Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Virological Methods, 42 (1993) 293–300

VIRMET 01480

Oligonucleotide probes in infectious bronchitis virus diagnosis and strain identification

Kemal Karaca[a], Peter Palukaitis[b] and Syed Naqi[a]

[a]*Department of Avian and Aquatic Animal Medicine, College of Veterinary Medicine,*
[b]*Department of Plant Pathology, Cornell University, Ithaca, NY (USA)*

(Accepted 25 November 1992)

Summary

Genomic RNA fingerprints of infectious bronchitis virus (IBV) strains M41 and Conn46 were prepared to identify T1 RNase-resistant oligonucleotides 'unique' to each of the two IBV strains. Such oligonucleotides were subsequently eluted from the gels and their nucleotide sequences determined. When oligonucleotide probes of those sequences were synthesized and used in a dot-blot hybridization assay, the probes lacked IBV strain-specificity and reacted with the RNAs of homologous as well as heterologous IBV strains. Based on these results, the methods used in this study need to be applied to a large number of oligonucleotide probes, to find one or a few that might be suitable as IBV strain- or serotype-specific oligonucleotide probes.

IBV strain M41; IBV strain Conn46; Monoclonal antibody; Nucleic acid probe; (Chicken)

Introduction

Infectious bronchitis virus (IBV) is the etiological agent of a highly contagious and economically significant respiratory disease of chickens (King and Cavanagh, 1991). The virus is a member of the Coronaviridae family of enveloped viruses containing single-stranded RNA of positive polarity. The IBV genome consists of approximately 27 kilobases (Boursnell et al., 1987) and codes for three structural proteins: peplomer (S) glycoprotein, membrane (M)

Correspondence to. S. Naqi, Department of Avian and Aquatic Animal Medicine, College of Veterinary Medicine Ithaca, New York, NY, USA.

glycoprotein and nucleocapsid (N) phosphoprotein (Cavanagh, 1981; Stern and Sefton, 1982).

IBV infection in chickens is currently diagnosed by virus isolation and identification, and the virus serotype is determined by virus neutralization (VN) assay using chicken sera of known specificity (Gelb, 1989). Since these methods are costly, laborious and often give inconclusive results, the search for better methods of IBV diagnosis and typing continues.

The two approaches that are being commonly investigated as alternatives to the currently used methods of IBV diagnosis and typing are: (1) the use of monoclonal antibodies (Mabs) (Koch et al., 1985; Waingright et al., 1989: Ignjatovic and McWaters, 1992); and (2) application of nucleic acid probes (Brown et al., 1984; Collisson et al., 1990; Andreasen et al., 1991; Lin et al., 1991; Zwaagstra et al., 1992). Although serotype- and strain-specific Mabs have been developed for IBV (Karaca et al., submitted; Koch et al., 1985; Mockett et al., 1984; Niesters et al., 1987), nucleic acid probes of similar specificities have not yet been produced.

Oligonucleotide probes have been used successfully for the detection and differentiation of specific nucleic acid molecules. Lin et al. (1987) used a 21-nucleotide (nt) synthetic oligonucleotide probe to detect hepatitis B virus DNA in human serum by dot blot hybridization. In another study, Robertson et al. (1987) applied a 50-nt probe to locate measles virus nucleocapsid gene in lymphocytes. Single nucleotide changes in the plasmid which contained the human interferon gene were detected by Alves et al. (1988) using a 25-nt probe. Alves and Carr (1988) also investigated point mutations in T24 cells using a 20-nt probe. Attempts to develop IBV strain- or serotype-specific nucleic acid probes have not yet been successful.

In this study we synthesized one oligonucleotide probe each against strain M41 and Conn46 belonging to IBV serotypes Massachusetts (Mass) and Connecticut (Conn), respectively. The approach we took was novel in the sense that we first performed T1 RNase fingerprinting to identify large oligonucleotide 'spots' unique to each of the two IBV strains (M41 and Conn46), and subsequently eluted and sequenced those oligonucleotides to synthesize complementary probes. The procedures applied and the results of hybridization studies are described.

Materials and Methods

Virus strains

IBV strains, Ark99 (belonging to serotype Arkansas), Conn46 and M41 were propagated in embryonating chicken eggs and purified using sucrose density gradients as described previously (Kusters et al., 1987).

Viral RNA extraction

Genomic RNAs from purified IBV virions were extracted as described

earlier (Karaca et al., 1990). RNA concentration and its integrity was estimated by electrophoresis using 0.8% agarose gels.

Oligonucleotide fingerprinting

RNase T1 digestion of viral RNA and fingerprint analysis of RNase T1-resistant oligonucleotides were performed as described (Palukaitis and Zaitlin, 1984). The fingerprints (pattern of separated oligonucleotides) of three strains were compared visually. In order to obtain sufficient labeling of the large oligonucleotides for sequence analysis, two cycles of ethanol precipitation were performed to remove most of the excess, smaller oligonucleotides, prior to 5′-end labeling of the oligonucleotides.

Elution and sequencing of RNase T1-resistant oligonucleotides

One oligonucleotide each was chosen from fingerprints of Conn46 and M41 for nucleotide sequence analyses (Fig. 1). To accomplish this, a piece of the gel containing the 'spot' of interest (following the second dimension electrophoresis) was cut, removed and the oligonucleotide eluted overnight in elution buffer (0.5 M ammonium acetate, 1% SDS and 1 mM EDTA). The oligonucleotide was subsequently recovered by ethanol precipitation in the presence of 20 μg of carrier RNA. The sequences of the recovered oligonucleotides were determined by partial enzymatic digestion with base-specific RNases and fractionation on 20% polyacrylamide gels as described (Haseloff and Symons, 1981).

Oligodeoxyribonucleotides complementary to oligoribonucleotides of Conn46 and M41 were synthesized at the Cornell Biotechnology Program

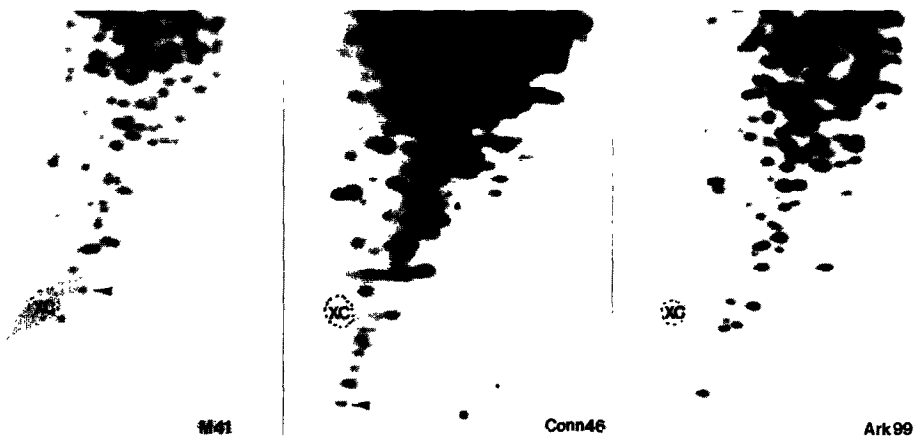

Fig. 1. RNase T1-resistant oligonucleotide fingerprints of genomic RNAs of IBV strains M41, Conn46 and Ark99. The ^{32}P-labeled oligonucleotides were separated by two-dimensional polyacrylamide gel electrophoresis in a 10% polyacrylamide gel in the first (horizontal) dimension at pH 3.5 and in a 20% polyacrylamide gel in the second (vertical) dimension at pH 8.3. Arrowheads show the oligonucleotides that were selected for sequencing and synthesis of oligonucleotide probes. XC, xylene cyanol dye marker.

Oligonucleotide Facility. Oligodeoxyribonucleotides were 5′-end labeled with [γ-^{32}P]ATP (specific activity 6000 mCi/mmol) (Amersham Corp., Arlington Heights, IL) with the aid of polynucleotide kinase, and purified by polyacrylamide gel electrophoresis (13% acrylamide and 8 M urea). The labeled oligodeoxyribonucleotides were eluted from the gels as described above and used as probes.

The nucleotide sequences of oligonucleotide probes to complementary RNase T1-resistant oligonucleotides from Conn46 and M41 are 5′-CAAA-GAAAAAGAATGTGAATGTTTTTATGGATTGTGAA-3′ and, 5′-CAAATGTTTGAAATGATGTTGTTGTGATTG-3′, respectively.

Dot-blot hybridization

Purified RNA samples (approximately 0.5 μg) from IBV strains Ark99, Conn46 and M41 and tobacco mosaic virus (TMV) were spotted onto nitrocellulose membranes. The membranes were baked and prehybridized as described (Palukaitis, 1986). They were subsequently hybridized with the oligonucleotide probes described above (approximately 10^6 cpm/ml) in the hybridization buffer (6 × SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0); 10 × Denhart solution; 1 mg/ml yeast RNA) by incubation at 50°C for 18 h. Filters were then washed three times with washing buffer (6 × SSC, 0.1% SDS) at room temperature, and then three times either at 50°C or 65°C for 5 min each. The membranes were dried and autoradiographed as described (Palukaitis, 1986).

Sequence alignment

The sequences of oligonucleotides were compared to genomic RNA sequence of IBV strain Beaudette by using a software program described by Devereux et al. (1984). According to this program 'Gap Weight: 2' was used and results were reported as percent similarities.

Results and Discussions

A comparison of nucleotide sequences of the oligonucleotides from Conn46 and M41 to IBV genomic RNA sequence of IBV strain Beaudette resulted in 71 and 84% similarities, respectively. These sequences were located within the genomic sequence of IBV between 5682–5719, and 12924–12953 nucleotides for oligonucleotides of Conn46 and M41, respectively. Both locations are found to be within the gene encoding an RNA polymerase.

The T1 RNase fingerprinting technique is based on the fact that T1 RNase cleaves an RNA strand 3′ of each guanosine (G) residue generating oligonucleotides of varying lengths. These oligonucleotides, 5′ end-labeled with a radioactive tracer (e.g., ^{32}P) and separated by two-dimensional gel electrophoresis, produce a unique (and reproducible) pattern of migration (fingerprint) for each specific RNA molecule. The fingerprint homology

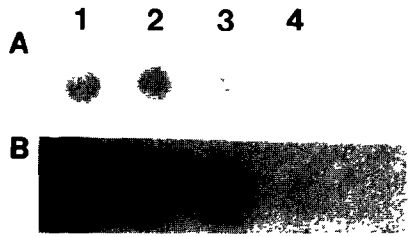

Fig. 2. A dot-blot hybridization analysis of IBV RNAs, using ^{32}P-labeled oligonucleotide probes to 'unique' RNase T1-resistant oligonucleotides of IBV strains Conn46 (A) and M41 (B). Samples (left to right) were 0.5 μg viral RNA from (1) Ark99, (2) Conn46, (3) M41 (all IBV strains) and (4) tobacco mosaic virus (TMV).

between viruses (including IBV isolates) has been used to assess their phylogenetic relatedness (Clewley et al., 1981; Kusters et al., 1987).

In this study we tested the hypotheses that: (1) the large T1 RNase-resistant oligonucleotides which appear unique to an IBV strain in a fingerprint, contain nucleotide sequences specific to that strain and (2) complementary probes made against those oligonucleotides specifically hybridize to the homologous strain.

In the sequencing of oligonucleotides, some difficulties were encountered initially. The amounts of RNA initially recovered by elution from the fingerprinting gels were not sufficient for sequencing. This was corrected by (1) increasing the amount of genomic RNA for RNase T1 digestion; and (2) enriching for the larger oligonucleotides prior to end-labeling. These steps led to incorporation of sufficient isotope into large oligonucleotides to permit nucleotide sequence analysis.

The results of the dot-blot hybridization experiments with the two probes synthesized during this study are presented in Fig. 2 A and B. Both probes reacted similarly with the purified RNAs of IBV strains Ark99, Conn46 and M41 under low stringency washing conditions, indicating the absence of strain specificity. However, when either the temperature of hybridization or the washing temperature was increased to 65°C there was no detectable signal even after long periods of exposures (results not shown).

We believe that the lack of strain specificity of the probes may have been influenced by the fact that T1 RNase-resistant oligonucleotides which appear specific to a strain of IBV may not have enough sequences unique to that strain to be used in hybridization studies for discrimination of IBV strains. In addition, the low G + C composition of the probes may have reduced the effective temperature range for discriminating well-matched from mismatched hybrids. Finally, the probes may have hybridized to themselves because of the palindromic nature of residues within the oligonucleotides which may have decreased the specificity of the hybridization and did not allow more stringent hybridization conditions. Although the actual reasons for the failure to generate strain- or serotype-specific oligonucleotide probes in this study are unclear, it appears that the use of nucleotide sequence information from T1

RNase-resistant oligonucleotides for the synthesis of strain-specific probes for IBV may not be suitable, without considerable effort to identify potential sequences that might yield serotype- or strain-specific probes.

Since the beginning of these studies, two other approaches have been described for detection of IBV RNA and identification of IBV serotypes. Each has met with limited success. The first is the use of cDNA clones, generated by a random priming method, as hybridization probes (Brown et al., 1984; Collison et al., 1990). The results of hybridization studies suggest that these probes can be used for detection of IBV RNA but can not be used for serotypic identification of IBV strains.

The second is the polymerase chain reaction (PCR)-based techniques. The first application of this technique to IBV was described by Andreasen et al. (1991) who reported amplification of a 1020-nt fragment from IBV strain Beaudette by using two primers to conserved regions. In another study, Lin et al. (1991) amplified 400 nt from the S2 genes of twelve strains of various IBV serotypes. Although, restriction enzyme analysis of amplified DNA showed different patterns that were consistent with serological typing, it was not clear why this consistency occurred, since serotypic determinants were shown to be located on the S1 subunit. In a similar study, Zwaagstra et al. (1992) amplified 438 nt from the N genes of 18 IBV isolates and sequenced 360 nt of each isolate. They showed that the sequences of the N genes were different between serologically indistinguishable strains.

All three methods, including the method described by us, were able to detect IBV RNA; however, none of them was able to detect serotypic differences. This suggests that nucleic acid detection methods may not be suitable for identification of IBV serotypes. This may due to the following: (1) as noted above cDNA clones are not suitable for discrimination because, while there may be variation between the strains, there is considerable homology between the strains of various IBV serotypes; (2) although considerable sequence data are available, design of universal IBV-specific primers that can be used for amplification of the S1 gene has not been achieved due to high sequence variability between the strains. Perhaps oligonucleotide hybridization probes based on sequences from the S1 gene would be more likely to discriminate strains belonging to different serotypes.

There are other reasons that may further hamper the development of serotypic nucleic acid detection methods. The most important is the difficulty in establishing a correlation between the nucleic acid sequences and VN determinants, probably due to the following: (1) the exact location of serotypic determinants remains to be determined; (2) it has been shown that VN epitopes that determine virus serotype are conformation dependent, and are usually formed by non-contiguous amino acids on the polypeptide chain; hence, amino acid changes in distant and different locations may cause changes in serotype of the virus; (3) modification of the proteins such as glycosylation may also cause changes that may not be detectable by nucleic acid probes. As a result we believe that although nucleic acid analysis methods are very valuable tools in

epidemiologic studies, nucleic acid detection methods may require further refinements in order to be used for identification of IBV serotypes.

References

Alves, A.M., Holland, D., Edge, M.D. and Carr, F.J. (1988) Hybridization detection of single nucleotide changes with enzyme labeled oligonucleotides. Nucleic Acids Res. 16, 8722.

Alves, A.M. and Carr, F.J. (1988) Dot blot detection of point mutations with adjacently hybridizing synthetic oligonucleotide probes. Nucleic Acids Res. 16, 8723.

Andreasen, J.R., Jackwood, M.W. and Hilt, D.A. (1991) Polymerase chain reaction amplification of the genome of infectious bronchitis virus. Avian Dis. 35, 216–220.

Boursnell, M.E.G., Brown, T.D.K., Foulds, I.J., Green, P.F., Tomley, F.M. and Binns, M.M. (1987) Completion of the sequence of the genome of coronavirus avian infectious bronchitis virus. J. Gen. Virol. 68, 55–77.

Brown, T.D.K., Cavanagh, D. and Boursnell, M.E.G. (1984) Confirmation of the presence of avian infectious bronchitis virus (IBV) using cloned DNA complementary to the 3′ terminus of the IBV genome. Avian Pathol. 13, 109–117.

Clewley, J.P., Morser, J., Avery, R.J. and Lomniczi, B. (1981) Oligonucleotide fingerprinting of ribonucleic acids of infectious bronchitis virus strains. Infect. Immun. 32, 1227–1233.

Collisson, E.W., Li, J.Z., Sneed, L.W., Peters, M.L. and Wang, L. (1990) Detection of infectious bronchitis viral infection using in situ hybridization and recombinant DNA. Vet. Microbiol. 24, 261–271.

Devereux, J, Haeberli, P. and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12, 387–395.

Gelb, J. Jr. (1989) Infectious Bronchitis. In: H.G. Purchase, L.H. Arp, C.H. Domermuth and J.E. Pearson (Eds), A laboratory Manual for Isolation and Identification of Avian Pathogens, 3rd Edn. Kendal/Hunt Publishing Company, Dubuque, pp. 124–127.

Haseloff, J. and Symons, R.H. (1981) Chrysanthemum stunt viroid: primary sequence and secondary structure. Nucleic Acids Res. 9, 2741–2752.

Karaca, K., Naqi, S., Palukaitis, P.P. and Lucio, B. (1990) Serological and molecular characterization of three enteric isolates of infectious bronchitis virus of chickens. Avian Dis. 34, 899–904.

King, D.J. and Cavanagh, D. (1991) Infectious bronchitis. In: B.W. Calnek, H.J. Barnes, C.W. Beard, W.M. Reid and H.W. Yoder, Jr. (Eds), Diseases of Poultry, 9th Edn., Iowa State University Press, Ames, pp. 471–484.

Koch, G., Hartog, L., Kant, A., Van Roozelar, D.J. and De Boer, G.F. (1985) Antigenic differentiation of avian infectious bronchitis virus strains employing monoclonal antibodies. Israel J. Vet. Med. 42, 89–97.

Kusters, J.G., Niesters, H.G.M., Bleumink-Plyum, N.M.C., Davelaar, F.G., Horzinek, M.C. and van der Zeijst, B.A.M. (1987) Molecular epidemiology of infectious bronchitis virus in the Netherlands. J. Gen. Virol. 68, 343–352.

Lin, Z., Kato, A., Kudou, Y. and Ueda, S. (1991) A new typing method for the avian infectious bronchitis virus using polymerase chain reaction and restriction enzyme fragment length. Arch. Virol. 116, 19–31.

Lin, H.J., Wu, P.C. and Lai, C.L. (1987) An oligonucleotide probe for the detection of hepatitis B virus DNA in serum. J. Virol. Methods 15, 139–149.

Mockett, A.P.A., Cavanagh, D. and Brown, T.D.K. (1984) Monoclonal antibodies to the S1 spike and membrane proteins of avian infectious bronchitis virus coronavirus strain Massachusetts M41. J. Gen. Virol. 65, 2281–2286.

Niesters, H.G.M., Bleumink-Pluym, N.M.C., Osterhaus, A.D.M.E., Horzinek, M.C. and Van der Zeijst, B.A.M. (1987) Epitopes on the peplomer protein of infectious bronchitis virus strain M41 as defined by monoclonal antibodies. Virology 161, 511–519.

Palukaitis, P. and Zaitlin, M. (1984) Satellite RNAs of cucumber mosaic virus: characterizat two new satellites. Virology 132, 426–435.
Palukaitis, P. (1986) Preparation and use of cDNA probes for detection of viral genomes. Me Enzymol. 118, 723–742.
Robertson, D.A.F., Guy, E.C., Zhang, S.L. and Wright, R. (1987) Persistent measles geno autoimmune chronic active hepatitis. Lancet ii, 9–11.
Stern, D.F. and Sefton, B.M. (1982) Coronavirus proteins: biogenesis of avian infectious bror virus proteins. J. Virol. 44, 794–803.
Zwaagstra, K.A., Van der Zeijst, B.A.M. and Kusters, J.G. (1992) Rapid detectior identification of avian infectious bronchitis virus. J. Clin. Microbiol 30, 79–84.