MOLECULAR TOOLS FOR THE STUDY, DIAGNOSIS, AND CONTROL OF INFECTIOUS BRONCHITIS VIRUS

by

SCOTT ANDREW CALLISON

(Under the Direction of MARK W. JACKWOOD)

ABSTRACT

We developed molecular tools for the study, diagnosis, and control of infectious bronchitis virus. A hammerhead ribozyme targeted to the IBV nucleocapsid mRNA was designed, synthesized, and *in vitro* analyzed. At a concentration of 0.5 or 10 μM, the ribozyme, designated IBVNRIBOZYME, effectively cleaved target RNAs *in trans*. Cleavage products were visualized by agarose gel analysis. The time course of the ribozyme reaction was monitored by agarose gel analysis and real-time RT-PCR. The amount of target RNA continually declined over a five-hour period.

We employed the staggered extension process (StEP) to shuffle the S1 genes from four infectious bronchitis virus (IBV) strains representing four unique serotypes. We produced 11 recombinat S1 genes. Each recombinant was unique and contained a full-length open reading frame. The average number of crossovers per recombinant was 5 and the average number of point mutations was 1.3. No recombinants contained sequences from all four parental genes, but several contained sequences from three of the parental S1 genes.

We developed a rapid diagnostic assay for differentiating IBV isolates, termed Sample to Residual Ratio Quantification (SRRQ) using RRT-PCR. We designed serotype specific chimeric oligonucleotides, one each for the Massachusetts, Connecticut, Arkansas, and Delaware/Georgia 98 serotypes and tested their ability to mediate cleavage of target RNA by RNase H. The specificity of each chimeric oligonucleotide was tested against homologous and heterologous strains of IBV. Our data showed that each chimeric oligonucleotide mediated cleavage of target RNA only from strains within the serotype that the chimeric was designed against. To validate the test, we performed a test on 15 samples without prior knowledge of their type. Our results correctly determined the serotype of each sample containing an IBV from within the serotypes for which a chimeric oligonucleotide was developed.

Our hammerhead riboyzme, novel S1 genes, and new SRRQ RRT-PCR assay will aid in the study, diagnosis, and control of IBV. The poultry industry will directly benefit from the molecular tools by allowing for the production of better vaccines and novel therapeutic molecules, as well as, allowing for more rapid differentiation of IBV isolates.

INDEX WORDS: Infectious bronchitis virus, Ribozyme, DNA shuffling, Real-time RT-PCR, Reverse genetics, S1 gene, nucleocapsid gene

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SCOTT ANDREW CALLISON

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SCOTT ANDREW CALLISON

Major Professor:	Mark W. Jackwood
Committee:	Pedro Villegas Maricarmen Garcia L. Jaso-Friedmann

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

I want to dedicate this dissertation to my family - Mom, Dad, Chris, Lori, and Alexis. Without your love and constant support nothing in my life would be possible. Thank you for always being there, through the laughter, through the tears, through the joy, and through the pain. My greatest love in life is each of you.

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CHAPTER 1

INTRODUCTION

Background of the study

Infectious bronchitis virus continues to cause a problem for the poultry industry despite intensive vaccination programs designed to control the spread of the virus and limit the severity of the disease it causes. The problem with controlling IBV is its inherent ability to rapidly change at the molecular level. Novel variant viruses continue to arise through point mutations (antigenic drift) and recombination (antigenic shift). Developing vaccines and diagnostic assays that keep pace with how fast the virus changes has proven difficult. Generally, traditional vaccines are only available for four or five serotypes, with the most common being for the Massachusetts and Arkansas serotypes. Protection afforded by the traditional vaccines against variant viruses is usually minimal. Therefore, a cross protective IBV vaccine or novel therapeutic molecules are needed.

Currently, many diagnostic tests exist for differentiating IBV isolates, including: monoclonal antibodies, dot blot, DNA probes, rapid plate hemagglutination test, SDS-PAGE polymorphism, direct S1 gene DNA sequencing, RT-PCR with serotype specific primers, and RT-PCR/RFLP. It has been shown that genotyping tests provide data that correlates well with the serotype of the virus. Due to their ease and correlation with serotype, genotype tests (DNA sequencing, serotype specific RT-PCR, and RT-PCR/RFLP) have become widely used. Although the genotyping tests are rapid, room

for improvement remains. In particular, elimination of post RT-PCR manipulations (gel electrophoresis, gel purification of DNA, restriction enzyme analysis, sequencing, etc.) would decrease the cost and overall assay time. Recent technological advances in the field of real-time nucleic acid amplification could be used to negate the need for post RT-PCR manipulations.

To better control IBV, several things are needed as follows: 1) a deeper understanding of the virus at the molecular level, including the function of all proteins, the genetic determinants of pathogenesis and tissue tropism, and mapping of all the virus neutralizing epitopes on the spike glycoprotein, 2) a rapid and reliable diagnostic assay that can determine the serotype of an IBV field isolate and 3) novel vaccines, including better strategies and systems for their development, and therapeutic molecules. To address these needs, we undertook several research projects with the following objectives.

Objective 1

Ribozymes are catalytic RNA molecules that can catalyze cleavage of a specific target RNA molecule *in trans*. They have great potential as antiviral and gene therapy agents. Indeed, antiviral ribozymes have been reported for viruses from several RNA virus families, including the *Coronaviridae* family. Currently, there are no published reports of a ribozyme targeted against IBV. We designed, synthesized, and analyzed *in vitro* a hammerhead ribozyme targeted to the nucleocapsid gene of IBV in hopes that it would be a novel antiviral therapeutic agent to control IBV infections.

Objective 2

Infectious bronchitis virus variants continue to arise due to point mutations and recombination. Specifically, changes in the spike gene lead to variants that are able to escape neutralization by the immune response of chickens stimulated by traditional vaccination. A cross protective IBV vaccine would be helpful in protecting chickens against these novel variants. Currently, a cross protective vaccine does not exist because the specific spike gene sequences needed to produce a cross protective virus are not known. Some published reports have narrowed down regions of the spike gene that encode neutralizing and serotype specific epitopes, but much remains unknown about the overall amino acid makeup and number of epitopes on a spike glycoprotein. In order to study and hopefully produce a spike gene sequence capable of being cross protective, we used the staggered extension process to shuffle the S1 portion of the spike gene from four different strains of IBV representing four different serotypes. We produced a library of novel S1 genes and began initial characterization of each gene.

Objective 3

Several diagnostic assays exist for the detection and differentiation of IBV field isolates. Genotyping tests, such as direct DNA sequencing, serotype specific RT-PCR, and RT-PCR/RFLP, are becoming more widely used. This is due to their good correlation with virus neutralization tests and ease with which the assays can be performed. Although accurate and reliable, all the assays involve some type of post RT-PCR manipulations that add to the time and cost. Using real-time RT-PCR, we

developed a novel real-time RT-PCR assay for differentiating IBV isolates from within five of the most common serotypes of IBV.

CHAPTER 2

LITERATURE REVIEW

Part I: Infectious bronchitis

INTRODUCTION

Infectious bronchitis (IB) is an extremely contagious viral infection of the upper-respiratory tract in poultry [1]. Generally, infections are self-limiting, uneventful, and cleared by the chicken immune response. Rarely, the infection may spread to the reproductive and renal systems, and lead to oviduct lesions in layers and kidney lesions, respectively [1, 2]. The respiratory manifestation of the disease is most common and characterized by tracheal rales, coughing, and sneezing [2]. In addition, young chicks may produce a nasal exudate and are more susceptible to mortality than mature chickens [1].

The morbidity and control of IB are of much greater economic importance than the mortality caused by the disease [1, 2]. Infectious bronchitis causes poor weight gain in broilers due to a decline in feed efficiency and is a component of mixed infections with *E. coli* and *Mycoplasma spp.* that ultimately warrant condemnation of broilers at processing. A decrease in egg production with a concurrent decline in egg shell quality in layers can also be attributed to the disease. Efforts to control IB by vaccination have been difficult and costly due to the high transmissibility and multiple serotypes of the etiologic agent [1].

HEALTH SIGNIFICANCE TO HUMANS

To date, IB is not known to be a human pathogen. In the late 1960's several novel viruses were isolated and morphologically characterized. These strains designated 229E and B814 had identical morphology when compared to infectious bronchitis virus strains (IBV) [3-5]. The 229E and B814 strains were shown to be antigenically distinct from known strains of IBV [6], providing evidence that coronaviruses infect humans but do not originate from avian species. However, in 1968 it was shown that people closely associated with poultry, had antibodies against IBV [7]. Approximately 40% of individuals with an average exposure of 15.5 years to poultry, had low titers of neutralizing antibody against IBV. The significance of this finding has never been understood, but three theories seem plausible. Possibly, antigenically related human coronaviruses can stimulate people to produce antibodies that cross-react with IBV, or IBV can cause a subclinical infection in humans that is asymptomatic and never diagnosed. The most likely explanation is that IBV can enter into the human respiratory tract where it cannot infect host cells, and the human immune system response clears the virus with minimal production of neutralizing antibodies.

HISTORY

A timeline of the early history and highlights of IB is listed below [1]:

1930 Respiratory disease associated with mortality of chickens seen in North Dakota, USA

1931 Cases in 1930 designated IB by Schalk and Hawn [8]

1936 Virus etiology established by Beach and Schalm [9]

1937 Cultivation of virus in embryonated chicken eggs by Beaudette and Hudson [10]

1940's A decline in egg production is associated with IB following the typical respiratory infection

1941 First attempt to prevent IB by controlled exposure of chickens to IBV

1956 Realization that virus has multiple serotypes after Connecticut (1951) isolate and Massachusetts (1941) isolate show no cross protection by Jungherr et al. [11]

1960's Kidney lesions shown to be associated with IB

WORLDWIDE DISTRIBUTION

Infectious bronchitis is distributed throughout the world and is reported from all countries where an intensive poultry industry has developed [2]. The reports of IB disease generally begin only a short time after the development of the poultry industry in a particular area. Not all incidence of IB are reported due to poor diagnostic and research facilities in some countries. It is probable that many IB infected flocks go unreported or undocumented.

Indeed, vaccinated flocks have outbreaks of IB because the causative agents isolated from the outbreaks are found to be serotypically distinct from the vaccine used for the flock [1]. New serotypes of IB virus have been identified since the 1950's in the U.S [11]. Beginning in the late 1970's, serotypes different from the Mass serotype were frequently identified in Europe [12-14].

PATHOGENICITY

The pathogenicity is reviewed by King in Diseases of Poultry and is summarized here [1].

General and influencing factors

Infectious bronchitis virus infects the respiratory tract of chickens and produces characteristic tracheal lesions. Usually, the chicken recovers without any lasting affects. However young birds can develop airsacculitis due to a secondary infection, or nephritis follows the respiratory infection. Several factors can contribute to the pathogenicity of an IBV infection, such as, environment, diet, secondary pathogens, and strain of the virus.

Virulence

The virulence of a strain can lead to different outcomes in an IBV infection. It has been shown in the laboratory that strains can become attenuated by successive passage in chicken embryos. The Beaudette strain is highly egg adapted and is apathogenic. It causes no detectable damage to the ciliated epithelium and has a diminished immunogenicity. In contrast, the Mass 41 strain is not as highly egg adapted and can destroy the ciliated epithelium in the trachea. Other virulent strains are known to be nephropathogenic [15]. They include the T strain from Australia that causes mortality and kidney lesions, the Italian strain, the U.S. Gray strain, and the U.S. Holte strain. Also, strains of IBV differ in their virulence for the reproductive tract in layers. Characteristic disease in these cases can cause as little as shell pigment change and as much as 5-20% egg production drops [2].

PATHOGENESIS AND EPIZOOTIOLOGY

Natural and experimental hosts

The chicken is the only animal that is known to be naturally infected by IBV and susceptible to disease [1, 2]. This susceptibility varies among breeds of chicken [1, 16]. There has been a report of isolation of IBV from pheasants showing characteristic signs of IB, suggesting that the pheasant is another natural host [17]. This has not been shown experimentally. There is serological data that suggests that turkeys may have been infected and there was a reported isolation of virus from magpies inoculated via the conjunctiva [2, 18]. Experimental inoculation of mammals has been tried with limited success [19]. This is not surprising due to the fact that coronaviruses typically cause clinical disease only in the species of isolation origin and replicate best in cultures obtained from that species [1, 20].

Sex and age

Both sexes are equally susceptible to IB [2]. The age of the chicken plays a large role in the outcome of an infection. Infectious bronchitis is most severe in young chickens, causing some mortality [1]. With increased age, comes increased resistance to infection and different disease manifestations [15, 16]. Mortality as high as 90% has been reported following 1-day-old inoculation, which drops to 20% when inoculation occurred after 6 weeks of age [2].

Transmission

Infectious bronchitis spreads rapidly and the most important route of spread is by aerosol [2]. A susceptible chicken placed in close contact with

infected chickens usually shows characteristic IB signs within 48 hours [1]. Although virus replicates mainly in the trachea, it can be isolated from other body tissues (lung, kidney, bursa) up to seven days post aerosol exposure [21]. Also of importance, is the fact that virus can be isolated from feces, the intestinal tract, and the cecal tonsils after the chicken has recovered from the clinical respiratory disease [2]. This provides another route of virus transmission by contamination of food, water, and litter.

Isolation of IBV from eggs and day-old chicks has been reported [2]. Therefore, vertical transmission of the virus is possible. Although the virus can be transmitted this way, experiments have demonstrated the ability to raise chickens free of IB from breeder flocks infected with IBV [1]. This suggests that vertical transmission of the virus may occur, but it has no real significance in transmission of the disease [2].

Incubation period

The incubation period of IB is between 18-36 hours depending upon inoculation route and dose [1, 22]. Infective egg fluid delivery as an aerosol usually produces tracheal rales within 24 hours, while natural spread has an incubation period of approximately 36 hours [1].

Clinical signs and lesions

Typical clinical signs of IB are tracheal rales, sneezing, coughing, poor weight gain due to poor feed efficiency in broilers, and a decline in egg production and quality in layers [1, 2]. Other signs include depression and huddling near a heat source, with all signs becoming less prominent with age [1].

Several clinical lesions are associated with IB [1, 2]. The most common lesion is the loss of ciliated epithelium in the trachea. Kidney manifestations of IB can cause nephritis. Airsacculitis may occur due to secondary infections of *E. coli* and *Mycoplasma spp*. Lastly, lesions in the oviduct of susceptible chickens may be the cause of non-layers.

Morbidity and mortality

All chickens in a flock become infected and show some type of morbidity. In contrast, mortality is variable and depends upon different factors [1]. Some are the virulence of the virus strain, age of the chicken, status of immunity, presence of maternal or active antibody, and other assorted environmental factors. Of these factors, age seems to be one of the most important. Mortality may be 25% or more in chickens less than 6 weeks of age.

Immunity

Two types of immunity are important in IB infections, active and passive. Active immunity confers protection for a recovered chicken when challenged with a homologous virus strain [1]. Protection against heterologous virus challenge tends to vary [23-26]. Some of the factors that complicate protection are the multiple serotypes of IBV, variation of strain virulence, and different manifestations of the disease [1].

Protection from IB infection can be evaluated in several ways and varies for different types of chickens and manifestations of the disease [1]. Respiratory protection is evaluated 3-4 weeks after infection or immunization by challenging with another virus via the respiratory route. Several different observations can be

used to establish a positive immunity to IB. They are as follows [1, 24]: failure to recover virus from the trachea 4-5 days post challenge, no virus isolation from kidney or oviduct, no clinical signs, no tracheal lesions, or tracheal ciliary activity.

Protection evaluation using mixed infections of IBV and *E. coli* have been used [16, 27]. This method has been used to characterize the virulence of different IBV strains. It has also provided evidence of greater cross-protection between different serotypes of virus than other methods. Protection against nephritis is judged by no mortality and protection of layers is judged by reduction or prevention of a decline in egg production [1].

Humoral and cell-mediated immune responses have been reported for IB infections [1]. The main humoral response is production of virus neutralizing and hemagglutination inhibiting antibody induced by the S1 glycoprotein and to some extent by the whole spike protein [28]. Also, antibody secreted into nasal secretions and the Harderian gland have been reported in the humoral response [1]. The humoral response contribution to the mechanism of IB protection is not fully understood because it has been shown that antibody was not essential for resistance to IB infection [29]. This suggests that cell-mediated immune responses play a role in IB infections. Indeed, cytotoxic lymphocyte activity has been reported [1].

Passive immunity is important for resistance to IB infections. Maternal antibody protected chicks from 1 day to 1 week of age but not at 2 weeks of age [1]. However, it can also reduce the reaction and efficacy of vaccines.

Interference is most prominent if the vaccine is of the same type as that used in the breeder chicken.

DIAGNOSIS

Diagnosis is reviewed by King in *Diseases of Poultry* [1] and by Cook in *The Coronaviridae* [2] and summarized here. The diagnosis of IB is based upon several factors. They include clinical history of flock, seroconversion or rising titers of antibody against IBV, antigen detection of IBV by immunofluorescence, virus isolation, and RT-PCR.

Isolation and identification of the causative agent

For isolation and identification of IBV, tissues from chickens within a flock or from sentinel chickens can be used [1]. The best tissue sample is the trachea, followed by the lung, kidney, oviduct, cecal tonsils, or cloaca. The cloaca is important because IBV is cleared from the trachea before the intestinal tract [1]. These tissue samples can be taken from live or dead birds. Sections or smears can be used to detect IBV antigen by IF [30-32].

Virus isolation is done by inoculating a tissue suspension into the allantoic sac of 9-to-12-day-old embryonated chicken eggs or tracheal organ cultures (TOC) [32]. Fluid harvested 48-72 hours after inoculation into either system can be used for identification purposes. A minimum of three and preferably five blind passages should be attempted without embryo death or ciliostasis in TOC before being considered negative. Cultures deemed positive can be further characterized by electron microscopy, differential diagnosis, and serology.

Serology

Serum antibody tests can be used to monitor flocks for vaccinal response and field infections. Multiple IBV serotypes make this method and subsequent analysis complex. Antibody is produced against group and type specific antigens. ELISA, immunofluorescence, and immunodiffusion can be used to determine group specific antigens, but not type specific antigens. In general, the primary antibody response to IB is type specific and the use of virus neutralization (VN) or hemagglutination inhibiting (HI) tests work well. A secondary antibody response to IB is less specific and the use of VN or HI tests is no better than group specific tests.

Routine serology has been done using VN tests (alpha, beta) in embryonated chicken eggs [33], TOC [25, 34], and cell cultures [35]. Hemagglutination inhibition tests [36] and ELISA [37] are also used regularly with both detecting antibody earlier than VN tests [1]. Either direct or indirect immunofluorescence has been used effectively, while immunodiffusion tends to be unreliable [1, 2].

TREATMENT

No specific treatment for IB exists, but some factors can be employed to reduce the severity of the disease [1]. Management factors include the addition of heat to eliminate cold stress, elimination of overcrowding, and maintenance of feed consumption [22]. Antibiotics can be used to reduce the chance of airsacculitis and electrolyte replacement has been effective in controlling nephritis in Australia [1].

PREVENTION AND CONTROL

The best prevention and control methods are proactive management procedures and immunization [1, 2, 22].

Immunization

Several types of vaccines can be used to protect chickens from IB [1, 2]. The most common vaccines used are live attenuated and inactivated oil emulsion, with subunit, virally vectored [38, 39] and DNA vaccines on the horizon. Live attenuated vaccines are used in broilers and as an initial vaccine for breeders and layers. Inactivated oil emulsion vaccines are used at point of lay in breeders and layers after priming with a live attenuated vaccine. Live attenuated vaccines are produced by serial passage of virus in embryonated chicken eggs [40]. There is a limit to the number of passages, as extensive passage has been shown to reduce immunogenicity [1]. One drawback to live attenuated vaccines is that back passages in chickens have proven to enhance virulence in some cases [41].

Selection of a vaccine strain should be made to represent the most prevalent antigenic types of virus in a specific geographical area [1, 2, 22]. The Mass serotype vaccine is widely used due to the initial isolation of IBV in many countries being of that serotype. Different serotype vaccines are only used when a new or antigenically distinct serotype becomes prevalent in a geographical area [1, 2].

Listed below are the vaccines commonly used in the U.S. and abroad (King, D., personal comm.).

U.S. - Mass, Holland, Connecticut (common)

JMK, Florida 18288, Arkansas (used regionally with license)

Netherlands - Holland, D274, D1466

U.K. - Mass, 4/91 attenuated

Australia - Strains of B and C subtypes, VicS

There are several methods of vaccine application [1]. Route of inoculation for live vaccines are eye-drop [42], intratracheal [43], intranasal, and embryonal [44]. Mass applications are given by aerosol, coarse spray, and in drinking water [1, 42, 43]. Although cheaper and more convenient, the dose is not always uniform. Furthermore, aerosol vaccination can cause severe reactions and drinking water vaccines can be inactivated by constituents of water [45]. Inactivated vaccines require much more effort and each chicken must be individually injected [1]. Vaccines of this type are given after priming with a live attenuated virus. The timing of immunization varies due to maternal antibody, type of vaccine, and application method. The most common time of immunization is 2 weeks of age, but 1-day-old vaccination has been successful [42, 43]. Combined IBV vaccines with Newcastle's disease virus are used [1].

Part II: Infectious bronchitis virus

ETIOLOGY

Classification of the viral agent

The causative agent of IB is infectious bronchitis virus (IBV) [1]. This virus belongs in the *Coronviridae* family, which contains the genuses Coronavirus and Torovirus [46]. Infectious bronchitis virus belongs in the Coronavirus genus along with turkey coronavirus and at least nine other viruses that infect mammals [20].

Morphology

The IB virion can be pleomorphic but is usually round in shape with a diameter that ranges from 90-200 nm [1]. A lipid bilayer, derived from host cell membranes, forms the outer envelope of the virion [20]. Extending from the viral envelope are club shaped surface projections (spikes) that are 20 nm in length and smaller projections of 7 nm in length [1]. The nucleocapsid complex of the virion is composed of the vRNA and nucleocapsid protein having helical symmetry [20]. The average density of viral particles is 1.15-1.18 g/ml in a sucrose gradient [1].

Sensitivity of IBV to physical and chemical agents

The sensitivity of infectious bronchitis virions to physical and chemical agents is listed below [1, 47, 48]:

Temperature:	56°C - 15 minutes (inactivates)
	45°C - 90 minutes (inactivates)
pH:	pH 3.0 - resistant

	pH 11.0 - sensitive
Ether:	Relatively stable
Sodium deoxycholate:	0.1% - 4°C - 18 hours (inactivates)
	0.2% - 20°C - 10 min. (inactivates)
Disinfectants:	50% chloroform (inactivates)
	Beta propiolactone (inactivates)
	Formalin (inactivates)
	Phenol (inactivates)
UV irradiation	Sensitive

MOLECULAR ASPECTS

Genome

The genome of IBV is a linear single stranded ribonucleic acid (RNA) polymer that associates with nucleocapsid protein to form the ribonucleoprotein complex (RNP) [1]. A 5' cap and 3' polyadenylated tail, along with the positive sense of the genome, renders the IBV genome infectious [20]. The complete genome of IBV is approximately 27,500 nucleotides and has been completely sequenced for the Beaudette strain [49]. A complete IBV genome contains at least 10 ORF's and is organized as follows: 5' - Pol 1a, Pol 1b, S, 3a, 3b, sM, M, 5a, 5b, N - 3' [46].

Major genes and proteins of IBV

There are five major genes within the IBV genome [20, 46]. They are the polymerase gene(s), which code(s) for the non-structural RNA dependent RNA

polymerase, and the four genes that code for the structural proteins as follows: spike glycoprotein, nucleocapsid protein, membrane glycoprotein, and the small membrane protein.

Polymerase gene(s) and protein(s)

Research efforts have failed to detect a virion-associated RNA polymerase, leading to the conclusion that the IBV polymerase must be translated from the positive sense vRNA that enters into the host cell. Two open reading frames have been reported, 1a and 1b [50]. The 1a ORF codes for a polypeptide of 441 kDa and the 1a/1b ORF codes for a polypeptide of 741 kDa, respectively [51]. Within the 1a protein, two overlapping papain like proteinase domains have been discovered [52, 53]. Also, a 3c-like proteinase [54] has been discovered and characterized. These proteinases appear to function in processing of the 741 kDa polyprotein produced by translation of the 1a/1b ORF into smaller, functional proteins. Putatively, the RNA dependent RNA polymerase and RNA helicase proteins are within the 1b ORF [55].

Spike gene and protein

The spike gene (mRNA 2) encodes the precursor spike glycoprotein that becomes the club shaped projections anchored in and extending from the viral envelope [20]. The gene is transcribed and translated as a large precursor S glycoprotein (S_0) of approximately 155,000 daltons, with the following order, NH_3-S1-S2-COOH [56]. This precursor protein is post-translationally cleaved by trypsin-“like” host cell enzymes at the cleavage site bearing the amino acid sequence X1-Arg-X2-Arg-Arg, where X1 represents His, Arg, or Tyr and X2

represents Ser, Phe, Ile, Arg, or His [57, 58]. The resultant protein subunits, S1 and S2, are 90,000 and 84,000 daltons, respectively [56, 59].

Formally, the mature spike glycoprotein within the viral envelope is composed of two to three subunits each of the S1 and S2 subunits that associate by weak non-covalent forces [59]. This research showed that intrapeptide and not interpeptide disulfide bridges are present within each subunit. Anchoring the spike glycoprotein in the membrane, the S2 subunit is approximately 625 amino acids in length. The polypeptide has 12 predicted N-linked glycosylation sites. Near the carboxy terminus, the S2 subunit has 44 non-polar amino acids that probably serve as the spike anchor.

Forming the globular head of the spike glycoprotein, the S1 subunit is approximately 520 amino acids in length with two hypervariable regions reported [60, 61]. Hypervariable region 1 is located within amino acids 38-51 and hypervariable region 2 is located within amino acids 99-115. The first 18 hydrophobic amino acids at the amino terminus probably serve as a signal sequence and do not appear on the mature S1 subunit [62]. The S1 polypeptide has approximately 16 predicted N-linked glycosylation sites [59].

Virus infectivity has been associated with the S1 subunit, as well as neutralizing epitopes within the defined hypervariable regions [61, 63]. Research has shown that virus retaining the S1 and not the S2 subunit portions of the spike glycoprotein induce neutralizing, serotype specific, and hemagglutination inhibiting antibodies [28]. It has been reported that these antibodies recognize a few amino acids involved in conformationally dependent epitopes within the first

and third quarters of the S1 subunit [61, 64]. Further research has shown that virus lacking the S1 subunit could attach to erythrocytes and chicken kidney cells as efficiently as intact virus [63]. There was a decline in infectivity, suggesting that the attachment process for the S1 subunitless virus was qualitatively different. The subsequent loss of infectivity was a due to the loss of some other spike function associated with the S1 subunit.

Complete spike protein has been expressed using a recombinant vaccinia virus system [38]. Antibodies raised against purified spike protein recognized spike protein expressed by the vaccinia virus system. Also, serum collected from recombinant vaccinia virus vaccinated mice was able to neutralize IBV infectivity in tracheal organ cultures as shown by ciliostasis tests. Other related work has reported that protective immunity has been achieved using a recombinant baculovirus vaccine [39].

Monoclonal antibody studies have defined antigenic domains on the spike glycoprotein of IBV. Specifically, eight epitope clusters were defined using monoclonal antibodies as follows: S1 subunit - S1A, S1B, S1C, S1D, S1E, S1F and S2 subunit - S2G and S2H [65]. Epitope clusters S1A to S1E and S2G were reported to moderately or strongly neutralize IBV, while the rest only weakly neutralized IBV. One monoclonal antibody against the S1D epitope inhibited the agglutination of erythrocytes. Monoclonal antibody studies have also been used to define an immunodominant region on the S2 subunit that induces neutralizing, but not serotype specific antibodies [66].

Sequencing studies of the S_0 gene and its deduced amino acid sequence have shown that there are two hypervariable regions within the gene and subsequent protein product [61]. Among closely related isolates with only 6% amino acid variation for the entire S1 subunit, the hypervariable regions 1 and 2 respectively have 29% to 40% amino acid variation. Specific amino acid positions that involved in virus neutralizing epitopes have been determined by a number of labs. Specifically, amino acid changes at positions 134, 304, and 386 have been reported to confer monoclonal antibody resistance for particular strains of IBV [61, 64].

Nucleocapsid gene and protein

The nucleocapsid gene (mRNA 6) encodes the nucleocapsid protein that is approximately 409 amino acids in length [20, 67]. It has a molecular weight of 50,000 daltons and is not N-linked glycosylated and due to its size and structure it probably has helical symmetry [50]. It has always been considered highly conserved between strains of a species, but recent reports have shown diversity between at least two distinguishable groups of nucleocapsid proteins [68]. The nucleocapsid protein associates with the vRNA in the virus core to form the ribonucleoprotein complex by binding to the positive sense 3' non-coding region at the end of the IBV genome [69]. Evidence suggests that this RNP complex associates with the membrane protein to facilitate virion assembly [67]. The immunogenicity of the nucleocapsid protein has been debated, but there is a report that epitopes mapped to the 120 carboxy-terminal residues may illicit CTL responses [70].

Membrane gene and protein

The membrane gene (mRNA 4) encodes the membrane glycoprotein that ranges from 225-230 amino acids in length and has a molecular weight between 23,000 and 34,000 daltons depending upon the degree of glycosylation [50, 71]. A model for the structure of the membrane protein has been proposed [50]. Three hydrophobic regions within the M glycoprotein are thought to be transmembrane domains. Approximately 9-20 amino acids of the amino terminus form an ectodomain that extends outside the viral membrane. There are two predicted N-linked glycosylation sites within the amino terminus. A 20-25 amino acid hydrophilic region within the carboxy terminus may associate with the vRNA in the virus core.

Envelope gene and protein

The envelope gene (mRNA 3c) encodes a small integral membrane protein that is approximately 109 amino acids in length and has a predicted molecular weight of 12,400 daltons [72]. Corse et al. have determined some characteristics of the protein [73]: a C-terminus located in the cytoplasm, a N-terminus that is translocated and contains an unused N-linked glycosylation site, and the protein is localized to the golgi complex in IBV infected cells. Physical interaction of the envelope and membrane proteins has been reported [74] and appears to be necessary for virion assembly and budding.

Recombination

Genetic recombination has been reported for many viruses, with the best example being influenza [75]. It has been hypothesized for IBV [20]. Several

studies have tried to answer the question about recombination in IBV. In 1990, Kusters et al., reported circumstantial evidence for recombination in IBV [76]. They hypothesized that the S gene of strain D207 and D1446 recombined to produce the S gene of strain UK/6/82. This was based on the fact that the first 3315 nucleotides showed >98% similarity to D207 but after that point there was only 57% similarity. The latter portion had >97% similarity to strain D1466 and only 73% similarity before that point.

In 1995, Kottier et al., coinfected embryonating eggs with two different strains of IBV, Beaudette and Mass 41 [77]. They found that recombination occurred to produce hybrid RNA's. Furthermore, Jia, et al., have reported that the strain CU-T2 has resulted from the recombination of three strains [78]. The recombinations reported were within the S1, S2, and N genes. Interestingly, half of the N gene was replaced by the N gene from the strain H-52, suggesting that recombinations among vaccine strains may contribute to the creation of IBV field variants.

In 1997, Wang, et al., were able to create chimeric IBV genomes with crossover sites in the S1 portion of the S_0 gene by co-infection with the strains Arkansas 99 and Massachusetts 41 [79]. The crossover sites were between nucleotides 50 and 155 of the S1 gene. Recombination was verified using heterologous RT-PCR primers that could not amplify mixed RNA from the strains Arkansas 99 and Massachusetts 41, but could amplify chimeric S_0 genes.

Lastly, Lee and Jackwood [80] studied the recombination of IBV by comparing sequence data for several strains of IBV that spanned from gene 3 to

the 3’ non-coding region. They determined that the intergenic (IG) consensus sequence (CTGAACAA or CTTAACAA) might serve as a recombination “hot spot”. Their sequence data analysis showed that the topology of phylogenetic trees for several strains of IBV changed depending upon the gene that was analyzed. They concluded that the IG region serves as a template-switching site for the viral polymerase that favors the recombination of whole genes instead of the recombination of parts of genes.

Viral replication and assembly

Virus replication has been reviewed in several papers and books and a summary is given here [1, 20, 81]. Upon entering a competent host cell, infectious bronchitis virions replicate in the cytoplasm of the cell. The process of replication is reported to be discontinuous and leader primed. Briefly, the positive sense genome is translated into the RNA dependent RNA polymerase, which then makes a full-length negative sense copy of the genome. This negative sense copy of the genome is used as a template to produce positive sense full-length genomes and a nested set of six 3' coterminal subgenomic mRNA's by leader primed transcription.

Leader primed transcription involves the synthesis of a leader sequence from the 3' end of the negative sense genome copy. The leader releases from the negative sense copy, while remaining attached to the RNA dependent RNA polymerase. This complex then moves farther down the negative template to sites that are recognized by the leader sequence. Once attached at the appropriate sites, transcription of each subgenomic mRNA begins. Only the unique 5' end of each

subgenomic mRNA is translated into the major structural proteins necessary for infectious virion production. The formation of an infectious virion is as follows:

1) Production of full-length positive sense genome
2) Translation of nucleocapsid protein in the cytoplasm that interacts with RNA genome to form ribonucleoprotein complex
3) Concurrent translation of spike protein, membrane protein, and small membrane protein by ER bound ribosomes
4) Interaction of membrane protein, localized within the ER and the Golgi complex, with ribonucleoprotein complex begins to form a bud
5) Final virion forms by budding from the ER and Golgi complex membranes
6) Virions accumulate in smooth vesicles, but release mechanism remains undefined

New virus can be visualized leaving the host cell at 3-4 hours after initial infection [1]. It is noteworthy that some debate as to these processes continues, and the finding of negative sense subgenomic mRNA's may lead to revisions of this replication strategy [20].

Laboratory host systems

Several host systems, chicken embryos, turkey embryos, cell culture, and organ culture have been used to grow IBV. A good review can be found in *Diseases of Poultry* by King [1] and is not presented here.

Strain/serotype classification

Several tests have been employed to place strains into serotypes. They are the virus neutralization test [25, 33-35], hemagglutination inhibition test [36], immunodiffusion, ELISA [82], immunofluorescence [31], nucleic acid probes [83, 84], T1 RNASE fingerprinting [12], RT-PCR [85], RT-PCR/RFLP [86, 87], SDS-PAGE polymorphisms [88] and DNA sequencing [89-93]. Procedures for the virus neutralization test, hemagglutination inhibition test, immunodiffusion test, ELISA, and immunofluorescence test can be found in the Laboratory Manual for the Isolation and Identification of Avian Pathogens and are summarized here.

Virus neutralization test

The virus neutralization test has been used in some form or another since 1974 to classify IBV serotypes. Classification of strains into a serotype requires the use of cross neutralization tests. In this procedure, two different virus strains are neutralized with homologous and heterologous antiserum separately. Cross neutralization tests have been performed in embryonated chicken eggs [33], tracheal organ cultures [25, 34], and chicken kidney cells [35]. Two different procedures, alpha and beta, have been developed:

Alpha - constant serum with variable virus

Beta - constant virus with variable serum

Briefly, monospecific antiserum is mixed with whole virus for an allotted amount of time. This mixture is then inoculated into embryonated chicken eggs, TOC, or chicken kidney cell cultures. These systems are evaluated differently.

Chicken embryos are evaluated one week after inoculation with death, dwarfing, curling, clubbed down feathers, and ureates representing non virus neutralization [1]. The Reed and Meunch formula is then used to calculate end point titers for homologous and heterologous neutralization. These cross neutralization titers can be used in the Archetti and Horsfall formula to calculate antigenic relatedness values that can classify viruses into the same, related, or unrelated serotype [94].

The formula is as follows:

$$R = (r_1 \times r_2)^{1/2}$$

R = antigenic relatedness

r_1 = heterologous virus $titer_2$/homologous virus $titer_1$

r_2 = heterologous virus $titer_1$/homologous virus $titer_2$

The limits for the Archetti and Horsfall antigenic relatedness value for determining virus serotype relatedness are as follows:

Same serotype =	0.80 to 1.00
Related serotype =	0.50 to 0.79
Non-related =	0.00 to 0.49

TOC is evaluated on the basis of the presence of tracheal ciliary activity. In the beta procedure, end point titers are chosen as the highest dilution of antiserum where there is tracheal ciliary activity. Cross neutralization titers can be used in the Archetti and Horsfall formula to determine antigenic relatedness as stated above.

Chicken kidney cell cultures are evaluated using the plaque reduction technique. In the beta procedure, the end point titer is the highest dilution of antiserum that produces a predetermined reduction in plaque size based upon plaque sizes produced by unneutralized virus. Cross neutralization titers can also be used to determine antigenic relatedness as done for eggs and TOC's.

Hemagglutination inhibition test

Certain strains of IBV agglutinate erythrocytes after treatment with phoshpolipase C (PLC) type 1. It was later determined that neuraminidase treatment was the actual cause of hemagglutination [95]. Since then this finding has been used to produce an HI assay that can determine the serotype of IBV. Briefly, antiserum is mixed with whole virus that has been treated with neuraminidase. The virus serotype is positively determined by the ability of the antiserum to inhibit hemagglutination of erythrocytes by the virus. Several researchers have evaluated this technique with variable results. It seems that the technique can detect antibodies earlier than by VN assays, but cross-reactions make the analysis of the results more complex [1].

Immunodiffusion test

The immunodiffusion or agar gel precipitin test is infrequently used except to identify new isolates. It is used in this case because it detects reactions with all group and type antigens. The precipitating antibodies used for this test are generally produced between days 8 to 20 post-infection. Therefore this test detects antibody in a limited time span, but any reaction suggests a recent infection. Briefly, the test is done by covering a slide with agar and making two

holes in the agar. Antiserum is placed in one hole while antigen (presumably virus) is placed in the other. The antibody and antiserum are allowed to diffuse into the agar and the formation of a precipitin line between the two holes is evidence of a positive reaction. Due to the generality of this test it is not used to classify strains/isolates into a serotype.

Enzyme linked immunosorbant assay

The enzyme linked immunosorbant assay (ELISA) has been used to diagnose IB infections. Several different procedures involving different monoclonal antibodies have been developed. Although widely used due to cost effectiveness and ease, the assay is best for group specific identification. Recently, there have been reports of monoclonal antibodies that are type and strain specific. Karaca, et al., prepared monoclonal antibodies against the strains Arkansas 99, Connecticut 46, and Massachusetts 41 [82]. The antibodies were placed into three categories, group specific, type specific, and strain specific. The monoclonal antibodies that were serotype specific were directed primarily against the S1 subunit. This report demonstrated a correlation between ELISA and VN tests, but the correlation was not 100%.

Immunofluorescence

The immunofluorescence test has been used to determine IB infections. It is another group specific test that is cheap, easy, and fairly reliable. Briefly, thin section smears of tissue are placed on a microscope slide and allowed to react with a group specific primary monoclonal antibody. Unbound antibody is then washed away. The slide is incubated with a secondary antibody specific for the

primary antibody. The secondary antibody is tagged with a fluorescent marker that can be visualized using a fluorescent microscope. A positive is easily discerned if the sample fluorescence is greater than the fluorescence seen in a negative control.

Nucleic acid probes

Nucleic acid probes have been developed to detect IB infections. Kwon, et al., developed a biotin-labeled DNA probe that was able to detect the genome of IBV from tracheal swabs taken from experimentally infected chickens [84]. Briefly, RNA was extracted from the tracheal swabs and used a template in a reverse transcriptase-polymerase chain reaction. The PCR product was then incubated with a biotin-labeled probe that could be visualized by colorometric assay. Strains of different serotypes were detected equally well. Also, EM analysis only found 4/8 positive samples, while the biotin-labeled probe found 8/8 positive samples. No clinical signs or specific antibody response was found, but histopathology lesions were observed. This suggests that this technique may be more sensitive and specific for the diagnosis of IB infections than EM, observing clinical signs, or serology.

T1 RNASE fingerprinting

T1 RNASE fingerprinting is a labor intensive assay that provides genomic information that is complex to analyze. Thus, it is not routinely used to characterize IBV. Briefly, the RNA genome of a virus is mixed with Ribonuclease T1. This enzyme cleaves RNA after guanine residues and a characteristic pattern can be obtained by two dimensional gel electrophoresis. If

done properly, it can be used to determine some evolutionary relationships between virus strains. Research groups have used this technique to place isolates/strains into broad genotype groups, which do not necessarily correlate with antigenic relatedness [12]. Therefore, this technique does not seem to be the best assay for serotyping.

RT-PCR

The reverse transcriptase-polymerase chain reaction (RT-PCR) has been used by several research groups to detect IB infections. As far back as 1993, RT-PCR primers have been used to amplify conserved regions of the IBV genome. Kwon, et al., have used primers flanking the M and N genes of IBV to produce an approximately 1,000 bp piece of DNA that is specific for IBV [86].

In 1996, Adzhar, et al., was able to use seven pairs of oligonucleotides specific for differing parts of the IBV genome that were specific for most known strains of IBV [96]. Falcone, et al., have also reported the ability to detect the N gene of the IBV genome using a nested RT-PCR that was quick, specific, and sensitive [85]. Although this method works well, it is only group specific and cannot be used to type a virus. One recent report claims that they have developed serotype specific primers. Keeler, et al., used 31 strains of IBV to identify conserved and variable regions within the S1 gene of IBV [97]. They used conserved areas to design degenerate primers and variable regions to design serotype specific primers. In a blind test with thirty reference strains, they found that their test correlated with VN test results. Of the thirty samples tested, 20 were found to be positive by RT-PCR and 85% of these agreed completely with

the VN test for the virus. This suggests that this technique is viable, but it may need some fine-tuning and will need to be updated as new serotypes become prevalent.

RT-PCR/RFLP

Reverse transcriptase/restriction fragment length polymorphism (RT-PCR/RFLP) has been used to successfully identify IB infections and to serotype the causative virus. Lin, et al., amplified part of the S2 gene of IBV and then analyzed this PCR product using restriction fragment length polymorphisms [87]. They suggested that this technique correlated with the VN test, but their data showed that it did not correlate with the currently recognized serotypes of IBV and representative strains from all serotypes were not included in the study.

Kwon et al., developed a similar test but amplified the entire S1 gene [86]. The PCR product was subjected to RFLP analysis with three enzymes, BstYI, HaeIII, and XcmI. The pattern created by analysis of the three enzyme products in contiguous lanes on an agarose gel, could be used to differentiate all strains tested into serotypes that correlated 100% with the VN test.

Another group has tried a derivative of this test using 5 different restriction enzymes to genetically group 25 IBV isolates in Taiwan [98]. There method was able to group these isolates into three genetic groups with a Massachusetts isolate belonging in a genetic group by itself. The efficacy of this method as a serotyping technique was not determined.

SDS-PAGE polymorphism

A SDS-PAGE polymorphism has been used to differentiate strains within the California serotype of IBV from strains belonging to other IBV serotypes. Case et al., found a novel protein polymorphism for the M protein in California serotype strains of IBV [88]. The protein polymorphism can be detected on Western blots using raw or concentrated infectious allantoic fluid. Due to this tests ability to differentiate strains of only one serotype from all other serotypes, the usefulness of this test needs to be determined farther.

DNA sequencing

Direct DNA sequencing of the different IBV genes has been abundantly reported. The sequencing procedure is generally the same for all genes and strains. Specific RT-PCR primers are used to amplify a region of interest and then this PCR product is directly sequenced by primer walking or by cloning and then sequencing. To date, the IBV S1 gene is the best predictor of serotype for IBV strains. As a general rule, strains that share similarity >85% for amino acid sequence are within the same serotype [92].

Exceptions to this rule do exist, for example, the strains Connecticut 46 and Florida 18288 share >96% amino acid similarity for their S1 genes, but remain serologically distinct [25]. It is thought that a few amino acid changes in key locations can cause a virus strain to be serotypically distinct from another virus that has a closely related S1 gene [64, 90]. For this reason, it is necessary to use DNA sequencing to complement other tests, such as VN, ELISA, or RT-PCR/RFLP, to definitively place an isolate/strain into a serotype.

Part III: Useful molecular tools for IBV research

RIBOZYMES

Ribozymes are catalytic RNA molecules [99]. These molecules catalyze the cleavage [100] or ligation of target RNA molecules [101, 102]. Ribozymes were first described by Cech's research group [103, 104]. Their study of *Tetrahymena thermophila* led to the discovery of RNA molecules able to self-catalyze the excision of an intron from a pre rRNA molecule.

Since the first description, several types of ribozymes have been discovered. The group I and group II intron ribozymes, which are primarily found in lower eukaryotes and some bacteria, have the capacity to self-splice by cleaving and religating strands of RNA. The RNase P ribozyme, along with a protein cofactor, is able to cleave a phosphodiester bond from a several different tRNA precursors [105]. The nucleolytic ribozyme group contains four members, the hammerhead, hairpin, hepatitis delta virus, and the Varkud satellite ribozymes. Each is unique in sequence and structure, but all are less than 150 nucleotides and essentially catalyze the same type of cleavage reaction [106]. Herein, we discuss the hammerhead ribozyme.

The secondary structure of the hammerhead ribozyme resembles the head of a hammer, hence its name [107]. These molecules are capable of catalyzing the cleavage of target RNA sequences *in cis* or *trans* [108]. They naturally occur in some plant viruses, viroids, and in RNA transcripts of satellite DNAs in newts and schistosome. Hammerhead ribozymes are composed of two components, a catalytic core and flanking sequences . The catalytic core is responsible for the catalytic

cleavage of the target RNA, while the flanking sequences determine the ribozyme specificity. Any RNA molecule can be cleaved by a hammerhead ribozyme as long as it contains a 5'-NUH-3' sequence, where N is any nucleotide and H is any nucleotide except G [109]. Another report claims that the target RNA sequence rule can be changed to 5'-NHH-3' [110], although the cleavage rate is lower when A or C resides in the second spot when compared to U.

The catalytic mechanism of the hammerhead ribozyme has been studied. Two possible catalytic mechanisms have been hypothesized. The first mechanism involves one Mg^{2+} ion and the second mechanism involves two Mg^{2+} ions [108]. Theoretical and experimental evidence supports the two Mg^{2+} ion model, but either way the ribozyme only positions the target RNA in such a conformation that the catalytic mechanism can occur between base 17 and 1.1 of the target RNA, when using the numbering system by Hertel *et al* [111].

Obviously, the ability of hammerhead ribozymes to target and catalyze the cleavage of almost any target RNA makes them very attractive therapeutic agents. Hammerhead ribozymes show great potential as antiviral therapeutic molecules. Published reports have shown that hammerhead ribozymes inhibited viral replication for three RNA viruses from different virus families; lymphocytic choriomeningitis virus (LCMV- *Arenaviridae*), mouse hepatitis virus (MHV - *Coronaviridae*), and human immunodeficiency virus (HIV - *Retroviridae*). Replication of LCMV in cell culture was inhibited by the stable expression of a hammerhead ribozyme targeted to the S segment of the viral genome. The hammerhead ribozyme decreased the production of infectious virus by 100X and the antiviral activity was shown to be

specific for LCMV [112]. Replication of MHV in cell culture was inhibited by the stable expression of either of two different hammerhead ribozymes targeted to the polymerase gene. Inhibition of acute and chronic viral infection was shown [113, 114]). Lastly, replication of HIV in cell culture was inhibited by the stable expression of a hammerhead ribozyme targeted to the tat gene. Specifically, CD4+ cells stably transduced by a pseudotyped retroviral vector containing the anti-tat hammerhead ribozyme were able to resist HIV infection for up to 20 days [115].

Hammerhead ribozymes could be used for gene therapy. In particular, hammerhead ribozymes are being investigated for genetic diseases that act in a dominant negative fashion, such as Marfan Syndrome [105]. This common connective tissue disorder stems from mutations in the fibrillin gene, with most of the mutations being ones that act in a dominant negative fashion [116]. Theoretically, a hammerhead ribozyme targeting mutant alleles causing Marfan Syndrome, while not targeting any non-mutant alleles, should help to ameliorate the disease.

While hammerhead ribozymes show great potential, some problems exist for their use as antiviral and gene therapy agents. One of the largest obstacles is finding an appropriate target site within a RNA molecule [109]. The target site region must have the NHH triplet and also be devoid of any secondary structure. Several methods exist for determining possible target sites. The easiest is the use of MFOLD, a computer program that uses algorithms based on thermodynamic data to predict secondary structure of RNA [117, 118]. Although the program can accurately predict regions of a RNA molecule lacking secondary structure, direct experimentation seems to work the best. Some researchers identify open regions

in a target RNA by hybridizing libraries of oligonucleotides to a labeled RNA transcript followed by subsequent RNase H digestion [119]. Obviously, any oligo that hybridized should mediate cleavage of the target RNA by RNase H. The cleavage can be assessed by any number of lab techniques, such as gel electrophoresis of cleavage products. Another technique is to bind target RNA to a microarray of oligos [120]. Hybridization of a particular oligo with the target RNA can be measured using microarray technology.

Another problem is how to effectively deliver enough of the ribozyme to the appropriate target cells and sites within a target cell. Basically, there are two forms of delivery, exogenous and endogenous [109]. For exogenous delivery, presynthesized ribozymes are directly placed onto the target cell. Animal and cell culture models exist for the exogenous delivery of hammerhead ribozymes without the use of a carrier that effectively reduced the expression of a target mRNA [121-123]. For the most part, the exogenous delivery of hammerhead ribozymes requires the use of a carrier molecule, such as lipofectamine, cationic lipids, or liposomes [109]. Endogenous delivery of ribozymes requires the use of a vector. Specifically, viral vectors appear to be the vectors of choice, with the most highly used viral vectors being retroviruses, adenoviruses, and adeno associated virus [109]. Retroviruses are favored because of their high transduction efficiency and their ability to stably integrate the ribozyme encoding gene into the genome of the cell. Another commonly used viral vector is the adenovirus. While able to deliver the ribozyme encoding gene into a target cell, adenoviruses only provide transient expression and stimulate a host immune

response. A likely alternative to the adenovirus is the adeno-associated virus. It is non-pathogenic, does not require actively dividing cells, and in humans, integrates itself into the genome at a specific site on chromosome 19.

Assuming effective delivery, another limitation may be the stability of the ribozyme. Nuclease resistant ribozymes have been reported [124-126]. In general, a modification at the 2' site of the ribose sugar, such as the addition of a fluoro, amino, or O-methyl group, renders ribozymes resistant to most nucleases [109]. This provides a longer half-life for the ribozyme.

Another obstacle may be that ribozymes lack the ability to hybridize with targets due to the secondary structure of mRNA or interaction of mRNA with accessory proteins or ribosomes [127]. One report showed that the interaction of target mRNA with ribosomes occluded the ability of a hammerhead ribozyme to gain access to its target site in an *E. coli* model [128]. When slow ribosome mutants were used, the hammerhead ribozyme was able to gain access to and cleave its target mRNA.

Lastly, ribozymes could be toxic to cells due to some nonspecific effect [129] or unexpected cleavage of a critical cellular RNA. Ribozymes will cleave any RNA containing a target sequence, so the specificity of the ribozyme is of utmost importance. Therefore, it seems necessary to continue to study all aspects of hammerhead ribozymes to better understand how to effectively deliver a stable ribozyme to a target cell that can gain access to its target RNA, specifically hybridize with it, and catalyze its cleavage. Although many hurdles remain, the promise of hammerhead ribozymes as effective antiviral and gene therapy molecules makes them

an attractive research area and hopefully their full potential will be realized in the future.

DNA SHUFFLING

Producing novel combinations of genetic material is a necessary process in the development of life and its adaptation to the prevailing environment. One of the most well-known examples of genetic recombination is the process of crossing over that occurs between homologous DNA chromosomes during gametogenesis in many organisms [130]. However, recombination is not limited to DNA. The genetic material, RNA, is capable of undergoing recombination, as well. Some RNA viruses, such as IBV, undergo significant amounts of recombination during the viral replication cycle [80]. In both examples, recombination events lead to novel genotypes, which may or may not lead to novel phenotypes, and in turn may produce a more or less fit virus for the current environment.

The bridge between genotype and phenotype is protein, a string of amino acids that folds together and performs some function or role, whether it be enzymatic or structural. Over millions of years, evolution and natural selection have fine tuned proteins to perform their particular function efficiently. Although evolution and natural selection are very successful at producing novel genetic combinations that lead to improved proteins, there are some shortcomings to the process as it pertains to the field of biotechnology. First, the time scale of millions of years is too slow, and second, natural processes do not always select for a protein with a desired quality that might be medicinally or technologically useful to humans.

Recent advances in biotechnology have made it possible to overcome both of the aforementioned shortcomings for evolution and natural selection. We now have the capability to produce new proteins with desired characteristics on a time scale suitable to human life. This idea, known as directed evolution, has been achieved through the use of a process termed DNA shuffling, which is the purposeful, but random, recombination of similar genes. The process uses *in vitro* techniques to produce novel genetic combinations, which in turn produce novel proteins that can be screened for desired characteristics.

W. P. Stemmer pioneered the concept of DNA shuffling [131]. His method of DNA shuffling involved the random fragmentation of similar genes, followed by reassembly of the fragments by primerless PCR. Basically, parental genes are randomly fragmented by treatment with Dnase I. The small fragments are isolated and used in a primerless PCR where the small fragments prime upon each other due to sequence similarity, leading to the recombination of parental genes into novel genes. Then, the novel genes are expressed and screened/selected for a desired characteristic. Genes that encode proteins that survive the screening/selection process are used as parental genes in another round of shuffling and the whole process is repeated over and over until a gene that encodes a protein with all of the desired characteristics is isolated.

In 1994, Stemmer tested the DNA shuffling technique in a beta-lactamase model system [131]. Several parental genes encoding beta-lactamase were shuffled and recombinants selected for on increasing concentrations of the antibiotic cefotaxime. After three rounds of shuffling and two rounds of back crossing with parental genes,

mutants were isolated with 32,000X increase in resistance to the antibiotic cefotaxime. Stemmer also applied DNA shuffling to produce a functional lacZα gene from two non-functional genes [132]. The two non-functional genes had improper stop codons in different sections of the gene. By applying DNA shuffling, he was able to produce a lacZα recombinant gene that lacked either of the improper stop codons. Therefore, the recombinant lacZα gene was able to express a whole functional protein. In the same report, Stemmer was able to create chimeras of interleukin 1 beta genes using human and murine genes as the parental templates.

Since the initial report of DNA shuffling, many researchers have applied Stemmer's method to produce novel genes/proteins with desired characteristics. Researchers have improved proteins for gene therapy, pharmaceuticals, and vaccines [133, 134]. The field of virology has also benefited from DNA shuffling. Two of the most interesting research articles involve the use of random fragmentation/primerless PCR to produce retroviruses with novel phenotypes. In 2000, Soong et al. shuffled six murine leukemia virus envelope genes and selected for a clone with a completely new tropism for Chinese hamster ovary (CHOK1) cells [135]. Characterization of the clone determined that the probability of naturally producing the particular envelope sequence was not likely. The researchers concluded that DNA shuffling was an effective process to produce viruses with novel and desirable characteristics. Using the same method, including viral strains, Powell et al. produced a murine leukemia virus (MLV) with improved stability and processing yields [136]. Apparently, retroviral vectors are sensitive to stress forces during purification and concentration. By shuffling the envelope gene sequence of six MLV's, viral clones were isolated

that showed no loss in titer as compared to parental clones that showed reduced titers of 30X – 100X during the manufacturing process.

In the past few years, new techniques for DNA shuffling have been invented. One of the most effective is the Staggered Extension Process (StEP). First reported by Zhao et al., the staggered extension process involves mixing equimolar amounts of parental DNA templates sharing sequence similarity and performing a modified PCR [137]. The modification is that the annealing/extension steps are combined and performed at a temperature ranging from 45 – 55º C for 5 –15 seconds. The extremely short annealing/extension times only allow the DNA polymerase to synthesize very short fragments of DNA every cycle of the PCR. The short fragments are denatured and allowed to reanneal to complimentary templates. Most of the time, the short fragments hybridize to different templates than the cycle before. This template switching recombines the parental templates. Repeated cycles of denaturation followed by annealing and short extension ultimately lead to full-length genes containing sequences from different parental templates. The novel genes can then be expressed and screened/selected for desired properties. This method has been applied to increasing the thermostability of subtilisin E enzymes [137]. By screening a StEP library created from five subtilisin E variants, researchers isolated an enzyme whose half-life at 65º C was 50X that of wild-type subtilisin E. Since its first report, StEP has been applied to several other proteins, including biphenyl dioxygenase [138], alpha-galactosidase [139], and 5-enolpyruvylshikimate-3-phosphate synthase [140]. The following website maintained by Dr. Francis Arnold's research group at the California Institute of Technology (where StEP was invented) lists the application

of several DNA shuffling methods to producing novel genes/proteins – http://www.che.caltech.edu/groups/fha/.

Another method for DNA shuffling is termed RACHITT or the random chimeragenesis on transient templates [141]. Basically, parental genes are randomly fragmented by treatment with Dnase I. Then, single stranded fragments are ordered, trimmed, and joined on a transient single stranded polynucleotide scaffold. This technique generated chimeric libraries with an average of 14 crossovers per gene. In particular, the researchers used the RACHITT technique to increase the rate and extent of biodesulfurization by monooxygenase.

Recently, an improvement upon the method of Stemmer has been reported by Miyazaki [142]. He replaced Dnase I with endonuclease V, which nicks uracil-containing DNA at the second or third phosphodiester bond 3' to uracil sites. Basically, PCR is performed in the presence of some dUTP (the concentration can be varied to adjust the length of desired fragments after endonuclease V digestion) and then the DNA products are cleaved by endonuclease V. The fragments are then used in a primerless PCR in which they prime upon each other to produce recombined full-length genes. Miyazaki proved the ability of the technique to shuffle DNA by recombining two truncated green fluorescent protein genes into functional full-length green fluorescent protein genes.

REAL-TIME AMPLIFICATION OF NUCLEIC ACIDS

The amplification of specific nucleic acid targets was developed by Mullis and Faloona in 1987 [143]. The process is known as the polymerase chain reaction or

PCR. It uses short oligonucleotide primers that flank a target area and a DNA polymerase to synthesize new strands of DNA that are copies of the target area. Briefly, PCR involves three steps; 1) denaturation of target templates, at temperatures above 90° C, to produce single stranded DNA molecules, 2) annealing of short oligonucleotides, at temperatures between 45° C – 72° C, that act as substrates for the DNA polymerase, and 3) extension of the annealed primers by a DNA polymerase, at temperatures between 68° C – 72° C, to produce new complementary strands of DNA to the target template. The three steps make up one cycle. Any number of cycles is performed, dependent upon the assay, to exponentially amplify target nucleic acids. Since its inception, PCR has become a widely used laboratory and diagnostic tool across many scientific disciplines [144].

Traditionally, PCR products are separated by gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination. When only qualitative data is needed about a PCR, gel electrophoresis analysis is enough. Quantitative data can be obtained from gel electrophoresis, but the methods are laborious and sometimes error prone. A better way to concurrently generate qualitative and quantitative data is by the use of real-time PCR. The technique of real-time PCR allows for detection of nucleic acid amplification as the PCR progresses by measuring the increase in fluorescence using a real-time PCR machine. In this manner, the identity and amount of a target nucleic acid can be determined.

In general, there are two methods for detecting the amplification of target nucleic acid in a sample. The first method employs a non-specific dsDNA binding dye, such as SYBR Green I (Molecular Probes: www.probes.com) [145, 146]. When

the dye binds to double stranded DNA, its inherit fluorescence is greatly enhanced and can be measured by the real-time PCR machine. An increase in fluorescence equates to an increase in dsDNA within the PCR tube. Although the dye is not useful in determining the presence of a specific sequence within the target template, it can be effectively used to quantify (Quantification analysis using known standards for comparison) and identify (Melting curve analysis) target templates [147].

Basically, quantification with SYBR Green I involves the amplification of an unknown nucleic acid and its comparison with the amplification of known standards. One pitfall for this type of analysis is that all dsDNA products (including primer dimer and non-specific reaction products) produced during PCR bind SYBR Green I and contribute to the overall fluorescence signal. Primer dimer and non-specific product contributions can be subtracted out of the overall signal by performing a melting curve analysis on the amplification products. By determining the amount of primer dimer and non-specific products produced as compared to specific products produced, the fluorescent signal can be adjusted and quantitative data obtained. A melting curve analysis is very simple. The fluorescence of the amplification products is measured as the temperature of the sample is slowly heated. When the dsDNA reaches its melting temperature, the strands denature causing a reduction in the fluorescence signal due to low fluorescence of SYBR Green I when bound to ssDNA. By plotting the negative first derivative of the fluorescence signal change with respect to temperature, a characteristic melting peak can be obtained for a dsDNA molecule. This data can be used to determine the identity of amplification products in a PCR. Therefore, melting curve analysis is a very powerful tool that is necessary to obtain

quantitative and qualitative data when using a non-specific dsDNA binding dye, such as SYBR Green I.

The second method employs fluorescently labeled DNA probes. The probes hybridize to certain sequences if present in the target template and hybridization detection can occur in any number of ways. This method can be used to quantify and identify specific sequences within target templates in a biological sample. For the basics of RRT-PCR and the myriad dye/probe based detection techniques, see the review by Mackay *et al*. [144]. Herein, only a few of the most common probe based detection systems are discussed.

The most commonly used sequence specific detection system is the TaqMan assay that uses a double-dye oligonucleotide probe [148]. Basically, a sequence specific probe is labeled at its 5' and 3' ends with fluorophore and a quencher, respectively. If specific for a complimentary sequence in the target nucleic acid, the probe binds to the amplicon during the annealing step of the PCR. As the DNA polymerase (Taq polymerase) extends the nascent strand of DNA toward the bound probe, the 5' exonuclease activity of Taq polymerase degrades the 5' end of the probe. The 5' end fluorophore is released from the probe. Normally, the quencher is able to accept energy from the fluorophore via fluorescence resonance energy transfer (FRET – described below) so that when excited by the machine, no fluorescence signal is measured, but due to the spatial separation caused by degradation of the probe, the fluorescence of the sample increases each cycle. This indicates the presence of a particular sequence within a target nucleic acid. This assay has been used to detect and quantify the presence of many viruses in biological samples,

including but not limited to, swine fever virus [149], hepatitis C virus [150], HIV-2 [151], west nile virus [152], bovine viral diarrhea virus [153], rift valley fever virus [154], and enteroviruses [155]. Also, it has been used to quantify gene expression for many genes, including but not limited to, human telomerase [156], human chorionic gonadotropin-beta [157], and tyrosine hydroxylase [158].

Hybridization probes are another common sequence specific detection system used in real-time PCR [159]. Again, this method depends upon FRET technology. In FRET, the excitation of one fluorophore by a particular wavelength of light causes the fluorophore to emit a wavelength of light that can excite a second fluorophore, which emits a different wavelength of light that can be measured. The hybridization probe method utilizes two probes. One probe is designated the anchor probe and labeled with a fluorophore at its 3' end, while the other is designated the detection probe and is labeled with a fluorophore at its 5' end. The fluorophores are chosen such that the emission spectrum of the anchor probe and the excitation spectrum of the detection probe overlap. During the annealing step of PCR or during a melting curve analysis, the two probes anneal to any complimentary target nucleic acid side by side. The machine emits a particular wavelength of light to excite the fluorophore on the anchor probe and then the amount of FRET between the two probes is measured. Measurement of FRET between the two probes during the annealing step of PCR can indicate the presence or absence of a particular sequence within the target nucleic acid. If not FRET, then the anchor or the detection probe or both were not able to hybridize to the target nucleic acid. Measurement of FRET between the two probes during a melting curve analysis can not only detect the presence or absence of a

particular sequence, but can also determine mutations within the target sequence because base pair mismatches between the target template and the detection probe cause it to melt off the target earlier than expected. Many genotyping tests are based upon this fact and are able to discriminate down to a 1 base pair mismatch.

In particular, Loparev et al. used hybridization probes to genotype wild-type and vaccine strains of varicella zoster virus [160]. Also, Parks et al. used hybridization probes for the detecting prevalent mutations that cause common thrombophilic and iron overload phenotypes [161]. Finally, Veistinen et al. used hybridization probes to quantify human ailos splice variants [162]. From these examples, it is easy to see that real-time PCR with hybridization probes is a flexible technique that can be used in many different areas of research.

Molecular beacons are another probe based detection system [163]. Basically, a molecular beacon is a just like a double-dye labeled probe. It is a single stranded probe that is labeled at its 5' and 3' ends with a fluorophore and a quencher, either end can be labeled with the fluorophore or the quencher. The difference is that the overall structure of the molecular beacon is that of a hairpin loop. The single stranded loop is complimentary to the target nucleic acid. The stem is formed by complementary base pairing of nucleotides non-specific for any target nucleic acid sequence. The stem holds the fluorophore and quencher in close proximity, which allows for the quenching of the fluorophore when excited by light from the real-time PCR machine. The way that molecular beacons work is that during the annealing step of PCR, the stem of the molecular beacon melts and allows for hybridization to a target nucleic acid. If hybridization occurs, the quencher no longer quenches the

fluorophore due to their spatial separation and the overall fluorescence increases. The increase in fluorescence indicates the presence of a particular sequence within the target nucleic acid. As for the hybridization probes, molecular beacons can be used to identify sequences within target nucleic acids, as well as, perform mutational analysis.

As for hybridization probes, molecular beacons are useful for any number of diverse assays and just a few are mentioned here. In particular, Belanger et al. used molecular beacons to detect shiga-toxin producing bacteria in feces [164]. Later, this same group applied molecular beacons to detect *C. difficile* from feces [165]. In the field of virology, a recent report tested Taqman and molecular beacons for the quantitative detection of Epstein-Barr virus and cytomegalovirus (CMV) [166]. They compared the performance of both probe types with a gel-based PCR method for the quantification of EBV and CMV in patients' samples. They concluded that the TaqMan and molecular beacon approaches were useful assays for the diagnosis and monitoring of EBV and CMV infections in patients. Lastly, molecular beacons have been used for genetic testing to detect Tay Sachs alleles [167] and p53 mutations [168].

There are some drawbacks to the use of either non-specific or specific detection methods. First, for non-specific detection formats, such as SYBR Green I, all dsDNA molecules will bind the dye and add to the fluorescence signal. This is not a problem during qualitative tests because specific and non-specific products are easily differentiated by melting curve analysis. SYBR Green I is difficult to use to obtain quantitative data. It is possible, but much more difficult. For the specific detection

systems, the labeled probes are expensive and have a relatively short shelf-life as compared to normal oligonucleotides. Also, the PCR becomes more complex with every new molecule included in the reaction. Finding the proper thermocycle protocol that can amplify the target template and at the same time detect the hybridization of probes can sometimes be difficult.

Even though real-time nucleic acid amplification has some drawbacks, the advantages are too great to ignore. The ability to identify and quantify a particular nucleic acid in a sample without any post PCR manipulations is tremendous. The detection formats for real-time nucleic acid amplification will continue to grow and should be helpful in many scientific fields, especially in viral diagnostics and in the understanding of genetic disease processes.

REVERSE GENETICS

In the field of molecular virology, reverse genetics is defined as the production of infectious virus from cDNA. The concept of reverse genetics began over twenty years ago with the production of QB phage from a cDNA copy of its RNA genome [169]. Basically, a cDNA copy of the RNA genome was made and cloned into PCRI. Upon introduction of the hybrid plasmid into *E. coli*, with the insert in either orientation, formation of bacteriophage occurred. A few years later, a cDNA copy of the whole poliovirus genome was cloned into the Pst I site of pBR322. When the hybrid plasmid was transfected into mammalian cells, infectious poliovirus was produced [170]. Since the initial reports, reverse genetics systems for positive sense RNA viruses have flourished. This is most likely due to the fact that transfection of

positive sense RNA into eukaryotic cells leads directly to viral protein expression (most importantly the viral polymerases) and viral replication. Reverse genetics systems exist for representative members of the following positive sense RNA virus families: *Flaviviridae* - hepatitis C [171], *Birnaviridae* – infectious bursal disease virus [172], *Reoviridae* - reovirus [173], *Togaviridae* – rubella virus [174], *Caliciviridae* – feline calicivirus [175], *Picornaviridae* – poliovirus [170] and foot and mouth disease virus [176], *Astroviridae* – human astrovirus [177], and *Coronaviridae* – transmissible gastroenteritis virus (TGEV) [178, 179], mouse hepatitis virus (MHV) [180], human coronavirus 229E (HCV 229E) [181] and avian infectious bronchitis virus (IBV) [182].

The concept of reverse genetics for negative sense RNA viruses is more complex. The negative polarity of their genomes disallows direct translation of the RNA into the required viral enzymes necessary for further replication. Therefore, the replicative enzymes most be supplied by expression plasmids containing cDNAs of the replicative enzymes. Although more challenging, reverse genetics systems for negative sense RNA virus families do exist and are reviewed by Neumann *et al*. [183].

This review focuses on a reverse genetics systems for Coronaviruses. The *Coronaviridae* family contains members that infect humans, dogs, cats, swine, mice, horses, and poultry. Viruses within the *Coronaviridae* family contain a non-segmented, single stranded, positive sense RNA genome that ranges from 23 kb to 32 kb. In general, the genome encodes for 4 to 5 structural proteins and an undetermined amount of non-structural proteins. The replication cycle of Coronaviruses is

straightforward. Upon attachment and entry into the target cell, the full-length genome is released into the cytoplasm and mRNA 1 is translated into the viral polymerase and accessory proteins necessary for replication. Once synthesized, the replicative enzymes produce a 3' coterminal nested set of 5-7 subgenomic mRNA's, each containing a unique 5' encoding region. Only the unique region of each mRNA is translated [20].

It is difficult to produce a reverse genetics system for Coronaviruses because of their large genome. Currently, no RT-PCR system exists that can amplify such a large RNA genome, so genomic cDNA must be produced in small overlapping pieces and ligated together. This strategy has been employed by several groups to produce reverse genetics systems for TGEV, MHV, HCV 229E and IBV [178-182].

Different reverse genetics systems have been used to produce viral RNA from cDNA. Two reverse genetic systems exist for TGEV. The first system produced by Almazan *et al*. [178] was based on cloning the whole viral genome as an infectious bacterial artificial chromosome (BAC). They positioned the whole genome between the cytomegalovirus (CMV) immediate early (ie) promoter and a hepatitis delta ribozyme sequence/bovine growth hormone termination and poly A signal. They transfected the whole BAC/TGEV cDNA construct into eukaryotic cells. Once inside the cell, the cellular RNA polymerases recognized the CMV ie promoter and produced an RNA molecule analogous to the positive sense RNA genome of TGEV, which could then replicate and produce infectious virus. One limitation of this system was the toxicity of some TGEV cloned sequences in *E. coli*, which necessitated more complex molecular manipulations before cDNA could be

transfected. Recently, the group reported an improvement of their reverse genetics system [184]. They stabilized the bacterial artificial chromosome containing their cDNA clone by the inclusion of intron sequences within regions of known toxicity. The intron sequences led to stabilization of the BAC in *E. coli*. After transfection of the DNA into eukaryotic cells, the intronic sequences were properly excised leading to infectious full-length RNA.

The second TGEV system produced by Yount *et al*. [179] was based on amplifying the whole genome in 6 overlapping pieces of DNA. The toxicity reported by the other TGEV reverse genetics system was overcome by amplifying that portion in two pieces. The overlapping DNA pieces were digested with restriction enzymes and religated by T4 DNA ligase, with a T7 promoter before the 5' end of the genome and a poly A tail at the 3' end of the genome. Next, RNA was *in vitro* transcribed using T7 polymerase and capped with GTP. The full-length RNA and nucleocapsid transcripts (produced from a nucleocapsid PCR product with a T7 promoter site at the 5' end) were transfected into eukaryotic cells . Once inside the cell, the RNA was translated and viral replication ensued. Yount *et al*. have successfully extended this system to MHV [180]. One limitation of this system is the number of cloning manipulations required to produce the full-length cDNA of the viral genome.

Thiel *et al*. reported the first reverse genetics system for a human coronavirus [181]. In particular, they made a cDNA copy of the HCV 229E genome and cloned it into the vaccinia virus genome behind a T7 promoter. They grew recombinant vaccinia virus in CV1 cells with a fowlpox helper virus. They isolated the recombinant DNA and synthesized RNA transcripts for the HCV 229E virus by T7

polymerase and capped with GTP. The capped RNA was transfected into MRC-5 cells and infectious virus was recovered.

Recently, Casais *et al.* reported a reverse genetics system for IBV [182]. Briefly, the whole IBV genome was cloned into a unique Not I site behind a T7 promoter within the vaccinia virus genome. The vaccinia virus genomic DNA including the IBV genome was restriction enzyme digested and transfected into eukaryotic cells previously infected with a recombinant fowlpox virus expressing T7 RNA polymerase. The T7 polymerase produced the full-length RNA genome of IBV from within the vaccinia virus genome and virus replication ensued. One limitation of this system will be separating the IBV from the helper virus used to drive the transcription of the IBV genomic RNA from the vaccinia virus genome. This problem must be dealt with if the resultant IBV is to be used in further experiments or vaccine production.

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CHAPTER 3

DESIGN, SYNTHESIS, AND *IN VITRO* ANALYSIS OF A HAMMERHEAD RIBOZYME TARGETED TO AVIAN INFECTIOUS BRONCHITIS VIRUS NUCLEOCAPSID MESSENGER RNA[1]

[1]Callison, S.A., D.A. Hilt, and M.W. Jackwood. To be submitted to *Avian Diseases*.

Abstract

Hammerhead ribozymes are catalytic RNA molecules that specifically cleave a target RNA molecule. Herein, we report the design, synthesis, and *in vitro* analysis of a hammerhead ribozyme targeted to the IBV nucleocapsid mRNA. At a concentration of 0.5 or 10 μM, the ribozyme, designated IBVNRIBOZYME, effectively cleaved target RNA's in trans (37º C, 10mM $MgCl_2$, 50 mM Tris). Cleavage products were visualized by agarose gel analysis. The time course of the ribozyme reaction was monitored by agarose gel analysis and relative quantitative RT-PCR. The amount of target RNA continually declined over a 5 hour period. A hammerhead ribozyme targeted to the IBV nucleocapsid mRNA may be a useful laboratory tool and may have therapeutic/prophylactic future applications.

Introduction

Ribozymes are catalytic RNA molecules that specifically cleave another target RNA molecule in cis or trans. Ribozymes occur naturally in pathogenic plant viroids (Hammann and Tabler 1999), viruses (Belinsky and Dinter-Gottlieb 1991), bacteria (Altman, Kirsebom et al. 1993), protozoans (Cech 1986), newts (Epstein and Gall 1987), and schistosomes (Ferbeyre, Smith et al. 1998). Many different types of ribozymes exist, including hammerhead, group I intron, hairpin and others (Doherty and Doudna 2000).

The focus of this study is on the hammerhead ribozyme. Hammerhead ribozymes are made up of two components, a catalytic core and flanking sequences (Fig. 1). The catalytic core is responsible for cleavage of the target RNA, while the flanking sequences determine the ribozyme specificity (Hammann and Tabler 1999). Hammerhead ribozymes have been constructed as antiviral molecules against viruses from families including, but not limited to, the *Arenaviridae* (Xing and Whitton 1992; Xing and Whitton 1993), *Retroviridae* (Jackson, Moscoso et al. 1998), *Birnaviridae* (Akin, Lin et al. 1999), *Paramyxoviridae* (Albuquerque-Silva, Milican et al. 1999), *Picornaviridae* (Macejak, Jensen et al. 2000), and *Coronaviridae* (Maeda, Mizutani et al. 1994).

Infectious bronchitis (IB) is an acute, highly contagious, upper respiratory tract disease in chickens. Clinical signs include tracheal rales, nasal exudate, coughing, and sneezing. The etiologic agent of IB is infectious bronchitis virus, a member of the *Coronaviridae* family. The virion is enveloped with club-shaped surface projections. The viral genome is a single stranded, positive sense, 27.5 kb RNA molecule. The genome codes for four major structural proteins: a spike glycoprotein, the integral

membrane glycoprotein, the envelope protein, and the nucleocapsid protein that associates with the RNA genome (King and Cavanagh 1991).

Due to the interaction of the nucleocapsid protein with the IBV viral genome (Zhou and Collisson 2000) and the proposed multiple roles of *Coronavirus* nucleocapsid protein in replication (Cologna, Spagnolo et al. 2000), we chose to target the mRNA encoding the nucleocapsid protein of IBV in trans with a hammerhead ribozyme. If functional *in vitro*, this molecule could inhibit the production of nucleocapsid protein *in vivo* and block IBV replication.

Materials and Methods

Virus. The IBV strain used in this study was Massachusetts 41 (Jungherr et al. 1956). The virus was propagated in 9 –11 day old embryonating chicken eggs (Senne 1998). After 48 hours of incubation, the allantoic fluid was harvested and stored at – 70º C until needed.

Nucleocapsid gene RT-PCR. Viral RNA was extracted using the High Pure RNA Isolation Kit (Roche Diagnostics Corp., Indianapolis, IN, USA) and used as template for RT-PCR (Titan One-Tube RT-PCR System, Roche Diagnostics Corp., Indianapolis, IN, USA). A primer set flanking the entire subgenomic nucleocapsid mRNA was used and the RT-PCR products were purified as previously described (Callison, Jackwood et al. 2001).

Nucleocapsid gene cloning. RT-PCR products were cloned (TOPO® XL PCR Cloning Kit, Invitrogen Corp., Carlsbad, CA, USA). and several clones were screened by sequencing for the complete nucleocapsid gene, including the 3' UTR of the IBV genome. One clone, designated N-9 was selected and used in all subsequent steps.

Nucleocapsid gene sequencing. The complete insert of the N-9 clone was sequenced (ResGen, Invitrogen, Corp., Huntsville, AL, USA) and assembled using MacDNASIS Pro V3.5 computer software (Hitachi Software Engineering Corp., San Bruno, CA, USA).

Ribozyme design. A search for possible hammerhead ribozyme target sequences was performed using MacDNASIS Pro V3.5 computer software (Hitachi Software Engineering Corp., San Bruno, CA, USA). Secondary structure predictions (Fig. 2) were performed using the MFOLD algorithm available over the Internet (http://bioinfo.math.rpi.edu/~mfold/rna/) (Mathews, Sabina et al. 1999; Zuker, Mathews et al. 1999). Regions with minimal secondary structure were defined using the single stranded plot option. Then, specific hammerhead target sites were analyzed within those regions. An average value for the ten nucleotides before and after the NUH target site was calculated. The target site and surrounding nucleotides having the highest average single strandedness was chosen for the hammerhead ribozyme target site.

Ribozyme synthesis. The hammerhead ribozyme was chemically synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA).

Target RNA production. Target RNA was synthesized with the RiboMax Large Scale RNA Production System – T7 (Promega Corp., Madison, WI, USA) using Hind III (New England Biolabs, Inc., Beverly, MA, USA) linearized N-9 clone DNA as the template. Production of correctly sized RNA was analyzed by agarose gel analysis. Briefly, 5.0 µl of RNA was mixed with 5.0 µl of TBE-Urea sample buffer (Bio-Rad, Hercules, CA, USA), heated to 95º C for 4 minutes, and placed on ice. The mixture was then loaded

onto a 1% agarose gel without formaldehyde (Liu and Chou 1990) and electrophoresed for approximately one hour at 80 volts in 1X TAE running buffer.

Ribozyme activity assay. A 100 µl reaction mix, containing target RNA - 20 µl, IBVNRIBOZYME – 0.5 µM, $MgCl_2$ – 10mM, Tris – 50 mM, and DEPC treated water added to the final volume, was set up. The reaction tubes were placed at 37º C for one hour. After incubation, 5.25 µl of 0.5 M EDTA was added, the tube was placed on ice, and the RNA was extracted as described in the RT-PCR section. Cleavage products were analyzed by agarose gel analysis.

Ribozyme time course assay. Six reaction tubes were set up, each one as stated above, except that only 15 µl of target RNA was used and the final IBVNRIBOZYME concentration was 10 µM. A time course assay was conducted with one of each of the six tubes representing time points 0, 1, 2, 3, 4, and 5 hours. The cleavage products were analyzed on a 1% agarose gel and by relative quantitative RT-PCR (LightCyclerTM RT-PCR Kit, Roche Diagnostics Corp., Indianapolis, IN, USA). For RT-PCR, one primer set flanked the target site of IBVNRIBOZYME, while the other set did not. The primer sets were used in a RT-PCR with the RNA extracted from each time point as the template.

Results

We produced target RNA from the cloned nucleocapsid gene from the Mass 41 strain of IBV using T7 RNA polymerase (Fig. 4). By agarose gel analysis, we observed cleavage of the target RNA (1,800 nt) into cleavage products (1,400 nt and 400 nt) in the reaction containing the IBVNRIBOZYME (Fig. 5). No cleavage was observed in the reaction setup that excluded the ribozyme.

To provide further evidence that IBVNRIBOZYME was actually catalytic, a time course experiment was performed. Both agarose gel analysis and relative quantitative RT-PCR showed a continual decline of full-length target RNA over time (Fig. 6, Fig. 7). Using primers that did not flank the ribozyme target site, no difference in the amount of target RNA was observed by relative quantitative RT-PCR, indicating that the target RNA was cleaved and not degraded (Fig. 8).

Discussion

In this study, we designed, synthesized, and analyzed a hammerhead ribozyme targeted against the nucleocapsid mRNA of IBV. During the course of our study, only one ribozyme was synthesized and analyzed. We believe that the MFOLD software program and careful analysis of the secondary structure prediction data was key in design and synthesis of an effective hammerhead ribozyme.

Cleavage at the proper target site was monitored by visualization of correctly sized cleavage products on an agarose gel. Further confirmation of proper cleavage at the target site was provided by relative quantitative RT-PCR using two different primer sets. One primer set flanked the cleavage site and showed a continual decline of full-length template RNA over time. The other primer set, which amplified a segment in the 3' end of the template RNA, showed no decline over time. The second primer set provided proof that amplification differences seen for the first primer set flanking the IBVNRIBOZYME target site were due to cleavage and not template RNA concentration differences or degradation.

We want to point out that cleavage of the target RNA was observed at time point 0 during the time course experiment. Only a minute passed between completion of making

the master mix and ribozyme inactivation by addition of EDTA to the tube. Therefore, it appears that the association/cleavage rates of the IBVNRIBOZYME are high. Further experimentation is needed to determine the optimal reaction conditions for the ribozyme and for calculating the association/cleavage rates.

It is reasonable to believe that the IBVNRIBOZYME will cleave target RNA *in vivo*. Since coronaviruses have a 3' coterminal nested set of mRNA's, the IBVNRIBOZYME should be able to cleave all the IBV mRNAs, including the full-length viral genome. Cleavage of the 3' end of the nucleocapsid and other IBV mRNA's may hinder IBV replication and packaging; nucleocapsid protein is believed to be important for both.

Ribozymes show great potential as antiviral therapeutic molecules. Published reports have shown that hammerhead ribozymes inhibited viral replication for three RNA viruses from different virus families; lymphocytic choriomeningitis virus (LCMV-*Arenaviridae*), mouse hepatitis virus (MHV - *Coronaviridae*), and human immunodeficiency virus (HIV - *Retroviridae*). Replication of LCMV in cell culture was inhibited by the stable expression of a hammerhead ribozyme targeted to the S segment of the viral genome. The hammerhead ribozyme decreased the production of infectious virus by 100X and the antiviral activity was shown to be specific for LCMV (Xing and Whitton 1993). Replication of MHV in cell culture was inhibited by the stable expression of either of two different hammerhead ribozymes targeted to the polymerase gene. Inhibition of acute and chronic viral infection was shown (Maeda et al 1994, Maeda et al. 1995). Lastly, replication of HIV in cell culture was inhibited by the stable expression of a hammerhead ribozyme targeted to the tat gene. Specifically, CD4+ cells

stably transduced by a pseudotyped retroviral vector containing the anti-tat hammerhead ribozyme were able to resist HIV infection for up to 20 days (Jackson et al. 1998).

Some problems do exist for the use of any ribozyme as an antiviral agent. One problem is how to effectively deliver enough of the ribozyme to the appropriate target cells and sites within a target cell. Assuming effective delivery, another limitation may be the stability of the ribozyme. Also, ribozymes may lack the ability to hybridize with targets due to the secondary structure of mRNA or interaction of mRNA with accessory proteins or ribosomes (Burke 1997). Lastly, ribozymes could be toxic to cells due to unexpected cleavage of a critical cellular RNA. Ribozymes will cleave any RNA containing a target sequence, so the specificity of the ribozyme is of utmost importance. Although many hurdles remain, the promise of ribozymes as effective antiviral molecules makes them an attractive research area and hopefully their full potential will be realized in the future.

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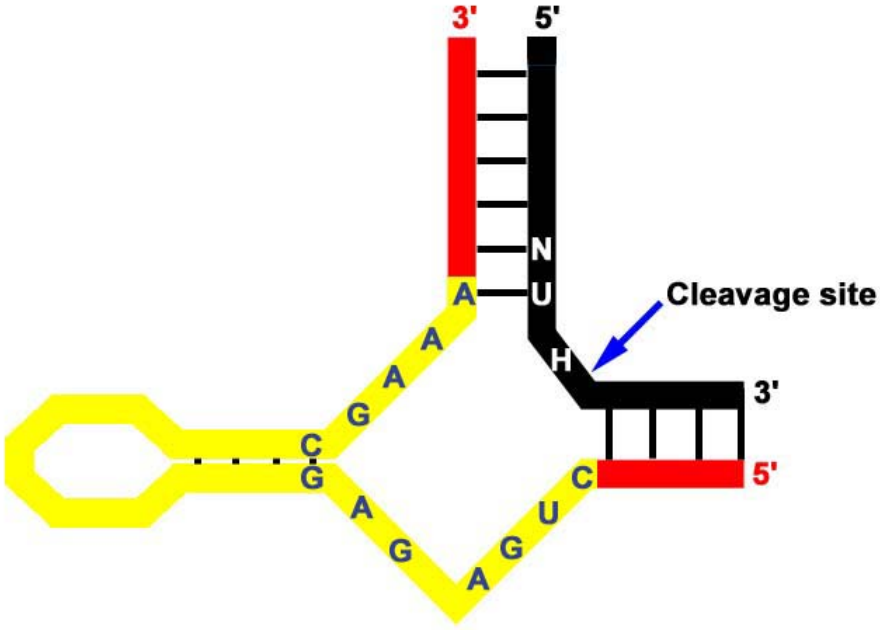
Fig. 3.1. Characteristic structure of a hammerhead ribozyme (yellow/red) when complexed with target RNA (black). The yellow section represents the catalytic core and the red sections are the flanking sequences. Blue nucleotides are conserved and white nucleotides represent the target site for the hammerhead ribozyme. The arrow marks the cleavage site. Black lines represent Watson-Crick base pairing of complementary nucleotides (Hammann and Tabler 1999).


3'
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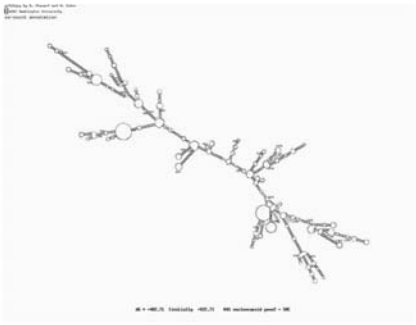
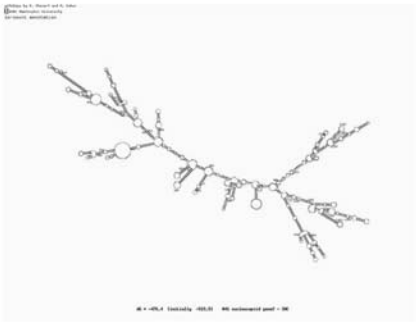
Cleavage site

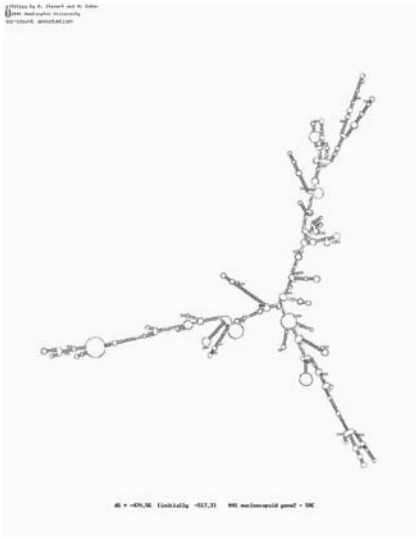
Fig. 3.2. Secondary structure predictions of IBV nucleocapsid subgenomic mRNA using MFOLD (Mathews, Sabina et al. 1999; Zuker, Mathews et al. 1999).



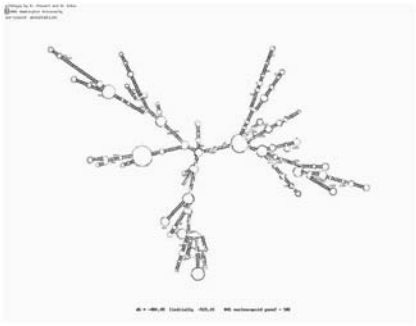
Prediction 1



Prediction 2



Prediction 3



Prediction 4

Fig. 3.3. Single strandedness plot for each nucleotide in IBV nucleocapsid subgenomic mRNA. The plot was generated by MFOLD using 33 individual secondary structure predictions. Black circles indicate regions with a high probability of no secondary structure. The nucleocapsid gene coding region is marked by the start and stop codons.

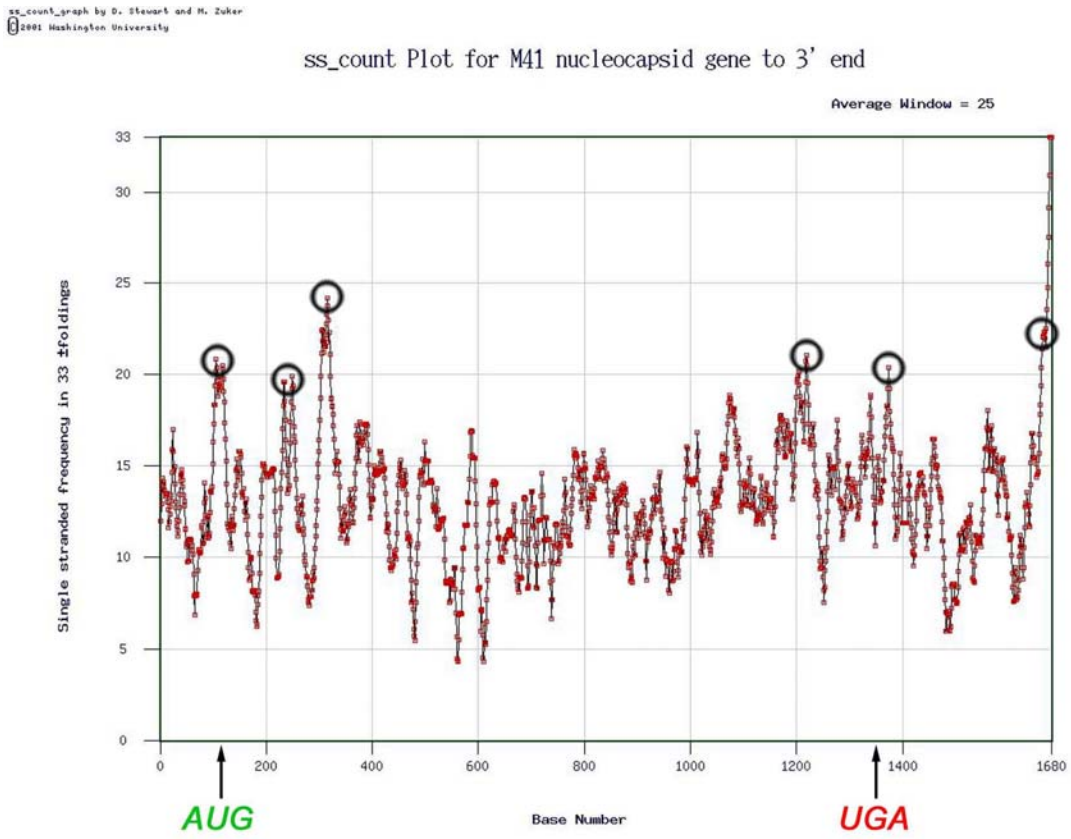

ss_count_graph by D. Stewart and M. Zuker
©2001 Washington University
ss_count Plot for M41 nucleocapsid gene to 3' end
Average Window = 25
Single stranded frequency in 33 ±foldings
Base Number
AUG
UGA

Fig. 3.4. Agarose gel analysis of target RNA produced from clone N-9 using T-7 *in vitro* run off procedure. Lane 1 = RNA ladder with bands of 9000, 7000, 5000, 3000, 2000, 1000, and 500 nt; Lane 2 = Target RNA from N-9 clone

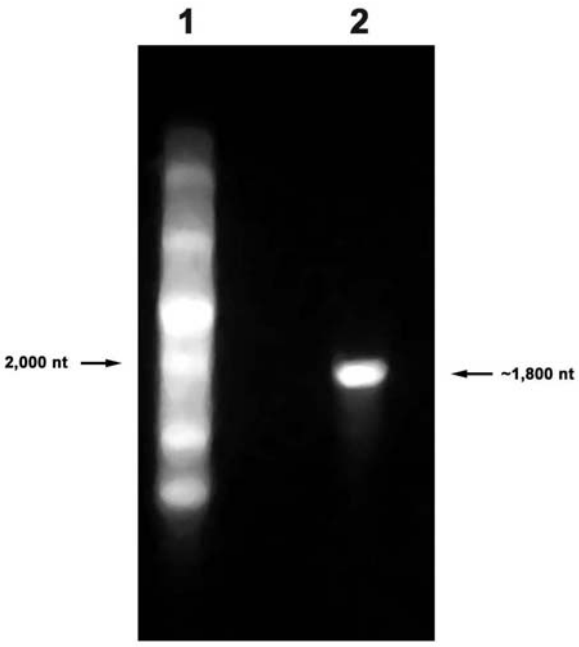

1
2
2,000 nt
~1,800 nt

Fig. 3.5. Cleavage of target RNA by IBVNRIBOZYME. Lane 1 = RNA ladder, see Fig. 4. for band sizes (New England Biolabs, Inc.); Lane 2 = Control RNA, kept on ice with no other chemicals added; Lane 3 = Target RNA incubated at 37º C for 1 hour with no ribozyme added; Lane 4 = Target RNA incubated at 37º C for 1 hour with IBVNRIBOZYME added

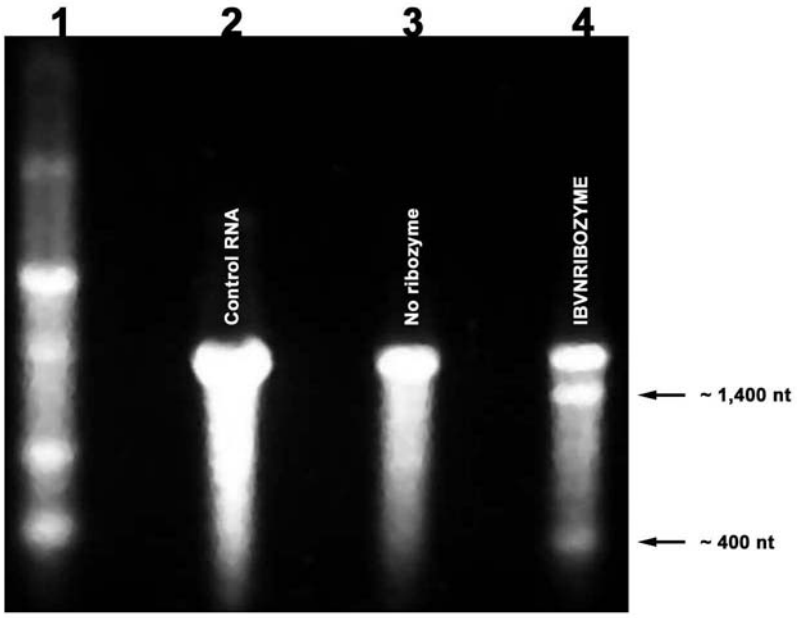

1
2
3
4
Control RNA
No ribozyme
IBVNRIBOZYME
~ 1,400 nt
~ 400 nt

Fig. 3.6. Visualization of IBVNRIBOZYME reaction over time. White arrows indicate cleavage products. Lane 1 = RNA ladder, see Fig. 4. for band sizes (New England Biolabs, Inc., Beverly, MA); Lane 2 = Control RNA; Lane 3-8 = Target RNA incubated with IBVNRIBOZYME for increasing amounts of time; Lane 9 = RNA ladder with bands of 6583, 4981, 3638, 2604, 1908, 1383, 955, 623, and 281 nt (Promega Corp., Madison, WI).

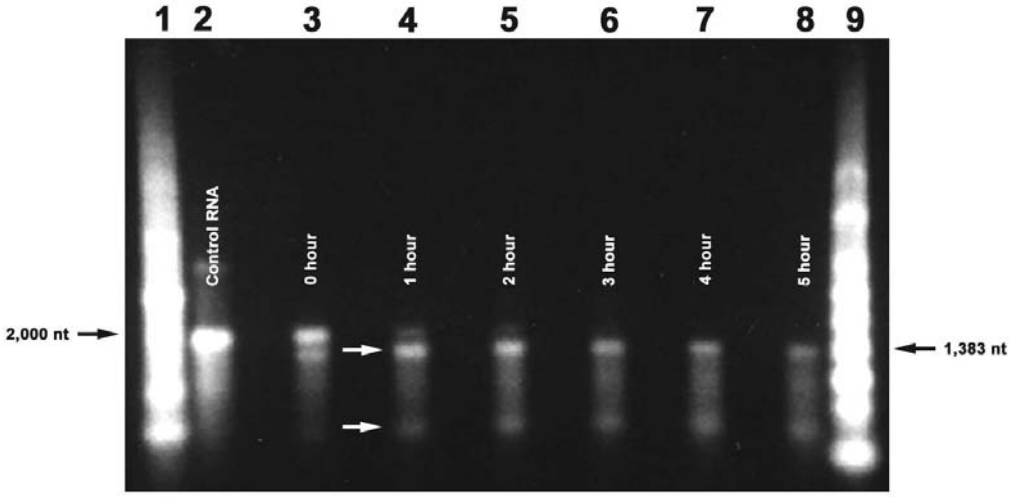

1 2 3 4 5 6 7 8 9
Control RNA
0 hour
1 hour
2 hour
3 hour
4 hour
5 hour
2,000 nt
1,383 nt

Fig. 3.7. Relative quantitative RT-PCR using template RNA taken from each time point of the time course assay. A - Schematic of template RNA showing the position of the ribozyme target site (red triangle) and primers (blue arrows). B - Amplification graph of template RNA taken from each time point. Fluorescence for each reaction was measured after each PCR cycle by the LightCycler™ instrument (Roche Diagnostics Corp., Indianapolis, IN). Amplification of the correct product was verified by melting curve and agarose gel analysis (data not shown).

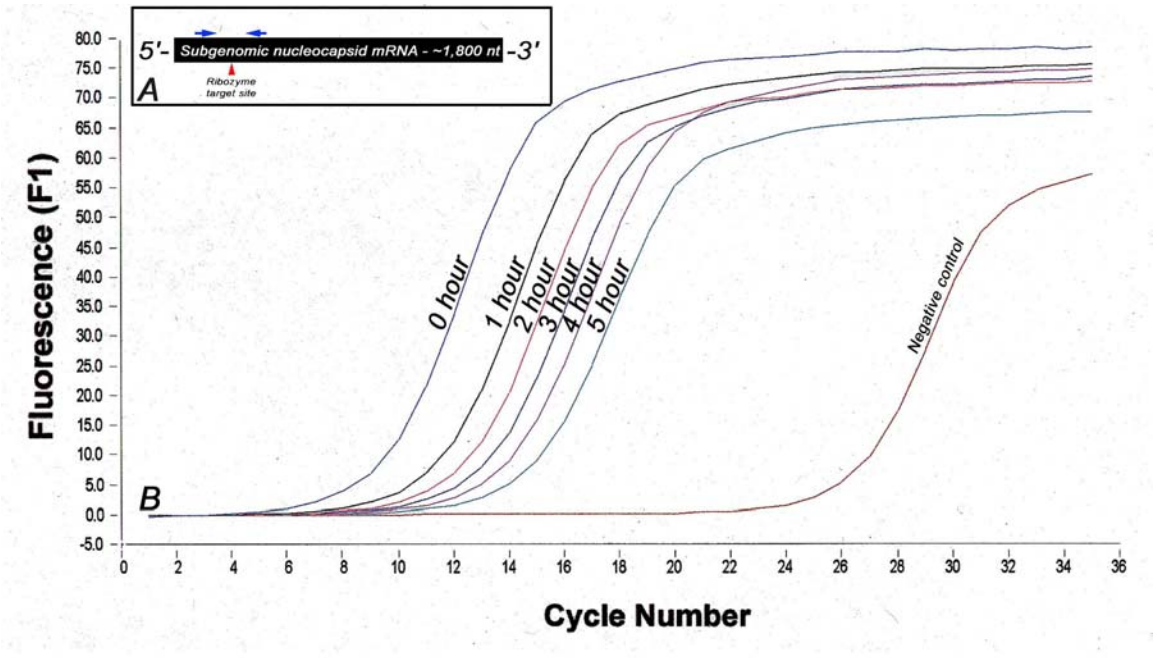
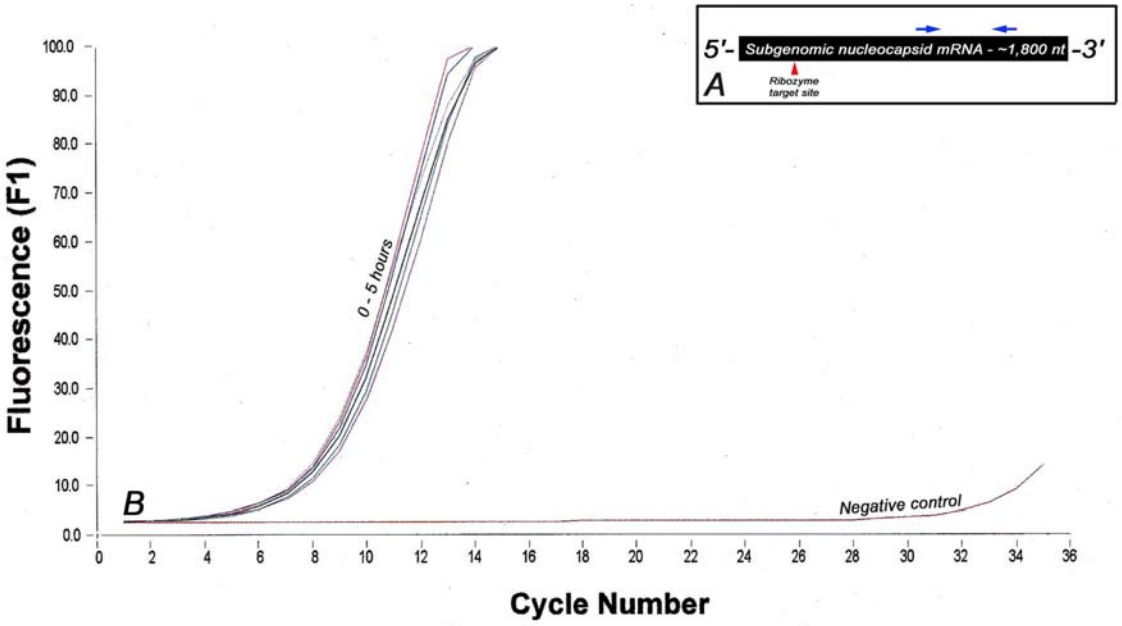

5′- Subgenomic nucleocapsid mRNA - ~1,800 nt -3′
Ribozyme target site
A
B
Fluorescence (F1)
Cycle Number
0 hour
1 hour
2 hour
3 hour
4 hour
5 hour
Negative control

Fig. 3.8. Relative quantitative RT-PCR using template RNA taken from each time point of the time course assay. A - Schematic of template RNA showing the position of the ribozyme target site (red triangle) and primers (blue arrows). B - Amplification graph of template RNA taken from each time point. Fluorescence for each reaction was measured after each PCR cycle by the LightCycler™ instrument (Roche Diagnostics Corp., Indianapolis, IN). Amplification of the correct product was verified by melting curve and agarose gel analysis (data not shown).


5′- Subgenomic nucleocapsid mRNA - ~1,800 nt -3′
A
Ribozyme target site
Fluorescence (F1)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0 - 5 hours
B
Negative control
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
Cycle Number

CHAPTER 4

CREATING NOVEL AVIAN INFECTIOUS BRONCHITIS VIRUS S1 GENES BY DNA SHUFFLING USING THE STAGGERED EXTENSION PROCESS[1]

[1]Callison, S.A., D.A. Hilt, and M.W. Jackwood. To be submitted to *Virus Genes*.

Abstract

We employed the staggered extension process (StEP) to shuffle the S1 genes from four avian infectious bronchitis virus (IBV) strains representing four unique serotypes (Massachusetts, Arkansas, Connecticut, and Delaware). Upon creating a shuffled S1 gene library, we randomly selected 25 clones and analyzed them by DNA sequencing. In total, eleven clones contained novel S1 gene recombinants. Each recombinant was unique and contained a full-length open reading frame. The average number of crossovers per recombinant was 5 and the average number of point mutations was 1.3, leading mostly to non-synonymous amino acid changes. No recombinant contained sequences from all four parental genes and no recombinant contained any sequence from the De072 strain.

Introduction

Infectious bronchitis virus (IBV) continues to cause disease in poultry flocks around the world. Although commercial poultry flocks are constantly vaccinated for IBV, outbreaks of infectious bronchitis still occur due to natural variant viruses that continue to arise. Thus, better vaccines are definitely needed for future control of IBV. Properties of a better IBV vaccine would be low virulence, high immunogenicity and most importantly, cross protection between different IBV strains/serotypes. Furthermore, a better strategy for developing vaccines that keeps pace with the rate of viral change in nature is needed.

Infectious bronchitis virus is a coronavirus that contains a large, single stranded, positive sense RNA genome that is 27.5 kb long. The genome encodes four major structural proteins: the membrane glycoprotein, the envelope protein, the nucleocapsid protein, and the spike glycoprotein, which contains virus-neutralizing and serotype specific epitopes [1]. The ability of IBV to rapidly change in nature is attributable to its method of replication. Infectious bronchitis virus undergoes genetic drift due to point mutations that occur during replication because the viral polymerase lacks proofreading ability. In addition, genetic shift occurs due to the recombination of two or more strains during replication. Both genetic drift and shift have been documented for IBV in the laboratory and in birds [2-7].

DNA shuffling is the process of purposeful, but random recombination of parental genes into novel recombinant genes. Basically, genes (parental) with some level of similarity are recombined in a test tube by any number of techniques, such that the newly created genes contain genetic information from some or all of the parental genes. The new recombinant genes can be expressed as protein and then selected or screened for a

desired property. Within the last ten years, researchers have used DNA shuffling to create new genes that encode novel or improved proteins [8-11]. As an example, Zhao et al., developed the Staggered Extension Process of StEP (Fig. 1) to shuffle different sequences encoding the subtilisin E protein and improved its half-life by at 65º C by 50 fold [12]. Briefly, they promoted recombination by using a modified polymerase chain reaction in which the annealing and extension steps were combined and allowed only brief periods of time for polymerization.

The long term goal of our work is 1) to develop a cross protective IBV vaccine and 2) to create a better vaccine development strategy for the future. Since the spike glycoprotein contains virus-neutralizing and serotype specific epitopes [13-15], we reasoned that shuffling the S1 portion of the spike glycoprotein gene of IBV strains from different serotypes might be a way to produce a spike glycoprotein with cross-protective properties that could be used as a vaccine. Also, shuffling the S1 sequences might be a way to study antigenic shift in IBV, and thereby lead to predictions of future variant IBVs. As a first step in obtaining our goals, we report herein the DNA shuffling of S1 genes from four different strains of IBV that represent four different serotypes using the StEP method.

Materials and Methods

Virus strains

The virus strains used in this study were Massachusetts 41 [16], Arkansas DPI [17], Connecticut 46 [16], and Delaware 072 [18]. All viral strains were propagated by allantoic sac inoculation of 9 – 11 day old embryonating chicken eggs. After 48 hours of incubation, the allantoic fluid was harvested and stored at –70º C [19].

RNA extraction, RT-PCR, and cloning of parental genes

Viral RNA was extracted from allantoic fluid using the High Pure RNA extraction kit (Roche Diagnostics Corporation, Indianapolis, IN). The S1 gene was amplified by RT-PCR as previously described [20, 21] using the Titan One Step RT-PCR kit (Roche Diagnostics Corporation, Indianapolis, IN). The 1,700 bp amplicon from each virus strain was gel purified on a 1% agarose gel using GenElute spin columns (Sigma-Aldrich Co, St. Louis, MO) and Microcon 30 columns (Millipore, Bedford, MA) per the manufacturers directions. The resultant DNA was cloned into TOPO XL per the manufacturers directions (Invitrogen Corp., Carlsbad, CA). Plasmid DNA was extracted from colonies using a plasmid miniprep kit (Qiagen, Inc., Valencia, CA). Plasmids containing inserts of the appropriate size were screened by DNA sequencing of the 5' and 3' ends. Clones containing S1 genes with appropriate 5' and 3' end sequences were fully sequenced (SeqWright, Houston, TX).

Preparation of parental template for use in StEP

Plasmid DNA for each parental S1 gene was isolated, linearized by digestion with Mlu I (New England Biolabs, Inc., Beverly, MA) and gel purified on a 1% agarose gel as stated earlier.

StEP reaction

The staggered extension process was performed as previously described [12]. Briefly, a 100 microliter PCR was assembled containing 0.05 picomoles of each linear parental template, 40 picomoles of each S1 primer, 50 microliters of a commercial PCR buffer mixture, Premix 7 (Epicentre, Madison, WI), and water up to 100 microliters. The reaction mixture was subjected to a thermocycle program consisting of 95º C – 5 min;

100 cycles of 95º C – 30 s, 55º C – 2 s; and 4º C hold until the products could be analyzed.

Cloning of StEP recombinants

StEP products were electrophoresed on a 1% agarose gel. The diffuse band around 1,700 bp was excised from the gel and purified as previously stated. The purified DNA was reamplified by PCR using 1 microliter as template in the same reaction mixture as for the StEP. The thermocycle program was 95º C – 2 min; 25 cycles of 95º C – 30 s, 45º C – 30 s, 72º C – 90 s; 4º C – infinity. After the reaction, the 1,700 bp amplicon was gel purified and cloned into TOPO XL according to the manufacturer's directions.

Characterization of StEP recombinant clones

Twenty-five colonies were randomly selected from the transformation reaction. Each clone was grown overnight in LB broth in the presence of kanamycin. Plasmid DNA was prepared from each clone as stated earlier and the inserts were sequenced (SeqWright, Houston, TX). Sequence data was analyzed using MacDNASIS Pro V3.5 (Hitachi Software Engineering Corp., San Bruno, CA) and Lasergene V3.12 (DNASTAR, Inc., Madison, WI).

Results

Creating a novel IBV S1 gene library using the StEP and initial screening of 25 clones

We shuffled the S1 gene of the Massachusetts 41, Arkansas DPI, Connecticut 46, and Delaware 072 IBV strains, which represent four different serotypes, using the StEP (Fig. 2). In total, 11 of the 25 clones examined contained recombinant S1 genes. Each of the recombinant genes had a unique sequence (Fig. 3) that maintained a full-length ORF from the ATG start site to the cleavage site. On average, each recombinant gene

contained 5 crossovers. Seven of the recombinants contained sequence from two of the parental genes, while four of the recombinants contained sequences from three of the parental genes. None of the recombinants contained sequences from all four of the parental genes. There was very little Arkansas DPI parental sequence and no Delaware 072 parental sequence in any of the recombinants.

Nucleotide and deduced amino acid similarity of recombinants to parental genes

The nucleotide similarity for each of the 11 recombinants, when compared with the Massachusetts 41 strain, ranged from 91.3% to 99.6%. The nucleotide similarity for each recombinant when compared with the Connecticut 46 strain, ranged from 90.4% to 98.8%. The nucleotide similarity for each recombinant when compared with the Arkansas DPI strain, ranged from 75.7% to 82.8% (Table 1). The amino acid similarity for each of the 11 recombinants, when compared with the Massachusetts 41 strain, ranged from 89.9% to 99.3%. The amino acid similarity for each recombinant when compared with the Connecticut 46 strain, ranged from 87.6% to 97.4%. The amino acid similarity for each recombinant when compared with the Arkansas DPI strain, ranged from 75.1% to 81.4% (Table 1).

Recombinant S1 deduced amino acid sequence analysis

The cleavage site sequence and the number of potential glycosylation sites were analyzed for each recombinant (Table 2). Each of the recombinants contained a cleavage sequence of the type X_1RX_2RR, where X_1 equals Arg, His, or Tyr and X_2 equals Ser, Phe, Ile, Arg, or His [22, 23]. The number of N-linked glycosylation sites ranged from 17 to 18. When secondary structure predictions of the amino acid sequence for each recombinant were performed using the Chou and Fasman algorithm [24], and compared to the parental

amino acid sequences, each recombinant was found to have a different secondary structure prediction (Fig. 4).

Recombinants containing sequences from three parental genes

The DS-2 and DS-16 recombinants contained parental sequence from Massachusetts 41, Connecticut 46, and Arkansas DPI (Fig. 5). The recombinant DS-2 had four crossovers and four point mutations, of which three were non-synonymous changes. The recombinant DS-16 had four crossovers and two point mutations, of which one was a non-synonymous change (Table 2).

Discussion

The StEP was used to produce recombinant S1 genes from four different strains of IBV, representing four different serotypes. We cloned the putative recombinant S1 genes and randomly selected 25 clones from the library for sequence analysis. Eleven of the clones contained recombinant S1 genes, while 14 did not. All eleven clones (44% of total clones) contained a unique S1 sequence. We may be able to improve this percentage by performing the StEP at a lower temperature and/or by decreasing the amount of time for annealing/extension. Lowering the temperature should increase the chance for recombination by slowing down the DNA polymerase and allowing for less similar sequences to hybridize, while decreasing the annealing/extension time would produce shorter fragments during each PCR cycle. Taking these steps should not only increase our number of unique recombinants, but it might also help to produce recombinants that contain sequences from all of the parental genes.

As stated earlier, there was very little Arkansas DPI sequence and no Delaware 072 sequence in the recombinant genes. This may be due to the lower level of similarity

between these two parental genes as compared to the Massachusetts 41 and Connecticut 46 S1 genes (Table 1). In fact, it may be impossible to recombine the Delaware 072 gene by the StEP because its S1 gene nucleotide similarity to the Massachusetts 41, Connecticut 46, and Arkansas DPI strains is 52.3%, 50.7%, and 48.7%, respectively. It is difficult to shuffle genes by the StEP with similarities below 70% (F. H. Arnold, personal communication).

Previous research has speculated that most natural recombination of infectious bronchitis viruses occurs in hot spots termed intergenic consensus sequences [5]. These areas are regions of similarity outside of the coding sequence for a gene. This normally leads to recombination of whole genes, not sections of genes, although some exceptions have been reported [2, 3]. Our research has shown that even under near ideal conditions for recombination, recombinants containing any Delaware 072 sequence could not be produced. It would seem logical to assume that natural Delaware 072 recombinants containing crossovers within the S1 gene coding sequence would be rare. Indeed, a recent report details a new serotype of IBV, designated GA98, which appears to have evolved from the Delaware 072 strain by antigenic drift [6]. Therefore, we believe that antigenic shift will continue to produce variants from the recombination of IBV strains with a S1 gene similarity above 70 –75%, while antigenic drift will continue to produce variants from IBV strains that are less than 70-75% similar to any other IBV strain. Obviously, this number has been determined from our data and should be considered an estimation, because exceptions in both cases are bound to naturally occur.

Although we did not produce any recombinants with sequences from all four parental S1 genes, we did produce several that contained sequences from three of the

parental S1 genes. In particular, recombinants DS-2 and DS-16 contained large S1 gene segments from parental strains, Massachusetts 41, Connecticut 46, and Arkansas DPI. The relatively low S1 amino acid sequence similarity for the DS-2 and DS-16 clones to any of the parental clones makes them good candidates for further serologic characterization. Interestingly, the crossover sites for DS-2 ensure that different parental sequences reside within regions known to play key roles in the formation of virus neutralizing epitopes [13-15, 25].

Since the virus neutralizing epitopes for IBV are conformationally dependent [13-15] it remains unknown as to whether hypervariable regions form neutralizing epitopes within themselves or whether they associate with other hypervariable regions to form neutralizing epitopes. Therefore, we cannot predict the antigenic outcome of having parental sequences from serologically distinct viruses within the different hypervariable regions. Furthermore, due to the known association of non-contiguous amino acids in the functional spike glycoprotein and the non-conservative point mutations within our recombinant S1 genes, the biological functionality and serological character of these genes remains questionable.

S1 amino acid sequence similarity between strains can be used as a predictor of serologic relatedness. In general, strains with S1 amino acid sequences similarities higher than 85% reside in the same serotype, although notable exceptions exist. Therefore, studying recombinant S1 clones with relatively low amino acid sequence similarity (< 85%) to parental clones might help to more clearly define regions within the spike glycoprotein that form serotype specific epitopes (Table 1).

It is our hope that in the future, the novel S1 genes will be characterized by expression/antibody binding studies and/or reverse genetics. The novel S1 genes from this study, along with future S1 genes created by DNA shuffling, may find utility in mapping serotype specific epitopes. In general, the S1 amino acid sequence similarity between strains remains a useful predictor of serologic relatedness. Strains with S1 amino acid sequences similarities higher than 85% usually reside in the same serotype, although notable exceptions exist [26]. Therefore, studying recombinant S1 clones with relatively low amino acid sequence similarity (< 85%) to parental clones might help to more clearly define regions within the spike glycoprotein that form serotype specific epitopes (Table 1). Also, a library of S1 genes with known serological characteristics may be helpful in developing future vaccines against variant viruses more rapidly and efficiently.

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Figure 4.1. Diagram of the Staggered Extension Process (StEP). The black and gray lines represent different parental templates. 1) Parental templates become denatured and gene specific primer anneals to one of two parental templates. 2) Annealed primers are extended by brief polymerase catalyzed primer extension. 3) Another cycle of StEP in which further extension occurs to fragments from step 2 (left side – no template switching, right side – template switching). 4) Appropriate number of StEP cycles performed until full-length genes are made. 5) Recombinant genes are cloned for further manipulation or analysis.


1
2
3
4
5

Figure 4.2. Representative gel analysis of Staggered Extension Process products. Lane 1 = DNA ladder 1 (Gene Choice, PGC Scientifics Corp., Frederick, MD), from top to bottom: 10000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1000, 800, 600, 400, 200; Lane 2 = Aliquot of StEP reaction after 20 cycles; Lane 3 = Aliquot of StEP reaction after 40 cycles; Lane 4 = Aliquot of StEP reaction after 60 cycles; Lane 5 = Aliquot of StEP reaction after 80 cycles; Lane 6 = Aliquot of StEP reaction after 100 cycles. The desired product is ~1700 bp. The bright band around 3000 bp represents parental template.

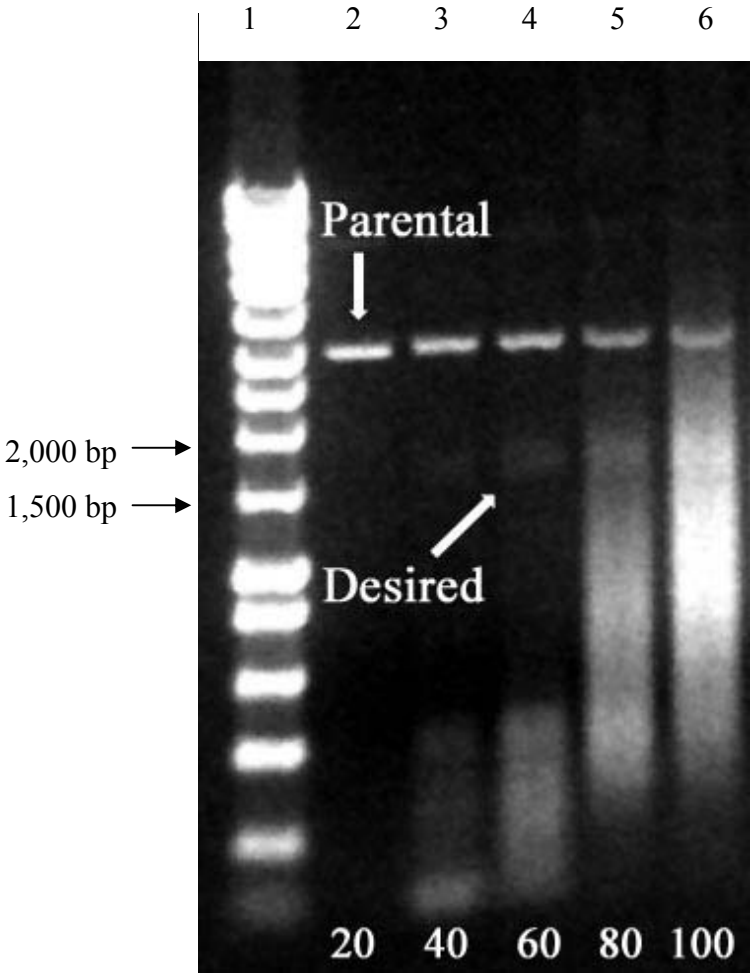

1
2
3
4
5
6
Parental
2,000 bp
1,500 bp
Desired
20
40
60
80
100

Figure 4.3. Comparison of recombinant S1 genes with S1 gene regions known to be involved in the formation of neutralizing and serotype specific epitopes (gray) [13-15]. Blue = Massachusetts 41 parental sequence; Green = Connecticut 46 parental sequence; Red = Arkansas DPI parental sequence.

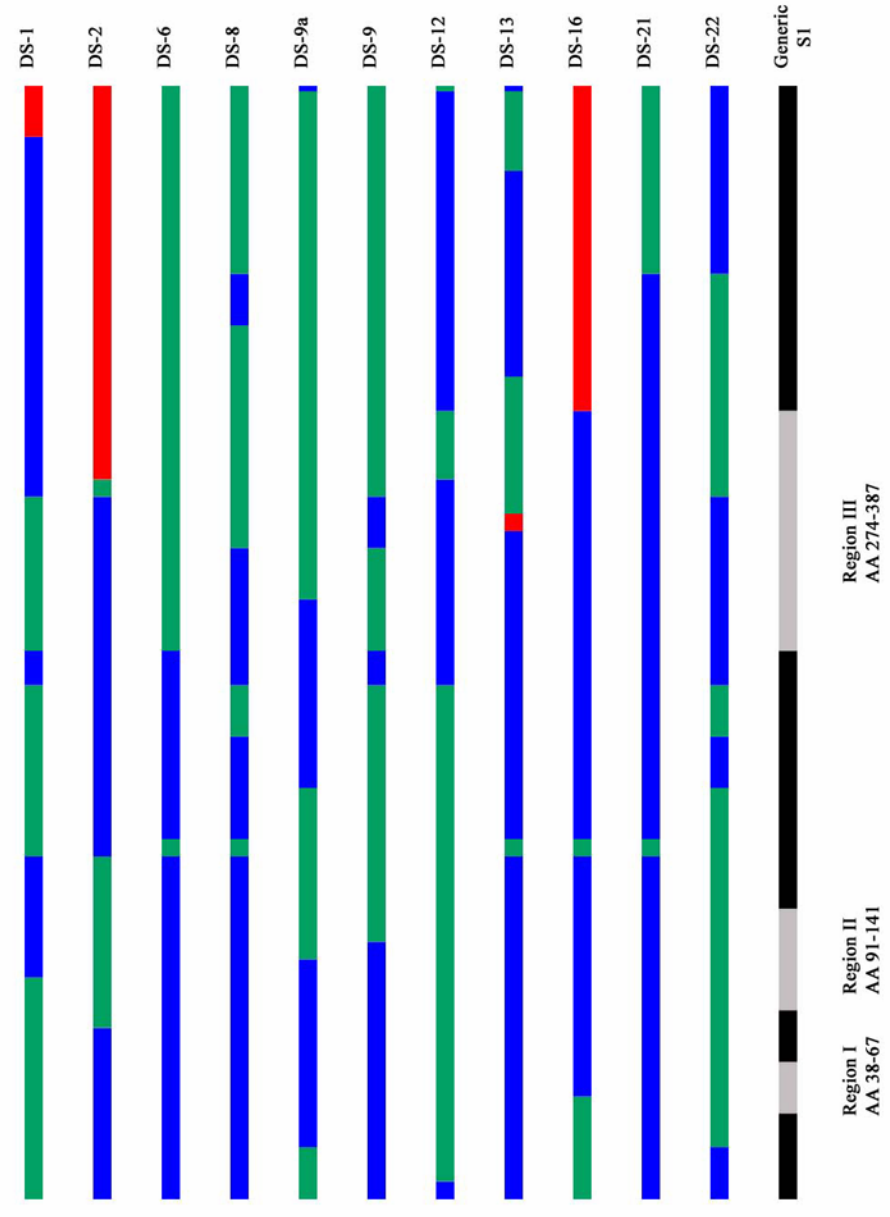
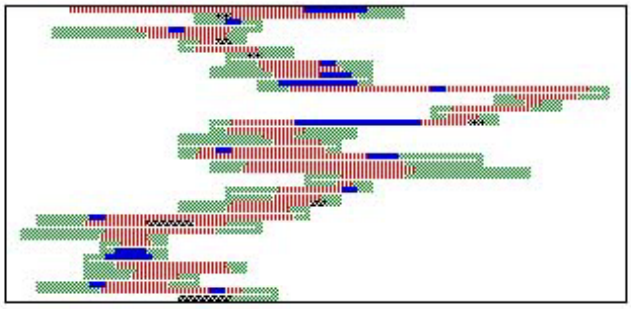
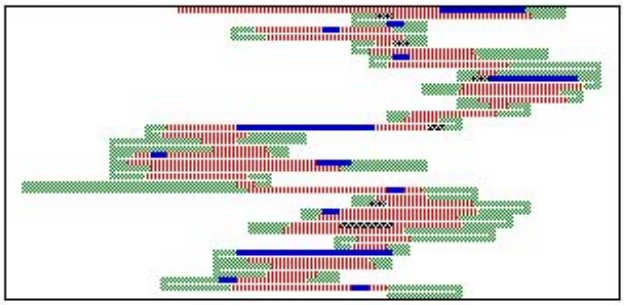

DS-1
DS-2
DS-6
DS-8
DS-9a
DS-9
DS-12
DS-13
DS-16
DS-21
DS-22
Generic S1
Region I AA 38-67
Region II AA 91-141
Region III AA 274-387

Figure 4.4. Secondary structure predictions of parental and recombinant S1 genes using the Chou and Fasman algorithm [23].

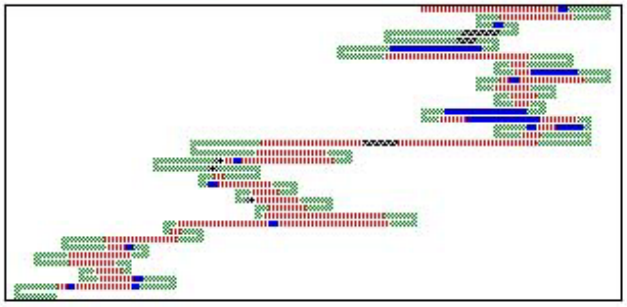
File: M41-5 AA
Size: 537 aa
Seq: 1 - 537
Function: Chou and Fasman
: HELIX : SHEET : TURN : COIL



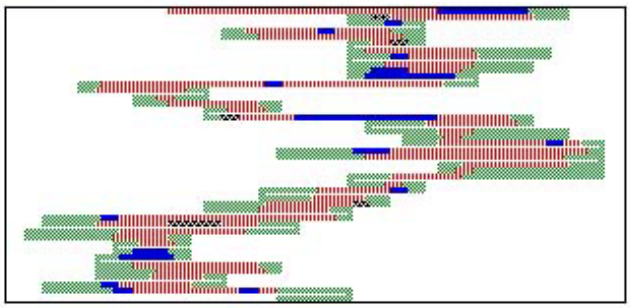
File: Conn-4 AA
Size: 534 aa
Seq: 1 - 534
Function: Chou and Fasman
: HELIX : SHEET : TURN : COIL



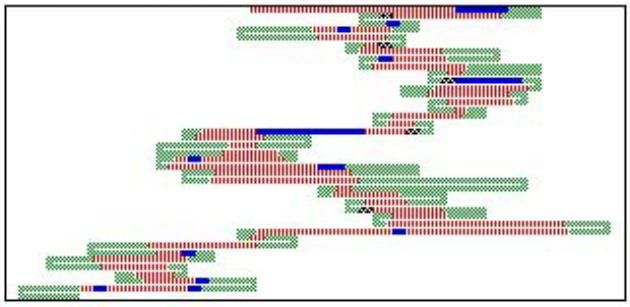
File: Ark-6 AA
Size: 544 aa
Seq: 1 - 544
Function: Chou and Fasman
: HELIX : SHEET : TURN : COIL



File: DS-1 AA
Size: 534 aa
Seq: 1 - 534
Function: Chou and Fasman
: HELIX : SHEET : TURN : COIL



File: DS-2 AA
Size: 537 aa
Seq: 1 - 537
Function: Chou and Fasman
: HELIX : SHEET : TURN : COIL



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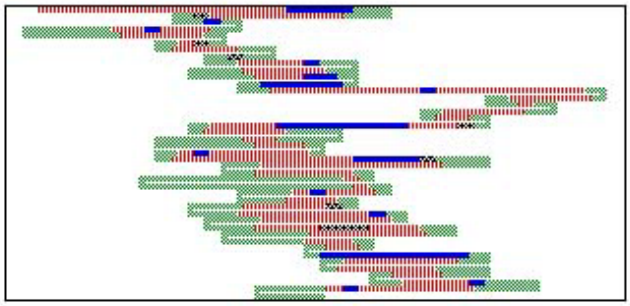
: HELIX : SHEET : TURN : COIL



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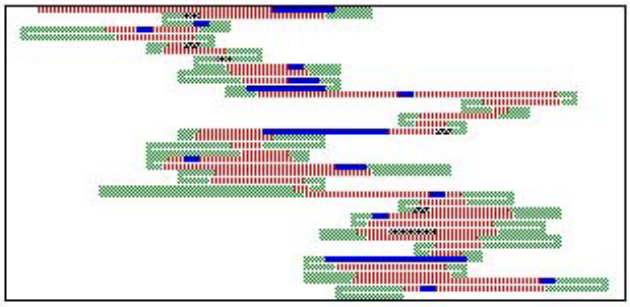
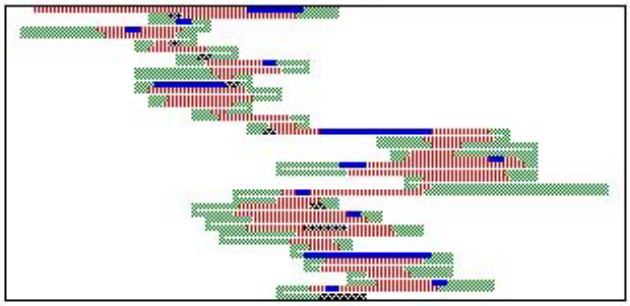
Function: Chou and Fasman
: HELIX : SHEET : TURN : COIL



File: DS-9a AA
Size: 537 aa
Seq: 1 - 537
Function: Chou and Fasman
: HELIX : SHEET : TURN : COIL



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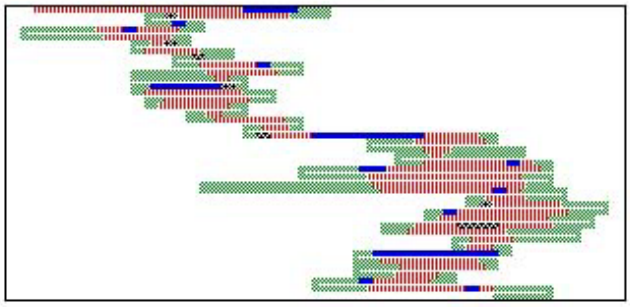
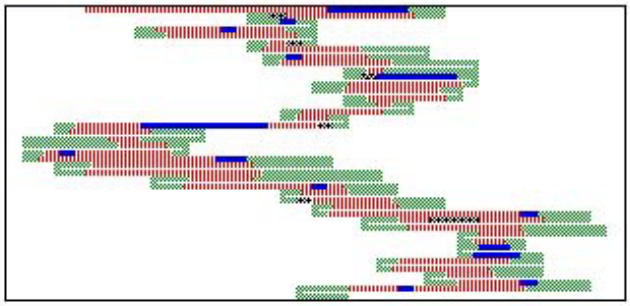
Function: Chou and Fasman
: HELIX : SHEET : TURN : COIL



File: DS-12 AA
Size: 534 aa
Seq: 1 - 534
Function: Chou and Fasman
: HELIX : SHEET : TURN : COIL



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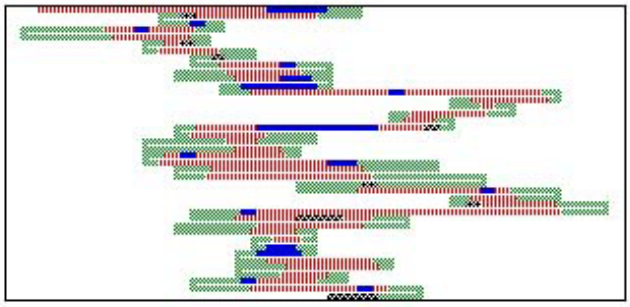
: HELIX : SHEET : TURN : COIL



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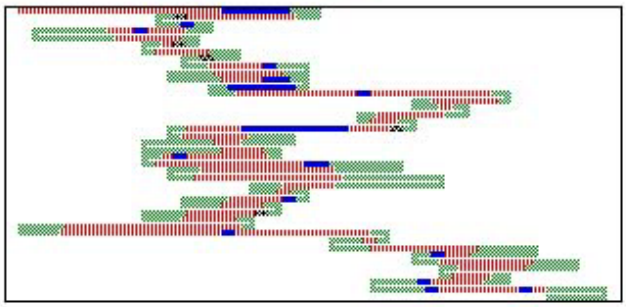
: HELIX : SHEET : TURN : COIL



File: DS-21 AA
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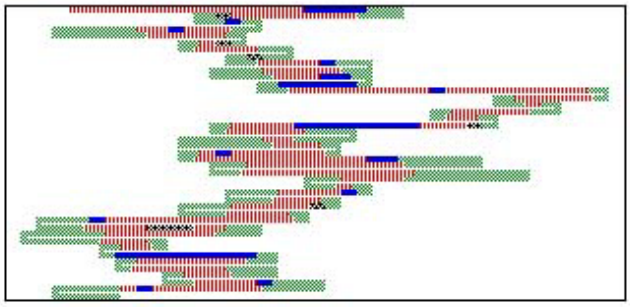
Function: Chou and Fasman
: HELIX : SHEET : TURN : COIL



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Size: 534 aa
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Function: Chou and Fasman
: HELIX : SHEET : TURN : COIL

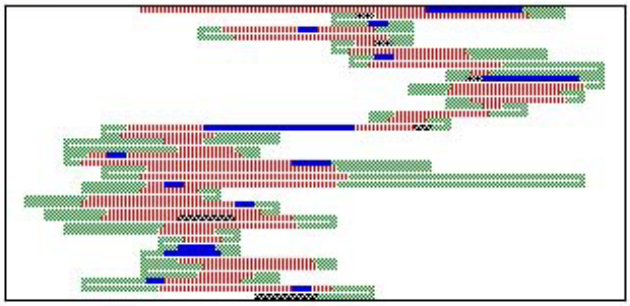


Table 4.1. S1 nucleotide and amino acid alignment pair distances

Nucleotide – percent similarity

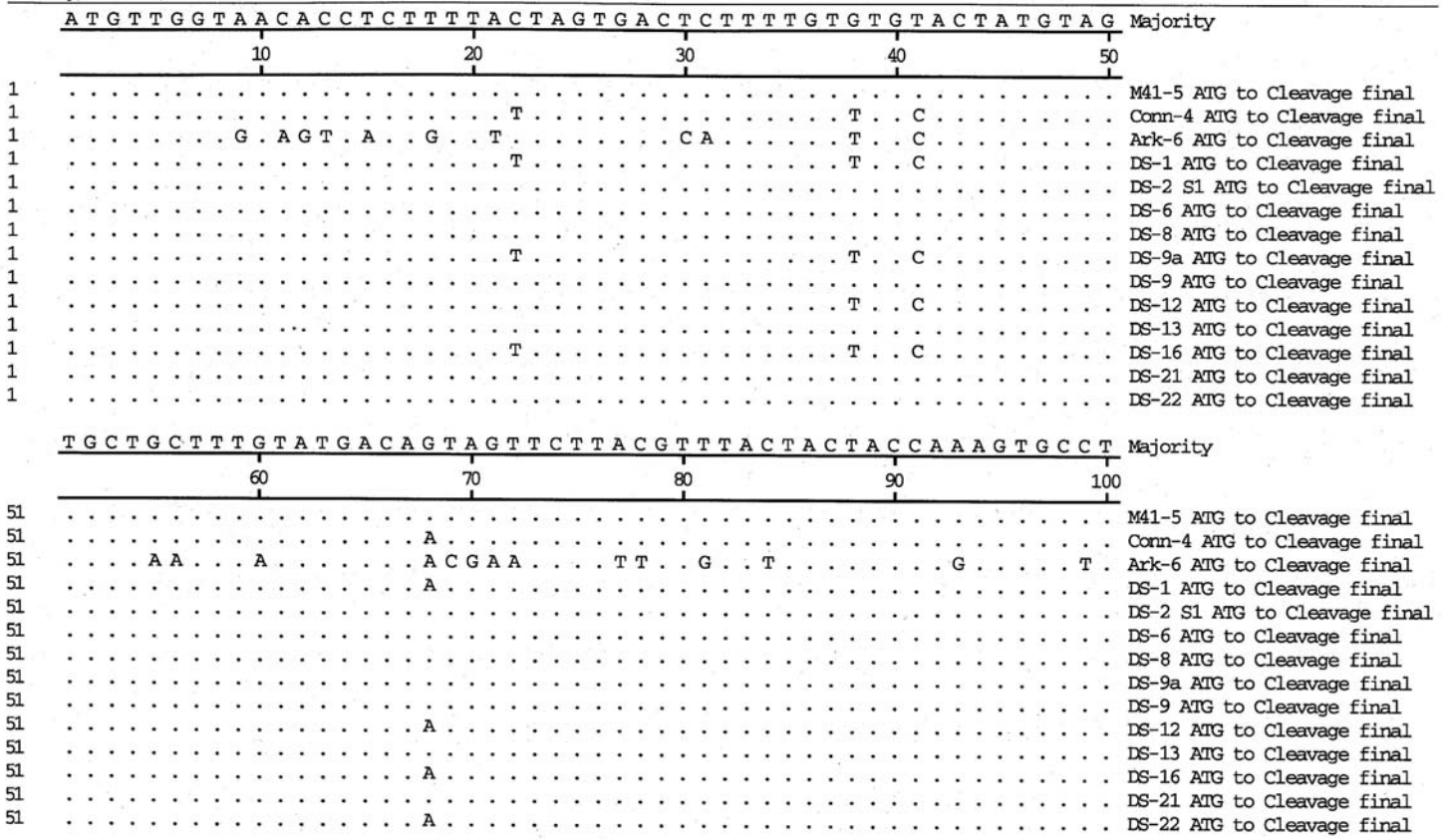
Deduced amino acid – percent similarity

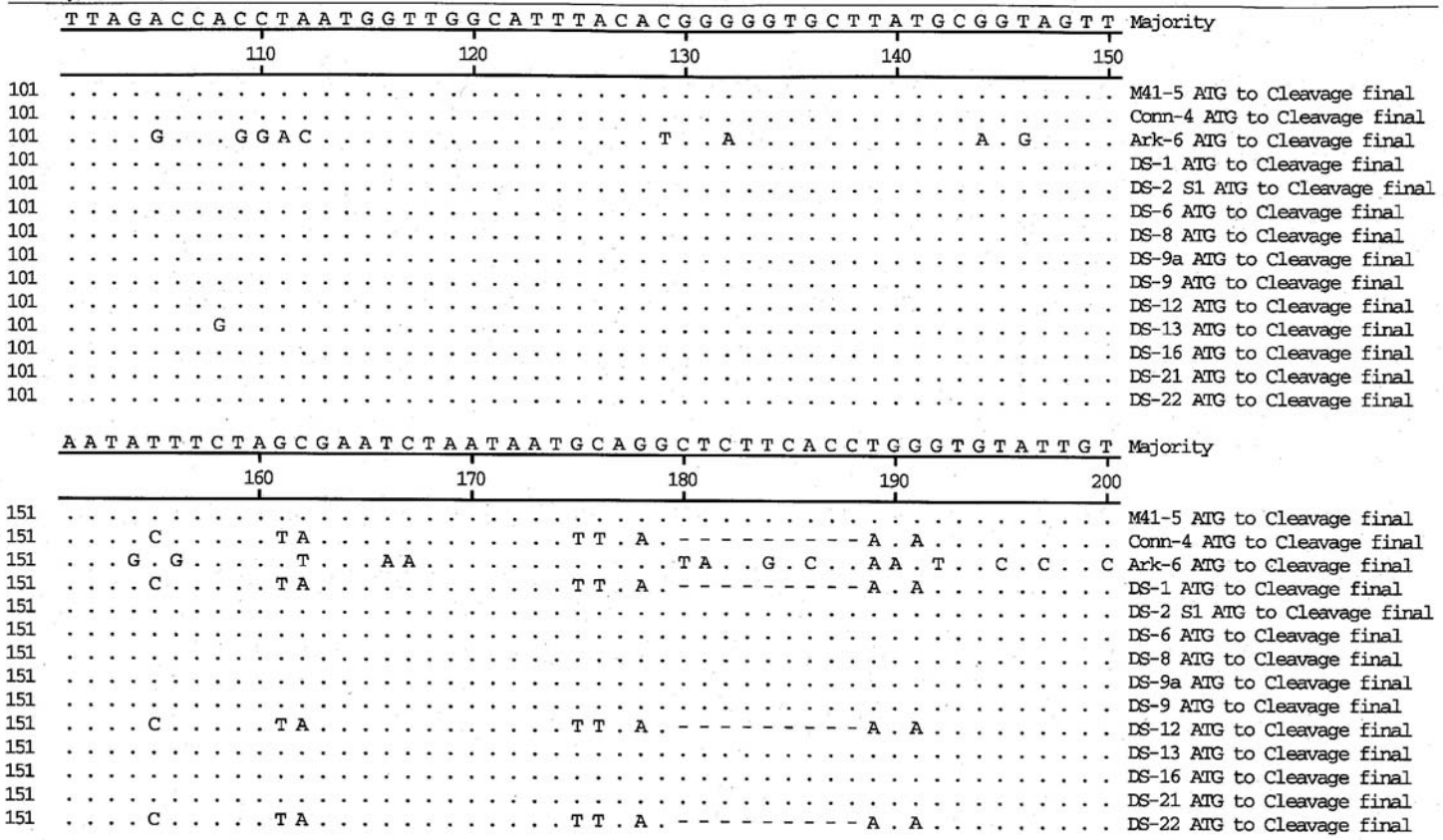
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1		94.9	76.3	96.9	91.3	98.1	98.4	97.2	97.1	96.5	99.1	93.6	99.6	95.9	**1**	**M41 S1**
2	90.1		75.5	97.3	90.8	96.5	96.3	97.7	97.9	98.1	95.2	90.4	95.3	98.8	**2**	**Conn46 S1**
3	75.4	75.7		76.0	82.8	75.9	76.1	76.5	76.1	75.7	76.5	82.4	76.2	76.0	**3**	**Ark DPI S1**
4	94.0	95.3	75.3		90.1	96.3	96.2	95.3	95.7	97.8	96.3	92.1	96.9	96.6	**4**	**DS-1 S1**
5	89.9	89.3	81.4	87.3		91.1	91.2	92.2	91.9	91.3	91.1	96.9	91.3	91.8	**5**	**DS-2 S1**
6	96.3	93.1	75.2	93.6	89.2		99.3	98.3	98.6	95.3	98.4	93.3	98.6	96.3	**6**	**DS-6 S1**
7	97.6	92.1	75.2	93.1	90.1	98.3		98.3	98.4	95.7	98.8	93.4	98.9	96.6	**7**	**DS-8 S1**
8	94.6	95.5	76.7	91.6	91.6	96.1	96.3		99.1	96.7	97.5	92.6	97.5	97.6	**8**	**DS-9a S1**
9	94.4	95.7	76.4	92.5	91.1	97.0	96.1	98.0		96.4	97.4	92.2	97.5	97.3	**9**	**DS-9 S1**
10	92.7	96.6	75.1	95.3	90.1	90.4	91.8	94.0	93.1		96.1	90.8	96.6	98.6	**10**	**DS-12 S1**
11	98.7	90.6	75.8	93.4	89.9	96.8	98.1	95.2	95.0	92.5		93.3	99.1	96.1	**11**	**DS-13 S1**
12	93.9	87.6	81.0	90.8	94.6	92.7	93.7	91.4	90.9	88.8	93.5		93.6	90.9	**12**	**DS-16 S1**
13	99.3	90.8	75.4	94.4	90.3	97.0	98.3	95.0	95.2	93.1	98.7	94.2		95.8	**13**	**DS-21 S1**
14	92.3	97.4	75.7	93.4	91.4	92.3	93.3	95.5	94.6	97.4	92.9	88.8	92.3		**14**	**DS-22 S1**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**		

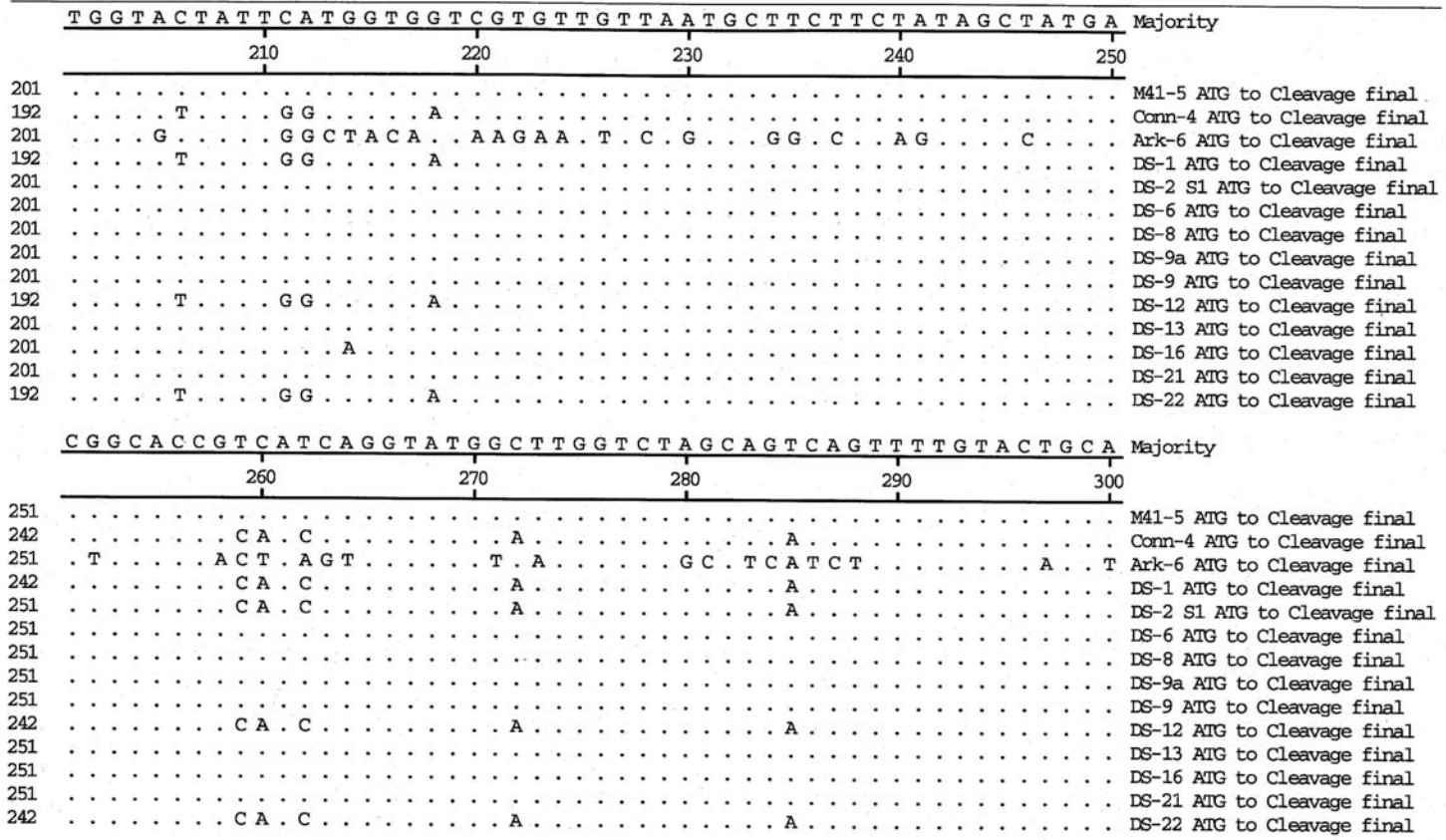
Table 4.2. Properties of parental and novel S1 gene recombinants.

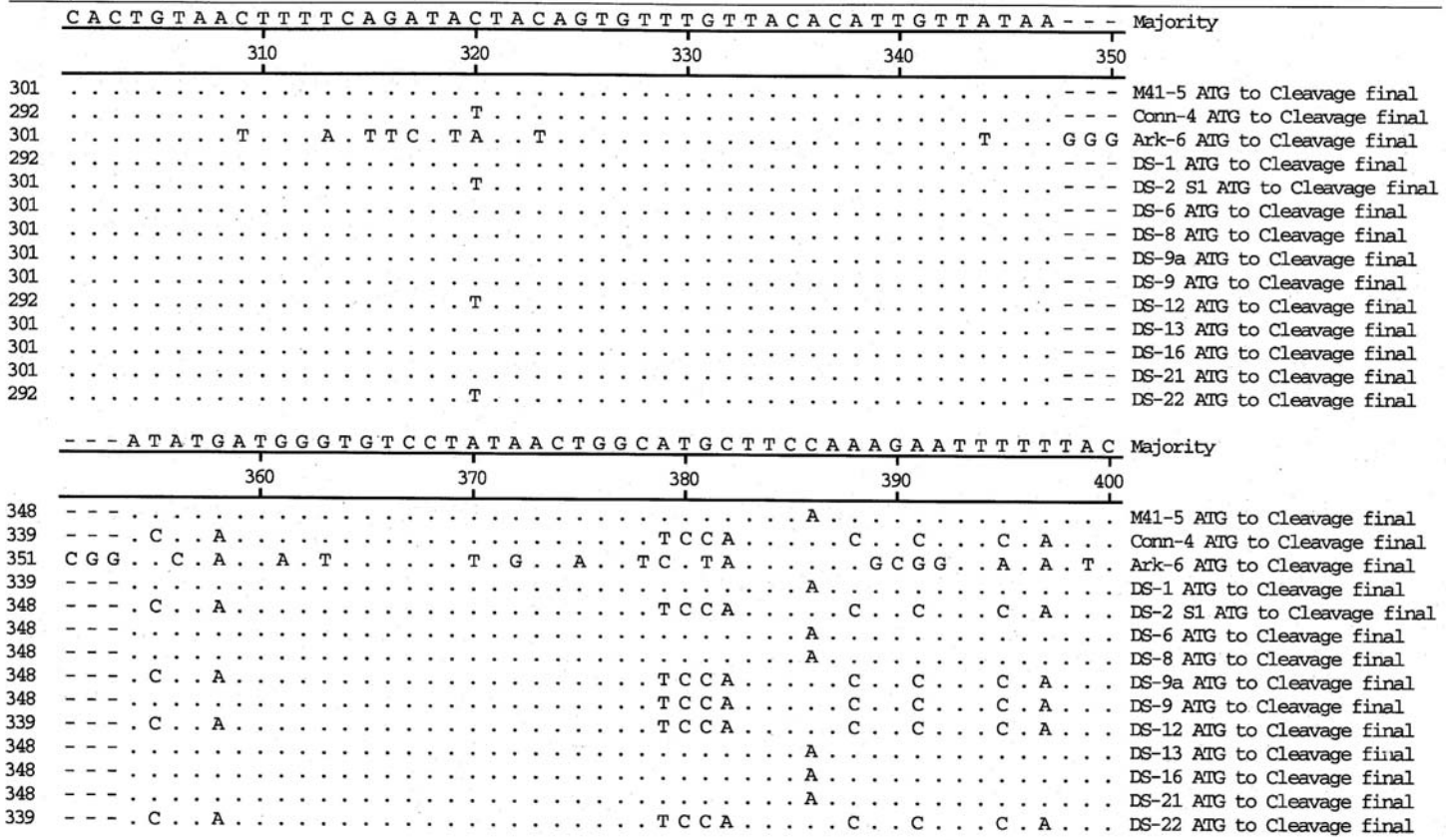
Clone name	Nucleotides/ Amino acids	Crossovers	Point mutations (non-synonmous changes)	Glycosylation sites	Cleavage sequence
Massachusetts 41	1611/537	NA	NA	17	RRFRR
Conneticut 46	1602/534	NA	NA	18	RRSRR
Arkansas DPI	1632/544	NA	NA	17	HRSRR
DS-1	1602/534	6	0	18	HRSRR
DS-2	1611/537	4	4(3)	17	HRSRR
DS-6	1611/537	3	2(2)	18	RRSRR
DS-8	1611/537	7	1(1)	17	RRSRR
DS-9a	1611/537	5	0	18	RRFRR
DS-9	1611/537	5	0	18	RRSRR
DS-12	1602/534	5	2(2)	17	RRSRR
DS-13	1611/537	7	1(0)	17	RRFRR
DS-16	1611/537	4	2(1)	17	HRSRR
DS-21	1611/537	3	0	17	RRSRR
DS-22	1602/534	6	2(1)	17	RRFRR

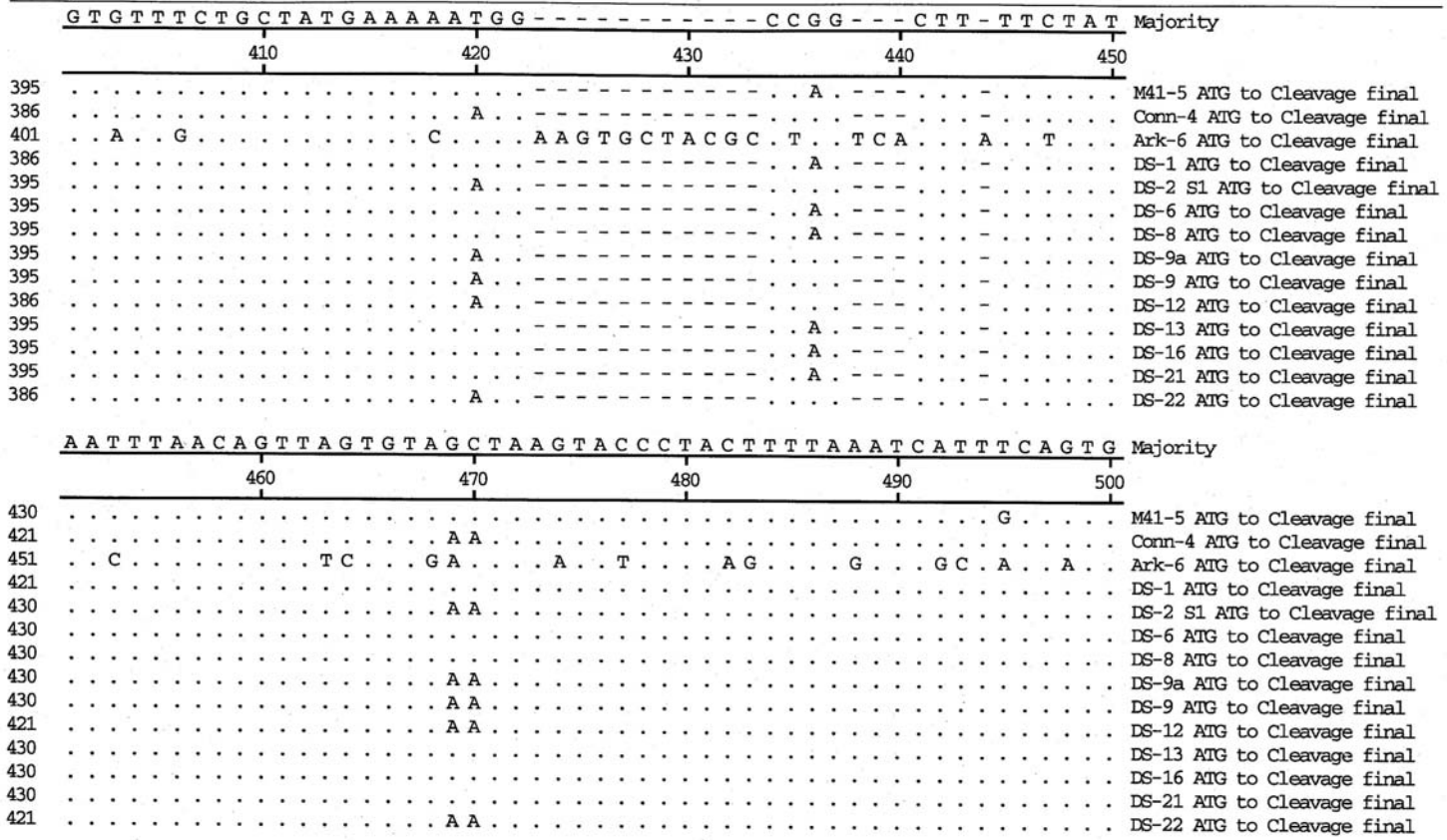
Figure 4.5. Nucleotide alignment for parental and recombinant S1 genes using the Clustal method with weighted residue weight table. Nucleotides matching the consensus sequence are hidden.

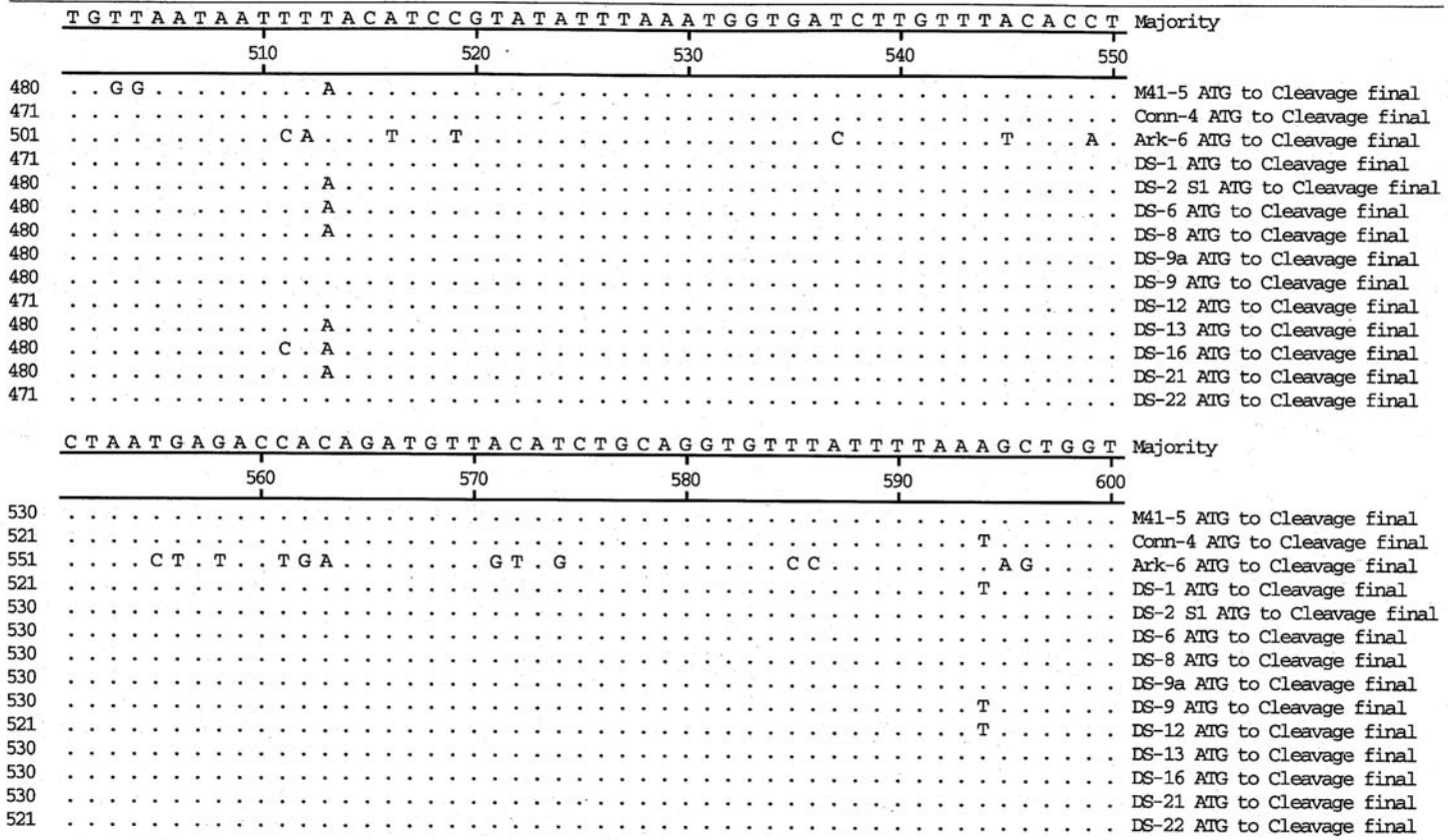

ATGTTGGTAACACCTCTTTTACTAGTGACTCTTTTGTGTGTACTATGTAG Majority
10
20
30
40
50
1 M41-5 ATG to Cleavage final
1 Conn-4 ATG to Cleavage final
1 Ark-6 ATG to Cleavage final
1 DS-1 ATG to Cleavage final
1 DS-2 S1 ATG to Cleavage final
1 DS-6 ATG to Cleavage final
1 DS-8 ATG to Cleavage final
1 DS-9a ATG to Cleavage final
1 DS-9 ATG to Cleavage final
1 DS-12 ATG to Cleavage final
1 DS-13 ATG to Cleavage final
1 DS-16 ATG to Cleavage final
1 DS-21 ATG to Cleavage final
1 DS-22 ATG to Cleavage final
TGCTGCTTTGTATGACAGTAGTTCTTACGTTTACTACTACCAAAGTGCCT Majority
60
70
80
90
100
51 M41-5 ATG to Cleavage final
51 Conn-4 ATG to Cleavage final
51 Ark-6 ATG to Cleavage final
51 DS-1 ATG to Cleavage final
51 DS-2 S1 ATG to Cleavage final
51 DS-6 ATG to Cleavage final
51 DS-8 ATG to Cleavage final
51 DS-9a ATG to Cleavage final
51 DS-9 ATG to Cleavage final
51 DS-12 ATG to Cleavage final
51 DS-13 ATG to Cleavage final
51 DS-16 ATG to Cleavage final
51 DS-21 ATG to Cleavage final
51 DS-22 ATG to Cleavage final

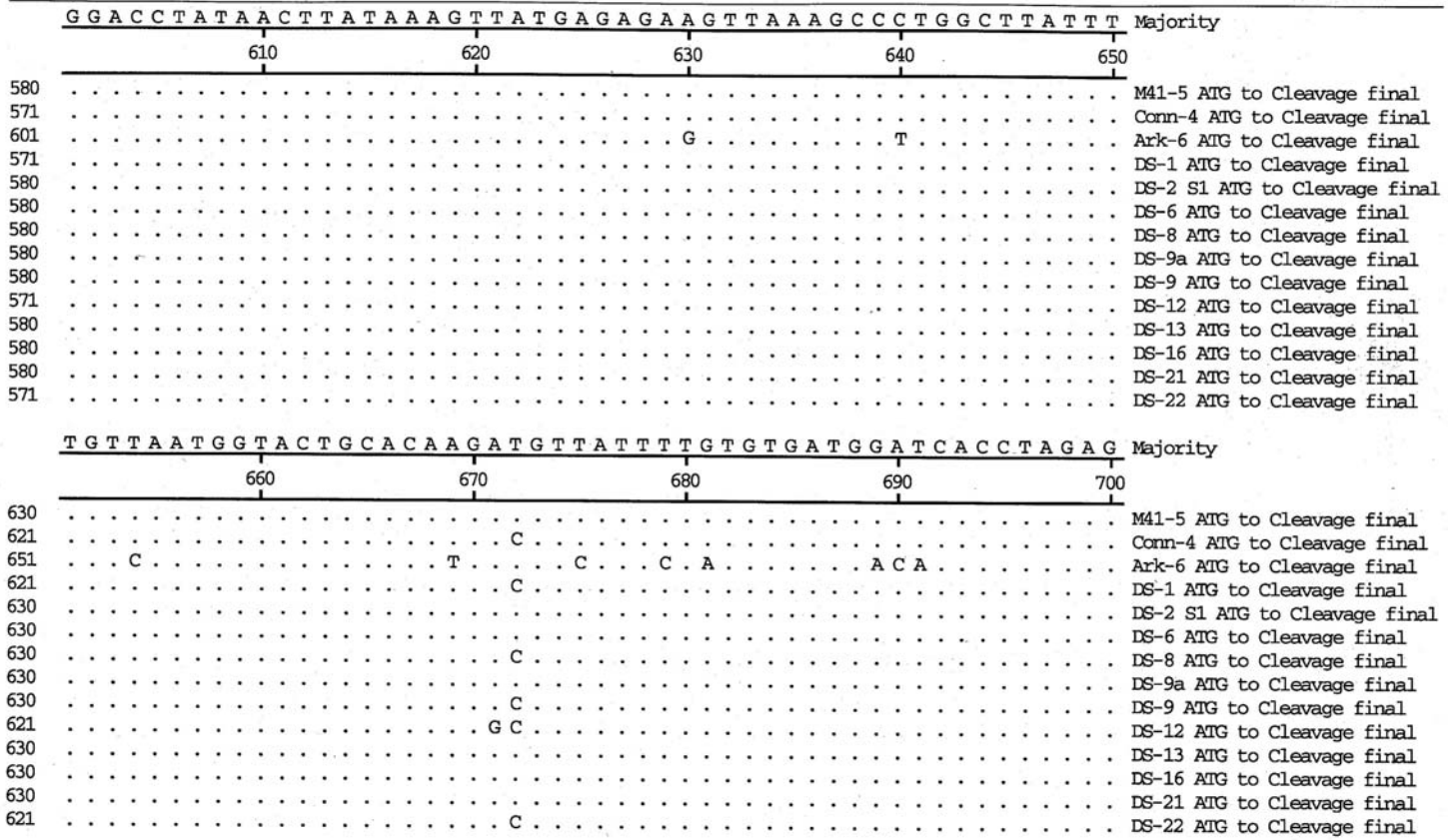

TTAGACCACCTAATGGTTGGCATTTACACGGGGGTGCTTATGCGGTAGTT Majority
110
120
130
140
150
101 M41-5 ATG to Cleavage final
101 Conn-4 ATG to Cleavage final
101 Ark-6 ATG to Cleavage final
101 DS-1 ATG to Cleavage final
101 DS-2 S1 ATG to Cleavage final
101 DS-6 ATG to Cleavage final
101 DS-8 ATG to Cleavage final
101 DS-9a ATG to Cleavage final
101 DS-9 ATG to Cleavage final
101 DS-12 ATG to Cleavage final
101 DS-13 ATG to Cleavage final
101 DS-16 ATG to Cleavage final
101 DS-21 ATG to Cleavage final
101 DS-22 ATG to Cleavage final
AATATTTCTAGCGAATCTAATAATGCAGGCTCTTCACCTGGGTGTATTGT Majority
160
170
180
190
200
151 M41-5 ATG to Cleavage final
151 Conn-4 ATG to Cleavage final
151 Ark-6 ATG to Cleavage final
151 DS-1 ATG to Cleavage final
151 DS-2 S1 ATG to Cleavage final
151 DS-6 ATG to Cleavage final
151 DS-8 ATG to Cleavage final
151 DS-9a ATG to Cleavage final
151 DS-9 ATG to Cleavage final
151 DS-12 ATG to Cleavage final
151 DS-13 ATG to Cleavage final
151 DS-16 ATG to Cleavage final
151 DS-21 ATG to Cleavage final
151 DS-22 ATG to Cleavage final

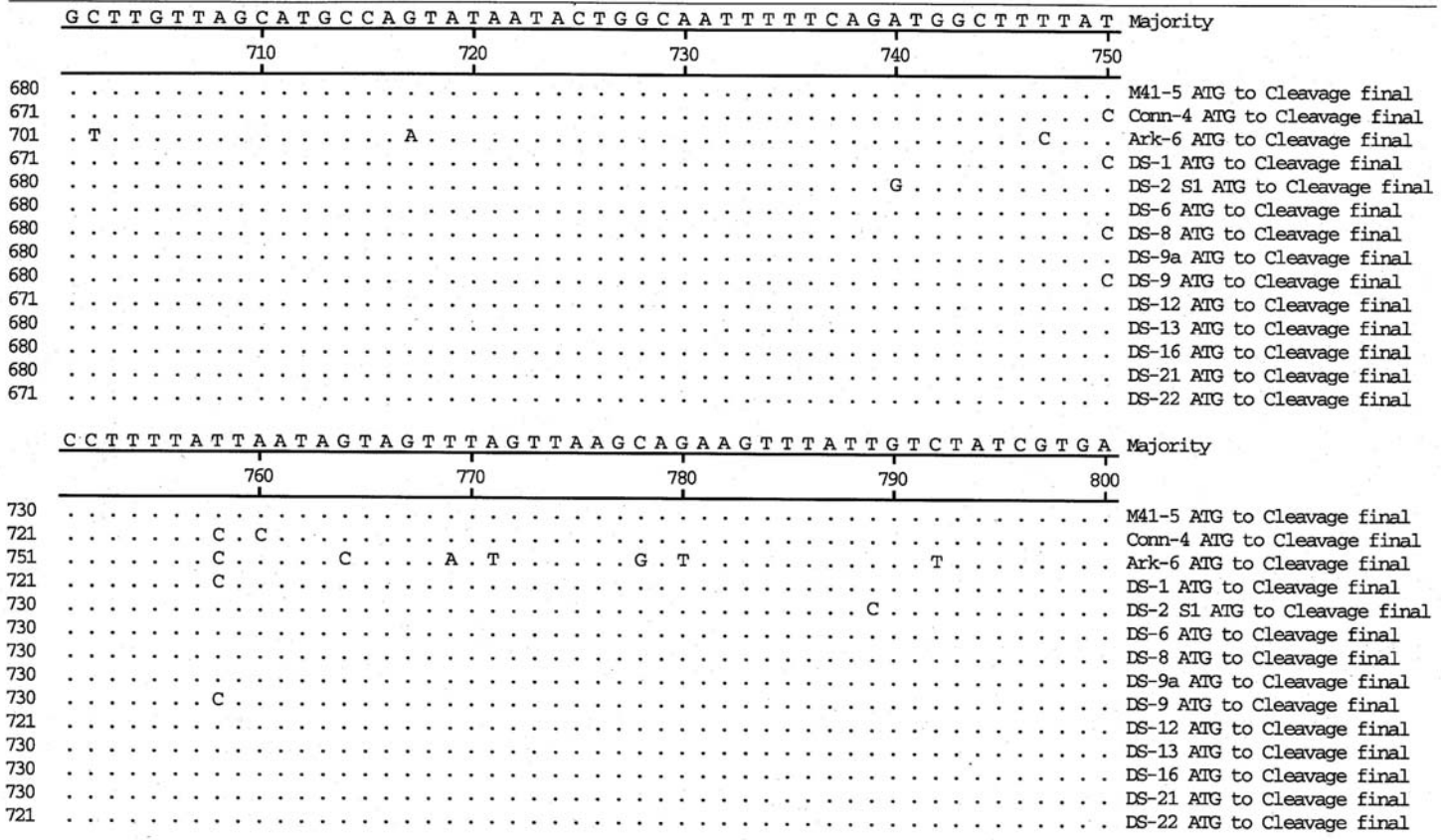

TGGTACTATTCATGGTGGTCGTGTTGTTAATGCTTCTTCTATAGCTATGA Majority
210
220
230
240
250
201 M41-5 ATG to Cleavage final
192 Conn-4 ATG to Cleavage final
201 Ark-6 ATG to Cleavage final
192 DS-1 ATG to Cleavage final
201 DS-2 S1 ATG to Cleavage final
201 DS-6 ATG to Cleavage final
201 DS-8 ATG to Cleavage final
201 DS-9a ATG to Cleavage final
201 DS-9 ATG to Cleavage final
192 DS-12 ATG to Cleavage final
201 DS-13 ATG to Cleavage final
201 DS-16 ATG to Cleavage final
201 DS-21 ATG to Cleavage final
192 DS-22 ATG to Cleavage final
CGGCACCGTCATCAGGTATGGCTTGGTCTAGCAGTCAGTTTTGTACTGCA Majority
260
270
280
290
300
251 M41-5 ATG to Cleavage final
242 Conn-4 ATG to Cleavage final
251 Ark-6 ATG to Cleavage final
242 DS-1 ATG to Cleavage final
251 DS-2 S1 ATG to Cleavage final
251 DS-6 ATG to Cleavage final
251 DS-8 ATG to Cleavage final
251 DS-9a ATG to Cleavage final
251 DS-9 ATG to Cleavage final
242 DS-12 ATG to Cleavage final
251 DS-13 ATG to Cleavage final
251 DS-16 ATG to Cleavage final
251 DS-21 ATG to Cleavage final
242 DS-22 ATG to Cleavage final

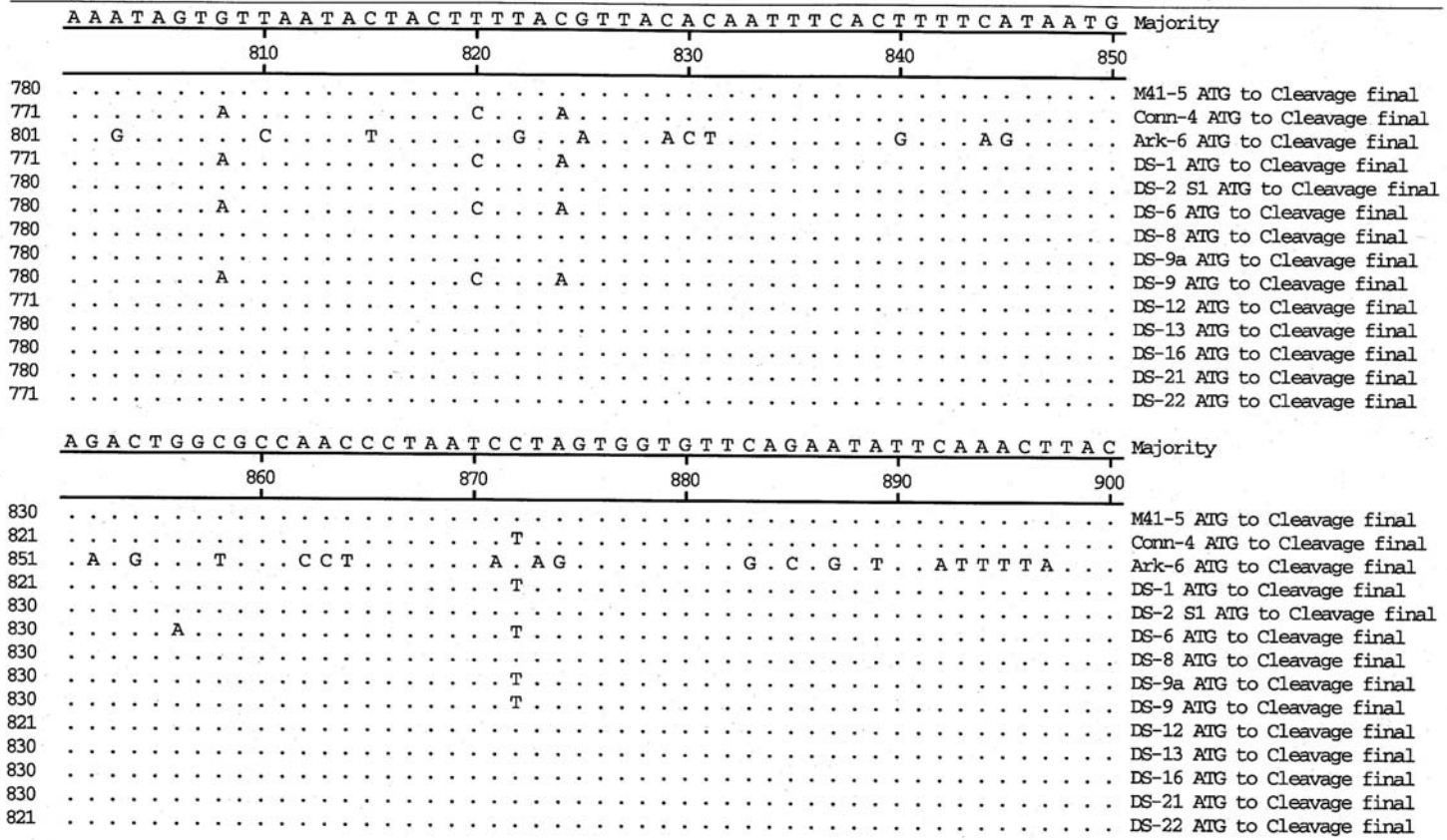

CACTGTAACTTTTCAGATACTACAGTGTTTGTTACACATTGTTATAA---
Majority
310
320
330
340
350
301 M41-5 ATG to Cleavage final
292 Conn-4 ATG to Cleavage final
301 Ark-6 ATG to Cleavage final
292 DS-1 ATG to Cleavage final
301 DS-2 S1 ATG to Cleavage final
301 DS-6 ATG to Cleavage final
301 DS-8 ATG to Cleavage final
301 DS-9a ATG to Cleavage final
301 DS-9 ATG to Cleavage final
292 DS-12 ATG to Cleavage final
301 DS-13 ATG to Cleavage final
301 DS-16 ATG to Cleavage final
301 DS-21 ATG to Cleavage final
292 DS-22 ATG to Cleavage final
---ATATGATGGGTGTCCTATAACTGGCATGCTTCCAAAGAATTTTTTAC
Majority
360
370
380
390
400
348 M41-5 ATG to Cleavage final
339 Conn-4 ATG to Cleavage final
351 Ark-6 ATG to Cleavage final
339 DS-1 ATG to Cleavage final
348 DS-2 S1 ATG to Cleavage final
348 DS-6 ATG to Cleavage final
348 DS-8 ATG to Cleavage final
348 DS-9a ATG to Cleavage final
348 DS-9 ATG to Cleavage final
339 DS-12 ATG to Cleavage final
348 DS-13 ATG to Cleavage final
348 DS-16 ATG to Cleavage final
348 DS-21 ATG to Cleavage final
339 DS-22 ATG to Cleavage final

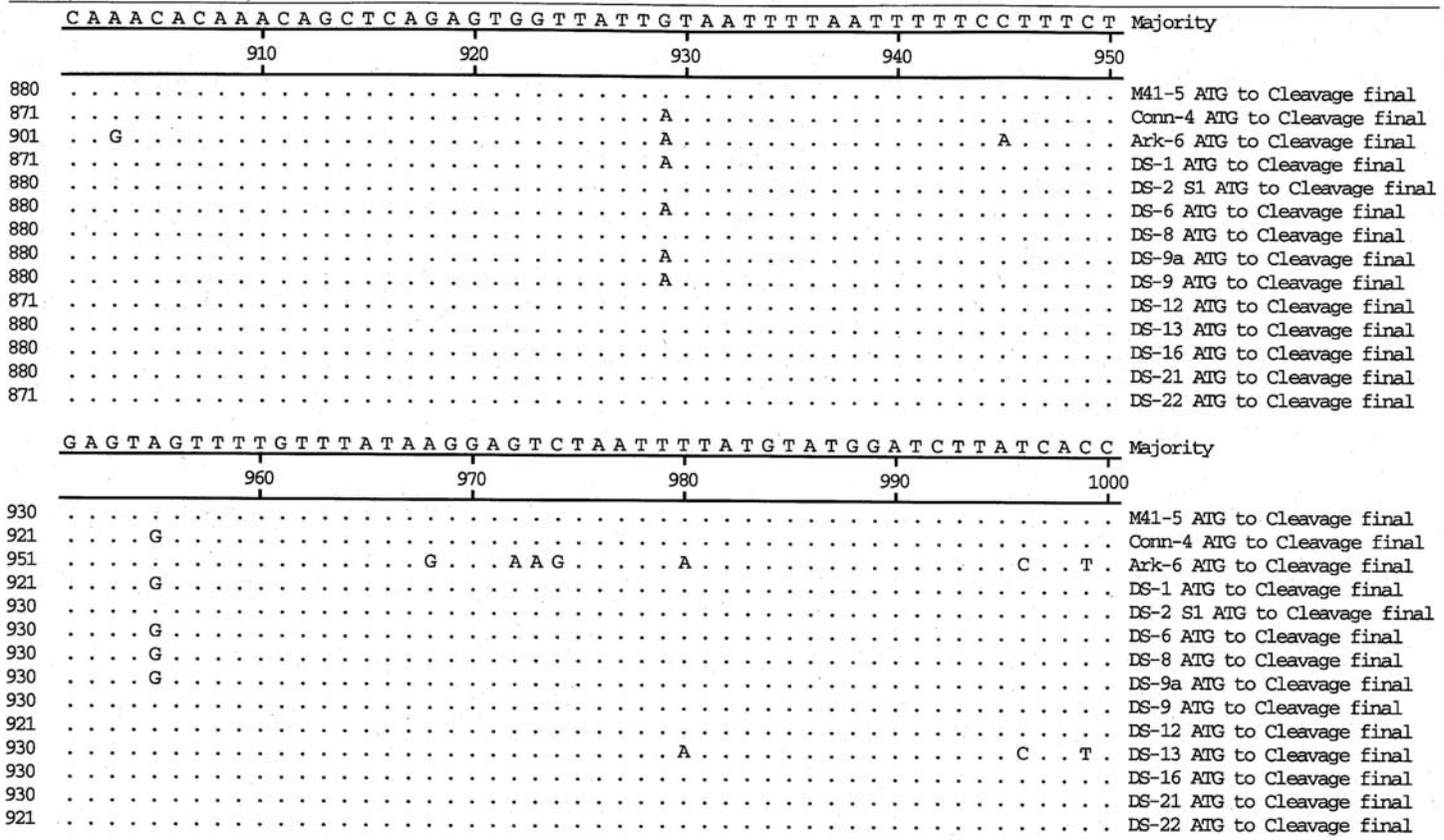

GTGTTTCTGCTATGAAAAATGG----------CCGG---CTT-TTCTAT Majority
410 420 430 440 450
395 M41-5 ATG to Cleavage final
386 Conn-4 ATG to Cleavage final
401 Ark-6 ATG to Cleavage final
386 DS-1 ATG to Cleavage final
395 DS-2 S1 ATG to Cleavage final
395 DS-6 ATG to Cleavage final
395 DS-8 ATG to Cleavage final
395 DS-9a ATG to Cleavage final
395 DS-9 ATG to Cleavage final
386 DS-12 ATG to Cleavage final
395 DS-13 ATG to Cleavage final
395 DS-16 ATG to Cleavage final
395 DS-21 ATG to Cleavage final
386 DS-22 ATG to Cleavage final
AATTTAACAGTTAGTGTAGCTAAGTACCCTACTTTTAAATCATTTCAGTG Majority
460 470 480 490 500
430 M41-5 ATG to Cleavage final
421 Conn-4 ATG to Cleavage final
451 Ark-6 ATG to Cleavage final
421 DS-1 ATG to Cleavage final
430 DS-2 S1 ATG to Cleavage final
430 DS-6 ATG to Cleavage final
430 DS-8 ATG to Cleavage final
430 DS-9a ATG to Cleavage final
430 DS-9 ATG to Cleavage final
421 DS-12 ATG to Cleavage final
430 DS-13 ATG to Cleavage final
430 DS-16 ATG to Cleavage final
430 DS-21 ATG to Cleavage final
421 DS-22 ATG to Cleavage final

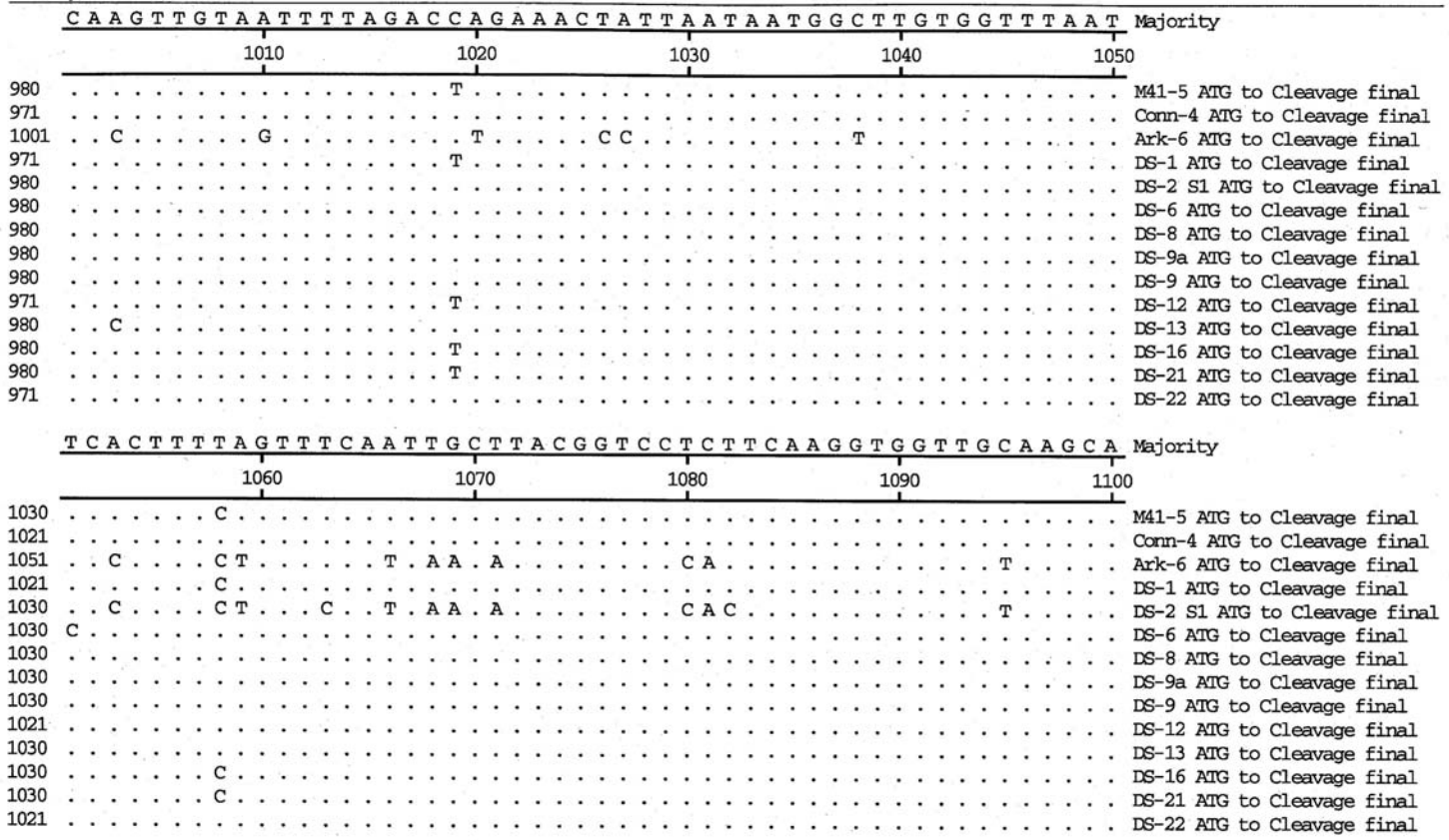

TGTTAATAATTTTACATCCGTATATTTAAATGGTGATCTTGTTTACACCT Majority
510
520
530
540
550
480 . . G G . . . . . A . . . . . M41-5 ATG to Cleavage final
471 . . . . . Conn-4 ATG to Cleavage final
501 . . . . . C A . . . T . . T . . . . . C . . . . . T . . . A . Ark-6 ATG to Cleavage final
471 . . . . . DS-1 ATG to Cleavage final
480 . . . . . A . . . . . DS-2 S1 ATG to Cleavage final
480 . . . . . A . . . . . DS-6 ATG to Cleavage final
480 . . . . . A . . . . . DS-8 ATG to Cleavage final
480 . . . . . DS-9a ATG to Cleavage final
480 . . . . . DS-9 ATG to Cleavage final
471 . . . . . DS-12 ATG to Cleavage final
480 . . . . . A . . . . . DS-13 ATG to Cleavage final
480 . . . . . C . A . . . . . DS-16 ATG to Cleavage final
480 . . . . . A . . . . . DS-21 ATG to Cleavage final
471 . . . . . DS-22 ATG to Cleavage final
CTAATGAGACCACAGATGTTACATCTGCAGGTGTTTATTTTAAAGCTGGT Majority
560
570
580
590
600
530 . . . . . M41-5 ATG to Cleavage final
521 . . . . . T . . . . . Conn-4 ATG to Cleavage final
551 . . . . C T . T . . T G A . . . . . G T . G . . . . . C C . . . . . A G . . . . Ark-6 ATG to Cleavage final
521 . . . . . T . . . . . DS-1 ATG to Cleavage final
530 . . . . . DS-2 S1 ATG to Cleavage final
530 . . . . . DS-6 ATG to Cleavage final
530 . . . . . DS-8 ATG to Cleavage final
530 . . . . . DS-9a ATG to Cleavage final
530 . . . . . T . . . . . DS-9 ATG to Cleavage final
521 . . . . . T . . . . . DS-12 ATG to Cleavage final
530 . . . . . DS-13 ATG to Cleavage final
530 . . . . . DS-16 ATG to Cleavage final
530 . . . . . DS-21 ATG to Cleavage final
521 . . . . . DS-22 ATG to Cleavage final

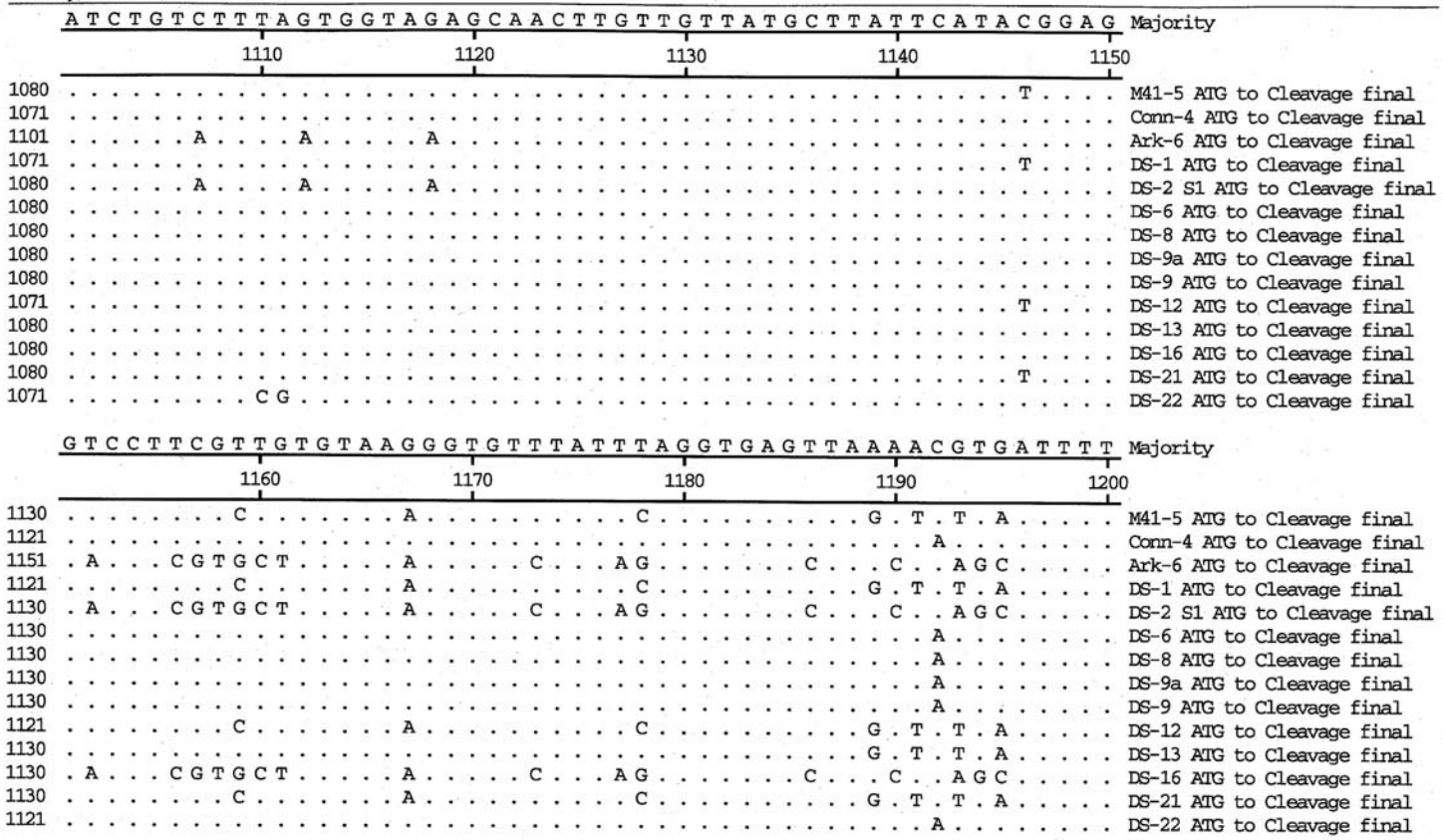

GGACCTATAACTTATAAAGTTATGAGAGAAGTTAAAGCCCTGGCTTATTT Majority
610
620
630
640
650
580 M41-5 ATG to Cleavage final
571 Conn-4 ATG to Cleavage final
601 Ark-6 ATG to Cleavage final
571 DS-1 ATG to Cleavage final
580 DS-2 S1 ATG to Cleavage final
580 DS-6 ATG to Cleavage final
580 DS-8 ATG to Cleavage final
580 DS-9a ATG to Cleavage final
580 DS-9 ATG to Cleavage final
571 DS-12 ATG to Cleavage final
580 DS-13 ATG to Cleavage final
580 DS-16 ATG to Cleavage final
580 DS-21 ATG to Cleavage final
571 DS-22 ATG to Cleavage final
TGTTAATGGTACTGCACAAGATGTTATTTTGTGTGATGGATCACCTAGAG Majority
660
670
680
690
700
630 M41-5 ATG to Cleavage final
621 Conn-4 ATG to Cleavage final
651 Ark-6 ATG to Cleavage final
621 DS-1 ATG to Cleavage final
630 DS-2 S1 ATG to Cleavage final
630 DS-6 ATG to Cleavage final
630 DS-8 ATG to Cleavage final
630 DS-9a ATG to Cleavage final
630 DS-9 ATG to Cleavage final
621 DS-12 ATG to Cleavage final
630 DS-13 ATG to Cleavage final
630 DS-16 ATG to Cleavage final
630 DS-21 ATG to Cleavage final
621 DS-22 ATG to Cleavage final

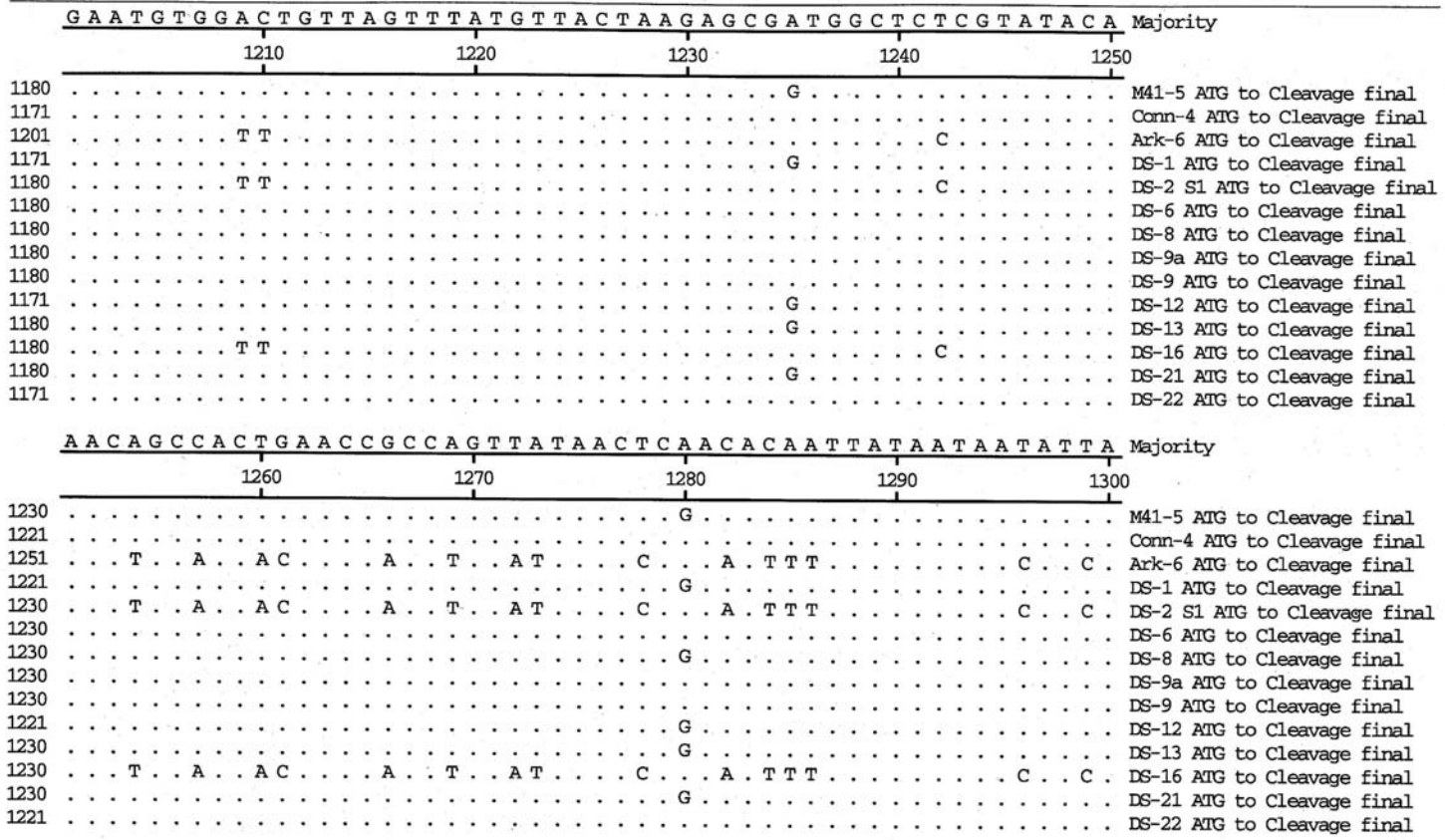

GCTTGTTAGCATGCCAGTATAATACTGGCAATTTTTCAGATGGCTTTTAT Majority
710
720
730
740
750
680 M41-5 ATG to Cleavage final
671 Conn-4 ATG to Cleavage final
701 Ark-6 ATG to Cleavage final
671 DS-1 ATG to Cleavage final
680 DS-2 S1 ATG to Cleavage final
680 DS-6 ATG to Cleavage final
680 DS-8 ATG to Cleavage final
680 DS-9a ATG to Cleavage final
680 DS-9 ATG to Cleavage final
671 DS-12 ATG to Cleavage final
680 DS-13 ATG to Cleavage final
680 DS-16 ATG to Cleavage final
680 DS-21 ATG to Cleavage final
671 DS-22 ATG to Cleavage final
CCTTTTATTAATAGTAGTTTAGTTAAGCAGAAGTTTATTGTCTATCGTGA Majority
760
770
780
790
800
730 M41-5 ATG to Cleavage final
721 Conn-4 ATG to Cleavage final
751 Ark-6 ATG to Cleavage final
721 DS-1 ATG to Cleavage final
730 DS-2 S1 ATG to Cleavage final
730 DS-6 ATG to Cleavage final
730 DS-8 ATG to Cleavage final
730 DS-9a ATG to Cleavage final
730 DS-9 ATG to Cleavage final
721 DS-12 ATG to Cleavage final
730 DS-13 ATG to Cleavage final
730 DS-16 ATG to Cleavage final
730 DS-21 ATG to Cleavage final
721 DS-22 ATG to Cleavage final

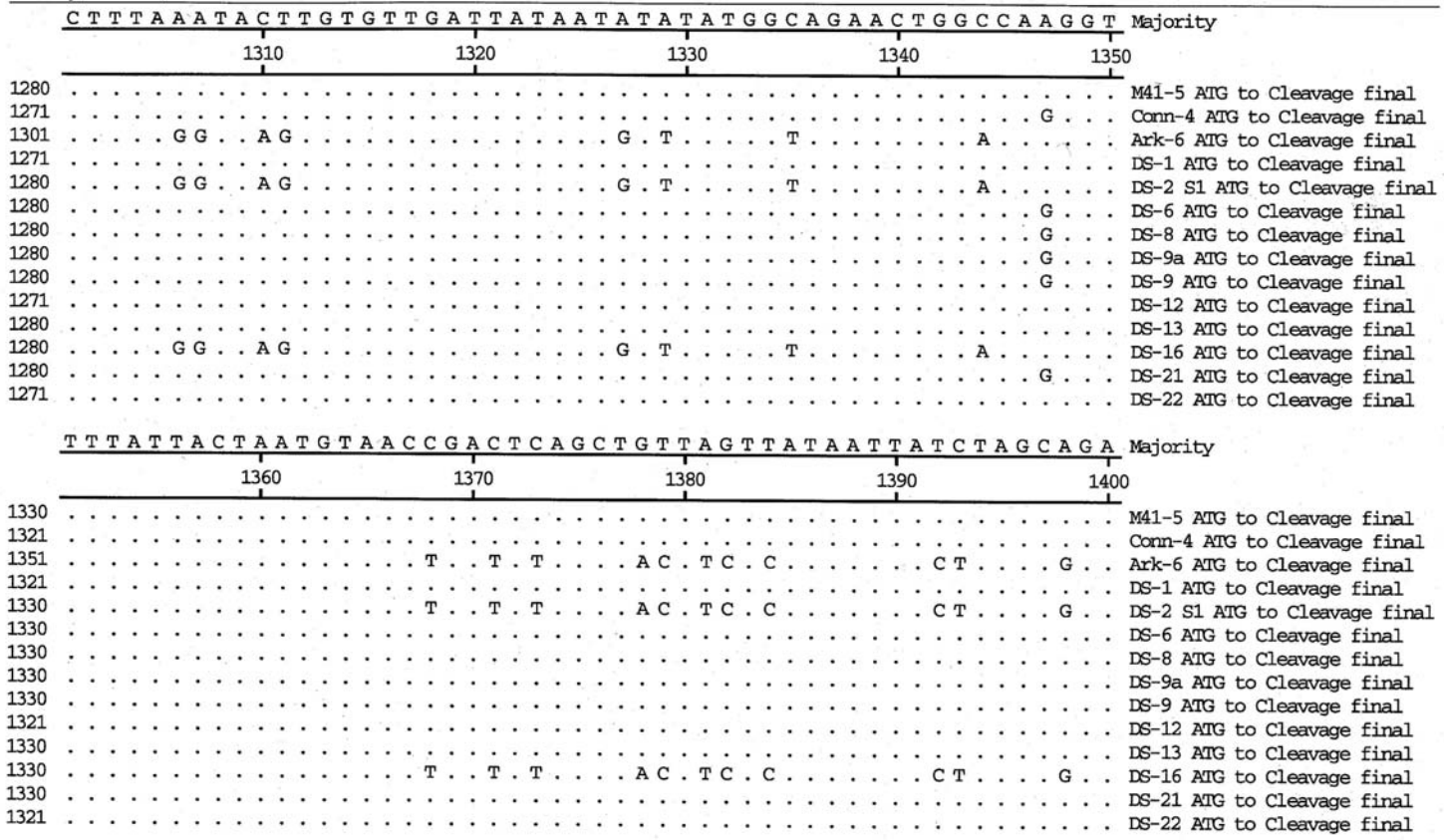

AAATAGTGTTAATACTACTTTTACGTTACACAATTTCACTTTTCATAATG Majority
810
820
830
840
850
780 M41-5 ATG to Cleavage final
771 Conn-4 ATG to Cleavage final
801 Ark-6 ATG to Cleavage final
771 DS-1 ATG to Cleavage final
780 DS-2 S1 ATG to Cleavage final
780 DS-6 ATG to Cleavage final
780 DS-8 ATG to Cleavage final
780 DS-9a ATG to Cleavage final
780 DS-9 ATG to Cleavage final
771 DS-12 ATG to Cleavage final
780 DS-13 ATG to Cleavage final
780 DS-16 ATG to Cleavage final
780 DS-21 ATG to Cleavage final
771 DS-22 ATG to Cleavage final
AGACTGGCGCCAACCCTAATCCTAGTGGTGTTCAGAATATTCAAACTTAC Majority
860
870
880
890
900
830 M41-5 ATG to Cleavage final
821 Conn-4 ATG to Cleavage final
851 Ark-6 ATG to Cleavage final
821 DS-1 ATG to Cleavage final
830 DS-2 S1 ATG to Cleavage final
830 DS-6 ATG to Cleavage final
830 DS-8 ATG to Cleavage final
830 DS-9a ATG to Cleavage final
830 DS-9 ATG to Cleavage final
821 DS-12 ATG to Cleavage final
830 DS-13 ATG to Cleavage final
830 DS-16 ATG to Cleavage final
830 DS-21 ATG to Cleavage final
821 DS-22 ATG to Cleavage final

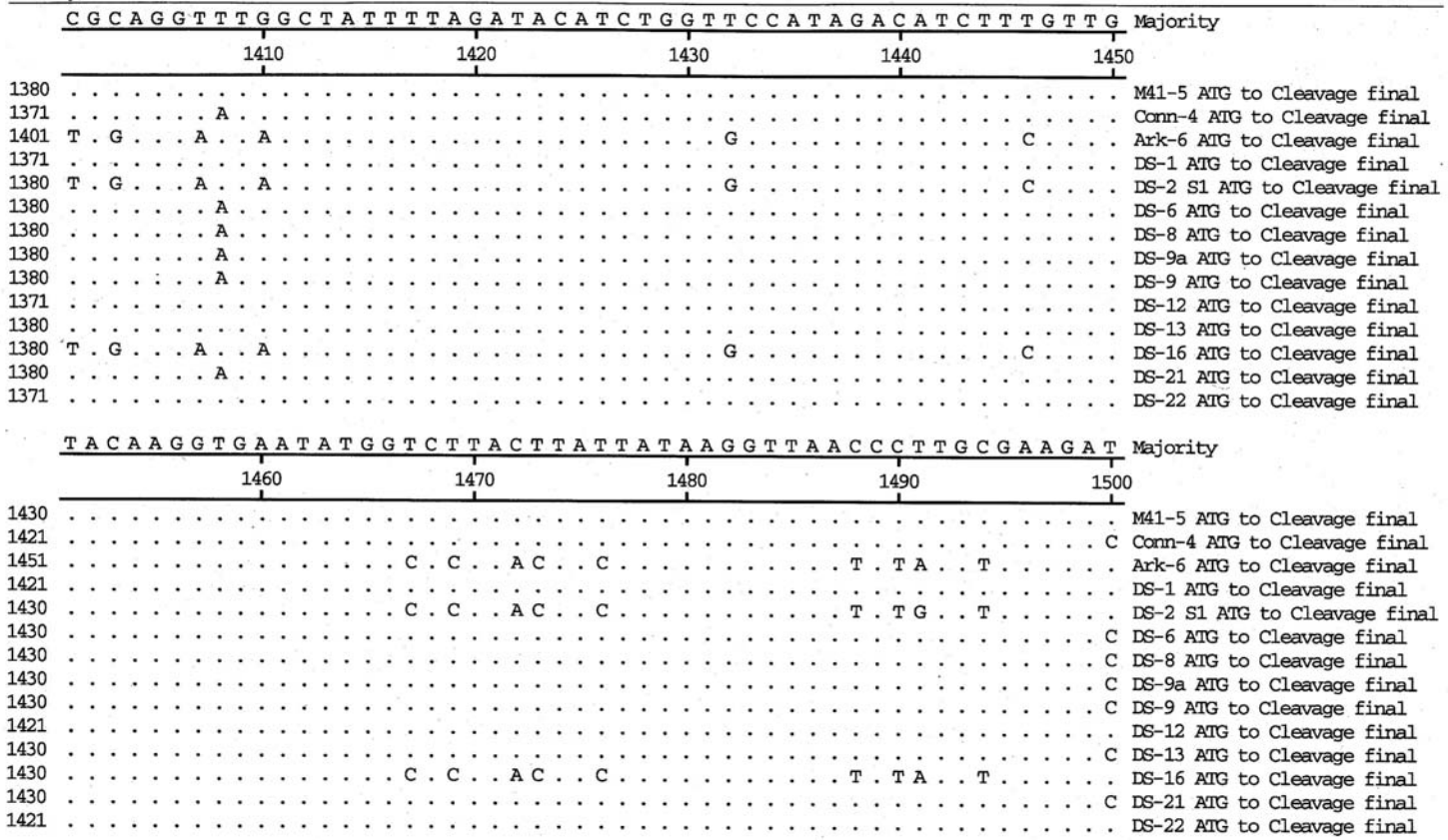

CAAACACAAACAGCTCAGAGTGGTTATTGTAATTTTAATTTTTCCTTTCT Majority
910
920
930
940
950
880 M41-5 ATG to Cleavage final
871 Conn-4 ATG to Cleavage final
901 Ark-6 ATG to Cleavage final
871 DS-1 ATG to Cleavage final
880 DS-2 S1 ATG to Cleavage final
880 DS-6 ATG to Cleavage final
880 DS-8 ATG to Cleavage final
880 DS-9a ATG to Cleavage final
880 DS-9 ATG to Cleavage final
871 DS-12 ATG to Cleavage final
880 DS-13 ATG to Cleavage final
880 DS-16 ATG to Cleavage final
880 DS-21 ATG to Cleavage final
871 DS-22 ATG to Cleavage final
GAGTAGTTTTGTTTATAAGGAGTCTAATTTTATGTATGGATCTTATCACC Majority
960
970
980
990
1000
930 M41-5 ATG to Cleavage final
921 Conn-4 ATG to Cleavage final
951 Ark-6 ATG to Cleavage final
921 DS-1 ATG to Cleavage final
930 DS-2 S1 ATG to Cleavage final
930 DS-6 ATG to Cleavage final
930 DS-8 ATG to Cleavage final
930 DS-9a ATG to Cleavage final
930 DS-9 ATG to Cleavage final
921 DS-12 ATG to Cleavage final
930 DS-13 ATG to Cleavage final
930 DS-16 ATG to Cleavage final
930 DS-21 ATG to Cleavage final
921 DS-22 ATG to Cleavage final

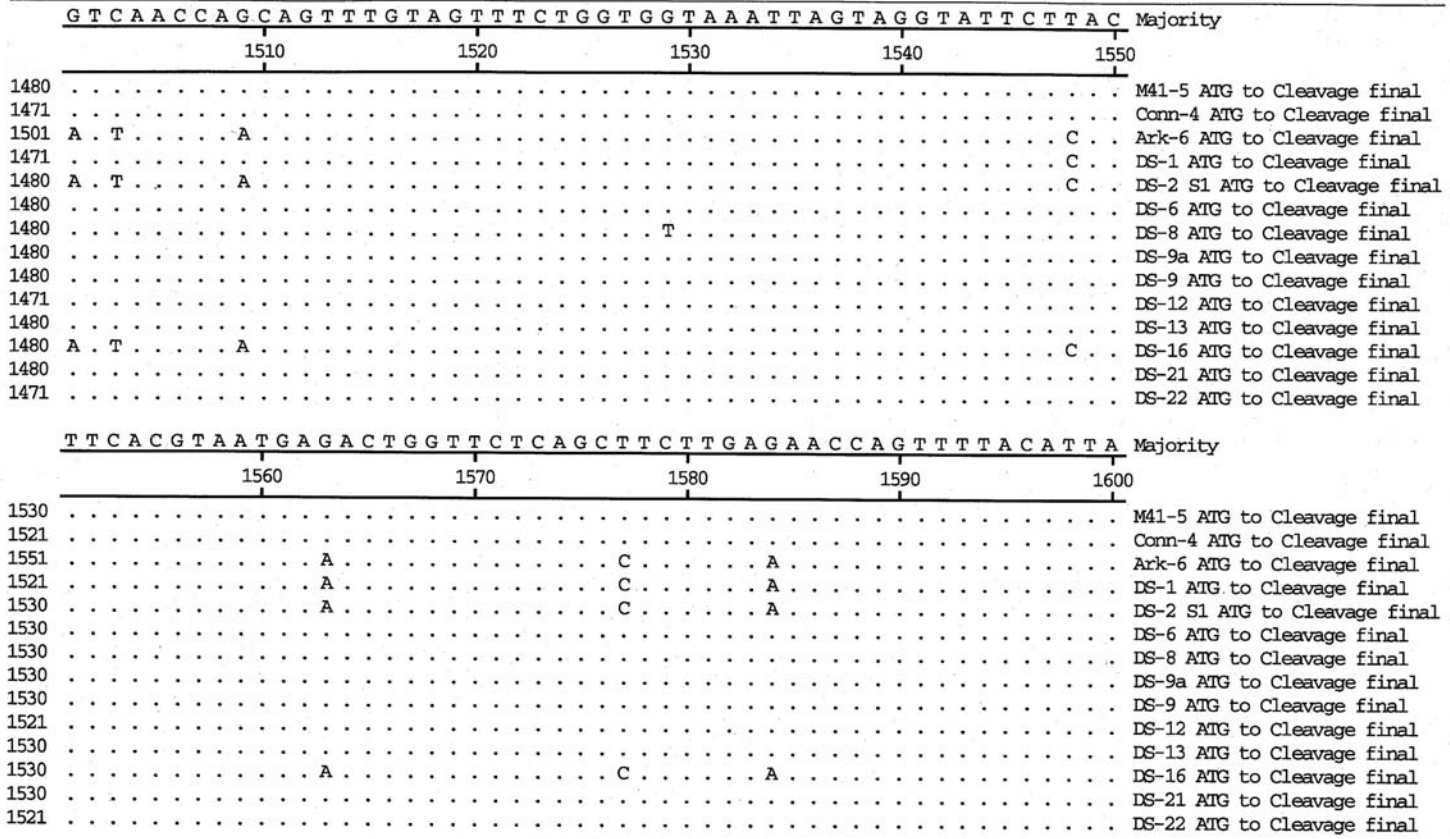

CAAGTTGTAATTTTAGACCAGAAACTATTAATAATGGCTTGTGGTTTAAT Majority
1010
1020
1030
1040
1050
980 M41-5 ATG to Cleavage final
971 Conn-4 ATG to Cleavage final
1001 Ark-6 ATG to Cleavage final
971 DS-1 ATG to Cleavage final
980 DS-2 S1 ATG to Cleavage final
980 DS-6 ATG to Cleavage final
980 DS-8 ATG to Cleavage final
980 DS-9a ATG to Cleavage final
980 DS-9 ATG to Cleavage final
971 DS-12 ATG to Cleavage final
980 DS-13 ATG to Cleavage final
980 DS-16 ATG to Cleavage final
980 DS-21 ATG to Cleavage final
971 DS-22 ATG to Cleavage final
TCACTTTTAGTTTCAATTGCTTACGGTCCTCTTCAAGGTGGTTGCAAGCA Majority
1060
1070
1080
1090
1100
1030 M41-5 ATG to Cleavage final
1021 Conn-4 ATG to Cleavage final
1051 Ark-6 ATG to Cleavage final
1021 DS-1 ATG to Cleavage final
1030 DS-2 S1 ATG to Cleavage final
1030 DS-6 ATG to Cleavage final
1030 DS-8 ATG to Cleavage final
1030 DS-9a ATG to Cleavage final
1030 DS-9 ATG to Cleavage final
1021 DS-12 ATG to Cleavage final
1030 DS-13 ATG to Cleavage final
1030 DS-16 ATG to Cleavage final
1030 DS-21 ATG to Cleavage final
1021 DS-22 ATG to Cleavage final

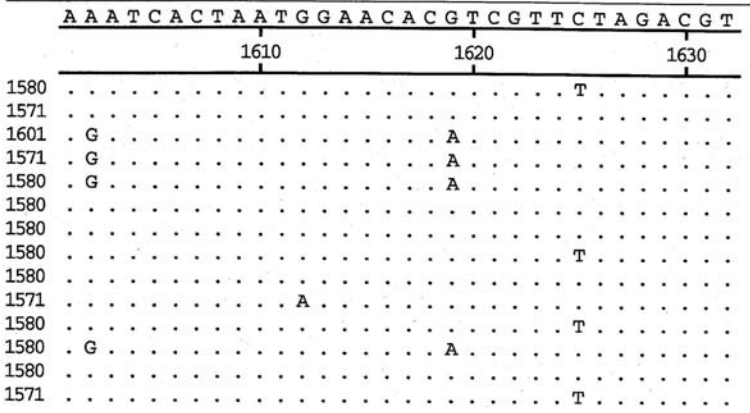

ATCTGTCTTTAGTGGTAGAGCAACTTGTTGTTATGCTTATTCATACGGAG Majority
1110
1120
1130
1140
1150
1080 M41-5 ATG to Cleavage final
1071 Conn-4 ATG to Cleavage final
1101 Ark-6 ATG to Cleavage final
1071 DS-1 ATG to Cleavage final
1080 DS-2 S1 ATG to Cleavage final
1080 DS-6 ATG to Cleavage final
1080 DS-8 ATG to Cleavage final
1080 DS-9a ATG to Cleavage final
1080 DS-9 ATG to Cleavage final
1071 DS-12 ATG to Cleavage final
1080 DS-13 ATG to Cleavage final
1080 DS-16 ATG to Cleavage final
1080 DS-21 ATG to Cleavage final
1071 DS-22 ATG to Cleavage final
GTCCTTCGTTGTGTAAGGGTGTTTATTTAGGTGAGTTAAAACGTGATTTT Majority
1160
1170
1180
1190
1200
1130 M41-5 ATG to Cleavage final
1121 Conn-4 ATG to Cleavage final
1151 Ark-6 ATG to Cleavage final
1121 DS-1 ATG to Cleavage final
1130 DS-2 S1 ATG to Cleavage final
1130 DS-6 ATG to Cleavage final
1130 DS-8 ATG to Cleavage final
1130 DS-9a ATG to Cleavage final
1130 DS-9 ATG to Cleavage final
1121 DS-12 ATG to Cleavage final
1130 DS-13 ATG to Cleavage final
1130 DS-16 ATG to Cleavage final
1130 DS-21 ATG to Cleavage final
1121 DS-22 ATG to Cleavage final


GAATGTGGACTGTTAGTTTATGTTACTAAGAGCGATGGCTCTCGTATACA Majority
1210
1220
1230
1240
1250
1180 . . . . . G . . . . . M41-5 ATG to Cleavage final
1171 . . . . . Conn-4 ATG to Cleavage final
1201 . . . . . TT . . . . . C . . . . . Ark-6 ATG to Cleavage final
1171 . . . . . G . . . . . DS-1 ATG to Cleavage final
1180 . . . . . TT . . . . . C . . . . . DS-2 S1 ATG to Cleavage final
1180 . . . . . DS-6 ATG to Cleavage final
1180 . . . . . DS-8 ATG to Cleavage final
1180 . . . . . DS-9a ATG to Cleavage final
1180 . . . . . DS-9 ATG to Cleavage final
1171 . . . . . G . . . . . DS-12 ATG to Cleavage final
1180 . . . . . G . . . . . DS-13 ATG to Cleavage final
1180 . . . . . TT . . . . . C . . . . . DS-16 ATG to Cleavage final
1180 . . . . . G . . . . . DS-21 ATG to Cleavage final
1171 . . . . . DS-22 ATG to Cleavage final
AACAGCCACTGAACCGCCAGTTATAACTCAACACAATTATAATAATATTA Majority
1260
1270
1280
1290
1300
1230 . . . . . G . . . . . M41-5 ATG to Cleavage final
1221 . . . . . Conn-4 ATG to Cleavage final
1251 . . . T . . A . . AC . . . . A . . T . . AT . . . . C . . . A . TTT . . . . . C . . C . Ark-6 ATG to Cleavage final
1221 . . . . . G . . . . . DS-1 ATG to Cleavage final
1230 . . . T . . A . . AC . . . . A . . T . . AT . . . . C . . . A . TTT . . . . . C . . C . DS-2 S1 ATG to Cleavage final
1230 . . . . . DS-6 ATG to Cleavage final
1230 . . . . . G . . . . . DS-8 ATG to Cleavage final
1230 . . . . . DS-9a ATG to Cleavage final
1230 . . . . . DS-9 ATG to Cleavage final
1221 . . . . . G . . . . . DS-12 ATG to Cleavage final
1230 . . . . . G . . . . . DS-13 ATG to Cleavage final
1230 . . . T . . A . . AC . . . . A . . T . . AT . . . . C . . . A . TTT . . . . . C . . C . DS-16 ATG to Cleavage final
1230 . . . . . G . . . . . DS-21 ATG to Cleavage final
1221 . . . . . DS-22 ATG to Cleavage final


C T T T A A A T A C T T G T G T T G A T T A T A A T A T A T A T G G C A G A A C T G G C C A A G G T Majority
1310
1320
1330
1340
1350
1280 M41-5 ATG to Cleavage final
1271 Conn-4 ATG to Cleavage final
1301 Ark-6 ATG to Cleavage final
1271 DS-1 ATG to Cleavage final
1280 DS-2 S1 ATG to Cleavage final
1280 DS-6 ATG to Cleavage final
1280 DS-8 ATG to Cleavage final
1280 DS-9a ATG to Cleavage final
1280 DS-9 ATG to Cleavage final
1271 DS-12 ATG to Cleavage final
1280 DS-13 ATG to Cleavage final
1280 DS-16 ATG to Cleavage final
1280 DS-21 ATG to Cleavage final
1271 DS-22 ATG to Cleavage final
T T T A T T A C T A A T G T A A C C G A C T C A G C T G T T A G T T A T A A T T A T C T A G C A G A Majority
1360
1370
1380
1390
1400
1330 M41-5 ATG to Cleavage final
1321 Conn-4 ATG to Cleavage final
1351 Ark-6 ATG to Cleavage final
1321 DS-1 ATG to Cleavage final
1330 DS-2 S1 ATG to Cleavage final
1330 DS-6 ATG to Cleavage final
1330 DS-8 ATG to Cleavage final
1330 DS-9a ATG to Cleavage final
1330 DS-9 ATG to Cleavage final
1321 DS-12 ATG to Cleavage final
1330 DS-13 ATG to Cleavage final
1330 DS-16 ATG to Cleavage final
1330 DS-21 ATG to Cleavage final
1321 DS-22 ATG to Cleavage final


CGCAGGTTTGGCTATTTTAGATACATCTGGTTCCATAGACATCTTTGTTG Majority
1410
1420
1430
1440
1450
1380 . . . . . M41-5 ATG to Cleavage final
1371 . . . . . A . . . . . Conn-4 ATG to Cleavage final
1401 T . G . . . A . . A . . . . . G . . . . . C . . . . Ark-6 ATG to Cleavage final
1371 . . . . . DS-1 ATG to Cleavage final
1380 T . G . . . A . . A . . . . . G . . . . . C . . . . DS-2 S1 ATG to Cleavage final
1380 . . . . . A . . . . . DS-6 ATG to Cleavage final
1380 . . . . . A . . . . . DS-8 ATG to Cleavage final
1380 . . . . . A . . . . . DS-9a ATG to Cleavage final
1380 . . . . . A . . . . . DS-9 ATG to Cleavage final
1371 . . . . . DS-12 ATG to Cleavage final
1380 . . . . . DS-13 ATG to Cleavage final
1380 T . G . . . A . . A . . . . . G . . . . . C . . . . DS-16 ATG to Cleavage final
1380 . . . . . A . . . . . DS-21 ATG to Cleavage final
1371 . . . . . DS-22 ATG to Cleavage final
TACAAGGTGAATATGGTCTTACTTATTATAAGGTTAACCCTTGCGAAGAT Majority
1460
1470
1480
1490
1500
1430 . . . . . M41-5 ATG to Cleavage final
1421 . . . . . C Conn-4 ATG to Cleavage final
1451 . . . . . C . C . . A C . . C . . . . . T . T A . . T . . . . . Ark-6 ATG to Cleavage final
1421 . . . . . DS-1 ATG to Cleavage final
1430 . . . . . C . C . . A C . . C . . . . . T . T G . . T . . . . . DS-2 S1 ATG to Cleavage final
1430 . . . . . C DS-6 ATG to Cleavage final
1430 . . . . . C DS-8 ATG to Cleavage final
1430 . . . . . C DS-9a ATG to Cleavage final
1430 . . . . . C DS-9 ATG to Cleavage final
1421 . . . . . DS-12 ATG to Cleavage final
1430 . . . . . C DS-13 ATG to Cleavage final
1430 . . . . . C . C . . A C . . C . . . . . T . T A . . T . . . . . DS-16 ATG to Cleavage final
1430 . . . . . C DS-21 ATG to Cleavage final
1421 . . . . . DS-22 ATG to Cleavage final


GTCAACCAGCAGTTTGTAGTTTCTGGTGGTAAATTAGTAGGTATTCTTAC Majority
1510
1520
1530
1540
1550
1480 M41-5 ATG to Cleavage final
1471 Conn-4 ATG to Cleavage final
1501 A.T....A.....C.. Ark-6 ATG to Cleavage final
1471 .....C.. DS-1 ATG to Cleavage final
1480 A.T....A.....C.. DS-2 S1 ATG to Cleavage final
1480 DS-6 ATG to Cleavage final
1480 .....T..... DS-8 ATG to Cleavage final
1480 DS-9a ATG to Cleavage final
1480 DS-9 ATG to Cleavage final
1471 DS-12 ATG to Cleavage final
1480 DS-13 ATG to Cleavage final
1480 A.T....A.....C.. DS-16 ATG to Cleavage final
1480 DS-21 ATG to Cleavage final
1471 DS-22 ATG to Cleavage final
TTCACGTAATGAGACTGGTTCTCAGCTTCTTGAGAACCAGTTTTACATTA Majority
1560
1570
1580
1590
1600
1530 M41-5 ATG to Cleavage final
1521 Conn-4 ATG to Cleavage final
1551 ...A.....C.....A..... Ark-6 ATG to Cleavage final
1521 ...A.....C.....A..... DS-1 ATG to Cleavage final
1530 ...A.....C.....A..... DS-2 S1 ATG to Cleavage final
1530 DS-6 ATG to Cleavage final
1530 DS-8 ATG to Cleavage final
1530 DS-9a ATG to Cleavage final
1530 DS-9 ATG to Cleavage final
1521 DS-12 ATG to Cleavage final
1530 DS-13 ATG to Cleavage final
1530 ...A.....C.....A..... DS-16 ATG to Cleavage final
1530 DS-21 ATG to Cleavage final
1521 DS-22 ATG to Cleavage final



Majority

M41-5 ATG to Cleavage final
Conn-4 ATG to Cleavage final
Ark-6 ATG to Cleavage final
DS-1 ATG to Cleavage final
DS-2 S1 ATG to Cleavage final
DS-6 ATG to Cleavage final
DS-8 ATG to Cleavage final
DS-9a ATG to Cleavage final
DS-9 ATG to Cleavage final
DS-12 ATG to Cleavage final
DS-13 ATG to Cleavage final
DS-16 ATG to Cleavage final
DS-21 ATG to Cleavage final
DS-22 ATG to Cleavage final

Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

CHAPTER 5

RAPID DIFFERENTIATION OF AVIAN INFECTIOUS BRONCHITIS VIRUS ISOLATES BY SAMPLE TO RESIDUAL RATIO QUANTIFICATION USING REAL-TIME REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION[1]

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[1]Callison, S.A., D.A. Hilt, M.W. Jackwood. To be submitted to the *Journal of Clinical Microbiology*.

Abstract

We developed a rapid diagnostic test for differentiating infectious bronchitis virus (IBV) isolates. The basis of the test is the cleavage of target RNA by RNase H mediated by sequence specific chimeric oligonucleotides followed by Sample to Residual Ratio Quantification (SRRQ) using RRT-PCR. We designed serotype specific chimeric oligonucleotides, one each for the Massachusetts, Connecticut, Arkansas, and Delaware/Georgia 98 serotypes and tested their ability to mediate cleavage of target RNA by RNase H. The specificity of each chimeric oligonucleotide was tested against homologous and heterologous strains of IBV. Our data showed that each chimeric oligonucleotide mediated cleavage of target RNA only from strains within the serotype that the chimeric was designed against. To validate the test, we performed a test on 15 samples without prior knowledge of their type. Our results correctly determined the serotype of each sample containing an IBV from within the serotypes for which a chimeric oligonucleotide was developed. We believe this assay will find future use as a rapid diagnostic method for determining the serotype of IBV isolates, a method for determining the purity of vaccines stocks, and should be applicable to any situation where determining the presence or absence of any target RNA is desired.

Introduction

Infectious bronchitis (IB) is a viral infection of the upper respiratory tract in chickens. It is of economic significance to the poultry industry due to the high morbidity and production losses associated with the disease. Clinical signs include tracheal rales, coughing, sneezing, poor weight gain in broilers, and a decline in egg-shell quality and egg quantity in layers. In addition, birds become susceptible to secondary pathogens, such as *E. coli* [1].

The etiologic agent of IB is infectious bronchitis virus (IBV). The virus belongs to the *Coronaviridae* family and Coronavirus genus. It is a positive-sense RNA virus with an envelope containing protruding spikes. These spikes mediate attachment to host cell receptors, are involved in membrane fusion, and contain conformationally dependent, serotype specific, and virus neutralizing epitopes [2-4].

There is no specific treatment for IBV. Prevention and control are through the use of modified live or killed vaccines. The vaccination strategy is dependent upon the serotype of IBV prevalent within a geographical area. Rapid and accurate diagnosis of the serotype of IBV involved in a field outbreak is necessary to establish an effective vaccine strategy for neighboring flocks.

Great strides have been made in the area of IBV diagnostics since the gold standard virus neutralization test was developed. Currently, many diagnostic tests exist for differentiating IBV isolates, including: monoclonal antibodies [5, 6] , dot blots [7], DNA probes [8, 9], rapid plate hemagglutination test [10], SDS-PAGE polymorphism [11], direct S1 gene DNA sequencing [12, 13], RT-PCR with serotype specific primers [14], and RT-PCR/RFLP [9, 15].

It has been shown that genotyping tests provide data that correlates well with the serotype of the virus [9, 12-14]. Due to their ease and correlation with serotype, genotype tests (DNA sequencing, serotype specific RT-PCR, and RT-PCR/RFLP) have become widely used. Although the genotyping tests are rapid, room for improvement remains. In particular, elimination of post RT-PCR manipulations (gel electrophoresis, gel purification of DNA, restriction enzyme analysis, sequencing, etc.) would decrease the cost and overall assay time. Recent technological advances in the field of real-time nucleic acid amplification could be used to negate the need for post RT-PCR manipulations.

Real-time RT-PCR (RRT-PCR) is a powerful technique that can determine the amount and identity of a target RNA template in a biological sample. In general, there are two methods for detecting the amplification of target RNA in a sample. The first method employs a non-specific dsDNA binding dye, such as SYBR Green I. When the dye binds to double stranded DNA, its inherit fluorescence is greatly enhanced and can be measured. Although the dye is not useful in determining the presence of a specific sequence within the target template, it can be effectively used to quantify (Quantification analysis) and identify (Melting curve) target templates [16]. The second method employs fluorescently labeled DNA probes (singly or doubly labeled). The probes hybridize to certain sequences if present in the target template and hybridization detection can occur in any number of ways [17-19]. This method can be used to quantify and identify specific sequences within target templates in a biological sample. For the basics of RRT-PCR and the myriad dye/probe based detection techniques, see the review by Mackay et al [20].

We developed a new method of real time RT-PCR termed Sample to Residual Ratio Quantification (SRRQ) that determines the presence or absence of a particular target RNA molecule within a biological sample. In the most simplified case, RNA is extracted from a biological sample, incubated in the presence of a complementary chimeric oligonucleotide (5'-DNA:2-O-Me-RNA-3'), and cleaved by RNase H at the site of hybridization [21, 22]. The total amount of RNA in the sample (uncleaved) and the amount of residual RNA following cleavage by RNase H is quantified by RRT-PCR. Then, the ratio of total RNA to cleaved RNA is calculated to determine the specificity of the chimeric oligonucleotide for the target RNA. In this way, specific chimeric oligonucleotides for each serotype of IBV can be used to type the virus. Herein, we report the use of SRRQ to differentiate IBV isolates belonging to one of four different serotypes (Fig. 1).

Materials and Methods

Virus strains. The virus strains used in this study are listed in Table 1. Briefly, virus was propagated in 9-11 day old embryonating eggs and the allantoic fluid was harvested 48 hours after inoculation and kept frozen at –70º C until needed [23].

RNA extraction. The High Pure RNA Isolation Kit (Roche Diagnostics Corp., Indianapolis, IN) was used to extract viral RNA from allantoic fluid per the manufacturer's directions.

Chimeric oligo design. Briefly, S1 gene sequence data for strains belonging to the Massachusetts, Arkansas, Connecticut, and Delaware/Georgia 98 serotypes were aligned using MacDNASIS Pro V3.5 (Hitachi Software Engineering Corp., San Bruno, CA). Conserved sequences within each serotype but not present in other serotypes were

identified. Complimentary serotype specific chimeric oligonucleotides [24, 25] to those conserved sequences with Tm values near 37º C were designed and synthesized (Integrated DNA Technologies, Inc., Coralville, IA).

Cloning of S1 genes. The S1 gene from the Massachusetts 41, Connecticut 46, Arkansas DPI, and Delaware 072 strains of IBV was cloned using standard molecular biology procedures. Briefly, the S1 gene from each virus was amplified by RT-PCR as previously described [26]. The resultant 1,720 bp amplicon was cloned into TOPO-XL (Invitrogen Inc., Carlsbad, CA) per the manufacturer's directions.

Preparation of RNA from cloned S1 genes. Runoff RNA transcripts were synthesized from each of the cloned S1 genes after linearization with Mlu I (New England Biolabs, Inc., Beverly, MA) using the T7 RiboMax Kit (Promega, Madison, WI) per the manufacturer's directions.

Cleavage of runoff RNA transcripts and agarose gel analysis. Four separate 20 microliter reactions containing 5 picomoles of one particular runoff RNA, 5 picomoles of one serotype specific chimeric oligonucleotide per tube, 1X RNase H reaction buffer and water up to 20 microliters were mixed in 0.2 mL PCR tubes. The mixtures were incubated at 70º C for 5 minutes and then cooled to 37º C, at which time 5 units of RNase H (New England Biolabs, Inc., Beverly, MA) were added. After a 1 hour incubation, the RNA from each tube was extracted as stated earlier. For agarose gel analysis, 5 microliters of extracted RNA was mixed with 5 microliters of Tris Borate EDTA(TBE)-Urea loading buffer (Biorad, Hercules, CA), heated at 95º C for 4 minutes, quick cooled on ice, and loaded onto a native 1X TBE agarose gel [27]. The RNA was electrophoresed at 80 volts (constant voltage) for approximately 1 hour, stained with

EtBr, visualized by UV transillumination and photographed using a Kodak EDAS 290 system (Eastman Kodak Co., Rochester, NY).

Sample RNA cleavage reaction for RRT-PCR analysis. Five microliters of RNA extracted from allantoic fluid was placed into each of five separate tubes, each containing 1X RNase H reaction buffer, 5 picomoles of one of 4 different serotype specific chimeric oligonucleotide (the fifth control tube received no chimeric oligo) and water up to 20 microliters. The tubes were incubate at 70º C for 5 minutes and then cooled to 37º C, at which time 5 units of RNase H were added. After a 1 hour incubation, the RNA from each tube was extracted as stated earlier and kept on ice until used as template in a RRT-PCR.

RRT-PCR. A set of RRT-PCR primers were synthesized that amplify a section of the IBV S1 gene containing the entire hypervariable region (HVR) I and most of HVR II. The 5' primer was NewS1Oligo5' [26] and the 3' primer (5'-AYMACARTGTGTMACAAA-3') was designated M41L328.

RRT-PCRs were assembled using a LightCycler™ RNA Amplification Kit (Roche Diagnostics Corp., Indianapolis, IN). Briefly, 20 microliter reactions were assembled, each containing 1X Reaction/SYBR Green I buffer mix, 5mM $MgCl_2$, 20 picomoles of each forward and reverse primer, 0.4 microliters of the polymerase enzyme mix, 10 microliters of template RNA and water up to 20 microliters. The reaction mixture was centrifuged into a LightCycler™ glass capillary tube and the RRT-PCR was performed per the manufacturer's directions. Briefly, the capillaries were subjected to the following thermocycle program: 42 C – 10 minutes; 95 C – 30 seconds; 45 cycles of 95 C – 0 seconds, 45 C – 10 seconds, 72 C – 20 seconds (Analysis mode: Quantification);

1 cycle of 95 C – 0 seconds, 65 C – 10 seconds, 95 C – 0 seconds (Analysis mode: Melting curve); and 40 C – 30 seconds. Quantification and melting curve analysis of each sample was conducted using the tools available in the LightCycler software version 3.5. The second derivative maximum option was used to calculate all crossing points to rule out investigator bias. Any experimental tube with a crossing point 2 cycles higher when compared to the control was considered to be susceptible to cleavage as mediated by that particular chimeric oligonucleotide.

Results

We designed four chimeric oligonucleotides, each complementary to a different serotype specific sequence within the S1 gene of IBV (Fig 2). Agarose gel analysis of cleavage products showed that each chimeric oligonucleotide specifically cleaved runoff RNAs from the homologous S1 gene, while not cleaving runoff RNAs from heterologous S1 genes. Figure 3 shows a representative agarose gel analysis of Massachusetts 41 S1 runoff RNA as cleaved after incubation with each chimeric oligonucleotide and RNase H. Cleavage analysis results for the Arkansas DPI, Connecticut 46, and Delaware 072 S1 runoff RNAs were similar (data not shown). In each case, specific cleavage of the S1 gene runoff RNA produced the correct size cleavage products as predicted from sequence data (see arrow, Fig. 3). There were no non-specific cleavage products for any of the chimeric oligonucleotides tested as determined by agarose gel analysis.

To further assess the specificity of each chimeric oligonucleotide, we tested their ability to cleave sample RNA extracted from 12 IBV strains representing 8 different serotypes. We performed SRRQ for each sample and the results of our analysis are summarized in Table 2. Briefly, all four chimeric oligonucleotides mediated the cleavage

of RNA extracted from strains of IBV in the appropriate serotype. No cross reactivity was observed for any of the chimeric oligonucleotides with strains from a heterologous serotype. Representative amplification graphs for Arkansas 99 and CAV 56b are shown in figure 4. The Arkansas 99 strain RNA was cleaved in the tube containing the Arkansas anti chimeric oligonucleotide (Fig. 4a) and RNA from the CAV 56b strain, which does not belong to any of the four serotypes for which a chimeric oligonucleotide was synthesized, was not cleaved (Fig. 4b). For some IBV strains not experimentally tested (Table 1), we examined the S1 gene sequence flanked by the RRT-PCR primer set, and no complementary regions to any of the chimeric oligonucleotides were present.

To validate our assay, we performed the SRRQ test on 15 allantoic fluid samples without prior knowledge to their grouping and found that we could accurately identify Massachusetts, Arkansas, Connecticut, and Delaware viruses in those samples (Table 3). Samples that showed no cleavage with any of the chimeric oligonucleotides or that were non-amplifiable with the primer set were listed as non-typeable. Each non-typeable strain was either an IBV from a serotype for which a chimeric oligonucleotide was not developed or an altogether different virus (Table 3).

Discussion

We have developed a rapid diagnostic test to identify IBV isolates within the Massachusetts, Arkansas, Connecticut, and Delaware/Georgia 98 serotypes. One chimeric oligonucleotide (Delaware) was designed to mediate cleavage of strains from the Delaware and Georgia 98 serotypes because it has been shown that strains from these two serotypes cross protect in birds [28]. The test relies on two things 1) the ability of sequence specific chimeric oligonucleotides to mediate cleavage of target RNA by RNase

H and 2) measurement of that cleavage by SRRQ using RRT-PCR. Based on this information, we differentiate the IBV isolate. Empirically, we assigned a difference of 2 cycles between uncleaved control RNA samples and cleaved RNA samples as the minimum for determining the specificity of the chimeric oligonucleotides (Fig. 5).

We only designed chimeric oligonucleotides for five of the most common serotypes of IBV. Thus, further characterization of non-typeable samples by RT-PCR/RFLP or DNA sequencing is required until other chimeric oligonucleotides are developed. It is interesting that we could not detect the presence of GAV92, an Ark-like strain of IBV. The complementary sequence for the Arkansas anti chimeric oligonucleotide differs by two bases at the 5' end of the chimeric oligonucleotide for the GAV92 strain (Fig. 2). Hence, the Arkansas chimeric oligonucleotide cannot mediate cleavage of this RNA. It might be necessary to design chimeric oligonucleotides specific for some unique strains of IBV.

Another limitation of this test may be its ability to diagnose dual infections. If a sample contains two IBVs in equal amounts, a chimeric oligonucleotide specific for either virus could only cleave half of the RNA within the sample. This would equate to a crossing point difference of only one. Obviously, improvements upon the variability of the test should allow for us to lower the crossing point difference number to a level where dual infections can be diagnosed with a high degree of confidence. A couple of test refinements could be 1) skipping the reextraction of the RNA after the cleavage reaction and 2) modifying the RT-PCR primers such that the RNase H cleavage reaction can be done in RT-PCR buffer. Not only would these refinements decease test variability, but

they would also decrease the time and cost of the test. Further effort to implement these improvements is on going.

We have observed that the starting RNA template concentration must be high enough so that the amplification reaction reaches the exponential phase by approximately 30 cycles. Reactions where the exponential phase occurs beyond 30 cycles are difficult to interpret because the primer dimer signal begins to mask the true product signal.

This IBV typing test is extremely fast. One RRT-PCR machine that can process 32 reactions is enough to type 6 samples in 3-4 hours or less. Future improvements could decrease the time to less than 2 hours. Also, this test does not require the use of fluorescently labeled probes, which can be expensive and generally have a short shelf life.

Another advantage of this test is that it has a built in control for detecting the presence or absence of IBV in each sample. The primer set developed for this test amplifies all IBV RNA's tested to date. Since an uncut control is always run for each sample, a positive reaction always denotes the presence of IBV, whether it is typeable by our test or not.

Finally, this test can be run without a RRT-PCR machine. The first step is to perform a regular RT-PCR on a particular sample to determine where the exponential phase of the amplification reaction occurs. Briefly, a sample of RNA is amplified by RT-PCR and aliquots are taken from the reaction every 5 – 10 cycles and separated on an agarose gel. The second aliquot showing the presence of a clear band of the proper size is then used as the number of total PCR cycles for the appropriate sample during the assay. Once the number of PCR cycles is determined for a particular sample, the

chimeric oligonucleotide cleavage reaction is run as stated, RNA is extracted, and used as template in a RT-PCR using the number of appropriate PCR cycles determined earlier. The reaction products are separated on an agarose gel and the intensity of the products is visualized. Any tube that contained chimeric oligonucleotide specific for the sample RNA will produce a product that is weaker than the uncut control. Using this information, determination of the sample serotype is possible. Although trickier and not as clear-cut, an experienced laboratory technician should be able to perform the test properly. The ability to use agarose gel analysis is a huge advantage of this test, since some laboratories lack sufficient funds to obtain expensive RRT-PCR machines. Thus, any lab that has a PCR machine and gel electrophoresis equipment can perform the test.

Future use of this assay as a rapid diagnostic method for determining the serotype of IBV isolates seems plausible. Although our goal was to create an assay for that purpose, it seems logical that this method could have other uses. For IBV, it could certainly find use as a tool for testing the purity of vaccine stocks. Due to the quasispecies of IBV and the ease of contamination of vaccine strains during manipulation, a test that can determine the level of purity for a particular sample should be helpful. Also, outside the realm of virology, this concept should be applicable to any situation where determining the presence or absence of any target RNA is desired.

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Figure 5.1. Diagram of Sample to Residual Ratio Quantification using RRT-PCR. Briefly, RNA is extracted from a biological sample, split into an appropriate number of tubes (Step 1), incubated in the presence of a complementary chimeric oligonucleotide (5'-DNA:2-O-Me-RNA-3'), and cleaved by RNase H at the site of hybridization (Step 2). The total amount of RNA in the sample (uncleaved) and the amount of residual RNA following cleavage by RNase H is quantified by RRT-PCR (Step 3). Then, the ratio of total RNA to cleaved RNA is calculated to determine the specificity of the chimeric oligonucleotide for the target RNA. In this way, specific chimeric oligonucleotides for each serotype of IBV can be used to type the virus.

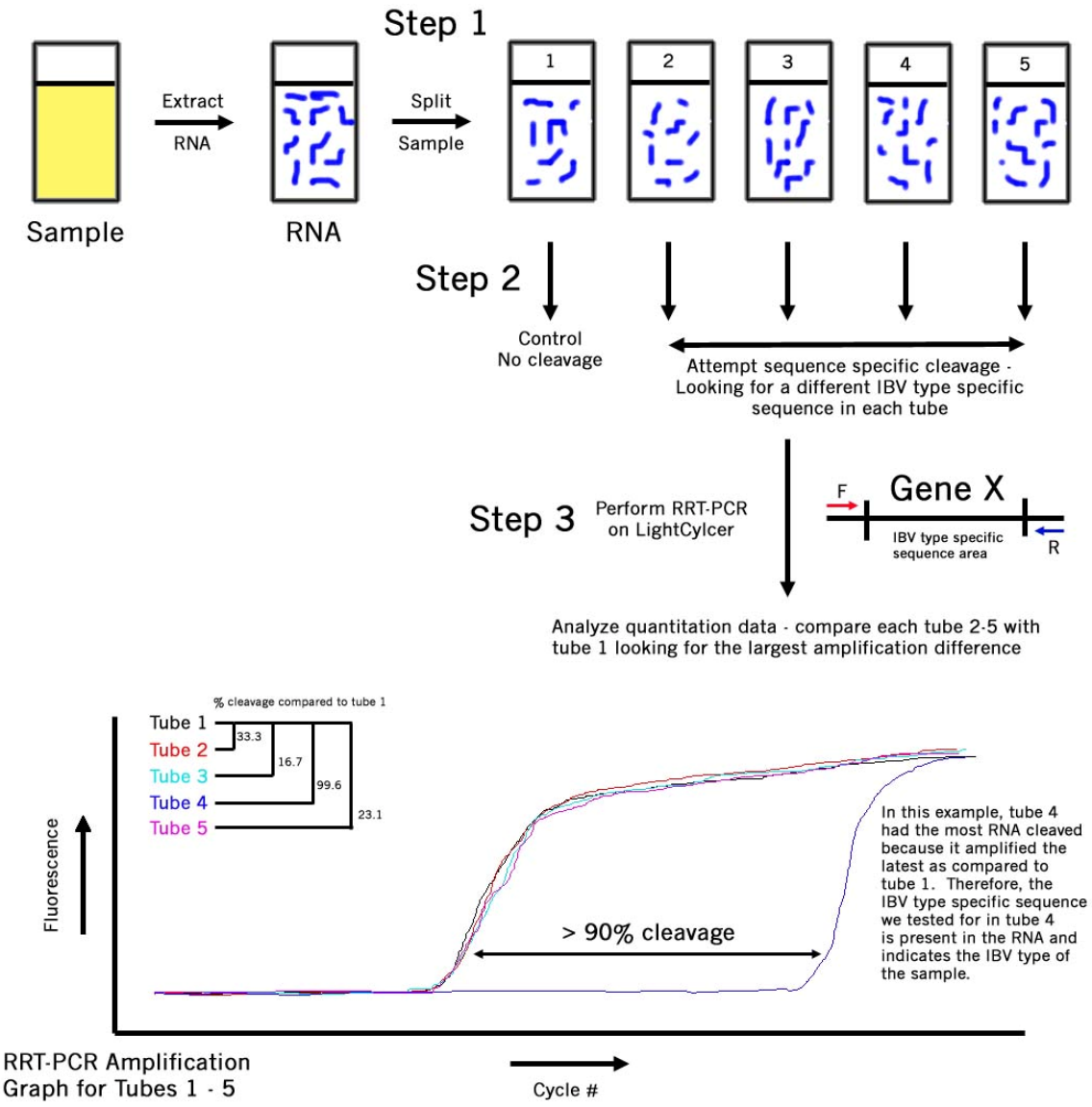

Step 1
Extract
RNA
Split
Sample
1
2
3
4
5
Sample
RNA
Step 2
Control
No cleavage
Attempt sequence specific cleavage -
Looking for a different IBV type specific
sequence in each tube
Step 3
Perform RRT-PCR
on LightCylcer
F
Gene X
IBV type specific
sequence area
R
Analyze quantitation data - compare each tube 2-5 with
tube 1 looking for the largest amplification difference
% cleavage compared to tube 1
Tube 1
Tube 2
Tube 3
Tube 4
Tube 5
33.3
16.7
99.6
23.1
Fluorescence
> 90% cleavage
In this example, tube 4
had the most RNA cleaved
because it amplified the
latest as compared to
tube 1. Therefore, the
IBV type specific sequence
we tested for in tube 4
is present in the RNA and
indicates the IBV type of
the sample.
RRT-PCR Amplification
Graph for Tubes 1 - 5
Cycle #

Table 5.1. Virus strains used in this study.

Strain	Serotype	Assayed[a]	Not assayed[b]	Accession #
Massachusetts 41	Massachusetts	X		M21883
Beaudette	Massachusetts	X		M95169
H52	Massachusetts		X	AF352315
H120	Massachusetts		X	M21970
Connecticut 46	Connecticut	X		L18990
Florida 18288	Florida	X		AF027512
Arkansas DPI	Arkansas	X		AF006624
Arkansas 99	Arkansas	X		L10384
3668-4	Arkansas	X		AF095702
GAV 92	Arkansas	X		U16157
CU-T2	Arkansas		X	U04739
Ark-like 4207	Arkansas		X	M. Jackwood
Delaware 072	Delaware 072	X		AF274435
GA/0470/98	GA 98	X		AF274437
CAV 56b	California variant	X		AF027509
15172C	Nebraska 95	X		M. Jackwood
15259	Nebraska 95	X		M. Jackwood
Gray	Gray		X	L14069
Holte	Gray		X	L18988
Iowa 609	Iowa		X	E. Collisson
JMK	JMK		X	L14070
PP14	PP14		X	M99483
SE17	SE17		X	M99484
4/91	793/B		X	AF093793

a = strains that were assayed by SRRQ using RRT-PCR
b = strains for which the first 300 bp of S1 gene were searched for sequences complimentary to any of the chimeric oligonucleotides instead of performing SRRQ using RRT-PCR assay

Figure 5.2. Design and synthesis of serotype specific chimeric oligonucleotides. A. S1 gene sequence alignments of IBV strains belonging to the Massachusetts and Arkansas serotypes were performed for the region flanked by the primer set NewS1OLIGO5'/M41L328. Only nucleotides 42-204 (ATG start site = 1) are shown. The unique serotype specific regions to which a complimentary chimeric oligonucleotide was synthesized are boxed (Red = Arkansas, Blue = Massachusetts). B. The structure of a representative chimeric oligonucleotide hybridized to a complimentary strand of RNA. Sequences in green are 2-O-Me RNA bases and sequences in purple are DNA bases. The arrow denotes the site at which RNase H will cleave the strand of RNA [21,22].

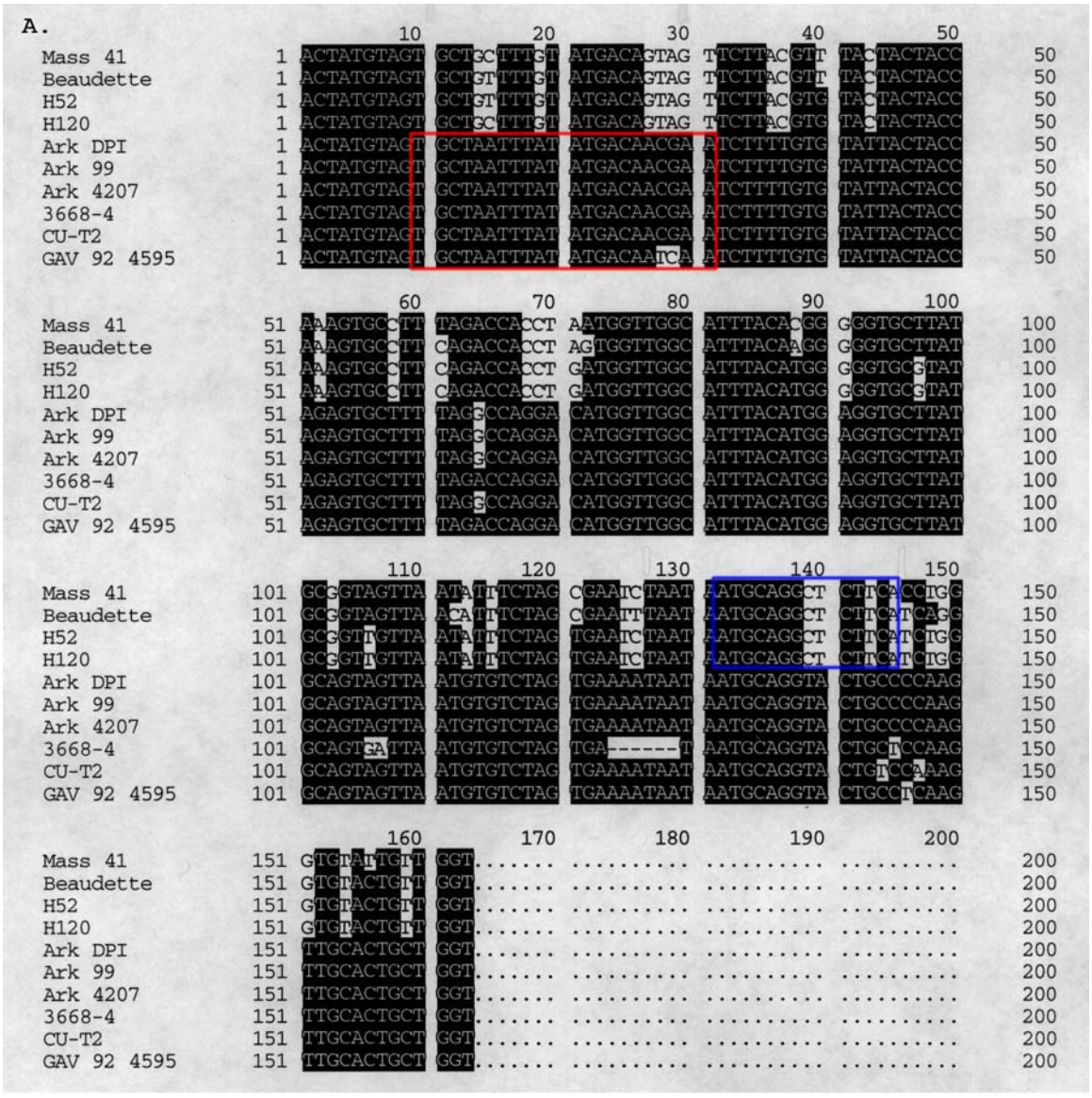

A.
Mass 41
Beaudette
H52
H120
Ark DPI
Ark 99
Ark 4207
3668-4
CU-T2
GAV 92 4595
B.
5'-
GCAUAGCAUGACAU
-3'
3'-CGUAUCGUACTGTA-5'

Figure 5.3. Native agarose gel analysis of Massachusetts 41 S1 runoff RNA cleavage as mediated by chimeric oligonucleotides specific for strains in the Massachusetts, Arkansas, Connecticut, and Delaware/Georgia 98 serotypes. Lane 1 = RNA ladder, sizes from top to bottom are 9000, 7000, 5000, 3000, 2000, 1000, and 500 bases (New England Biolabs, Beverly, MA); Lane 2 = Uncleaved Massachusetts 41 S1 runoff RNA; Lane 3 = Massachusetts 41 S1 runoff RNA incubated with Massachusetts anti and RNase H; Lane 4 = Same as lane 3 except, Arkansas anti used; Lane 5 = Same as lane 3, except Connecticut anti used; Lane 6 = Same as lane 3, except Delaware anti used. Arrows indicate cleavage products of ~ 1500 and 300 bases.

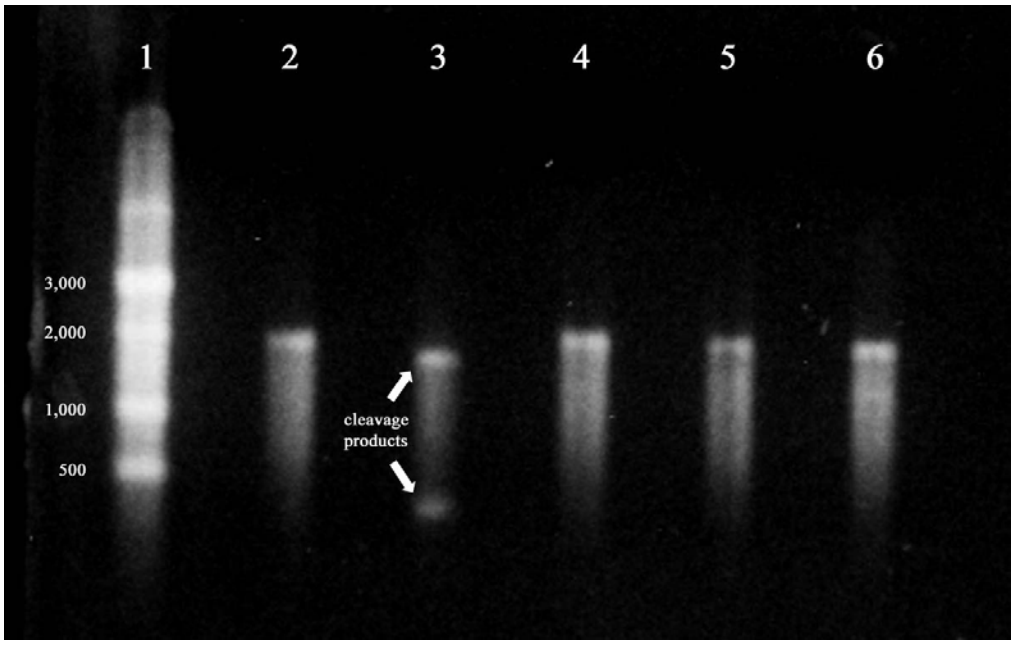

1
2
3
4
5
6
3,000
2,000
1,000
500
cleavage
products

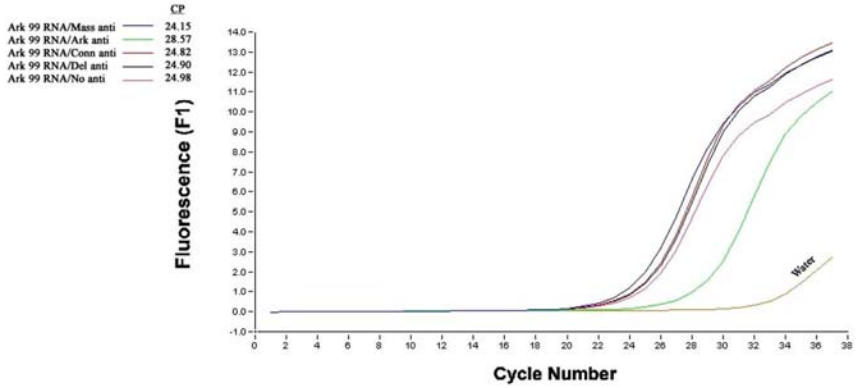
Table 5.2. Sample to Residual Ratio Quantification using RRT-PCR results for known IBV strains.

Strain	Serotype[a]	Anti Massachusetts	Anti Arkansas	Anti Connecticut	Anti Delaware	Assay serotype
Massachusetts 41	Massachusetts	+[b]	-[c]	-	-	Massachusetts
Beaudette	Massachusetts	+	-	-	-	Massachusetts
Connecticut 46	Connecticut	-	-	+	-	Connecticut
Florida 18288	Florida	-	-	-	-	Not typeable
Arkansas DPI	Arkansas	-	+	-	-	Arkansas
Arkansas 99	Arkansas	-	+	-	-	Arkansas
3668-4	Arkansas	-	+	-	-	Arkansas
GAV 92	Arkansas	-	-	-	-	Not typeable
Delaware 072	Delaware	-	-	-	+	Delaware
0470	GA98	-	-	-	+	Delaware
CAV 56b	California Variant	-	-	-	-	Not typeable
15172 C	Nebraska 95	-	-	-	-	Not typeable

a = serotype as determined by virus neutralization or RT-PCR/RFLP
b = a plus sign denotes that the sample RNA incubated with the particular chimeric oligonucleotide amplified at least 2 cycles later as compared with the uncleaved control RNA
c = a negative sign denotes that the sample RNA incubated with the particular chimeric oligonucleotide amplified no later than 1.99 cycles as compared with the uncleaved control RNA

Figure 5.4. Representative Sample to Residual Ratio Quantification (SRRQ) amplification graphs and data analysis. A. SRRQ for IBV strain Arkansas 99. The crossing point for each tube was calculated using the second derivative maximum option. Only the RNA incubated with the Arkansas anti chimeric oligonucleotide had a crossing point > 2 cycles as compared to the uncleaved control. Therefore, we determined the RNA to be from an IBV in the Arkansas serotype. B. SRRQ for IBV strain CAV 56b. The crossing point for each tube was calculated using the second derivative maximum option. No RNA incubated with any of the chimeric oligonucleotides had a crossing point > 2 cycles as compared to the uncleaved control. Therefore, we determined the RNA to be from a non-typeable IBV serotype.

A.


CP
Ark 99 RNA/Mass anti 24.15
Ark 99 RNA/Ark anti 28.57
Ark 99 RNA/Conn anti 24.82
Ark 99 RNA/Del anti 24.90
Ark 99 RNA/No anti 24.98
Fluorescence (F1)
Cycle Number
Water

B.

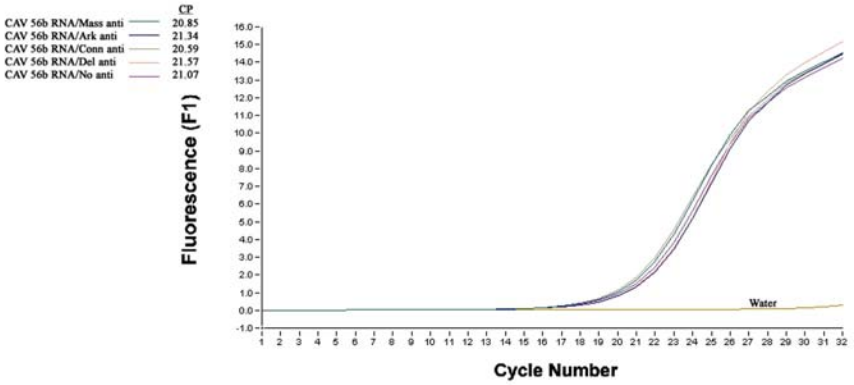
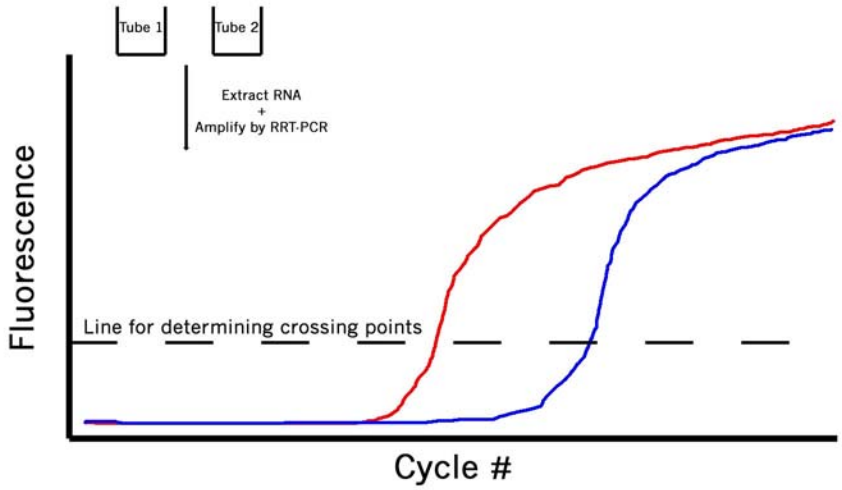

CP
CAV 56b RNA/Mass anti 20.85
CAV 56b RNA/Ark anti 21.34
CAV 56b RNA/Conn anti 20.59
CAV 56b RNA/Del anti 21.57
CAV 56b RNA/No anti 21.07
Fluorescence (F1)
Cycle Number
Water

Table 5.3. Sample to Residual Ratio Quantification using RRT-PCR results for 15 samples tested without prior knowledge.

Sample	Strain	Serotype[a]	Anti Massachusetts	Anti Arkansas	Anti Connecticut	Anti Delaware/Georgia 98	Assay serotype
1	15259	Nebraska 95	-[b]	-	-	-	Not typeable
2	CAV 56b	California Variant	-	-	-	-	Not typeable
3	De 072	Delaware	-	-	-	+[c]	Delaware/GA98
4	GAV 4595	Arkansas	-	-	-	-	Not typeable
5	Massachusetts 41	Massachusetts	+	-	-	-	Massachusetts
6	Arkansas 99	Arkansas	-	+	-	-	Arkansas
7	Florida 18288	Florida	-	-	-	-	Not typeable
8	Beaudette	Massachusetts	+	-	-	-	Massachusetts
9	GA/0470/98	Georgia 98	-	-	-	+	Delaware/GA98
10	Connecticut 46	Connecticut	-	-	+	-	Connecticut
11	Arkansas DPI	Arkansas	-	+	-	-	Arkansas
12	Connecticut 46	Connecticut	-	-	+	-	Connecticut
13	De 072	Delaware	-	-	-	+	Delaware/GA98
14	TCV	Turkey coronavirus	-	-	-	-	Not typeable
15	Massachusetts 41	Massachusetts	+	-	-	-	Massachusetts

a = serotype as determined by virus neutralization or RT-PCR/RFLP
b = a negative sign denotes that the sample RNA incubated with the particular chimeric oligonucleotide amplified no later than 1.99 cycles as compared with the uncleaved control RNA
c = a plus sign denotes that the sample RNA incubated with the particular chimeric oligonucleotide amplified at least 2 cycles later as compared with the uncleaved control RNA

Figure 5.5. Sample to Residual Ratio Quantification math. For any two tubes containing the same target nucleic acid in different amounts, the ratio of starting template between the two samples can be determined. Basically, a crossing point for each tube is obtained by RRT-PCR. Then, the crossing point difference between the two tubes is calculated. Using this calculation, a ratio of starting template for tube 1:tube 2 can be established using the following formula: $T_1:T_2 = 2^{cpd}:1$ In this example, the crossing point of tube 1 (uncleaved sample RNA) was 15 and the crossing point for tube 2 (sample RNA cleaved by chimeric oligonucleotide and RNase H) was 18.3. Using the above formula, the ratio of starting template for tube 1:tube 2 was 10:1. This ratio means that there was 90% less starting RNA template in tube 2, i.e. it was cleaved by RNase H due to hybridization of RNA with a sequence specific chimeric oligonucleotide.



	CP
Tube 1	15.0
Tube 2	18.3

Ratio of T1:T2 = 2^{cpd}:1

Ratio of T1:T2 = $2^{3.3}$:1

Ratio of T1:T2 = 10:1

CHAPTER 6

DISCUSSION

Development of a hammerhead ribozyme against IBV

Hammerhead ribozymes are catalytic RNA molecules capable of catalyzing the cleavage of target RNA molecules *in trans*. This property makes hammerhead ribozymes attractive candidates for development as antiviral molecules. We designed a hammerhead ribozyme targeted to the nucleocapsid gene of the IBV genome. We used MFOLD, a computer program that uses thermodynamic algorithms to predict the secondary structure of RNA sequences, to choose our target site. A region with a high probability of single strandedness was determined and we synthesized a complementary hammerhead ribozyme to the region. Cloned nucleocapsid gene from the Massachusetts 41 strain of IBV was used to synthesize runoff RNAs. Specific cleavage of this nucleocapsid RNA was demonstrated by agarose gel analysis and real-time RT-PCR.

We reasoned that targeting and cleaving the nucleocapsid gene might have an antiviral affect on IBV infection. The nucleocapsid protein may be necessary for replication of the viral genome because it appears to hinder viral replication when not present. Also, the target site of our hammerhead ribozyme is within all the IBV mRNAs due to their 3' coterminal nested set arrangement. Its been hypothesized that sequences within the 3' end of the full-length RNA genome might be required for efficient packaging of the genome into new virions, so cleaving these 3' end sequences from the full-length genome might decrease the efficiency of new virion development. As yet, we

have not tested the ability of our hammerhead ribozyme to hinder IBV replication in a cell culture model because we lack a cell culture adapted Massachusetts 41 strain, but current work is proceeding towards that goal.

Creating novel IBV S1 genes

Currently, a cross protective vaccine for IBV does not exist. We lack the ability to rationally design a spike gene that would encode a spike glycoprotein with cross protective properties. Little is known about sequences that form neutralization and serotype specific epitopes. Studies with monoclonal antibody neutralization escape mutants have identified spike gene regions and a few specific amino acids involved in epitope formation, but much remains unknown. Therefore, a new strategy for developing novel spike genes with a desired property is needed that does not require knowing a lot of information beforehand.

DNA shuffling is a concept that can be used to produce novel genes with desired properties without prior knowledge of how to produce the gene. We used the concept of DNA shuffling as a way to create novel S1 genes for IBV that might encode cross protective spike glycoproteins. In particular, we shuffled the S1 genes from four different strains of IBV that represented four different serotypes of IBV using the staggered extension process. We cloned the shuffled S1 genes and characterized 25 random clones by DNA sequencing. In total, 11 clones were recombinants that contained an average of 5 crossovers and 1.3 point mutations. Each clone was unique and maintained a full-length open reading frame. We were unable to produce any recombinants that contained sequences from all four of the parental genes; therefore, StEP parameters may need to be adjusted to promote more recombination. We envision

that a shuffled IBV S1 gene library will be helpful in epitope mapping studies and in the production of novel vaccines with cross protective properties.

Differentiation of IBV isolates using RRT-PCR

There is no specific treatment for IBV. Prevention and control are through the use of modified live or killed vaccines. The vaccination strategy is dependent upon the serotype of IBV prevalent within a geographical area. Rapid and accurate diagnosis of the serotype of IBV involved in a field outbreak is necessary to establish an effective vaccine strategy for neighboring flocks. Currently, many diagnostic tests exist for differentiating IBV isolates, including: monoclonal antibodies, dot blot, DNA probes, rapid plate hemagglutination test, SDS-PAGE polymorphism, direct S1 gene DNA sequencing, RT-PCR with serotype specific primers, and RT-PCR/RFLP.

It has been shown that genotyping tests provide data that correlates well with the serotype of the virus. Due to their ease and correlation with serotype, genotype tests (DNA sequencing, serotype specific RT-PCR, and RT-PCR/RFLP) have become widely used. Although the genotyping tests are rapid, room for improvement remains. In particular, elimination of post RT-PCR manipulations (gel electrophoresis, gel purification of DNA, restriction enzyme analysis, sequencing, etc.) would decrease the cost and overall assay time. Recent technological advances in the field of real-time nucleic acid amplification could be used to negate the need for post RT-PCR manipulations.

We developed a novel diagnostic assay termed sample to residual ratio quantification (SRRQ) using RRT-PCR. The basis of the assay is on the ability of serotype specific chimeric oligonucleotides to mediate cleavage of target RNA by RNase

H. Measurement of the cleavage mediated by each serotype specific chimeric oligonucleotide as compared to uncleaved control RNA identifies the virus type. Using this assay, we can differentiate IBV isolates from within five different serotypes without any post RT-PCR manipulations. The specificity of the assay was tested by analysis of 12 strains of IBV representing 8 different serotypes. For each strain tested, SRRQ using RT-PCR accurately determined the serotype of any strain from within one of the five serotypes for which a chimeric oligonucleotide was developed.

To validate the assay, we analyzed 16 allantoic fluid samples without prior knowledge of virus presence or serotype. Our assay accurately determined the serotype of any strain from within one of the five serotypes for which a chimeric oligonucleotide was developed. This novel assay should find future use as a rapid screening diagnostic assay for IBV field isolates, as well as, a general assay for detecting the presence or absence of any RNA molecule within a biological sample.