5-2002

Analysis and Diagnostic Implications of the Fusion and Attachment Protein Genes of Respiratory Syncytial Viruses of Ruminants

Nasser Zakaria Eleraky
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To the Graduate Council:

I am submitting herewith a dissertation written by Nasser Zakaria Eleraky entitled "Analysis and Diagnostic Implications of the Fusion and Attachment Protein Genes of Respiratory Syncytial Viruses of Ruminants." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

L. N. D. Potgieter, Major Professor

We have read this dissertation and recommend its acceptance:

Stephen Kania, Melissa Kennedy, Karla Mattesson, Sarel Van Amstel

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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ANALYSIS AND DIAGNOSTIC IMPLICATIONS OF THE FUSION AND ATTACHMENT PROTEIN GENES OF RESPIRATORY SYNCYTIAL VIRUSES OF RUMINANTS

A Dissertation

Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Nasser Z. Eleraky

May 2002

DEDICATION

This dissertation is dedicated to the memory of my father Mr. Zakaria Eleraky (1924-1999) who gave me love and support all his life.

Also, I dedicate this dissertation to my mother, my wife and my children.

ACKNOWLEDGMENTS

First,I would like to express my deep appreciation to my major professor Dr. Leon Potgieter for his guidance, advice, help and endless support throughout the research. I learned many valuable things from him either in virology or in life. Also, I would like to thank other members of my committee; Dr. Stephen Kania, Dr. Melissa Kennedy, Dr. Karla Mattesson and Dr. Sarel Van Amstel for their help and guidance. Special Thanks to Dr. Stephen Kania for sharing his laboratory experience and helping in the research.

I would also like to thank Dr. Mellissa Kennedy and Dr. Vina Diderich who thought me the Laboratory Virology. Special thanks to my wife Eman for her continuous encouragement and support. She took care of everything to save my time and my effort for work.

ABSTRACT

Respiratory syncytial viruses (RSVs) in ruminants are classified into two subgroups, ovine RSV and bovine RSV. Although ovine RSV infects cattle, its contribution to bovine respiratory tract disease has not been established which is an important issue for vaccine development in cattle.

Diagnosis by virus isolation or serology has low or variable sensitivity and/or specificity and PCR has been recommended as a rapid and sensitive technique for RSV detection. Prior to this study, a laboratory test to differentiate between bovine and ovine RSVs did not exist.

First, the nucleotide sequence of the ovine RSV fusion (F) gene was determined and compared with representative strains of bovine RSV and human RSV subgroups A and B. The ovine RSV F gene has 85% and 72-73% nucleotide identity with those of bovine RSV and human RSV respectively. The predicted amino acid sequence of the ovine RSV F gene has 94% and 83-84% amino acid identity with those of bovine RSV and human RSV respectively.

Two RT-PCR assays have been developed for simultaneous detection of bovine and ovine respiratory syncytial viruses. One, a set of primers amplified a 426 bp fragment of either bovine or ovine RSV F gene (RT-PCR F). PCR-F products could be distinguished by EcoRI or BstYI restriction endonuclease cleavage. In the other assay, a set of primers amplified a 542 bp fragment of either ovine or bovine RSV G gene (RT-PCR G). EcoO109I and RsaI restriction enzymes were used to differentiate between the ovine and bovine PCR-G products. Sequencing of the PCR products confirmed the fidelity of both assays.

The two assays were evaluated using eight bovine RSV isolates, one ovine RSV, one bighorn sheep RSV, one caprine RSV, two human RSV isolates and several viruses associated with bovine respiratory tract disease.

The two PCR assays described in this study potentially constitute sensitive and specific means for simultaneous detection of bovine and ovine RSV and differentiation between them. However, RT-PCR F followed by the appropriate restriction enzyme cleavage may be superior to RT-PCR G to discriminate between the two ruminant RSV subgroups and for determining the relative contribution of ovine and bovine RSV to the pathogenesis of bovine respiratory tract disease. This is relevant to the complete understanding of RSV epidemiology and immunoprophylaxis.

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Part I

Literature Review

Respiratory Syncytial Virus Infections in Ruminants

Abstract

Bovine respiratory syncytial virus (BRSV) has a worldwide distribution in the cattle population and is associated with calf pneumonia in many countries. Ovine respiratory syncytial virus (ORSV), caprine respiratory syncytial virus (CRSV) and bighorn sheep RSV are isolates associated with respiratory tract diseases in sheep, goats and Bighorn sheep respectively. Some studies indicated the possibility of interspecies transmission of bovine and ovine RSVs. This review presents updated information on respiratory syncytial viruses with an emphasis on those that affect ruminants.

History

Respiratory syncytial virus (RSV) was first isolated in 1956 from a laboratory chimpanzee during an outbreak of illness resembling the common cold and was named chimpanzee coryza agent (Morris et al., 1956). Shortly thereafter, it was isolated from two children with respiratory tract disease in Baltimore, Maryland. Similar investigations followed in many parts of the world that indicated RSV was associated with lower respiratory tract disease in people (Beem et al., 1960; Chanock et al, 1961; Suto et al., 1965; Tyrrell, 1965). The virus is designated respiratory syncytial virus which reflects the characteristic effect of the virus on infected cells; the formation of large multinucleated cells (syncytia) (Chanock and Finberg, 1957).

A similar virus to human respiratory syncytial virus (HRSV) had been suspected to exist in cattle after detection of specific antibody in bovine serum that inhibited HRSV growth in cell culture (Taylor-Robinson and Dogget, 1963; Dogget et al., 1968). Bovine

respiratory syncytial virus (BRSV) then was isolated for the first time from conjunctivo-nasal specimens from two diseased animals in Switzerland (Paccaud and Jacquier, 1970). At the same time, bovine RSV was isolated in 1970 from animals involved in an outbreak of severe respiratory tract disease in Japan that included more than 40,000 cattle of all ages (Inaba et al., 1970a; Inaba et al., 1970b; Inaba et al., 1972). Bovine RSV was first isolated in US by two independent investigators in 1974 from cattle in Iowa and Missouri (Rosenquist, 1974; Smith et al., 1974).

A sheep isolate of respiratory syncytial virus was characterized in 1983 (Everman et al., 1985). A goat respiratory syncytial virus isolate was first reported in 1979 (Smith et al., 1979). RSV was isolated from bighorn sheep in 1986 (Spraker et al., 1986).
In 1985, subgrouping of human respiratory syncytial viruses into two antigenic types (A and B) was defined by reaction patterns with monoclonal antibodies (Anderson et al, 1985; Mufson et al., 1985).

Similarly, it has been proposed that ruminant RSVs be divided into 2 subgroups, one representing bovine RSV and the other representing ovine RSV, based on analysis by RNAse protection of the G glycoprotein transcripts and sequence comparison of ovine and bovine RSV-G protein genes (Alansari and Potgieter, 1993; Alansari et al. 1999).

Viral properties

RSV virions grown in cell culture are heterogeneous in size and shape, consisting of pleomorphic spherical particles that range from 80-350 nm in diameter and are up to 10 μm in length (Armstrong et al., 1962; Norrby et al., 1970; Bachi and Howe, 1973; Bachi, 1988). RSV virion consists of a nucleocapsid contained within a lipid envelope.

The nuclocapsid contains the single strand of negative sense genomic viral RNA (Huang and Wertz, 1982). Virions assemble at the plasma membrane of infected cells and mature by budding, during which the intracellular nucleocapsid is packaged within a viral envelope that is derived directly from the host cell plasma membrane (Norrby et al., 1970; Bachi and How, 1973; Bachi, 1988). The envelope contains spike like projections that are approximately 11-20 nm in length and are spaced at 6-10 nm interval (Norrby et al., 1970; Bachi and How, 1973; Bachi, 1988).

RSV virions are extremely labile. However, infectivity can be stabilized by sucrose or magnesium sulphate (Ueba, 1978; Fernie and Gerin, 1980). BRSV is sensitive to low PH, ether, chloroform and is destroyed by heating at 56C for 30 minutes (Inaba et al., 1970; Paccaud and Jacquier, 1970; Rosenquist, 1974). The infectivity titer of a BRSV suspension decreases by about one log per 24 hours at 37C. The virus remains infectious for at least 10 years when stored in liquid nitrogen (Wellemans, 1990).

The RSV genome (strain A2) is a single strand of negative sense RNA (Huang and Wertz, 1982). Genomic RNA is neither capped nor polyadenylated (Mink et al., 1991). Genomic RNA is tightly associated with N protein. The 3' end of genomic RNA consists of a 44-nucleotide extragenic leader region that is presumed to contain the major viral promoter (Mink et al., 1991). The leader region is followed by the ten viral genes in the order 3' Ns1-Ns2-N-P-M-Sh-G-F-M2-L 5' (Collins et al., 1986; Collins et al., 1987). The last gene, L, is followed by a 155-nucleotide extragenic trailer region (Mink et al., 1991). The first nine RSV genes are non-overlapping and are separated by intergenic regions. The last two RSV genes, the M2 and L genes overlap (Collins et al., 1987).

Three of the ten proteins are transmembrane surface proteins: the attachment protein (G), the fusion protein (F) and the small hydrophobic protein (SH). Two proteins are non-glycosylated virion matrix proteins (M and M2). Three proteins associated with genomic RNA to form the viral nucleocapsid: the nucleoprotein (N), the phosphoprotein (P) and the large catalytic subunit of the RNA polymerase (L). The two nonstructural proteins (NS1 and NS2) accumulate in infected cells but are present in virions in trace amounts (Collins et al., 1996).

Replication

Attachment and entry: The G protein mediates attachment of the virion to host cells (Levine et al., 1987). The F protein mediates viral penetration into cells by fusion of the viral envelope with the plasma membrane of the host cell. This has been directly observed for RSV by video microscopy (Bachi, 1988). After penetration, the viral envelope becomes incorporated into the cell surface (Routledge et al., 1987). The nucleocapsid is released into the cytoplasm, and transcription is mediated by the viral RNA polymerase. All events in the RSV replication cycle occur in the cytoplasm without nuclear involvement (Kisch et al., 1962). Interaction between RSV proteins and cellular receptors has been studied recently. RSV-G protein interacts with heparin and cellular heparan sulfate (Krusat and Streckert, 1997). Also, RSV-F interacts independently with heparin/ heparan sulfate suggesting that RSV-F protein can facilitate virus attachment and infectivity in the absence of RSV-G protein (Feldman et al., 2000). Interaction of RSV and ICAM-1 on human epithelial cells has been studied recently suggesting that

ICAM-1 facilitates RSV entry and infection of human epithelial cells by binding to F protein (Behara et al., 2001).

Transcription and translation: RSV genes are transcribed in their 3' to 5' order from a single promoter near the 3' end of the genome (Dickens et al., 1990). The polymerase contacts genomic RNA within the nucleocapsid in the leader region and begins transcription. The polymerase completes transcription in a sequential stop-start mechanism guided by the gene-start and the gene–end signals. The result is the synthesis of a series of subgenomic mRNA which appear to be exact colinear copies of the genes, with no evidence of mRNA editing, splicing, or other modifications (Collins et al., 1996). The RSV mRNAs are capped at the 5' end and polyadenylated at the 3' end (Barik, 1993). A number of events occur at the gene junctions; polyadenylation and termination of the completed mRNA, movement of the polymerase across the intergenic region, re-initiation at the next gene and capping and methylation of the nascent mRNA (Collins et al., 1996). The intergenic regions do not contain important signals, and their length difference does not seem to be important. Thus, the gene start and gene end signals appear to be the only critical signals at the gene junction (Collins et al., 1996). The relative abundance of each RSV mRNA decreases with the distance of its gene from the promoter, presumably due to polymerase fall-off during sequential transcription (Dickens et al., 1990). In addition to the ten unique mRNAs, a number of less abundant, larger poly-transcripts are generated by transcriptional read–through of two or more adjacent genes. This probably occurs because the polymerase sometimes fails to recognize a gene-end signal and continues transcribing the next gene until it encounters the next gene-end signal. These poly-transcipts likely are not essential gene products (Collins et al., 1996).

The L gene start signal lies 68 nucleotides upstream of M2 gene –end signal, resulting in overlap (Collins et al., 1987). The presence of the M2 gene end-signal within the L gene results in a high frequency of premature termination of L gene transcription (Collins et al., 1996). Full length L mRNA is much less abundant, presumably full length is generated when the polymerase fail to recognize the M2 gene-end motif (Collins et al., 1996).

RNA replication: RNA replication is achieved by a switch from the stop-start mode of mRNA synthesis to the anti-termination read-through mode (Collins et al., 1996). This results in synthesis of positive sense replicative intermediate (RI) RNA that is an exact complementary copy of genomic RNA. The latter then serves as a template for synthesis of progeny genomes (Collins et al., 1996).

Virion morphogenesis: Progeny RSV virions mature by budding through areas of the plasma membrane that contain localized accumulations of viral spikes (Armstrong et al., 1962; Norrby et al., 1970; Bachi and How, 1973; Bachi, 1988).

Viral proteins

F protein: The fusion protein of paramyxoviruses is responsible for viral penetration and syncytium formation of infected cells (Collins, 1991). RSV-F protein was identified by immunoprecipitation with monoclonal antibodies that inhibits syncytium formation in cell culture (Walsh and Hruska, 1983).

Experiments were done to determine whether F protein alone mediates fusion. It was concluded that efficient fusion was obtained only with the co-expression of all three surface proteins F, G, and SH (Heminway et al., 1993). The SH and G proteins might be

important for placing F in an optimal configuration with regard to the target membrane or they might participate more directly in the conformational changes involved in fusion (Collins et al., 1996). In a recent study, recombinant bovine RSVs lacking either the G gene or SH gene and recombinants without both genes were generated. The deletion mutants were fully competent for multicycle growth in cell culture suggesting that the SH and G genes are not essential for replication (karger et al., 2001). Also, RSV-F protein may interact independently with heparin/ heparan sulfate suggesting that RSV-F can facilitate virus attachment and infectivity in the absence of RSV-G protein (Feldman et al., 2000).

The RSV F protein is synthesized as a precursor F0, which is cleaved by a cellular protease to generate two disulphide–linked subunits, NH2-F2-F1-COOH (Walsh et al., 1985). The BRSV F mRNA consists of 1899 nucleotides and has a single major open reading frame which codes for a polypeptide of 574 amino acids with a molecular weight of 63.8 KDa (Lerch et al., 1991). Residues 1-26 of this polypeptide represent NH2 terminal signal sequence and residues 131-136 represent a site for proteolytic cleavage to generate the disulphide-linked F1 and F2 subunits. Residues 522-549 represent the hydrophobic trnsmembrane anchor sequence (Lerch et al., 1991).

The ovine RSV F gene has 85% and 72-73% nucleotide identity with those of bovine RSV and human RSV respectively (Eleraky et al., 2001). The predicted amino acid sequence of the ovine RSV F gene has 94% and 83-84% amino acid identity with those of bovine RSV and human RSV respectively (Eleraky et al., 2001). Bovine and ovine RSV F genes have nearly the same level of nucleotide and amino acid identity with

human RSV F gene (Walravens et al., 1990; Lerch et al., 1991; Himes and Gershwin, 1992; Eleraky et al., 2001).

The molecular weights of the F2 fragments in HRSV (long strain) and some BRSV isolates (A51908 and Mdx) were approximately 20 KDa whereas those of CRSV and other BRSV isolates (FS-1, VC-464) were 15.5 KDa. The molecular weight of the F1 was 48 KDa in all isolates examined (Mallipidi and Samal, 1993 b). This variation in the size of F2 polypeptides may be due to the differences in the extent of glycosylation (Mallipidi and Samal, 1993 b).

The fusion protein is superior to the attachment protein G in inducing neutralizing antibodies (Olmsted et al., 1986; Westenbrink et al., 1989) and for eliciting cytotoxic T cells (Pemberton et al., 1987; Cannon and Bangham, 1989). Most neutralizing antibodies have been mapped to the fusion glycoprotein F (Arbiza et al.; 1992, Lopez et al., 1990; Lounsbach et al. 1993; Paradiso et al., 1991;Scopes et al., 1990; Taylor et al., 1992; Walsh et al., 1986; and West et al., 1994). Many experimental studies suggest that the RSV fusion protein is the major protective antigen (Wertz et al., 1987; Stott et al., 1987; Olmsted et al., 1986 and Olmsted et al., 1988). Two neutralizing fusion-inhibiting bovine monoclonal antibodies directed at different epitopes on the fusion protein of RSV protected the lungs of gnotobiotic calves from RSV infections (Thomas et al., 1998). A conserved neutralization site (173 STNKAVVSLS 182) has been identified in BRSV and HRSV F proteins (Langediijk et al., 1998). This site is located on the N-terminus of F1, adjacent to the hydrophobic, putative fusion related region (Langediijk et al., 1998). This site is also conserved in Ovine RSV fusion protein (Eleraky et al., 2001). It has been proposed that this linear conserved epitope may be a potential candidate for a peptide-

based vaccine, which can induce neutralizing antibodies against all RSV groups and subgroups (Langediijk et al., 1998).

Ruminant RSV have been grouped into two subgroups based on the reactivity of three monoclonal antibodies specific to BRSV-F protein (Pastey and Samal, 1997) supporting the existence of antigenic variation among ruminant RSVs (Pastey and Samal, 1997). The F protein of BRSV was expressed in a baculovirus vector (Himes and Gershwin, 1992; Pastey and Samal, 1998) and was proposed as an antigen for detecting antibodies to BRSV in enzyme immunoassay (Pastey and Samal, 1998).

G protein: Antibodies specific to RSV-G protein inhibited the adsorption of virions to the cells, whereas antibodies to the F protein inhibited syncytium formation but not virus adsorption (Levine et al., 1987). Therefore, G protein is the viral attachment protein. G protein lacks hemagglutination and neuraminidase activity (Richman et al., 1971).

G protein is the most divergent viral protein among RSV strains (Lerch et al., 1990; Alansari and Potgieter, 1993). A 53% amino acid identity between the G protein of subgroups A and B of HRSV exists (Johnson et al., 1987) and is similar to that between the G protein of ORSV and BRSV [62%] (Alansari and Potgieter, 1993). BRSV G protein has 29-30% amino acid identity with that of HRSV (Lerch et al., 1990). ORSV G protein has 21-29% amino acid identity with that of HRSV. G proteins of ORSV and BRSV differ substantially from that of HRSV. Analysis of the G glycoproteins clearly segregates ruminant RSV isolates from human RSV isolates and the relationship between A and B subgroups of HRSV is analogous to that between BRSV and ORSV isolates (Alansari and Potgieter, 1993).

The central hydrophobic region of the ectodomain encompassing amino acids 166-186 may be a putative receptor binding site in the HRSV G protein (Johnson et al., 1987**)**. This region is not conserved in the ORSV or BRSV G protein and only six out of the 13 amino acids in this region were conserved among all RSV strains (Mallipedi and Samal, 1993).

SH protein (Small hydrophobic region): SH protein contains a central core of hydrophobic amino acids flanked by short N-terminal and C-terminal domains that each contains a potential acceptor site for N-linked carbohydrate (Collins and Wertz, 1985). The function of the SH protein is unknown, but its status as an integral membrane protein suggests that it might be involved in attachment, penetration, uncoating and/or virion morphogenesis (Collins et al., 1996). SH protein greatly enhances membrane fusion when it is co-expressed with the F and G protein (Heminway et al., 1994). The SH gene and its encoded protein differ significantly among human, bovine, and ovine RSV isolates (Anderson et al., 1992; Collins et al., 1990; Samal and Zamora, 1991; Alansari and Potgieter, 1994a).

Nucleocapsid-associated protein N, P, and L: RSV has three nucleocapsid-associated proteins N, P, and L. N protein is the RNA binding major nucleocapsid protein, P protein is a phosphoprotein, and L protein is a large polymerase subunit (Collins et al., 1996). RSV L protein is similar in size to its rhabdovirus and paramyxovirus counterparts and contains six conserved segments, which presumably represent functional domains (Stec et al., 1991). Comparison of amino acid sequences of L protein of BRSV with those of HRSV A2 and avian pneumovirus revealed 84% and 64% identity respectively (Yunus et al., 1999). Comparison of RSV-N protein with the

N-terminal 400 amino acids of the N proteins of various non-segmented, negative strand viruses revealed that all contain three segments of similar sequence and predicted secondary structure (Barr et al., 1991). N protein is the most conserved protein between HRSV and ruminant RSV isolates (Alansari and Potgieter, 1994b). ORSV P protein has 90% and 81% identity with those of BRSV and HRSV respectively (Alansari and Potgieter, 1994b).

Matrix proteins (M and M2): Pneumoviruses differ from other non-segmented, negative strand RNA viruses with respect to their matrix proteins. The former have two matrix proteins M and M2, whereas the latter have only one (Huang et al., 1985). Pneumovirus M protein corresponds to the M protein of other paramyxoviruses, but M2 may not have a counterpart in other paramyxoviruses (Collins et al., 1996). The M protein of non-segmented, negative-strand viruses likely have two general functions; rendering the nucleocapsid transcriptionality inactive before packaging and mediating association of the nucleocapsid with the nascent envelope (Collins et al., 1996). In pneumoviruses, these functions of the M protein may be shared between the M and M2 proteins (Collins et al., 1996). The ORSV M protein has 97% and 90% amino acid identity with those of BRSV and HRSV respectively (Alansari and Potgieter, 1994b). The level of amino acid identity between M2 of ORSV and those of BRSV and HRSV was 93% and 82% respectively (Alansari and Potgieter, 1994b). The M and N proteins may be the most conserved protein among ruminant RSV isolates (Alansari and Potgieter, 1994b).

Nonstructural proteins NS1 and NS2: The functions of NS1 and NS2 proteins are unknown. They may have roles in regulating RNA synthesis or in virion

morphogenesis (Collins et al., 1996). A NS2-deficient BRSV replicated autonomously and could be passaged demonstrating that NS2 is not essential for virus replication in cell culture. However, growth of the mutant was considerably slower than that of the wild type and the final infectious titer was reduced indicating that NS2 provides a supporting factor required for full replication capacity (Buchholz et al., 1999). Recent work suggested that bovine respiratory syncytial virus nonstructural proteins NS1 and NS2 have the potential to cooperatively antagonize alpha/beta interferon-induced antiviral response (Schlender et al., 2000). The amino acid sequence of BRSV NS1 and NS2 have been compared to those of ORSV and HRSV A and B. The amino acid identity between BRSV and ORSV is higher than that between BRSV and HRSV (Pastey and Samal, 1995). That supports also segregation of ruminant RSV isolates from human isolates.

Classification

RSVs belong to the family Paramyxoviridae, subfamily Pneumovirinae, genus Pneumovirus (Collins et al., 1996). The pneumoviruses are classified as a separate genus because the nucleocapsid diameter is narrower (12-15nm) than that of paramyxoviruses (18nm) and because of the lack of detectable viral hemagglutination activity for RSV (Kingsbury et al , 1978; Richman et al 1971). The pneumoviruses, like the morbilliviruses, lack a neuraminidase (Collins et al., 1996). Respiratory syncytial viruses are enveloped, single stranded, nonsegmeneted, negative-sense RNA viruses. The pneumoviruses encode a larger number of mRNAs (ten) than paramyxoviruses (six or seven) although the length of the genomic RNA are similar (Collins et al., 1996). The

genes NS1, NS2, SH and M2 of pneumoviruses have no counterparts in most of the paramyxoviruses (Collins et al., 1996).

RSV subgrouping: Two antigenic subgroups, A and B, were defined for HRSV on the basis of reaction patterns with monoclonal antibodies (Mufson et al., 1985; Anderson et al., 1985; Gimenez et al., 1986, Hendry et al., 1986). Antigenic diversity of HRSV is associated with extensive degree of amino acid sequence divergence (53%) between the G protein of the two subgroups (Johnson et al., 1987).

The attachment glycoprotein G of BRSV differs antigenically from that of HRSV, whereas the bovine RSV F, N, M, and P proteins cross-react with those of human RSV (Lerch et al., 1989). Heterogeneity among 19 BRSV strains has been studied by using monoclonal antibodies specific for the G protein. Although two antigenic subgroups were identified (Furze et al., 1994), sequence data revealed a high degree of amino acid identity (85%) among the strains of suggested subgroups of BRSV (Furze et al., 1997; Prozzi et al., 1997). These findings are in sharp contrast to HRSV subgrouping, where only 53% identity between members of A and B subgroups exists (Johnson et al., 1987). Therefore, BRSV isolates may constitute a continuum of slightly different strains within a single subgroup rather than distinct subgroups (Prozzi et al., 1997, Stine et al., 1997).

Subsequent work resulted in a proposal that ruminant RSVs be divided into 2 subgroups, one representing bovine RSV and caprine RSV, and the other representing ovine RSV. This was based on analysis by RNAse protection of the G glycoprotein transcripts using two (bovine and ovine) antisense radiolabelled RNA probes (Alansari et al., 1999). Moreover, the ovine and bovine RSV G glycoprotein genes have nucleotide identity (70%) and amino acid identity (62%) nearly similar to those that exist between

the two human RSV subgroups (67% and 53% respectively). (Alansari and Potgieter, 1993). This supported the ruminant RSV isolates subgrouping hypothesis. Some other studies on the F protein of bovine and ovine RSV have supported this hypothesis. In one study, antigenic variation between bovine and ovine RSV with monoclonal antibodies to the F protein of bovine RSV was reported (Pastey and Samal, 1997). In another study, the level of identity between ovine and bovine RSV F genes (85%) and their predicted proteins (94%) are close to those found between the two human subgroups A and B, 80% nucleotide identity and 90% amino acid identity (Eleraky et al., 2001).

Clinical disease

Human: HRSV infection is a major cause of respiratory tract disease in infants and young children. The disease is characterized by pneumonia and bronchiolitis (Avila et al., 1989; Tsutsumi et al., 1991). In older children and adults, HRSV causes moderate to severe upper respiratory tract symptoms (Finger et al., 1987; Agius et al., 1990).

Cattle: BRSV is considered to be one of the most important causes of respiratory tract disease in cattle (Inaba et al., 1972; Stott et al., 1980; Verhoeff et al., 1984; Baker et al., 1997; Caldow et al., 1993). Severe respiratory disease has been reported in calves less than 6 months of age (Bryson et al., 1978; Pirie et al., 1981; Kimman et al., 1988; Vanderpoel et al., 1993). Severe respiratory disease affecting adult cattle has been recorded also (Harrison et al., 1985; Castlman et al., 1985; Elvander et al., 1996; Ellis et al., 1996). The symptoms include cough, nasal discharge, conjunctivitis and lacrimation. The disease is associated with pyrexia and in some cases signs of lung emphysema may occur after 2-3 days. Some animals may have difficulty in breathing that may be

accompanied by dry cough with rapid and shallow breathing. Abdominal respiration sometimes develops (Wellemans, 1990).

Sheep and goats: Ovine RSV was isolated from a one-year-old ewe with rhinitis (Evermann et al., 1985). Experimental infection of three month-old lambs with ORSV induced mild conjunctivitis. Clinical signs of pneumonia were not observed, but gross and microscopic evidence of pulmonary inflammation did develop. Lung lesions were more severe when ORSV infection was accompanied with *Mannheimia hemolytica* (Evermann et al., 1985). Signs of clinical illness, pulmonary lesions, and seroconversion occurred in lambs experimentally inoculated with a BRSV isolate (Lehmkuhl and Cutlip 1979; Sharma and Woldehiwet, 1991). RSV infection is widespread in free-ranging Bighorn sheep populations and RSV may be an important factor in the Bighorn sheep pneumonia-complex (Dunbar et al., 1985). RSV was isolated from Bighorn sheep with respiratory symptoms (Spraker et al.,1986; Evermann et al., 1994). Caprine RSV was isolated from a herd of pygmy goats with respiratory disease (Smith et al., 1979). Natural infection with BRSV has been recorded in eight cases in a herd of 50 Murciana kids from an intensive goat farm in Spain. In two of the eight cases, concurrent infection with *Mannheimia hemolyica* A occurred (Redendo et al., 1994).

Pathogenesis and epidemiology:

The means of transmission of BRSV is mostly by contact with respiratory secretions from infected cattle and by the aerosol route (Baker et al., 1997). Once a herd in a region is affected, the disease rapidly spreads from farm to farm (Wellemans, 1990). Mortality may reach 20% (Wellemans, 1990). Outbreaks of respiratory disease associated

with BRSV peak in autumn and winter (Baker et al., 1985; Van der Poel et al., 1993), but some epidemics have occurred in spring and summer (Wellemans, 1990). BRSV infection occurs in both dairy and beef cattle (Baker et al., 1997). Infections resulting in severe clinical disease are mostly observed in calves under 6 months of age (Bryson et al. 1978; Kimman et al. 1988, Pirie et al 1981; Van der Poel et al. 1993). Severe signs of respiratory tract infections also have been observed in adult cattle (Castleman et al., 1985; Harrison et al. 1985; Elvander, 1996; Ellis et al. 1996). Severe clinical signs in adult cattle likely relate to a primary exposure to the virus (Baker et al., 1997). The factors responsible for severity of the disease are unknown, but concurrent viral, bacterial and mycoplasmal infections, housing and management conditions, weather conditions and passively acquired antibody titer may have roles in outcome of the disease (Van der poel et al., 1994).

Maternal antibodies can be detected in calves for up to about three months (Baker et al., 1986 a**)**. Although maternally derived antibodies do not effectively prevent the disease, they may reduce severity of the disease in colostrum-fed calves (Belknap et al., 1991). Mucosal and systemic IgA responses are inhibited in calves when maternally derived antibodies are present at the time of infection (Kimman et al., 1987). Humans of all ages are regularly reinfected with human RSV (Glizen et al., 1986). Similarly, cattle are regularly reinfected by bovine RSV (Van der Poel et al., 1993).

The existence of persistent infections of BRSV have been suspected because of re-occurrence of BRSV in closed populations (Van der Poel et al., 1993). This led to a hypothesis that BRSV may persist in some cows during summer and serve as a source of new outbreaks in autumn or winter (Van der Poel et al., 1993). Experimental studies to

support this hypothesis revealed serological evidence of persistence of BRSV in cattle but the virus or its RNA has not been detected in these populations (Van der Poel et al., 1997).

Immunologic response has been implicated in the pathogenesis of BRSV infection. Disease severity correlated with production of BRSV-specific IgE in experimentally infected calves suggesting that type I hypersensitivity response can have a role in BRSV infection (Stewart and Gershwin, 1989a, b).

The role of non-bovine species in the epidemiology of BRSV infections has been investigated. Sera from cattle, nine non-bovine species and people that had contact with cattle were examined for BRSV-specific antibodies in a BRSV G-peptide ELISA. BRSV-specific antibodies have been found in all cattle tested, goats (27.5%) and occasionally in some other animals (horses, roe, cat, and dog). That study suggested that beside cattle, only goats may have a role in the epidemiology of BRSV (Van der Poel et al., 1995). A serological survey of pigs in Northern Ireland using BRSV antigens revealed 41% positive rate by immunofluorescence (Allan et al., 1998). In that study, the immunofluorescent-staining pattern observed with the majority (63%) of BRSV-reactive pig sera was similar to that observed with known BRSV-reactive bovine sera. The other immunoreactive pig sera stained BRSV infected cell culture in an atypical pattern suggesting that more than one serotype of a porcine pneumovirus may exist (Allan et al., 1998). Antibodies to BRSV have been found in sheep (Berhiaume et al., 1973; Adair et al., 1984). Experimental infection of lambs with BRSV produced mild clinical signs and some pulmonary lesions (Lehmkuhl and Cutlip, 1979; Sharma and Woldehiwet, 1991). Existance of two related but antigenically distinguishable RSV types in sheep has been

suggested, one of them is similar to bovine strains (Adair and McFerran, 1987). Inoculation of ORSV into calves and deer fawns resulted in mild to moderate clinical respiratory disease in calves but not in fawns. Lesions were detected and ORSV was re-isolated from the lower respiratory tract of calves and fawns (Bryson et al., 1988).

Subgroup-specific peptide-based ELISA, derived from the unique central hydrophobic region of the ovine and bovine G-glycoprotein respectively, has been used to determine the prevalence of antibodies against ovine and bovine subgroup strains of RSV in cattle. The prevalence was 56-60% for the bovine strains and 15.9% for the ovine strain (Grubbs et al., 2001 a; Grubbs et al., 2001 b). Recently, PCR assay targeting F gene has been recommended to detect both bovine and ovine RSV and simultaneously differentiate between them (Eleraky et al. 2001). This assay may be useful to understand the epidemiology of RSV in ruminants.

Pathology

Lesions that have been associated with BRSV natural and experimental infection include lung consolidation, interstitial pneumonia, lung edema and emphysema. Microscopically, interstitial pneumonia, interstitial edema, necrotic bronchiolitis and multinucleated syncytial epithelial cells on bronchial and alveolar walls have been reported (Bryson et al., 1983; Castlemann et al., 1985; Kimman et al., 1989; Appel and Heckert, 1989).

Immune response

Several experimental studies suggested that the RSV fusion protein F is the major protective antigen (Wertz et al., 1987; Stott et al., 1987; Olmsted et al., 1986; Olmsted et al., 1988). However, the G protein also has a role in eliciting a protective immunity (Taylor et al., 1997; Plotnicky- Gilquin et al., 2000).

Although maternally derived antibodies do not effectively prevent disease, they may reduce severity of disease in colostrum-fed calves (Belknap et al., 1991). Mucosal and systemic Ig A responses are inhibited in experimentally infected calves when maternally derived antibodies are present at the time of infection (Kimman et al., 1987).

Specific neutralizing antibody responses and positive lymphocyte proliferative responses were detected in calves experimentally infected with BRSV (knott et al., 1998). Data from a recent study suggests that most BRSV experimentally infected calves experience an increase in specific IgM and IgG1 titers about 6-10 days after infection with BRSV (Uttenthal et al., 2000). The IgM titer was transient, lasting for only 5-10 days. IgA was detected concomitantly with IgM but at a lower level. Production of IgG2 anti-BRSV antibodies was detected three weeks after infection (Uttenthal et al., 2000). CD8+ T lymphocytes may have a central role in the recovery of calves from RSV infection (Taylor et al., 1995, Gaddum et al., 1996; Thomas et al., 1996). The demonstration of CD8+ CTL in the lungs at a time when bovine RSV was being cleared implicated the importance of these cells in recovery from infection (Gaddum et al., 1996). The depletion of CD8+ T-lymphocytes with a CD8-specific monoclonal antibody was associated with an enhanced pneumonic consolidation in calves experimentally infected

with BRSV. However, calves depleted of the CD4+ subpopulation also had enhanced macroscopic lesions but with less active histological lesions (Thomas et al., 1996). In one study, BRSV significantly suppressed lymphocytes response to phytohemagglutinin in experimentally infected lambs (Sharma and Woldehiwet, 1991).

Immunization

In contrast to modified-live virus vaccines, inactivated vaccines stimulate low concentration of virus neutralizing antibodies and high concentration of non-neutralizing antibodies (Ellis et al., 1995). Certain inactivation processes may alter functionally important epitopes on BRSV envelope glycoproteins, leading to production of predominantly non-neutralizing antibodies in immunized cattle (Ellis et al., 1995). In the late 1960s, a formalin-inactivated vaccine not only failed to protect vaccinated children against RSV but also sensitized vaccinated children who suffered more serious disease after infection than unvaccinated children (Kim et al., 1969). However, vaccinal serum antibodies elicited by inactivated vaccine was not associated with disease enhancement following experimental infection in calves with BRSV (Mohanty et al., 1981). In another study, an inactivated BRSV vaccine elicited clinical protection against subsequent experimental infection with virulent virus and decreased the severity of pulmonary lesions in vaccinated animals (Ellis et al., 2001). Efficacy was similar to that of modified live virus vaccine (Ellis et al., 2001).

BRSV vaccines can be administered to pregnant cattle during late gestation to induce a high level of BRSV specific antibodies in colostrum (Ellis et al., 1996 a).

Several modified live virus BRSV vaccines are available and most contain BRSV in combination with other viral and bacterial pathogens (Baker et al., 1997). Vaccination with BRSV modified virus vaccines significantly reduced the clinical disease and pulmonary pathology in calves (Verhoeff et al., 1984; West et al., 1999). In vitro assays of cellular immunity more consistently correlated with vaccine-induced protection than presence of post-vaccination serum antibody (West et al., 1999).

A peptide representing amino acids 174-187 of BRSV G glycoprotein has been used to induce protection in mice against BRSV challenge (Bastein et al., 1999).

Immunization with a recombinant F protein, expressed in insect cells, with Quail A adjuvant induced neutralizing antibodies and protection in mice against BRSV challenge (Walravens et al., 1996). Recombinant vaccinia viruses expressing the F, G or N protein of BRSV induced resistance to BRSV challenge in calves (Taylor et al., 1997). A bovine Herpesvirus 1 (BHV1) vector vaccine carrying a gene encoding the BRSV G protein induced protection in calves against BRSV and BHV1 infections (Schrijver et al., 1997). Significant protection against RSV infection was induced in mice vaccinated with DNA encoding the F or G protein of RSV (Bembridge et al., 2000). Immunization using plasmid DNA encoding the RSV G glycoprotein induced protective effects in mice and cotton rats (Li et al., 2000).

Diagnosis

Virus isolation: BRSV is labile outside the host and is thought to lose viability during transport to a laboratory. BRSV is thermolabile and is sensitive to freeze- thawing (Baker, 1992). Successful isolation of BRSV in cell culture may require multiple

subpassages (Baker, 1992). BRSV isolation is not recommended as a routine procedure because its isolation is difficult and prolonged (Edwards et al., 1984).

Antigen detection: BRSV was detected in bovine nasal swabs by enzyme immunosorbent assay designed to detect HRSV (Osorio et al., 1989). BRSV has been detected using Immunofluorescence in lung tissues from experimentally and naturally infected calves and nasopharyngeal smears from experimentally infected calves (Thomas and Stott, 1981). Immunofluorescence also has been used to detect BRSV in lung lavage samples from epizootics of bovine respiratory tract disease (Kimman et al., 1986).

Serology: Many serological tests have been developed and used in BRSV diagnosis as complement fixation test (Takashi et al., 1975), microtiter serum neutralization test (Rossi and Kiesel, 1974), indirect fluorescent antibody test (Potgieter and Aldridge, 1977), immunodiffusion (Zygraich and Wellemans, 1981), indirect hemagglutination test (Martin, 1983) and ELISA (Gillette, 1983). Also, ELISA has been used to detect IgM to BRSV by using monoclonal antibodies to bovine IgM as the trapping antibody (Westenbrink and Kimman, 1987). A microneutralization ELISA was developed to detect specific antibodies to BRSV in cattle sera using a monoclonal antibody to the F protein of the virus (Ellis et al., 1995). Subgroup specific-peptide-based ELISA were used to determine the prevalence of antibodies against ovine and bovine subgroup isolates of RSV in cattle (Grubbs et al., 2001).

Reverse transcription-Polymerase chain reaction (RT-PCR) assays : Reverse transcription-polymerase chain reaction (RT-PCR) assays targeting sequences of the F protein gene (Oberst et al., 1993; Vilcek et al., 1994; West et al., 1998; Larsen et al., 1999), G protein gene (Vilcek et al.,1994), nucleoprotein gene (Valarcher et al., 1999)

have been used to detect BRSV. PCR assays may be more sensitive than IF (Vilcek et al., 1994; Valarcher et al., 1999) and ELISA (Larsen et al., 1999; Valarcher et al., 1999) and as specific as IF and ELISA (Valarcher et al., 1999) for the detection of bovine RSV infections. RT-PCR based on amplifying part of G gene was used to distinguish between A and B subgroups of HRSV **(**Gottschalk et al., 1996 **).** In a recent study, RT-PCR assay targeting a specific fragment (426 bp) of F gene has been recommended to differentiate bovine and ovine RSV (Eleraky et al., 2001).

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Part II

The ovine respiratory syncytial virus F gene sequence and its diagnostic application

Abstract

Ruminant respiratory syncytial viruses are classified into two subgroups, ovine RSV and bovine RSV. Although ovine RSV infects cattle, its contribution to bovine respiratory tract disease has not been established which is an important issue for vaccine development in cattle. Diagnosis by virus isolation or serology has low or variable sensitivity and/or specificity and PCR has been recommended as a rapid and sensitive technique for RSV detection. A simple procedure has been developed to detect and identify bovine and ovine RSVs. First, the nucleotide sequence of the ovine RSV fusion (F) gene was determined and compared with representative strains of bovine RSV and human RSV subgroups A and B. The ovine RSV F gene has 85% and 72-73% nucleotide identity with those of bovine RSV and human RSV respectively. The predicted amino acid sequence of the ovine RSV F gene has 94% and 83-84% amino acid identity with those of bovine RSV and human RSV respectively. Then, PCR primers targeting a specific F gene fragment of bovine and ovine RSV were designed. The primers represented bases 85-103 and the complementary sequence to bases 510-493 of the ovine RSV F gene. A similar PCR product (426bp) was obtained on agarose gel electrophoresis from bovine RSV and from ovine RSV. The products, however, were unique to the parent virus and could be distinguished by EcoRI or MspI restriction endonuclease cleavage. EcoRI cleaved the ovine product into 2 bands (285 bp and 141 1bp) but failed to affect the bovine RSV PCR product. However, MspI cleaved the bovine product into 2 bands (229 bp and 197 bp), but had no effect on the ovine product. Also, this assay did not amplify any PCR product with human RSV. RT-PCR followed

by restriction enzyme digestion is a useful and practical approach for detection and differentiation of ruminant respiratory syncytial viruses.

Introduction

Respiratory syncytial viruses are major causes of severe respiratory tract disease in young children [5] and calves.[7, 26, 14] Ovine RSV was isolated from a one-year-old ewe with rhinitis.[11] RSV also was isolated from an eight-month-old bighorn lamb with respiratory symptoms.[29]

Respiratory syncytial viruses are classified in the genus Pneumovirus, which is included in the family Paramyxoviridae. They are enveloped, single stranded, negative-sense RNA viruses that replicate in the cytoplasm and mature by budding from the cell membrane. The genome is non-segmented, consisting of 10 genes that are transcribed to 10 unique mRNA. Each mRNA encodes a unique protein (NS1, NS2, N, P, M, SH, G, F, M2, and L).

The G (attachment protein) and F (fusion protein) are the two major surface glycoproteins encoded by respiratory syncytial viruses. RSV F protein was identified when F-specific monoclonal antibodies inhibited syncytium formation in cell culture.[34] The F glycoprotein is synthesized as an inactive precursor, Fo, that is cleaved by a cellular protease to generate two disulfide linked subunits, NH2-F2-ss-F1-COOH.[10.]The fusion protein may be superior to the attachment protein G in inducing neutralizing antibodies [22, 36] and cytotoxic T cells.[9, 25] Furthermore, the F protein induces fusion-inhibiting antibodies that correlate well with protection.[30] A conserved neutralization site

(173 STNKAVVSLS 182) has been identified on F protein in all known BRSV and HRSV strains.[15]

It has been proposed that ruminant RSVs be divided into 2 subgroups, one representing bovine RSV, and the other representing ovine RSV, based on analysis by RNAse protection of the G glycoprotein transcripts, [3] nucleotide sequence of ORSV-G protein gene [2] and reactivity of ruminant RSVs to monoclonal antibodies specific to BRSV-F protein.[24]

The predicted BRSV-F protein shares 80.5% overall amino acid identity with the HRSV F protein with 89% identity within the F1 polypeptide but only 68% identity within the F2 polypeptide.[17] HRSV subgroup B (15837 strain) and subgroup A (A2 strain) have 89% overall amino acid identity of their respective F proteins.[13]

Experimental infection with ovine RSV in calves resulted in mild to moderate clinical respiratory disease.[8] It has been suggested that two related but antigenically distinguishable RSV types are present in sheep, one of which is similar to bovine strains based on differences in fluorescent Ab staining of bovine RSV virus infected cells by ovine and bovine sera.[1] Antibodies specific for either ovine or bovine subgroup strains of RSV were detected in cattle by using a subgroup-specific G-peptide ELISA, suggesting that ovine RSV does infect cattle.[12]

Whether ovine RSV contributes to bovine respiratory disease or not is an important issue regarding RSV epidemiology and vaccine development in cattle. BRSV isolation is very difficult because of its lability [28] and viral detection by IF [31] and ELISA [23, 27] are of low or variable sensitivity and specificity. PCR assays may be more sensitive than IF [33] and ELISA [16] for the detection of BRSV in cattle with acute respiratory disease.

PCR assays have been recommended as a rapid and sensitive means for detection of BRSV infection in cattle.[33, 21,16] The nucleotide sequence analysis of the ovine RSV F gene has not been done. In this study, the authors report its nucleotide sequence and its predicted protein. Based on this information, a PCR test targeting a specific fragment in the F gene of both bovine RSV and ovine RSV has been developed. The purpose of this test is to detect both BRSV and ORSV in infected cattle and concurrently to differentiate between them.

Materials and methods

Nucleotide sequence of F gene and F-M2 intergenic region of ovine RSV:

1- Virus and cells: Ovine respiratory syncytial virus strain WSU 83-1578 [a] was grown on Madin Darby Bovine Kidney (MDBK) cells with Dulbeco's modified Eagles medium[b] supplemented with 2% fetal bovine serum [c] and antibiotic-antimycotic. [c]

2- RNA extraction and quantification: Ovine RSV was grown until 50-70% CPE developed. RNA was extracted with Trizol reagent [c] according to the protocol of the manufacturer. In brief, infected cells were collected from a 75-cm^2 flask and pelleted cells were suspended in one ml Trizol and incubated for 5 minutes at room temperature. Then, after adding 0.2 ml chloroform, incubating for 2-3 minutes at room temperature and centrifuging at 12,000 xg for 15 minutes at 2-8 ^oC, the aqueous upper phase was obtained. RNA was precipitated by adding 0.5 ml isopropyl alcohol, incubating for 10 minutes at room temperature and centrifuging at 12,000 xg for 10 minutes at 2-8 ^oC. The RNA pellet then was washed with 1 ml of 75% ethanol and centrifuged at 7,500 xg for 5

minutes at 2-8 ^{o}C. The RNA pellet finally was air dried and dissolved in RNase free water. The RNA concentration was quantified in a spectrophotometer at A260 wave length.

3- Primers: Two primers were developed and synthesized [c] for using in RT-PCR (figure 1). The F25 primer used for first strand cDNA synthesis (5' TGATCTCATATTTGCAGGG 3') represents the complementary sequence of bases 43-25 of the published sequence of M2 gene of ovine RSV.[4] The second primer, F19, (5'GAGACAAGGGTGTATATAG 3') representing part of the published intergenic sequence between F and G genes of ovine RSV [19] was used with the F25 primer for amplification.

4- First strand cDNA synthesis: Synthesis of cDNA was carried out in 20 ul reaction volumes by using superscript II Rnase H^{-} reverse transcriptase [c] according to the manufacturer's protocol. Briefly, two picomoles of F25 primer, 4-5 µg of total RNA and up to 12 µl of water were heated to 70^{o}C for 10 minutes and chilled quickly on ice. Then, four µl of 5x first strand buffer [c], two µl of 0.1 M dithiothritol (DTT) [c] and one µl of 10 mM dNTP mix [c] were added and incubated at 42^{o}C for two minutes. One µl (200 units) of superscript II then was added and incubated at 42 C for 50 minutes followed by heating at 70 C for 15 minutes to inactivate the reaction.

5- Amplification of ovine RSV F gene and F-M2 intergenic region: Elongase enzyme mix [c] was used according to the manufacturer's directions for amplifying the F gene of ovine RSV. In brief: For 50 µl-reaction volume, two mixtures were used. Mixture one consisted of one ul of 10 mM dNTP mix [c], one µl of 10 uM F19 primer, 1µl of 10 µM of F25 primer, one µl of cDNA template and water to 20 ul. Mixture two consisted of two

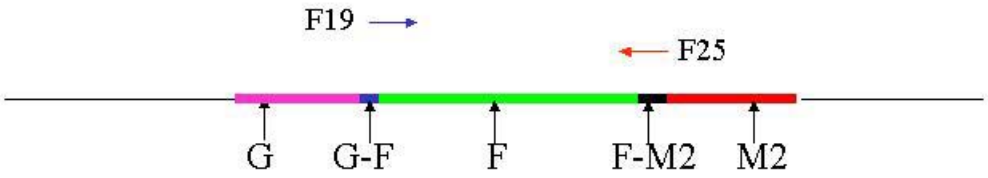

Figure 1: Primers used to amplify ovine RSV F gene and F-M2 intergenic region

µl of 5x buffer A [c], eight µl of 5x buffer B [c], two µl of elongase enzyme mix and water to 30 ul. Mixtures one and two then were gently mixed together in one tube and placed in a thermal cycler [d] using the following conditions: denaturation at 95C for 1.5 minutes, then a three-step cycling program consisting of annealing at 50 ^{o}C for 30 seconds, extension at 72 ^{o}C for one minute and denaturation at 95 ^{o}C for one minute and repeated 30 times. At the end of the last cycle, the temperature was held at 50 C for two minutes followed by 72 C for 10 minutes.

6- Gel extraction of the PCR product: PCR amplified products were electrophoresed in 1% agarose gel in 1x TAE buffer. The band representing F gene and F-M2 region of ovine RSV was extracted from the gel using a gel extraction kit.[e]

7- Sequencing of the PCR product: Sequencing was done at the University of Tennessee Molecular Biology Research Facility.[f] Sequencing was done in both directions by using the F19 and the F25 primers. The initial sequencing data were used to design additional primers for sequencing the flanking regions (figure 2 and table 1). This strategy was repeated to obtain the sequence of the whole product by assembly of overlapping regions.

Detection of ruminant RSV (bovine and ovine RSV) and differentiation between them by using RT-PCR: RT-PCR targeting a specific fragment in both bovine and ovine RSV F genes was used to detect both bovine and ovine RSV and to differentiate between them.

1- Virus and cells: Ovine respiratory syncytial virus strain WSU 83-1578 [a] and bovine respiratory syncytial virus 391-2 [g] were grown on MDBK cells as described before. Other isolates of bovine RSV (375 [a], FS1-1 [a], NMK-7 [a], MN [i], and NY191285 [i]),

Figure 2: Sequencing strategy of ORSV F gene and F-M2 intergenic region

F19 primer was used to get 1a fragment sequence located in the 5' end and F25 primer was used to get 2a fragment located in the 3' end. From 1a and 2a fragments, additional primers were designed (F238 and F255 from 1a fragment and F410 and F503 from 2a fragment). This strategy was repeated until sequence of the whole product was obtained by assembly of overlapping regions. 1a, 1b, 1c, 1d, 1e, 1f, 1g, 2a, 2b and 2c represent the overlapping fragments. The primers used for sequencing of each fragment are between parentheses. The arrows indicate the direction of amplification.

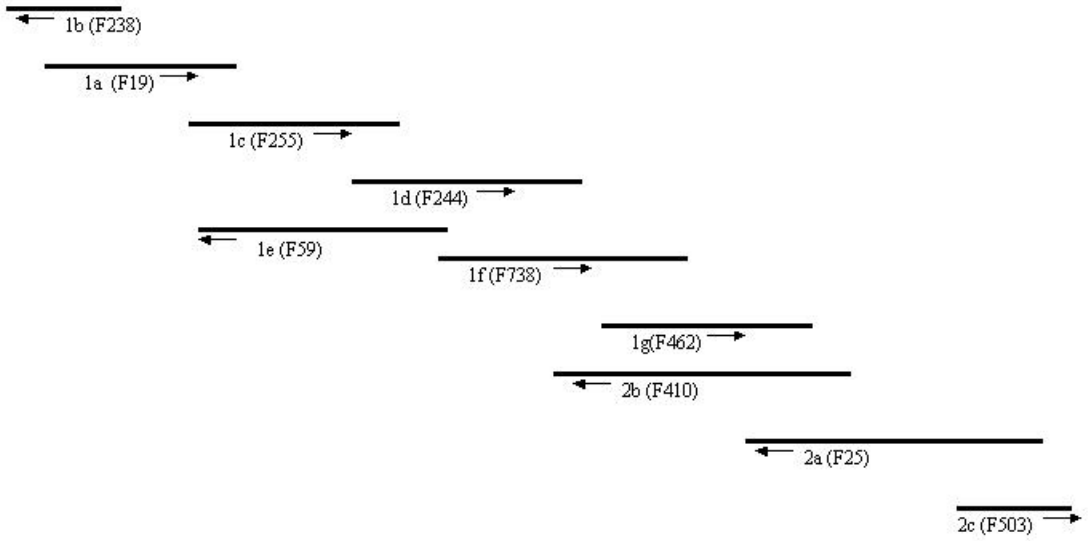
1b (F238)
1a (F19)
1c (F255)
1d (F244)
1e (F59)
1f (F738)
1g(F462)
2b (F410)
2a (F25)
2c (F503)

Table 1: Primers used for sequencing of ORSV F gene and F-M2 intergenic region

Primers used to sequence the positive sense regions (1a, 1c, 1d, 1f, 1g and 2c)	Primers use to sequence the negative sense regions (2a, 2b, 1e and 1b)
F19 (GAGACAAGGGTGTATATAG)	F25 (TGATCTCATATTTGCAGGG)
F255 (AGCAGTACTGATTCAAAGG)	F410 (CACTAAGCTGATCTTTTCC)
F244 (TTCTCACTAGCAAAGTGC)	F59 (AGGCATATCATTAATCAGTG)
F738 (GAATTTAGTATAAATGCTGG)	F238 (ACCTTTGAATCAGTACTGC)
F462 (TACTGCCAAATATGACTGC)	
F503 (CATGATCTGAACCTCAAAC)	

human RSV subgroup A (strain A2 [g]), and human RSV subgroup B (strain B8/60 [g]) were included in the study. Human RSV strains were grown on vero cells.

1- RNA extraction and quantification were done as described above.

2- Primers: Two primers were developed and synthesized.[c] The F103 primer (5' TGCCAAAACATAACAGAAG 3') was used for cDNA synthesis. It represents a conserved sequence in both ovine and bovine RSV F genes; bases 85-103 of ovine RSV F gene (determined in this study) and bases 86-104 of bovine RSV F gene.[17] The other primer; F493 (5' TTTATTCACCTCTCCCTC 3') represents a conserved sequence in both ovine and bovine RSV F genes. It is complementary to bases 510-493 of the ovine RSV F gene (determined in this study) and bases 511-494 of bovine RSV F gene.[17] These primers (F493 and F103) were used for amplification of specific fragment of F genes of both bovine and ovine RSV.

3- **PCR:** Taq DNA polymerase [c] was used for amplification of a 426 bp fragment of F gene of both bovine and ovine RSV according to the manufacturer's recommendations. In brief, the following components were added for a final reaction volume of 100 µl: Ten µl of 10x PCR buffer [c], three µl of 50 mM Mgcl2 [c] , two µl of 10 mM dNTP mix [c], one µl of 10 uM F 493 primer, one µl of 10uM F103 primer, one µl taq DNA polymerase , two µl of cDNA and 80 µl of water. Amplification was done using the same conditions mentioned before except using of 58^{o}C for annealing instead of 50^{o}C.

4- Restricticton enzyme cleavage and electrophoresis: EcoRI [c] and MspI [c] restriction enzymes were used separately to cleave each of the 426 bp amplified products of bovine and ovine RSV F genes. Four µl of unpurified PCR product, four µl of water, one µl of the restriction enzyme used and one µl of the buffer supplied with the enzyme

were added together and incubated for one hour at 37 C. After incubation, electrophoresis was done using three percent high-resolution agarose.[h]

Results

Nucleotide sequence of the ovine RSV F gene and F-M2 intergenic region:

Figure 3 shows the PCR product representing the F gene, the F-M2 intergenic region and the M2 gene 5' end of ovine RSV. After extraction of this, approximately 2000 bp, PCR product from the gel, its sequence was determined (Fig. 4).

A comparison of this sequence (Gene Bank accession number AF334398) with representative published sequences of bovine RSV and human RSV F genes [13,17, 18] indicated that the sequence from 1-1896 bases most likely represents the F gene of ovine RSV (Fig.4a) and the sequence from 1897-1955 bases represents F-M2 intergenic region (Fig.4b). The sequence from 1956- 1992 bases was identical to bases 1-37 of the 5' end of the published sequence of M2 gene of ovine RSV.[4] The ovine RSV F gene has 85%, 73% and 72% nucleotide identity with those of bovine RSV, [17] human RSV subgroup A [18] and human RSV B subgroup [13] respectively (Fig.4a and Table 2). The coding sequence of ovine RSV gene, represented by bases 13-1737, is more conserved than the non-coding sequence (1738-1896). However, the 3' end of the non-coding region (bases 1887-1896) is highly conserved (Fig.4a and Table 2).

The open reading frame of ovine RSV F gene predicts a polypeptide of 574 amino acids. The predicted amino acid identity between the F protein of ovine RSV and those of bovine RSV, [17] human RSV subgroup A [18] and human RSV subgroup B [13] is 94%, 83%

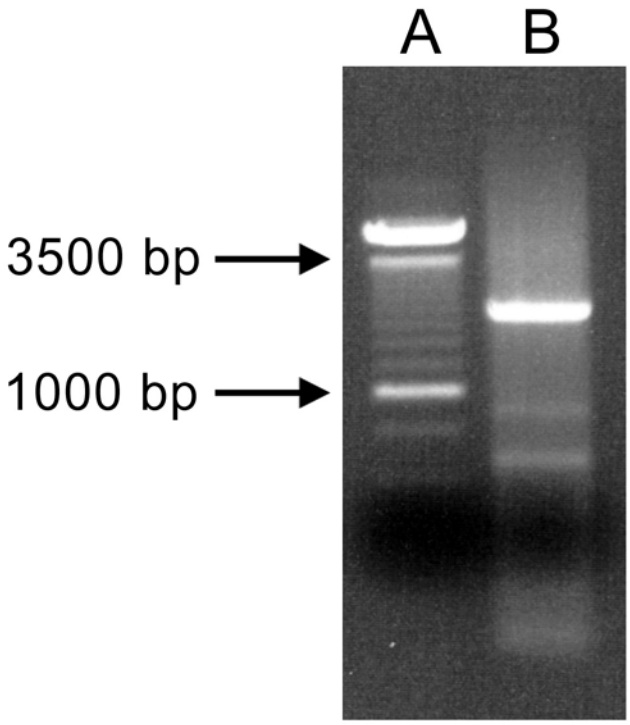

Figure 3: The PCR product representing the ovine RSV F gene, the F-M2 intergenic region and the 5' end of the M2 gene (1992 bp) is in lane B. The 250 bp DNA ladder is in lane A.

Figure 4: Nucleotide sequence of ovine RSV F gene and F-M2 intergenic region and comparison with other RSVs.

(a) Comparison of nucleotide sequence of ovine RSV F gene strain WSU 83-1578 with those of bovine RSV (strain 391-2), human RSV(A) Long strain and human RSV(B) strain 18537. The dots above the sequence are spaced every 10 nucleotides and the number of the last nucleotide for each line is given on the right end of the line. Only the non-identical bases are indicated for the bovine and human RS viruses. (b) Nucleotide sequence of F-M2 intergenic region (1897-1955)and the M2 gene 5' end (1956-1992).

(a)

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                    .         .         .         .         .         .
ORSV (83-1578) .GGGGCAAATAAAATGGCGACAATAGCCATGAAGATAATCACCAGCACCATCTTTATCTC 59
BRSV (391-2)   NNNNNNN TA GG      G   C        G   G    T     TT     C      60
HRSV (Long)    G   CA  TA C     A TTGCC AT C C  AGC  ATG A TT    CAA CC  G  60
HRSV (18537)   NN  NN  TA CC    A TTGC GAT CAC G TC  GTG A T TT C AAC C TG  60

                    .         .         .         .         .         .
ORSV (83-1578) TATCTCTGTATTGCATATCTCTTTGTGCCAAAACATAACAGAAGAATTTTATCAATCAAC 119
BRSV (391-2)     C  A A GACA      A    A                                   120
HRSV (Long)     GCAGTCAC   TTG T TG   CTA T        C  T                    120
HRSV (18537)    G TAA  C    T CC  A C CAA T  G        T  G  G     C     G   120

                    .         .         .         .         .         .
ORSV (83-1578) ATGTAGTGCAGTTAGTAGAGGTTATCTCAGTGCATTACGAACTGGATGGTACACAAGTGT 179
BRSV (391-2)      C                      T         A            T         180
HRSV (Long)       C           C A   C     T     TC  A       T     T  T      180
HRSV (18537)                  C         T T     T   A    A  T     T  C      180

                    .         .         .         .         .         .
ORSV (83-1578) GGTGACAATAGAATTGAGCAACATACAAAAAAATGTATGTAGCAGTACTGATTCAAAGGT 239
BRSV (391-2)   A  A        G         A        G     G    AA             A   240
HRSV (Long)    TA A  T        A  T  T  CA GG     AAG    ATG A  A   G C      240
HRSV (18537)   CA A           A  T  T   A  G   CCAA   C ATG A     CA T  A   240

                    .         .         .         .         .         .
ORSV (83-1578) AAAATTGATAAAGCAAGAGTTAGAAAGATATAATAATGCAGTAGCTGAGTTACAGTTGCT 299
BRSV (391-2)   G     A          AC G        C  C         ATA  A  G    CA   300
HRSV (Long)        C       A     A     T A      A     T   A A  A  G         300
HRSV (18537)       C T     A     A     T AG     G         A A  A      C A   300

                    .         .         .         .         .         .
ORSV (83-1578) CATGCAAAATACACCGTCTATCACCAACAGGGCCAAAAGAGGAATATCAGAGTTGGTTCA 359
BRSV (391-2)   T         GA    G  TC TT  GT  A  A        G   C        A A   360
HRSV (Long)            GC     AG AGCA A   TC A    G     A C  C  AG   TA GA  360
HRSV (18537)   T        C  G  AG  GC  A    C      G     A GC C  C   ACA GA  360

                    .         .         .         .         .         .
ORSV (83-1578) CTATACAAAGAATTCCACTAAGAGATTTTATGGTCTAATGGGCAAAAAAAGAAAAAGGAG 419
BRSV (391-2)   T       GA  C  T  A             GT          G  G     G      420
HRSV (Long)    T      CTC  CAAT  C  A A ACCA   TAAC T AA    G     G     A   420
HRSV (18537)      C    TC   A    A  A ACC AA   TATC   AA    G     G   C A   420

                    .         .         .         .         .         .
ORSV (83-1578) GTTCTTAGGGTTCTTGCTTGGTATAGGGTCTGCTATTGCGAGTGGAATAGCAGTGTCTAA 479
BRSV (391-2)   A  T     A        A     T  A          A     TG         C   480
HRSV (Long)    A  TC T  T  T   T A   G T  A     A  C  C     C CT  T  A      480
HRSV (18537)   A  TC G  C      T A   G    A     A  A  A     T     T  A  C   480

                    .         .         .         .         .         .
ORSV (83-1578) AGTATTACATTTAGAGGGAGAGGTGAATAAAATTAAGAATGCACTGCTATCTACAAATAA 539
BRSV (391-2)       C    CC G                       A             C         540
HRSV (Long)    G  CC G  C     A     A     C  G  C  A G   T  A     C     C   540
HRSV (18537)       T     CC T  A     A     C     C  A     TT  T G         C   540

                    .         .         .         .         .         .
ORSV (83-1578) AGCAGTGGTTAGTCTATCTAATGGAGTCAGTGTTCTCACTAGCAAAGTGCTTGATCTAAA 599
BRSV (391-2)         A          C        T     C  T          A            600
HRSV (Long)    G  C  A  C  CT    A        T     CT A  C         T A  C  C   600
HRSV (18537)       T  A  C        A     G         T A  C         T A     C   600

                    .         .         .         .         .         .
ORSV (83-1578) AAATTATATAGACAAAGAGCTTTTACCTAAAGTCAACAATCATGATTGTAGGATTTCCAA 659
BRSV (391-2)   G  C                 C          T                    A       660
HRSV (Long)       C        T   C AT G       TT  G  T  G  AAGC  C  A  A  A   660
HRSV (18537)   G     C   A T  CCGAT A     C T   A  TC A  GAGC   C C  C      660
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                    .         .         .         .         .         .
ORSV (83-1578) TATAGAAACTGTAATAGAATTCCAACAAAAAAACAATAGATTGTTAGAGATTGCCAGGGA 719
BRSV (391-2)   C          G                                   A     T      720
HRSV (Long)                G     G           G     C   C AC        A      720
HRSV (18537)   C  T     A  T           G  G TG  T GC        G  A  CA    A   720

                    .         .         .         .         .         .
ORSV (83-1578) ATTTAGTATAAATGCTGGTGTCACTACACCTCTAAGCACATACATGTTGACCAATAGTGA 779
BRSV (391-2)          G           A T  C     C  C  T                       780
HRSV (Long)           G T     A     A         G       T        A  T        780
HRSV (18537)          G T     A     A  A      T       T        A  A  C     780

                    .         .         .         .         .         .
ORSV (83-1578) ATTACTTTCACTGATTAATGATATGCCTATAACAAATGACCAGAAAAAATTAATGTCAAG 839
BRSV (391-2)         A     A                     G        A     GC          840
HRSV (Long)        T G   T A  C                        T        G        C A 840
HRSV (18537)   G     A   T    C                                             840

                    .         .         .         .         .         .
ORSV (83-1578) TAATGTTCAAATAGTTAGGCAACAAAGTTATTCTATTATGTCGGTGGTCAAAGAAGAGGT 899
BRSV (391-2)                 C  A     G        C        A                  900
HRSV (Long)    C                A  G        C     C     CA AA A     G  A   900
HRSV (18537)   C        G     A                  C     TA AA A  G     A   900

                    .         .         .         .         .         .
ORSV (83-1578) TATAGCCTATGTTGTACAATTACCGCTTTATGGGGTGATAGATACACCTTGTTGGAAAAT 959
BRSV (391-2)   C     T             G  TA       A  T     C  C  C         C  960
HRSV (Long)    CT    A     A          A  A     T                       T  960
HRSV (18537)   CC T  A          GC    TA C     T  A             C      T  960

                    .         .         .         .         .         .
ORSV (83-1578) ACATACCTCTCCGTTATGTACTACTGATAACAAAGAGGGATCAAATATTTGCTTAACTAG 1019
BRSV (391-2)      C        A     C  C        T     A  G     C  C           1020
HRSV (Long)       C  A  C  TC       A  CA C CA     A  G     C  C  T     A   1020
HRSV (18537)      C  A  A  TC    C  C  CA C T      A             T     A   1020

                    .         .         .         .         .         .
ORSV (83-1578) AACAGATCGTGGGTGGTACTGTGACAATGCAGGTTCTGTGTCTTTTTTCCCACAAGCAGA 1079
BRSV (391-2)   G               T              C                     G      1080
HRSV (Long)       T  CA A  A                  A  A  A     C          T   1080
HRSV (18537)   G  T   A A  A     T     T       A  A  A  C  C       G  T   1080

                    .         .         .         .         .         .
ORSV (83-1578) AACATGTAAAGTACAATCAAATAGAGTATTCTGTGATACAATGAACAGTTTAACTTTACC 1139
BRSV (391-2)   G        G                G       C                 C G   1140
HRSV (Long)                T     G   C       T     C               A     1140
HRSV (18537)   T  T  C        G  C   C       T     C  T            A     1140

                    .         .         .         .         .         .
ORSV (83-1578) CACTGATGTTAATTTGTGCAATACAGACATATTTACTGCCAAATATGACTGCAAAATAAT 1199
BRSV (391-2)   T          C  A     C  T       C A A A  G       T        1200
HRSV (Long)    A G   A  A   C C      GTT       C A C         T  T     T   1200
HRSV (18537)   A G   A  C GCC T  T  C  T       C A T   G             T   1200

                    .         .         .         .         .         .
ORSV (83-1578) GACATCTAAAACTGACATAAGTAGCTCTGTGATCACCTCACTAGGAGCTATTGTATCATG 1259
BRSV (391-2)                                   A  T   A                  1260
HRSV (Long)       T  A     A  TG    C     C  T     A  T       C     G      1260
HRSV (18537)         A     A        C     A  A  T  T  T  T       A  G      1260

                    .         .         .         .         .         .
ORSV (83-1578) CTATGGCAAAACAAAATGCACTGCTTCCAACAAAAATCGTGGAATCATTAAGACTTTTTC 1319
BRSV (391-2)         G  G        T  A     T  T                A           1320
HRSV (Long)               T     T  A  A     T                A     A      1320
HRSV (18537)         A     T           A     T           G  T  A     A      1320
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                    .         .         .         .         .         .
ORSV (83-1578) CAATGGGTGTGATTATGTATCAAACAAGGGAGTTGATACTGTATCTGTAGGTAATACATT 1379
BRSV (391-2)                              A         C           T     C   C  1380
HRSV (Long)    T  C              C      T  A  G  A  C     G           C      1380
HRSV (18537)   T     T     C     G        A     A        G  A  G  C  C  T   1380

                    .         .         .         .         .         .
ORSV (83-1578) ATATTATGTAAATAAGCTAGAAGGGAAAGCACTTTATGTAAAAGGTGAGCCAATTATTAA 1439
BRSV (391-2)                         G           C   A    G     A           1440
HRSV (Long)                    A      C   AGT  C             A     A  A   1440
HRSV (18537)      C        C     G     C   AAC              G  A  T  A  A   1440

                    .         .         .         .         .         .
ORSV (83-1578) TTATTACGATCCACTGGTGTTCCCCTCAGATGAATTCGATGCTTCAATTGCTCAAGTCAA 1499
BRSV (391-2)      C  T        A     T  T  T     G  T     A        C     A   1450
HRSV (Long)     TC  T  C   T A  A        T        T     A     AT           1450
HRSV (18537)      C  T     T  A     T  T  T     G  T     A     AT           1450

                    .         .         .         .         .         .
ORSV (83-1578) TGCAAAAATAAACCAGAGCTTAGCTTTTATTCGTCGATCTGACAAGTTACTTCATAGTGT 1559
BRSV (391-2)                  A   C G     C  A           TG         C      1560
HRSV (Long)      AG  G  T        T     A         AA   C  TG A   T A   CA    1560
HRSV (18537)     A      C  T  A  T               A       TG A     A    A    1560

                    .         .         .         .         .         .
ORSV (83-1578) AGATGTAGGAAAATCTACCACAAATGTAGTGATTACTACCATTATCATAGTAATAGTTGT 1619
BRSV (391-2)                  C              A        T     T     G         1620
HRSV (Long)     A   CT  T     A         A CA    A     T  A  T     G  TA A   1620
HRSV (18537)    A  ACT  C        T      A TA    A     A     T        CA     1620

                    .         .         .         .         .         .
ORSV (83-1578) TATTGTATTGATGTTGATAGCTGTGGGGTTACTTTTTTACTGTAAGACTAAAAGTACACC 1679
BRSV (391-2)   AG GA    A     A        A  A     G             C GG     T   1680
HRSV (Long)    A  AT G  ATCA  A  T     T  AC G  CC A         G C G   C      1680
HRSV (18537)   AG AT G  ATCA  A      A T  T     G  G  T  C  AG C    AC      1680

                    .         .         .         .         .         .
ORSV (83-1578) CATCATGTTAGGAAAAGATCAGCTTAGTGGCATCAATAATCTTGCTTTTAGCAAGTGAAG 1739
BRSV (391-2) T      C       G            T     C      T C     T  A    A   1740
HRSV (Long)    AG   CAC  A C  G     A  G     T  A      A    A     T  C    T 1740
HRSV (18537)   AG T CAC  A C     C  A  A     A         A    A  C     A AG C 1740

                    .         .         .         .         .         .
ORSV (83-1578) TGTAAAATATTCATGATCTGAACCTCAAACTCATCTACAACCTGAGTCAGAAATATCTAT 1799
BRSV (391-2)     C T   G  T CA    A       G A    AA TGTGA    CT A TT   TA   1800
HRSV (Long)    AAA  T GCAC TAATCA  TT T AC  TGGT TACT T TGCTCAT   C ACC ATC 1800
HRSV (18537)   AAA  . CTACTTAATCA  TTT AAC   AATCTGCTG   ACCAATCCC  ATCAAC  1799

                    .         .         .         .         .         .
ORSV (83-1578) ACATACCAGAACCCCACCCACCAAGAC..TCACACATTTGTTGGATCTTGAACCACCCAA 1857
BRSV (391-2)       T A A GTT T T  G       CTG  TTTT   A CA G    AT TA G T   1860
HRSV (Long)    TATC TTG  TTTT TTAA ATCT  ACT   TCG AACTC TATCTA A     T TC  1860
HRSV (18537)   TA C A  A TAT..TT A  ATCATAGCA  GG TGAATCATTTC  CAT T  TG T  1857

                    .         .         .
ORSV (83-1578) CCATACATGCTACATTTAGCTTCATGTCCATAGTTATAT 1896
BRSV (391-2)     T          C C    C     TG          1899
HRSV (Long)     TTACAC AT TA G AGAT C TA  TT          1899
HRSV (18537)     TACACAA   AGC AGATC TCAACT       C   1896
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(b)

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                    .         .         .         .         .         .
               AAAAATGTTATAATAGCACCCCATAATTGAACAAATTAATGACCAACCAATTAGAAAGTG 1956
                    .         .         .         .         .         .
               GGGCAAATATGTCACGAAGAAATCCCTGCAAATATG 1992
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Table 2: Percentage of nucleotide identity among F protein genes of respiratory syncytial viruses [ORSV strain WSU 83-1587, BRSV strain 391-2, HRSV (A) Long strain and HRSV (B) strain 18537]

	ORSV X BRSV	ORSV X HRSV (A)	ORSV X HRSV (B)	BRSV X HRSV (A)	BRSV X HRSV (B)	HRSV (A) X HRSV (B)
Signal peptide	77	43	44	37	36	61
F2	83	72	73	73	71	82
F1	88	79	78	78	78	84
Coding sequences	87	76	75	75	75	82
Non-coding sequence	67	46	45	45	38	53
Overall F gene	85	73	72	73	72	80

Fig. 5: Comparison of the predicted amino acids of the ORSV F protein with those of BRSV strain 391-2, HRSV (A) Long strain and HRSV (B) strain 18537. The dots above the sequence are spaced every 10 amino acids and the number of the last amino acid for each line is shown to be the right end of the line. Only the non-identical amino acids are indicated for the bovine and human RS viruses. The amino terminal hydrophobic domain (presumptive signal peptide) and the carboxy terminal domain (membrane anchorage domain) are underlined. The hydrophobic sequence at the N terminus of F1 is denoted by double underline. Potential N-linked glycosylation sites are highlighted and cysteine residues are indicated by solid triangles.

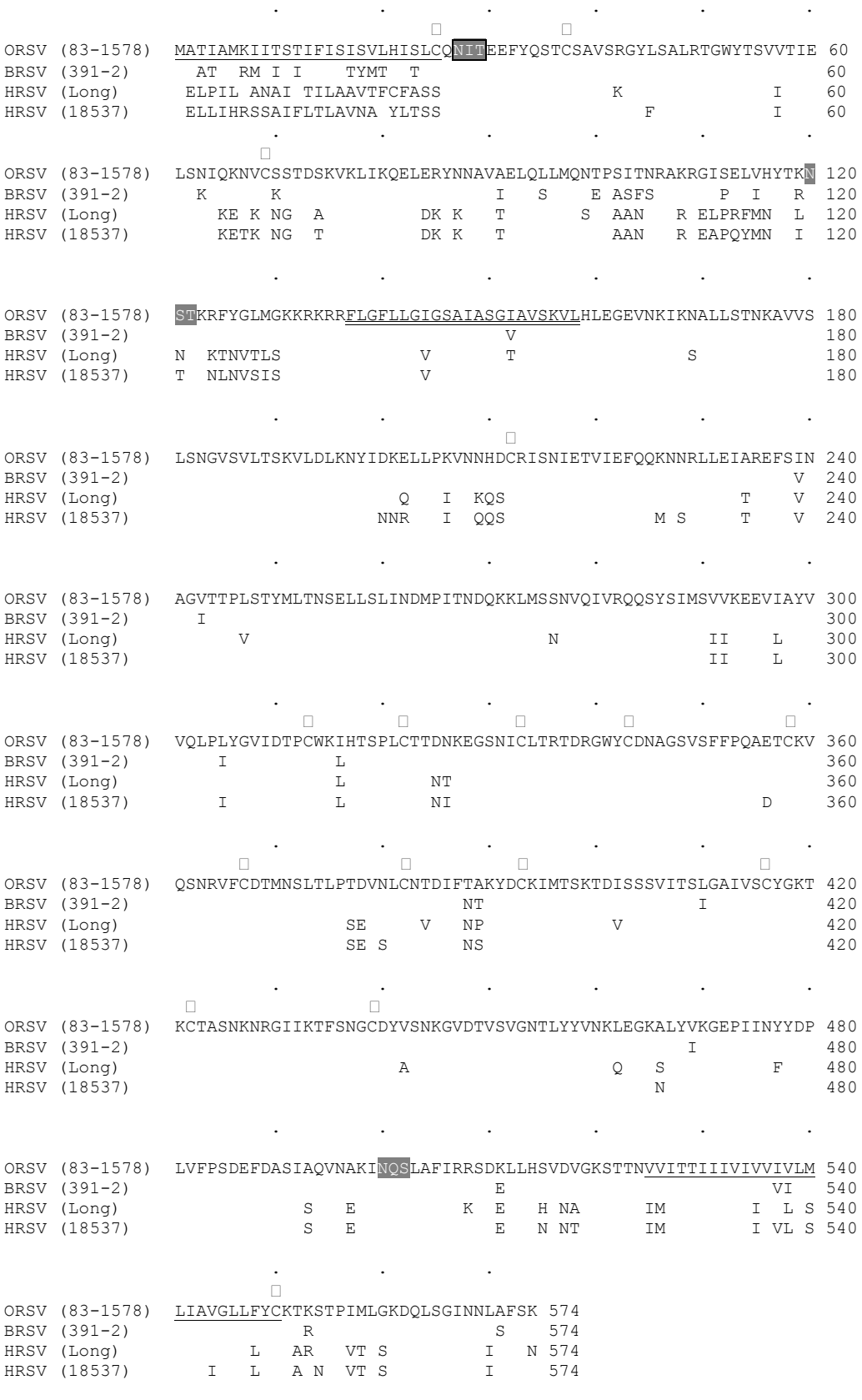
ORSV (83-1578) MATIAMKIITSTIFISISVLHISLCQNITEEFYQSTCSAVSRGYLSALRTGWYTSVVTIE 60
BRSV (391-2) AT RM I I TYMT T 60
HRSV (Long) ELPIL ANAI TILAAVTFCFASS K I 60
HRSV (18537) ELLIHRSSAIFLTLAVNA YLTSS F I 60
ORSV (83-1578) LSNIQKNVCSSTDSKVKLIKQELERYNNAVAELQLLMQNTPSITNRAKRGISELVHYTKN 120
BRSV (391-2) K K I S E ASFS P I R 120
HRSV (Long) KE K NG A DK K T S AAN R ELPRFMN L 120
HRSV (18537) KETK NG T DK K T AAN R EAPQYMN I 120
ORSV (83-1578) STKRFYGLMGKKRKRRFLGFLLGIGSAIASGIAVSKVLHLEGEVNKIKNALLSTNKAVVS 180
BRSV (391-2) V 180
HRSV (Long) N KTNVTLS V T S 180
HRSV (18537) T NLNVSIS V 180
ORSV (83-1578) LSNGVSVLTSKVLDLKNYIDKELLPKVNNHDCRISNIETVIEFQQKNNRLLEIAREFSIN 240
BRSV (391-2) V 240
HRSV (Long) Q I KQS T V 240
HRSV (18537) NNR I QQS M S T V 240
ORSV (83-1578) AGVTTPLSTYMLTNSELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSVVKEEVIAYV 300
BRSV (391-2) I 300
HRSV (Long) V N II L 300
HRSV (18537) II L 300
ORSV (83-1578) VQLPLYGVIDTPCWKIHTSPLCTTDNKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKV 360
BRSV (391-2) I L 360
HRSV (Long) L NT 360
HRSV (18537) I L NI D 360
ORSV (83-1578) QSNRVFCDTMNSLTLPTDVNLCNTDIFTAKYDCKIMTSKTDISSSVITSLGAIVSCYGKT 420
BRSV (391-2) NT I 420
HRSV (Long) SE V NP V 420
HRSV (18537) SE S NS 420
ORSV (83-1578) KCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNKLEGKALYVKGEPIINYYDP 480
BRSV (391-2) I 480
HRSV (Long) A Q S F 480
HRSV (18537) N 480
ORSV (83-1578) LVFPSDEFDASIAQVNAKINQSLAFIRRSDKLLHSVDVGKSTTNVVITTIIIVIVVIVLM 540
BRSV (391-2) E VI 540
HRSV (Long) S E K E H NA IM I L S 540
HRSV (18537) S E E N NT IM I VL S 540
ORSV (83-1578) LIAVGLLFYCKTKSTPIMLGKDQLSGINNLAFSK 574
BRSV (391-2) R S 574
HRSV (Long) L AR VT S I N 574
HRSV (18537) I L A N VT S I 574

Table 3: Percentage of amino acid identity among F proteins of respiratory syncytial viruses. [ORSV strain WSU 83-1587, BRSV strain 391-2, HRSV (A) Long strain and HRSV (B) strain 18537]

	ORSV X BRSV	ORSV X HRSV (A)	ORSV X HRSV (B)	BRSV X HRSV (A)	BRSV X HRSV (B)	HRSV (A) X HRSV (B)
Signal peptide	56	12	8	4	12	40
F2	89	70	70	68	68	87
F1	97	89	89	89	89	93
F protein	94	82	82	81	82	90

and 84% respectively (Fig. 5 and Table 3).

The ovine RSV F1 subunit has 97% and 89% amino acid identity with those of bovine RSV [17] and human RSV A&B [18, 13] respectively, whereas the F2 subunit identity is somewhat lower, 89% and 70%, respectively (Fig. 5 and Table 3). The predicted signal peptide amino acid sequence of ovine RSV F protein shows extensive divergence from those of bovine and human RSV (Fig. 5 and Table 3). The positions of cysteine residues in ovine RSV F protein are conserved with respect to bovine and human RSV except in one position (residue 25), which is conserved only in ovine and bovine RSV (Fig. 5). Amino acid residues 131-136 in the ovine RSV F protein sequence is identical to the proposed cleavage signal in bovine and human RSV F proteins.[17, 13] Hydrophobic residues (137-158) likely represent the proposed amino terminus of F1 subunit after cleavage, while hydrophobic residues 525-550 may represent the proposed membrane anchor. Three potential sites for N-linked glycosylation have been found in the predicted ovine RSV F protein; 2 in F2 subunit (residues 27 and 120) and one in F1 subunit (residue 500) (Fig. 5).

Detection of ruminant RSV and differentiation between its two subgroups:

An RT-PCR assay targeting the F gene resulted in amplification of a 426 bp product in both bovine and ovine RSV (Fig. 6). Nucleotide sequencing of both products (data not given) confirmed the fidelity of this PCR assay. Digestion of both products by EcoRI restriction endonuclease cleaved the ovine PCR product into 2 bands (285 bp and 141 bp), but did not affect the bovine product (Fig.6). In contrast, MspI restriction endonuclease cleaved the bovine PCR product into 2 bands (229 bp and 197 bp), but had no effect on the ovine product (Fig.6).

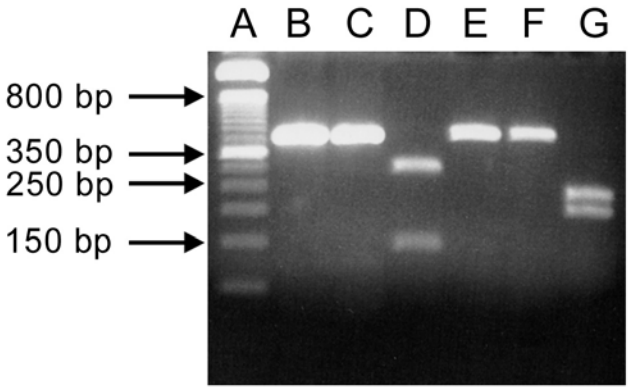

Figure 6: Detection of bovine and ovine RSV and differentiation between them by RT-PCR followed by restriction enzyme cleavage. Lane A contains the 50 bp DNA ladder. Lanes B and C represent the PCR products obtained from ovine and bovine RSV respectively (426 bp). Lane D contains the cleavage products (285 bp and 141 bp) of the ovine RSV PCR product after EcoRI treatment. Lane E contains the bovine RSV PCR product (426 bp) after EcoRI treatment. The ovine RSV PCR product (426 bp) after MspI treatment is represented in lane F. Cleavage products of the bovine RSV PCR product (229 bp and 197 bp) after MspI treatment are in lane G.

All bovine RSV isolates tested (375, FS1-1, NMK-7, MN, and NY191285) gave the same PCR product and the same cleavage pattern obtained with bovine RSV strain 391-2. All of these bovine isolates could be differentiated from the ovine RSV. No PCR products resulted when human RSV subgroup A (strain A2), and human RSV subgroup B (strain B8/60) were used in the assay.

Discussion

Ovine RSV F gene has 85% and 72-73% nucleotide identity with those of bovine RSV and human RSV respectively. The predicted amino acid sequence of the ovine RSV F gene has 94% and 82% amino acid identity with those of bovine RSV and human RSV respectively.

The level of identity between ovine and bovine RSV F genes (85%) and their predicted proteins (94%) are close to those found between the two human subgroups A and B, 80% nucleotide identity and 90% amino acid identity (Table 2 and 3). This supports the existence of two ruminant subgroups, one representing bovine RSV and the other representing ovine RSV.[3, 2, 24]

The level of identity between ovine and human RSV F predicted proteins (82%) is similar to that found between bovine and human RSV (81-82%). The positions of the 3 potential N-linked glycosylation sites in ovine RSV F predicted protein coincide with that found in bovine and human RSV.[17, 13] But, human RSV strains have 2-3 additional sites in the F2 peptide.[13, 6] The difference in the extent of glycosylation of F2 is responsible for the difference in electrophoretic mobility of F2. [17,20]

A novel neutralization site (residues 173-182) has been mapped in bovine RSV F protein by using a neutralizing monoclonal antibody against F protein.[15] This site is conserved in the ovine sequence as well as in the human RSV F proteins (Fig. 5). This conserved linear epitope may be a potential candidate for peptide-based vaccine against all groups and subgroups of RSV.[15]

PCR assays have been used in several studies for detection of bovine RSV. [33, 21, 35, 32, 16.] In one study specific bovine RSV primers amplified bovine RSV strains, but did not amplify ovine RSV. PCR assay used in a previous study was able to detect the bovine RSV, but not the ovine RSV.[21] It has been found that ovine RSV does infect cattle.[12] The PCR assay described in the present study has the capacity to detect both bovine and ovine RSV by using one set of primers and subsequent differentiation between them by a simple procedure.

The two primers used in this PCR assay represent conserved sequences in ovine and bovine RSV F genes that are not conserved in the human RSV F gene. This allowed clear differentiation between ruminant RSV and human RSV. RSV PCR assays depend upon detection of viral RNA or mRNA transcripts that are present in active infections, whereas serological tests depend upon detection of antibodies, which develop during the convalescent phase of infection. Therefore, an advantage of PCR tests over serological tests is that earlier detection of infection is possible. Also, conventional serological diagnosis is dependent upon paired serum samples taken during the acute and convalescent phases of the disease and, unlike the PCR test in the present study, are not capable of discriminating between the ruminant RSV subgroups. This PCR test can by used to detect both bovine and ovine RSV in cattle and may be useful for determining the

contribution of ovine RSV in the pathogenesis of bovine respiratory tract disease. Moreover, data from some previous studies indicated that PCR assays are more sensitive than IF [33] and ELISA [16] for the detection of BRSV antigen.

Sources and manufacturers

a. Provided by Dr Howard Lehmkuhl, National Animal Disease Laboratory, Ames, Iowa.

b. Bio-Whittaker, Walkersville, MD.

c. Gibco-BRL, Gaithersburg, MD.

d. Perkin Elmer Gene Amp PCR system 9600, Perkin Elmer Inc., Norwalk, CA.

e. QIA quick gel extraction kit, Qiagen Inc., Valencia, CA.

f. Using an ABI prism dye terminator cycle sequencing reaction kit and an ABI 373 DNA sequencer, Perkin Elmer Inc., Foster city, CA.

g. Provided by Dr. Gail Wertz, Dept. of Microbiology, Univ. of Alabama Medical School, Birmingham, AL.

h. Sigma Chemicals Co., St. Louis, MO.

i. Provided by Dr. John Baker, Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan.

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Part III

RT-PCR for simultaneous detection of bovine and ovine respiratory syncytial viruses

Abstract

Whether ovine RSV contributes to bovine respiratory disease is relevant to the complete understanding of RSV epidemiology and vaccine development in cattle and sheep. Currently a laboratory test to differentiate between bovine and ovine RSVs does not exist. Two RT-PCR assays have been developed for simultaneous detection of bovine and ovine respiratory syncytial viruses. One, a set of primers amplified a 426 bp fragment of either bovine or ovine RSV F gene (RT-PCR F). PCR-F products could be distinguished by EcoRI or BstYI restriction endonuclease cleavage. In the other assay, a set of primers amplified a 542 bp fragment of either ovine or bovine RSV G gene (RT-PCR G). EcoO109I and RsaI restriction enzymes were used to differentiate between the ovine and bovine PCR-G products. Sequencing of the PCR products confirmed the fidelity of both assays. The two assays were evaluated using eight bovine RSV isolates, one ovine RSV, one bighorn sheep RSV, one caprine RSV, two human RSV isolates and several viruses associated with bovine respiratory tract disease. The two PCR assays described in this study potentially constitute sensitive and specific means for simultaneous detection of bovine and ovine RSV and differentiation between them. However, RT-PCR F followed by the appropriate restriction enzyme cleavage may be superior to RT-PCR G to discriminate between the two ruminant RSV subgroups and for determining the relative contribution of ovine and bovine RSV to the pathogenesis of bovine respiratory tract disease. This is relevant to the complete understanding of RSV epidemiology and immunoprophylaxis.

Keywords: RSV; diagnosis; PCR

Introduction

Respiratory syncytial viruses (RSVs) are major causes of severe respiratory tract disease in young children (Avila et al., 1989) and calves (Bryson et al., 1978; Pirie et al.,1981; Kimman et al., 1988). Ovine RSV was isolated from a one-year-old ewe with rhinitis (Evermann et al., 1985). Bighorn sheep RSV was isolated from respiratory disease outbreak in a captive herd of Bighorn sheep (Evermann et al., 1994).

Respiratory syncytial viruses are classified in the genus Pneumovirus, which is included in the family Paramyxoviridae. The G (attachment protein) and F (fusion protein) are the two major surface glycoproteins encoded by respiratory syncytial viruses.

The ovine RSV F gene and its encoded protein have 85% nucleotide identity and 94% amino acid identity with that of bovine RSV (Eleraky et al., 2001). While, the ovine RSV G gene and its encoded protein share 70% and 62% nucleotide and amino acid identity to the equivalent gene and its encoded protein, respectively, of bovine RSV (Alansari and Potgieter, 1993).

It has been proposed that ungulate RSVs be divided into 2 subgroups, one representing bovine RSV, and the other representing ovine RSV, based on analysis of ruminant RSVs by RNAse protection of the G glycoprotein transcripts (Alansari et al., 1999), nucleotide sequence of ORSV-G protein gene (Alansari et al., 1993) and reactivity of ungulate RSVs to monoclonal antibodies specific to BRSV-F protein (Pastey and Samal, 1997).

Experimental infection with ovine RSV in calves resulted in mild to moderate clinical respiratory disease (Bryson et al., 1988). It has been suggested that two related but antigenically distinguishable RSV types are present in sheep, one of which was

similar to bovine strains and the other had a different fluorescence pattern (Adair and McFerran, 1987). Antibodies specific for either ovine or bovine subgroup strains of RSV were detected in cattle by using a subgroup-specific G-peptide ELISA, suggesting that ovine RSV also infects cattle (Grubbs et al., 2001).

Whether ovine RSV contributes to bovine respiratory disease or not is relevant to the complete understanding of RSV epidemiology and vaccine development in cattle and sheep. BRSV isolation is difficult because of its lability (Smith et al., 1974). PCR assays may be more sensitive than IF (Vilcek et al., 1994; Valarcher et al., 1999) and ELISA (Larsen et al., 1999; Valarcher et al., 1999) and as specific as IF and ELISA (Valarcher et al., 1999) for the detection of bovine RSV infections. PCR assays have been recommended for diagnosis of bovine RSV infection in cattle (Oberst et al., 1993; Vilcek et al., 1994; Valarcher et al., 1999; Larsen et al., 1999).

Currently a laboratory test to differentiate between bovine and ovine RSVs does not exist. We developed and evaluated two PCR assays; one targeting the F gene (RT-PCR F) and the other targeting the G gene (RT-PCR G) of bovine RSV and ovine RSV to simultaneously detect bovine and ovine RSV and concurrently to differentiate between them.

Materials and Methods

Viruses and cells: Ovine respiratory syncytial virus isolate WSU 83-1578 (Provided by Dr Howard Lehmkuhl, National Animal Disease Laboratory, Ames, Iowa) and bovine respiratory syncytial virus 391-2 (Provided by Dr. Gail Wertz, Dept. of Microbiology, Univ. of Alabama Medical School, Birmingham, AL) were used to

inoculate Madin Darby Bovine Kidney (MDBK) cells grown with Dulbeco's modified Eagles medium supplemented with 2% fetal bovine serum.

RNA extraction and quantification: Ovine and bovine RSV were grown until a 50-70% cytopathic effect developed. RNA was extracted with Trizol reagent (Gibco-BRL, Gaithersburg, MD) according to the protocol of the manufacturer. In brief, infected cells were collected from a 75-cm^2 flask, and suspended in one ml Trizol and incubated for 5 minutes at room temperature. Then, after adding 0.2 ml chloroform, incubating for 2-3 minutes at room temperature and centrifuging at 12,000 xg for 15 minutes at 2-8 ^oC, the aqueous upper phase was obtained. RNA was precipitated by adding 0.5 ml isopropyl alcohol, incubating for 10 minutes at room temperature and centrifuging at 12,000 xg for 10 minutes at 2-8 ^oC. The RNA pellet then was washed with 1 ml of 75% ethanol and centrifuged at 7,500 xg for 5 minutes at 2-8 ^oC. The RNA pellet then was air dried and dissolved in Rnase-free water. The RNA concentration was quantified in a spectrophotometer at A260 wavelength.

Primers for RT-PCR F: Two primers were developed and synthesized (Gibco-BRL, Gaithersburg, MD) for amplification of a 426 bp F gene fragment of bovine and ovine RSV (Fig. 1). The F103 primer (5'TGCCAAAACATAACAGAAG 3') was used for cDNA synthesis. It represents a conserved sequence in ovine and bovine RSV F genes [bases 85-103 of ovine RSV F gene (Eleraky et al., 2001) and bases 86-104 of bovine RSV F gene (Lerch et al., 1991)]. The other primer, F493 (5' TTTATTCACCTCTCCCTC 3'), represents also a conserved sequence in ovine and bovine RSV F genes. It is complementary to bases 510-493 of the ovine RSV F gene (Eleraky et al., 2001) and bases 511-494 of bovine RSV F gene (Lerch et al., 1991).

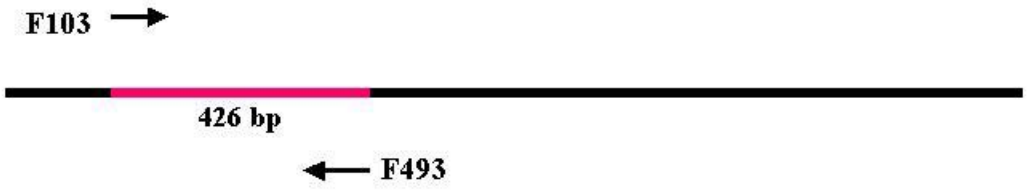

Figure 1: Primers used for RT-PCR F

- **F103 primer** was used for cDNA synthesis.
- **F493 primer** was used with F103 for amplification.

Figure 2: Primers used for RT-PCR G

•**G164 primer** was used for cDNA synthesis.

•**G672 primer** was used with G164 for amplification

Primers for RT-PCR G: Another set of primers, derived from the published sequence of ovine RSV G gene (Alansari and Potgieter, 1993), was synthesized and used for amplification of 542 bp G gene fragment of ovine and bovine RSV (Fig. 2). One primer [G164 (5' AGCCCTAGCAATGATAAC 3')] representing bases 147-164 of ovine RSV G gene was used for cDNA synthesis. The second primer [G672 (5' GACTGGTTCTGTGGTGG 3')] represents the complementary sequence of bases 688-672 of the ovine RSV G gene.

First strand cDNA synthesis: Synthesis of cDNA was done in 20 µl reaction volumes by using superscript II Rnase H^- reverse transcriptase (Gibco-BRL, Gaithersburg, MD) according to the manufacturer's protocol. Briefly, two pico moles of the specific primer, 4-5 µg of total RNA and up to 12 µl of water were heated to 70^{o}C for 10 minutes and chilled quickly on ice. Then, four µl of 5x first strand buffer (Gibco-BRL, Gaithersburg, MD), two µl of 0.1 M DTT (Gibco-BRL, Gaithersburg, MD) and one µl of 10mM dNTP mix (Gibco-BRL, Gaithersburg, MD) were added and incubated at 42^{o}C for two minutes. One µl (200 units) of superscript II then was added and incubated at 42 C for 50 minutes followed by heating at 70 C for 15 minutes to inactivate the reaction.

PCR: In brief, the following components were mixed for a final reaction volume of 100 µl: ten µl of 10x PCR buffer (Gibco-BRL, Gaithersburg, MD), three µl of 50 mM Mgcl2 (Gibco-BRL, Gaithersburg, MD), two µl of 10 mM dNTP mix (Gibco-BRL, Gaithersburg, MD), one µl of 10 uM of each specific primer, one µl taq DNA polymerase (Gibco-BRL, Gaithersburg, MD), two µl of cDNA and 80 µl of water. Amplification was done under the following conditions: denaturation at 95^{o}C for 1.5 minutes, then a three-step cycling program (repeated 30 times) consisting of annealing at 58^{o}C for 30 seconds,

extension at 72^{o}C for one minute and denaturation at 95^{o}C for one minute followed by holding the temperature at 50^{o}C for two minutes and then 72^{o}C for 10 minutes.

Sequencing of the PCR products: Sequencing was done at the University of Tennessee Molecular Biology Research Facility by using an ABI prism dye terminator cycle sequencing reaction kit and an ABI 373 DNA sequencer, Perkin Elmer Inc., Foster City, CA.

Restrictiction enzyme cleavage and electrophoresis: EcoRI (Gibco-BRL, Gaithersburg, MD), and BstYI (New England BioLabs, Beverly, MA) restriction enzymes were used separately to cleave each of the 426 bp-amplified products (PCR F) of ovine and bovine RSV F genes. EcoO109I and RsaI restriction enzymes (Gibco-BRL, Gaithersburg, MD) were used separately to cleave each of the 542 bp PCR products (PCR G) of bovine and ovine RSV G genes. Four µl of unpurified PCR product, four ul of water, one µl of the restriction enzyme used and one µl of the buffer supplied with the enzyme were mixed together and incubated for one hour at 37 ^{o}C except with BstYI (60 ^{o}C). After incubation, electrophoresis was done in three percent high-resolution agarose (Sigma Chemicals Co., St. Louis, MO).

Evaluation studies: The two assays were evaluated with the following additional RSV isolates: bovine RSV (isolates 375, FS1-1, NMK7, MN, CA, NY191285 and 1156R), caprine RSV, Bighorn sheep RSV isolate (WSU 87-6750), human RSV subgroup A (strain A2), human RSV subgroup B (strain B8/60). These isolates kindly were provided by Dr Howard Lehmkuhl, National Animal Disease Laboratory, Ames, Iowa; Dr. Gail Wertz, Dept. of Microbiology, University of Alabama Medical School, Birmingham, AL; Dr. John Baker, Department of Large Animal Clinical Sciences,

College of Veterinary Medicine, Michigan State University, East Lansing, MA; and Dr. James Evermann, Washington Animal Disease Diagnostic Laboratory, Pullman, WA. Also, they were tested with several viruses associated with bovine respiratory tract disease including bovine herpesvirus 1, bovine parainfluenza 3, bovine virus diarrhea virus, bovine coronavirus, and bovine adenovirus 3. These strains were provided by the Clinical Virology Laboratory, College of Veterinary Medicine, University of Tennessee, Knoxville, TN. MDBK line cells were used for growing of bovine RSV strains, bovine herpesvirus 1, bovine parainfluenza 3, bovine virus diarrhea virus and bovine adenovirus 3. HRT line cells were used for growing of bovine coronavirus. Secondary goat turbinate cells and VERO line cells were used for growing of caprine RSV and human RSV respectively.

Also, the two RT-PCR assays were done using decreasing amounts of total RNA extracted from infected cells (4 μg, 2 μg, 1 μg, 0.5 μg, 0.25 μg, 0.13 μg, 0.06 μg and 0.03 μg)

Results

RT-PCR F: The RT-PCR assay targeting the F gene resulted in amplification of a 426 bp (PCR F) with ovine RSV strain WSU 83-1578 and bovine RSV strain 391-2 (fig. 3B, 3c). Nucleotide sequencing of both products confirmed the fidelity of this assay (Fig. 4). Digestion of both products by EcoRI restriction endonuclease cleaved the ovine PCR product into 2 bands, 285 bp and 141 bp (fig. 3D) but did not affect the bovine product (fig.3E). In contrast, BstYI restriction endonuclease cleaved the bovine PCR product into 2 bands, 361 bp and 65 bp (fig. 3F), but had no effect on the ovine product (fig. 3G).

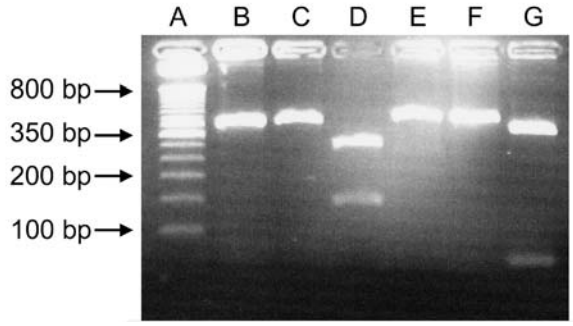

Figure 3: RT-PCR F followed by restriction enzyme cleavage. Lane A contains the 50 bp DNA ladder. Lanes B and C represent the PCR F products obtained from ovine and bovine RSV respectively (426 bp). Lane D contains the cleavage products (285 bp and 141 bp) of the ovine RSV PCR F product after EcoRI treatment. Lane E contains the bovine RSV PCR F product (426 bp) after EcoRI treatment. The ovine RSV PCR F product (426 bp) after BstyI treatment is represented in lane F. Cleavage products of the bovine RSV PCR F product (361 bp and 65 bp) after BstyI treatment are in lane G.

a)

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  1  CAATCAACAT GTAGTGCAGT TAGTAGAGGT TATCTCAGTG CATTACGAAC
 51  TGGATGGTAC ACAAGTGTGG TGACAATAGA ATTGAGCAAC ATACAAAAAA
101  ATGTATGTAG CAGTACTGAT TCAAAGGTAA AATTGATAAA GCAAGAGTTA
151  GAAAGATATA ATAATGCAGT AGCTGAGTTA CAGTTGCTCA TGCAAAATAC
201  ACCGTCTATC ACCAACAGGG CCAAAAGAGG AATATCAGAG TTGGTTCACT
251  ATACAAAGAA TTCCACTAAG AGATTTTATG GTCTAATGGG CAAAAAAAGA
301  AAAAGGAGGT TCTTAGGGTT CTTGCTTGGT ATAGGGTCTG CTATTGCGAG
351  TGGAATAGCA GTGTCTAAAG TATTACATTT AGAGGGAGAG GTG
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b)

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  1  CAATCAACAT GCAGTGCAGT TAGTAGAGGT TATCTTAGTG CATTAAGAAC
 51  TGGATGGTAT ACAAGTGTAG TAACAATAGA GTTGAGCAAA ATACAAAAGA
101  ATGTGTGTAA AAGTACTGAT TCAAAAGTGA AATTAATAAA GCAAGAACTG
151  GAAAGATACA ACAATGCAGT AATAGAATTG CAGTCACTTA TGCAAAATGA
201  ACCGGCTTCC TTCAGTAGAG CAAAAAGAGG GATACCAGAG TTGATACATT
251  ATACAAGAAA CTCTACAAAG AGATTTTATG GGTTAATGGG CAAGAAGAGA
301  AAGAGGAGAT TTTTAGGATT CTTGCTAGGT ATTGGATCTG CTGTTGCAAG
351  TGGTGTAGCA GTGTCCAAAG TACTACACCT GGAGGGAGAG GTGAATAAAA
```

Figure 4: Nucleotide sequence of the amplified PCR F products of ovine RSV (a) and bovine RSV (b).

RT-PCR G: The PCR assay targeting the G gene resulted in production of a 542 bp (PCR G) product with ovine RSV strain WSU 83-1578 and bovine RSV isolate 391-2 (fig.5B, 5C). Sequencing of the PCR products confirmed the fidelity of this assay (Fig. 6). EcoO109I treatment resulted in production of two bands, approximately the same size (275 bp and 267 bp) with the ovine RSV product only (fig.5D) and did not affect the bovine RSV product (fig.5E). RsaI treatment produced two bands (391 bp and 151 bp) with the bovine RSV product only (fig.5G) and had no effect on ovine RSV (fig. 5F).

Evaluation studies: All bovine RSV isolates tested (375, FS1-1, NMK7, MN, CA, NY191285 and 1156R) were successfully amplified by the two assays and gave the same cleavage pattern obtained with bovine RSV strain 391-2. All of these bovine isolates could be differentiated from ovine RSV by RT-PCR F and RT-PCR G followed by the appropriate restriction enzyme treatment (Table 1). The caprine RSV gave the same pattern obtained with the bovine RSV isolates with both PCR assays and was differentiated from the ovine RSV (Table 1). Amplification products resulted with the bighorn sheep RSV isolate WSU 87-6750 in the two assays. However, the RT-PCR F product yielded the same cleavage pattern as with ovine RSV isolate WSU 83-1578 with EcoRI, but the RT-PCR G product was not cleaved with EcoO109I or RsaI (Table 1). No PCR products resulted in either assay with human RSV subgroup A (strain A2), human RSV subgroup B (strain B8/60), bovine herpesvirus 1, bovine parainfluenza virus 3, bovine virus diarrhea virus, bovine coronavirus or bovine adenovirus 3 (Table 1). PCR F and PCR G products with ovine and bovine RSV isolates were detected by using as little as 0.06 μg and 0.03 μg respectively of total RNA (Table 2).

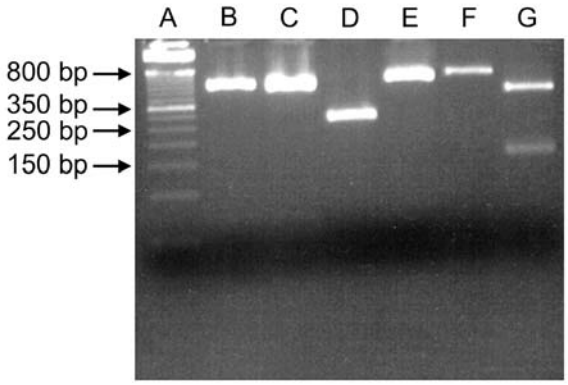

Figure 5: RT-PCR G followed by restriction enzyme cleavage. Lane A contains the 50 bp DNA ladder. Lanes B and C represent the PCR G products obtained from ovine and bovine RSV respectively (542 bp). Lane D contains the cleavage products of the ovine RSV PCR G product having approximately the same size (275 bp and 267 bp) after EcoO109I treatment. Lane E contains the bovine RSV PCR G product (542 bp) after EcoO109I treatment. The ovine RSV PCR G product (542 bp) after RsaI treatment is represented in lane F. Cleavage products of the bovine RSV G product after RsaI treatment (391 bp and 151 bp) are in lane G.

a)

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  1  CTTACCATAA CAGCCATCAT TTATATTAGC ACAGGAAACA CAAAAGCCAA
 51  ACCCATGCCT ACACCAACAA TTCAGATCAC CCAACAGTTC CAAAACCACA
101  CCTCTCTGCC TCCCACAGAA CACAACCATA ACTCTACTCA CTCTCCAACT
151  CAAGGCACCA CATCACCCCA CACTTTCGCC GTAGATGTCA CCGAAGGAAC
201  TGCATACTAC CACTTGACCC ACAAAACTCA AGGCGGTAAA ACCAAAGGCC
251  CTCCTACTCC ACATGCCACA AGGAAACCCC CCATCAGTTC ACAGAAGAGC
301  AATCCCTCCG AAATTCAACA AGATTACAGT GACTTTCAAA TACTTCCCTA
351  TGTGCCCTGC AACATATGTG AAGGTGACTC TGCTTGTTTA TCCCTCTGTC
401  AAGATAGATC CGAGAGCATA CTGGATAAAG CTCTAACAAC CACCCCCAAA
451  AAAACTCCAA AACCCATGAC CACCAAAAAG CCAACC
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b)

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  1  CATCACTCGT CATCACAGCC ATTATTTACA TTAGTGTGGG AAATGCTAAA
 51  GCCAAGCCCA CATCCAAACC AACCATCCAA CAAACACAAC AGCCCCAAAA
101  CCATACCTCA CCATTTTTCA CAGAGCACAA CTACAAATCA ACTCACACAT
151  CAATTCAAAG CACCACACTG TCCCAACTAC TAAACATAGA CACTACTAGA
201  GGAATTACAT ATGGTCACTC AACCAACGAA ACCCAAAACA GAAAAATCAA
251  AGGCCAATCC ACTCTACCCG CCACCAGAAA ACCACCAATC AATCCATCGG
301  GAAGCATCCC CCCTGAAAAC CATCAAGACC ACAACAACTT CCAAACACTC
351  CCCTATGTGC CTTGCAGTAC ATGTGAAGGT AATCTTGCTT GCTTATCACT
401  CTGCCATATT GAGACGGAGA GAGCACCAAG CAGAGCCCCT ACAATCACCC
451  TCAAAAAGAC TCCAAAACCC AAAACCACTA AAAAGCCAAC CAAGA
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Figure 6: Nucleotide sequence of the amplified PCR G products of ovine RSV (a) and bovine RSV (b).

Table 1: Evaluation of the potential specificity RT- PCR F and RT-PCR G

	PCR F	PCR F + EcoRI	PCR F + BstYI	PCR G	PCR G + EcoO109I	PCR G + RsaI
Bovine RSV 8 strains§	426 bp	426 bp	361 bp, 65 bp	542 bp	542 bp	391 bp, 151 bp
Caprine RSV	426 bp	426 bp	361 bp, 65 bp	542 bp	542 bp	391 bp, 151 bp
Ovine RSV	426 bp	285bp, 141 bp	426 bp	542 bp	275 bp, 267 bp	542 bp
Bighorn sheep RSV	426 bp	285 bp, 141 bp	426 bp	542 bp	542 bp	542 bp
Human RSV (A&B)	No product			No product		
Other viruses*	No product			No product		

§ 391-2, 375, FS1-1, NMK7, MN, CA, NY191285 and 1156R

* Viruses associated with bovine respiratory tract disease; bovine parainfluenza virus 3, bovine herpesvirus 1, bovine virus diarrhea virus, bovine coronavirus, and bovine adenovirus

Table 2: Evaluation of the potential sensitivity of RT-PCR F and RT-PCR G assays

Total RNA used	PCR F product	PCR G product
4 µg	+	+
2 µg	+	+
1 µg	+	+
0.5 µg	+	+
0.25 µg	+	+
0.13 µg	+	+
0.06 µg	+	+
0.03 µg	No product	+

Discussion

PCR assays have been used in several studies for detection of bovine RSV (Oberst et al., 1993; Vilcek et al., 1994; Valarcher et al., 1999; Larsen et al., 1999). In the present study, the bovine RSVs tested (8 isolates from different sources) reacted identically and could be differentiated from ovine RSV in both PCR assays described in this study. Moreover, no PCR products were detected with human RSV, bovine parainfluenza virus 3, bovine herpesvirus 1, bovine virus diarrhea virus, bovine coronavirus, and bovine adenovirus in these two assays.

BstyI used in cleavage of bovine RSV F products in this study may be superior to MspI used in a previous study (Eleraky et al., 2001). BstyI cleaved all the PCR F products obtained from the eight bovine RSV isolates, but some isolates (CA and 1156R) resisted cleavage by MspI. The MspI target sequence does not appear to be well conserved (data not shown).

Caprine RSV gave the same pattern as bovine RSV did in both assays and could be differentiated from ovine RSV. This confirms previous studies suggesting that caprine RSV is closely related to bovine RSV isolates (Alansari et al., 1999).

Nucleotide sequencing of amplified fragments of F and G genes of Bighorn sheep isolate WSU 87-6750 (data not shown) indicated very high level of identity with ovine RSV isolate WSU 83-1578 suggesting that the Bighorn RSV may be considered a member of the ovine RSV subgroup. However, unlike ovine RSV isolate WSU 83-1578, the bighorn sheep RSV strain WSU 87-6750 resisted cleavage using EcoO109I following RT-PCR G due to a point mutation in the cleavage site (data not shown), but they had the same cleavage pattern with EcoRI of their respective F gene amplified products.

It has been proposed that at least 2 ruminant subgroups exist: one represented by RSV isolated from cattle and goat and the other represented by ovine RSV (Alansari et al., 1999). The two PCR assays described in our study constitute potential sensitive and specific means for simultaneous detection of both bovine and ovine RSV and differentiation between them. RT-PCR F followed by the appropriate restriction enzyme cleavage may be superior to RT-PCR G to classify ruminant RSV into two subgroups because it resulted in the expected pattern for all the viruses tested. However, as indicated earlier RT-PCR G followed by EcoO109I treatment did not give the expected cleavage pattern with Bighorn RSV strain WSU 87-6750. Moreover, F gene is highly conserved (>95%) among different bovine RSV strains (Pastey and Samal, 1993), whereas the G gene is less conserved. According to one study, the nucleotide sequence identity among G gene of different bovine RSV strains was 88-100% (Elvander et al., 1998). RT-PCR F has the potential to determine the relative contribution of ovine RSV and bovine RSV to the pathogenesis of bovine respiratory tract disease. This information would be relevant to the complete understanding of RSV epidemiology and immunoprophylaxis. Further studies are required to determine sensitivity and specificity of the two assays to detect bovine and ovine RSVs in clinical samples from infected animals.

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Part IV

Summary

Summary

Two antigenic subgroups A and B were defined for human respiratory syncytial virus (HRSV) isolates on the basis of their reaction patterns with monoclonal antibodies (Mufson et al., 1985; Anderson et al., 1985; Gimenez et al., 1986, Hendry et al., 1986). Antigenic diversity of HRSV is associated with an extensive degree of amino acid sequence divergence (53%) of the G protein between the two subgroups (Johnson et al., 1987).

The attachment glycoprotein G of bovine respiratory syncytial virus (BRSV) differed substantially from that of HRSV, whereas the bovine RSV F, N, M, and P proteins cross-reacted antigenically with those of HRSV (Lerch et al., 1989). Early work suggested that heterogeneity among 19 BRSV isolates exists based on reactivity with monoclonal antibodies specific for G protein and two antigenic subgroups were identified (Furze et al., 1994). However, sequence data revealed a high degree of amino acid identity (85%) between the strains of the putative BRSV subgroups (Furze et al., 1997; Prozzi et al., 1997). These findings were in sharp contrast to the HRSV antigenic subgroups, where there is only 53% identity between A and B subgroups (Johnson et al., 1987). It has been suggested that BRSV isolates form a continuum of slightly different strains within a single subgroup rather than distinct subgroups (Prozzi et al., 1997, Stine et al., 1997).

However, an RSV isolated from sheep was clearly distinguished from BRSV but was more closely related to BRSV than to HRSV (Alansari and Potgieter, 1993; Mallipeddi and Samal, 1993; Alansari and Potgieter, 1994a; Alansari and Potgieter, 1994b; Pastey and Samal, 1995; Alansari et al., 1999). It has been proposed that ruminant

RSVs be divided into 2 subgroups, one representing bovine RSV, and the other representing ovine RSV, based on analysis by RNAse protection of the G glycoprotein transcripts and sequence comparison of ovine and bovine RSV-G protein genes (Alansari and Potgieter, 1993; Alansari et al. 1999). Furthermore, variation in the reactivity of bovine and ovine RSV with some monoclonal antibodies to the F protein of bovine RSV (Pastey and Samal, 1997) supported this classification.

Some previous studies indicated possibility of interspecies transmission of bovine and ovine RSVs. Experimental infection of lambs with BRSV produced mild clinical signs and some pulmonary lesions (Lehmkuhl and Cutlip, 1979; Sharma and Woldehiwet, 1991). Adair and McFerran, 1987 suggested that two related but antigenically distinguishable RSV types may infect sheep, one of them may have been BRSV. Inoculation of ORSV into calves and deer fawns resulted in mild to moderate clinical respiratory disease only in calves. Lung lesions were detected in calves and fawns and ORSV was reisolated from the lower respiratory tract of inoculated calves and fawns (Bryson et al., 1988). Subgroup-specific, peptide-based ELISA derived from the unique central hydrophobic region of the ovine and bovine G-glycoprotein, respectively were used to determine the prevalence of antibodies against ovine and bovine subgroup strains of RSV in cattle. The prevalence was 56-60% for the bovine strains and 15.9% for the ovine strain (Grubbs et al., 2001 a; Grubbs et al., 2001 b).

Although these studies suggested that ovine RSV participates in the pathogenesis of bovine respiratory tract disease, Confirmation of this hypothesis has not been possible because a laboratory test differentiating between bovine and ovine RSV isolates did not exist. The purpose of the present study was to develop reverse transcription-poymerase

chain reaction (RT-PCR) assays for simultaneous detection of bovine and ovine RSVs and differentiation between them. RSV isolation is inefficient because of its lability (Smith et al., 1974). PCR assays for RSV may be more sensitive than immunofluorescence (IF) (Vilcek et al., 1994; Valarcher et al., 1999) and ELISA (Larsen et al., 1999; Valarcher et al., 1999) and as specific as IF and ELISA (Valarcher et al., 1999) for the detection of BRSV infections. PCR assays have been recommended for diagnosis of bovine RSV infections in cattle (Oberst et al., 1993; Vilcek et al., 1994; Valarcher et al., 1999; Larsen et al., 1999). RSV PCR assays depend upon detection of viral RNA or mRNA transcripts that are present in active infections, whereas serological tests depend upon detection of antibodies, which develop during the convalescent phase of infection. Therefore, an advantage of PCR tests over serological tests is that earlier detection of infection is possible. Also, conventional serological diagnosis is dependent upon paired serum samples taken during the acute and convalescent phases of the disease and are not capable of discriminating between the ruminant RSV subgroups.

In this study, two structural protein genes were chosen as targets for the two PCR assays; the fusion protein (F) for one assay and the attachment glycoprotein (G) gene for the other assay. The F protein is more conserved ,whereas the G protein gene is the most divergent viral protein among RSV strains (Lerch et al., 1990; Himes and Gershwin, 1992; Alansari and Potgieter, 1993). The nucleotide sequences of G protein genes of BRSV and ORSV have been determined. But, the nucleotide sequence of F protein gene is known only for BRSV. Therefore, the nucleotide sequence of ovine RSV F gene had to be determined. For this purpose, two primers (F25 and F19) were derived from the 5' end of ovine RSV M2 gene and G-F intergenic region respectively (Alansari and Potgieter,

1994; Mallipeddi and Samal, 1993). Sequencing was done in both directions, 5' to 3'by using the F19 primer, and 3' to 5' by using the F25 primer. The initial sequencing data were used to design additional primers for sequencing the flanking regions. This strategy was repeated to obtain the sequence of the whole product by assembly of overlapping regions.

The ovine RSV F gene has 85%, 73% and 72% nucleotide identity with those of bovine RSV, human RSV subgroup A and human RSV B subgroup respectively. The predicted amino acid identity between the F protein of ovine RSV and those of bovine RSV, human RSV subgroup A and human RSV subgroup B is 94%, 83% and 84% respectively.

Based on this information, a PCR test targeting a specific fragment (426 bp) in the F gene of both bovine RSV and ovine RSV has been developed (RT-PCR F). Also, another assay amplifying 542 bp of both bovine and ovine RSV G genes has been developed (RT-PCR G). The primers used in both assays were derived from conserved sequences in bovine and ovine RSVs, bur that are not conserved in human RSV. This allowed amplification of both bovine and ovine RSV, but not human RSV. Also, the primers used did not have any homology with the sequences derived from other viruses causing bovine respiratory tract disease; bovine parainfluenza, bovine virus diarrhea, bovine coronavirus, bovine herpesvirus and bovine adenovirus. Sequencing of the PCR products derived from ORSV and BRSV confirmed the validity of the two assays.

In RT-PCR-F assay, the PCR products obtained from bovine and ovine RSVs could be distinguished by EcoRI treatment that resulted in two bands (285 and 141 bp)

with ORSV and one band (426 bp) with BRSV or BstYI treatment that resulted in one band (426 bp) with ORSV and 2 bands (361 and 65 bp) with.BRSV.

In RT-PCR G assay, PCR products obtained from bovine and ovine RSVs could be distinguished by EcoO109I treatment that resulted in two bands (275 and 267 bp) with ORSV and one band (542 bp) with BRSV or RsaI treatment that resulted in one band (542 bp) with ORSV and two bands (391 and 151 bp) with BRSV.

The two assays were evaluated with eight different bovine RSV isolates (391-2, 375, FS1-1, NMK7, MN, CA, NY191285 and 1156R), ovine RSV isolate WSU 83-1578, Bighorn sheep RSV isolate 87-6750, caprine RSV isolate, two human RSV isolates (A2 and B8/60) and several other viruses associated with bovine respiratory tract disease (bovine parainfluenzavirus, bovine virus diarrhea virus, bovine coronavirus, bovine herpesvirus and bovine adenovirus). All bovine RSV isolates tested were successfully amplified by the two assays and resulted in an identical cleavage pattern. All of these bovine isolates could be differentiated from ovine RSV by RT-PCR F and RT-PCR G followed by the appropriate restriction enzyme treatment. The results with caprine RSV were similar to those obtained with the bovine RSV isolates in both PCR assays.The bovine/caprine isolates were clearly differentiated from the ovine RSV isolate. These results confirm previous studies suggesting that caprine RSV is closely related to bovine RSV isolates (Alansari et al., 1999). Amplification products were produced with the Bighorn sheep RSV isolate WSU 87-6750 in the two assays. The RT-PCR F product yielded the same cleavage pattern as the ovine RSV isolate WSU 83-1578 with EcoRI, but the RT-PCR G product from the Bighorn isolate was not cleaved with EcoO109I or RsaI. Sequence data of the target region indicated that this was due to a point mutation in

the cleavage site. Nucleotide sequencing of amplified fragments of F and G genes of Bighorn sheep isolate WSU 87-6750 indicated very high level of identity with ovine RSV isolate WSU 83-1578 suggesting that the Bighorn RSV may be considered a member of the ovine RSV subgroup.

Both assays gave a constant pattern with the eight bovine RSV isolates and the caprine isolate that were clearly distinguished from the ovine RSV. Moreover, No PCR products resulted in either assay with human RSV subgroup A (strain A2), human RSV subgroup B (strain B8/60), bovine herpesvirus 1, bovine parainfluenza virus 3, bovine virus diarrhea virus, bovine coronavirus or bovine adenovirus 3. This information reveal that the two assays constitute a potential specific means for simultaneous detection and differentiation of BRSV and ORSV.

PCR F and PCR G products with ovine and bovine RSV isolates were detected by using as little as 0.06 ug and 0.03 ug respectively of total RNA indicating that the two assays may be sensitive to detect very low amount of viral RNA.

RT-PCR F followed by the appropriate restriction enzyme cleavage may be superior to RT-PCR G to classify ruminant RSV into two subgroups because it resulted in the expected pattern for all the viruses tested. However, as indicated earlier, RT-PCR G followed by EcoO109I treatment did not give the expected cleavage pattern with Bighorn RSV strain WSU 87-6750. Moreover, F gene is highly conserved (>95%) among different bovine RSV strains (Pastey and Samal, 1993), whereas the G gene is less conserved. According to one study, the nucleotide sequence identity among G gene of different bovine RSV strains was 88-100% (Elvander et al., 1998). RT-PCR F has the potential to determine the relative contribution of ovine RSV and bovine RSV to the

pathogenesis of bovine respiratory tract disease. This information would be relevant to the complete understanding of RSV epidemiology and immunoprophylaxis. Further studies are required to determine sensitivity and specificity of the two assays to detect bovine and ovine RSVs in clinical samples from infected animals.

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VITA

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