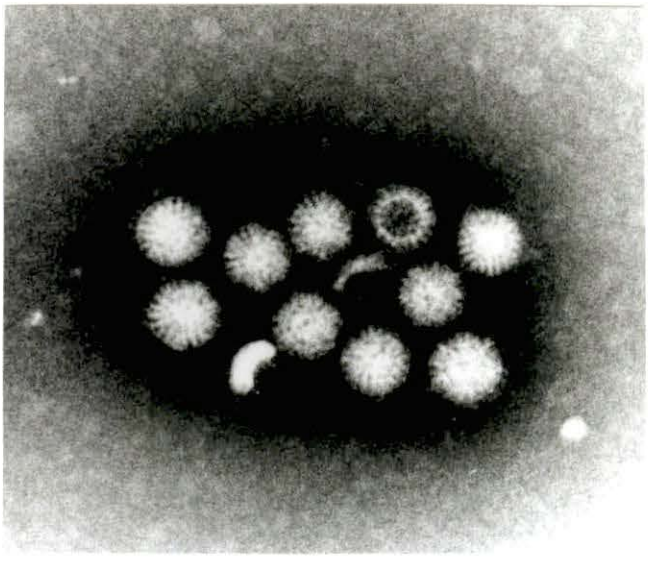

Fig. 2. Immune electron micrograph of negatively stained group D rotavirus particles (130,910x magnification).

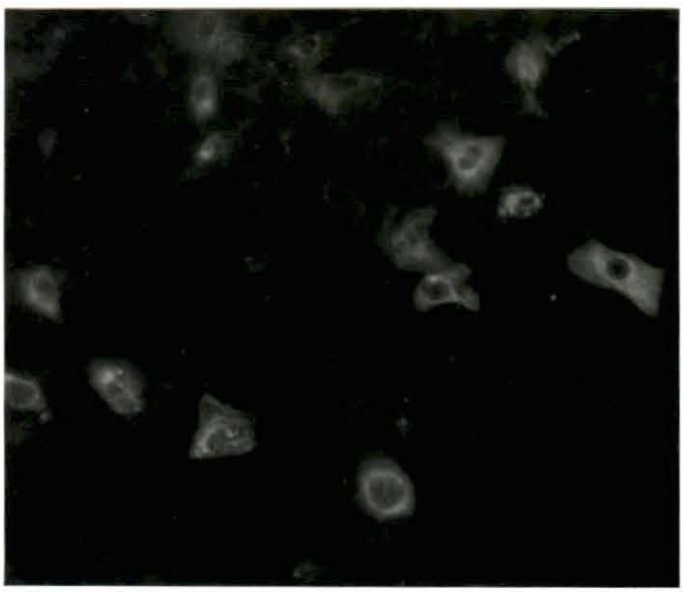

Fig. 3. Fluorescein conjugated rabbit anti-chicken IgG reacting with anti-group A antibody that is bound to MA-104 cells infected with group A rotavirus.

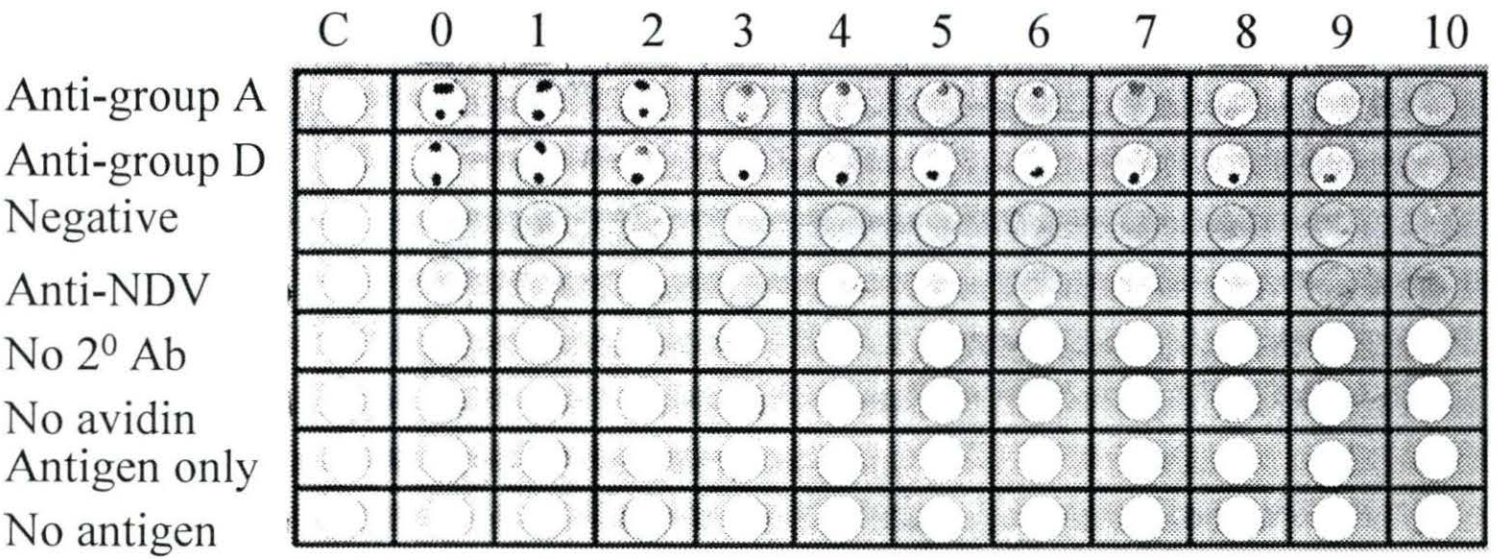

Fig.4. Enzyme-linked immunosorbent assay showing cross reaction between group A rotavirus and group D rotavirus. Each disk has group A rotavirus on top and group D rotavirus on the bottom. Column 1 is control. Other columns are labelled as the reciprocal $\log_2$ dilution x100.

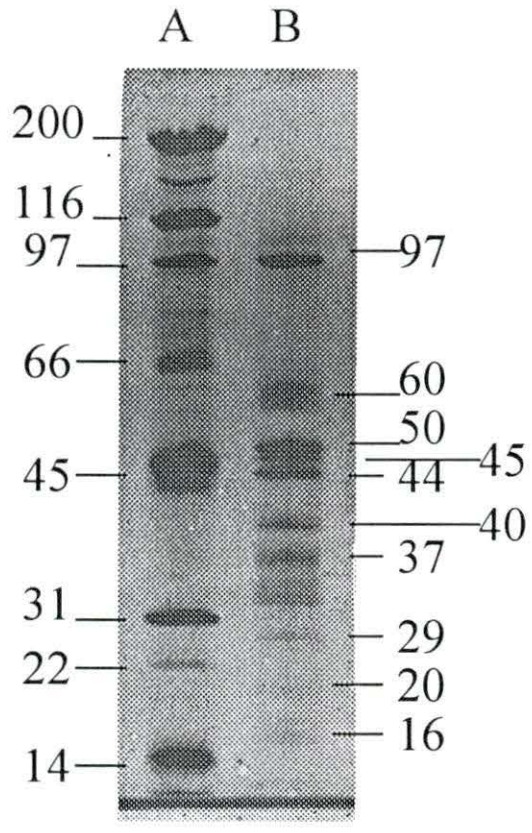

Fig.5. Polyacrylamide gel electrophoresis of the polypeptides from a group D rotavirus (column B). Column A represents marker proteins.

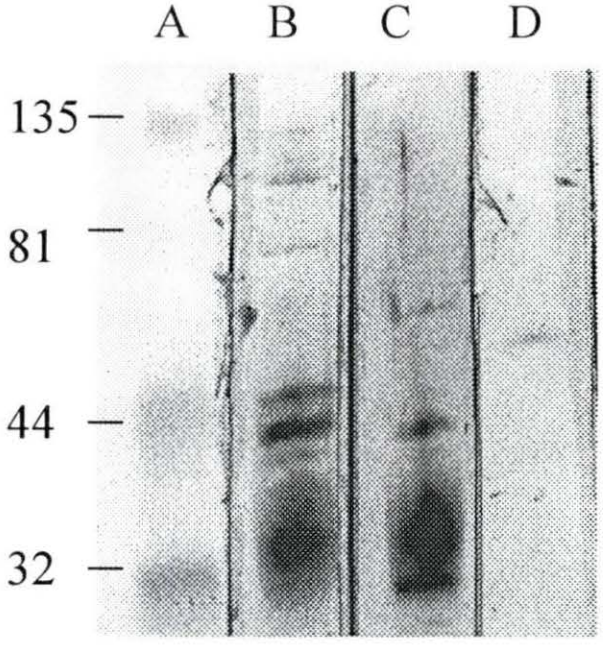

Fig. 6. Western blot of group A rotaviral proteins with prestained marker proteins in column A. Columns; B: group A antiserum, C: group D antiserum, D: group A and group D negative serum.

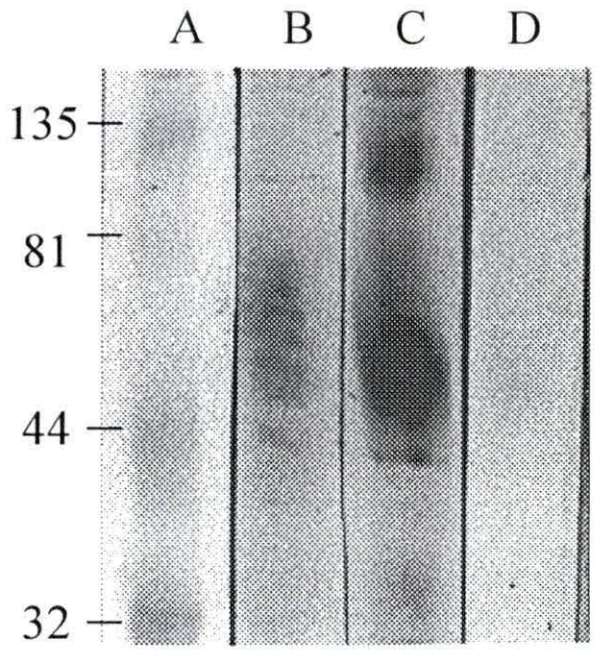

Fig. 7. Western blot of group D rotaviral proteins with prestained marker proteins in column A. Columns; B: group A antiserum, C: group D antiserum, D: group A and group D negative serum.

DISCUSSION

Although the polypeptides of avian group A rotaviruses have been characterized (5), information about the viral proteins of avian group D rotaviruses have not been reported in the literature. In this study the viral polypeptides of a group D rotavirus were separated and identified. Results, from dissociation and electrophorectic separation of the D polypeptides, revealed 10 viral proteins that ranged in molecular mass from 16kD to 97kD (16, 20, 29, 37, 40, 44, 45, 50, 60, and 97kD). Group A rotaviral structural proteins (VP1-VP7) range in molecular mass from 37kD to 125kD. The group D proteins were not further characterized at this time. The purpose for their identification was for antigenicity studies.

This study explored the antigenic relatedness of the group D rotavirus and a group A rotavirus. Group D rotaviruses are believed to play a major role in viral diarrhea of some poultry, especially in pheasants and turkey poults (14,15). It is important to futher characterize the group D rotaviruses to better understand their role in enteric disease. It has been noted that the group A rotaviruses also play a role in viral diarrhea (7). The avian group A rotaviruses, unlike the group D rotaviruses, can be propagated in vitro. This study took advantage of the ability to grow the group A rotavirus in the MA104 cell line. This virus was then antigenically compared to the group D rotaviruses that had been propagated in pheasant chicks.

Results of the agar gel immunodiffusion supported work done previously (2) that showed no cross reaction between these two different serogroups. In the work done by Devitt and Reynolds (2), it was reported that no cross reaction occurred using immune electron microscopy techniques.

The ELISA results revealed a cross reaction between the two rotaviruses. This technique demonstrated a two way cross reaction with the anti-group D serum reacting with

group A rotavirus up to a 1:400 antiserum dilution. Anti-group A serum reacting with group D rotavirus up to a 1:800 antiserum dilution. The antiserum used was from hyperimmunized turkeys which had a very high homologous titer of the respective group A or group D rotaviral antibodies. The cross reaction observed between the antisera from the two rotaviral groups occurred at relatively high dilutions. This fact supports the significance of the cross reaction and decreases the likelihood of nonspecific cross reactivity.

This antigenic relatedness was further explored using western blot studies. The data from this work once again supported that these two serogroups were antigenically related. This work demonstrated that the group D antiserum cross reacted with two group A viral proteins. These two proteins represented VP7 (37kD) and VP6 (45kD) which are believed to be responsible for virus neutralization and represent the group/subgroup antigen respectively. This finding could be expected since the group D antiserum at high concentrations neutralized the group A rotavirus. The group A antiserum also cross reacted with group D viral proteins. The proteins represented a block ranging from 44-62kD and a protein of approximately 100kD. The individual proteins were difficult to distinguish and identify due to poor separation distance and resolution. This reveals a need for future work that could potentially utilize a larger, more conventional size gel or monoclonal antibodies to the avian group A rotaviruses. The Phast Gel^{R} was chosen because it minimizes the amount of reagents needed for polypeptide separation and detection, and greatly decreases the amount of time normally needed for these procedures. A conventional size gel might provide additional gel length that would allow greater distance between each individual rotaviral protein and possibly better resolution. Use of monoclonal antibodies would increase the ability to interpret the exact group D protein with which the group A antibody is cross reacting and to which group A protein it is related.

This study also utilized a one-way serum virus neutralization. The inability to propagate the group D rotavirus in a continuous cell line necessitated the one-way assay. The results once again supported that antigenic relatedness of the group A and group D rotaviruses exists. A neutralization titer was calculated to help interpret the data. This titer was the reciprocal of the highest dilution of serum that caused an 80% reduction in the fluorescent foci compared to the virus control. Although the anti-group A serum showed the greatest neutralization titer, the anti-group D serum at high concentrations also appeared to have the capability to neutralize group A rotavirus. The neutralization titer of the anti-group D serum was more than two dilutions greater than the control serum (rota A and rota D negative serum). The group A and group D negative serum, at the dilutions used, was not able to cause an 80% reduction in FFU, even at the highest serum concentration.

The results from this study revealed a definitive antigenic cross reaction between the group A and group D rotavirus. In addition, the viral polypeptides of the group D rotavirus were identified. Information about the antigenic relatedness of different serogroups may be beneficial in strategies in the prevention of rotaviral disease.

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APPENDIX

Table 1. Results from three one-way serum-virus neutralization assays using group A rotavirus and anti-group A serum. The values in the table represent the number of fluorescent forming units (ffu) counted in each well.

no serum	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
Trial 1										
22	1	2	4	1	2	3	5	13	19	31
36	3	3	2	8	5	5	5	14	32	42
18	2	0	2	4	12	3	4	20	25	39
31	4	3	4	5	6	3	3	11	24	36
23	0	6	3	7	1	6	2	12	13	27
Trial 2										
156	5	8	12	13	11	19	31	35	62	>80
121	7	6	9	14	14	15	26	39	46	>80
134	4	8	5	9	9	15	32	33	66	>80
Trial 3										
160	5	8	11	7	11	13	18	36	53	>80
148	5	7	9	10	12	17	24	38	63	>80

Table 2. Results from three one-way serum-virus neutralization assays using group A rotavirus and anti-group D serum. The values in the table represent the number of fluorescent forming units (ffu) counted in each well.

no serum	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
Trial 1										
32	4	2	6	8	20	25	30	35	>35	>35
35	3	8	11	10	21	23	26	30	>35	>35
29	4	6	12	14	20	18	31	37	>35	>35
28	6	7	7	10	13	14	26	28	>35	>35
23	3	6	10	12	18	18	21	24	>35	>35
Trial 2										
162	6	10	10	32	57	>80	>80	>80	>80	>80
141	9	11	12	26	56	>80	>80	>80	>80	>80
170	9	8	10	39	51	>80	>80	>80	>80	>80
Trial 3										
166	10	12	12	27	64	>80	>80	>80	>80	>80
161	6	12	11	31	49	>80	>80	>80	>80	>80
144	9	10	14	39	54	>80	>80	>80	>80	>80

Table 3. Results from three one-way serum-virus neutralization assays using group A rotavirus and group A and group D negative serum. The values in the table represent the number of fluorescent forming units (ffu) counted in each well.

no serum	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
Trial 1										
77	16	33	34	20	32	37	36	45	55	70
62	21	18	23	26	24	44	41	43	57	68
122	19	21	25	45	49	47	43	47	48	61
101	18	20	19	25	41	39	36	39	48	63
95	19	25	26	30	34	39	42	45	49	61
Trial 2										
129	43	49	>50	>50	>50	>50	>50	>50	>50	>50
138	36	38	>50	>50	>50	>50	>50	>50	>50	>50
125	35	42	>50	>50	>50	>50	>50	>50	>50	>50
Trial 3										
110	24	34	>50	>50	>50	>50	>50	>50	>50	>50
97	23	22	>50	>50	>50	>50	>50	>50	>50	>50

GENERAL CONCLUSIONS

The purpose of this study was to explore the antigenic relatedness of a group A and a group D rotavirus. In previous studies, these two rotavirus groups had been shown to be distinctly different groups based on serologic assays and electropherotyping. The present study, using ELISA, virus neutralization and western blot techniques, revealed an antigenic cross reaction between these two groups of avian rotavirus.

In this study, one-way serum virus neutralization revealed that the group D anti-serum was able to neutralize group A rotavirus. Although the neutralization titer for the group D anti-serum was lower than for the group A anti-serum, this serum did show neutralizing ability that exceeded the group A and group D negative serum. The ELISA and western blot techniques both revealed a two way cross reaction between the group A and group D rotaviruses. The cross reactions that were observed with the ELISA occurred at relatively high dilutions of the antiserum providing a high degree of confidence that the reaction was not nonspecific reactivity. Western blot results revealed a reaction of group D antiserum with group A rotaviral proteins at 37kD and 45kD. These proteins may represent VP7 and VP6 respectively. The VP7 protein is believed to be responsible for group A rotaviral neutralization. This may explain the neutralization of group A rotavirus by group D antiserum. The group A antiserum also cross reacted with group D viral proteins ranging from 44-62kD and a protein at 100kD. Although the group D proteins were identified, they

were not further characterized. At this time it is not possible to identify which group D rotaviral proteins are represented at these molecular masses.

A problem encountered in this research involved the western blot technique. This technique at first would not produce homologous or heterologous reaction between the group A and group D rotaviruses and their respective antisera. This problem was solved by deleting the use of beta-mercaptoethanol from the procedure and carefully monitoring the amount of time that the viruses incubate at 100C in the 5% SDS solution.

This study also utilized polyacrylamide gel electrophoresis (PAGE) to separate the group D rotaviral polypeptides. SDS-PAGE revealed 10 group D rotaviral proteins that ranged in molecular mass from 16kD to 97kD (16, 20, 29, 37, 40, 44, 45, 50, 60, and 97kD). There have also been 10 major polypeptides identified in group A rotaviruses. These group A polypeptides range in molecular mass from 125kD to 26kD (Kang et al., 1987).

This study supports an antigenic relatedness of group A and group D rotaviruses and provides for future research on avian rotaviral vaccines and cross reaction between different rotaviral serogroups. The new information concerning the antigenic relatedness of different rotaviral serogroups may be beneficial in identifying those proteins important in developing strategies for prevention and control that will take advantage of a heterotypic rotaviral immune response.

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