

**Identification of amino acids involved in a serotype  
and neutralization specific epitope within the s1 subunit  
of avian infectious bronchitis virus**

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**Summary.** Localization of neutralizing, serotype specific epitopes of infectious bronchitis virus has been difficult because these epitopes are conformationally dependent. We identified amino acids involved in a serotype specific, conformationally dependent epitope by analysis of the S1 gene of 13 monoclonal antibody-neutralization-resistant mutants. Substitutions in the predicted amino acid sequence of these mutants were located at residues 304 and/or 386. Most of the substitutions at residue 304 were from threonine to isoleucine, whereas the substitutions at residue 386 were from arginine to proline, histidine, cysteine, or tryptophan. Based on this data, it appears that AA residues at 304 and 386 on the S1 glycoprotein are involved in a virus neutralizing serotype specific epitope.

### **Introduction**

Avian infectious bronchitis virus (IBV), the etiologic agent of infectious bronchitis (IB), causes an upper respiratory tract disease in chickens. In addition to respiratory disease, IBV may cause damage to the reproductive tract, and some strains cause nephritis and urolithiasis. Economic losses are due to poor weight gains in broilers, egg production losses, and decreased egg quality in layers and breeders [14]. Multiple IBV serotypes complicate vaccination programs due to a lack of cross protection between serotypes [6, 10, 21].

Infectious bronchitis virus, a member of the Coronaviridae family, contains the following structural proteins – a phosphorylated nucleocapsid protein, a membrane glycoprotein, a small membrane protein, and a spike glycoprotein. The spike glycoprotein is post-translationally cleaved into S1 and S2 subunits [14]. The S1 subunit forms a globular structure anchored to the viral membrane by the S2 subunit [2]. The S1 subunit is associated with virus neutralization, hemagglutination (HA), membrane fusion, and attachment [3, 4, 15, 18, 19].

The total number of neutralizing epitopes on the S1 subunit of the spike glycoprotein is not known. However, based on data obtained using monoclonal antibodies (Mab), there appears to be at least three to five neutralizing, conformationally dependent epitopes on the S1 subunit in different IBV strains [13, 15, 20]. One or more of these epitopes is a serotypic determinant [13]. For production of molecularly engineered vaccines, it is important to localize areas of the S1 gene encoding these conformationally dependent epitopes.

Mab-neutralization-resistant (NR) mutants have been used to identify amino acids (AA) involved in conformationally dependent epitopes in the spike protein of IBV [5, 12]. Based on AA substitutions in Mab-NR mutants, the S1 subunit was divided into three regions associated with neutralizing, conformationally dependent epitopes. These are designated I (residues 38–67), II (residues 91–141), and III (residues 274–387) [12, 16]. Region I, corresponding to hypervariable region (HVR) I, was associated with a M41 strain-specific, neutralizing, and HA epitope due to a substitution at residue 63 [5]. Other regions of AA variation, HVR II and residues 269–365, were associated with neutralizing epitopes in regions II and III, respectively [12].

The objective of this study was to identify AA involved in the serotype-specific, neutralizing epitope on the Arkansas (Ark) DIP strain of IBV. This extends the work of Kant et al. [12] and Cavanagh et al. [5] by identifying specific AA involved in an epitope [5] that is neutralizing and serotype specific.

## Materials and methods

### *Virus strains and propagation*

In this study, the Ark DPI strain was used [7]. The Ark DPI strain was adapted to replicate in primary chicken embryo kidney cells (CEKC) by 11 serial passages (designated Arkp11) until cytopathic effects (CPE) were observed. Virus was propagated in developing specific pathogen free (SPF) chicken embryos at 9 to 11 days of incubation, or on monolayers of primary CEKC [8, 11]. Monolayers were grown in M199 culture medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL), 40% of F-10 nutrient mixture (Gibco BRL), 2% tryptose phosphate broth (Gibco BRL), 2.7% of a 7.5% sodium bicarbonate solution (Gibco BRL), 50 mM HEPES (Gibco BRL), 50 µg/ml of gentamicin sulphate (Gibco BRL), and 2.5 µg/ml of fungizone (Atlanta Biologics, Norcross, GA, USA). Monolayers were maintained with the M199 culture media supplemented as above except 1% calf serum (Gibco BRL) was used.

### *Monoclonal antibody and hemagglutination inhibition test*

Monoclonal antibody 1318, previously described, neutralizes the Ark serotype of IBV, and is specific for a conformationally dependent epitope on the S1 subunit [13]. Hemagglutination inhibition (HI) tests were conducted by procedures previously described using Mab 1318 and the Ark 99 strain obtained from SPAFAS (Norwich, CT, USA) [7].

### *Production of Mab-NR mutants*

The experimental design was based on the work reported by Cavanagh et al. and Kant et al. [5, 12]. Briefly, Mab-NR mutants were selected by mixing virus ( $1.4 \times 10^6$  plaque forming

units (PFU)/ml) and Mab 1318 (undiluted, 1 ml neutralizes  $1 \times 10^6$  PFU of virus) in equal volumes, and incubating at 25 °C for 1 h [11]. The chorioallantoic sac of 11-day-old developing chicken embryos was inoculated with 0.2 ml of the mixture, and incubated for 48 h at 37 °C in a humidified incubator. Chorioallantoic fluid was collected, and diluted 1:100 with growth medium. Ten-fold serial dilutions of Mab 1318 were mixed with an equal volume of chorioallantoic fluid, and incubated at 25 °C for 1 h. The mixture was inoculated on a monolayer of CEKC, and incubated at 37 °C for 1 h before the agar overlay was added. Plaques were suspended in 0.5 ml media, and stored at -70 °C. Some Mab-NR mutants were plaque-purified twice before viral stocks were made in CEKC.

#### *Identification of Mab-NR mutants*

Reverse transcriptase-polymerase chain reaction (RT-PCR) with primers MIBVPCR and NIBVPCR amplify a conserved region in IBV confirming Mab NR mutants as IBV [1]. Reverse transcriptase-polymerase chain reaction using primers S1OLIGO5' and S1OLIGO3', and restriction fragment length polymorphism (RFLP) of the S1 gene was used to serotype the mutants [17].

#### *Neutralization experiments*

The titer of each Mab NR mutant and Arkp11 was determined by limiting dilution in a CEKC microtiter assay. Ten-fold serial dilutions of the Mab-NR mutants were inoculated (0.05 ml) onto CEKC monolayers with 0.15 ml of M199 culture medium supplemented with 1% calf serum. The titers were determined by the reciprocal of the last dilution with CPE.

For virus neutralization, Mab 1318 was diluted in 10-fold serial dilutions, and added to equal volumes of 10 tissue culture infectious dose 50 (TCID<sub>50</sub>) of the mutants. The mixture was incubated for 1 h at 25 °C, and 100 µl were added to CEKC in 96-well tissue culture plates. Monolayers were observed for 4 days for CPE.

#### *DNA sequencing and sequence analysis*

Reverse transcriptase PCR with the primers S1OLIGO5' and S1OLIGO3' was used to amplify the S1 gene [17]. For sequencing, PCR product from six or more reactions for each sample was combined and purified using Microcon 30 columns (Amicon Inc., Beverly, MA). Double stranded DNA was sequenced using the Prism DyeDeoxy terminator cycle sequencing kit as recommended by the manufacturer (Applied Biosystems, Branchburg, NJ, USA). The majority of automated sequencing was conducted at the USDA Southeastern Poultry Research Facility (Athens, GA, USA), while some was conducted at the Molecular Genetics Instrumentation Facility University of Georgia, Athens, GA, USA). OLIGO version 4.0 (National Biosciences, Inc., Plymouth, MN, USA) was used to design sequencing primers synthesized at the Molecular Genetics Instrumentation Facility, to various regions within the S1 gene of Arkp11.

Nucleic acid and predicted AA sequences of the mutants were compared to Arkp11 and Ark DPI. Analysis of the sequence data and secondary structure predictions were conducted using MacDNASIS ProV3.0 software (Hitachi Software Engineering Co., Ltd., San Bruno, CA, USA).

## Results

### *Isolation, identification, and neutralization of Mab-NR mutants*

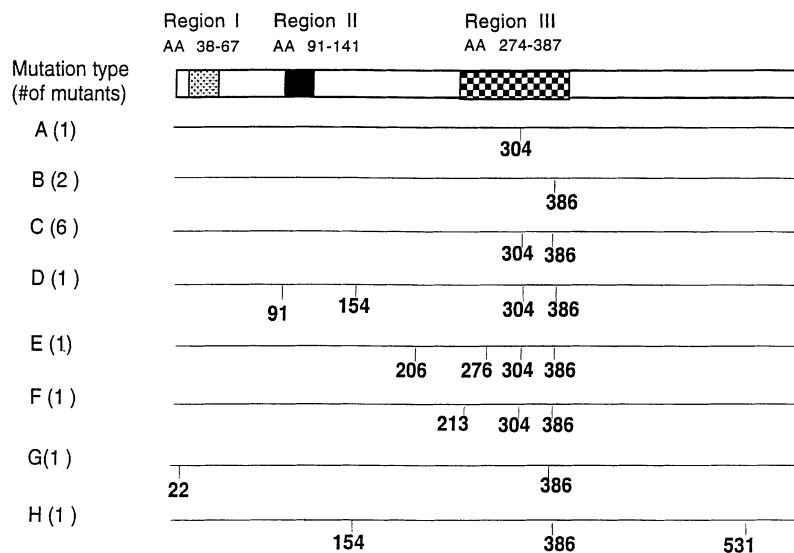
One hundred twenty-four Mab-NR mutants were plaque-purified from CEKC. Of these 124 mutants, 14 were plaque-purified again, and amplified in CEKC for characterization.

All 14 mutants were identified as IBV by RT-PCR using the primers MIBVPCR and NIBVPCR. The expected 1.7 kilobase PCR product was observed in mutants after RT-PCR with primers S1OLIGO5' and S1OLIGO3'. Restriction fragment length polymorphism patterns of all mutants using restriction endonucleases BstYI, HaeIII, and XcmI were characteristic of the Ark serotype [17]. In the microtiter neutralization assay, Mab 1318 at a dilution of  $2 \times 10^5$  neutralized 10 TCID<sub>50</sub> of Arkp11, but neutralization was not observed for any of the mutants. Hemagglutination inhibition was not observed for any of the mutants using Mab 1318.

### *Sequence analysis*

Polymerase chain reaction products from the S1 gene of Ark DPI, Arkp11 and 14 Mab NR mutants were sequenced. After 11 passages in CEKC, comparison between Ark DPI and Arkp 11 showed one point mutation at nucleotide 976 translating to an AA substitution from asparagine to tyrosine at residue 326.

Sequence comparisons between the parent virus, Arkp11, and selected mutants, allowed for grouping of the mutants by type from A to H (Fig. 1). Type



**Fig. 1.** The S1 subunit is divided into three regions designated by Koch et al. [15]. Lines represent S1 mutant types and the number of mutants each type is in parenthesis. Numbers represent the AA substitutions observed in Mab NR mutants in which 304 and 386 were the most frequently observed substitutions. (Type A = NR-25; Type B = NR-1 and NR-11; Type C = NR-5, NR-9, NR-19, NR-20, NR-22, NR-28; Type D = NR-23; Type E = NR-27, Type F = NR-26; Type G = NR-24; Type H = NR-29)

**Table 1.** Common nucleotide and AA substitutions in Mab NR mutants

Virus	Nucleotide 911	Nucleotide 1156	Residue 304	Residue 386
ArkDPI	A-ACA-G	T-CGT-G	Q-T-A	P-R-G
Arkp11	A-ACA-G	T-CGT-G	Q-T-A	P-R-G
1	A-ACA-G	T-TGT-G	Q-T-A	P-C-G
5	A-ATA-G	T-CCT-G	Q-I-A	P-P-G
9	A-ATA-G	T-CAT-G	Q-I-A	P-H-G
11	A-ACA-G	T-TGT-G	Q-T-A	P-C-G
18	A-ACA-G	T-CGT-G	Q-T-A	P-R-G
19	A-ATA-G	T-TGT-G	Q-I-A	P-C-G
20	A-ATA-G	T-CAT-G	Q-I-A	P-H-G
22	A-ATA-G	T-CAT-G	Q-I-A	P-H-G
23	A-ATA-G	T-CAT-G	Q-I-A	P-H-G
24	A-ACA-G	T-TGT-G	Q-T-A	P-C-G
25	A-ATA-G	T-CGT-G	Q-I-A	P-R-G
26	A-ATA-G	T-TGT-G	Q-I-A	P-C-G
27	A-ATA-G	T-CCT-G	Q-I-A	P-P-G
28	A-ATA-G	T-CCT-G	Q-I-A	P-P-G
29	A-ACA-G	T-TGG-G	Q-T-A	P-W-G

A consisted of one mutant containing one AA substitution at residue 304. Type B consisted of two mutants containing a single AA substitution at residue 386. Type C consisted of six mutants containing AA substitutions at both residues 304 and 386. Types D, E, and F consisted of one mutant each, containing AA substitutions at residues 304 and 386 along with other substitutions throughout the S1 subunit. Types G and H consisted of one mutant each, containing an AA substitution at residue 386 along with other substitutions throughout the S1 subunit. One mutant, NR-18, was not grouped because no AA substitutions in the S1 subunit were observed.

The most common AA substitutions were at residues 304 and/or 386 (Table 1). Transitions caused substitutions at residue 304 replacing threonine (Thr), uncharged polar side chain, with isoleucine (Ile), nonpolar side chain. Transversions or transitions resulted in AA substitutions at 386. Transversions caused substitutions are residue 386 replacing arginine (Arg), charged polar side chain, with proline (Pro), nonpolar side chain. Transitions caused substitutions at residue 386 replacing Arg with histidine (His), charged side chain, or Arg with cysteine (Cys), uncharged polar side chain. Two transitions occurred in NR-29 (type H) causing a substitution at residue 386 in which Arg was replaced with tryptophan (Trp), nonpolar side chain.

Silent mutations were observed in several mutants at nucleotides 402, 822, 1302, 1335, 1344, 1621, 1623. No predicted N-linked glycosylation sites (Asn-X-Thr/Ser) were gained or removed.

### *Secondary structure and glycosylation*

Due to the conformationally dependent nature of the epitope examined in this study, changes in the secondary structure of Mab-NR mutants may be important. The secondary structure for the S1 subunit was predicted by the Robson method using DNASIS ProV3.0. Comparisons between the parent, Arkp11, and Mab-NR mutants resulted in four different secondary structures (data not shown). Those conformational differences were observed between parent and mutant viruses that had either Pro, Cys, or Trp at residue 386.

None of the AA substitutions observed in Mab-NR mutants caused addition or subtraction of the 16 predicted N-linked glycosylation sites (Asn-X-Thr/Ser) in the S1 subunit for the Ark serotype.

### **Discussion**

Monoclonal antibody-NR mutants were isolated after mixing Mab 1318 with Arkp11. The S1 gene was sequenced and compared to the parent virus, Arkp11. Most of the predicted AA substitutions were observed at residues 304 and 386.

In previous research, AA substitutions in IBV Mab-NR mutants were divided into three regions associated with neutralizing epitopes. These regions were designated as I (residues 38–67) associated with the HVR I for the Mass serotype and HA, II (residues 97–141) associated with the HVR II for the Mass serotype, and III (residues 274–387) associated with a neutralizing epitope [16] (Fig. 1). In our research, substitutions at residues 304 and 386 correspond to region III designated by Koch et al. [15]. Since Mab 1318 is serotype-specific, did not inhibit HA, and the predicted AA substitutions were observed at 304 and 386, we associate region III with a serotype-specific, neutralizing epitope that is not involved in HA.

Some of the Mab-NR mutants reported herein had more than two AA substitutions. Koch et al. [15] or [16] also reported that multiple substitutions were observed in some of their Mab mutants.

Predicted secondary structures, used to formulate general ideas about native protein structure, showed conformational differences between the parent virus and mutants containing either Pro, Cys, or Try at residue 386 (data not shown). Changes in the predicted glycosylation sites on the spike glycoprotein, which has been reported to be important in virus attachment to host cells [22], were not observed.

Interestingly, NR-18 did not have any AA substitutions in the S1 subunit. Similarly, in mouse hepatitis virus (MHV) Mab-NR mutants selected with a Mab specific for the S1 subunit did not have any substitutions in S1, though some were observed in S2. In MHV, no major conformational changes were observed, and it was suggested that residues in the S2 subunit interact with residues in the S1 subunit [9]. Further analysis of NR-18 may provide more information about interactions between S1 and S2 for IBV.

In summary, we identified that residues 304 and 386 are involved in a virus-neutralizing, serotype-specific epitope on the S1 subunit of IBV using Mab-NR

mutants. Localization of virus-neutralizing serotype-specific epitopes from other serotypes of IBV would contribute to our understanding of neutralizing epitopes on the S1 subunit, and possibly facilitate the construction of molecularly engineered vaccines.

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