P153820.PDF [Page: 1 of 52]

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TITLE

DEVELOPMENT AND EVALUATION OF A NON-RADIOACTIVE, COLORIMETRIC, MEMBRANE-BASED GENE PROBED ASSAY FOR NEWCASTLE DISEASE VIRUS

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Development and Evaluation of a Non-Radioactive, Colorimetric, Membrane-Based Gene Probe Assay for Newcastle Disease Virus

BY

Douglas E. Bader* and Glen R. Fisher

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P153820.PDF [Page: 5 of 52]

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SUFFIELD REPORT 617

DEVELOPMENT AND EVALUATION OF A NON-RADIOACTIVE, COLORIMETRIC, MEMBRANE-BASED GENE PROBE ASSAY FOR NEWCASTLE DISEASE VIRUS

BY

Douglas E. Bader* and Glen R. Fisher

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ACKNOWLEDGEMENTS

Thanks to Dr. L. Nagata for his assistance in isolating NDV B1 RNA for these studies.

ABSTRACT

A non-radioactive, membrane-based, colorimetric gene probe assay was developed for Newcastle disease virus (NDV) to evaluate the potential of gene probes as tools for the identification of biological agents. Two gene probes for the NDV major nucleocapsid protein gene were evaluated, namely, (i) a digoxigenin-labelled, double-stranded DNA fragment of approximately 673 base pairs designated as dig-NDVNP673 and (ii) a 5'-fluorescein-labelled, single-stranded oligonucleotide probe of 20 bases, designated as NDVNP-PB4. Detection limits of 10^5-10^6 molecules of NDV target DNA were achieved using dig-NDVNP673, however this probe was found to cross-react with bacterial plasmid DNA (pBR328), even under highly stringent assay conditions (Tm-1.4°C). The oligonucleotide probe, NDVNP-PB4, did not cross-react with pBR328 DNA, even under low stringency conditions (Tm-29°C), and generated a detection limit of 10^7-10^8 molecules for NDV target DNA. However, the oligonucleotide probe cross-reacted with herring sperm DNA and lambda bacteriophage DNA under these conditions. When the stringency was increased during the post-hybridization wash step, from Tm-29°C to Tm-13°C, cross-reactivity was eliminated without compromising assay sensitivity. This study gave us an indication of the level of sensitivity one can expect to achieve using a colorimetric, membrane-based system for short and long gene probes and demonstrated the importance of screening gene probes for specificity. This work will serve as a benchmark for comparison to other gene probe assay systems that are under investigation.

EXECUTIVE SUMMARY

Title

Bader, D.E. and Fisher, G.R. "Development and Evaluation of a Non-Radioactive, Colorimetric, Membrane-Based Gene Probe Assay for Newcastle Disease Virus". DRES Suffield Report No. 617, December 1994.

Introduction

The Canadian Forces (CF) have a basic requirement to be able to function within a biological warfare (BW) environment. Early warning detection-identification is the first line of defence for such a scenario. The Defence Research Establishment Suffield has been tasked to develop a field-based BW detection-identification system. Because of the diversity in the nature of the BW agents that could be used against the CF (bacteria, viruses, microbiological toxins, human effector molecules), it is currently impossible to apply a single technology that is capable of identifying all potential BW threat agents. Therefore, several technologies are being evaluated including mass spectrometry, capillary electrophoresis, antibody-based technologies, and gene-based technologies.

Gene-based technologies address not only the classical biological agents (eg. virus, bacteria, rickettsia, fungi), but also newly emerging threat agents that could arise as a consequence of advances in molecular biology and biotechnology, for example, innocuous organisms containing cloned biological toxin genes or liposomes containing infectious viral nucleic acid.

This study describes the development and evaluation of gene probes (short pieces of genetic material that bind to complementary nucleic acid sequences) as tools for agent identification. More specifically, this study describes the development and evaluation of a non-radioactive, membrane-based, gene probe assay for the detection and identification of RNA-based viruses using the viral simulant Newcastle disease virus (NDV).

Results

The first part of the report describes experiments intended to evaluate a commercial gene probe assay in terms of sensitivity and compares assay sensitivity to that of a first-generation gene probe for NDV developed in-house. The lower detection limit for the NDV probe was found to be 10^5-10^6 target molecules, which was 10-fold less sensitive than the commercial probe for its target DNA. Both probes were found to be cross-reactive, even under fairly stringent experimental conditions, indicating significant sequence homology.

The second part of the report describes the design of a second-generation NDV gene probe and evaluation with respect to cross-reactivity and sensitivity. This probe did not cross-react with the commercial DNA source even under low stringency conditions, indicating that this probe is a better candidate for use as an NDV-specific probe. The lower detection limit for this gene probe was 10^7-10^8 molecules of target DNA. Since target nucleic acid sequences were amplified 10^5 fold prior to detection using *in vitro* DNA amplification techniques, the combined detection limit (amplification and gene probe detection) was 10^2-10^3 target molecules which is equivalent to 100-1000 NDV particles.

Significance of Results

The results from this study show that low level detection of viruses is possible through the combination of nucleic acid amplification and gene probe detection technologies. This study has improved our understanding of the use of gene probes for identification and enhanced our capability to design and test gene probes for BW agents. This study has led to the development of a good probe/target model system with which to pursue other critical gene probe studies. Gene-based technologies will play an important and necessary role in BW identification strategies developed for the Canadian Forces.

Future Goals

Further research is required to improve gene-based identification strategies for the field including studies to assess DNA amplification/gene probe techniques on environmental samples and evaluation of assay formats that are less technically demanding, more easily automated and therefore more likely to be adapted into a fieldable system. Studies have already been initiated with respect to the latter and include (i) evaluation of a microplate-based gene probe assay and (ii) coupling gene probes to biosensors, specifically, the light addressable potentiometric sensor.

TABLE OF CONTENTS

LIST OF FIGURES

GLOSSARY OF TERMS AND ABBREVIATIONS

agarose gel electrophoresis: the procedure by which DNA fragments are separated based on size by molecular sieving through agarose in an electric field.

amplification: to make multiple copies of a DNA sequence.

amplicon: a DNA fragment of defined size generated by PCR amplification.

AP: abbreviation for alkaline phosphatase, an enzyme which catalyzes the hydrolysis of 5'phosphate groups.

bp: an abbreviation for base pairs of DNA .

dATP: 2'-deoxy-adenosine-5'-triphosphate.

dCTP: 2'-deoxy-cytidine-5'-triphosphate.

dGTP: 2'-deoxy-guanosine-5'-triphosphate.

dTTP: thymidine-5-triphosphate.

dUTP: 2'-deoxy-uridine-5'-triphosphate.

dNTP: a generic abbreviation for deoxynucleotide triphosphates.

DEPC: diethyl pyrocarbonate, used to inactivate RNAses (enzymes that degrade RNA).

dig: short form for digoxigenin, a steroid which occurs naturally in Digitalis purpurea and Digitalis lanata plants.

dig-11-dUTP: digoxigenin-11-2'-deoxy-uridine-5'-triphosphate, a nucleotide analog which is incorporated into DNA gene probes by gene labeling techniques, allowing the labelled DNA to be selected for detection.

dig-NDVNP673: a 673 bp gene fragment of the NDV NP gene which is labeled with digoxigenin and used as a gene probe.

dig-pBR328: bacterial plasmid DNA which is labeled with digoxigenin and used as a gene probe.

DNA: 2' deoxyribonucleic acid, the genetic coding sequence of cells.

Dot blot assay: a gene probe assay in which the target DNA is applied and fixed directly to a solid support (nylon or nitrocellulose membrane) using a vacuum filtration device in the form of circular spots or dots. The sample is then hybridized with a gene probe.

EDTA: ethylene diamine tetraacetic acid.

EGTA: ethyleneglycol-bis (β -aminoethyl ether) tetraacetic acid.

Gene probe: a piece of genetic material ranging in size from several nucleotides to thousands of nucleotides, that contains a detectable marker molecule and binds to a target gene sequence through complementary molecular interaction (hybridization).

IPTG: isopropylthio-β -d-galactopyranoside, an inducer of the enzyme β-galactosidase.

LB: an abbreviation for Luria-Bertani, a bacterial growth media containing yeast extract, tryptone and sodium chloride.

LMP agarose: abbreviation for low melting point agarose, used to purify DNA of a specific size following DNA fragment separation.

NBT/X-phosphate™: Nitroblue tetrazolium/phosphate compound, a yellow soluble substrate which is converted to blue colored insoluble product in the presence of the enzyme alkaline phosphatase.

NDV: abbreviation for Newcastle disease virus, a virus of the Paramyxoviridae virus family whose natural host is the chicken and whose genetic material is single-stranded RNA.

NDVNP673: the designated name for the 673 bp DNA fragment of Newcastle disease virus nucleocapsid protein gene that was generated by RT-PCR.

NDVNP-PB4: a 5'- fluoresceinated 20 base oligonucleotide probe.

NDVNP-PR5: a PCR primer of 20 bases used with NDVNP-PR6 primer to generate a 673 bp fragment of the NDV NP gene in a reverse transcription polymerase chain reaction.

NDVNP-PR6: a PCR primer of 20 bases used with NDVNP-PR5 primer to generate a 673 bp fragment of the NDV NP gene in a reverse transcription polymerase chain reaction.

NP: abbreviation for nucleocapsid protein.

pBR328: an autonomous, self-replicating, extrachromosomal, circular DNA molecule of bacterial origin, which is used for subcloning and contains antibiotic resistance genes.

PCR: abbreviation for polymerase chain reaction, a technique for making multiple copies of a size-specific DNA fragment.

Primer: a short single-stranded gene probe used in PCR which is required by the polymerase enzyme to initiate polymerization activity (extension of the primer strand while using the complementary target strand as a template).

Restriction: the process by which the phosphodiester backbone is cut or nicked between two adjacent nucleotides of the DNA molecule with a restriction enzyme, usually at a sequence-specific location.

Restriction enzymes: enzymes which recognize sequence specific sites on the DNA molecule and nick the DNA.

RNA: ribonucleic acid.

RT: abbreviation for reverse transcription, the process by which RNA is converted to a single-stranded DNA copy.

RT-PCR: abbreviation for reverse transcription polymerase chain reaction, the process by which RNA is converted to multiple copies of double-stranded DNA fragment of defined size or length.

SDS: sodium dodecyl sulfate or sodium lauryl sulfate.

Southern Blot: the procedure by which denatured DNA is transferred from agarose gel to a nylon filter where it is hybridized with a gene probe.

SSC buffer (1x): 0.15M sodium chloride, 0.15M sodium citrate buffer pH 7.0

Tm: abbreviation for melting temperature, the midpoint of the temperature range over which double-stranded DNA is denatured to single strands.

UV: abbreviation for ultraviolet.

INTRODUCTON

We have initiated studies to evaluate the potential of gene probes as tools for the identification of biological agents. Gene probes are nucleic acid molecules that bind to complementary nucleic acid sequences (RNA or DNA) in a sequence specific manner, through the process of hybridization. They can be used for identifying organisms at almost all levels of the taxonomic classification level (family, order, genus, species and strain). They can be generic when directed at highly conserved sequences that cross different classes of organisms or they can be highly specific. In a BW context, gene probes are usually directed against a virulence factor or some signature sequence which distinguishes it from related non-pathogenic strains. While gene probes can be used to detect the presence of and indicate the identity of conventional biological agents (eg. viruses and bacteria), they can also be used to identify genes that have been transferred naturally or by human design into foreign vehicles (eg. cloning of BW toxin genes into innocuous organisms or liposome-encapsulation of infectious viral nucleic acid). While gene probes cannot be used to detect toxins directly, it is possible to detect signature genes or fragments thereof, if present in a sample, for example, a crude toxin preparation.

Gene probes are labelled either with radioisotopes (eg. ^{32}P) or with non-radioactive haptens (eg. biotin, digoxigenin, enzymes, fluorescent moieties). Radioactive probes, although extremely sensitive, have short shelf-lives and pose health and safety hazards especially in a field operational setting. Non-radioactive probes are preferred since they do not have these limitations. Therefore, this study looked at using non-radioactive probes.

In trying to gain experience with gene probe assays (probe design strategies, assay formats, assay sensitivities, etc.), we have chosen to begin initial studies with BW simulants. Consequently NDV has been chosen as a model virus for two reasons. First, NDV has been developed as a BW viral simulant for use in field experiments at DRES [1]. Second, many BW viral threat agents are RNA viruses. Since the genomic nucleic acid component of NDV is RNA, NDV serves as a useful model for RNA viruses.

NDV belongs to the Paramyxoviridae family of viruses[1]. The NDV viral genome consists of a single, non-segmented, negative-sense RNA molecule of approximately 15,000 bases [4]. The complete genome of NDV has been sequenced. The genome contains a set of six or more genes covalently linked in tandem. These include the L gene (large auxiliary nucleocapsid protein), the HN gene (haemagglutinin/neuraminidase protein), the F_o gene (membrane fusion protein), M gene (matrix protein), P/C gene (auxiliary nucleocapsid proteins) and the NP gene (major nucleocapsid protein).

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[1] For current review of information on the structure and function of NDV and other related viruses see [2,3].

The nucleocapsid comprises the viral RNA and three nucleocapsid proteins: NP, L and P. The NP protein is the major nucleocapsid protein which is translated from a monocistronic mRNA of approximately 1641 nucleotides to give a protein of 515 amino acids (57.8 kilodaltons). The NP protein is tightly bound to the viral RNA and plays a primary structural role in stabilizing the nucleocapsid. The carboxy- terminal third of the NP protein probably plays a role in binding the L and P proteins, which are believed to play a role in viral RNA synthesis as well as other enzymatic functions such as methylation, capping and polyadenylation of mRNA. We have chosen the NP gene for gene probe design because it has been suggested that the nucleocapsid structure unit is an obligatory requirement in the template activity of negative-stranded RNA viruses[2]. As such, the NP gene, being a major component of the nucleocapsid structure, should be a good target with which to design gene probes for negative-stranded RNA viruses.

MATERIALS AND METHODS

Virus Strains

Newcastle disease virus attenuated vaccine strain B1 was used as a source of virus for genomic RNA isolation. Purified stock virus was obtained from DRES collections and previously prepared by inoculating chicken embryos, isolating the allantoic fluid, and purifying the virus by continuous sucrose density gradient centrifugation.

Synthetic Oligonucleotides

The RT-PCR primers used in this study were designed to amplify a 673 base pair region of the NP gene which included part of the coding sequence and part of the untranslated region upstream of the translational start signal, based on sequence information from NDV strain D26 [5]. The RT-PCR primers were prepared by the Regional DNA Synthesis Laboratory (University of Calgary, AB) and were designated as NDVNP-PR5 (5' OH-acagagaattcg-taagttac-OH 3') and NDVNP-PR6 (5' OH- gctgtctcatctgcagtcat-OH 3').

The oligonucleotide gene probe used in this study was selected based on a sequence alignment of representative viral NP gene sequences from viruses in the Paramyxoviridae family around a region within the 673 fragment. This region was 100% conserved among two strains of NDV (D26 and Beaudette C) but only 15-30% conserved among the other sequences. The probe was prepared by the Regional DNA Synthesis Laboratory (University of Calgary, AB) and was designated as NDVNP-PB4 (5' Fluorescein-gctcctcgcg-gctcagactc-OH 3'). The fluorescein moiety was detected by α-fluorescein-alkaline phosphatase Fab antibody (Boehring Manheim Canada (BMC) Montreal, Quebec).

Commercial DNA

Target DNA used in the commercial gene probe assay was BamHI linearized pBR328 (BMC, Montreal, Quebec). The commercial probe material was digoxigenin-pBR328 (5.2 ng/µL) labelled by the random primer method (BMC, Montreal, Quebec). Lambda phage DNA used in this study was obtained as a Hind III restriction enzyme digest (Promega-Fisher, Edmonton, AB).

Isolation and Purification of NDV B1 Genomic RNA

Genomic RNA from NDV strain B1 was isolated and purified for subsequent RT-PCR generation of a 673 base pair fragment which was used as a source of target DNA for probe assays and was labelled with digoxigenin for use as a gene probe.

NDV B1 RNA was purified using procedures similar to those outlined elsewhere [6]. Briefly, 0.5 mL of gradient-purified NDV B1 (2.6×10^{10} pfu/mL) was mixed with 2.0 mL of guanidinium solution (4 M guanidinium isothiocyanate, 20 mM sodium acetate-pH 5.2, 0.1 mM dithiothreitol, 0.5% N-lauryl sarcosine). The solution was drawn several times through a 20 gauge needle and transferred to a clean tube. This solution was layered onto 1.5 mL of 5.7 M CsCl solution in polyallomer ultracentrifuge tubes and centrifuged at 35,000 rpm in a Beckman SW-55 rotor for 12-20 h at 18°C. The supernatant was removed carefully, the tube was inverted and allowed to stand for 5-10 min to remove remaining liquid. The RNA pellet was re-suspended in 400 µL of TES solution (10 mM Tris-Cl pH 7.4, 5 mM EDTA, 1% SDS) for 5 min at room temperature and then transferred to a clean microcentrifuge tube. The sample was then extracted once with 1 volume of phenol-chlorofom and the aqueous layer (RNA) was removed. A 1/10 volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol was added, mixed and allowed to sit at -70°C (Ultra-Low Freezer, Forma Scientific) for 30 min to precipitate the RNA. The sample was subjected to centrifugation in an Eppendorf benchtop microcentrifuge for 30 min at 14,000 x g. The pellet was resuspended in 400 µL of nuclease-free water or DEPC-treated water. A portion of this material was stored at -20°C and -70°C. The remainder was precipitated with 3 M sodium acetate and 95% ethanol as above and stored in this form at -20°C and -70°C. The RNA was quantitated by measuring the absorbance at 260 nm and 280 nm.

Preparation of NDVNP673 DNA by RT-PCR

NDVNP673 DNA was prepared from purified RNA by reverse transcription PCR as follows. A 500 µL master mix containing all components except template RNA was prepared. The master mix contained 50 mM KCl; 10 mM Tris pH 9.0; 0.1% Triton X-100; 0.2 mM dNTP mixture of dATP:dTTP:-dGTP:dCTP (Bio/Can, Mississauga, Ontario); 0.2 µM NDVNP-PR5; 0.2 µM NDVNP-PR6; 0.2 µM Rnasin (Fisher-Promega, Edmonton, Alberta); 0.025U/uL AMV Reverse transcriptase (Life Sciences, St. Petersburg, Florida) and 0.025 U/µL Taq Enzyme (Bio/Can, Mississauga, Ontario). Four µL of a 10^{-1} dilution of stock RNA (2.7×10^{10} molecules) were added to 400 µL of template-free master mix, mixed and then four 100 µL volumes were aliquotted into separate oil-less PCR tubes (Bio/Can, Mississauga, Ontario).

Negative controls were prepared by aliquotting three 30 µL volumes of remaining template-free master mix into separate oil-less PCR tubes. Prior to placing the PCR tubes into a programmable DNA thermal cycler (Perkin Elmer Cetus), the cycler was allowed to warm up for 30 min and the diagnostic "auto-tune" program was performed. A drop of heavy oil (Sigma, St. Louis, Missouri) was added per well and the thermal cycler was pre-warmed to 42°C. The PCR tubes were incubated at 42°C for 1 h for the reverse transcription reaction. The following PCR thermal cycling reaction was then initiated: 94°C for 5 min (1 cycle); 94°C for 1min + 47°C for 2 min + 72°C for 3 min (30 cycles); 72°C for 7 min (1 cycle); 4°C hold. An aliquot from each tube was mixed with a 1/10 volume of 10x gel loading buffer (0.1% bromophenol blue, 1.0% SDS, 0.1 M EDTA pH 8.0, 50% glycerol) and then loaded onto a 1% agarose gel (0.75 g of agarose in 75 mL of electrophoresis buffer: 40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.2) containing 0.5 µg/mL ethidium bromide. The samples were subjected to horizontal agarose gel electrophoresis under the conditions of 50-60 V for 1-2 h at room temperature in electrophoresis buffer without ethidium bromide. The gel was visualized by UV illumination on a UV light box (Fotodyne). High quality samples were pooled and subjected to horizontal agarose gel electrophoresis under the conditions of 50-60 V for 2-3 h at 4°C through a 2% low melting point agarose gel (Gibco-BRL, Burlington, Ontario). The gel was briefly exposed to UV light (prep setting) to visualize the amplified 673 bp band for excision. The excised gel fragments were placed in 1.5 mL microcentrifuge tubes and a 1/3 volume of TE 10/1 buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) was added, based on an estimation of the volume of the excised gel material using a specific density of 1.0 g/mL. The tubes were placed in a 65°C waterbath for 15 min to melt the agarose. The tubes were cooled to room temperature for 2-5 min. A 1/3 volume of water-saturated phenol was added, vortexed for 30 sec and then centrifuged at 16,000 x g for 5 min. The top aqueous layer was transferred to new tubes and the extraction was performed again. The top aqueous layer was extracted several times with an equal volume of dry n-butanol (vortexed for 30 sec then centrifuged for 1 min at 16,000 x g for each extraction). The extractions were repeated until the total volume was 1/10 the starting volume. The DNA was precipitated by adding a 1/10 volume of 3 M sodium acetate (pH 5.0) and 2 volumes of 95% ethanol, followed by incubation at -20°C for 1 h or at -70°C for 30 min. The precipitate was pelleted by centrifugation at 16,000 x g for 30 min and then washed once with 70% ethanol, vacuum-dried (10-30 min) and finally re-suspended in TE 10/1 buffer or nuclease-free water. The DNA was analyzed for quality and quantity by running a sample on a regular 1% agarose gel at room temperature. The concentration of sample DNA was obtained by comparing band intensities to molecular weight markers of known concentration (λ DNA digested with Hind III).

Preparation and Analysis of dig-NDVNP673 Probe Material

Purified NDVNP673 DNA was labelled with digoxigenin-11-dUTP using the random primer digoxigenin labelling and detection kit purchased from BMC according to their protocol but with some modifications. Briefly, up to 1 μg of purified DNA (1-15 μL) was denatured for 10 min in a boiling water bath and then chilled rapidly on ice for 5 min. The following components were added to the denatured DNA: 2 μL of 10x hexanucleotide mixture (random hexanucleotide primers); 2 μL of 10x dNTP labelling mixture (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM dig-11-dUTP, pH 6.5 (20°C)); 1 μL of Klenow enzyme (2 Units/μL) and nuclease-free H_2O to a final volume of 20 μL. This reaction mixture was allowed to incubate at 37°C for 16-20 h. The labelled probe was separated from unincorporated dig-11-dUTP and random primers by the spun-column technique [7]. The purification column consisted of a 1 mL syringe packed with 0.05 mL of siliconized sterile glass wool, followed by packing with pre-swelled sterile Sephadex G-50 to give bed volumes ranging from 0.7 to 1.2 mL for sample volumes between 50 μL and 100 μL. The column was placed inside a plastic 15 mL snap-cap tube, capped and centrifuged for 60 sec in a benchtop IEC clinical centrifuge, at setting 4. The column was removed to a clean 15 mL snap cap tube and the entire sample to be purified was placed onto the top of the column. The column was spun as above for 80 sec. The effluent was transferred to a clean 1.5 mL microcentrifuge. The probe material was quantitated by dot blot analysis of serial dilutions of the probe material against a known concentration of digoxigenin-labelled pBR328 DNA from the BMC kit. The procedure for quantitating digoxigenin labelled NDVNP673 was the same as that described below for detecting target DNA, except that the pre-hybridization and hybridization steps were omitted.

Dot Blot Assay

The procedures for hybridization and detection of digoxigenin labelled probes (dig-NDVNP673 and dig-pBR328), described below, were based on the protocols provided in the digoxigenin random primer labelling and detection kit from Boehringer Mannheim Canada (Montreal, Quebec). For the fluorescent oligonucleotide probe, NDVNP-PB4, the procedures were similar except α-fluorescein-AP was used in place of α-digoxigenin-AP.

Concentrated stocks of target DNA were denatured to single strands by heating at 100°C for 10 min followed by chilling on ice for 5 min. Dilutions of the denatured target DNA were prepared with nuclease-free water or DNA dilution buffer supplied in the BMC kit (the dilution buffer contained herring sperm carrier DNA at a concentration of 50 μg/mL). Five to ten μL of each DNA sample, were applied in triplicate to positively charged nylon membranes (BMC, Montreal, Quebec), which were previously pre-wetted with 200 μL of sterile 3x distilled H2O per well, under vacuum using a 96 well vacuum filtration manifold (BioRad). After spotting the samples, the membranes were removed from the vacuum filtration manifold and allowed to air dry for 20-30 min. The membranes were exposed to UV light (preparative setting) for 5 min by placing membranes face down on a transillumination UV light table

(Fotodyne). The membranes were placed into hybridization bags containing 0.2 mL/cm^2 hybridization solution (5x SSC, 0.1% Na- salt N-lauroylsarcosine, 0.02% SDS, 1% BMC blocking reagent, no probe) and incubated in a water bath shaker at the hybridization temperature of interest for 1-2 h. The hybridization solution was removed and replaced with fresh hybridization solution 0.05 mL/cm^2 containing gene probe material. Double-stranded probe material (dig-NDVNP673 and dig-pBR328) was always denatured by heating at 100°C for 10 min and chilling on ice for 5 min. The membranes were incubated at the hybridization temperature of interest for various lengths of time in a water bath shaker. The membranes were washed twice for 5 min in 0.5 mL/cm^2 of wash buffer no. 1 (2x SSC, 0.1% SDS) at room temperature. This was followed by two 5-15 min washes in 0.5 mL/cm^2 of wash buffer no. 2 (0.1x SSC, 0.1% SDS) at temperatures at or above room temperature. All subsequent washes were done at room temperature. The membranes were prepared for detection by first rinsing briefly (1 min) in buffer no. 3 (100 mM Tris, 150 mM NaCl, pH 7.5). This was followed by washing in 1 mL/cm^2 of buffer no. 4 (0.5% BMC blocking reagent in buffer no. 3) for 30 min. The membranes were then washed in 1 mL/cm^2 of buffer no. 5 (buffer no. 4 + anti-digoxigenin-alkaline phosphatase Fab conjugate at a concentration of 150 mU/mL) for 30 min. This was followed by two washes in 1 mL/cm^2 of buffer no. 3 for 15 min each and then equilibration in 1 mL/cm^2 of buffer no. 6 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 5 min. The membranes were incubated in the dark with 0.1 mL/cm^2 of color solution (buffer no. 6 containing 0.34 mg/mL NitroBlue Tetrazolium and 0.17 mg/mL X-phosphate^{TM} (BMC, Montreal, Quebec)). Color development was monitored and was allowed to develop anywhere from 1 to 24 hours at which time the reaction was quenched by removing the membrane from the color development solution and washing the membrane in 1 mL/cm^2 of buffer no. 7 (10 mM Tris, 1 mM EDTA, pH 8.0).

Southern Blot Analysis

Samples to be analyzed by southern blotting were first subjected to electrophoresis on 1% agarose gels to separate the DNA fragments. After run times of 1.5 to 2 h, the DNA was denatured to single strands by soaking the gels in 0.5 M NaOH (3 changes for 10 min each) with shaking. The gels were then transferred to 0.5 M Tris-HCl pH 7.0 (3 changes for 10 min each) with shaking, followed by 2x SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) for 30 min. The wick, membrane and 3M Whatman filters were presoaked in 2x SSC. The transfer was allowed to go overnight (16-24 h) using 10x SSC buffer. The membrane was air-dried for 30 min and the DNA was fixed on the membrane by exposure to UV light for 5 min on a UV light box (preparative setting). The membranes were probed in the same manner as described for the direct dot blot assay.

Melting Temperature Calculations

Estimates of the melting temperatures (Tm) for the NDV probes used in this study were determined from the following formulae:

NDVNP673

$Tm = 81.5 + 16 \log [Na^+] + 0.41 (\%GC) - 0.65 (\% \text{ formamide}) - (675/n) - \% \text{ mismatch}$ [8].

where $[Na^+]$ =molar sodium ion concentration
=0.0165 M (0.1x SSC)
=0.83M (5x SSC)
GC= 45%
% formamide = 0
n = complexity = 673
mismatch (assumed to be 0)

NDVNP-PB4

Tm values were calculated by "Nearest-Neighbour Method" using Oligo: Primer Analysis Software program version 4.1 from National Biosciences [9].

RESULTS

Preparation and Analysis of LMP Purified NDVNP673 DNA

Genomic RNA was isolated from NDV B1, purified and analyzed by measuring absorbance at 260 nm and 280 nm using a single beam spectrophotometer (Pye Unicam). The RNA preparation was found to have a concentration of 540 ng/mL based on Abs_{260} measurements with an $Abs_{260/280}$ ratio of 1.14. Since pure RNA has an $Abs_{260/280}$ ratio of 2.0, this preparation was about 57% pure and thus the concentration of RNA, as determined from the Abs_{260} readings, were not highly accurate. Nonetheless, the RNA was used in RT-PCR reactions to generate material for probe studies.

A rangefinding experiment was performed to determine the quantity of starting template material to use for large scale RT-PCR. Samples from this experiment were analyzed by agarose gel analysis. The results of this analysis are shown in Figure 1. The 673 bp fragment was present for all dilutions of the starting material tested, as evidenced by the presence of a band at the position between the molecular weight marker bands of 564 and 2021 bp (lanes 5-16). The negative control samples, which contained all components except template RNA, showed no 673 bp band at this position (lanes 2-4). The level of amplification for the 673 bp fragment was estimated to be 1.4×10^5. For comparison purposes, a PCR reaction using a commercial PCR kit (Perkin Elmer-Applied Biosystems, Mississauga, Ontario) was performed yielding an amplification efficiency of about 5×10^5.

Based on these results, a 10^{-1} dilution of starting template was used to prepare NDVNP673 DNA on a larger scale (Figure 2). The amplified material was purified by LMP agarose gel melting method and was found to generate a distinct band on a 1% agarose gel with a concentration of about 46 ng/µL. This material was used as target material for NDV gene probe assays and was labelled with digoxigenin for use as a double-stranded NDV gene probe.

Preparation and Analysis of dig-NDVNP673

Purified NDVNP673 DNA was labelled with digoxigenin-11-dUTP using the random primer method. The labelled material was quantitated by comparing serial dilutions to control digoxigenin-labelled pBR328 DNA of known concentration as shown in Figure 3. The concentration of dig-NDVNP673 from this preparation was estimated to be about 5.3 ng/µL. Dig-pBR328, supplied in the commercial kit, was 5.2 ng/µL, indicating that we had achieved labelling efficiencies equivalent to those of the commercial supplier.

Gene Probe Assays Using Dig-NDVNP673

Prior to performing gene probe assays for NDV, assays were performed using standardized reagents and protocols from a commercial kit (BMC, Montreal, Quebec), to see if we could obtain the level of detection claimed by the manufacturer using their system. According to the manufacturer, the lower detection limit was claimed to be 0.1 pg or 1.9×10^4 molecules of denatured, double-stranded, bacterial plasmid pBR328 DNA in the presence of $>1 \times 10^4$ pg of herring sperm DNA under the standard conditions of 26 ng/mL of digoxigenin-labelled pBR328 hybridized at 68°C for 16 h; 2x15 min washes in 0.1x SSC/0.1% SDS at 68°C; 150 mU/mL anti-digoxigenin antibody; 24 h color development. We were able to achieve the level of detection of 1.9×10^4 molecules under the conditions set by the manufacturer from time to time but only by direct visualization of the membranes as photodocumentation of membranes resulted in a 10-fold loss of resolution (Figure 4). We typically observed detection limits between 1.9×10^4 and 1.9×10^5 molecules.

Assays for the detection of NDVNP673 DNA using dig-NDVNP673 probe were then performed under the same conditions as above (pBR328/dig-pBR328 was run in parallel as a control). The results are shown in Figure 5 and indicate a lower detection limit of 1.9×10^6 molecules for NDVNP673 DNA. Sensitivity limits for NDVNP673 DNA/dig-NDVNP673 in subsequent trials resulted in detection limits of 10^5-10^6 molecules and were always 10-fold less sensitive than parallel assays for pBR328/dig-pBR328.

We were interested in investigating the effect of herring sperm DNA on assay sensitivity since it was included in the dilution buffer, supposedly to act as a carrier for very small quantities of target DNA. Detection limits were the same when nuclease-free water was used in place of the dilution buffer (Figure 6), indicating that herring sperm DNA did not improve assay sensitivity, under the conditions used.

Cross-Reactivity of dig-NDVNP673 for pBR328 DNA

In an effort to assess the size range of dig-NDVNP673 and dig-pBR328 probe material, both probe preparations were subjected to agarose gel electrophoresis (in duplicate) and transferred to positively-charged nylon membranes for southern blot analysis. One membrane was probed with dig-NDVNP673, the other with dig-pBR328 (Figure 7). For dig-pBR328 (lane 5), the majority of the labelled material was in the size range of 400-1500 bp. There did not appear to be a preponderance of labelled material within this size range for dig-NDVNP673 (lane 3). Instead, the labelled material appeared from about 300 bp to greater than 23000 bp with about equal distribution.

The southern blots indicated considerable cross-reactivity since dig-NDVNP673 probe generated a strong signal for pBR328 DNA (Figure 7-I, lane 4) and dig-pBR328 probe generated a strong signal for NDVNP673 DNA (Figure 7-II; lane 2). The degree of cross-reactivity was considered quite significant given the fact that the assay was carried out under highly stringent conditions[2]. Note that, in this experiment, neither probe bound to herring sperm DNA (lane 6) as evidenced previously in the negative controls of the direct dot blot assay results.

Gene Probe Assays Using NDVNP-PB4

Design of the oligonucleotide probe, NDVNP-PB4, was based on sequence information for the NDVNP673 fragment region. A sequence alignment of NP gene sequences from representative viruses in the Paramyxoviridae family around a region within the 673 bp fragment was performed. The results of the sequence alignment are shown in Figure 8. In the figure, the alignment is shown for nine different sequences representing the three different genuses of the Paramyxoviridae family (paramyxovirus, morbillivirus and pneumovirus) and 8 different species: NDV D26 [10]; NDV Beaudette C [11]; Sendai virus [12]; Parainfluenza type 2 [13]; Parainfluenza type 3 [14]; Parainfluenza type 4a and 4b [15]; Measles virus [16] and Respiratory syncytial virus [17]. The alignment indicated that the nucleotide sequence was 100% conserved between the two NDV strains (D26 and Beaudette C) but only 15-30% conservation among the other sequences, suggesting that this region may be a suitable region with which to design an NDV-specific gene probe. The conserved sequence, was selected for synthesis and was designated as NDVNP-PB4. The fluorescein moiety was incorporated onto the 5' end of the oligonucleotide during the synthesis.

When NDVNP-PB4 was used to probe NDVNP673 DNA and pBR328 DNA in a southern blot assay, there was no cross-reactivity with pBR328 DNA under low stringency conditions (Figure 9). In addition, the southern blot indicated that the majority of the dig-NDVNP673 probe material was in the size range of 700-800 bp. To ensure that the absence of signal for pBR328 DNA was not due to insufficient target being transferred from the gel to the membrane, a dot blot analysis was performed. The results of this assay are

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[2] Based on a calculated Tm value of 69.4°C and a hybridization incubation temperature of 68°C, the stringency is 1.4°C below the calculated Tm (Tm -1.4°C).

presented in Figure 10. The presence of a signal for pBR328 DNA suggested cross-reactivity with NDVNP-PB4, but because the signal did not decrease as the amount of pBR328 decreased, it was suspected that the probe hybridized to the herring sperm DNA that was present in the dilution buffer. When nuclease-free water was used as the diluent to replace herring sperm DNA, there was no signal for pBR328 indicating that NDVNP-PB4 did indeed cross-react with herring sperm DNA at low stringency (Tm- 28.4°C). These results also showed that the lower detection limit for NDVNP673 DNA using NDVNP-PB4 was 10^7 molecules under low stringency conditions (Tm-28.4°C). The cross-reactivity of NDVNP-PB4 probe for herring sperm DNA was unexpected. Attempts to eliminate cross-reactivity by increasing the stringency of the assay were carried out. Because of the limited amounts of herring sperm DNA in the kit, the stringency studies were performed on λ bacteriophage DNA, since NDVNP-PB4 also cross-reacted with λ bacteriophage DNA (Figure 11).

Stringency Studies of NDVNP-PB4

Initially we decided to increase the stringency during the hybridization step rather than the post-hybridization wash step. Figure 12 shows the results of four incremental increases in temperature during the hybridization step. Even at high stringency, (Tm-0.4°C), there was still cross-reactivity with λ phage DNA. We then decided to increase stringency during the post-hybridization washes. The cross-reactivity was successfully eliminated at a stringency of Tm-13.5°C (Figure 13) without seriously compromising sensitivity.

DISCUSSION

This study was intended to evaluate a non-radioactive, colorimetric, membrane-based gene probe assay for NDV relative to a commercially developed assay for bacterial pBR328 DNA. Initial comparisons were based on comparing sensitivity limits but it became apparent during the course of the study that the specificity of the two gene probes (dig-pBR328 and dig-NDVNP673) was questionable, since the pBR328 probe cross-reacted with NDVNP673 DNA and the NDVNP673 probe cross-reacted with pBR328 DNA even under highly stringent conditions (at or near the calculated Tm of the probes). This led to the design of a single-stranded, fluoresceinated oligonucleotide probe for NDV (NDVNP-PB4). Under low stringency conditions, we found that NDVNP-PB4 did not bind to pBR328 DNA, but did bind to herring sperm DNA (present in the DNA dilution buffer) and to λ phage DNA. Consequently, ways to eliminate this cross-reactivity were examined by increasing the stringency of the DNA:DNA binding event during the hybridization reaction and the post-hybridization washing step. Altering the stringency during the hybridization step, even at a stringency close to the calculated Tm (Tm-0.4°C), did not eliminate cross-reactivity, most likely due to the fact that stringency was relaxed between the hybridization step and the wash step. When the stringency was increased during the post-hybridization washes, the cross-reactivity was successfully eliminated at Tm-13.5°C, without compromising sensitivity.

The cross-reactivity problems experienced in this study, highlights an area of potential concern for gene probe identification. If identification is based solely on the binding event of the probe with target, and false positives are to be kept to a minimum, the probe must be specific for its target. This necessitates screening gene probes for all known interfering sequences, a labour intensive and time-consuming effort. Despite the futility of screening all known sequences with each and every gene probe designed, there are approaches which can be taken to add confidence to gene probe-based identification. One could increase the level of confidence in the assay by including more than one probe or by including additional identification technologies. For example, PCR could be used to amplify a distinct fragment of the intended target gene (as was done in this study in which RT-PCR was used to generate NDVNP673 target DNA from NDV B1 RNA), which would then be analyzed for size, based on molecular separation technologies (agarose gel electrophoresis, capillary electrophoresis). If this information is coupled to gene probe analysis, (eg. southern blot analysis), the confidence level for identification is improved. The probability of a heterologous DNA sequence generating a fragment of the correct size following PCR amplification, as well as generating a signal using a gene probe against the amplified fragment, are less than using either technology alone. PCR not only helps to improve specificity for identification purposes, but it also increases the sensitivity of detection by a considerable degree. In this study, using the NDVNP-PB4 gene probe, we were able to detect 10^7-10^8 molecules of NDV DNA. Given that a single NDV particle contains 1 copy of the NP gene target sequence, at best we would be able to detect about 10^7-10^8 virus particles (living or dead). If however, one incorporates PCR amplification into the assay, the limit of detection would be lower. Note that we were able to demonstrate an amplification level of about 10^5 fold for NDV. Therefore, the combination of PCR amplification with a colorimetric, membrane-based gene probe assay, could allow for the detection of between 100-1000 virus particles. Sensitivities could be improved even further by (i) including a second round of PCR amplification (using fresh reagents and nested primers); (ii) using direct fluorescence detection (as opposed to a colorimetric system); (iii) designing gene probes for multicopy gene targets (eg. ribosomal RNA genes).

With respect to sensitivity limits, we experienced about a 10-fold variance in assay sensitivity between trials. This variance could possibly be attributed to (i) differences in buffer preparations; (ii) lot-to-lot variation in detector antibody-enzyme activity; (iii) denaturation of antibody-enzyme conjugate activity with time; (iv) lot-to-lot variation of nylon membranes[3]. For pBR328, we were able to achieve a lower detection limit of 10^4 molecules of

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[3] We have found up to 10 fold differences in sensitivity between membrane lots from the same manufacturer.

denatured double-stranded pBR328 DNA in the presence of $>10^4$ pg of heterologous DNA (herring sperm DNA) using a digoxigenin labelled pBR328 probe, as claimed by the manufacturer using their kit and reagents. Detection limits were always found to be 10-fold poorer for dig-NDVNP673 than for the pBR328 system, under the same assay conditions. This finding may be a result of the difference in the thermal stabilities between the two probes due to differences in their nucleotide composition. Of interest in this study was the fact that assay sensitivity was not affected by the presence or absence of carrier DNA.

With respect to assay sensitivity for the fluorescent oligonucleotide probe, NDVNP-PB4, we were able to achieve a lower detection limit of 10^7-10^8 molecules at a stringency of Tm-13°C (no cross reactivity with λ DNA). This is about 100-fold less sensitive than that obtained for the dig- NDVNP673 probe. It was expected that the NDVNP-PB4 probe would not be as sensitive for two reasons. First, NDVNP-PB4 contains one fluorescein molecule per probe molecule while NDVNP673 probe contains approximately 27 digoxigenin molecules per probe molecule[4]. Second, NDVNP-PB4 is a single-stranded probe while dig-NDVNP673 is double-stranded. As such, there are effectively twice the number of target molecules for the NDVNP673 gene probe as there are for the single-stranded oligonucleotide probe. Sensitivity comparisons based on this data is tenuous however, since the NDVNP673 probe cross-reacted with pBR328 DNA under high stringency (Tm-1.4°C) while NDVNP-PB4 did not (Tm -13.4°C). That is, for dig-NDV673, higher stringencies would be required to eliminate cross-reactivity which, in turn, could reduce sensitivity of the dig-NDVNP673 assay and consequently, the magnitude of the difference in sensitivity between the two probes. Furthermore, the difference in sensitivity may not be a significant issue if other factors, such as assay speed and specificity, are considered. While a time course evaluation of hybridization time versus sensitivity/specificity was not done in this study, theoretically, the NDVNP-PB4 probe should bind more quickly than the NDVNP673 probe.

This study indicates the level of sensitivity which may be achieved using a membrane-based system for short and long probes and serves as a benchmark for comparison of other assay systems that are under investigation.[5] This study also showed the importance of screening gene probes for specificity and how specificity can be manipulated by altering assay stringency.

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[4] Boehringer Mannheim product literature indicates that the labeling efficiency using the method described in this study should be 1 digoxigenin molecule for every 20-25 bases.

[5] We are evaluating a colorimetric, microplate-based gene probe assay and a dual probe gene probe assay for the Light Addressable Potentiometric Sensor-Threshold Unit.

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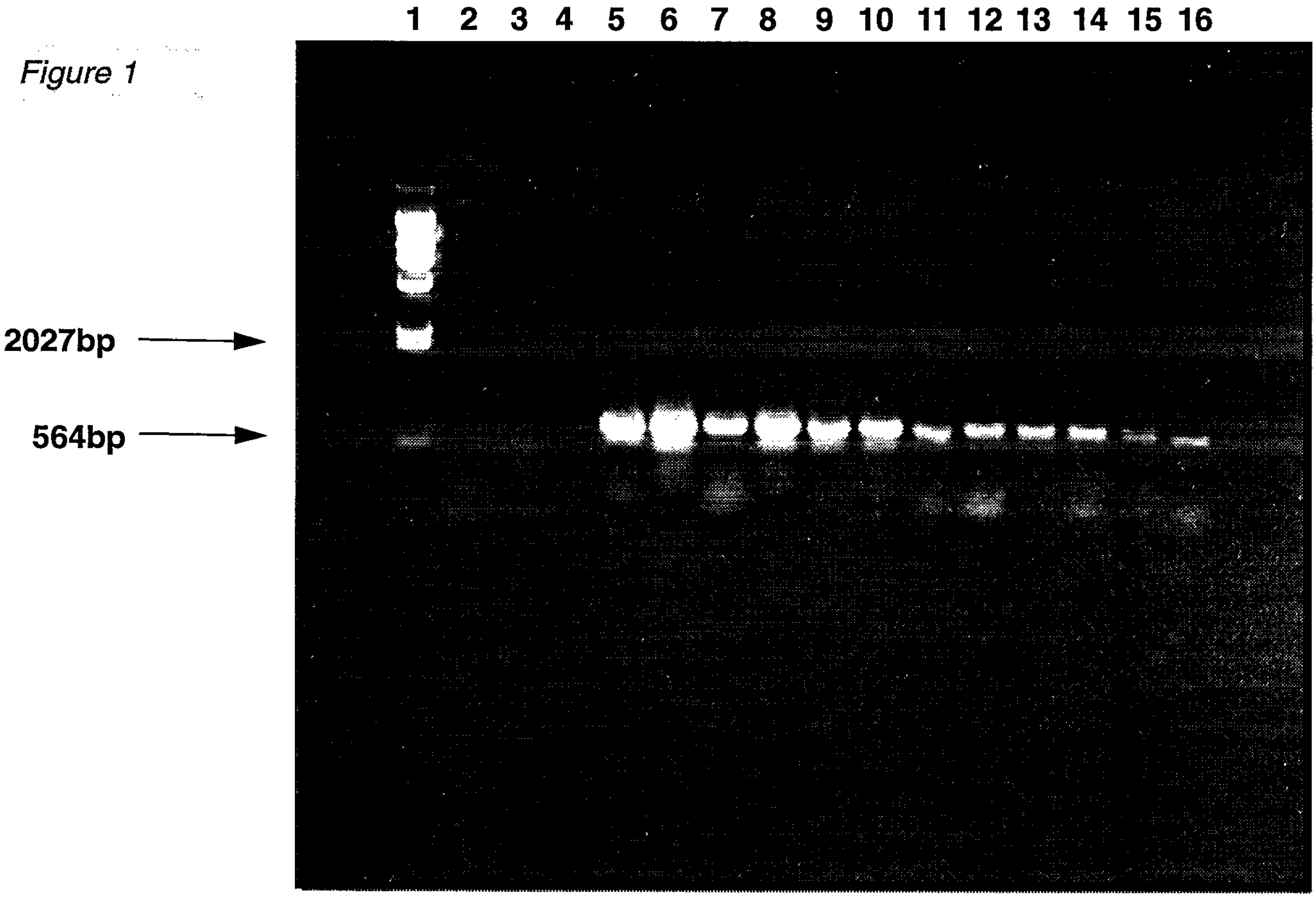
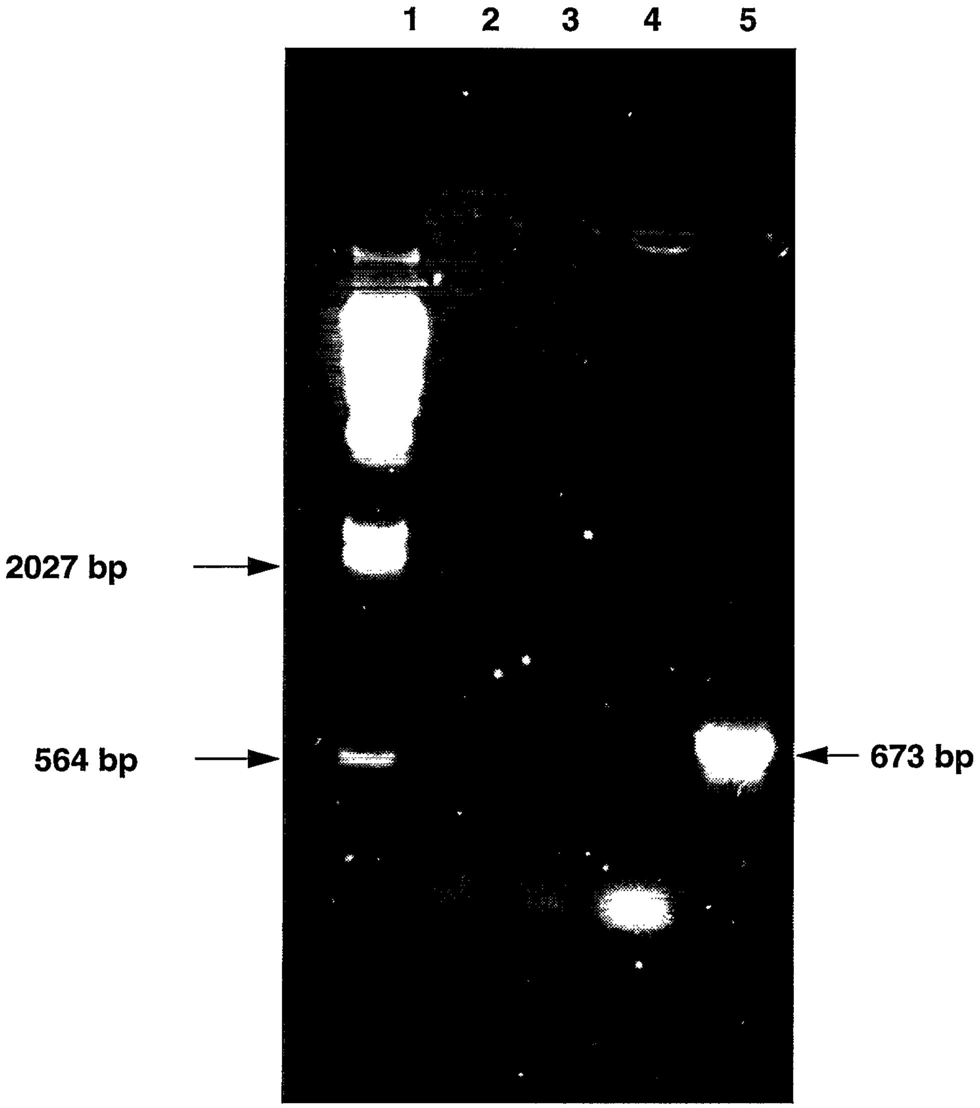
Figure 1



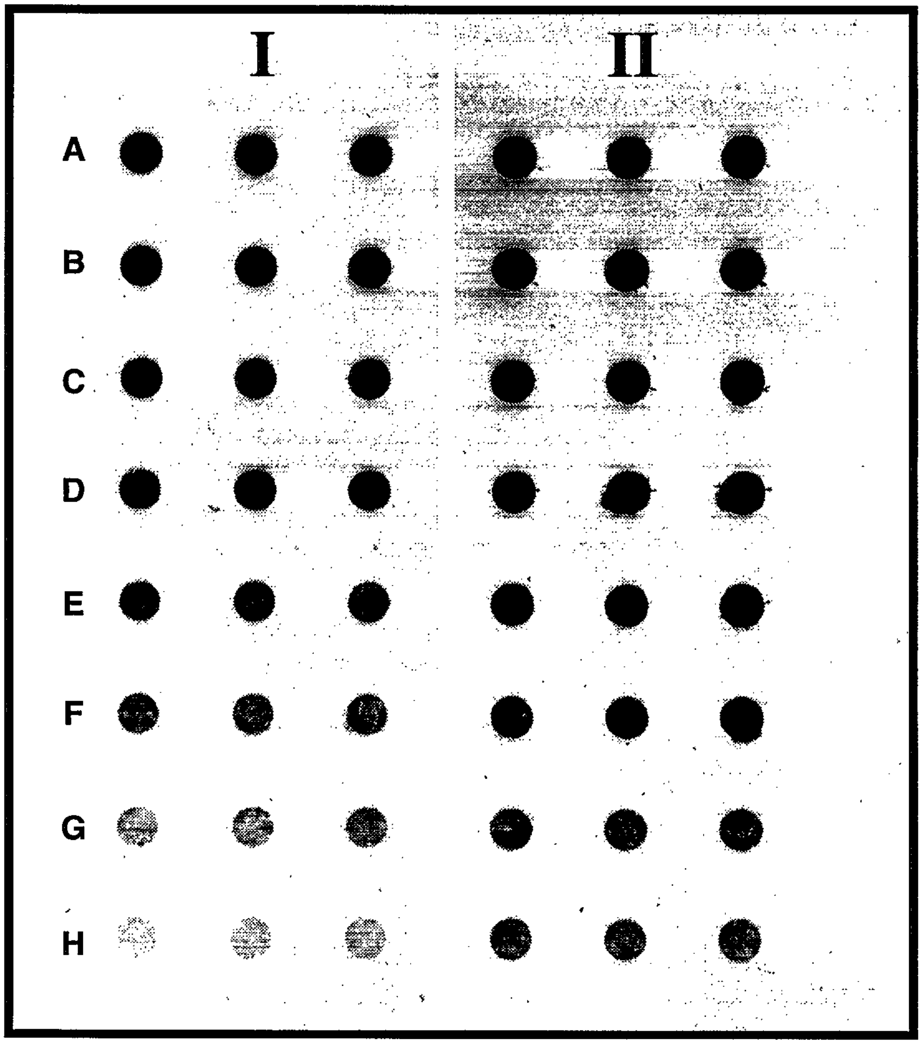
Horizontal agarose gel analysis of reverse transcription-PCR using NDV B1 RNA as the template. **Lane 1**: Hind III digested lambda DNA mwt markers (1.0 µg). The molecular weights of the marker bands from top to bottom are 23130, 9416, 6682, 4361, 2322, 2027, 564, and 123 bp (may not always be visible). **Lanes 2-4**: no template RNA. **Lanes 5-16**: 6.7×10^{10} to 6.7×10^{5} RNA molecules in duplicate 10-fold decrements.

Figure 2



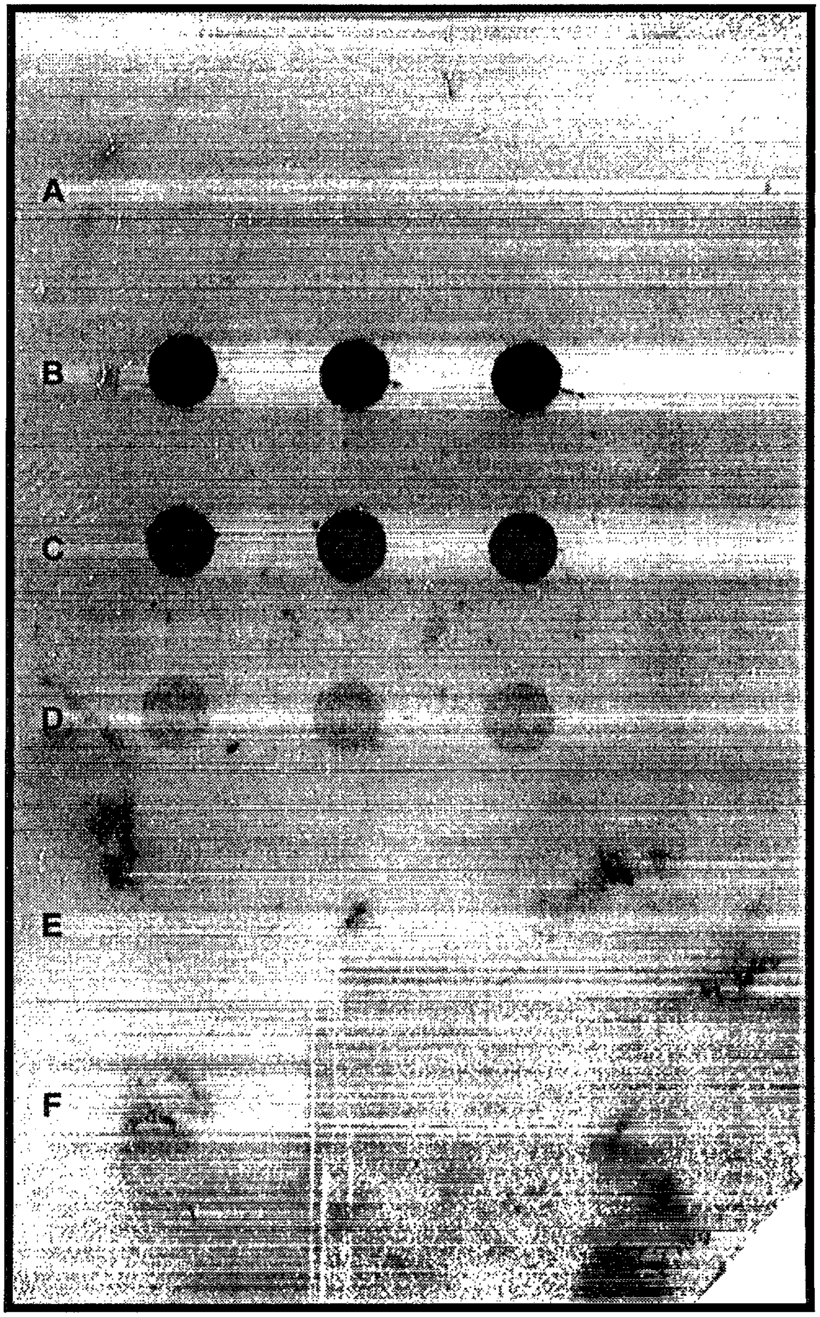
Horizontal agarose gel analysis of large scale RT-PCR using NDV B1 RNA as the template. **Lane 1**: Hind III digested lambda DNA mwt markers (1.0 µg). The molecular weights of the marker bands from top to bottom are 23130, 9416, 6682, 4361, 2322, 2027, 564, and 123 bp (may not always be visible). **Lanes 2-4**: Negative controls (no RNA template). **Lane 5**: 6.7×10^9 molecules of RNA template.

Figure 3



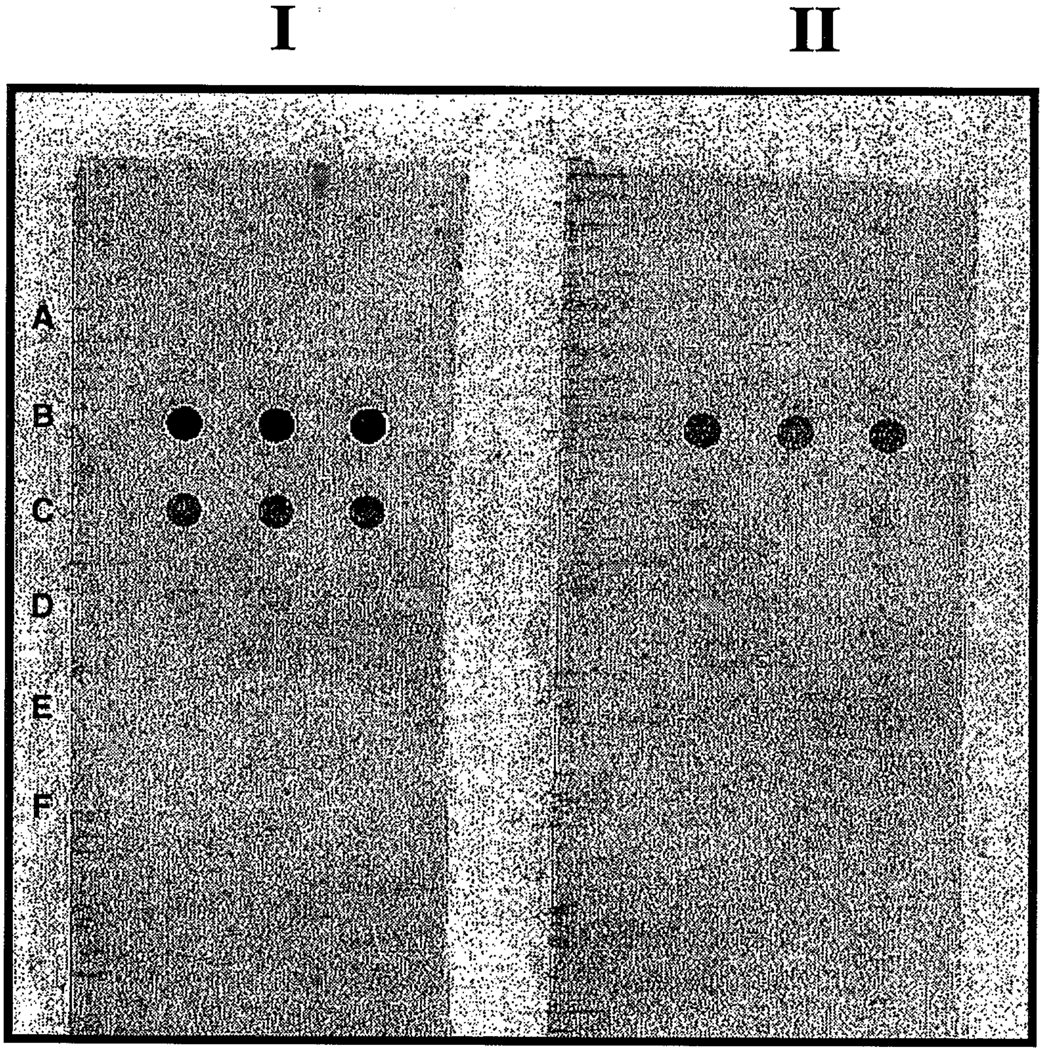
Dot blot quantitation of digoxigenin-labelled NDVNP673 DNA probe material. **(I) dig-pBR328**: Row A - H: 8 pg/L - 0.062 pg/L in 2- fold decrements. **(II) dig-NDVNP673**: Row A - H: 1/500 - 1/64000 dilutions in 2-fold decrements.

Figure 4



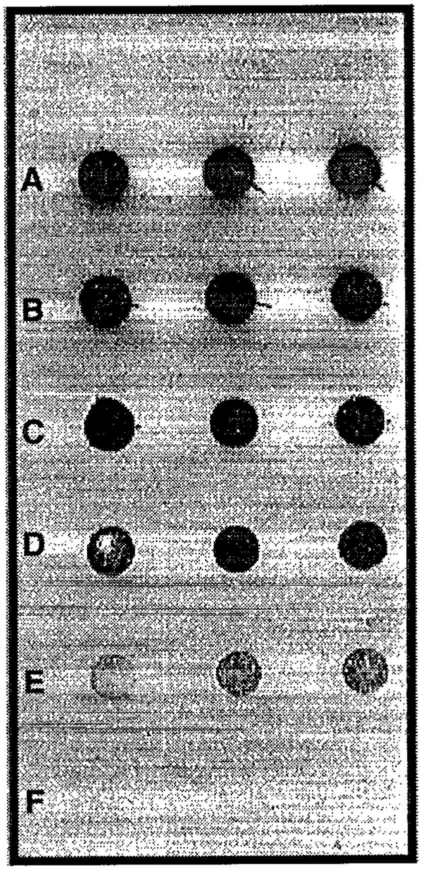
Dot blot assay for pBR328/dig-pBR328. Conditions include 16.5h hybridization at 68°C with 26 ng/mL probe; 2x15 min washes at 68°C in 0.1xSSC/0.1%SDS; 24h color development. **Row A**: negative control (DNA dilution buffer containing 500 ng herring sperm DNA). **Rows B - F**: $1.9\text{x}10^7$ to $1.9\text{x}10^3$ molecules of pBR328 in 10-fold decrements.
Stringency = Tm-1.4°C.

Figure 5



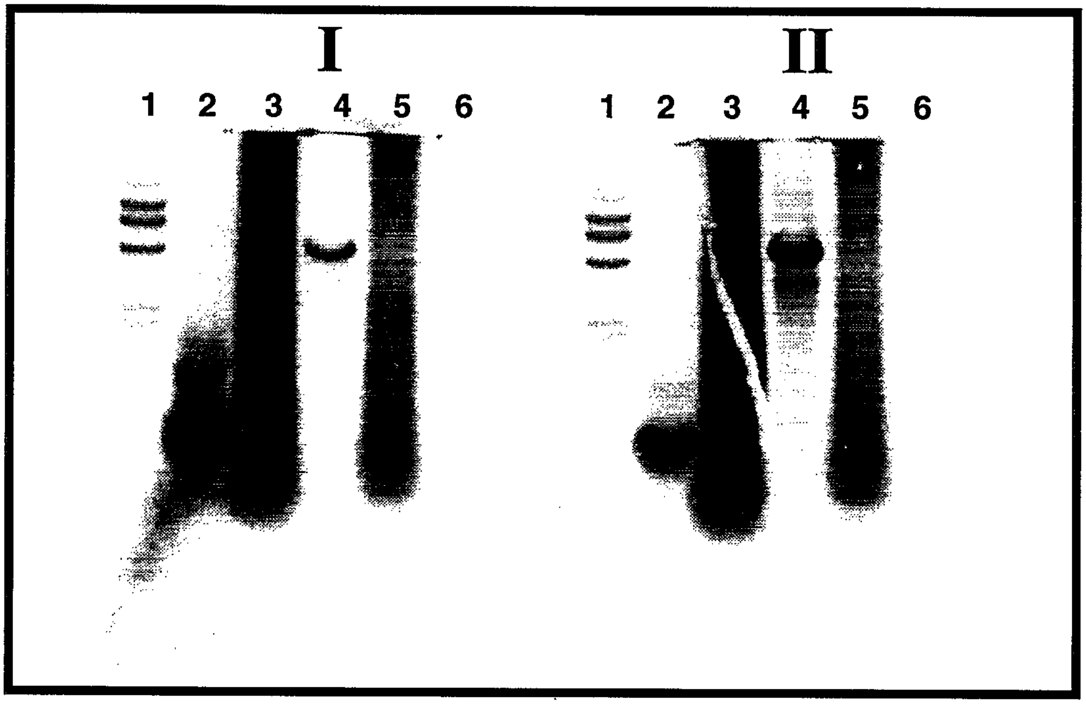
Comparison of assay sensitivities between (I) pBR328/dig-pBR328 and (II) NDVNP673/dig-NDVNP673 under identical hybridization conditions. Conditions include 16.5h hybridization at 68°C with 26 ng/mL probe; 2x15 min washes at 68°C in 0.1xSSC/0.1%SDS; 24h color development. **Row A:** 500 ng of herring sperm DNA per spot. **Rows B-F:** 1.9×10^7 to 1.9×10^3 molecules in 10-fold decrements.
Stringency = Tm-1.4°C

Figure 6



Dot blot assay for NDVNP673/dig-NDVNP673 without herring sperm DNA as diluent. Conditions include 16.5h hybridization at 68°C with 26 ng/mL probe; 2x15 min washes at 68°C in 0.1xSSC/0.1%SDS; 24h color development. **Row A - F**: 1.9×10^{10} to 1.9×10^{5} molecules of NDVNP673 DNA in 10-fold decrements. Stringency = Tm-1.4°C.

Figure 7



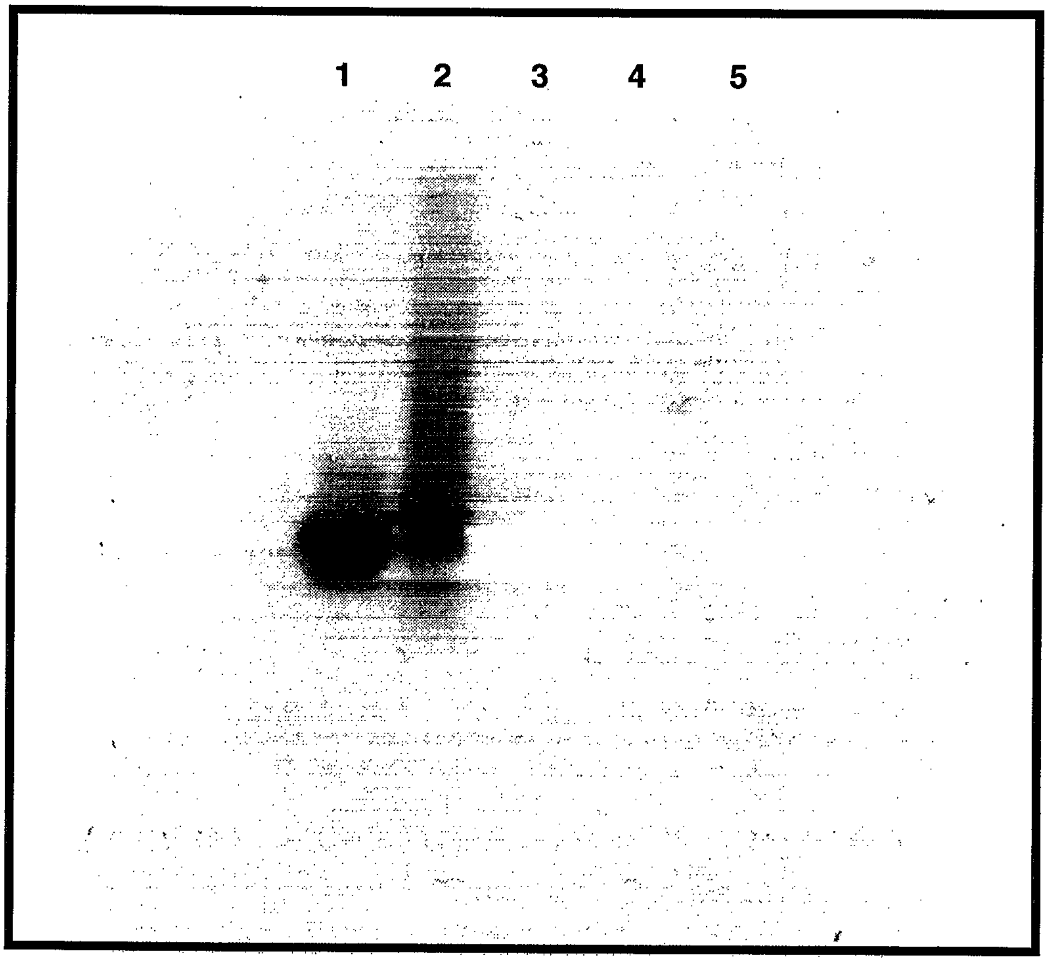
Southern blot analysis of **(I)** dig-NDVNP673 and **(II)** dig-pBR328 probe material. Conditions include 16.5h hybridization at 68°C with 26 ng/mL probe; 2x15 min washes at 68°C in 0.1xSSC/0.1%SDS; 0.5h color development. **Lane 1**: dig-λ-Hind III DNA mwt markers (90 ng). **Lane 2**: NDVNP673 DNA ($1.5x10^{11}$ molecules). **Lane 3**: dig-NDVNP673 ($1.3x10^{11}$ molecules). **Lane 4**: pBR328 ($1.7x10^{10}$ molecules). **Lane 5**: dig-pBR328 ($1.8x10^{9}$ molecules). **Lane 6**: herring sperm DNA (455 ng).
Stringency = Tm-1.4°C.

Figure 8

		% homology
NDV (D26)	GCT CCT CGC GCG TCA GAC TC	100
NDV (Beaudette C)	GCT CCT CGC GCG TCA GAC TC	100
Sendai	ATT TAG CTC TAG GAG GAG CG	30
Parinfluenza type 2	ATT TAC TAT ACA ACA GGA GC	30
Parinfluenza type 3	ACG TAG GCA AGA AAA CAT AA	15
Parinfluenza type 4a	GTT TCT TCA AAC AAC TGA AG	20
Parinfluenza type 4b	ATT TCT TCA AAC AAC TGA AG	15
Measles	GTT CAA AAG AAA CAA GGA CA	25
Respiratory syncytial virus	TAC ACT CAA CAA AGA TCA AC	25

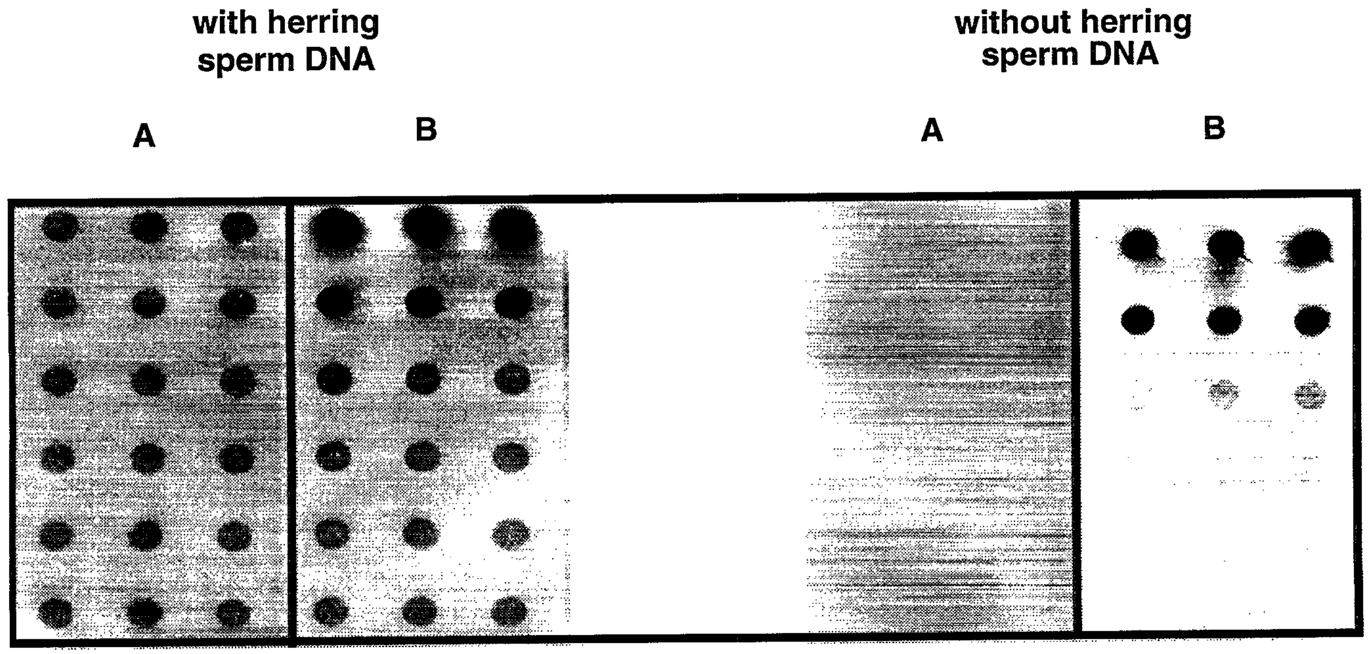
Alignment of a region within the NP gene sequence for different species within the Paramyxoviridae family. Conserved nucleotides relative to NDV sequences are underlined.

Figure 9



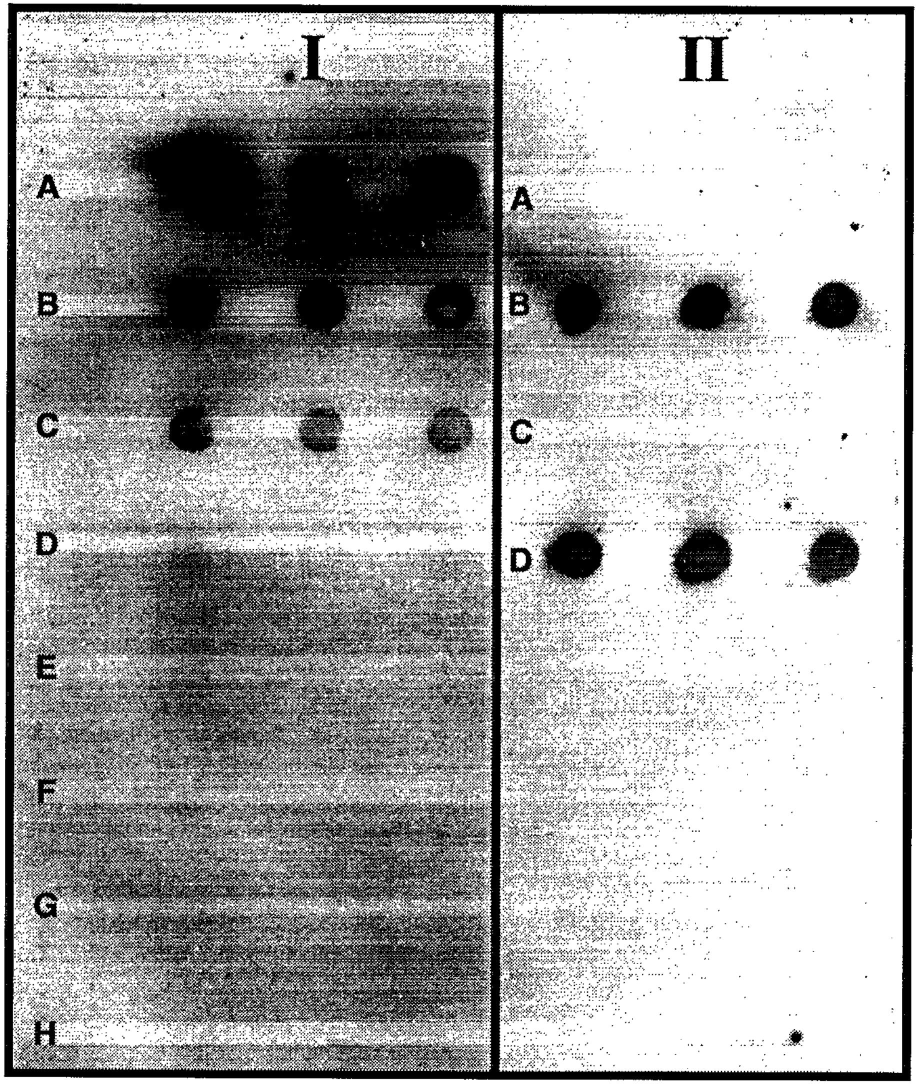
Southern blot analysis of NDVNP673 DNA and pBR328 DNA with NDVNP-PB4. Conditions include 2h hybridization at 58°C with 80 ng/mL probe; 2x5 min washes at room temperature in 0.1xSSC/0.1%SDS; 17h color development. **Lane 1**: NDVNP673 DNA ($1.5x10^{11}$ molecules). **Lane 2**: dig-NDVNP673 ($1.3x10^{11}$ molecules). **Lane 3**: pBR328 ($1.7x10^{10}$ molecules). **Lane 4**: dig-pBR328 ($1.8x10^{9}$ molecules). **Lane 5**: herring sperm DNA (455 ng).
Stringency = Tm-27.2°C.

Figure 10



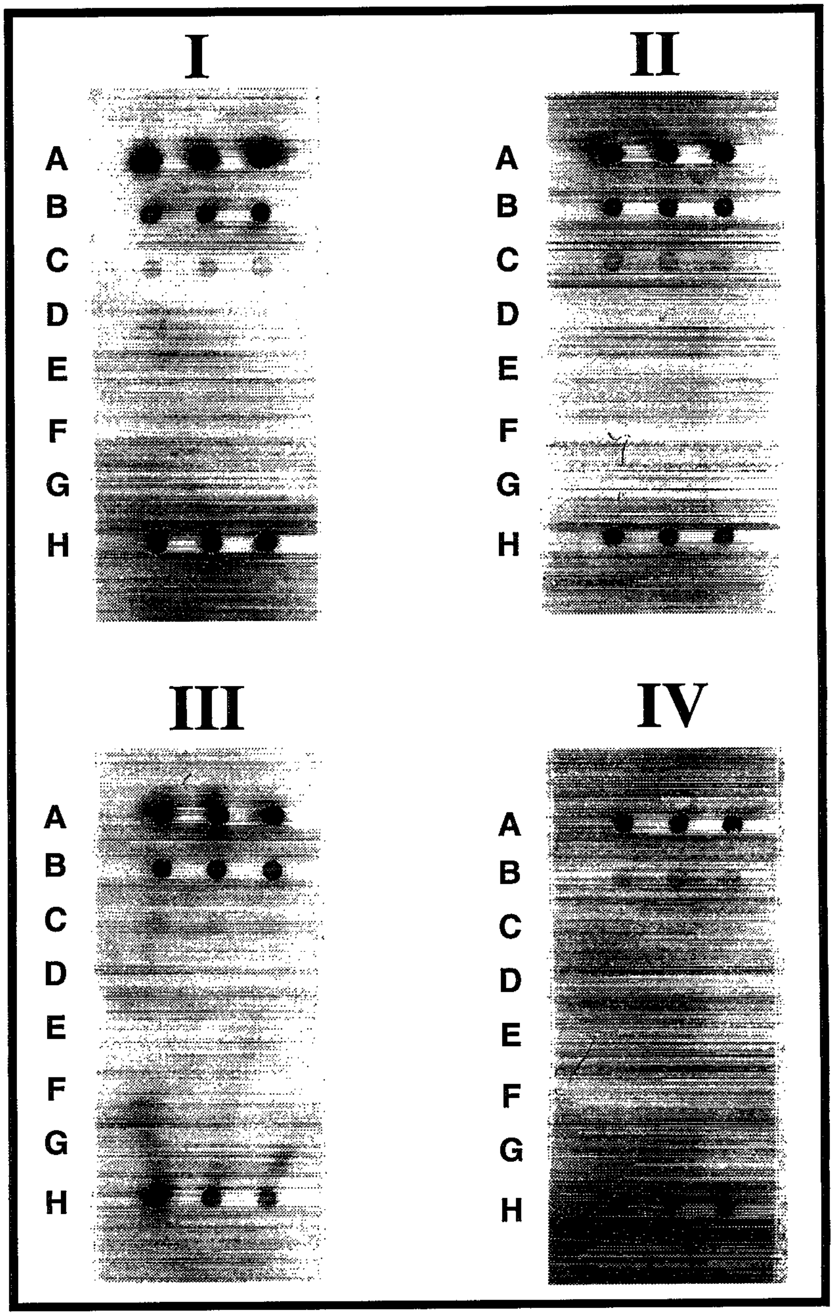
Dot blot assay using NDVNP-PB4 as probe with and without herring sperm DNA as diluent. Conditions include 2h hybridization at 58°C with 200 ng/mL probe; 2x5 min washes at room temperature in 0.1xSSC/0.1%SDS; 16h color development. **A**: pBR328 DNA: 3.7×10^{10} to 3.7×10^{5} molecules in 10-fold decrements. **B**: NDVNP673 DNA: 3.1×10^{10} to 3.1×10^{5} molecules in 10-fold decrements. Stringency = Tm-28.4°C.

Figure 11



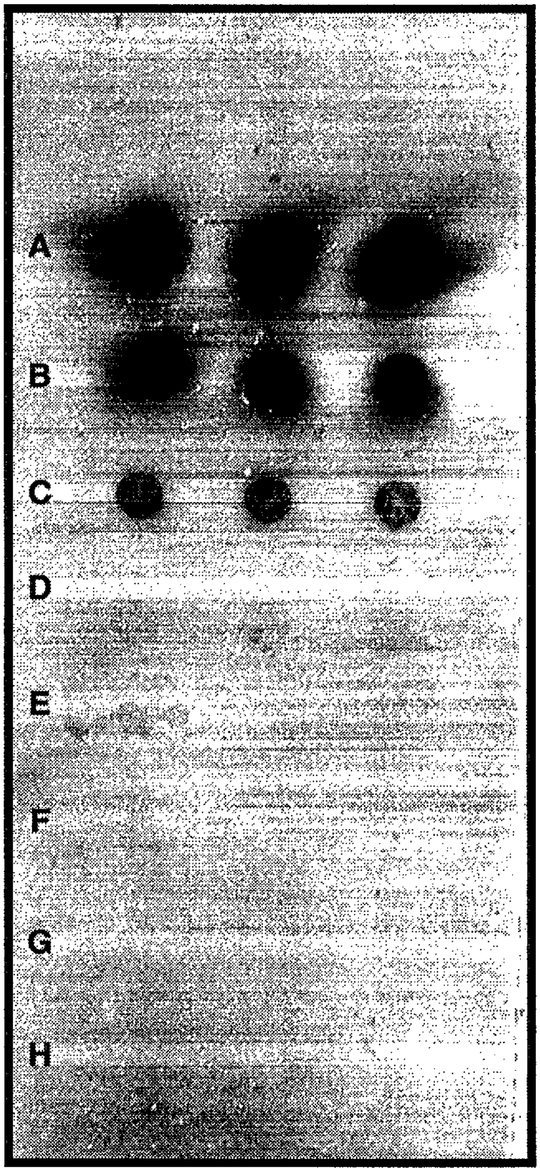
Dot blot assay showing cross-reactivity of NDVNP-PB4 with herring sperm DNA and λ phage DNA. Conditions include 2h hybridization at 58°C with 200 ng/mL probe; 2x5 min washes at room temperature in 0.1xSSC/ 0.1%SDS; 16h color development. **Column I, Rows A-H**: NDVNP673 DNA from 3.7×10^{10} to 3.7×10^{3} molecules in 10-fold decrements. **Column II Row A:** blank, **Row B**: λ phage-HindIII DNA (3.1×10^{10} molecules); **Row C**: pBR328 (3.1×10^{10} molecules); **Row D**: herring sperm DNA (250 ng). Stringency = Tm-28.4°C.

Figure 12



Effect of increasing stringency during hybridization on cross-reactivity of NDVNP-PB4 probe for λ DNA. (I) Tm-23.4°C; (II) Tm- 18.4°C; (III) Tm-13.4°C; (IV) Tm-0.4°C. Conditions include 2h hybridization at increasing temperatures; 200 ng/mL probe; 2x5 min washes at room temperature in 0.1xSSC/0.1%SDS; 16h color development. **Rows A-G**: NDVNP673 DNA from 3.1×10^{10} to 3.1×10^{4} molecules in 10-fold decrements. **Row H**: λ phage-Hind III DNA (3.1×10^{10} molecules).

Figure 13



Effect of increasing stringency during post-hybridization wash step on cross-reactivity of NDVNP-PB4 probe for λ DNA. Conditions include 2h hybridization at 58°C; 200 ng/mL probe; 2x5 min washes at 45°C in 0.1xSSC/ 0.1%SDS; 16h color development. **Rows A-G**: NDVNP673 DNA from 3.1×10^{10} to 3.1×10^{4} molecules in 10-fold decrements. **Row H:** λ phage-Hind III DNA (3.1×10^{10} molecules). Stringency = Tm-13.4°C.

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A non-radioactive, membrane-based, colorimetric gene probe assay was developed for Newcastle disease virus (NDV) to evaluate the potential of gene probes as tools for the identification of biological agents. Two gene probes for the NDV major nucleocapsid protein gene were evaluated, namely, (i) a digoxigenin-labelled, double-stranded DNA fragment of approximately 673 base pairs designated as dig-NDVNP673 and (ii) a 5'-fluorescein-labelled, single-stranded oligonucleotide probe of 20 bases, designated as NDVNP-PB4. Detection limits of 10^5-10^6 molecules of NDV target DNA were achieved using dig-NDVNP673, however this probe was found to cross-react with bacterial plasmid DNA (pBR328), even under highly stringent assay conditions (Tm-1.4°C). The oligonucleotide probe, NDVNP-PB4, did not cross-react with pBR328 DNA, even under low stringency conditions (Tm-29°C), and generated a detection limit of 10^7-10^8 molecules for NDV target DNA. However, the oligonucleotide probe cross-reacted with herring sperm DNA and lambda bacteriophage DNA under these conditions. When the stringency was increased during the post-hybridization wash step, from Tm-29°C to Tm-13°C, cross-reactivity was eliminated without compromising assay sensitivity. This study gave us an indication of the level of sensitivity one can expect to achieve using a colorimetric, membrane-based system for short and long gene probes and demonstrated the importance of screening gene probes for specificity. This work will serve as a benchmark for comparison to other gene probe assay systems that are under investigation.

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