DETECTION OF VIRUSES CAUSING BOVINE RESPIRATORY DISEASES

By

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DETECTION OF VIRUSES CAUSING BOVINE

RESPIRATORY DISEASE

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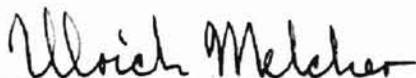

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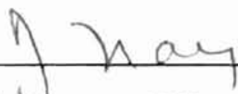

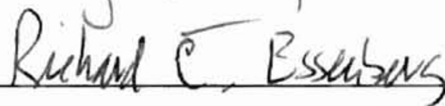

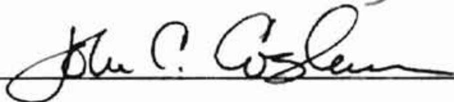

Dean of the Graduate College

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NOMENCLATURE

μL	microliter
AT	adenine and thymine
AP	alkaline phosphatase
bp	base pair
CCD	charge-coupled device
cy	cyanine
cm^2	square centimeter
ddH2O	double-distilled water
DIG	digoxigenin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
cDNA	complementary DNA
dNTP	deoxyribonucleoside triphosphate
dCTP	deoxycytidine triphosphate
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
ER	endoplasma recticulum
kb	kilo base pair
mg	milligram

ml	milliliter
nt	nucleotides
PCR	polymerase chain reaction
PMT	photomultiplier tube
RNA	ribonucleic acid
mRNA	messenger RNA
RT	reverse transcription
u	unit
UV	ultra violet

Introduction

Bovine respiratory disease

Bovine respiratory disease (BRD) is an important disease of cattle, and an estimated $3 billion is spent annually for its prevention and treatment[1,2]. Although it has been extensively investigated in recent years, BRD remains a major cause of economic loss in cattle in US.

BRD is caused by a variety of viral and bacterial agents[4]. *Pasteurella hemolytica* and *Pasteurella multocida* are bacteria frequently isolated from BRD cases[2,3]. These bacteria are commonly found in the nasopharyngeal area of healthy animals[2]. Although the bacteria cannot enter the lower respiratory tract and cause pneumonia under normal conditions, a relatively small number of bacteria leads to severe pneumonia under certain predisposing conditions; these include presence of a respiratory viral infection and/or stresses brought about by climatic, nutritional changes, shipment, overcrowding, and/or weaning[2,3]. Viruses commonly implicated in BRD are bovine herpes virus types 1 (BHV-1) and BHV-3, bovine viral diarrhea virus (BVDV), bovine parainfluenza 3 (PI-3) virus, and bovine respiratory syncytial virus (BRSV) [1]. Other viruses less frequently associated with BRD include bovine adenovirus, bovine rhinovirus, bovine coronavirus, bovine reovirus, bovine enterovirus, alcelaphine herpesvirus-1, BHV-4, and BHV-5[1,3,5,6,7,8]. Various factors must therefore be present to cause BRD and it is believed that virus infection, stresses, and immunosuppressive agents lead to bacterial pneumonia.

Bovine herpes virus

BHV-1 belongs to the virus family of Herpesviridae and subfamily of Alphaherpesvirinae[17,22,29]. Herpes virus is spherical or pleomorphic with diameter of 150-

200 nm and consists of an envelope and an icosahedral capsid[29]. The length of BHV-1 DNA is 135 kilobase pair (kb) [4,42]. The viral genome is a linear double stranded DNA subdivided into a long segment (106 kb) and a short segment (10 kb) flanked by internal and terminal inverted repeats (11 kb each) [16,18]. Following adsorption to a host cell receptor via the peplomers of the envelope, the nucleocapsid penetrates by fusion or phagocytosis[11]. Replication occurs in the nucleus and BHV-1 virus acquires an envelope by budding through nuclear membrane[11]. BHV-1 causes respiratory and reproductive disorders leading to infectious bovine rhinotracheitis/pustular vulvovaginitis in cattle and abortion[4,19]. This virus causes inflammatory and necrotizing lesions in the upper respiratory tract, trachea, and conjunctiva[3]. To control viral infection, vaccination is used. Modified live vaccine (MLV) is useful against BHV-1, and it starts to protect the cattle 48 hours after vaccination[2]. MLV may be used safely, but it is difficult to administer because of post-vaccinal disease, which can occur if the vaccine virus regains virulence[2,39]. Killed vaccine is also available, but it is expensive and takes longer to initiate the protection, and has a shorter protection period of about a year. So, use of killed vaccine is not common for protection against BHV-1[2].

Bovine viral diarrhea virus

BVDV is a single-stranded linear RNA virus of the *Pestivirus* genus within the family *Flaviviridae*[9-11,13]. It is a small (50 nm) spherical icosahedral capsid with enveloped virus and the genome is positive sense[13,28]. The length of BVDV RNA is 12.3 kb. BVDV is slightly AT rich, and has one long ORF 3898 codons long starting from nt 386. BVDV also has several very short ORFs (< 30 nt) [10]. Two types of BVDV (type 1 and 2) are identified. BVDV enters cells by receptor-mediated endocytosis that delivers

the viral genome to the cytosol[10]. Replication occurs in cytosol, the assembly of BVDV takes place in the ER and Golgi, and then BVDV acquires an envelope in the lumen of the vesicular compartment[10]. This virus is a common pathogen of calves. Its immuno-suppressive ability[20]. BVDV is consequently associated with a sporadically occurring, highly fatal, and enteric disease that is termed mucosal disease[9]. Infection during pregnancy can result in abortion, teratogenic defects, or birth of persistently infected animals. Persistently infected animals serve to spread BVDV in the herd[15]. Sometimes bovine fetal serum from persistently infected calves contaminate cell cultures for years without detectable alteration of cell physiology and metabolism[10]. There are 140 licensed BVDV vaccines in the US. Since live vaccine and killed vaccine require at least 3 weeks and 4 weeks after vaccination to take effect, respectively[4], it is necessary to identify the virus infecting the cattle at an early stage before the infection extends to the entire herd. There is evidence that vaccination does not fully protect against BVDV[4]. There is the need, therefore, to quickly identify the BVDV infection and isolate the infected bovine from the herd.

Diagnostic methods

The common methods to diagnose BHV-1 and BVDV viral infection are virus isolation in cell culture, immunofluorescent antibody tests (IFA), necropsy, and serologic tests[17,21]. However, those tests present some disadvantages. Virus isolation tests are not very sensitive because some viruses grow with difficulty, and the tests may take 1 to 3 weeks to complete[17]. Virus identification by serology and necropsy methods require either recovery or fatality of a bovine. This delays initiation of the treatment for other cattle in the herd to avoid infection by these viruses. Immunoperoxidase plate assay for

diagnosis of BVDV can handle large number of samples simultaneously, but this method also takes 5 to 7 working days to complete[12].

It is important to identify viruses infecting cattle quickly and accurately, since diagnosis early in the course of infection makes it possible to choose the right nutrition, treatments (such as isolation of infected animals), and medication (such as vaccination). Speedy and accurate diagnosis of BRD is essential for immediate action against BRD virus infection.

Real-time PCR

Currently two commercial systems for real-time PCR are available. They are the ABI PRISM 7700 Sequence Detection System (Perkin-Elmer, Foster city, CA) and the LightCycler (Roche, Indianapolis, IN). Real-time PCR made quantitative PCR faster and it also reduces the chance of contamination[46-49].

A fluorogenic probe is prepared to be specific to an internal region of the PCR product and s labeled with a reporter fluorescent dye [FAM (6-carboxy-fluorescein)] at the 5' end and a quencher fluorescent dye [TAMRA (6-carboxy-tetramethyl-rhodamine)] at the 3' end[46-49]. When the probe is intact, fluorescent energy transfer occurs and the reporter dye emission is absorbed by the quencher dye. During the extension phase of PCR, a fluorogenic probe is cleaved by the 5' nuclease activity of the DNA polymerase, separating the reporter dye from the quencher dye and generating a sequence-specific signal (518 nm in peak fluorescent emission) [48]. With each cycle, additional reporter dye molecules are cleaved from their respective probes. The fluorescence intensity is monitored during the PCR[49].

The real-time PCR system uses specially designed reaction tubes that remain closed throughout the PCR amplification and detection process, reducing the chance of contamination[49]. Results are available one minute after completion of thermal cycling[49] and the whole process is done in 30 minutes[47] to one hour[46]. Portable battery-powered devices can be used[46] and the real-time PCR is the most possible method to be automated. Since the real-time PCR is PCR primers with fluorogenic probe, it is necessary to establish multiplex PCR conditions to use real-time PCR for the detection of two viruses or more, simultaneously.

Multiplex PCR

Multiplex PCR is used for simultaneous amplification of two or more DNA fragments in the same reaction[23,24], and is used for DNA testing, including analyses of deletions, mutations and polymorphisms[23]. The multiplex PCR is one assay that can be used to diagnose viruses causing BRD. As compared to single template PCR, multiplex PCR can reduce the total number of tubes, the cost for the reagents, and possible contamination[24].

The first single template PCR for BVDV and BHV-1 were described in 1990[43] and 1993[45], respectively. No multiplex PCR related to BHV-1 has been described thus far, but several were published for BVDV; two were for distinguishing among border disease virus (BDV), BVDV-1, and BVDV-2[25-27]. One of them also distinguished hog cholera virus[26] from BVDV. Those studies succeeded in diagnosing BDV and BVDV in multiplex PCR without RNA extraction[25,26]. This multiplex PCR without RNA extraction made it possible to diagnose the infection about 8 hours after blood collection[26].

However, multiplex PCR is not suitable to diagnose all viruses causing BRD because at least 13 viruses are known to cause BRD. Multiplex PCR under these conditions is expected to be difficult due to three reasons. Firstly, competition among the multiplex PCR products makes it difficult to amplify viruses which are present in small amounts in the sample or have low amplification efficiency during PCR[32,33]. Secondly, separation and identification of PCR products by size by gel electrophoresis will be difficult. This is because there are at least 13 fragment sizes to identify on the gel. The need to identify the fragment sizes put restrictions on the primer design. Thirdly, primer-dimers and primer-artifacts will be a problem[32]. It is possible that those products might interact with PCR products and make PCR products of unexpected sizes.

Microarray technology

The most commonly used microarray technique is that in which a microscopic array of cDNA molecules is immobilized on solid surfaces (i.e. nylon, nitrocellulose, and glass) as hybridization targets for biochemical analysis[14,50]. The array of cDNAs is printed on solid surfaces as targets and the mRNAs are enzymatically fluorescently labeled as cDNA probes. These cDNAs are hybridized on the microarray and un-incorporated labeled probes are washed away. Thus, fluorescently labeled targets which are complementary to targets are bound to targets. The fluorescence is detected by confocal laser scanning and charge-coupled device (CCD) imaging[14,50]. This method was developed for accurate measurement of the expression of the corresponding genes[50]. Array densities are 400 to 250,000 cDNAs per cm^2. This allows quantitative estimation of the expression of many genes in one single hybridization[14]. GSI Lumonics, Watertown, CA (a maker of microarray scanning device) developed a four-color fluorescence

detection scheme, which allows up to four kinds of probes to be used in one expression analysis. Today, fluorescently labeled DNA or RNA from biological sources is used as probe and a complementary sequence is used as target[14]. The applications of microarrays have been extend to expression analysis, polymorphism detection, DNA resequencing, and genotyping on a genome scale[14].

Affymetrix, Santa Clara, CA developed on-chip photolithographic synthesis, GeneChip technology[51]. It can make miniaturized, high-density arrays of oligonucleotide probes by light-directed chemical synthesis[51]. First, the protecting groups are removed and generate free hydroxyl groups at positions illuminated by UV light passing through the photolithographic mask. Then, 5'-protected phosphoramidite is added to deprotected sites, and oligonucleotides are synthesized by repeating this process[52].

In this project, I have attempted to devise a fast and simple diagnosis method for clinical users. Fast diagnosis of bovine respiratory disease is essential to control and prevent infection of other bovines[12]. By knowing the virus or viruses infecting the cattle, the rancher can decide to isolate the infected individual or vaccinate the whole herd to prevent infection to others. High sensitivity and a rapid procedure are required to achieve this purpose. Further, the method of diagnosis must be user friendly. At least 13 viruses cause bovine respiratory disease and it will be useful if a single PCR can detect all BRD viruses. In this research, I attempted to develop a method for the simultaneous detection of BHV and BVDV by microarray technique and PCR. First, I attempted multiplex PCR to diagnose the virus infections of BRD. Next, I attempted to develop a ViSA (Virus Signature Amplification) card method and a ViSH (Virus Signature Hybridization) chip method. Both methods are based on microarray technologies and they have the potential

to provide simultaneous diagnosis of thousands of viruses. Mosaic Technologies, Inc. is also working with Bridge amplification technologies which are similar to ViSA card method.

Bridge amplification technologies

Mosaic Technologies, Inc. has devised bridge amplification technology[30-32]. They immobilized acrylamide-modified oligonucleotides on many kinds of media (i.e. nylon beads, optical fibers, and glass plates) and performed immobilized PCR. The "Bridge" of bridge amplification technologies comes from the shape when the extension product from one primer binds to another primer during the annealing step[32]. Many primer sets are placed on the media separately. This procedure should make it possible to diagnose tens to hundreds of individual amplification reactions in a single reaction mixture and vessel[32]. The Mosaic Technologies' primer attachment method is co-polymerization of acrylamide-modified oligonucleotide into a polyacrylamide co-polymer[31]. The benefit of this method is that it can avoid cross-contamination from amplified products since all amplified products are bound to the media. This system will be easier to automate and the use of real-time PCR is possible. Furthermore, this method costs less than a single template PCR for each primer since it can test many primers in a single assay [32].

Materials and Methods

Reagents

KlenTaq, wild-type Taq DNA polymerase missing an N-terminal portion[44], and PC2 buffer (50 mM Tris-HCl pH 9.1, 16 mM ammonium sulfate, 3.5 mM $MgCl_2$, and 150 µg/ml BSA) were purchased from Ab Peptides, Inc., St. Louis, MO. FluoroLink Cy3-dUTP and Cy5-dCTP (5-Amino-propargyl-2'-deoxycytidine 5'-triphosphate coupled to Cy3 or Cy5 florescent dye) in 10 mM phosphate buffer, pH 7.0 was purchased from Amersham Pharmacia Biotech, Arlington Heights, IL. Digoxigenin-3-O-succinyl-aminocaproyl-[5-(3-aminoallyl)-2'-deoxyuridine-5'-triphosphate] tetralithium salt (DIG-dUTP), CSPD Ready-to-use (chemiluminescent substrate), Blocking reagent, and alkaline phosphatase conjugated anti-digoxigenin (Anti-DIG-AP conjugate) were purchased from Boehringer Mannheim, Indianapolis, IN. Taq DNA polymerase in Buffer B, 10x PCR buffer, and 10 mM $MgCl_2$ was purchased from Promega, Madison, WI. 10x PCR buffer contains 10 mM Tris-HCl, 50 mM KCl, and 0.1 % Triton X-100. AP buffer consisted of 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 100 mM $MgCl_2$. Sodium borohydride solution was purchased from Fisher Scientific, Pittsburgh, PA. Maleate buffer consisted of 100 mM maleic acid, pH 7.5, and 150 mM NaCl. 20x SSC consisted of 3 M NaCl and 0.3 M sodium citrate. Stop reagent consisted of 50% glycerol, 10 mM EDTA, pH 8.0, and 0.025% bromphenol blue.

Materials

Nylon membrane (Hybond-N, 0.45 micron, 137 mm) was also purchased from Amersham Pharmacia Biotech, Arlington Heights, IL. Silylated glass plates (cat.# CSS-100) were purchased from CEL Associates, Inc., Houston, TX. PCR select-II and III were

purchased from 5 prime→3 prime, Inc., Boulder, CO. GeneAmp RNA PCR kit containing MuLV reverse transcriptase, RNase inhibitor, and random hexamers were purchased from Perkin Elmer, Foster city, CA. 100 bp DNA ladder was purchased from Life Technologies, Rockville, MD. Frame-Seal incubation chambers for sealing reactions on slides were purchased from MJ Research, Inc., Watertown, MA. Array it UniHyb was purchased from Telechem International, Sunnyvale, CA. Array it Hybridization cassette was also a product of Telechem International, and was kindly provided by Dr. Cushman.

Machines

ScanArray 3000 (GSI Lumonics, Watertown, CA), also provided by Dr. Cushman was used to scan the slides. PCR cyclers were PTC-100 made by MJ research, Inc. PCR cycler for slides was provided by Dr. Verchot, Department of Entomology and Plant Pathology. UV cross-linking was performed in a Stratalinker (Stratagene, La Jolla, CA). Vacuum oven was GP100 115 made by Savant, Farmingdale, NY. for the vacuum part and model 5831 made by Napco, Tualatin, OR. for the oven part. Speed Vac (sc110A) was made by Savant. Gel Doc 1000 was made by Bio-Rad, Hercules, CA. HL-4 rotor of a GLC-2 centrifuge was made by Kendoro laboratory products, Newtown, CT.

Templates

All templates were provided by Dr. d'Offay. BHV was propagated from clinical tissue samples in primary bovine fetal kidney cells and in Madin Darby bovine kidney cells (MDBK) [41]. Then, DNA was extracted from infected MDBK cells and purified by ethidium bromide-sodium iodide equilibrium gradient centrifugation[41]. DNA-containing

gradient fractions were dialyzed against TE buffer (10 mM Tris, 1 mM EDTA), ethanol precipitated, and resuspended in TE buffer[41].

To isolate BVDV, supernatant fluid from BVDV infected cells was mixed with guanidine isothiocyanate lysis buffer, sodium acetate, phenol, and chloroform[27]. Then, the mixture was centrifuged and the aqueous phase precipitated with isopropanol. The resultant precipitate was washed with ethanol and dissolved in water[27].

Primers

Pairs of primers are required for each amplification of viral nucleic acid by PCR. Table 1 lists primers used for each amplification in this project.

Table 1 primers

Virus	Primer	Primer sequence (5'-3')	Genome position
BHV-1	766 InBHV1 665	(T)CAACCTCGTCGTGTGCACCCTC (22 bp) TAGACCCCAGTTGTGATGAATGC (23 bp) TAGAGCTCGCGGCACTTGAGCG (22 bp)	63775-63796 63994-64015 64059-64080
BHV-2	4109 InBHV2 4110	(T)GCGGCGGCGGAGTCTGGCTTTGAGG (24 bp) TACCAGAAGTCCGACAAG (18bp) TCGCTGATGTTGTTCGGAGGGAGGTTGA (28 bp)	2416-2439 2569-2586 2810-2837
BRD and BVDV 1st amplification	P1 P2	TAACAAACATGGTTGGTGCAACTGGT (26 bp) (T)CTTACACAGACATATTTGCCTACGTTCCA (29 bp)	1424-1449 2221-2250
BRD 2nd amplification	TS1 P2	TATATTATTTGGAGACAGTGAATGTAGTAGCT (32 bp) (T)CTTACACAGACATATTTGCCTACGTTCCA (29 bp)	1684-1716 2221-2250
BVDV-2 2nd amplification	TS2 P2	TGGTTAGGGAAGCAATTAGG (20bp) (T)CTTACACAGACATATTTGCCTACGTTCCA (29 bp)	1802-1821 2221-2250
BVDV-1 2nd amplification	TS3 P2	TGGGGGTCACTTGTCGGAGG (20bp) (T)CTTACACAGACATATTTGCCTACGTTCCA (29 bp)	2027-2045 2221-2250

Note: (T) denotes an additional T added when 5' amino-linked oligonucleotides were made.

Two 22 bp primers, 665 and 766, were designed by Ely, R.W. et al. from a conserved region of published nucleotide sequences of BHV-1 thymidine kinase genes[5]. Sullivan, D. G. et al designed P1 and P2 to share maximum homology with BVDV-1, BVDV-2, and border disease virus (BDV). They designed type specific primers TS1, TS2, and TS3 to anneal specifically to BDV, BVDV-2, and BVDV-1 respectively[27]. InBHV1 and InBHV5 were designed by Kibenge F. S. B. et al[40]. Primer 4109, 4110, and InBHV2 were designed by Dr. d'Offay from the sequence of BHV-2 (unpublished).

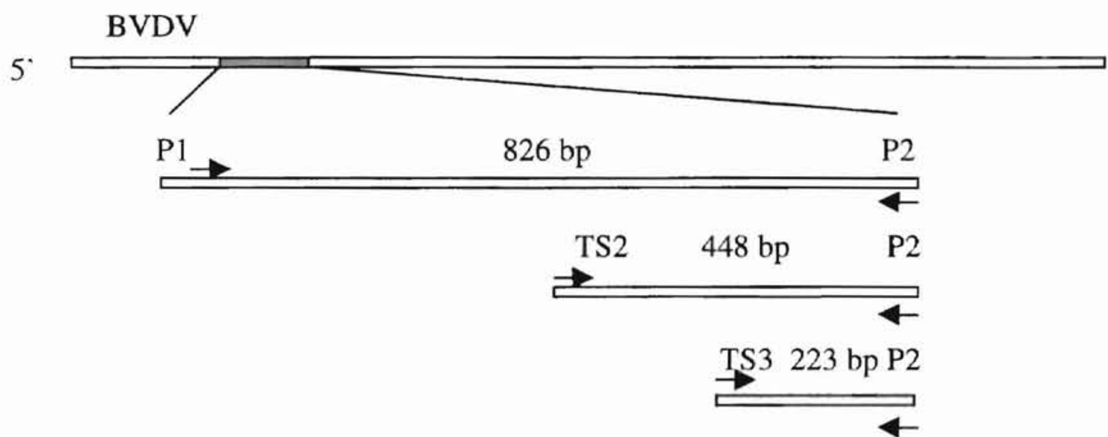

Fig. 1 Diagram of BVDV specific primers

Nested PCR for BVDV

BVDV amplification was performed by nested PCR. Since BVDV is an RNA-containing virus, reverse transcription (RT) is carried out first. Next, the first PCR was performed to amplify all types of BVDVs, and then the second (nested) PCR was done using the diluted first PCR product as template to amplify specific types of BVDVs.

Detection of soluble PCR products by agarose gel electrophoresis

Soluble PCR products were analyzed by agarose gel electrophoresis. 1.0 and 1.5 % agarose gels for electrophoresis contained 0.5 mg/ml ethidum bromide for detection of DNA. Samples loaded consisted of 5 μL PCR product, 4 μL stop reagent, and 10 μL ddH_2O. 100 bp ladder was used to determine product sizes. DNA was detected by Gel-Doc 1000.

Invariant conditions for PCR

Unless mentioned otherwise, I used the following conditions. The volume of PCR mixture was 50 μL total in most soluble PCR experiments but for the experiments using Cy5 the volume was 25 μL total.

Specific for BHV amplification

PCR mixture consisted of 2.5 u Taq DNA polymerase, 1 μL each of 1 mg/mL 665 and 766 primers (Table 1), 1x PCR buffer, 2 % DMSO, 1.5 mM $MgCl_2$, and 0.2 mM dNTP. PCR cycle was 94 °C for 3 minutes followed by 40 cycles of 94 °C, 68 °C, and 72 °C for 1 minute each, and then, 72 °C for 10 minutes.

Specific for BVDV amplification

First, reverse transcription (RT) was performed. The conditions for RT were 25 u MuLV reverse transcriptase, RNase Inhibitor 10 u, 1 μL of 1 mg/mL P2 primer, and 0.32 mM dNTP with total 10 μL. This mixture was heated to 42 °C for 15 minutes, and 99 °C for 5 minutes, then cooled to 4 °C for 5 minutes.

Next, the first PCR was performed. PCR mixture consisted of 10 μL of RT mixture, 2.5 u Taq DNA polymerase, 1 μL of 1 mg/mL P1 primer, 1x PCR buffer, 1.5 mM $MgCl_2$, and 0.2 mM dNTP. PCR cycle was 94 °C for 2 minutes followed by 35 cycles of 94 °C, 55 °C, and 72 °C for 1 minute each, and then, 72 °C for 10 minutes.

Then, the second PCR (nested PCR) was performed. PCR mixture consisted of 1 μL of 1:500 diluted 1st PCR mixture, 2.5 u Taq DNA polymerase, 1 μL each of 1 mg/mL TS1, TS2, TS3, and P2 primer, 1x PCR buffer, 1.5 mM $MgCl_2$, and 0.2 mM dNTP. PCR cycle was 94 °C for 2 minutes followed by 30 cycles of 94 °C, 50 °C, and 72 °C for 1 minute each, and then, 72 °C for 10 minutes.

ViSA card method and ViSH chip method

ViSA card method used PCR amplification on the primer-attached media, such as nylon membranes or glass slide. One set of primers needed for PCR amplification are mixed and spotted on the media. At the denaturing temperature (about 94 °C) of PCR cycle, templates are denatured and separated into two strands. Then, at the annealing temperature (about 50 °C), templates attach onto primers complementary to the template. Next, at the extension temperature (72 °C), PCR products, which are complementary to templates, are made by polymerase activity. By repeating this cycle, primers, attached on the media, change into PCR products.

ViSH chip method uses hybridization on the primer-attached media. Each primer, which is complementary to the PCR products, is spotted separately on the media. PCR products are hybridized with complementary primers onto the media.

Attachment of primers on a nylon membrane

0.2 µL of 10 mg/mL mixed primers were spotted on a nylon membrane and treated with 120.0 mJ UV by UV crosslinker. (Alternatively a vacuum oven was used with for 4 hours at 80 °C). Then, the nylon membrane was washed with 0.2% SDS and 0.2x SSC solution for 10 minutes twice, and with 0.2x SSC solution for 10 minutes four times. This nylon membrane was used for PCR immediately after the washing steps.

Attachment of primers on a silylated glass slide

0.5 µL of 20x SSC was added to 4.50 µL of mixed primer, and the mixture was spotted on a silylated glass slide. This slide was dehydrated for at least 4 hours, then washed with 0.2% SDS solution for 1 minute and with ddH_2O for 1 minute twice. Next, it was incubated in sodium borohydride solution for 5 minutes, and washed with 0.2% SDS solution for 1 minute and with ddH_2O for 1 minute twice. This slide was kept in the dark and room temperature until used.

DIG or Cy labeled PCR product

DIG labeled product was produced from 0.7 mM DIG-dUTP under the PCR conditions mentioned above. Cy labeled product was produced from 1 mM Cy3 dUTP or Cy5 dCTP in the PCR conditions mentioned above.

A detection method for DIG

Nylon membranes or glass slides were washed in 2x SSC and 0.2% SDS solution for 5 minutes twice and in 0.2x SSC solution for 15 minutes twice. Then, they were neutralized in maleic buffer for 5 minutes and in 1% blocking buffer for 30 minutes.

Next, they were incubated in 1:5000 Anti-DIG-AP conjugate in 1% blocking buffer for 30 minutes, and washed in maleic buffer for 15 minutes twice and in AP buffer for 2 minutes. At last, binding of conjugate was detected by X-ray film using chemiluminescent substrate according to manufacturer's instructions.

PCR product attachment and detection method for Cy3 and Cy5

PCR in Frame-Seal

Two Frame-Seals were applied to make spaces to apply the PCR mixture on a primer-attached slide, and this slide was kept at room temperature over night. Then, a 25 µL of PCR mixture, which was mentioned above but without primers, was applied to the Frame-Seal chambers and sealed by a plastic sheet supplied with Frame-Seal. Next, the slide was placed on a thermal cycler.

Hybridization in Frame-Seal

One or two Frame-Seals were applied on a primer-attached slide, and this slide was kept at room temperature overnight. Then, hybridization solution consisting of one volume of PCR product and four volumes of UniHyb hybridization solution was applied on this slide, covered by a Frame-Seal sheet and kept in an oven at 50 ºC or 68 ºC for 4 hours.

Hybridization in the hybridization cassette

1.25 µL of hybridization solution (one volume of PCR product and four volumes of UniHyb hybridization solution) per 1 cm^2 of glass cover area was applied on glass cover and this glass cover was placed on the primer-attached slide in a Hybridization

cassette with 5 μL hybridization solution for humidity. This cassette was placed in a heated water bath for 4 hours.

Detection method for fluorescence

Glass slides were washed in 2xSSC and 0.2% SDS solution for 5 minutes and in 0.05xSSC solution for 1 minute. Then, they were dried by spinning at 500 to 1000 rpm in a HL-4 rotor of a GLC-2 centrifuge. Presence of Cy3 and Cy5 was determined by Scanarray 3000.

PCR select II and III

PCR select-II or III was used to remove unincorporated dNTPs, primers, and primer-dimers. PCR select-II was used to remove up to a 100 bp product, and PCR select-III was used to remove up to a 300 bp product. 5 μL of PCR product was taken from tube and diluted to 50 μL with ddH_2O. PCR select-II or III column was inverted several times to resuspend gel, and centrifuged at 1000xg for 1 minute using a HL-4 rotor of a GLC-2 centrifuge. The collection tube was emptied and the column was centrifuged at 1000 xg for 5 minutes. Then, the collection tube was discarded, 50 μL of diluted PCR product was applied into the column, and centrifuged at 1000xg for 3 minutes. The collected buffer was placed in speed vac and centrifuged for 2 hours. The dried sample was mixed with 5 μL ddH_2O.

Oligonucleotide (primer) detection by YOYO-1

1 μL of 1:1000 dilution of YOYO-1 in TAE buffer (4 mM Tris-acetate, 0.1 mM EDTA, pH 8.2) were applied to the nylon membrane on which primers were

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immobilized. One hour later, the nylon membrane was washed with water briefly and dried. The hybrids of YOYO-1s and primers fluoresce yellow under UV lamp.

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Results and Discussion

Detection of specific viruses by soluble PCR of a part of the virus genome and analysis of the result of amplification by agarose gel electrophoresis is well established. Thus, we started optimizing the conditions for soluble PCR for each single BRD virus. This optimization process was necessary to perform multiplex PCR, which amplifies and detects more than one virus, since each amplification had a different optimum range of annealing temperatures, Mg^{++} concentrations and other conditions. It was also important to use these data to determine conditions compatible with both amplifications for use as starting points for the PCR conditions of ViSA cards.

Soluble PCR results

First, BHV-1 amplification was performed by the soluble PCR. PCR cycle was 94 °C for 3 minutes followed by 40 cycles of 94 °C, 68 °C, and 72 °C for 2 minutes each, and then, 72 °C for 10 minutes (72 °C for 2 minutes instead of 1 minute in invariant conditions for BHV-1 amplification described in Materials and Methods). At 10 % DMSO, BHV-1 amplification was detected as 302 bp DNA fragments at 1.0 and 1.5 mM $MgCl_2$ but not at 0.5 and 2.0 mM. Next, DMSO volume ratios 0, 2, 4, 6, 8, and 10 % were tested with 5.0 u Taq DNA polymerase. Expected size DNAs were identified on the agarose gel only from 2, 4, and 6 % DMSO PCR products. All PCR products, including no-template control product, showed unexpected fragments of less than 100 bp size after gel electrophoresis. These fragments, often refereed to as primer-artifact or primer-dimer[36], appeared in most PCR reactions for BHV and BVDV. The primer-dimer or

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primer-artifact is caused from self-annealing or dimerization of primer 665 and 766 in BHV-1 amplification.

Multiplex PCR, in which more than one amplification occurs simultaneously in one tube, requires one set of conditions that allows all of the desired amplifications to occur. Thus, I tested whether BVDV was amplified in the first PCR at 1.5 mM $MgCl_2$, a concentration at which BHV was amplified (2.0 mM $MgCl_2$ is a designated concentration for this amplification). First, BVDV-1 first amplification was tested at 1.5 and 2.0 mM $MgCl_2$. Although 2.0 mM $MgCl_2$ produced a larger amount of the expected size PCR product, 1.5 mM $MgCl_2$ was enough to identify BVDV-1 amplification.

Also, BVDV-2 amplification was tested at 1.5 mM $MgCl_2$ and obtained after second amplification with 1.5 mM $MgCl_2$. Therefore, both BHV and BVDV (including first and second amplification of BVDV) can be amplified at 1.5 mM $MgCl_2$.

Next, I tried to amplify each virus in the same PCR conditions. BHV-1, BVDV-1, and BVDV-2 were amplified in the first RT-PCR conditions for BVDV, whose annealing temperature was 55 °C, (described in Materials and Methods) with 10 % DMSO. All tubes contained primers for BVH-1 (665 and 766) and first PCR of BVDV (P1 and P2).

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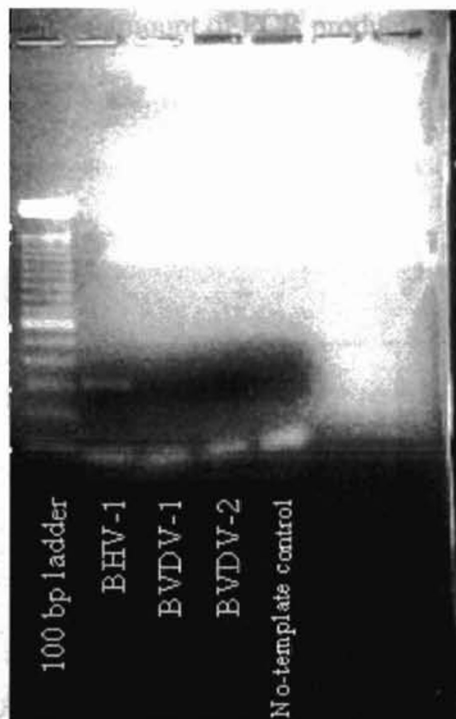


Fig. 2-1 Gel electrophoresis analysis of first PCR amplification products formed under identical conditions from BHV-1, BVDV-1, and BVDV-2 templates

From left, lane 1: 100 bp ladder. Lane 2: BHV-1. Lane 3: BVDV-1 template. Lane 4: BVDV-2 template. Lane 5: no-template control.

Fig. 2-1 shows first amplification of nested PCR results. In this experiment, a tube that contained BHV-1 as a template showed a light band of the expected BHV-1 PCR fragment size (302 bp). The faintness of the band was probably due to a difference in optimum annealing temperatures between BHV-1 and BVDV-1 amplifications. BHV-1 amplification was optimal at 68 °C as an annealing temperature, but this experiment used 55 °C as an annealing temperature since BVDV amplifications were optimal at 55 °C for a first amplification. The tubes containing BVDV-1 and BVDV-2 did not show any products in agarose electrophoresis after the first PCR. In most PCR, first amplification

of BVDV did not produce visible amount of PCR products and PCR products were seen after nested PCR.

Fig. 2-2 Gel electrophoresis analysis of second (nested) PCR amplification products formed under identical conditions from BHV-1, BVDV-1, and BVDV-2 templates

From left, lane 1: 100 bp ladder. Lane 2: BHV-1 product from first PCR (Fig. 2-1). Lane 3: BVDV-1 product from first PCR. Lane 4: BVDV-2 product from first PCR. Lane 5: no-template control product from first PCR. Lane 6: no-template control (H_2O).

In Fig. 2-2, the second (nested) PCR results, PCR conditions for BVDV were used with 1:500 dilution products of the first PCR as template. Both a tube with BVDV-1 template and a tube with BVDV-2 template showed expected size products (224 bp and 448 bp, respectively) after agarose gel electrophoresis. This second PCR did not contain

DMSO and contained primers only for BVDV second PCR (P2, TS1, TS2, and TS3). Thus, no amplification of BHV-1 was expected since there are no BHV-1 specific primers, and no BHV-1 product was seen in the gel shown in Fig. 2-2.

Since I succeeded in single virus amplification of BHV-1, BVDV-1, and BVDV-2 in the same PCR conditions and reagents, I tested whether the individual reactions could be multiplexed. BRD is often caused by two or more viruses infected simultaneously. When two or more viruses and appropriate primers were mixed in one tube, no or only one virus was amplified and detected with MJ Research's thermal cycler while multiplex PCR was successful with a thermal cycler made by Perkin Elmer. One possibly important difference between the two cycler is that for the latter a few drops of oil are added to PCR tubes to avoid evaporation, while in the MJ Research cycler no such addition is necessary. In this experiment, RT was performed in RT conditions mentioned in "Materials and Methods" with BHV-1 and BVDV-2 as templates. I prepared three tubes; one BVDV-2 only template tube with only P1 primer (a BVDV primer), one BHV-1 only template tube with 766 and 665 primers (BHV-1 primers), and one mixture of BVDV-2 and BHV-1 templates tube with P1, 766, and 655 primers (a BVDV primer and BHV primers). First RT-PCR was performed in BVDV RT-PCR conditions with a BVDV primer, P2, used in RT, with 2 % DMSO. Primers P1, 766, and 665 were added after the RT reaction.

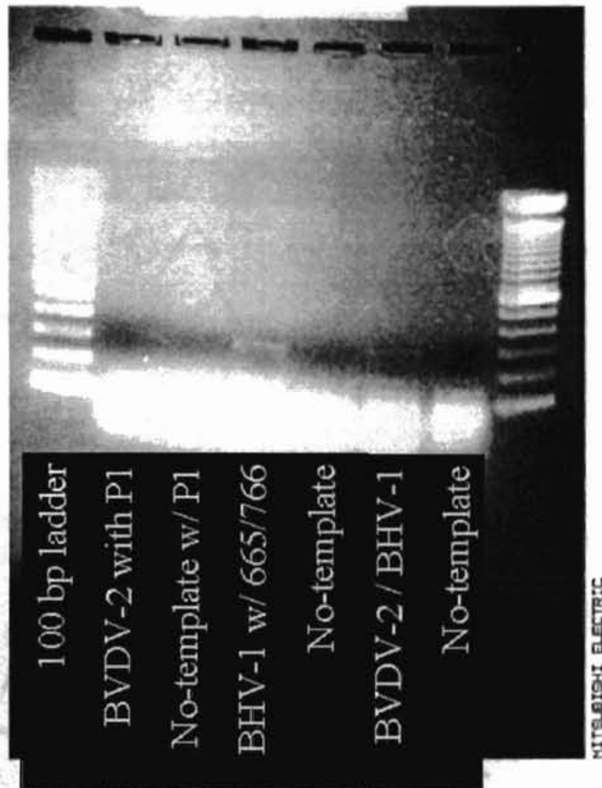


Fig. 3-1 Gel electrophoresis analysis of first PCR amplification products from mixed BHV-1 and BVDV-2 templates

From left, lane 1: 100 bp ladder. Lane 2: BVDV-2 template with P1 primer. Lane 3: no-template control with P1 primer. Lane 4: BHV-1 template with 665 and 766 primers. Lane 5: no-template control with 665 and 766 primers. Lane 6: mixed templates of BHV-1 and BVDV-2 with P1, 665, and 766 primers. Lane 7: no-template control with P1, 665, and 766 primers.

Fig. 3-1 shows results after the first amplification of nested PCR. An expected size BHV-1 product (302 bp) showed up weakly on an agarose gel in the tube that contained a mixture of BHV-1 and BVDV-2 templates while the BHV-1 only tube produced a strong 300+bp band and the BVDV-2 only tube did not produce any detectable products. Second PCR was performed in BVDV second PCR conditions with

P2, TS1, TS2, and TS3 primers in all tubes and the 1:500 dilution products of the first PCR as templates.

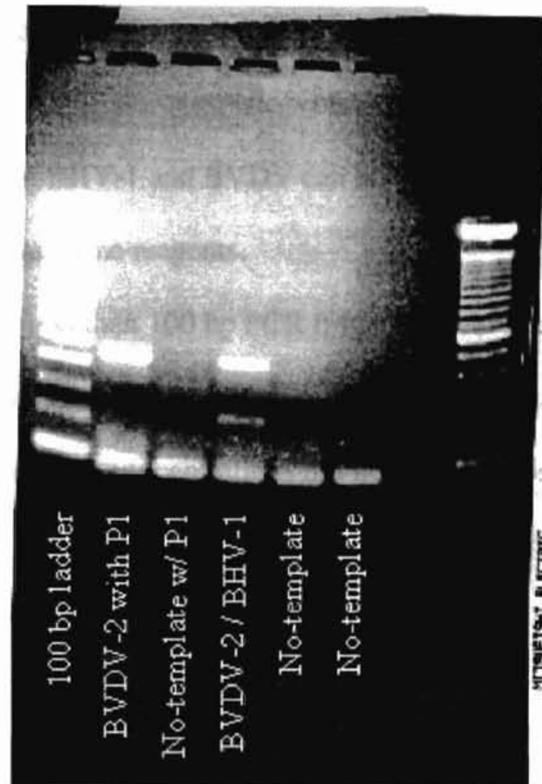


Fig. 3-2 Gel electrophoresis analysis of second (nested) PCR amplification products from mixed BHV-1 and BVDV-2 templates

From left, lane 1: 100 bp ladder. Lane 2: BVDV-2 template from Fig. 3-1 lane 2. Lane 3: no-template control from Fig. 3-1 lane 3. Lane 4: mixed templates of BHV-1 and BVDV-2 from Fig. 3-1 lane 6. Lane 5: no-template control from Fig. 3-1 lane 7. Lane 6: no-template control, H_2O.

Second (nested) PCR, Fig. 3-2, produced two products in the tube that contained a mixture of BHV-1 and BVDV-2 templates. One was the expected size BVDV-2 product

(448 bp). Another was of an unexpected size (about 200+bp). The BVDV-2 only tube produced the expected size BVDV-2 product and the BHV-1 only tube did not produce any detectable products. Since 200+bp band was produced only in amplifications with the mixture of BVDV-2 and BHV-1 templates, this product was probably an artifact due to interaction of BVDV primers or template with BHV-1. This experiment and previous experiments show that BHV-1 and BVDV can be diagnosed in the same nested PCR conditions and with the same reagents.

In most PCR, less than 100 bp PCR fragments were seen on the gel. Those were primer-dimers or primer-artifacts made from primer-primer interactions. Changing of Mg^{++}, primer, and template concentrations did not reduce those products.

Nylon membrane supported insoluble PCR (ViSA card)

Multiplex PCR produced an artifact and lower amplification in the soluble PCR of the previous section because primers will interact with templates and PCR products in unintended ways if many pairs of primers are mixed. To solve this problem, I started the ViSA card. It is not necessary to mix many primers on the ViSA card since each pair of primers were spotted separately and each virus amplification occurs only at a primer spot at which both primers are complementary to the viral nucleic acid, so that we expected more specific simultaneous amplification for many viruses. (See "Methods and Materials" section for detail.)

Either UV crosslinking or a vacuum oven was used to attach primers on nylon membranes. These membranes, when used in PCR, could detect the presence of viral nucleic acid (Fig. 4-1 and 4-2).

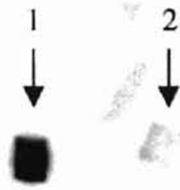


Fig. 4-1 ViSA card detection with BVDV-2 template

Autoradiographic image of nylon membrane containing TS2 and P2 primers and amplified in the presence (1) or absence (2) of BVDV-2 template at 0 % DMSO.

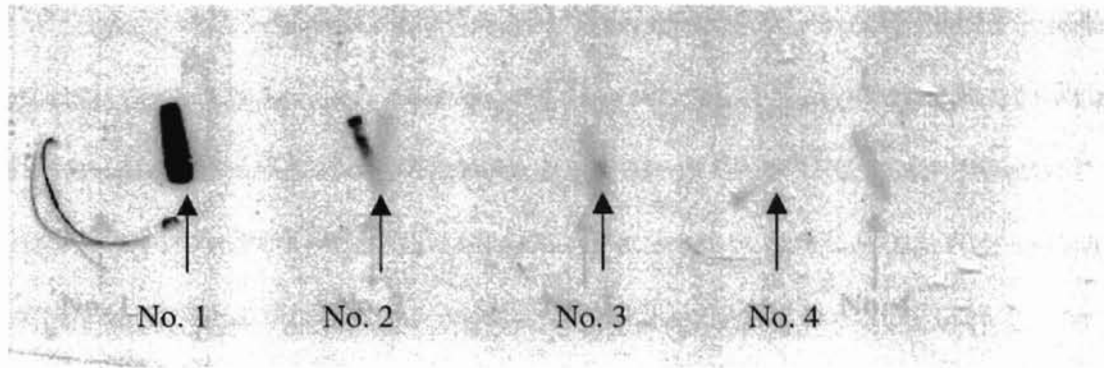
Fig. 4-1 shows a ViSA card that detected BVDV-2. In this experiment, 1µL of 1mg/ml primers for BVDV-2 second PCR was spotted on the nylon membrane and the membrane baked for 4 hours in a vacuum oven. These nylon membranes were inserted directly, without washing, in 0.5 mL tubes and BVDV second PCR mixture was added into the tubes. One tube also received the diluted PCR product of BVDV-2 amplification. Chemiluminescence was used for the detection (See materials and methods). The card reacted with BVDV-2 template produced a definite spot on X-ray film after 15 minutes of exposure to the film while the control card reacted without BVDV-2 template produced a very faint spot. No-template controls often produced a spot. This indicates that DIG can attach on the membrane directly but not with high affinity.

Fig. 4-2 ViSA card detection with BHV-1 template

Autoradiographic image of nylon membrane containing 665 and 766 primers and amplified in the presence (1) or absence (2) of BHV-1 template at 2 % DMSO.

Fig. 4-2 shows a ViSA card produced with UV crosslinked primers. The ViSA card with which BHV-1 was used as a template clearly produced a definite spot in X-ray film after 3 minutes exposure. The card incubated without BHV-1 template did not produce a spot. A washing step (see Materials and Methods) before PCR was needed to obtain this result. Without this washing step, the negative control (without template) showed up faintly, and sometimes it was difficult to identify a difference between positive control (with template) and negative control.

BHV amplification in soluble PCR required at least 2 % DMSO, and BVDV was amplified with up to 10 % DMSO in soluble PCR. However, DMSO inhibited BHV-2 amplification in nylon membrane supported ViSA card.



ViSA card	No. 1	No.2	No. 3	No. 4
% DMSO	0%	1%	2%	0%
template	+	+	+	-

Fig. 5 BHV-2 detection on nylon membrane at various DMSO concentrations

Autoradiographic image of nylon membrane containing 4109 and 4110 primers and amplified in the presence or absence of BHV-2 template with various % DMSO.

Fig. 5 shows the effect of DMSO in BHV-2 amplification. 0.2 μL of 1 mg/ml mixed primers, 4109 and 4110, were spotted on nylon membrane. The PCR experiment was performed with 0, 1, and 2 % DMSO in each tube. ViSA card without DMSO produced a very dark spot, while that at 1 % DMSO produced a faint one and that at 2 % DMSO produced almost no spot on the X-ray film after 30 minutes exposure. The no-template control produced a spot as weak as 2 % DMSO did. Thus, BHV-2 amplification on the nylon membrane showed inhibition by DMSO.

No. 1 No. 2 No. 3 No. 4

ViSA card	No. 1	No.2	No. 3	No. 4
% DMSO	0%	4%	2%	0%
template	-	+	+	+

Fig. 6 BHV-1 detection on nylon membrane at various DMSO concentrations

Autoradiographic image of nylon membrane containing 665 and 766 primers and amplified in the presence or absence of BHV-1 template with various % DMSO.

Fig. 6 shows the effect of DMSO in BHV-1 amplification. 0.2 μL Mixed primers of 1 mg/ml 665 and 766 were spotted on nylon membrane. When BHV-1 was used for a template, all ViSA cards with 0, 2, and 4 % DMSO produced a spot. When no-template control was added, no spot was seen. Immobilized BHV-1 amplification showed no inhibition. Since both BHV-1 and BHV-2 show amplification without DMSO and BHV-2 shows inhibition by DMSO, it is not desirable to use DMSO in ViSA card. Moreover, Fig. 4-1 showed the detection of BVDV-2 without DMSO. Thus, DMSO is not required for the detection of BHV and BVDV on the ViSA card.

Spotting of 0.2 μL of primer solution made only a small spot on the ViSA card and did not cover all the nylon membrane even when the membrane was small. As mentioned in "Material and Methods" section, PCR products are extended from primers

and all primers are attached on the ViSA card. Thus, no PCR products should be outside of the spots made when primers were spotted. However, most X-ray films showed that a spot covered all the membrane. The spot on X-ray film is due to DIG (from DIG-dUTP used for labeling the PCR product.) Anti-DIG-AP attaches to DIG and then Anti-DIG-AP causes a chemiluminescent reaction with other substrates. Possible explanations for the DIG reaction product covering all the membrane are: the spotted primers diffuse from the place of spotting to cover the whole membrane; somehow DIG became attached not only on the primer but also on the nylon membrane directly; anti-DIG-AP attached on something else besides DIG; and PCR products were released from the membrane and reattached at random locations. To test whether primers are removed from the nylon membrane to produce a spot outside of where 1 mg/ml primers are spotted I used YOYO-1 to check the primer position after spotting. YOYO-1 reaction revealed a spot as small as the place wetted after primer solution was spotted, even after washing steps. This confirmed that the place primer attached to was the same as the place wetted during primer spotting. However, 0.1 mg/ml primer on nylon membrane was not detected by YOYO-1, and I could not test whether primer was removed during the PCR cycle since YOYO-1 detects the PCR products as well as the primers and PCR products are accumulated during PCR cycles. At this point, I am not sure that primers stay at the same position during PCR cycle or only small amounts of primers (not detectable amount by YOYO-1) are detached and reattached around area where primers were originally attached. The second two possibilities are probably impossible since most no-template controls show nothing on X-ray film. If DIG or Anti-DIG can attach on nylon membrane directly, there should have been spots on the no-template control, too. The remaining possibility is that PCR product becomes attached on the nylon membrane directly.

Thus, I tested whether PCR product can attach on nylon membrane without primers. Soluble PCR was performed with BHV-1. This PCR mixture contained primers, nylon membrane, and DIG-dUTP in the solution. The nylon membrane was removed from solution after the PCR cycles, washed, and allowed to expose X-ray film as ViSA card. A spot, which covered all the membrane, was seen without immobilized primer on the nylon membrane. No-template control had no spot on the ViSA card. This indicated PCR products can attach on the nylon membrane during PCR cycles and these bonds are strong enough not to wash away during washing steps.

Although the explanation for the spot covering all the membrane is unclear, the background problem (a dark place on X-ray film around where the primer should be) could be controlled by changing the primer and template concentrations (Fig. 7 and 8).

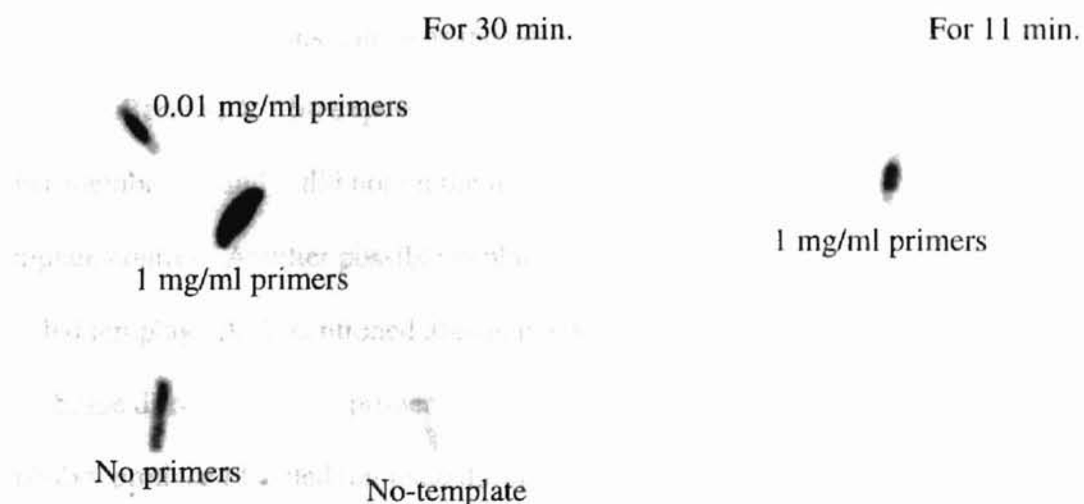


Fig. 7 BHV-2 detection by ViSA card using different primer concentrations

Autoradiographic image of nylon membrane containing 4109 and 4110 primers and amplified in the presence or absence of BHV-2 template with various primer concentrations. The picture on the left shows the image after 30 minutes exposure. Right one shows the image after11 minutes exposure.

Fig. 7 shows the effect of dilution of primers on BHV-2 amplification. The first membrane (Fig. 7, no-primers) had no primer spotted and was submitted to amplification with non-diluted BHV-2 template. The second membrane (Fig. 7, 1 mg/ml primers), which received 1 mg/ml primers (4109 and 4110), and the third membrane (Fig. 7, 0.01 mg/ml primers), which received 0.01 mg/ml primers, were amplified with 1:10 diluted BHV-2 template. The fourth membrane had 0.01 mg/ml primers and was subjected to PCR without BHV-2 template (Fig. 7, no-template). After 11 minutes of exposure to X-ray film, the membrane with 1 mg/ml primers produced a spot in the center of ViSA card and other ViSA cards did not produce a clear image. After 30 minutes, the membrane with 0.01 mg/ml primers produced a spot in the center of ViSA card, too. These spots (1 mg/ml and 0.01 mg/ml) were in places primers were spotted. No-template control weakly darkened the film all over the membranes. No-primer control clearly showed up and was weaker than membrane with 0.01 mg/ml primer. This was an unexpected result which has two possible explanations. One was the unbalanced distribution of chemiluminescent substrate. Because X-film exposure was long (30 minutes), the substrate dried up on the other membranes and it did not on the no-primer control. Thus, it became darker than no-template control. Another possible explanation is due to non-specific attachment of labeled template. As I mentioned above, labeled PCR product could attach on the membrane directly. The no-primer control can not produce the PCR product but it probably produced labeled template during the PCR cycles. Then, the labeled template attached on the membrane directly.

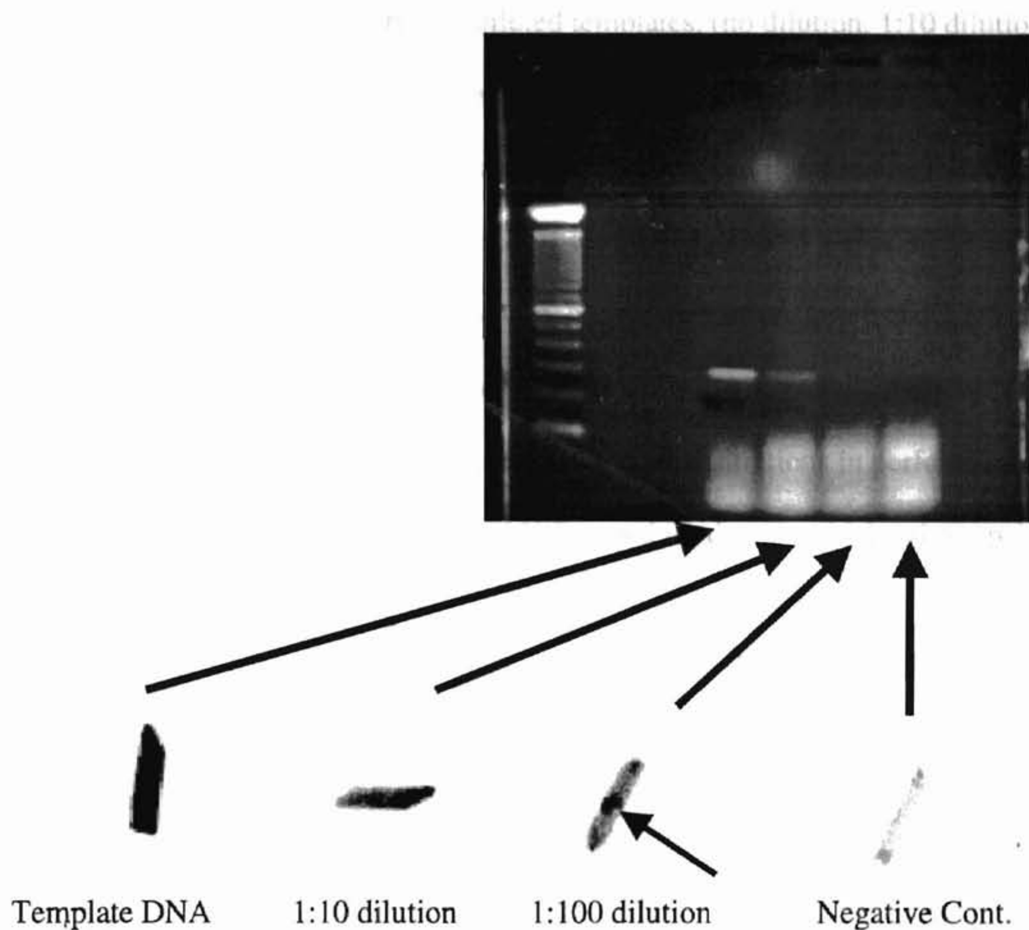


Fig. 8 Detection of diluted BHV-1

(Top) Gel electrophoresis result of diluted BHV-1 PCR results. From left, lane1: 100 bp ladder. Lane 2: original BHV-1. Lane 3: 1:10 diluted BHV-1. Lane 4: 1:100 diluted BHV-1. Lane 5: no-template control.

(Bottom) Autoradiographic image of nylon membrane containing 665 and 766 primers and amplified in the presence or absence of BHV-1 template with various template concentrations. Arrow marks position of a darker spot in the center of strip.

Nylon membrane supported ViSA cards showed a higher sensitivity than soluble PCR (Fig. 8). Reactions used BHV-1 for a template. 0.1 μL 1 mg/ml primers (665 and 766) were spotted on nylon membrane and UV-crosslinked. Soluble PCR was tested under the same PCR conditions except for addition of primers. When soluble and ViSA

card PCR were performed with a series of diluted templates, (no dilution, 1:10 dilution, and 1:100 dilution), all dilutions produced spots in ViSA cards but soluble PCR produced a detectable band in agarose gel only at no and 1:10 dilution, but it did not at 1:100 dilution. On the other hand, 1:100 diluted BHV-1 template produced a spot in the area where the primers were actually spotted while higher concentrations produced a more disseminated reaction (Fig. 8 top). Thus, these results showed higher sensitivity of ViSA card and the importance to control primer and template concentrations in performing multiplex PCR on the nylon membrane based ViSA card to identify which primer pairs reacted to the viruses.

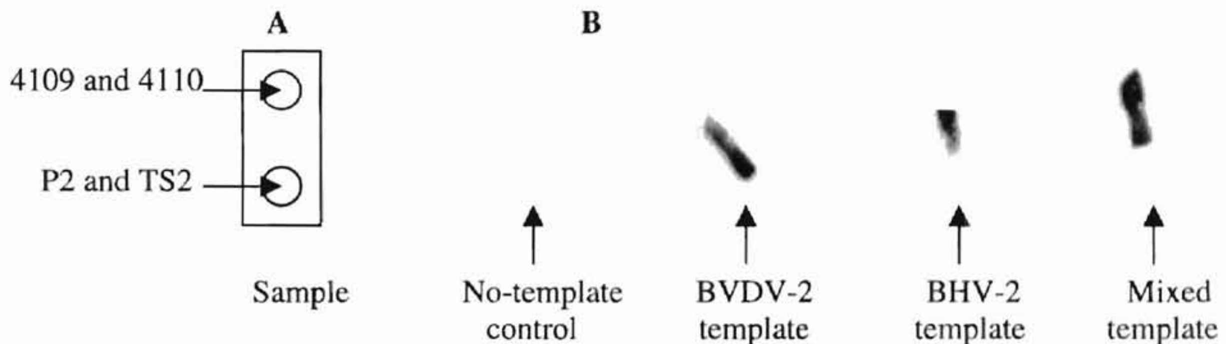


Fig. 9 Multiplex immobilized PCR on ViSA card (nylon membrane)

A is a diagram of placement of primer pairs on the card. B is autoradiographic image of nylon membrane containing primers as described in A and amplified in the presence or absence of BHV-2 and BVDV-2 template.

Fig. 9 shows a multiplex PCR result with BHV-2 and BVDV-2. On the bottom half of nylon membranes 1 mg/ml mixed primers for BVDV-2 (P2 and TS2) were spotted

and 1 mg/ml primers for BHV-2 (4109 and 4110) were spotted on the top half. Each PCR tube contained one ViSA card and the PCR mixture with DIG-dUTP. One tube contained two templates (BHV-2 and BVDV-2). A pair of tubes contained one or the other template. The final tube contained no-template. BVDV-2 template was a 1:500 dilution of first amplification product in soluble PCR. After 7 minutes of exposure to X-ray film, ViSA card with two templates produced two spots, one for each primer pair spotted, while those with one template produced one spot at the appropriate place and the one with no-template did not produce any spots.

Silylated glass plate supported ViSA card and ViSH chip results

A ViSA card is a nylon membrane or glass plate on which primers are immobilized and PCR is performed. Since all PCR primers are on the card, all PCR products will be attached on a ViSA card. A ViSH chip is a glass plate on which oligonucleotides are immobilized to serve as hybridization targets for PCR. PCR products for ViSH chip are prepared separately in soluble PCR.

ViSA card results

First, DIG-anti DIG detection was used with silylated glass plate ViSA cards. Fig. 10 shows two slides with 0.5 μL of 10 mg/ml primers, P2 and TS2, that were amplified with and without 1:500 dilution of first BVDV-2 PCR products as template. The card with the template produced weak but darker spots than the one without template on the X-ray film. This result suggested that amplification of BVDV-2 occurred. However, it was not easy to identify the difference between with and without the template and it took too long to obtain a result since the amplification with DIG was weak and required about

6 hours of exposure to X-ray film. Since DIG label did not inhibit the amplification on the soluble PCR and nylon membrane supported ViSA card, the detection of amplification must have been poor due to the use of the silylated glass slide.

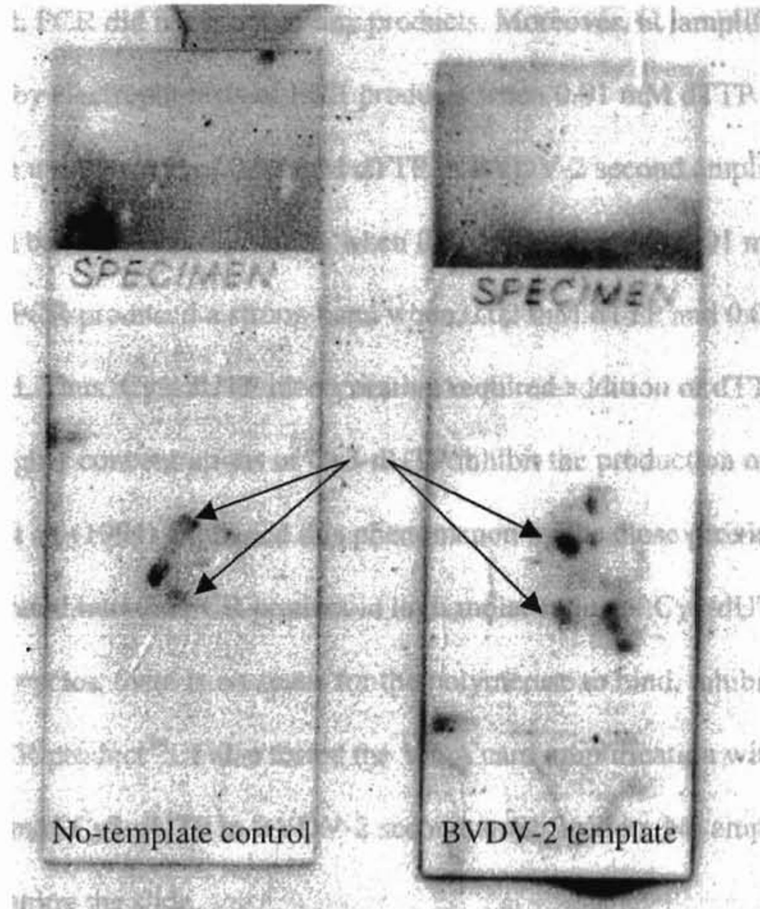


Fig. 10 Detection of BVDV-2 by ViSA card (glass slide)

Autoradiographic image of glass slide containing P2 and TS2 primers and amplified in the presence or absence of BVDV-2 template. Arrow marks position of primers were immobilized.

However, there was no better way to detect the tiny amount of DIG labeled PCR product. Thus, I decided to try Cy dyes (Cy3-dUTP and Cy5-dCTP) with the ScanArray 3000. Before the ViSA card experiments with Cy dye were started, I tested whether amplification with Cy3-dUTP required conditions in the soluble PCR which differed

from DIG labeled or other soluble PCR conditions. The first BVDV-2 PCR product was diluted and amplified with Cy3-dUTP under second amplification conditions of BVDV-2 including up to 4 % DMSO. When soluble PCR conditions but with Cy3-dUTP instead of dTTP were used, PCR did not produce any products. Moreover, no amplification detected in soluble PCR by electrophoresis of PCR products when 0.01 mM dTTP and 0.02 mM Cy3-dUTP were used instead of 0.02 mM dTTP in BVDV-2 second amplification. PCR produced a faint band on electrophoresis when 0.01 mM dTTP and 0.01 mM Cy3-dUTP were used, and PCR produced a strong band when 0.02 mM dTTP and 0.01 mM Cy3-dUTP were used. Thus, Cy3-dUTP incorporation required addition of dTTP. This result indicates that higher concentrations of Cy3-dUTP inhibit the production of PCR product.

Zhu, Z et al. (1994) explained this phenomenon by the close proximity of Cy3-dUTPs incorporated into the PCR product in high molar ratios of Cy3-dUTP to dTTP, so that, in the next cycles, there is no space for the polymerase to bind, inhibiting the formation of PCR product[34]. I also tested the ViSA card amplification with 0.02 mM dTTP and 0.01 mM Cy3-dUTP in BVDV-2 second amplification. No amplification was detected by scanning the slide.

According to Voss, H. et al. (1997), Cy5 dye is incorporated less with AmpliTaq than with Klentaq[35]. There were three possibilities; one was that no amplification occurred on the silylated glass plate supported ViSA card with Cy3 label. Another was that PCR product was produced on the ViSA card but it could not produce a spot by scanning since not enough Cy3 dyes were incorporated into PCR product. A third possibility was that product was released from the slide during high temperature in PCR. If the second one were right, Klentaq would be more likely to produce a spot detectable by scanning. Although Klentaq is derived from Taq polymerase, Klentaq has different

optimal conditions from Taq polymerase. BVDV amplification with Taq polymerase required 1.5 to 2.0 mM $MgCl_2$ and 1xPCR buffer, and BHV amplification required 1.0 to 1.5 mM $MgCl_2$ and 1xPCR buffer. However, the amplification with Klentaq was better with no $MgCl_2$ added and 1xPC2 buffer (contained a final concentration of 3.5 mM $MgCl_2$) than any other $MgCl_2$ concentration and buffer.

Glass slides were scanned after the primer attachment process and washing steps to confirm the attachment of primers on the silylated glass plate. In this experiment, 5' aminolink primer was spotted on the center of a silylated glass plate and non-aminolink primer was spotted on the bottom half of the same silylated glass plate. Under the highest sensitivity setting of ScanArray (Laser power at 100 % and PMT at 100 %), aminolink primer produced a spot on the glass plate while no-aminolink primer did not. Aminolink primer itself does not emit fluorescence, and the spot was due to the rough surface of the glass plate in the area the primer was spotted. This indicates something on the glass plate makes the surface rough and this something must be the aminolink primer since no-aminolink primer did not produced a spot under the same conditions.

Next, I attempted PCR amplification on the glass-plate supported ViSA card with Cy5 and Klentaq. I have never achieved detectable PCR amplification on the silylated glass plate. Thus, second possibility (Cy3 incorporation) was not the reason why no amplification was detected on the glass slide.

ViSH chip results

When Cy5 labeled PCR product was incubated with a primer-attached glass slide (ViSH chip) at 50 °C for overnight, it produced a high background in the inside of the frame-seal chamber. A similar high background resulted from PCR on the ViSA card.

Background problem was solved by applying 1 % blocking reagent on the slide before use.

A ViSH chip containing spots of P2 and TS2 primers was treated with 1 % blocking reagent after primer was immobilized on the slide and was washed with 0.2 % SDS solution for 1 minute, and then, with ddH_2O for 1 minute twice. The upper part of the ViSH chip was incubated at 50 °C overnight with Cy5-labeled PCR product formed by soluble PCR without template, and the lower part was incubated with Cy5-labeled PCR product made with BVDV-2 template. Fig. 11-1, scanned at 60 % laser power and 50 % PMT, shows the spot in the lower part was stronger than the one in the upper part. Thus, blocking reagent avoided the attachment of Cy5 or PCR product directly to the glass slide and PCR product was attached only to the primer. The same result was acquired from ViSH chip, which was treated with 1 % blocking reagent immediately after 4 hours of rehydration and washing steps. PCR was also performed with blocking reagent treated ViSA card, but no PCR product was produced on the ViSA card.

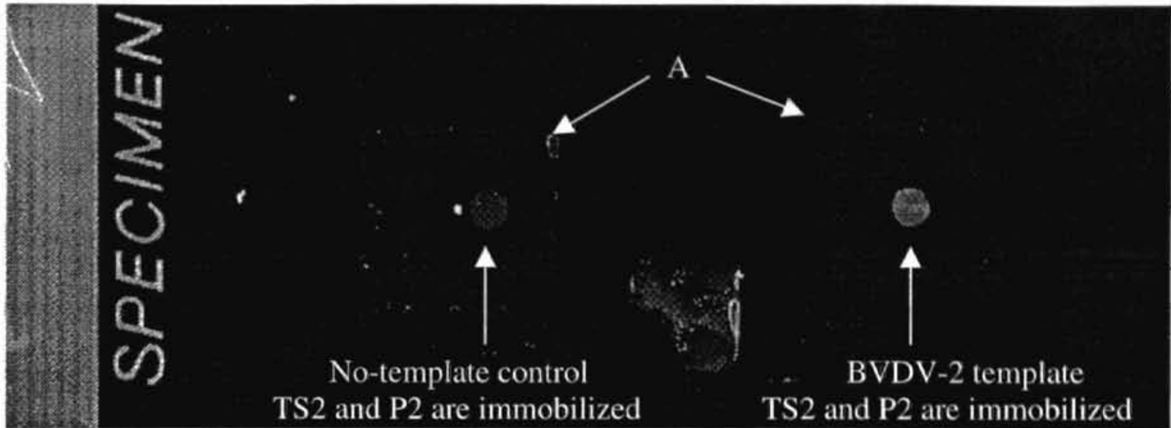


Fig. 11-1 BVDV-2 detection on ViSH chip without UniHyb

Scanned image of glass slide containing P2 and TS2 primers and hybridized in the presence or absence of Cy5 labeled BVDV-2 product using hybridization reagent. Arrow marks "A" faint white area which indicates the area covered by the PCR solution and the Frame-Seal.

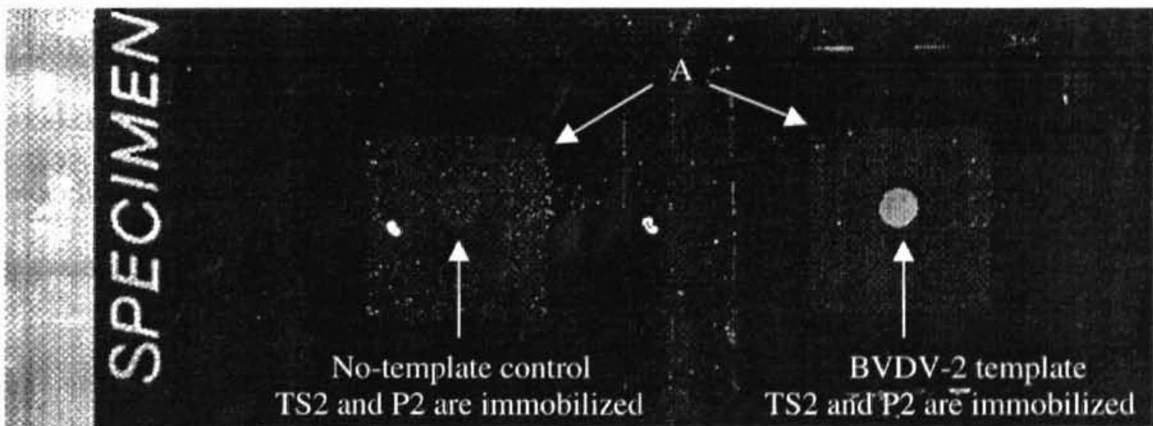


Fig. 11-2 BVDV-2 detection on ViSH chip with UniHyb

Scanned image of glass slide containing P2 and TS2 primers and hybridized in the presence or absence of Cy5 labeled BVDV-2 product using UniHyb. Arrow marks "A" faint white area which indicates the area covered by the PCR solution and the Frame-Seal.

ViSH chip with UniHyb as a hybridization solution

The previous section demonstrated hybridization or annealing of the PCR product on the ViSH chip without a hybridization solution. After that, I started to hybridize the PCR product on the ViSH chip using UniHyb. These ViSH chips showed higher contrast between positive and negative (no-template) control in hybridization of PCR product than ViSH chip without UniHyb in Fig. 11-1.

The process to attach primers is the same between two, but the ViSH chip without using UniHyb was treated with 1 % blocking reagent (Fig. 11-1) and the ViSH chip with using UniHyb was not treated with blocking reagent prior to use (Fig. 11-2). In one experiment, 5 μL Cy5 labeled BVDV-2 PCR product was hybridized to a ViSH chip with 20 μL UniHyb for 4 hours at 50 °C. Fig. 10-2 shows the scanning result of the ViSH chip with UniHyb. The scanning conditions are same for Fig. 11-1 and Fig. 11-2. The contrast between positive and no-template control is bigger in the ViSH card with UniHyb (Fig. 11-2) than one without UniHyb (Fig. 11-1). The ViSH chip with UniHyb produced weaker fluorescence on no-template control and stronger fluorescence with template than blocking reagent treated ViSH chip without UniHyb. Thus, the use of UniHyb can make it easier to identify the virus.

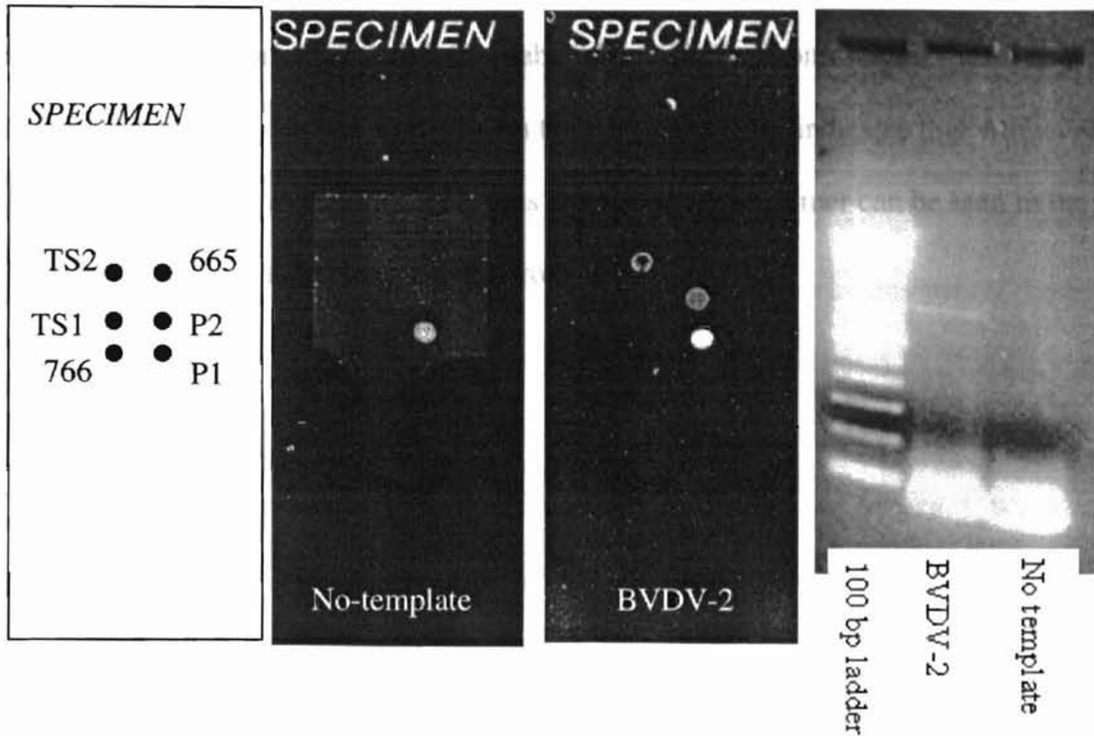


Fig. 12 BVDV-2 detection on the ViSH chip with 6 different oligonucleotides

From left, image of ViSH chip with oligonucleotides positions, no-template control, BVDV-2 control, and gel electrophoresis result for labeled PCR products. Gel electrophoresis result, from left, lane 1 100 bp ladder, BVDV-2 template product, and no-template control.

Next, TS2, TS1, P1, P2, 665, and 766 were spotted separately on the ViSH chip, and hybridized with Cy5 labeled BVDV-2 first amplification product for 4 hours at 50 °C (Fig. 12). Since BVDV-2 first amplification product contained P2 and P1 primers sequence at the extremities and the sequence complementary to TS2 primer in the middle of PCR product, these three produced spots on the ViSH chip. Other primers were not seen as fluorescent spots on the ViSH chip scanning result. Both no-template control and BVDV-2 template amplification product contained P2 and P1 as PCR primers and less

than a 100 bp sized product which probably was caused by primer dimer formation. Another chip was hybridized with a Cy5-labeled no-template control amplification product. This chip produced a spot only on the P1 primer. This indicates that artifact or primer dimer is due only to P1 primer. This artifact or primer dimer can be seen in the soluble PCR result as a less than 100 bp product.

Conclusions

In this research, I established soluble multiplex PCR conditions for distinguishing between BHV-1 and BVDV-2. No diagnostic method using multiplex method for distinguishing between BHV-1 and BVDV is published yet. This multiplex PCR is useful since both BHV-1 and BVDV are a major cause of BRD, and the treatments for the herd will differ depending on the type of virus infection. By using multiplex PCR, instead of single template PCR, we can reduce reagent costs as well as possible contamination[37]. Although I did not test wider applications, the diagnosis of BHV-1, BVDV-1, BVDV-2, and BDV will be possible since the multiplex PCR I used included the primer pairs for all the viruses listed above and PCR conditions were set up for all.

As mentioned in the introduction, multiplex PCR for typing BVDV without RNA extraction has been published [25,26]. In my research, I did not perform PCR without RNA extraction for BVDV but it would help to reduce the time for diagnosis to less than 8 hours after a blood sample is collected.

Though Multiplex PCR method was established for BHV-1 and BVDV, I expect that it will be more difficult to add more viruses for a simultaneous diagnosis due to three reasons (see introduction). Firstly, the PCR product interacts with primers and other products. Secondly, because of various sizes of PCR products, it will be difficult to read the electrophoresis gel. Lastly, there is the competition among the PCR products. Real-time PCR is a very fast method and can be automated. However, since there are more than 13 infectious agents in BRD, real-time PCR must be multiplexed or separated into 13 single virus detections. Thus, I tried to develop the ViSA card method.

A ViSA card can amplify a fragment of virus DNA with immobilized primer sets and detect the amplified products by chemiluminescence or fluorescence scanning for

fluorescently labeled product. Both bridge amplification technologies, which was established by Mosaic Technologies, Inc. and the ViSA card have common benefits: use of less reagents and the ability to avoid cross-contamination. However, nylon-based ViSA cards can be created more easily and less expensively than bridge amplification technology which uses acrylamide-modified oligonucleotides as immobilized primers. The ViSA card method uses UV crosslinking or vacuum oven to immobilize primers on a nylon membrane. With the ViSA card, I succeeded in detecting all BHV-1, BHV-2, BVDV-1, and BVDV-2 in single template PCR, and a mixture of BHV-2 and BVDV-1 in multiplex PCR (all data not shown). Differentiating between BVDV types was successful but it was not entirely satisfactory due to high background (data not shown). The high background problem was caused by non-specific binding of DIG labeled PCR products to the primers and the no-primer-attached area. When detecting the type of BVDV, the BVDV-1 PCR product bound non-specifically with unexpected primers for BVDV-2 (TS1) and BDV (TS3). All nylon membrane based ViSA cards produced high background levels when primer or virus template concentrations were high. This problem can be partially solved by reducing the primer or template concentration. I did not establish the method to determine the optimum concentration of the primer and template for the ViSA card. It will be take some time to optimize the reaction for nylon membrane-based ViSA card method. This background problem must be solved before further uses of the ViSA cards can be made.

Glass plate based ViSA card can be scanned with high sensitivity by laser scanning microscopy. However, amplification efficiency on the glass plate was extremely low. It can detect BHV-1, but it was not clear enough compared to the nylon membrane based ViSA card. This seems to be a common problem when we work with immobilized

PCR. Boles, T. C. et. al. wrote that the amplification efficiency was 1.2 to 1.3 per cycle in bridge technologies and about 1.9 per cycle in soluble PCR[32]. I used silylated glass slide and borohydride treatment to attach primers on the slide. This method has been used for microarray technologies[38] which normally employs hybridization temperature normally up to 70 °C. In the ViSA card method, the temperature is elevated up to 95 °C for the denaturing step. This high temperature probably broke the bonds between primer and support, and reduced the amplification efficiency on glass slide supports.

The ViSH chip method showed clearer results compared to the ViSA card. The ViSH chip with UniHyb hybridized with two out of three BVDV-2 primers and no other primers when it was incubated with Cy5 labeled BVDV-2 first PCR product. This experiment also revealed that primer-dimer or primer artifacts, which were made during soluble PCR, were made from P1 primer since the ViSH chip reacted only with P1 and TS2 primers. For the ViSH chip, blocking reagent was essential to reduce background problems. The ViSH chip without UniHyb and blocking reagent treatment had a much stronger background than the one with blocking reagent treatment without UniHyb. The ViSH chip with UniHyb had stronger positive reaction and weaker negative reaction. Thus, UniHyb will be useful for further experiments. Furthermore, a universal method to make Cy labeled PCR products for all 13 viruses that cause BRD to use the ViSH chip for diagnosis of BRD is needed. DNA chip technology uses thousands of oligonucleotides as probes. This is to assure that the detected result is not from unexpected contamination. It is also useful for the ViSH chip and the ViSA card to make sure the detection of virus is correct by checking with many sets of oligonucleotide. GenChip technology will be very useful in constructing the ViSH chip with high density oligonucleotides.

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