BARD

FINAL REPORT

PROJECT NO. US-3068-98

**Identification of Avian Infectious Bronchitis Virus
Variant Serotypes and Subtypes by PCR Product
Cycle Sequencing for the Rational Selection of
Effective Vaccines**

J. Gelb, Jr., Y. Weisman, B. Ladman, R. Meir

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BARD Final Scientific Report

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Project Title: Identification of Avian Infectious Bronchitis Virus Variant Serotypes and Subtypes by PCR Product Cycle Sequencing for the Rational Selection of Effective Vaccines

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)	0	0	1	1
Submitted, in review, in preparation	1	1	1	3
Invited review papers	0	0	0	0
Book chapters	0	0	0	0
Books	0	0	0	0
Master theses	0	1	0	1
Ph.D. theses	0	0	0	0
Abstracts	1	1	0	2
Not refereed (proceedings, reports, etc.)	0	0	0	0

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

None _____

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1	0	2
Longer Visits (Sabbaticals)	0	0	0	0

Description of Cooperation:

The complementary core competency of each lab was integral to the completion of the objectives. The ability of Kimron to perform and analyze RFLP patterns was essential in comparing molecular identification techniques, given Delaware's lack of experience with RFLP. Both the USA and Israeli researchers performed vaccination-challenge experiments with their native field isolates. The use of shared of protocols for assessing immunity provided more consistent basis of comparison of the results. It is clear that this research could not have been completed without contributions of both Kimron and Delaware.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
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Issued (allowed)	0	0	0	0
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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.
genotyping, spike glycoprotein, restriction fragment length polymorphism, coronavirus, chicken,

Abbreviations commonly used in the report, in alphabetical order:

Ark = Arkansas
Conn = Connecticut
DACS = direct automated cycle sequencing
DE = Delaware
IB = infectious bronchitis
IBV = infectious bronchitis virus
Mass = Massachusetts
MD = Maryland
PA = Pennsylvania
RFLP = restriction fragment length polymorphism
RT-PCR = reverse transcription - polymerase chain reaction
S1 = S1 subunit of the spike glycoprotein

Budget: IS: \$103,000

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Signature
Principal Investigator

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ABSTRACT

Objectives

1. Determine the serotypic identities of 40 recent IBV isolates from commercial chickens raised in the USA and Israel.
2. Sequence all IBV field isolates using PCR product cycle sequencing and analyze their S1 sequence to determine their homology to other strains in the Genbank and EMBL databases.
3. Select vaccinal strains with the highest S1 sequence homology to the field isolates and perform challenge of immunity studies in chickens in laboratory trials to determine level of protection afforded by the vaccines.

Background

Infectious bronchitis (IB) is a common, economically important disease of the chicken. IB occurs as a respiratory form, associated with airsacculitis, condemnation, and mortality of meat-type broilers, a reproductive form responsible for egg production losses in layers and breeders, and a renal form causing high mortality in broilers and pullets. The causative agent is avian coronavirus infectious bronchitis virus (IBV). Replication of the virus' RNA genome is error-prone and mutations commonly result. A major target for mutation is the gene encoding the spike (S) envelope protein used by the virus to attach and infect the host cell. Mutations in the S gene result in antigenic changes that can lead to the emergence of variant serotypes. The S gene is able to tolerate numerous mutations without compromising the virus' ability to replicate and cause disease. An end result of the virus' "flexibility" is that many strains of IBV are capable of existing in nature. Once formed, new mutant strains, often referred to as variants, are soon subjected to immunological selection so that only the most antigenically novel variants survive in poultry populations.

Many novel antigenic variant serotypes and genotypes have been isolated from commercial poultry flocks. Identification of the field isolates of IBV responsible for outbreaks is critical for selecting the appropriate strain(s) for vaccination. Reverse transcriptase polymerase chain reaction (RT-PCR) of the S1 subunit of the envelope spike glycoprotein gene has been a common method used to identify field strains, replacing other time-consuming or less precise tests. Two PCR approaches have been used for identification, restriction fragment length polymorphism (RFLP) and direct automated cycle sequence analysis of a diagnostically relevant hypervariable region were compared in our BARD research.

Vaccination for IB, although practiced routinely in commercial flocks, is often not protective. Field isolates responsible for outbreaks may be unrelated to the strain(s) used in the vaccination program. However, vaccines may provide varying degrees of cross-protection vs. unrelated field strains so vaccination studies should be performed.

Conclusions

- RFLP and S1 sequence analysis methods were successfully performed using the field isolates from the USA and Israel. Importantly, the S1 sequence analysis method enabled a direct comparison of the genotypes of the field strains by aligning them to sequences in public databases e.g. GenBank.

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- Novel S1 gene sequences were identified in both USA and Israel IBVs but greater diversity was observed in the field isolates from the USA. One novel genotype, characterized in this project, Israel/720/99, is currently being considered for development as an inactivated vaccine.
- Vaccination with IBV strains in the US (Massachusetts, Arkansas, Delaware 072) or in Israel (Massachusetts, Holland strain) provided higher degrees of cross-protection vs. homologous than heterologous strain challenge. In many cases however, vaccination with two strains (only studies with US strains) produced reasonable cross-protection against heterologous field isolate challenge.

Implications

- S1 sequence analysis provides numerical similarity values and phylogenetic information that can be useful, although by no means conclusive, in developing vaccine control strategies.
- Identification of many novel S1 genotypes of IBV in the USA is evidence that commercial flocks will be challenged today and in the future with strains unrelated to vaccines. In Israel, monitoring flocks for novel IBV field isolates should continue given the identification of Israel/720/99, and perhaps others in the future.
- Strains selected for vaccination of commercial flocks should induce cross-protection against unrelated genotypes. Using diverse genotypes for vaccination may result in immunity against unrelated field strains.

ACHIEVEMENTS

Significance and Impacts

Infectious bronchitis is difficult to control because of the numerous strains (serotypes and genotypes) of the virus, IBV, which have been identified around the world. Strains arise via mutation of field and or vaccine strains and selection pressures such as partial immunity. Control of the disease is achieved through vaccination. Four serotypes (Massachusetts, Connecticut, Arkansas and Delaware 072) of the virus are available for use as live vaccines in the USA. Application of combinations of two strains may induce varying degrees of cross-protection against heterologous and homologous field strains. Furthermore, autogenous inactivated vaccines are used in the USA, but are economically practical only for breeders and layers. In contrast, only one live IBV vaccine strain, the Massachusetts H120 is available in Israel. Immunity following vaccination with only a single strain in Israel provides a comparatively narrow spectrum of immunity.

Forty IBV field isolates, 20 each from commercial poultry in the USA and Israel were identified and representative viruses were used in vaccination efficacy studies. The research also compared the two available gene based methods for identifying field isolates, restriction fragment length polymorphism (RFLP) and S1 gene sequencing.

Some field isolates from the USA and Israel were genetically similar to vaccine strains. In these instances, vaccination with the homologous genotype induced immunity in chickens. Thus, in these instances, vaccination with the appropriate strain can be recommended to control the disease.

However, most of the isolates from the USA were highly novel and unrelated to vaccinal strains as determined by S1 sequencing. Furthermore, many of the novel isolates from the USA were not the same, indicating they most likely had different origins.

In Israel, several additional isolates of the previously described Variant (Var) I and Var II genotypes were identified. Furthermore, a new, previously unreported S1 genotype, Israel/720/99, was discovered and another isolate, Israel/885/00, of the same genotype as Israel/720/99 was recovered from poultry with the renal form of the disease.

The genetic techniques used in this study, RFLP and S1 sequencing, were successfully tested by the diversity of the novel variant IBVs included in panels of the field isolates from the USA and Israel. The S1 sequencing method has been now extensively used to identify numerous isolates from North and South America, Europe, and Asia. However, isolates from the Middle East had not been evaluated prior to this research. IBV field isolates from different geographic areas are often unique to the region. The ability of the PCR primers to amplify novel Israeli isolates indicates their wide applicability for IBV genotyping.

RFLP technology is also capable of identifying a wide range of IBV isolates. Isolates are identified on the basis of restriction enzymes to cleave specific sequence motifs of the PCR products. Genotypes may be grouped into RFLP groupings. However interpretation of RFLP patterns requires observation of banding patterns and as such may be subjective. Unlike sequence analysis, RFLP results cannot be used to establish the relatedness of a field isolate and a known virus. Using the sequencing method, S1 sequences can be downloaded from public databases and directly compared with the field isolate sequence to generate similarity values and phylogenetic relationships.

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Control of the novel field isolates using selected vaccinal serotypes was also evaluated. Vaccination with Massachusetts + Arkansas strains provided chickens cross-protection against many of the novel isolates from the USA. The Massachusetts + Arkansas vaccine combination has previously been shown by our laboratory (Univ. of Delaware) to offer cross-protection against unrelated serotypes. A limitation to the use of the Massachusetts + Arkansas vaccine is that it cannot be used unless the Arkansas strain is licensed in the state or country. Vaccination with the H120 strain to control the novel genotype Israel/720/99 however did not protect chickens. The Israeli poultry industry is exploring the feasibility of controlling this genotype using an inactivated autogenous vaccine.

Many novel variants from the USA were from commercial layer flocks. Variant serotypes represent biosecurity risks to neighboring poultry farms and may be spread via manure (fertilizer) application, exchange of contaminated egg packing materials, and movement of spent fowl to processing plants.

Details of Cooperation

As specified by the BARD proposal, Kimron accomplished the objective of evaluating RFLP analysis for identifying IBV and University of Delaware performed the direct automated cycle sequencing method. As it became evident that the sequencing method gave information that was preferable to RFLP, Delaware assisted Kimron in developing sequencing capabilities. In fact, a new isolate, Israel/885/00, obtained during the course of the BARD research was sequenced at Kimron, then sent to Delaware for confirmation. The isolate is under consideration for development into a killed autogenous vaccine for use by the poultry industry in Israel to control the renal form of IB. The adoption of the S1 sequencing technology by Kimron will continue to benefit agriculture in Israel.

The complementary core competency of each lab was integral to the completion of the objectives. The ability of Kimron to perform and analyze RFLP patterns was essential in comparing molecular identification techniques, given Delaware's lack of experience with RFLP. Both the USA and Israeli researchers performed vaccination-challenge experiments with their native field isolates. The use of shared protocols for assessing immunity provided more consistent basis of comparison of the results. It is clear that this research could not have been completed without contributions of both Kimron and Delaware.

APPENDIX

BARD Grant No. US-3068-98

Identification of Avian Infectious Bronchitis Virus Variant Serotypes and Subtypes by PCR Product Cycle Sequencing for the Rational Selection of Effective Vaccines

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<i>Submitted manuscript</i> Identification and Characterization of a Novel Nephropathogenic Infectious Bronchitis Virus in Israel. R. Meir, E. Rosenblut, S. Perl, N. Kass, G. Ayali, E. Hemsani, and S. Perk. Submitted to <i>Avian Diseases</i> .	Pages 7-14
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A Comparison of Three Israeli Infectious Bronchitis Virus Isolates by Genotyping and Virus Neutralization Test

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Abstract

The significance of different restriction fragment length polymorphism (RFLP) patterns was tested by comparing three Israeli infectious bronchitis (IB) field isolates to the H120 vaccine strain. The tested viruses, IS/236, a Massachusetts like variant, IS/234, (Variant I group), and IS/223, (Variant II group) each had a distinct RFLP pattern. Comparison of the S1 protein sequence of IS/236, IS/234 and IS/223 to the vaccine strain revealed 99.3%, 79%, and 86.2% respective identities. Virus neutralizing index for IS/236, IS/234 and IS/223 when tested against H120 anti sera was 0.8, 0.7, and 0, respectively. Results demonstrated a) the use of RT/PCR/RFLP as a reliable and rapid way of screening IB outbreaks, and b) the necessity to reevaluate the use of a single serotype vaccine for control of the disease.

Introduction

Infectious bronchitis (IB) is a highly contagious upper respiratory disease of economic importance (3). The disease is controlled with serotype specific vaccines (10). In Israel the only authorized vaccine is H120, a Massachusetts serotype (8). The etiologic agent, infectious bronchitis virus (IBV) is a member of the *Coronaviridae* family. The virion has a single stranded positive-sense RNA, and contains four major structural proteins: a nucleocapsid protein (N), a membrane glycoprotein (M), an envelope protein (E) and a spike glycoprotein (S) (3). The S protein contains two subunits, S1, and S2. The S2 subunit anchors the spike in the membrane whereas the S1 subunit is involved in the induction of neutralizing, serotype specific antibodies (2-4, 11). IBV is known for its high frequency mutations and RNA recombination, which leads to the emergence of new serotypes (7, 13). That, and the need to evaluate vaccine efficacy were the reasons necessitating the use of rapid and reliable methods vis a vis local IBV strains for diagnosis and serotyping of the causative agents.

Typing of Israeli field isolates by reverse transcriptase-polymerase chain reaction-restriction fragment length polymorphism (RT-PCR-RFLP) (6) revealed the existence of Massachusetts-like isolates as well as two other molecular groups, designated the names variant I and variant II. Three field isolates, representing each group, were compared to the vaccine strain in use in this country by means of RT-PCR-RFLP, sequence analysis

of the PCR amplicons of the S1 gene and the translated proteins, and by the virus neutralization test.

Materials and Methods

Viruses: Three field isolates were IS/236, a Massachusetts like variant; IS/234, a member of the variant I group; and IS/223, a member of the variant II group. The vaccine virus was H120, a live, attenuated Massachusetts strain (Abik, Israel). All viruses were propagated in 9-11 day old embryonated SPF eggs. After 48 h of incubation post injection eggs were chilled and the allantoic fluid was harvested.

RNA Extraction and RT-PCR-RFLP: Total RNA extraction was performed using a commercial kit (Bio 101, La Jolla, Ca, USA). RT-PCR-RFLP was performed as previously described (6). Briefly, a 20 μ l RT reaction consisting of 2 μ l buffer (x10) (Tris HCL 200mM, KCL 500mM, bovine serum albumin 0.5 mg/ml), 2.4 μ l of 80 mM $MgCl_2$, 2 μ l mixture of four deoxynucleoside triphosphates (10 mM each), 40 units Rnasin (Promega Corporation), 250 ng S1OLIGO3' primer (5' -CATAACTAACA TAAGGGCAA- 3'), 4 μ l suspended RNA was incubated at 65 ° C followed by chilling on ice for 5 min. M-MLV -reverse transcriptase (200 units) (New England BioLabs) was added followed by incubation at 37° C for 1 h and 95° C for 5 min.

A PCR reaction mixture contained 20 μ l of the RT reaction, 8 μ l buffer, 12 μ l $MgCl_2$, 250 ng, S1OLIGO5' (5' TGAAA ACTGAACAAAAGA CA -3'), and Taq DNA polymerase (5 units) (Promega Corporation) in a total of 100 μ l. Amplification was carried out at 35 cycles of 94° C for 1 min, 55° C for 1 min, 74 ° C for 1 min, proceeded by 94° C for 1 min, 55° C for 1 min, and 74° C for 5 min. The products of approximately 1700 bp were electrophoresed on 1.5 % agarose gel (Emresco,) containing ethidium bromide (0.5 μ g/ml).

For the RFLP test, the amplicon was digested by three restriction enzymes, *Bst*YI, *Hae*III, and *Xcm*I (New England BioLabs,) according to manufacturers instructions, and the products were separated in a 2% agarose gel, stained with ethidium bromide.

Sequence analysis: The amplified cDNA of all three isolates was purified and concentrated with JETsorb purifying kit (Genimed Inc), and directly sequenced using the BigDye Terminator Cycle Sequencing Kit from ABI (DNA Sequencing Unit, Weizmann Institute, Rehovot, Israel). Molecular analyses were conducted using MEGA2 version 2.1 (5). Protein sequence similarities of IS/236, IS/234, IS/223, and H120, were calculated using the pairwise distances mode.

Sequence accession numbers: We sequenced directly the S1 gene of the field isolates, and the sequence of IS/236 was submitted to GenBank (accession number AY135205). The isolates IS/234 and IS/223 were also sequenced by Callison et al. (1) in a comparative study of isolates from outside the United States and submitted to GenBank (accession numbers AF093795, and AF093796). The sequence of H120 was obtained through GenBank database (accession number M21970).

Virus neutralization (VN). A group of 5 SPF chicks, 7 weeks of age, was infected with the vaccine strain, virus titer EID_{50} 10^4 , by administering 0.2 ml/per bird into the trachea. A second uninfected group served as control. Both groups were reared in positive pressure isolators. After two weeks vaccinated birds were injected intra muscular with 0.2 ml live vaccine with same titer per bird. Blood samples were collected two weeks after the second inoculation and the serum was inactivated at 56° C for 30 min. VN was conducted using the _-

neutralization procedure, with serial dilutions of serum tested against standard dose of virus ($EID_{50}10^3$). Residual virus was assayed in chicken embryos (12). The neutralizing index (NI) was calculated by the Reed and Muench method (9).

Results

We analyzed three IBV field isolates representing different molecular groups according to their RFLP patterns (Fig. 1). The vaccine strain had identical RFLP as the field Massachusetts type (Fig1A). IS/234 (Variant I) had distinctly different TFLP when digested with *Bst*Y I and *Hae* III (Fig 1B) and IS/223 had no restriction sites for both *Bst*Y I and *Xcm* I (Fig1C). Compared to H120, field isolates IS/236, IS/234, and IS/223 revealed 99.3%, 79%, and 86.2% identities respectively (table 1).

Table 1. S1 protein sequence identity (in %)

		1	2	3	4
H120	1		99.3	79.0	86.2
IS/236	2			79.0	85.9
IS234	3				88.3
IS223	4				

The NI was 0.8 for IS/236, 0.7 for IS/234, and 0 for IS/223.

Discussion

The distinct differences in RFLP patterns for the three field isolates are supported by the differences in protein sequences. Based on these data, there is a question of protection provided by H120 alone. The NI test demonstrates that the vaccine in use can provide protection against field isolates of the same strain and may cross protect against variant I group isolates. However, no neutralization antibodies are detected against a virus from variant II group. In addition, our results demonstrate the necessity to test the assumption that different RFLP patterns reflect different serotypes. We examined three isolates with different RFLPs, and differences in S1 protein sequences. Yet IS/236 (Mass type) and IS/234 (variant I) yielded similar NI (0.8, and 0.7) whereas IS/ 223 (variant II) was not neutralized. Sequencing of the S1 protein provides an important tool for monitoring the emergence of new variants but further tests are necessary for a serologic classification, as indicated by the VN results

In conclusion this study confirms the use of RT/PCR/RFLP as a reliable and rapid way of screening IB outbreaks. It points out the necessity to reevaluate the use of a single

serotype vaccine. It may be economically sound to consider the introduction of new vaccines for better control of the disease.

Acknowledgment

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Figure 1. Agarose gel electrophoresis of PCR-amplified S1 genes and RFLP patterns of three Israeli field isolates. Panel A – IS/236 (Mass like); panel B – IS/243 (variant I); panel C - IS/223 (variant II). M- molecular weight marker VI, pBR 328 DNA digested with *Bg I* + pBR 328 DNA digested with *Hinf I* (Boehringer Mannheim, Germany), from upper band, 2176 bp, 1766 bp, 1230 bp, 1033 bp, 653 bp, 517 bp, 453 bp, 394 bp, 298 bp, 234 bp, and 220 bp. Lane 1 – PCR amplified product (uncut); lane 2 - digestion with *BstY I*; lane 3 – digestion with *Hae III*; lane 4 – digestion with *Xcm I*.