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DIFFERENTIAL IDENTIFICATION OF PORCINE TRANSMISSIBLE GASTROENTERITIS CORONAVIRUS AND BOVINE ENTERIC CORONAVIRUS USING CLONED NUCLEIC ACID PROBES

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Linda J. Shockley

June 1986

This thesis

is dedicated to

my parents, for their

love and confidence

and to my neice, Shaunnie,

for her smiles and laughter

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ABSTRACT

Cloned bovine coronavirus (BCV) and transmissible gastroenteritis virus (TGEV) cDNAs were used as quantitative hybridization probes to detect viral RNA. Insert DNA was cleaved from the cloning vector, purified by electroelution, and then nick-translated with ^{32}P-dCTP to a specific activity of 10^8cpm/µg of DNA. As standards, purified quantified BCV and TGEV RNAs were denatured in high salt, formaldehyde and detergent and then blotted with a 96-well minifold dot blot apparatus onto nitrocellulose. A similar dot blot methodology was applied to cell culture supernatant fluids and fecal samples. After hybridization and autoradiography, it was observed that each probe detected only RNA from its homologous virus to a sensitivity level of 25 pg of RNA per dot. No crossreactivity between the two coronaviruses was observed, even at RNA concentrations of 10 ng per dot. The probes were specific, since they did not hybridize to a variety of heterologous virus RNAs. BCV and TGEV RNA could also be readily detected in cell culture supernatant fluids and in stool samples by this procedure. The volume of sample required was small (1-10 µl), sample manipulations were few, and multiple samples could be analysed simultaneously. The probes will be useful for routine diagnosis of BCV and TGEV infection and for research into the pathogenesis of chronic disease.

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PART I: LITERATURE REVIEW

CHAPTER I

INTRODUCTION

Coronaviruses were originally grouped together because of their characteristic appearance in electron micrographs (Tyrrell et al., 1968). Large, club-shaped glycoprotein peplomers extend from the viral surface, surrounding the virion with its distinctive halo or corona. These viruses are also enveloped and ether-labile and have a genome of ribonucleic acid (Pensaert and Callebaut, 1978). In 1975, a study group classified them as a separate family, the Coronaviridae, which is monogeneric (Tyrrell et al., 1975). This brings the total of the known animal RNA viruses to eleven.

Coronaviruses cause many diseases in a wide range of hosts, as seen in Table I.1.1. The diseases in food animals cause significant economic loss because of the high mortality rate in young animals (House, 1978). Infections may be either acute or chronic. Coronaviruses readily establish persistent infections in tissue culture (Lucas et al., 1977; Stohlman et al., 1979). In addition, coronaviruses have been implicated in chronic human disease. They have been isolated from the brain tissues of multiple sclerosis victims (Burks et al., 1980) and have been seen in electron microscopy of kidney tissue from patients with chronic nephropathy (Apostalov et al., 1975) and in feces of children with sprue (Baker et al., 1982).

It is now possible to prepare cloned, complimentary DNA (cDNA) of specifically defined sequence from viral RNA with recombinant DNA techniques. The cDNA can then be used to detect quantitatively the

Table I.1.1. Listing of the most characterized coronaviruses[a]

Virus	Abbreviation	Natural Host
Avian infectious bronchitis virus	IBV (AIBV)	Chicken
Bovine enteric coronavirus	BCV	Calf
Canine coronavirus	CCV	Dog
Feline infectious peritonitis virus	FIPV	Cat
Human respiratory coronavirus OC43	HCV-OC43	Human
Human respiratory coronavirus 229E	HCV-229E	Human
Murine (mouse) hepatitis virus	MHV	Mouse
Porcine transmissible gastroenteritis virus	TGEV	Pig
Porcine hemagglutinating encephalomyelitis virus	HEV	Pig
Parker's rat coronavirus	RCV	Rat
Rat sialodacryoadenitis virus	SDAV	Rat
Turkey coronavirus	TCV	Turkey

[a]From the review by Wege et al., 1982.

presence of RNA virus nucleic acids in body fluids and in tissue (Brahic et al., 1984). This is a powerful technique which can be applied both to the diagnosis of coronavirus infections and to the study of the virus' role in chronic diseases. The chronic disease caused by persistent infections of RNA viruses may be the result of subtle mechanisms. This is demonstrated by a report that viral nucleic acids, but not viral proteins or intact virions, could be detected in mouse brains which were being demyelinated after infection with mouse hepatitis coronavirus (Lavi et al., 1984).

The specific aims of the present study were (1) to determine the specificity and sensitivity of hybridization probes prepared from cloned coronavirus cDNA in the detection of coronavirus RNA and (2) to establish the use of these hybridization probes to identify and quantify viral nucleic acids in cell culture fluids and body fluids. The methodology for this uses characterized (i.e., totally sequenced) cloned cDNA which has been prepared from the genome of two enteric coronaviruses of animals: the bovine enteric coronavirus (BCV) and the porcine transmissible gastroenteritis virus (TGEV). As a long range objective, others may be able to use these hybridization probes to diagnose and study the pathogenesis of coronavirus-caused acute enteritis in animals and humans and to test the hypothesis that coronaviruses are causally related to chronic disease in man. A brief review of the literature applicable to this research follows in Chapters II and III.

CHAPTER II

OVERVIEW OF CORONAVIRUS STRUCTURE AND PATHOGENESIS

Morphology

In electron micrographs, negatively-stained coronavirions appear pleomorphic, with diameters ranging from 60 to 220 nm (Pensaert and Callebaut, 1978). They have club-shaped surface projections which are typically arranged as a corona on the outside of the particle. These peplomers are 12 to 24 nm long and may be only loosely attached to the surface (McIntosh, 1974). In thin sections, the virus envelope consists of inner and outer electron-dense shells separated by a translucent space. The inner shell in negatively stained avian infectious bronchitis virus (IBV) appears as a tongue-shaped membraneous structure (Bingham and Almeida, 1977).

When virions are disrupted with a mild detergent, the internal ribonucleoprotein (RNP) can be seen as a long strand of 1 to 2 nm in diameter (Davies et al., 1981) or as a helix condensed into coiled structures of 10 to 20 nm in diameter (Macnaughton et al., 1978; Caul et al., 1979). These two forms of ribonucleoprotein apparently represent different states of relaxation. Coronaviruses are the first positive-stranded RNA virus shown to have a helical ribonucleoprotein as its genome (Siddell et al., 1982). A simplified model of a coronavirus is shown in Figure I.2.1.

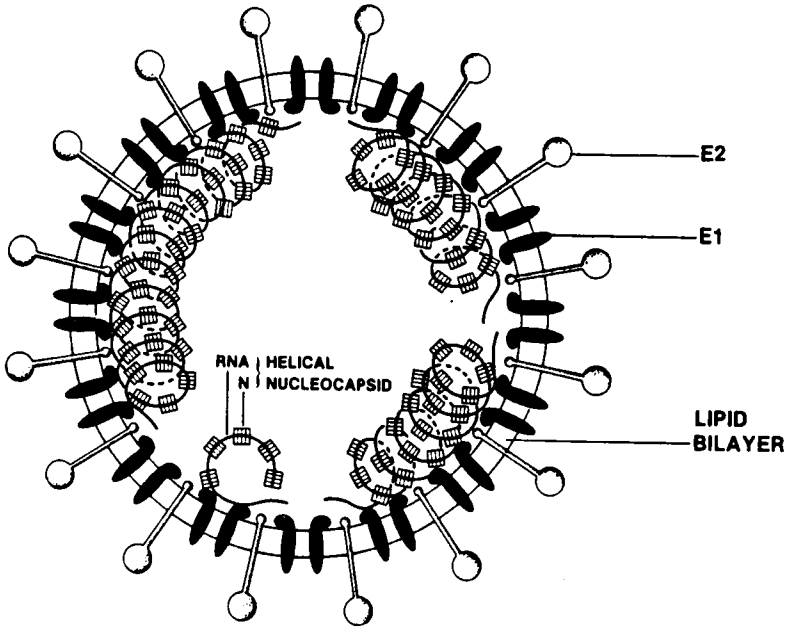


Figure I.2.1. Model of a coronavirus. From Sturman and Holmes, 1983. The viral nucleocapsid is a long, flexible helix composed of the positive-strand genomic RNA and many molecules of the phosphorylated nucleocapsid protein, N. The viral envelope includes a lipid bilayer derived from intracellular membranes of the host cell and two viral glycoproteins, E1 and E2. The peplomers are composed of E2. The matrix glycoprotein E1 penetrates through the lipid bilayer and interacts with the nucleocapsid within the virion.

Structural Proteins

The protein which encapsidates the genomic RNA is a basic protein of 50 to 60 K molecular weight. This nucleocapsid (N) protein is phosphorylated and rich in lysine and arginine residues (Sturman, 1977; Stohlman and Lai, 1979). The N genes of MHV A59 (Armstrong et al., 1983), MHV JHM (Siddell, 1983), IBV (Boursnell and Brown, 1984), BCV (Lapps, 1986), and TGEV (Kapke, 1985) have been cloned and sequenced. The helical nucleocapsid, formed when many molecules of N protein interact with the RNA, lies within a lipoprotein envelope. Embedded in the lipid bilayer of the envelope are the two other structural proteins of coronaviruses, M (or E1) and P (or E2).

M protein, the smaller of the two, is a glycoprotein of 20 to 30 K molecular weight. It is a transmembrane protein which has several unusual properties compared to the P protein and glycoproteins of other RNA viruses. Although the M protein functions similarly to the matrix proteins of myxoviruses and rhabdoviruses, it is O-glycosylated at serine or threonine residues instead of being N-glycosylated at asparagine residues. M protein apparently transverses the viral envelope multiple times (for review see Sturman and Holmes, 1983). The M genes of MHV-A59 (Armstrong et al., 1983), IBV (Boursnell et al., 1985), BCV (Lapps, 1986), and a large portion of the M gene for TGEV (Kapke, 1985) have been cloned and sequenced. The corresponding amino acid sequence suggests that M crosses the lipid bilayer by means of two hydrophobic domains and also that a large domain lies beneath the bilayer (Armstrong et al., 1984). Only the small, glycosylated amino

terminal region is exposed on the outer surface of the virions (Rottier et al., 1984).

The M protein performs several functions. It is required for formation of the viral envelope by directing virion budding into the smooth endoplasmic reticulum (Rottier et al., 1981, Sturman, 1981). The nucleocapsid probably binds to the cytoplasmic domain of M; this anchors the flexible helix to the growing viral envelope (Sturman et al., 1980). The stabilization of the nucleocapsid caused by this interaction may inhibit translation or transcription of the genomic RNA. M protein may also contain sites for interaction with P protein, the peplomeric glycoprotein (Sturman and Holmes, 1983).

P is the structural protein which forms the characteristic club-shaped peplomers on the surface of coronaviruses. A glycoprotein of 150 to 200 K molecular weight, P is anchored to the viral envelope by a short hydrophobic region (Cavanaugh, 1981); the rest of the molecule is outside the envelope. Palmitic acid is covalently attached at or near the hydrophobic domain (Schmidt, 1982). P is N-glycosylated at the nitrogen of asparagine residues, as are the glycoproteins of large, negative-strand RNA viruses. The protein contains a number of free sulfhydryl groups and intramolecular disulfide bonds; both are necessary for the protein to retain its native conformation and biological function (Garwes and Pocock, 1975).

One of the functions of the P glycoprotein is to serve as the attachment site which binds the virion to the surface receptors of the cell (Holmes et al., 1981). Cell mediated immunity is directed against the peplomers on the plasma membrane of coronavirus-infected cells

(Welsh et al., 1983). Additionally, antibodies to P can neutralize viral infectivity (Collins et al., 1982). In the mouse hepatitis virus, cleavage of this glycoprotein into two 90 K polypeptides by host cell proteases activates cell fusion (Holmes et al., 1984).

All coronaviruses contain the three structural proteins just discussed. An additional structural protein has been described for those mammalian coronaviruses which cause agglutination of mouse, rat, or chicken red blood cells (Mebus et al., 1973). A hemagglutinin (H) has been identified and characterized for HEV (Callabaut and Pensaert, 1980), OC43 (Hogue et al., 1984) and BCV (King et al., 1985). The molecular weight of this protein ranges from 130 to 140 K. In both BCV and OC43, the hemagglutinin is composed of two apparently identical disulfide-linked subunits of 65 K; each is N-glycosylated (Hogue, 1986). The diagram in Figure I.2.2 shows the relationship among the three structural proteins in the virion of TGEV and the four structural proteins in the virion of BCV.

Viral RNA

The coronavirus genome is a nonsegmented, linear molecule of single stranded RNA. The molecular weight of the RNA strand has been estimated for most coronaviruses; it ranges from 5.4×10^6 to 6.9×10^6, which corresponds to 16 to 21 kilobases (Siddell et al., 1982). Thus, this genome is the largest single-stranded one known for an animal RNA virus. The RNA is polyadenylated at the 3' end and is infectuous (Schrochetman et al., 1977; Brian et al., 1980). This

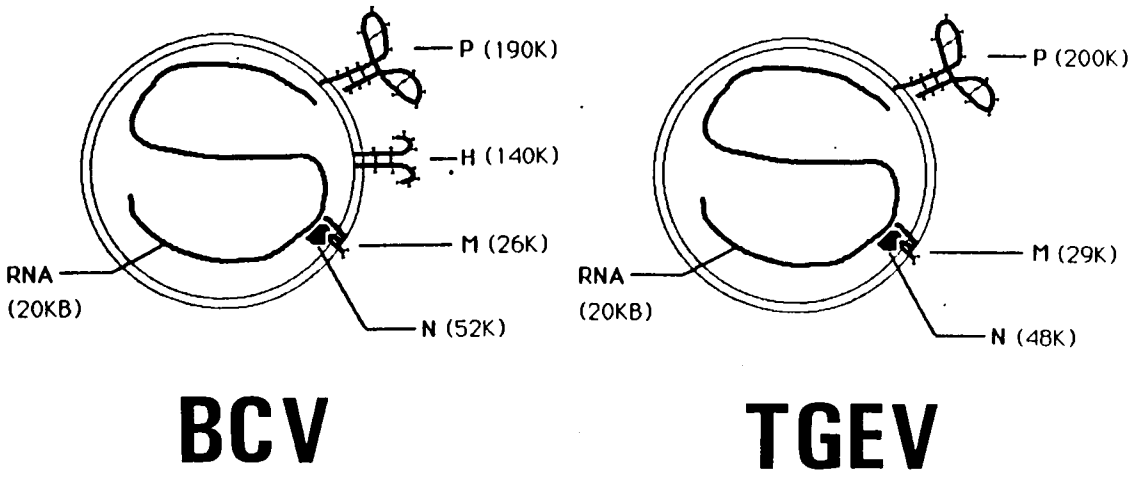


Figure I.2.2. Structural proteins of BCV and TGEV. P: peplomeric glycoprotein (also known as E2). M: matrix glycoprotein (also known as E1). N: phosphorylated nucleocapsid protein. H: hemagglutinating glycoprotein.

infectivity means that coronaviruses are positive-stranded RNA viruses, a distinction shared only by three other virus families (Baltimore, 1971). The genome of the mouse hepatitis virus (MHV) has also been shown to be capped at the 5' end (Lai and Stohlman, 1981; Lai et al., 1982a).

After the virus penetrates the cell and uncoats, the genomic RNA acts as a messenger RNA for the synthesis of virus-specific, RNA-dependent RNA polymerase (Brayton et al., 1982; Dennis and Brian, 1982). The genome is then transcribed by this enzyme, forming a complementary, full-length, minus-strand template with a poly (U) tail at the 5' end (Lai et al., 1982b). From the template, new genomic RNA and five to six species of subgenomic mRNA are transcribed (Lai et al., 1981). All of these RNAs are capped and polyadenylated. The combined sizes of these subgenomic species greatly exceed the total size of the genome, indicating that subgenomic RNAs share some sequences. T1 oligonucleotide mapping showed that subgenomic RNAs form a nested set of overlapping molecules with common 3' ends (Spaan et al., 1982; Stern and Kennedy, 1980). Each mRNA has all the nucleotide sequences of the next smaller mRNA plus an additional gene at the 5' end. Figure I.2.3 shows this nested set pattern.

In vitro translation of mRNAs from MHV, IBV, and TGEV indicate that only the 5' proximal sequence of each mRNA species is translated, yielding a single polypeptide (Siddell et al., 1980, 1981; Leibowitz et al., 1982; Stern and Sefton, 1982; Jacobs et al., 1986). In these three systems, the smallest RNA codes for the nucleocapsid protein. In both MHV and TGEV, the next smallest codes for the M (or E1)

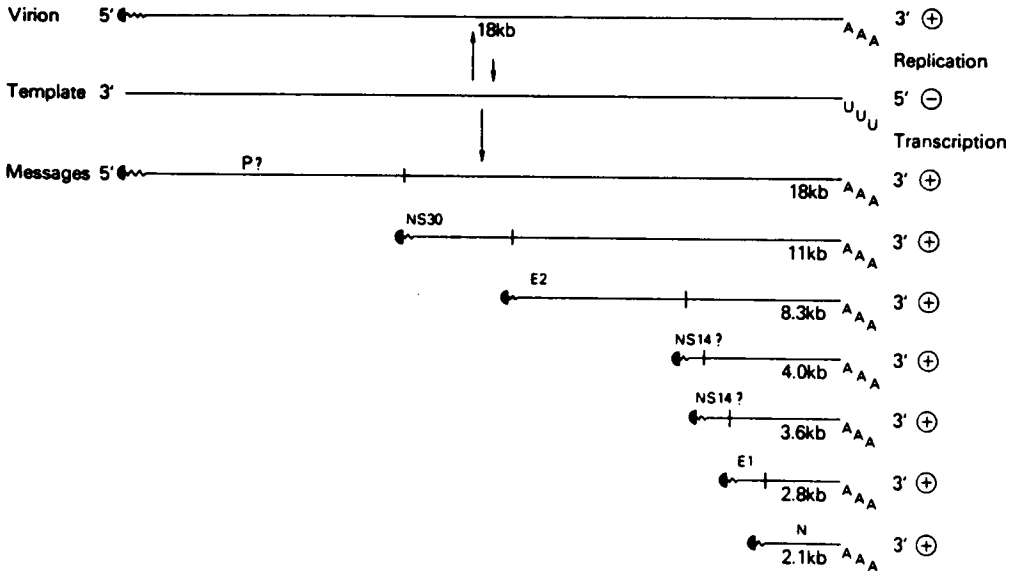


Figure I.2.3. Schematic of coronavirus transcription and replication. The scheme illustrates the nested set arrangement of mRNAs which share common 3' ends and their relationship to the genome. The size of the mRNAs are indicated. The leader sequence (~~) occurs at the 5' end of each mRNA and the genome. All mRNAs and the genome are capped (●~~—) and polyadenylated (AAA). Only the 5' end (to the short vertical line) is translated. The translation product of each mRNA is indicated above its 5' gene (taken from Dubois-Dalcq et al., 1984).

glycoprotein; in IBV, the third smallest codes for this protein. The P (or E2) glycoprotein is encoded by the second largest mRNA in TGEV AND IBV and by the third largest in MHV. The other mRNAs code for non-structural proteins; Table I.2.1 summarizes the data.

Homologies Between BCV, TGEV, MHV, and IBV

Studies in this laboratory indicate that BCV and TGEV appear to be similar to the MHV system. The sequences have been determined for 2.45 Kb of the 3' end of BCV (Lapps, 1986) and for 2.11 Kb of the 3' end of TGEV (Kapke, 1985). Analysis of the coding regions of these nucleotides has revealed both homologies and divergences in the proteins encoded by N and M genes of MHV, BCV, TGEV and IBV. Figure I.2.4 compares the gene maps of these four coronaviruses.

The N protein of BCV has an amino acid sequence homology of 70% when compared to the N proteins of both MHV A59 (Armstrong et al., 1983) and MHV JHM (Siddell, 1983). Thus, BCV and MHV appear to be closely related; this is consistent with antigenic studies of the two coronaviruses (Pederson et al., 1978). The previous antigenic data showed no antigenic relatedness between BCV and either TGEV or IBV. When the amino acid sequence of the MHV N protein is compared with IBV and TGEV, a sequence homology of only 27% is seen with IBV (Boursnell and Brown, 1984) and of 26% with TGEV (Kapke, 1985). The above data suggests that BCV has only recently diverged from MHV but is highly diverged from IBV and TGEV.

When the M proteins of BCV and MHV are examined, the two are found to be very similar (Armstrong et al., 1984; Lapps, 1986). The BCV M

Table I.2.1. Comparison of the intracellular RNAs of TGEV, MHV, and IBV and their translation products[a]

RNA[b]	Mol wt (10^6) and translation product[c]		
	TGEV	MHV	IBV
1/F	7.8	5.6, NS	8.1
2		4.0, NS	
3/E	2.8, E2	3.0, E2	2.6, E2
4/D	1.3, NS	1.4, NS	1.5
5/C	1.1	1.2, NS	1.3, E1
6/B	0.9, E1	0.9, E1	0.9, NS
7/A	0.6, N	0.8, N	0.8, N

[a]From Jacobs et al., 1986.

[b]Numbers indicate RNAs for TGEV and MHV; letters indicate corresponding RNAs for IBV.

[c]Translation products: E2, peplomer protein; E1, matrix protein; N, nucleocapsid protein; NS, nonstructural protein.

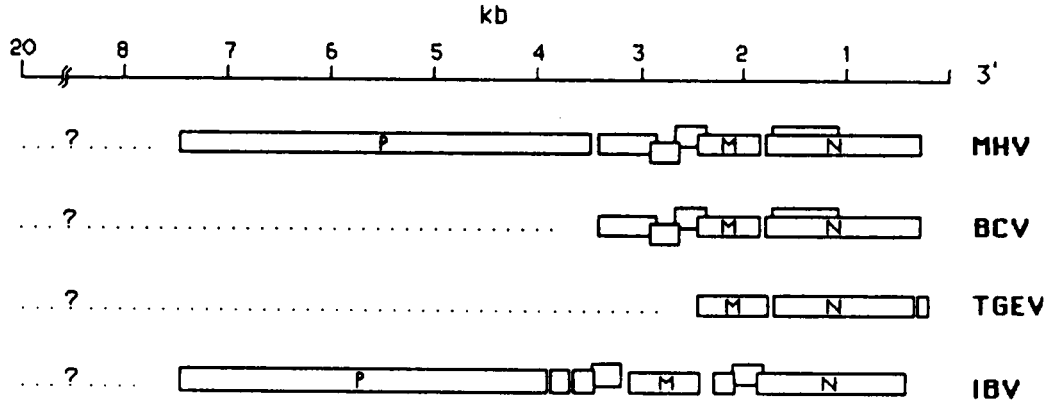


Figure I.2.4. Gene maps of the 3' end of MHV, BCV, TGEV and IBV. Open reading frames are indicated by rectangles. N: nucleocapsid protein gene. M: matrix protein gene. P: peplomeric protein gene.

protein contains 230 amino acids; MHV M protein is 229 amino acids long. More than 86% of the amino acids show perfect sequence homology. Since another 6% show only conservative changes, the overall functional amino acid sequence homology for these two proteins could be considered to be 92%. This high degree of homology is to be expected when the specialized functions of the M protein are considered. The M protein of TGEV may also show a high degree of homology with the BCV M protein when its M gene is cloned and sequenced.

TGEV has been shown by one group (Jacob et al., 1986) to have five subgenomic mRNAs and by another in this laboratory (Dennis and Brian, 1982) to have nine species. Analysis of the recently sequenced 3' end of the TGEV genome provides evidence to support the presence of the smallest 240 K mRNA described by Dennis and Brian (Kapke, 1985). The first 1700 bases sequenced apparently code for two genes. One of these genes encodes a 382 amino acid protein which has similar properties to the N proteins described for other coronaviruses. To the 5' side of this gene are sequences of part of the M gene, an arrangement seen in both BCV and MHV. Flanking the N gene on its 3' side is an open reading frame which encodes a small, hydrophobic protein of 78 amino acids (Kapke, 1985). Such an arrangement of genes has not been seen in any other coronavirus.

Pathogenesis

Coronaviruses have a wide distribution in nature. They cause a spectrum of diseases in a wide variety of hosts. Table I.2.2 shows

Table I.2.2. Spectrum of diseases caused by different coronaviruses[a]

Type of disease	Virus/Host										
	MHV mouse	RCV rat	SDAV rat	TCDV turkey	TGEV pig	HEV pig	BCV bovine	IBV chicken	CCV dog	FIPV cat	HCV man
Encephalitis	+					+				+	
Enteritis	+			+	+	+	+		+	+	
Hepatitis	+									+	
Adenitis	+		+								
Nephritis	+				+			+		+	
Pancreatitis	+										
Peritonitis	+									+	
Respiratory infections	+	+		+	+	+		+		+	+

[a]Adapted from Tyrrell, 1981.

their natural hosts and the diseases which they cause. The two most common diseases are enteritis and respiratory infections (Tyrrell, 1981). Thus, they usually replicate in either the respiratory or gastrointestinal tracts of susceptable hosts. Transmission of the virus is normally through aerosol or fecal contamination. Although most coronaviruses cause clinical disease in the species from which they were isolated, natural and experimental transmission to other, unrelated species is possible (Siddell et al., 1983). Such transmission usually results in either inapparent infections or in diseases not normally seen in the natural host (Wege et al., 1982).

The infections of the respiratory tract by HCV, IBV, RCV and MHV and of the gastrointestinal tract by BCV, TGEV, CCV, TCV and some MHV strains are generally acute (Siddell et al., 1983). The pathophysiological events which lead to clinical symptoms are due to the cytocidal infection of the target cells, epithelial cells of the intestine or ciliary epithelium of the respiratory tract (Wege et al., 1982). With respiratory infections, a local immune response leading to the secretion of IgA is usually sufficient to combat the acute stage of illness; the development of humoral immunity then prevents spreading of the disease to other organs (Siddell et al., 1982).

The majority of the enteropathogenic coronaviruses selectively infect absorptive cells of the intestinal mucosa (Pensaert, 1970). This causes atrophy of the villi, which leads to diarrhea, acidosis, and dehydration. The severity of disease, which depends upon many factors, varies from mild, transient enteritis to a rapidly progressing, fatal diarrhea (Siddell et al., 1983). Production of

secretory antibody provides the major defense against enteric infection, as it does against respiratory infection (Pensaert and Callebaut, 1978). In neonates, maternal antibodies provided by colostrum and milk are an additional protection against enteritis (Doughi and Storz, 1977).

A quite different mechanism of acute coronavirus infection has been described for the hemagglutinating encephalitis virus (HEV). HEV causes vomiting and wasting disease, sometimes accompanied by encephalomyelitis (Wege et al., 1982). The virus first infects the respiratory system and intestine, from which it spreads along peripheral nerves to the brain stem. Lesions in the brainstem lead to vomiting. Infection of the neurons which regulate the peristaltic functions can result in paralysis of the ileum, leading to wasting (Andries and Pensaert, 1980).

Coronaviruses can also readily induce persistent infection in both animals (Robb and Bond, 1979) and in cell cultures (Lucas et al., 1977; Stohlman et al., 1979). The salient feature of the persistent cell culture infections is that, in most instances, the majority of cells did not contain viral antigens; but all the cells were resistant to superinfection by wild-type virus. Persistent infections in animals often lead to disease of a subclinical or chronic nature. Little is known of the molecular mechanisms important in the development and maintenance of persistent infections, in vivo or in vitro.

However, some factors which influence the outcome of the infections in animals have been described. In several murine systems, the host's genetic background determines resistance (Wege et al., 1982).

This expression of genetic resistance is manifested by the mature macrophage. Virus replicates well in macrophages of sensitive mice, but viral replication in resistant mice is restricted (Stohlman et al., 1982). Viral replication is also influenced by interferon and complement, as well as prostaglandins secreted by macrophages. Secretory functions may be impaired during chronic infections (Virelizier et al., 1976).

Persistent infections of the central nervous system can be induced in rodents by the neurotropic MHV JHM strain, especially if attenuated virus is used (Stohlman et al., 1982). Mild lesions of demyelination in the brain are detectable by electron microscopy, even in the absence of acute disease. Viral antigens have not been detected in neuronal tissue, but viral nucleic acid has been detected in situ hybridization (Lavi et al., 1984). Such infections are of interest as experimental models for virus-induced demyelinating diseases in humans.

A small percentage of animals infected with feline infectious peritonitis virus (FIPV) develop fatal disease after a long incubation period. A persistent infection is apparently established in macrophages and the cells of the reticuloendothelial system of the animals (Siddell, 1983). Experimental results suggest that the pathological changes observed during the chronic infection are caused by an immune complex disease (Pederson, 1976). The disease develops much faster in seropositive kittens than in seronegative ones. In these seropositive animals, organs develop lesions containing viral antigen bound to IgG and complement. Deposits of immunoglobulins and complement complexes are found in the renal glomeruli of these cats.

In order to identify the interactions between coronavirus and host which permit these different types of persistent infections, much more information about the molecular mechanism of pathogenesis is needed. What host cell functions are used by the virus during replication? What controls are exerted on coronavirus transcription and translation? Persistent infections depend on the expression of viral genes, the functional impairment of certain host cells, and interaction of viral components with the host's immune system (Wege et al., 1982). The molecular details of this combination of factors are of great interest and will further the understanding of persistent diseases in animals and humans.

CHAPTER III

NUCLEIC ACID HYBRIDIZATION COMPARED TO OTHER METHODS OF VIRUS DETECTION

Diagnosis of a viral infection can be made in one of three basic ways: (1) detection of an immune response to the virus, (2) detection of the infectious virus, or (3) detection of components of the virus. The first, serological diagnosis, is not appropriate for studies of virus in cell culture or chronic viral infections. This brief review, then, will consider only methods for direct detection of viruses or viral components, with primary emphasis on RNA-DNA hybridization.

The oldest and perhaps most sensitive method is isolation of the virus, usually in cell culture, but sometimes in organ culture, eggs, or experimental animals (Richman et al., 1984). This method offers a sensitivity unmatched by any other, since a single infectious virion can provide a positive result if an appropriate susceptable cell culture is available. Because the causative agent is recovered and propagated, there can be unambiguous identification of the virus (Joklik, 1985). Perhaps most importantly, virus isolation can detect new agents as well as known viruses whose presence was not suspected. Unfortunately, the method is slow, expensive, and requires technical expertise and sophistication (Richman et al., 1984). It necessitates the use of appropriate cell lines or primary cell culture for different virus families. Unfortunately, certain viruses, such as the coronaviruses, are not readily grown by available cell culture techniques (McIntosh, 1974).

Another method of detecting the virus itself is electron microscopy. With the development of negative staining (Brenner and Horne, 1959), viruses could be visualized in cell or tissue culture preparations or in clinical specimens. Larger viruses and those with a geometric construction are the easiest to visualize (Almeida and Waterson, 1980). Although coronaviruses have distinctive surface projections, their identification by EM presents some problems. There are many artifacts in clinical specimens, such as endoplasmic reticulum, bacteria, mycoplasma, and cristae of mitochondria, which, when disrupted, may produce spherical fragments that frequently display a fringe of surface projections (Almeida, 1983). Thus, coronavirus may be identified when none are actually present.

The lower limit of sensitivity in EM ranges from 10^5 to 10^7 virus particles per gram of feces; this can be decreased by a factor of ten if specific antisera are used to cluster the virus (Obert, 1981). Careful attention to detail in specimen preparation is necessary to achieve this level of sensitivity. A specimen can be examined for the presence of any virus rather than one particular type of virus. However, for routine viral diagnosis, EM is too expensive and insensitive. The more recently developed techniques of immunofluorescence and enzyme immunoassay, especially with monoclonal antibodies, are better for the routine aspects of viral diagnosis. Electron microscopy is still valuable, though, to obtain information from unusual specimens and to diagnose those viruses for which no other method as yet exists (Almeida, 1983).

Since most viruses produce many more structural components than are actually incorporated into new virions, detection of viral components such as structural proteins and nucleic acids may be a more sensitive means of diagnosis. Immunofluorescence microscopy and a recently developed modification, immunoperoxidase microscopy, are two techniques used to detect viral antigens (Gardner and McQuillin, 1980). The first requires a fluorescence microscope, the second, a light microscope. Both techniques are highly dependent on the reagents used and cannot be automated. Significant technical problems are encountered in the preparation of suitable specimens, autofluorescence, and nonspecific binding of antibody (Richman et al., 1984). Though their reliability has been improved recently by the introduction of monoclonal antibodies (Yolken, 1983), better methods are needed because of their relative insensitivity and inefficiency.

Enzyme-linked, immunosorbant assays (ELISA) are more sensitive, have an objective end point and can easily be used for a large number of samples or even automated. Some of the disadvantages of ELISA are the carcinogenicity of certain substrates, the expense of the automated equipment, and an unacceptable level of sensitivity for many purposes (Yolken, 1982). The many variations of ELISA already may have reached the limit of their sensitivity. This limit is their inability to discriminate between the soluble colored compound produced by enzyme that is bound specifically to viral antigen and that produced by enzyme bound nonspecifically (Ekins, 1981). A different approach is necessary for increased sensitivity, the detection of another viral component, nucleic acids.

The detection of nucleic acids as a means of virus diagnosis has several advantages over the methods described above. With sequences which are unique to a specific viral genome, a DNA probe can have a very high degree of specificity. Conversely, in families of related species, sequences common to most can be used as probes to identify a broader spectrum of viruses. The high avidity of complementary nucleic acid strands for each other makes hybridization assays very sensitive, resulting in the detection of fewer than one copy of virus genomic RNA per cell (Brahic and Haase, 1978). With the recent advances in molecular cloning, the required quantities of standardized reagents, for the detection of any virus, can be prepared relatively easily (Maniatis et al., 1982). Since coronaviruses are single-stranded (ss)RNA viruses, the rest of this review will consider methods for the immobilization and detection of viral RNA on solid supports.

More than twenty years ago, it was discovered that ssDNA could be bound to nitrocellulose (NC) and then hybridized to a ssDNA or RNA probe (Nygaard and Hall, 1963). Initially, it was reported that RNA would not bind to NC (Gillespie and Spiegelman, 1965). Subsequent investigations determined that RNA in very concentrated salt solutions would bind to NC (DeLarco and Guroff, 1973). It was first thought that only those RNA molecules with a sufficiently long poly (A) tail could bind. This method thus appeared to be selective for messenger RNA, but did not include the small percentage of mRNA which is tailless. It was later reported that, after denaturing in glyoxal, RNA of all types could be immobilized to NC in concentrated salt solutions (Thomas, 1980). This provides a different explanation for the results of

DeLarco and Guroff. The mRNA did not bind to NC strictly because of the poly (A), but because with the tail, which usually did not form secondary structures with the rest of the molecule, it was more denatured than RNA with short or no terminal poly (A).

Another advance in binding RNA to NC was the development by White and Bancroft of a method for immobilizing total RNA from cytoplasmic extracts (White and Bancroft, 1982). When these extracts were dot blotted to NC with a 96-well blotting apparatus, the RNA would still efficiently hybridize to a complementary probe in spite of the presence of other macromolecules. It was later learned that detergents, which were used in the above extraction method, suppress DNA-NC and protein-NC interactions but do not interfere with RNA-NC interactions (Bresser et al., 1983a). Thus, this method could be used to rapidly extract RNA from numerous samples and blot them simultaneously to NC.

A similar dot blot method was developed by Bresser and Gillespie using NaI and detergent to denature and bind RNA (Bresser et al., 1983b). By lowering the temperature of the denaturing solution, immobilization of mRNA is favored while immobilization of proteins, DNA and nonmessenger RNA is suppressed. Also, binding of mRNA is so strong that baking of the NC is not required; thus, the RNA is biologically active. Multiple samples can also be processed with the protocol.

Both of the above procedures have advantages over other methods of sample preparation for detection of viral RNA. The quantity of sample material required is small. For example, 2.5×10^4 cells, 1/100 of a rat pituitary (less than 100 µg of tissue), or 1 µl of fecal material

is sufficient for the assay. There is minimal manipulation of samples prior to analysis, since the RNA is not first purified by phenol extraction or ethanol precipitation. After preparation, samples may be frozen and later blotted. The NC filters can themselves be stored after blotting; immediate probing is not necessary.

The virus-specific DNA probe, prepared in quantity with recombinant DNA techniques, can be labeled by a number of methods. The most common is nick translation, in which DNase and DNA polymerase are used to incorporate ^{32}P-labeled deoxynucleoside triphosphates into the DNA molecule (Rigby et al., 1977). A probe with a specific activity of 1-2 x 10^8 cpm/µg of DNA can be generated with this method. At this activity, a sensitivity level of 10-50 pg of RNA per dot can be detected (Parker and Stark, 1979; Gillespie and Bresser, 1983). After hybridization of the probe with RNA-blotted NC, radiolabel bound to the RNA after washing is detected by autoradiography.

Dot blot methodology and molecularly cloned cDNA probes have been used by many researchers in a wide variety of studies of RNA viruses. This research includes: (1) analysis of genome subunit reassortment among bunyaviruses (Pringle et al., 1984), (2) detection of rotavirus in stool (Flores et al., 1983), (3) detection of enterovirus in cell culture (Hyypia et al., 1984), (4) comparison of genomic homologies in Coxsackievirus (Tracy, 1985), and (5) diagnosis of rotavirus infection (Lin et al., 1985). The probes were very specific and sensitive, with levels of detection of viral RNA as low as 8 pg per dot.

One possible way to further improve the sensitivity of detection is to prepare hybridization probes by in vitro transcription of cloned

cDNA. This has been done with cloning vectors which contain the DNA polymerase promotor for the Salmonella typhimurium bacteriophage SP6; cloned DNA adjacent to this promotor can be transcribed in vitro into RNA transcripts with high efficiency (Green et al., 1983) (see Figure I.3.1). Up to 10 µg of ssRNA can be obtained from 1 µg of vector DNA per reaction. These RNA transcripts can be radiolabeled to specific activities of 6×10^8 cpm/µg of RNA and allow for up to tenfold greater sensitivity than nick-translated probes in hybridization detection of RNA in blots (Melton et al., 1984) or in situ (Cox et al., 1984). Thus, amounts of less than 1 pg of RNA can be detected after exposure of X-ray film to the blots for 48 hours.

Although ^{32}P-labeled cDNA probes have a greater sensitivity in detecting viral RNA than methods of detecting viral antigen, there are disadvantages associated with their use (Richman et al., 1984). Handling and disposal of ^{32}P reagents can be hazardous and expensive and require special permits. Because of the short half-life of ^{32}P, labeled probes are only usable for two weeks. In order to keep background low, no more than 10^6 cpm of probe per square centimeter of blot should be used; this results in hybridization times of at least six hours. Also, autoradiography requires exposure of X-ray film to the blots for 24 to 72 hours to obtain high sensitivity.

Many of these disadvantages can be overcome by a nonradioactive labeling method such as biotinylation. This method involves the incorporation of biotinylated nucleotides (bio-11-UTP) into cDNA by standard techniques (Langer et al., 1981). The biotin-labeled DNA, after

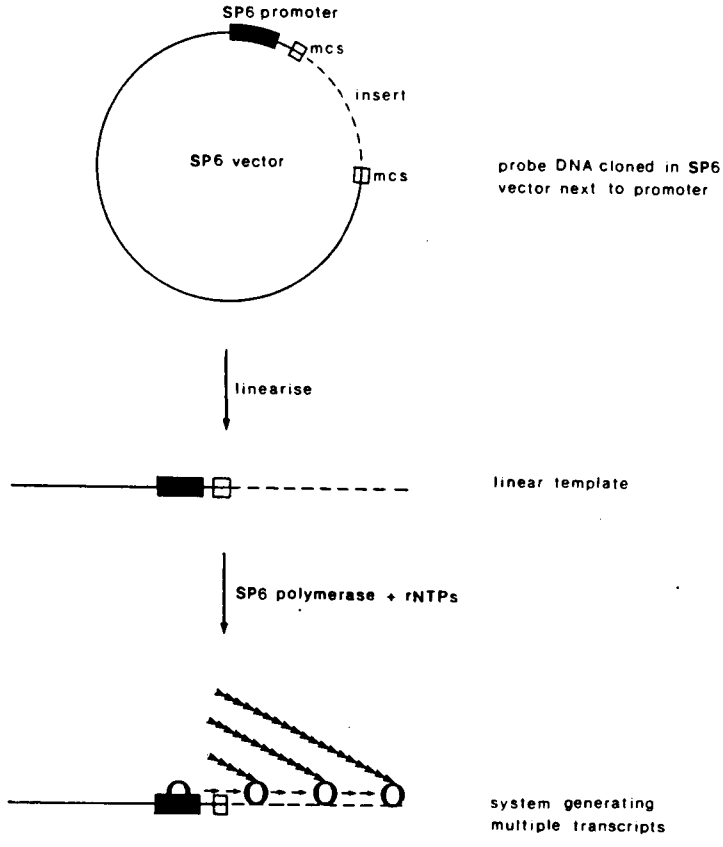


Figure I.3.1. The SP6 polymerase system. From Arrand, 1985. MCS: multiple cloning site. ➤➤➤➤➤➤: labelled transcript. O : SP6 polymerase. →→ : direction of transcription. rNTPs: ribonucleoside triphosphates.

hybridization, is detected with avidin or strepavidin which have been chemically coupled to an enzyme catalyzing a colormetric reaction. Biotin binds this enzyme complex; the biotinylated DNA is then visualized by development of the color reaction (Brigati et al., 1983). Biotin-labeled probes have a long shelf-life--exceeding one year. Probe concentrations of up to 1 µg/ml can be used, thus reducing hybridization time to one hour (Leary et al., 1983). The color development phase, which is short when compared to the time required for autoradiography, takes only two to three hours. Concentrations of 1 to 5 pg of RNA per dot have been detected with biotinylated probes.

For the detection of coronaviruses, nucleic acid hybridization appears to be the method of choice. Although virus isolation in cell culture is the most sensitive procedure, coronaviruses are difficult to grow in primary culture. EM and methods of viral antigen detection are either too cumbersome and expensive for routine use or lack sensitivity. With further developments in cloning vectors, labeling techniques, and sample preparation protocols, nucleic acid hybridization will likely become even more sensitive and efficient. In addition to diagnosis of viral infections, nucleic acid hybridization may be used to investigate chronic diseases in which coronaviruses are suspect.

CHAPTER IV

RATIONALE FOR PRESENT WORK

Researchers in this laboratory have, over the past eight years, worked to develop an understanding of the molecular structure and replication of two enteric coronaviruses, the bovine enteric coronavirus (BCV) and the porcine transmissible gastroenteritis virus (TGEV). These two viruses were chosen for several reasons. When the antigenic relationships among coronaviruses are considered, the viruses can be placed in four antigenic subgroups (Pederson et al., 1978; Robb and Bond, 1979a). As Figure I.4.1 shows, BCV and TGEV belong to separate antigenic subgroups of mammalian coronaviruses and so do not appear to have any antigenic similarities. BCV additionally is one of three hemagglutinating coronaviruses; the others are HEV and HCV OC43.

Both BCV and TGEV are economically important as disease producers in food animals. Both preferentially infect neonates and cause high morbidity and mortality in young animals. BCV is a major cause of neonatal bovine enteritis and is responsible for nearly 25% of all deaths due to enteritis in developed countries (House, 1978). Vaccines of virus attenuated through cell culture passage are ineffective in combating these diseases.

There have been reports which indicate that both viruses may be zoonotic. Coronavirus particles were visualized in renal-biopsy tissues from patients with chronic nephritis (Apostolov et al., 1975). This disease, endemic (Balkan) nephropathy, occurs almost exclusively

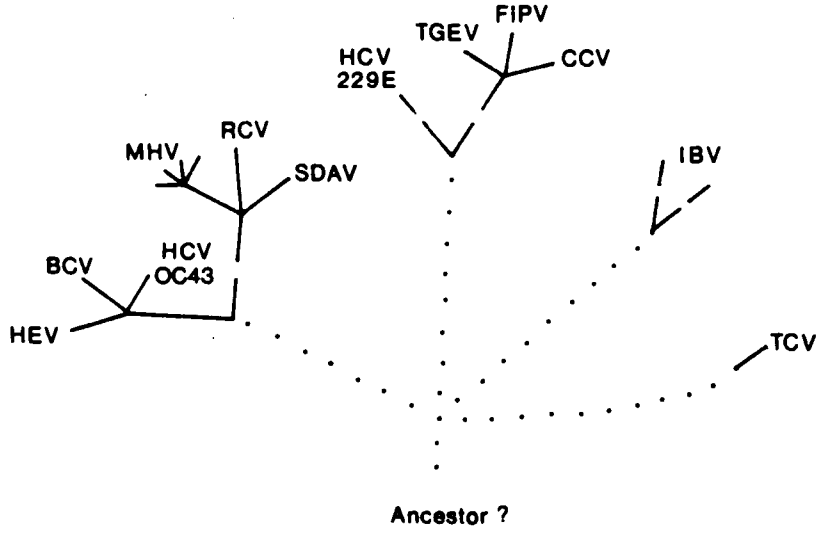


Figure I.4.1. Tentative coronavirus family tree based on antigenic interrelatedness. Data for this figure comes primarily from the review by Wege et al., 1982 (figure taken from Brian et al., 1983). Solid lines indicate evidence for cross-neutralizing activity; dashed lines indicate immunological evidence (other than neutralizing) for cross reactivity; dotted lines indicate possible relatedness with little or no supporting data. Viruses include: the porcine hemagglutinating encephalomyelitis virus (HEV), bovine coronavirus (BCV), human coronavirus OC43 (HCV OC43), murine hepatitis virus (MHV), rat coronavirus (RCV), rat sialodacryoadenitis virus (SDAV), human coronavirus 229E (HCV 229E), porcine transmissible gastroenteritis virus (TGEV), feline infectious peritonitis virus (FIPV), canine coronavirus (CCV), avian infectious bronchitis virus (IBV), and turkey bluecomb disease coronavirus (TCV).

among people in close contact with swine, thus implicating TGEV. BCV may cause acute gastroenteritis in humans. A researcher, after experimentally infecting calves with BCV, became ill with severe diarrhea (Storz and Rott, 1981). Coronavirus particles were later identified in his stool by electron microscopy. It may be possible for BCV and TGEV to serve as models for human enteric coronaviruses which cannot yet be isolated by present cell culture techniques.

The biological behavior of coronaviruses suggests that they may be involved in chronic disease, both of animals and humans, for which no cause is yet known. Although difficult to isolate in primary cell culture, when the proper permissive cell line is determined, coronaviruses readily establish persistent infection. This has been done with BCV and TGEV. Persistent infections with MHV, causing chronic hepatitis and a demyelinating encephalitis, have been described (Wege et al., 1982). As mentioned earlier, coronaviruses have been implicated in chronic human diseases, such as multiple sclerosis (Burks et al., 1980) and sprue (Baker et al., 1982). BCV has been detected in feces of clinically normal animals (Crouch et al., 1985).

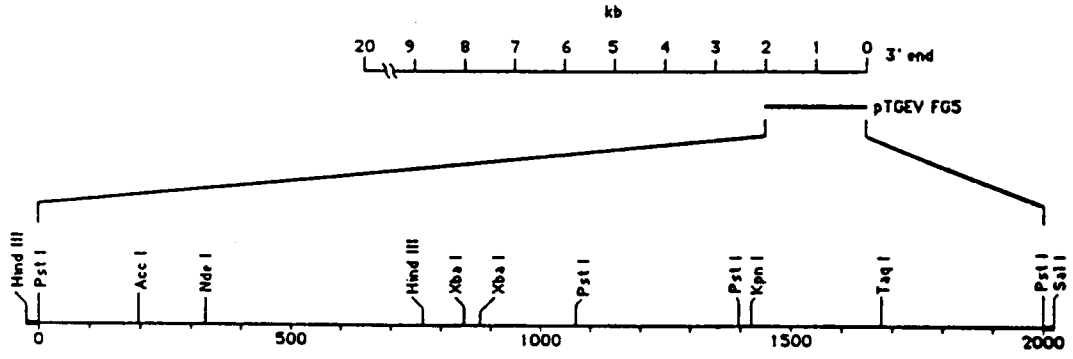
Considering all these findings, there is a profound need for a rapid and sensitive method to detect both BCV and TGEV. For this purpose, nucleic acid hybridization probes to detect coronaviral RNA will be beneficial. Such probes can be used for the rapid diagnosis of acute clinical infection in animals. They even may be useful in the diagnosis of human enteric viral infections, especially of human coronaviruses which have not yet been successfully cultured (Resta

et al., 1985). When a TGEV or BCV zoonosis is suspected, the probes can be used to confirm the presence of these viruses. Perhaps most importantly, these nucleic acid probes can test the hypothesis that TGEV and BCV, or other coronaviruses closely related to them, can cause chronic disease in animals and humans as a result of persistent infections.

For both TGEV and BCV, cDNA clones 1 Kb to 4.2 Kb in length have been prepared (Kapke, 1985; Lapps, 1986). Figure I.4.2 shows the restriction endonuclease maps for selected clones of BCV and TGEV. For the development of hybridization probes, clones FG5 and MN3, which represent the first 2.0 Kb of TGEV and 2.2 Kb of BCV, respectively, are the sequences of choice for virus species specificity. From analysis of the sequences contained in these two clones, the following was determined: (1) This region in the TGEV genome codes for a previously uncharacterized hydrophobic protein, the N protein, and a small portion of the M protein. (2) In BCV, the same region codes only for the N protein and part of the M protein. (3) When the amino acid sequences of the two N proteins were compared, a homology of 26% was found. (4) However, because of different codon use, no nucleotide sequence homology is found (above that expected from radomness). There should be no cross-hybridization between the two clones. Figure I.4.3 shows the position of the clones relative to the gene map of each virus.

The nucleocapsid and matrix proteins are highly conserved in all strains of the mouse coronavirus (Cheley et al., 1981). There will probably be a similar conservation of these proteins within each

a.



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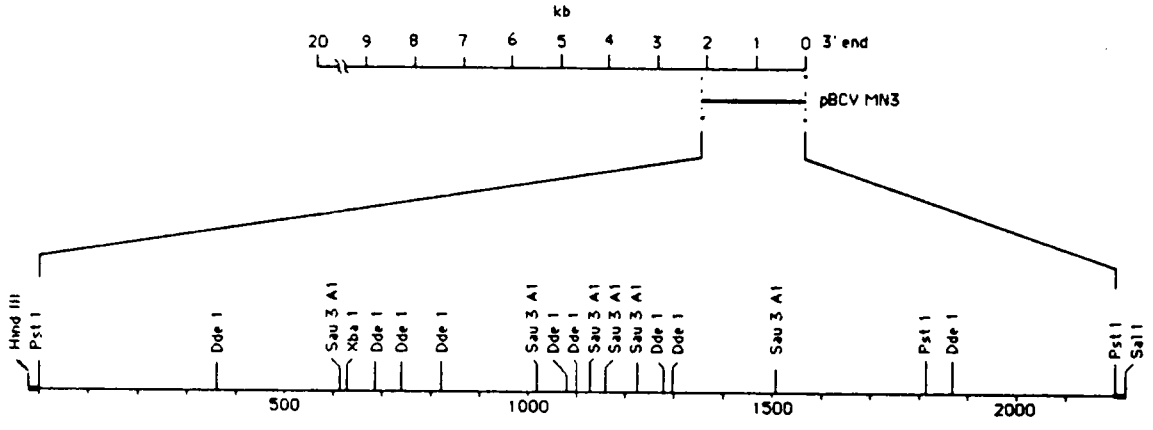
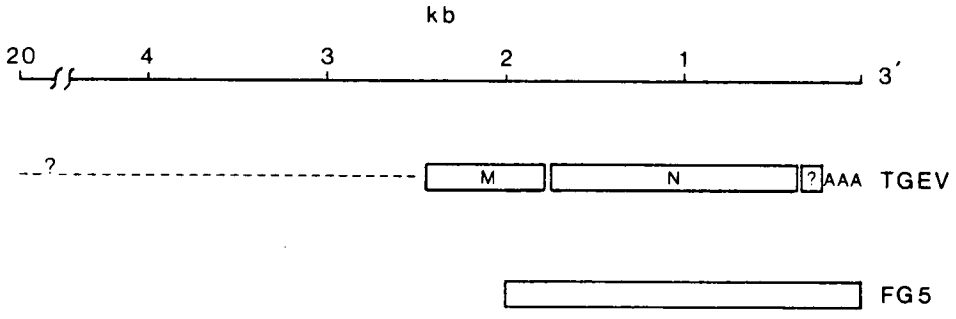


Figure I.4.2. Restriction endonuclease maps of TGEV and BCV cDNA clones. (a) TGEV clone FG5 (From Kapke, 1985). (b) BCV clone MN3 (From Lapps, 1986).

a.



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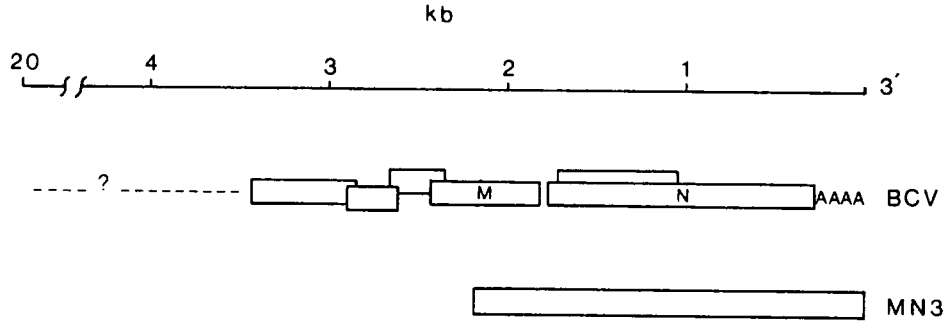

Figure I.4.3. Schematic diagram of the relationship of the cloned sequences to genomic RNA. (a) Gene map of TGEV as determined by nucleotide sequencing and subsequent analysis of encoded proteins (Kapke, 1985) and 2.0 Kb clone FG5. (b) Gene map of BCV as determined by nucleotide sequencing and analysis of encoded proteins (Lapps, 1986) and 2.2 Kb clone MN3. M: matrix glycoprotein gene; N: nucleocapsid protein gene; AAA: noncoding region and poly (A) tail.

species of coronavirus. Additionally, genomic RNA and all subgenomic mRNAs contain the sequences for the N gene. Therefore, hybridization probes prepared from clones FG5 and MN3 should be highly specific for their respective RNAs as well as sensitive in detection of any homologous mRNA species.

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PART II: USE OF CLONED HYBRIDIZATION PROBES FOR DETECTION OF PORCINE TRANSMISSIBLE GASTROENTERITIS CORONAVIRUS AND BOVINE ENTERIC CORONAVIRUS

CHAPTER I

INTRODUCTION

Both the bovine enteric coronavirus (BCV) and the porcine transmissible gastroenteritis virus (TGEV) are coronaviruses, a group of single-stranded, nonsegmented, enveloped viruses of positive polarity (Wege et al., 1982). BCV and TGEV infections cause significant economic loss as a result of young animal mortality, but neither is yet effectively controlled by vaccination. Each belongs to a separate antigenic subgroup of mammalian coronavirus and they appear not to share antigens (Pederson et al., 1978). Both may be zoonotic; TGEV may cause chronic nephritis in humans (Apostolov et al., 1975) and BCV is implicated in acute gastroenteritis (Storz and Rott, 1981). The biological behavior of coronaviruses suggests that they may be involved in chronic diseases of animals and humans. In cell culture, persistent infection can be readily established with both BCV and TGEV. Persistent infections with accompanying disease are well documented for the mouse hepatitis coronavirus (Wege et al., 1982). There have also been reports which suggest that coronaviruses may be involved in chronic human diseases such as multiple sclerosis (Burks et al., 1980), chronic nephropathy (Apostolov et al., 1975), and sprue (Baker et al., 1982).

Using recombinant DNA techniques, cDNA of specifically defined sequence has been prepared from TGEV and BCV genomic RNA (Kapke, 1985; Lapps, 1986). Clone pTGEV-FG5, which represents the first (3') 2.02 Kb sequence of the 20 Kb genome of TGEV, and clone pBCV-MN3, which

represents the first (3') 2.2 Kb sequence of the 20 Kb BCV genome, were selected for use in this study. In both genomes, this region encodes the complete nucleocapsid (N) protein and a large portion of the matrix (M) protein, based on an analysis of the primary structure of these sequences (Kapke, 1985; Lapps, 1986). The nucleocapsid and matrix proteins are highly conserved among the mouse coronavirus strains (Cheley et al., 1981; Lai and Stohlman, 1981). Therefore, DNA coding for these proteins were the sequences of choice for developing diagnostic hybridization probes for virus species specificity. There is a 26% sequence homology in the amino acids of the BCV and TGEV N proteins; but, because of different codon usage, there is no nucleotide sequence homology above that expected from randomness. Thus, probes prepared from these clones should be specific and therefore distinguish between the two coronaviruses.

Both BCV and TGEV are excreted in large quantities in the stools of infected animals. However, either the low degree of sensitivity or the extensive time involved in current methods of coronaviral detection, namely, electron microscopy, virus isolation, and immunofluorescence, prohibit routine rapid enteric coronavirus diagnosis. In this study we describe the use of cloned BCV and TGEV cDNA as hybridization probes to detect and differentiate their respective viral RNAs. With dot blots of uniform size on nitrocellulose, both probes could detect as little as 25 pg of homologous RNA. No crossreactivity between the two coronaviruses was observed even at RNA concentrations of 10 ng per dot. Using a two-step denaturation procedure, we were

able to detect RNA in virus obtained from cell cultures or fecal specimens. Results of electron microscopy, virus isolation, immunofluorescence and dot blot hybridization analysis were compared on fecal swabs taken from a litter of piglets experimentally infected with TGEV.

CHAPTER II

MATERIALS AND METHODS

Cells and viruses. Cell cultures and viruses TGEV, BCV, murine hepatits virus (MHV) A59, vesicular stomatitis virus (VSV) and New Castle disease virus (NDV) were grown as previously described (Brian et al., 1980; Dennis and Brian, 1978).

Preparation of virion RNA samples. Methods used for infection of cells, virus purification and virion RNA extraction have been described previously (Dennis et al., 1982; King et al., 1982). For these studies, virus (BCV, TGEV, or MHV) was purified from clarified supernatant fluids by pelleting through a barrier of 37% sucrose (wt/wt) made up in TMEN (100 mM Tris maleate [pH 6.0] - 100 mM NaCl-1 mM EDTA) prior RNA extraction. Virion RNA was extracted using the proteinase K-SDS-phenol method (Brian et al., 1980). After ethanol precipitation, RNA was resuspended in water and quantitated by absorbance with a Perkin-Elmer 554 spectrophotometer.

For filter binding, known quantities of RNA were added to a buffer containing 10 mM Tris (pH 7.0)-1 mM EDTA-0.5% Nonident P-40 (Shell). An equal volume of freshly prepared denaturing solution consisting of two parts 37% (w/w) formaldehyde and three parts 20X SSC (1X SSC = 150 mM NaCl - 15 mM trisodium citrate) was then added and the mixture was heated at 60°C for 15 minutes. Dilutions of RNA were made with 15X SSC using either 0.6 ml siliconized polypropylene tubes or a 96-well siliconized microtiter plate. RNA was handled at all times in

baked or autoclaved siliconized containers and prepared in solutions made from diethylpyrocarbonate-treated water.

Preparation of cell culture virus samples. To prepare cell culture-grown virus, cells were infected at a multiplicity of approximately 1 virus per cell and harvested at 17, 48, and 10 hours post-infection respectively for TGEV, BCV, and MHV. Cell culture supernatant fluids were clarified by centrifugation at 2,000 X g for 10 minutes and used directly for sample preparation. To lyse virions, 0.1 volume of 5% NP40 was added and allowed to mix for 10 minutes on ice (White and Bancroft, 1982). After centrifugation for 3 minutes at 0°C, 5000 rpm, supernatants were removed and treated with an equal volume of denaturing solution and heated as described above.

Preparation of fecal specimens. Individuals in a litter of 12 3-day-old pigs were given 5 ml inoculum by stomach tube of a given dilution of virus stock. Virus stock was small intestinal contents containing Miller strain TGEV that had undergone 11 passages in gnotobiotic piglets subsequent to plaque purification in cell cultures (Frederick et al., 1976). Pigs 1 and 2 were inoculated with a 10^{-2} dilution; pigs 3 and 4 with a 10^{-3} dilution, and pigs 5 and 6 with 10^{-6} dilution. Fecal specimens were taken with dry cotton swabs and stored at -80°C until use. To prepare samples, 1.0 ml of sterile EBSS (Earle's Balanced Salt Solution) was added to the tube containing the swab. After the tube was agitated vigorously, an aliquot was removed, clarified by microfuge centrifugation for 30 sec, and treated with an equal volume of denaturing solution and heat as described above.

Preparation of TGEV and BCV cDNA probes. For both TGEV and BCV, cDNA clones representing the 3' end of their genomes have been prepared and characterized (Kapke, 1985; Lapps, 1986). The cDNA was cloned by a modified method of Gubler and Hoffman (1983) into plasmid pUC9. E. coli strain JM103 was the host. Insert-containing plasmid was harvested from cultured bacteria, and DNA was extracted and purified on CsCl gradients (Maniatis et al., 1982). Insert DNA was cleaved from the plasmid with restriction endonucleases Bam Hl and Hind III which cut in the multiple cloning region. BCV clone MN3 yielded the insert as a single 2.2 Kb fragment, while the TGEV clone FG5 yielded two fragments of 1.3 and 0.7 Kb. Fragments were recovered by preparative electrophoresis in 1% agarose gels and electroelution (Maniatis et al., 1982). cDNA was labeled to specific activities of 1-2 x 10^8 cpm per µg by nick-translation with ^{32}P dCTP (Rigby et al., 1977). Before they were used for hybridization, probes were denatured by heating for 5 minutes at 95-100°C and then chilled on ice.

RNA dot blotting and molecular hybridization. Nitrocellulose (BA85; Schleicher and Shuell, Inc.) was soaked in DEPC-treated water and equilibrated in 20X SSC before application of samples. Nitrocellulose was supported by two sheets of blotting paper in a 96-hole Minifold dot-blot apparatus (Schleicher and Schuell, Inc.). Samples were applied in a volume of 100 µl per well. Samples of purified RNA were allowed to set for 30 seconds before light suction was applied. With all other samples, strong suction was used as the samples were added. The nitrocellulose sheet was then air dried and baked at 76°C

for 90 minutes in a vacuum oven to fix the RNA (Thomas, 1980). Hybridization was carried out essentially as described by Thomas (1980), but without dextran sulfate. Blots were incubated for 4-24 hours at 42°C in sealed plastic bags containing a solution of 50% formamide-5X SSC-50 mM Na_2HPO_4 (pH 6.5)-0.2% SDS-1X Denhardt's solution (0.02% of bovine serum albumin-0.02% polyvinylpyrrolidone-0.02% Ficoll)-250 µg/ml sheared, denatured salmon sperm DNA. After prehybridization, fluids were removed and fresh solution (1 ml/cm^2) was added to the bag followed by 10^6 cpm/cm^2 of the denatured cDNA probe. The bag was sealed and incubated at 42°C for 16-24 hours. After hybridization, nitrocellulose was washed four times with 2X SSC-0.1% SDS for 5 minutes at room temperature, then twice with 0.1X SSC-0.1% SDS for 15 minutes at 50°C. Dried blots were exposed to Kodak AR X-ray film at -70°C, with intensifying screen, for 24-48 hours.

CHAPTER III

RESULTS

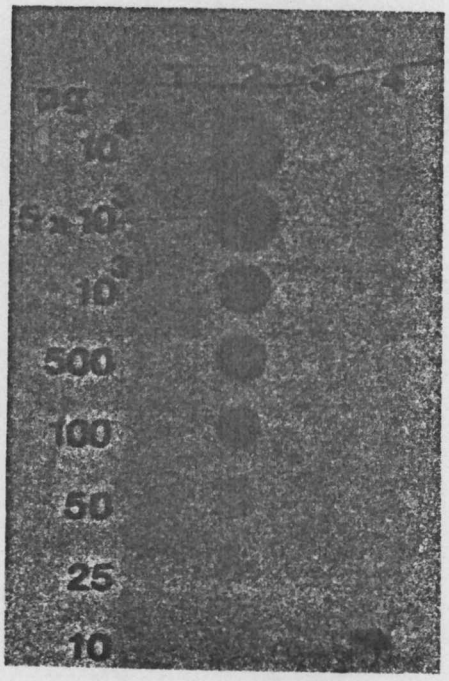
Specificity and Sensitivity of cDNA Probes

To determine the specificity and sensitivity of each probe for its respective RNA, purified BCV, TGEV, and yeast transfer RNA were denatured as described earlier. Dilutions were then prepared, and samples ranging from 10 pg to 10 ng were dotted onto nitrocellulose. As seen in Figure II.3.1, each probe was highly specific for its homologous RNA since no cross reactivity with heterologous RNAs was observed. Both probes were able to detect as little as 25 pg of RNA. Dot blots of 10 ng of MHV, NDV, and VSV RNA were probed by both BCV and TGEV cDNAs; no detectable hybridizations was observed at any wash stringency used (Figure II.3.2).

Detection of Viral RNA in Cell Culture Fluids

Clarified cell culture fluids from TGEV, BCV, and MHV infected cells were treated and denatured as described earlier. A series of tenfold dilutions were made into 15X SSC, and 100 µl of each sample was dotted onto nitrocellulose. As Figure II.3.3 illustrates, each probe was specific for its respective RNA. Virus dilutions of 10^{-3} (equivalent to 0.1 µl of supernatant fluid) are visible. However, as seen in Figure II.3.3B, the BCV probe may begin to hybridize with MHV RNA at concentrations greater than 10 ng/dot. In other blots, both probes failed to hybridize to VSV cell culture fluids (data not shown).

A.



B.

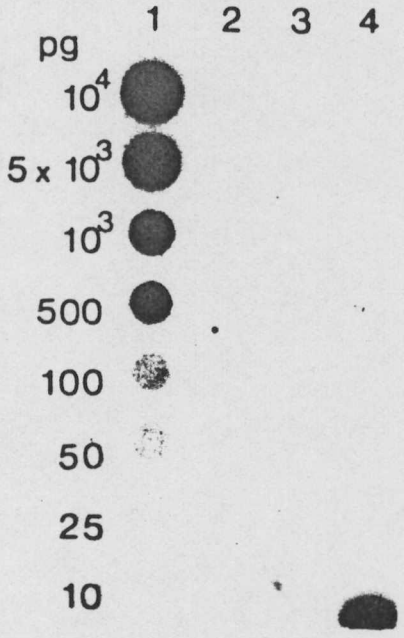


Figure II.3.1. Comparison of the sensitivity and specificity of TGEV and BCV cDNA probes for different viral RNAs. Colums: 1, BCV RNA dilutions; 2, TGEV RNA dilutions; 3, Yeast transfer RNA dilutions; 4, 100 µl of 15X SSC per dot. (A) TGEV probe. (B) BCV probe. RNAs were denatured as decribed in materials and methods and applied to nitrocellulose at the concentrations shown in a volume of 100 µl per dot. Duplicate filters were baked, prehybridized, hybridized to ^{32}P-labeled probes (specific activity, 1-2 x 10^8 cpm/ug), washed and autoradiographed at -70°C for 48 hrs with an intensifying screen.

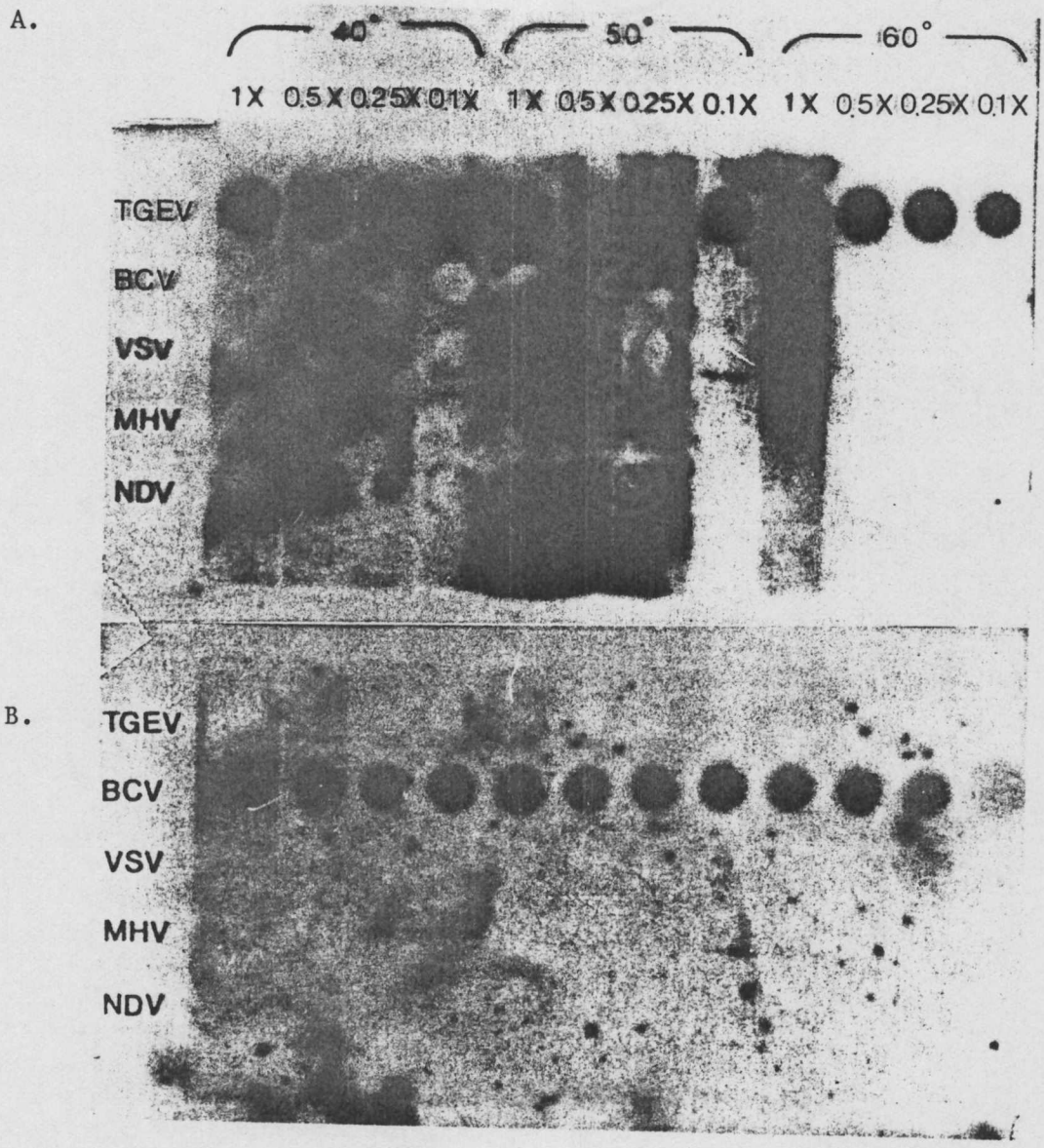


Figure II.3.2. Effect of wash stringency on hybridization. (A) TGEV probe. (B) BCV probe. Purified RNAs from TGEV, BCV, VSV, MHV, and NDV were denatured as described; 10 ng of each in a volume of 50 µl was applied to BA85 nitrocellulose in a series of 12 replicate dots. Duplicate nitrocellulose sheets were baked, prehybridized, and hybridized to ^{32}P-labeled probes. The sheets were initially washed at room temperature in 2X SSC/0.1% SDS for 4 x 5 minutes. Then each sheet was cut into 12 identical strips. The individual strips were washed for 2 x 15 minutes at the temperature and SSC concentration (+0.1% SDS) indicated above each column and autoradiographed at -70°C for 48 hours with intensifying screen.

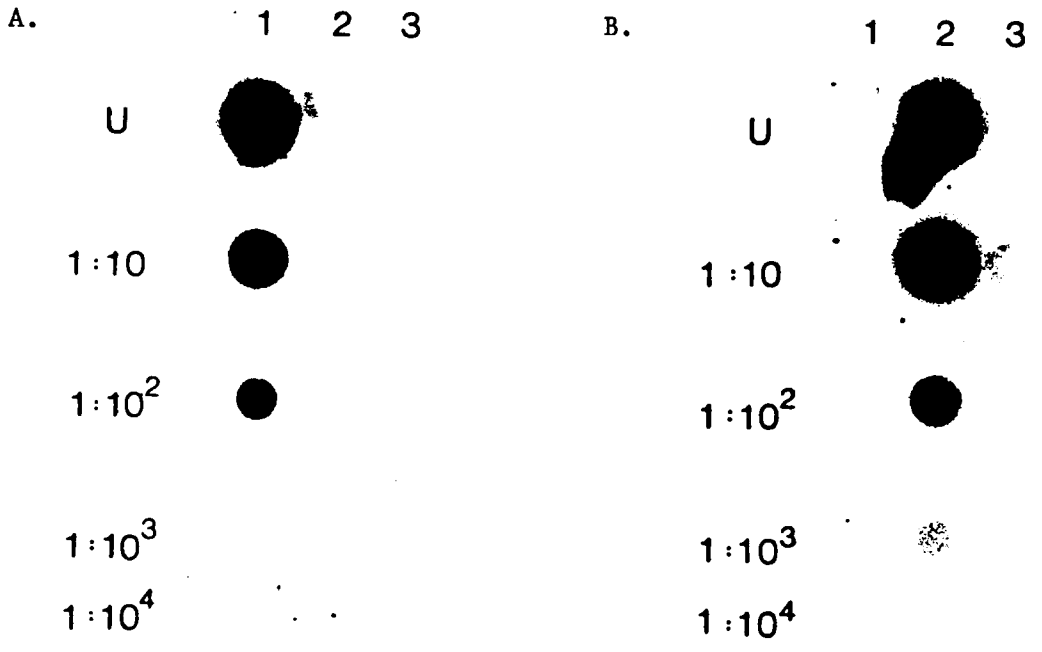


Figure II.3.3. Detection of viral RNA in dilutions of cell culture supernatants. Columns: 1, TGEV; 2, BCV; 3, MHV. (A) TGEV probe. (B) BCV probe. The supernatants from tissue cultures of the three viruses were clarified by centrifugation at 10,000 rpm for 10 mins. Equal volumes of supernatant were incubated with the formaldehyde/20X SSC denaturing solution as described, then serially diluted 1:10. One hundred µl of each sample was filtered through BA85 nitrocellulose. After the filters were probed with the nick-translated cDNAs, they were autoradiographed at -70°C for 48 hrs, with (TGEV) and without (BCV) intensifying screens.

Detection of TGEV RNA in Fecal Samples

A litter of piglets was experimentally infected per os with TGEV. Fecal swabs were taken prior to infection and at 18, 30, 44, 90, and 144 hours postinfection. Although the fecal material contained on the swab was often less than a gram, the quantity of virus excreted during an infection was large enough to be readily detected by the dot-blot hybridization assay (Figure II.3.4). No viral RNA was detectable until more than 18 hours after infection. Only two of the six piglets (3 and 6) survived to 144 hours postinfection. Because of the viscosity of the undiluted sample, three samples (4U and 6U at 90 hrs; 6U at 144 hours) partially or completely peeled off the nitrocellulose during processing. That problem was not seen with the tenfold dilutions. A comparison of these dot blot hybridization results with those obtained by electron microscopy, virus isolation and immunofluorescence is shown in Table II.3.1.

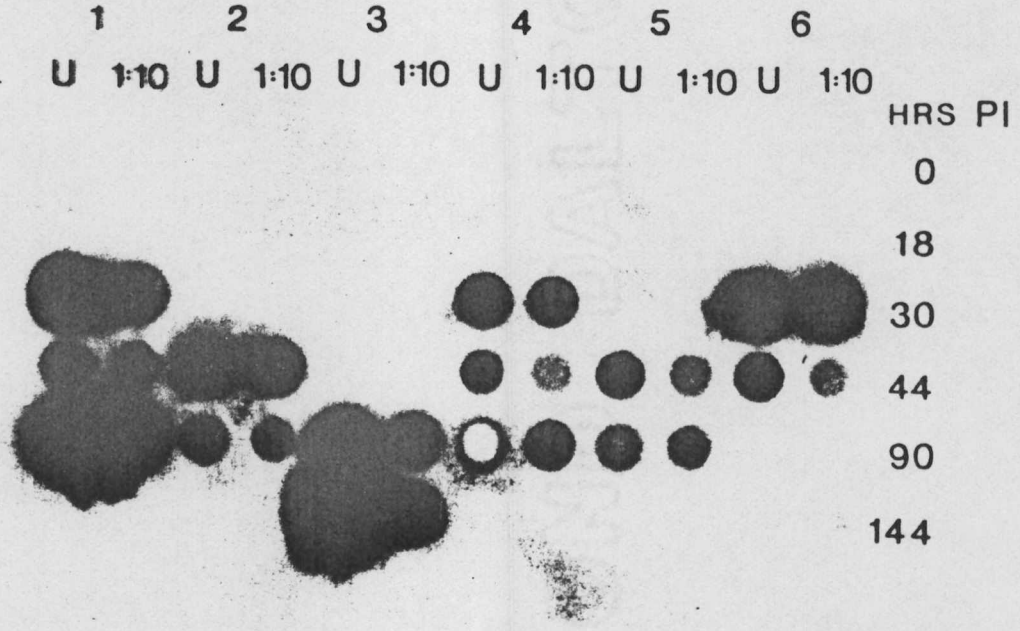


Figure II.3.4. Dot blot hybridization detection of viral RNA from fecal swabs of a litter of piglets experimentally infected with TGEV. Fecal swabs were taken prior to and at intervals after infection with TGEV. Swabs were suspended in 0.6 ml of EBSS and vortexed. From each tube, 315 µl of the fecal suspension were removed and treated with 35 µl of 5% NP40 for 10 minutes on ice. After clarifying for 3 minutes at 5000 rpm, the supernatant was incubated with equal volumes of denaturing solution, as described. One hundred µl of each sample was dotted on BA85 nitrocellulose, along with 100 µl of a ten-fold dilution. The filter was then hybridized with the ^{32}P-labeled TGEV probe, as described earlier, and autoradiographed at -70°C for 24 hours with intensifying screen.

Table II.3.1. Comparison of dot-blot hybridization with other methods of detection of TGEV from fecal swabs

Specimen no. and hrs. P.I.	Results of virus detection by: Virus isolation[a] EM	CPE	IF	Dot blot hybridization[b]
1. 0	-	-	Neg	-
18	ND	±	Neg	-
30	+	++	Pos	++++
44	-	+++	Pos	++
90	+	+	Pos	++++
2. 0	-	-	Neg	-
18	+	-	Neg	-
30	-	±	Neg	-
44	+	++	Pos	+++
90	+	-	Neg	++
3. 0	-	-	Neg	-
18	+	±	Neg	-
30	ND	++	Neg	-
44	+	+++	Neg	-
90	+	+++	Pos	++++
144	+	+++	Pos	++++
4. 0	-	-	Neg	-
18	-	-	Neg	-
30	+	+++	Pos	++
44	+	+++	Pos	+
90	+	+++	Pos	++
5. 0	-	-	Neg	-
18	ND	-	Neg	-
30	+	±	Neg	-
44	ND	++	Pos	+
90	ND	±	Pos	+
6. 0	-	-	Neg	-
18	+	-	Neg	-
30	+	++	Pos	++++
44	-	++++	Pos	++
90	-	±	Neg	+
144	ND	-	Neg	+

[a]Swine testicle cells were innoculated with the fecal specimens. The cells were evaluated for cytapathic effect after three days and tested with TGEV-specific fluorescent antibody.

[b]Estimated viral RNA concentrations: -, <25 pg/dot (equivalent to <9 ng/ml of feces); +, 25 to 1000 pg (equivalent to 9 to 360 ng/ml of feces); ++, 1 to 10 ng/dot (equivalent to 360 to 3600 ng/ml of feces); +++, 10 to 50 ng/dot (equivalent to 3600 to 1.8×10^4 ng/ml of feces); ++++, >50 ng/dot (equivalent to 1.8×10^4 ng/ml of feces).

Abbreviations: EM, electron microscopy, CPE, cytopathic effect; IF, immunofluorescence; ND, not done.

CHAPTER IV

DISCUSSION

In this study, we have assessed the specificity and sensitivity of ^{32}P-labeled cDNA clones of BCV and TGEV in the detection of RNA in dot blot hybridizations. Both probes were specific for their homologous RNA and no cross reactivity was noted. The sensitivity of detection, 25 pg/dot, was comparable to that seen in other studies (Bradsma and Miller, 1980; Flores et al., 1983; Hyypia et al., 1984). Also, neither probe hybridized to MHV, NDV, or VSV RNAs at concentrations of 10 ng/dot. However, when MHV RNA concentrations approached 500 ng, in cell culture supernatant preparations, some hybridization with the BCV probe was observed. These two species share about 70% amino acid homology in the nucleocapsid protein (Lapps, 1986), so there may well be short segments of nucleotide homology in the N protein genes. But for hybridization between the BCV probe and MHV RNA to be detectable, the MHV RNA must be present in large excess.

The TGEV probe was also very effective in detecting viral RNA in fecal samples, as shown by an analysis of a series of swabs from an experimentally infected litter of pigs. These swabs were also assayed by another laboratory for the presence of TGEV, using electron microscopy and virus isolation in cell culture followed by immunofluorescence (Table II.3.1). In general, hybridization correlated very well with the infectivity assay. With three cases (2, 90 hours; 6, 90 and 144 hours), the dot blots were positive while the cell cultures and

immunofluorescence were negative. Since all these specimens were taken late in infection, when the levels of bile salts and other proteolytic enzymes in the gut are high, the virus, though still present, may have lost infectivity. The correlation between EM and the other two methods was not as strong. The quantity of sample was insufficient for examination in six cases. In three cases (2, 18 hours; 3, 18 hours; 6, 18 hours), EM was positive when the other methods were negative.

The procedure used to prepare the fecal specimens for dot blotting was short and involved few handling steps. Phenol extraction or ethanol precipitation was not required before the samples are blotted onto nitrocellulose. The other macromolecules present in the sample did not appear to mask the presence of the viral RNA or cause false positive results. With this technique, many samples may be processed simultaneously; one blot can accommodate 96 dots. Alternatively, samples may be frozen, either before or after processing, and then blotted at a convenient time.

Nucleic acid hybridization has some advantages over the two other methods currently used to diagnose coronavirus, electron microscopy and virus isolation. For routine processing of numerous fecal samples, electron microscopy is too expensive, cumbersome, and insensitive and requires a relatively large sample (Almeida, 1983). Viral isolation, while highly specific and able to detect a wide range of viruses, is often slow, expensive, and requires technical expertise and sophistication which may not be available in a small veterinary laboratory (Richman et al., 1984). Also, many coronaviruses are difficult to isolate and grow by normal cell culture techniques.

There are problems associated with the use of ^{32}P-labeled probes, however. Special permits and techniques are required in handling radioactive material. Additionally, ^{32}P has a half life of only 14 days; this affects both the sensitivity and shelf life of the probe reagent. The use of nonradioactive DNA labels, such as biotin (Langer et al., 1981), would alleviate this drawback. With such a biotinylated probe, this hybridization diagnostic methodology should, in addition to its specificity and sensitivity, be a rapid and feasible tool for clinical use.

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