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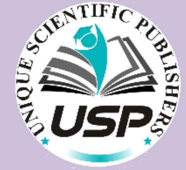
P-ISSN: 2304-3075; E-ISSN: 2305-4360

International Journal of Veterinary Science

www.ijvets.com; editor@ijvets.com



Short Communication https://doi.org/10.47278/journal.ijvs/2024.159

Evidence for Antibody Dependent Enhancement for an Avian Coronavirus

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Article History: 24-421 Received: 12-Feb-24 Revised: 22-Mar-24 Accepted: 26-Mar-24

ABSTRACT

Avian infectious bronchitis (IB) is caused by a gamma coronavirus and is a highly infectious disease of chickens that is problematic for commercial chicken producers around the world. A major problem for managing this disease is the ability of the infectious bronchitis virus (IBV) to mutate and generate new variants and serotypes that yield current vaccines less protective. Numerous new serotypes and variants have emerged that are unique to geographical regions around the globe. Antibody dependent enhancement (ADE) is a phenomenon in which either non-neutralizing antibodies, or suboptimal (i.e., low) levels of neutralizing antibodies, facilitate entry into cells and promote increased viral replication. In this short communication, we report preliminary *in vitro* evidence of ADE using a chicken egg embryo model and a vaccine strain of IBV. To our knowledge, ADE has neither been reported nor explored for any viral disease of birds.

Key words: Antibody dependent enhancement, Infectious bronchitis virus, Chicken coronavirus

INTRODUCTION

Avian infectious bronchitis (IB) is a highly infectious viral disease that is problematic for commercial chicken producers around the world. The disease occurs primarily in chickens and infects broilers, layers, and breeder birds of all ages (Jackwood and de Wit 2020). Clinically, infectious bronchitis virus (IBV) causes respiratory, reproductive, and kidney diseases (Jackwood and de Wit 2020). In broiler chickens, IBV causes respiratory disease and increases flock morbidity (i.e., decreased weight gains, decreased feed efficiency, and increased processing condemnations) and mortality. In laying chickens and breeder birds, IBV causes reproductive disease resulting in decreased egg production, misshapen eggs, poor egg quality, etc. Avian infectious bronchitis virus is an enveloped, single-stranded RNA, gamma coronavirus (Jackwood and de Wit 2020). Although commercial vaccines are available for controlling the disease, the ability of the IBV to mutate and generate new variants and serotypes can yield current vaccines less protective. These new mutant IBV strains emerge in various geographical regions making IB difficult to diagnose and problematic to control (Jackwood 2012). The mechanisms by which new strains and serotypes of IBV emerge have been reviewed and reported (Jackwood 2012; Toro et al. 2012). The report by Toro et al. (2012), addresses the fact that IBV is of moderate virulence (and relatively low lethality) and is highly infectious and lends itself to a high rate of mutation. The report identifies two mechanisms responsible for IBV evolution. The first mechanism, termed by the authors as "generation of genetic diversity", involves the rapid replication, high mutation rate, and recombinant events characteristic of coronaviruses (Jackwood 2012; Toro et al. 2012). These events are followed by the second mechanism of "selection". Selection of the emerging viruses involves numerous host factors such as immune responses, viral affinity for cell receptors and other physical and biochemical conditions (Toro et al. 2012).

Antibody dependent enhancement (ADE) is a phenomenon in which either non-neutralizing antibodies, or suboptimal (i.e. low) levels of neutralizing antibodies, facilitate entry into cells and promotes increased viral replication (Morens 1994; Takada and Kawaoka 2003). The first reports of ADE are credited to Hawkes (1964) and Hawkes and Lafferty (1967) in the 1960s whereby, ADE was observed *in vitro* using immune avian antisera to neutralize a homologous flavivirus in chick cells. At the time, the results were largely ignored by the scientific community (Morens 1994). Some 10 years later, epidemiology studies were conducted that established the connection between *in vitro* ADE, as demonstrated by

Cite This Article as: Reynolds DL, Simpson EB and Hille MM, 2024. Evidence for antibody dependent enhancement for an avian coronavirus. International Journal of Veterinary Science 13(5): 707-711. https://doi.org/10.47278/journal.ijvs/2024.159

Hawkes, and severe dengue disease in humans (Halstead and O'Rourke 1977; Morens 1994). Subsequent investigations documented ADE and dengue disease and provided insight on the mechanisms of ADE and the pathogenesis of dengue disease (Morens 1994; Takada and Kawaoka 2003). ADE has been documented to occur with other viruses including Ebola virus, HIV, togaviruses, arteriviruses, picornaviruses, paramyxoviruses, rhabdoviruses, arenaviruses, orthomyxoviruses, reoviruses, parvoviruses, papovaviruses, poxviruses, herpesviruses and coronaviruses (Takada and Kawaoka 2003). Those coronaviruses in which ADE have been demonstrated include feline infectious peritonitis virus (Olsen et al. 1992) and Middle East respiratory syndrome coronavirus (MERS-CoV) (Houser et al. 2017). Recently, much effort has been directed towards COVID-19 vaccine development and this has raised concerns regarding ADE with respect to suboptimal levels of antibody in response to COVID-19 vaccines / vaccination and to preexisting cross-reacting (but non-neutralizing) antibody levels from other coronavirus infections (Fierz and Walz 2020; Iwasaki and Yang 2020). Additionally, recent reports indicate mutations in SARS-CoV-2 and the potential for new emerging strains to occur (Holland et al. 2020).

Five mechanisms are described as the molecular basis for ADE when sera contains either non-neutralizing or suboptimal levels of antibody to neutralize the virus in question (Takada and Kawaoka 2003; Kulkarni 2019; Wan et al. 2020). The first mechanism is referred to as Fc receptor (FcR) -dependent ADE and is an interaction between the virus-bound antibody and the Fc receptor found on the host cell. A second mechanism occurs in a similar manner through the complement receptors (C1r, C1q, C1s, C3) of cells and immune sera containing antibodies. A third mechanism is similar to the complement receptor in which the complement receptors (C1q, C1r) induce a conformational change in the virus-antibody complex and allows for attachment to the host cell C1q receptor. A fourth mechanism is by the antibody providing a conformational change in the virus-antibody complex which facilitates fusion to the host cell membrane. A fifth mechanism occurs after the virus has entered the cell via the FcR-dependent mechanism and further viral replication suppresses host cell antiviral gene expression (Takada and Kawaoka 2003).

To our knowledge ADE has neither been reported nor explored for any avian / poultry virus (disease). IBV is an ideal candidate for exploring ADE in the avian species because of the large populations of poultry occurring worldwide in specific geographic locales that are vaccinated with different commercially available vaccines, the different serotypes and variants that exist within these geographical regions, and the emergence of new variant IBV strains within these locales. We hypothesize that ADE occurs in avian IBV infections and plays a significant role in the emergence of new serotypes through the host immune mechanism of IBV selection (discussed above). In this short communication we report *in vitro* evidence of ADE occurring with the avian coronavirus, IBV.

MATERIALS AND METHODS

Ethical statement

This study was conducted at the University of Nebraska–Lincoln. Those protocols utilizing animals were approved by the Institutional Animal Care and Use Committee (Project ID 2114 and 1874).

Birds, eggs and blood samples

Specific pathogen free (SPF) layer-type chickens were hatched from SPF eggs obtained from VALO BioMedia (Adel, IA, USA) and reared in a biocontainment 2 facility. Eggs were obtained from the same source and incubated in our facility to obtain chicken embryos. Blood samples were collected from SPF birds by the wing-vein method.

Virus

The live virus vaccine strain (Massachusetts Type, Merial, Inc., Athens, GA) was used in this study. The Massachusetts strain of IBV was egg adapted by reconstituting the live virus vaccine in phosphate buffered saline (PBS) and inoculating the virus preparation into 9–12-day old embryonating eggs by the chorioallantoic (CA) route as previously described.(Lukert 1966; Hitchner 1973; Gelb and Jackwood 2008). The IBV was passaged three times in embryonating eggs and then titrated in egg embryos prior to usage in the trials described below. A virus neutralization test using constant homologous virus and diluted sera employing both positive and negative control sera was performed to ensure the expected level of sensitivity and specificity of the IBV antisera and to provide a serologic titer to compare with the ELISA results (see below).

Generation of IBV antibodies

Hyperimmune avian antisera from SPF birds vaccinated with the Massachusetts IBV serotype was obtained. Briefly, the birds were vaccinated with the Massachusetts IBV serotype that had been egg adapted (see above). Six three-week-old SPF birds were vaccinated subcutaneously with approximately 100 egg infectious dose 50% (EID_{50}) per injection/bird. Another group of six hatch mates were vaccinated with sterile PBS and used for generating negative control sera. The birds were booster vaccinated at six and nine weeks of age. Antibody titers were monitored using IBV ELISAs (IDEXX kits, IDEXX Laboratories, Inc., Westbrook, ME, USA) and were performed in the Nebraska Veterinary Diagnostic Center (NVDC). Blood was collected at 9 and 12 weeks of age by the wing-vein method. Sera was separated from the blood by low-speed centrifugation and pooled from each group of birds which was then stored at -80C. The antibody titer (i.e., ELISA titer) was determined for each pool of antisera prior to storage and prior to use.

Virus detection

IBV was detected from chicken egg embryos by two methods. First, embryonic death and lesions were observed as previously reported (Gelb and Jackwood 2008). Second, a quantitative polymerase chain reaction (qPCR) was used to detect and quantify the IBV. The IBV qPCR procedures were conducted by the NVDC using the Infectious Bronchitis virus RNA Test Kit (BioChek (UK) Ltd., Berkshire, UK) and the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Experimental design

Trial 1. Trial 1 was conducted to obtain a serum neutralization titer of the antiserum which had an ELISA titer of 9566. The egg propagated IBV was diluted with PBS and aliquoted into separate tubes. The immune antiserum was diluted two-fold beginning at 1:100 dilution through 1:3200 dilution. Negative serum was also included at dilutions of 1:100 and 1:200 as was a PBS only inoculum (null group). The eggs were inoculated with six EID_{50} and evaluated at 72 hours post-inoculation for virus infection using embryonic lesions and the qPCR assay (see above). The numbers of egg embryos per treatment group are presented in Table 1. The method of Reed and Muench was used to calculate an endpoint titer as previously described (Villegas 1998).

Trial 2. The egg propagated IBV was diluted with PBS to provide one EID_{50} and aliquoted into separate tubes and either PBS or diluted IBV immune antiserum (diluted with PBS) was added to each tube. The immune antiserum was found to have an ELISA titer of 4655 and was diluted two-fold beginning at 1:32 through 1:256. Ten-day-old SPF chicken embryos were then inoculated by the CA route with either 0.2mL of PBS + virus or 0.2mL of the diluted antiserum + virus preparations. The eggs were evaluated at 72 hours post-inoculation using embryonic lesions and the qPCR assay. The numbers of egg embryos per treatment group are presented in Table 2.

Trial 3. Trial 3 was conducted similarly to trial 2 whereby, the egg propagated IBV was diluted with PBS to provide one EID_{50} and aliquoted into separate tubes. To the viral aliquots either PBS or diluted IBV immune antiserum (diluted with PBS) was added. The immune antiserum was found to have an ELISA titer of 7463 and was diluted two-fold beginning at 1:100 through 1:800. Ten-day-old SPF chicken embryos were then inoculated by the CA route with either 0.2mL of PBS + virus or 0.2mL of the diluted antiserum + virus preparations. The eggs were evaluated at 72 hours post inoculation using embryonic lesions and the qPCR assay. The number of egg embryos per treatment group are presented in Table 3.

Statistical analysis

The Chi-square and Fisher's exact tests were used to determine statistical significance and equality.

RESULTS AND DISCUSSION

The report on ADE by Hawkes and Lafferty (1967) states "Immunological enhancement can only be observed when the test virus has a low EOP in the assay system, since enhancement basically represents an increased efficiency in the initiation of infection by the virus. Greater enhancement being observed when the EOP of the virus was low." In the report by Hawkes and Lafferty (1967) EOP (efficiency of plating) refers to a low viral inoculum and equates to a low egg infectious dose 50% (EID_{50}) used in the present study.

Trial 1 was performed to establish a serum-virus neutralization end point titer of the IBV anti-serum obtained from chickens receiving the second booster IBV vaccinations. The ELISA titer of the antiserum used in this trial was 9566. In this trial, a viral inoculum of six EID_{50} was used. Additionally, the effects (or lack thereof) of IBV negative chicken sera, obtained from unvaccinated SPF chickens, was evaluated. The results of trial 1 are presented in Table 1 and Fig. 1. The results of trial 1 are typical (and expected) in a serum-virus neutralization assay. The PBS only (i.e., no virus) inoculated group had no infected embryos (or dead embryos) indicating the CA inoculation procedure had no negative effects on the embryos or trial results. It was determined that the virus inoculum devoid of antisera infected 13 of 15 embryos or 86.7%. The negative serum, at both dilutions, neither inhibited nor enhanced viral replication and was deemed to have no effect. The positive IBV antiserum dilutions provided viral neutralization at the lower dilutions, but the neutralization effects waned as the dilutions were increased. Although the 1:1600 and 1:3200 dilution groups had higher percentages of infected embryos than the virus inoculated group, there was no statistically significant difference between them. The endpoint titer was calculated using the method by Reed and Muench (Villegas 1998) and was found to be log 3.64 or 4,365 when expressed arithmetically.

The results of trial 2 are displayed in Table 2 and Fig. 2. Trial 2 was the first preliminary trial to determine if ADE occurs with IBV. The results of trial 2, as depicted in Fig. 2, indicate an enhanced viral replication. That is, there was increased (i.e., enhanced) viral replication in the 1:128 antiserum dilution compared to the viral inoculum devoid of antiserum. Unfortunately, the numbers of egg embryos utilized in trial 2 were not sufficient for any meaningful statistical evaluation. However, the results were indicative of ADE occurring with IBV and prompted us to perform trial 3. Note that the ELISA titer of the IBV antisera used in trial 2 was 4655. This IBV anti-serum was prepared from blood obtain after the first IBV booster vaccination of the SPF chickens.

Table 1: Results of a serum-virus neutralization assay with infectious bronchitis virus

Group	Serum Dilution	N	IBV Neg	IBV Pos	
				No.	%
PBS no IBV	-	15	15	0	0.00
PBS + IBV	-	15	2	13	86.7
Neg* serum + IBV	1:100NS	15	1	14	93.3
Neg serum + IBV	1:200NS	15	0	15	100.0
Pos** serum + IBV	1:100PS	15	15	0	0.0
Pos serum + IBV	1:200PS	11	10	1	9.1
Pos serum + IBV	1:400PS	13	11	2	15.4
Pos serum + IBV	1:800PS	14	8	6	42.9
Pos serum + IBV	1:1600PS	15	0	15	100.0
Pos serum + IBV	1:3200PS	15	1	14	93.3

*Negative serum; **Positive IBV antiserum

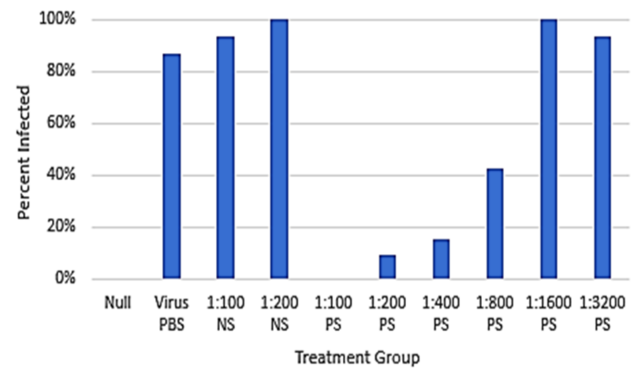

Fig. 1: Results of trial 1. IBV serum-virus neutralization assay using six EID_{50}. Null=uninoculated embryos, NS=Negative sera, PS=Positive IBV antisera.

Table 2: Results of preliminary trial indicating occurrence of antibody dependent enhancement (ADE)

Group	Serum Dilution	N	IBV Neg	IBV Pos	% Pos
PBS + IBV	-	5	2	3	60.0
Pos serum* + IBV	1:32	12	10	2	16.7
Pos serum + IBV	1:64	12	8	4	33.3
Pos serum + IBV	1:128	12	3	9	75.0
Pos serum + IBV	1:256	12	7	5	41.7

*Positive IBV antiserum; Neg=Negative; Pos=Positive.

Table 3: Results of second trial demonstrating antibody dependent enhancement (ADE)

Group	Serum Dilution	N	IBV Neg	IBV Pos	% Pos
PBS + IBV	Virus	20	6	14	70.0
Pos serum* + IBV	1:100	20	20	0	0.0
Pos serum + IBV	1:200	20	16	4	20.0
Pos serum + IBV	1:400	20	2	18	90.0
Pos serum + IBV	1:800	20	1	19	95.0

*Positive IBV antiserum; Neg=Negative; Pos=Positive.

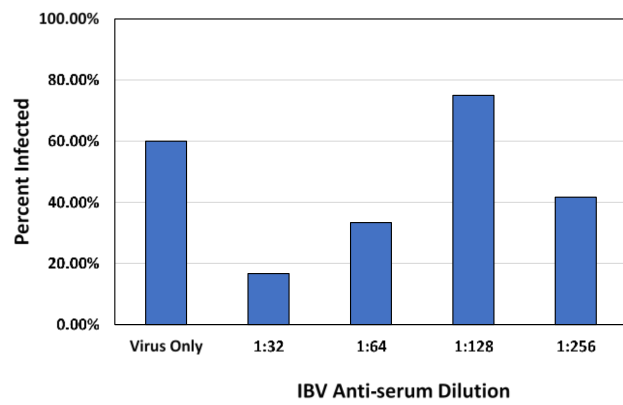

Fig. 2: Results of trial 2. IBV serum-virus neutralization assay using a low virus inoculum displaying antibody dependent enhancement.

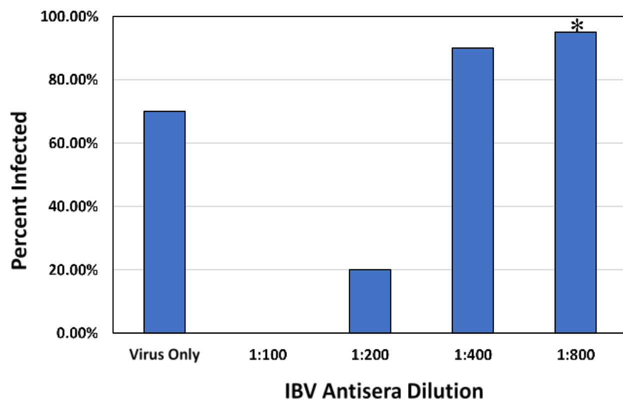

Fig. 3: Results of trial 3. IBV serum-virus neutralization assay using a low virus inoculum displaying antibody dependent enhancement. *P<0.05

The results of trial 3, demonstrating ADE, are displayed in Table 3 and Fig. 3. It should be noted that the IBV antiserum used in trial 3 had an ELISA titer of 7463. The results of trial 1 whereby, the IBV antiserum with an ELISA titer of 9566 and resulting in an endpoint serum-virus neutralization titer of 4,365, led to the conclusion that using IBV antiserum dilutions in trial 3 from 1:100 through 1:800 would result in an adequate range. Retrospectively, an additional dilution (or two) would have been advantageous. The IBV inoculum in trial 3 was slightly higher than one EID_{50} as evidenced by the 70% infection rate of the virus inoculum group. However, the IBV antiserum dilution of 1:800 treatment group had more positive embryos and was statistically significantly higher than the number of positive embryos in the virus inoculum devoid of antiserum group (P=0.0457). Table 4 contains the results of the statistical comparisons performed on trial 3 data. Statistical analysis among the five groups (one control and four treatment groups) showed significant difference at least with one group from the remainder (Chi-square value=58.99, df=4, P=4.729e-12). To detect the different group(s), instead of a pairwise comparison, only the differences in the treatment group in comparison with the control group were evaluated. The Fisher's exact test was used, because it is preferable to the chi-squared test in the situation with relatively small sample sizes. The findings show that the infection percentage in the group of 1:800 is statistically higher than that in the control group, while a moderate difference, albeit nearly significant (P=0.1176), was observed for the group of 1:400. Results are summarized in Table 4. Note that it was hypothesized to have a higher infection percentage in those groups with higher dilution factors, which is indicative of the occurrence of antibody-dependent enhancement phenomenon. Due to this reason, a one-sided hypothesis was tested for the 1:400 and 1:800 dilution groups.

Table 4: Statistical significance of trial 3 using the Fisher's exact test

Group	Control	1:100	1:200	1:400	1:800
Test hypothesis	-	Two sided	Two sided	Greater	Greater
P value	-	3.34e-06	0.0036	0.1176	0.0457*

*Statistically higher than the control group (P<0.05).

In this study homologous antiserum was used with the homologous virus. It was evident that for ADE to occur, a low viral inoculum paired with an optimum antibody dilution (i.e., high enough not to cause neutralization yet having adequate antibody to facilitate ADE) are essential conditions. With good vaccination practices and excellent vaccines, the likelihood of these optimum conditions to occur *in vivo* to allow ADE in commercial poultry production does not seem plausible. However, the world production of commercial chickens number in the billions with the U.S. producing an estimated 9.17 billion broiler chickens in 2022 (USDA and NASS 2023). The use of live IB vaccines is widely practiced and has been attributed to generating IBV variants (Toro 2021). Recognizing that there is an enormous global chicken population and that the use of various live IBV vaccines (and various vaccine strategies) may result in inconsistent immunity of birds, it is reasonable to assume these factors may play an important role in increasing the probability of ADE occurring. Clearly, more work is warranted to further explore and document ADE with IBV and other avian viruses. However, this initial report documents ADE occurring *in vitro* and the potential for ADE to occur *in vivo*. ADE should be considered as another significant factor for the propagation of new IBV serotypes in chicken populations. Therefore, it behooves poultry producers to properly immunize and monitor their flocks for the prevention and control of IB.

Conclusion

This report demonstrates ADE occurring *in vitro* with the avian infectious bronchitis virus and discusses the potential for ADE to occur in commercial poultry. ADE should be considered as another significant factor for the emergence of new IBV serotypes in chicken populations.

Acknowledgements

The authors acknowledge the statistical expertise and consultation provided by Professor Katherine Hanford at the University of Nebraska–Lincoln. Support for this project was provided in part by the USDA multistate research project, NC1180.

Author's Contribution

DLR: Principal investigator; concept, study design, data analysis, writing manuscript, securing project funding. EBS: Research manager; laboratory protocols and techniques, data collection, manuscript review. MMH: Veterinary pathologist; confirming embryo lesions, manuscript review.

Conflict of interest statement

The authors declare no conflict of interest.

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