Arch Virol (1994) 136: 309–323

Archives of Virology

Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis

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Accepted January 26, 1994

Summary. A polymerase chain reaction-based assay capable of detecting a broad range of pestiviruses from pigs, cattle, or sheep was developed. Of six sets of primers selected from different parts of the pestivirus genome, the best results were provided by a pair from the highly conserved 5′ non-coding region which gave amplification with all 129 isolates tested. This panel consisted of 33 isolates from pigs, 79 from cattle, and 17 from sheep. Differentiation between the viruses was achieved by cutting the PCR-amplified products with the restriction endonucleases AvaI and BglI. Using this procedure it was possible to distinguish at least 3 genogroups; group 1 (HCV) contained 32 of the pig isolates, group II (BVDV) contained all the cattle isolates tested plus 6 sheep isolates and group III (BDV) contained 11 sheep isolates and 1 pig isolate.

Introduction

Pestiviruses cause economically important diseases of pigs, cattle, and sheep [33]. The genus *Pestivirus* has been classified recently in the family *Flaviridae* [48] and consists of hog cholera virus (HCV), bovine viral diarrhoea virus (BVDV) and border disease virus (BDV) of sheep. These pathogens are responsible for substantial losses and their effective control relies on accurate laboratory diagnosis.

The current detection of pestiviruses in clinical specimens is based on direct virus isolation in cell culture or the detection of viral antigen by immunological methods such as immunohistochemistry and immunoassay. These techniques can be time-consuming and, although HCV can be discriminated from the

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ruminant pestiviruses using a panel of monoclonal antibodies (Mabs), the distinction between BVDV and BDV isolates is less clear [16].

The introduction of in vitro amplification of nucleic acids by the polymerase chain reaction (PCR) [44] has led to the development of faster, more sensitive and specific laboratory tests for the detection of microorganisms, especially viruses [5]. Several investigators have reported the utilisation of the reverse transcriptase-PCR (RT-PCR for the amplification of pestivirus RNA [1, 4, 6, 7, 21, 23, 27, 28, 40, 42, 45, 47, 49]. The primers for PCR have been selected from different regions of the pestivirus genome to amplify specific viruses or to attempt to detect all pestiviruses. The differentiation of the pestiviruses is desirable in order to understand further the epidemiology of these agents which will aid control and eradication programmes of the diseases they cause.

The publication of several complete sequences for pestivirus genomes (BVDV strains NADL [9], Osloss [38] and SD-1 [13]; and HCV strains Alfort [30] and Brescia [34]) has given more detailed information on the pestivirus genomic organisation. The comparison of these five sequences has allowed the identification of conserved regions in the 12.5 Kb RNA genomes. In this paper we report detection of a broad range of pig, cattle and sheep pestiviruses by RT-PCR using six sets of PCR primers. In addition, it has been possible to differentiate at least three genogroups of pestiviruses by restriction endonuclease digestion of the amplified product from the single most successful primer pair.

Materials and methods

Virus isolates

The 33 porcine pestiviruses were selected from the panel of reference strains held at CVL, Weybridge to represent antigenically divergent isolates from around the world: Germany (7), Britain (5), Japan (4), Malaya (4), Belgium (3), Brazil (3), France (2), Netherlands (2), USA (2) and Italy (1). All viruses were stored at −70 °C as infected PK-15 cell culture supernatants.

Of the 79 pestiviruses isolated from cattle three (NADL, Osloss and Oregon C24V) were cytopathic (CP) reference strains and two were non-cytopathic (NCP) viruses used as reference strains at Moredun Research Institute namely G982 [3] and KY1203 [24]. A sixth cattle pestivirus was a Czechoslovakian vaccine strain, while the remaining 73 pestiviruses were field isolates from cattle in Scotland. They had been isolated in secondary bovine embryonic kidney cells as previously described [2] from specimens submitted for virus diagnosis from apparently healthy cattle or those suffering congenital, enteric or respiratory infections. The isolates were selected randomly from viruses collected between 1979 and 1993 which had been stored at −70 °C as infected cell cultures at low passage level. Twenty three of the field isolates showed evidence of cytopathic effect in cell cultures. Nineteen of these isolates were from cattle with clinical signs of enteric disease strongly suggestive of mucosal disease, one was from a bull with respiratory disease and no history was given with the other three.

Sheep were the source of 17 pestiviruses tested. These included the Weybridge NCP reference strain [19], Moredun CP and NCP reference strains [46] and the NCP AV-2 virus isolated from a case of Aveyron disease in France [8]. The other 13 pestiviruses were NCP isolates from field cases of border disease in the U.K. They had been isolated in

secondary ovine or bovine fetal cells between 1981 and 1988 and stored at −70 °C as infected cell cultures at low passage levels.

Isolation of RNA

Two techniques were used for extraction of total RNA from infected cell cultures:

Phenol/chloroform/proteinase K method

To 500 µl of cell culture fluid were added 50 µl of 10% SDS and 5 µl of proteinase K (20 mg/ml-Sigma) and the mixture was incubated at 56 °C for 25 min. RNA was extracted with 500 µl of a phenol (saturated with water): chloroform (1:1 vol/vol) mixture. The aqueous layer was precipitated with 2 volumes of EtOH held at −20 °C overnight, and centrifuged to pellet the RNA. The pellet was resuspended in 10 µl of sterile, double distilled H_2O and stored at −20 °C.

GITC/phenol method

To 100 µl of cell culture fluid were added 500 µl of extraction buffer (4 M GITC-guanidium isothiocyanate (Serva), 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M β-mercapto-ethanol), 50 µl 2 M sodium acetate (pH 4), 500 µl of phenol saturated with water and 100 µl chloroform. After extraction 500 µl isopropylalcohol were added to the water phase and RNA was precipitated for 1 h at −20 °C. Pellet was dissolved in 500 µl of extraction buffer and the RNA was reprecipitated using 500 µl of isopropylalcohol. The RNA pellet was dissolved in 4 µl of sterile H_2O and stored at −20 °C.

Primer design

Six sets of the primer pairs were used for PCR. Their sequences, position in the NADL strain of the BVDV genome and the expected size of amplified products are indicated in

Table 1. Nucleotide sequence position in NADL strain of BVDV of the primers used for RT-PCR, and length of the expected amplification product

Primer	Sequence (5′ → 3′)	Position in NADL	Product size in base pairs
324	ATG CCC$^{T}_{A}$TA GTA GGA CTA GCA	108–128	288 bp for 324/326
325	AGA GGC TAG CCA TGC CCT TAGT	97–118	
326[a]	TCA ACT CCA TGT GCC ATG TAC	395–375	299 bp for 325/326
928	GTT AGT GGC AGC ACT CAT AGA GCT GAA CT	4615–4643	
929[a]	TAA GGT CGT AGG GAA CCT CAC CGG GGC TCT	5557–5528	943 bp
H1	CAG GGT CAA AGG ACT ACC ACT ATG A	6990–7014	
H2[a]	AGT CTC CAC TAC CTG CTG GTT CCC	7441–7418	452 bp
L1	GAT TTC AAG GGG ACT TTTTT	9893–9912	
L2[a]	ACA TCT CCT ACT AAG TAG TA	10098–10079	206 bp
S1	GAC ACA AG$^{T}_{C}$GCA GGC AACA	11417–11435	
S2[a]	AG$^{C}_{T}$GGG TTC CAG GA$^{A}_{G}$ TAC AT	11865–11846	449 bp

[a] Antisense primer

Table 1. Primers 324, 325, 326 were selected from the highly conserved 5′ non-coding/non-structural coding regions of the pestivirus genomes. Primers 928/929 flank a 944 bp region of a p54 coding sequence of BVDV and included an insertion sequence in the NADL and Osloss strains. The H1/H2 primers are shortened PCR primers published by Hooft van Iddekinge et al. [23] and they flank a 452 bp region in p80. The L1/L2 primers were published by Lopez et al. [28] and cover a 206 bp region from the 3′ end of the BVDV genome. Finally, the S1/S2 primers were also selected from the 3′ end of pestivirus genomes at Moredun. The selection of primers 324, 325, 326, S1 and S2 from highly conserved regions of the published pestivirus genome BVDV strains NADL, Osloss and SD-1 and the HCV strains Alfort and Brescia was performed by the use of the computer alignment programmes of GCG [15] and Clustal V packages [22]. All primers were synthesized by Oswel DNA Service, Edinburgh.

RT-PCR

Synthesis of cDNA

Each cDNA reaction consisted of 2 µl of extracted RNA, 5 µl sterile H_2O and 1 µl antisense primers (final concentration −1 µM) or 1 µl (0.02U) random hexamers (Pharmacia). Mixtures were denatured at 65 °C for 5 min and then cooled on ice for 5 min. Then 1 µl of 'RNA Guard' (Pharmacia), 2.5 µl 10 × RT (final concentration −50 mM Tris, HCl, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiotreitol, pH 8.5), 2 µl of each 2.5 mM dNTP (Pharmacia), 1 µl (12U) avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) and sterile H_2O were added to give a final volume of 25 µl. The reactions were incubated for 1.5 h at 37 °C and the cDNA used immediately for PCR or stored at −20 °C.

PCR

The amplification of a cDNA by PCR was carried out in a total volume of 25 µl containing 2.5 µl 10 × reaction buffer (final concentration −10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatine; pH 8.3), 2 µl of each primer (final concentration −1 µm) 2 µl of each 2.5 mM dNTP (Pharmacia), 2 µl cDNA and 2 µl (1U) Taq DNA polymerase (Boehringer Mannheim). The reaction was overlaid with 30 µl of mineral oil (Sigma) and heated in a thermocycler for 35 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min.

PCR using Thermus thermophilus *(Tth) DNA polymerase*

Total reaction mixture (25 µl) contained 2.5 µl 10 × Tth reaction buffer (final concentration −10 mM Tris-HCl, pH 8.3, 90 mM KCl), 2.5 µl 8.5 mM $MnCl_2$, 2 µl of each primer (final concentration −1 µM), 2 µl of each 2.5 mM dNTP (Pharmacia), 2 µl of RNA sample and 2 µl (5U) Tth DNA polymerase (Perkin Elmer-Cetus). The solution was overlaid with 30 µl of mineral oil (Sigma) and heated at 70 °C for 15 min (RT step). In vitro amplification was carried out in the same tube using the Hybaid thermocycler and a two step thermal profile: denaturation at 95 °C for 1 min and annealing/extension at 60 °C for 1 min for 40 cycles.

The specificity of the RT-PCR was validated by including control uninfected cell culture fluids as well as harvests of bovine herpesvirus type 1 infected cell cultures.

Digestion of the amplified products by restriction enzymes

The amplified products obtained using the 324/326 primers were digested by AvaI or Bg1I according to manufacturer's protocol (Boehringer Mannheim). A typical reaction contained 5 µl of amplified product in a 25 µl reaction and approximately 8–10U of enzyme per 1 µg of DNA was used.

Agarose gel electrophoresis

The electrophoretic analysis of the PCR products was carried out in 2% agarose gel using 40 mM Tris-acetate, 1 mM EDTA, pH 7.5 at 100 V for 45 min. Visualisation of the ethidium bromide-stained DNA bands was performed using a UV transluminator and gel images recorded with video camera and thermal printer (UV Products Ltd, Cambridge, U.K.).

PAGE electrophoresis

Some samples were analysed by polyacrylamide electrophoresis in 10% PAGE gels run in 36 mM Tris, 1 mM EDTA, 30 mM sodium dihydrogen orthophosphate pH 7.8 at 15 V/cm of gel for 1 h. DNA bands were visualised by the silver staining method of Herring et al. [20].

Results

Preliminary experiments were directed at optimising the most critical steps of RT-PCR; the isolation of RNA and synthesis of cDNA. The comparison of a conventional phenol/chloroform/proteinase K method with a GITC/phenol method for isolation of RNA showed that both methods provided very similar in vitro amplification results (Fig. 1A). No significant differences were found in the yield of RT-PCR when cDNA was synthesised using antisense primers (Fig. 1B, tracks 1, 3, 5) or random hexamers (Fig. 1B, tracks 2, 4, 6). On the basis of these results all further RT-PCR experiments were carried out using RNA template extracted by the technically simpler phenol/chloroform/proteinase K method and cDNA synthesis was by random hexamer priming. Changes of annealing temperature in the range 46–56 °C had little influence on the yield of PCR and all primers were annealed at 56 °C.

The pestivirus laboratory strains and field isolates were analysed by RT-PCR using six sets of primers selected along the pestivirus genome. The amplified

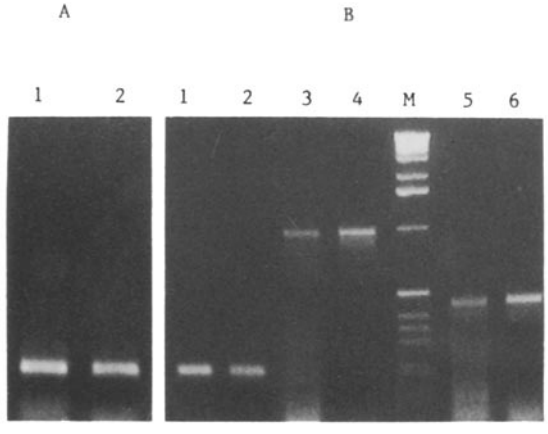

Fig. 1. Influence of method of isolation of RNA and synthesis of cDNA on the efficiency of RT-PCR. Isolation of BVDV NADL RNA (**A**) was by phenol/chloroform/proteinase K method (*1*) or GITC/phenol method (*2*). Synthesis of cDNA (**B**) was carried out using antisense primers (*1*, *3*, *5*) or random hexamers (*2*, *4*, *6*). Primers: L1/L2 (**A**, *1* and *2*, **B**, *1* and *2*), 928/929 (**B**, 3 and 4), H1/H2 (**B**, *5* and *6*). *M* 1kb ladder (BRL)

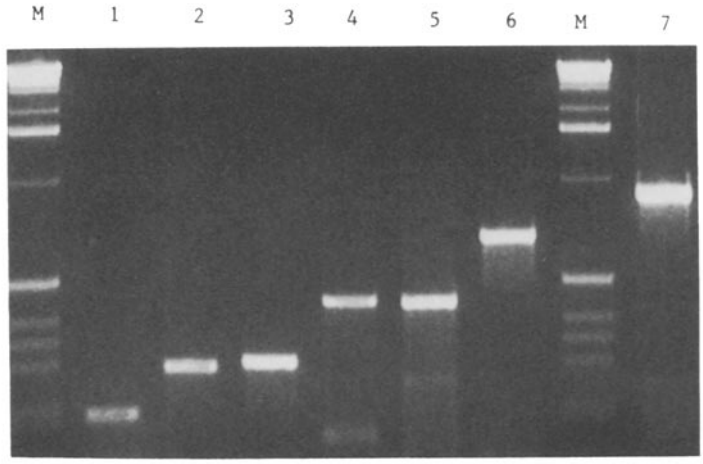

Fig. 2. Typical agarose gel electrophoresis of the RT-PCR amplification products derived from field isolates of bovine pestiviruses and NADL strain (*7*) of BVDV using various sets of primers. Primers L1/L2 (*1*), 324/326 (*2*), 325/326 (*3*), S1/S2 (*4*), H1/H2 (*5*), 928/929 (*6*), 928/929-NADL (*7*). *M* 1kb ladder (BRL)

products obtained from positive RT-PCR experiments showed the expected size of electrophoretic bands with all pestiviruses dependent on the position of the primers as shown in Fig. 2. Expected product sizes for the primer pairs was: L1/L2-206 bp, 324/326-288 bp, 325/326-299 bp, S1/S2-449 bp, H1/H2-452 bp.

The 928/929 primers were selected from a genomic region where cellular insertions occur in the NADL and Osloss strains of BVDV. With the Oregon C24V reference strain and 16 of the 23 CP field isolates an eletrophoretic band of approximately 700 bp was observed as a result of RT-PCR in 928/929 amplified positive samples (Fig. 2, track 6), this size being approximately 250 bp shorter than the 943 bp amplified product detected with the NADL and Osloss strains of BVDV. Three of the 16 positive CP field isolates contained larger bands in addition to the 700 bp amplified product. The first of these (L598), containing 2 additional bands of approximately 900 bp and 1000 bp, was a CP virus isolated from a pooled nasal/ocular swab from an eighteen month old bull with a clinical history of pyrexia, cough and nasal discharge. The second (Q3080/1), was a CP isolate from a retropharyngeal lymph node of a 3 month old calf with mucosal erosions in the upper alimentary system, which gave one extra band of approximately 900 bp. A third CP field isolate (R1569) gave an extra band around 800 bp in length. This isolate was from a pooled spleen/lymph node specimen submitted from a 5 month old calf with a clinical and post-mortem history of mucosal disease. The most likely explanation for the multiple bands was that each of these 3 isolates contained a mixture of CP and NCP viruses.

As shown in Table 2 the most reliable amplification was obtained with primer pair 324/326 which amplified RNA from all of the 129 pestivirus strains and field isolates tested.

Table 2. Summary of RT-PCR amplification positive results for detection of 129 pestiviruses originating from cattle, pigs and sheep

Species of origin of pestivirus	Primers 324/326	325/326	S1/S2	H1/H2	928/929	L1/L2
Cattle (79)	79	79	75	72	48	57
Pig (33)	33	31	32	ND	0	ND
Sheep (17)	17	7	8	4	3	ND

ND Not determined

All 79 bovine isolates were also detected by the 325/326 primers while the other primer pairs amplified the majority of bovine isolates. The S1/S2 primer pair failed to amplify 4 isolates, the H1/H2 failed with 7 isolates, L1/L2 with 22 isolates and 928/929 with 31 isolates. The isolates which failed to be amplified by the 928/929 primer pair consisted of 7 CP and 24 NCP isolates.

Of the porcine isolates only two failed to be amplified with 325/326 primers and S1/S2 detected all but one isolate. There was no amplification with the 928/929 primers. The two isolates negative with the 325/326 primers were both UK isolates designated congenital tremor and 87/6. The latter of these two isolates was also negative with the S1/S2 primers.

The sheep-derived pestiviruses gave the most heterogenous amplification results with the different primers. The full results for the ovine pestiviruses with five of the primer sets are shown in Table 3. Four viruses (Weybridge, B1056, R2727 and D861) were comparable to the bovine viruses giving a PCR positive result with 4 or 5 sets of primers. Aveyron AV-2 gave a positive result with 3 primer sets, while 5 viruses (173157, Lees, Moredun NCP, 135661 and 59386) were positive with only 2 sets of primers. A further 7 viruses (Moredun CP, A1870, G2048, JH 2816, G1305, L991 and SP 137/4) were positive only with the 324/326 primers.

A simpler one step RT-PCR using Tth DNA polymerase which was recently reported by Young et al. [50] was also examined for amplification of selected bovine and ovine isolates using various sets of primers. A comparison between these results is shown in Table 4. Taq DNA polymerase amplified BVDV (isolate 7446) with all six sets of primers but amplification using Tth DNA polymerase was successful only with 324/326, 325/326 and S1/S2 primers. In vitro amplification of ovine isolate BDV (strain G1305) was positive for both enzymes with the 324/326 primers.

The possibility of differentiating pestiviruses by restriction site analysis was investigated with the 288 bp products generated by the 324/326 primers. Amplified products from all the porcine and ovine isolates and 25 randomly selected bovine isolates were digested with BglI and AvaI restriction enzymes. Examples of the results obtained with BglI and AvaI digestion are presented in Fig. 3. The restriction enzyme BglI cut only the porcine isolates (track 3) giving

Table 3. The RT-PCR amplification positive results for the detection of 17 pestiviruses isolated from sheep

Virus designation	Primers 324/326	325/326	S1/S2	H1/H2	928/929
Weybridge Group					
Weybridge[a]	+[b]	+	+	+	+
B1056	+	+	+	+	+
R2727	+	+	+	+	+
D861	+	+	+	+	−
Aveyron AV-2	+	+	+	−	−
173157	+	+	−	−	−
Moredun Group					
Moredun CP[a]	+	−	−	−	−
Moredun NCP	+	−	+	−	−
135661	+	−	+	−	−
59386	+	−	+	−	−
A1870	+	−	−	−	−
G2048	+	−	−	−	−
JH2816	+	−	−	−	−
G1305	+	−	−	−	−
L991	+	−	−	−	−
1374	+	−	−	−	−
Lees	+	+	−	−	−

[a] Two different stocks of each reference strain gave identical results

[b] The 324/326 amplified products of the 6 viruses in the Weybridge group were all cut by the restriction enzyme AvaI. None of the amplified products of the 11 viruses in the Moredun group was cut by this enzyme

expected fragments of 247 bp and 41 bp in length. One porcine isolate (UK 87/6) was not cut with enzyme BglI giving the same result as the bovine and ovine isolates (Tracks 1 and 2) which were not digested.

When the restriction enzyme AvaI was used to digest the product of the 324/326 primers, digestion occurred with all 25 of the bovine isolates tested (Fig. 3, track 6) and 31/33 of the porcine isolates (Fig. 3, track 4) giving the expected 171 bp and 117 bp fragments. One of the porcine PCR products. (Japanese Kanagawa strain) not digested with AvaI had been digested with BglI while the other porcine isolate not digested was UK 87/6 which had not cut with BglI.

When the ovine 324/326 primer amplification products were digested with AvaI, six viruses (Weybridge group – see Table 3) were cut giving fragments similar to those of the cattle viruses (Fig. 3, track 6) while the products of 11 viruses (Moredun group – Table 3) were not cut (Fig. 3, track 5).

The results of all the RE analyses, summarised in Table 5, provide evidence that pestiviruses can be divided into at least 3 genogroups. Group 1 (HCV),

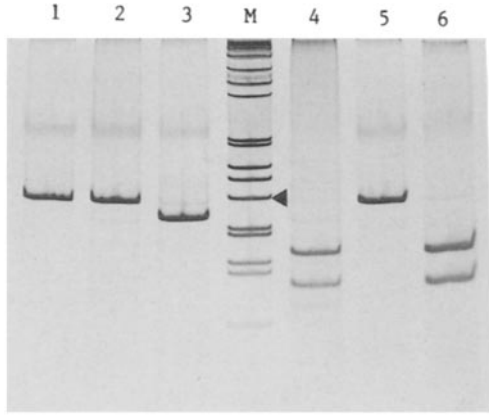

Fig. 3. Differentiation of pestiviruses by digestion of a 324/326 RT-PCR product using the restriction enzymes Bg1I (T *1* to *3*) or AvaI (T *4* to *6*). *M* is a 1 kb ladder (BRL); the arrowed band has a size of 298 bps and the bands below it have sizes 220, 200, 154, 142 and 75 bps. A representative amplification product obtained from cattle isolates or the 6 ovine viruses in the Weybridge group is shown in T *1* and *6*. A representative product from the 11 ovine viruses in the Moredun group is shown in *2* and *5*, while porcine isolates are represented in *3* and *4*

Table 4. Comparison RT-PCR of BVDV and BDV using Taq and Tth DNA polymerase

Primers	324/326	325/326	S1/S2	H1/H2	L1/L2	928/929
			BVDV, isolate 7446			
Taq DNA polymerase	+	+	+	+	+	+
Tth DNA polymerase	+	+	+	−	−	−
			BDV, ovine isolate G1305			
Taq DNA polymerase	+	−	−	−	−	−
Tth DNA polymerase	+	−	−	−	−	−

+ Positive amplification, − no amplification

containing 32 (not UK 87/6) of the 33 porcine isolates, are cut by Bg1I and mostly by AvaI. Group 2 (BVDV) which contained all the bovine isolates tested plus the Weybridge group of 6 ovine isolates are cut only by AvaI, and Group 3 (BDV) which contained the 11 ovine isolates of the Moredun group plus the one porcine isolate UK 87/6, and which are not cut by either enzyme.

Table 5. Summary of results of RE digestion of the amplified product from primer pair 324/326

Species from which isolated	No. tested	Digestion with BglI	Digestion with AvaI	No digestion with either endonuclease
Cattle	25	0	25	0
Pig	33	32	31	1
Sheep	17	0	6	11

Discussion

Detection of pestiviruses by PCR requires the selection of appropriate primers. Due to high mutation frequencies among pestivirus isolates [39] it is difficult to predict primers which will amplify all virus strains. This is particularly true of BD viruses which appear serologically distinct from HCV and BVDV but for which only limited sequence data are available [12, 29, 42]. Many authors have developed PCR assays for detecting BVDV and other pestiviruses selecting primers from conserved regions of different parts of published sequences of the laboratory strains of BVDV and HCV. For instance, primers were selected from gp 53 [21, 42], gp 55 [42], p80 [21, 23, 47], gp 48 [4], 5'non-coding [6, 25] or 3' end [28] of the BVDV genome. The accumulated data suggest that the 5'non-coding part of the pestivirus genome, which probably contains sequence elements important for replication, is the most useful region from which to select PCR primers that detect the maximum number of isolates. Ridpath et al. [40] used primers from this region which amplified all BVDV isolates tested but only a minority of porcine and ovine isolates. Wirz et al. [49] selected primers from the 5' region which amplified all 57 isolates tested but their primers gave only a product of 72–74 bps which is relatively difficult to detect.

To find PCR primers which will detect the maximum number of pestivirus isolates we have compared the results of RT-PCR with six sets of primers selected from four different regions of the pestivirus genome. Two sets of primers, H1/H2 and L1/L2, were taken from published PCR assays [23, 28] which provided promising positive results with several pestivirus strains. Using computer comparison of NADL, Osloss, SD-1, Alfort and Brescia sequences we have selected S1/S2 primers from the highly conserved 3' end of the pestivirus genome and 324, 325 and 326 primers from the 5' non-coding/non-structural coding region of the genome. The 326 antisense primer came from a 33 nucleotide region which is totally conserved among published sequences of pestivirus strains and which has also been used by other workers [40, 49]. The sense 324, 325 primers have been selected from an upstream conserved region to give a PCR product of approximately 300 bp which is easily resolvable by agarose gel electrophoresis. To determine the possible occurrence of cellular insertions in pestivirus isolates, we have chosen the 928/929 primers which bracket the regions of these insertions in NADL and Osloss strains of BVDV.

Our results confirmed that the selection of primers from the 5′-noncoding region of the genome is best for the detection of pestiviruses with one of our primer pairs giving positive amplification with all isolates tested. The H1/H2 and S1/S2 primers, selected from p80 or 3′ end, respectively were able to detect most cattle isolates. It seems that some isolates of pestiviruses from sheep represented by the Weybridge reference strain are closer to BVDV since, as with the cattle isolates, 4–5 sets of primers were able to amplify their genomes. The isolates of BDV which aligned with the Moredun reference strain of ovine virus gave negative results with all primers except the 324/326 pair (Table 3). This result agrees well with earlier findings that ovine pestiviruses can be subdivided antigenically into viruses that resemble BVDV (Weybridge group) and those that are distinct as represented by the Moredun reference strain [35, 36].

On the basis of cell culture experiments there are two biotypes of bovine pestiviruses, CP and NCP [17, 26]. In an attempt to explain the basis of cytopathogenicity at the molecular genetic level, some authors have speculated that CP strains arise as a result of a genomic mutation of NCP strains [10], resulting from insertion of cell-derived RNA into the p125 gene or genomic duplication in the p80 region [31, 37]. Since the occurrence of cellular insertions has not been confirmed in all CP BVDV strains [11, 14, 18, 37] the origin of cytopathogenicity of some BVDV isolates is more complex. Of the 73 bovine field isolates examined, 23 showed evidence of cytopathogenicity in BEK cells. Using the 928/929 primers a 700 bp fragment (i.e. around 250 bp less than the DNA amplified from the NADL strain) was detected in 13 of the 23 CP viruses suggesting that large p125 cellular insertions were absent. Three other CP isolates of BVDV yielded longer PCR products. These observations suggest that while cytopathogeniticity can be associated with insertions in this region of the genome, either these primers are not suitable for the detection of all insertions or other mutagenic mechanisms may be involved.

The thermostable Tth DNA polymerase has a reverse transcriptase activity which is active at an elevated temperature (60 °C) [32]. The use of this higher temperature is advantageous for synthesis of cDNA if the target RNA contains a high degree of secondary structure. A simple, 'one tube' method RT-PCR for amplification of RNA using Tth DNA polymerase has been reported recently [50]. We examined this modified RT-PCR to determine if high temperature reverse transcription could increase the number of isolates detected. In our hands, Taq DNA polymerase amplified more genomic regions from BVDV isolates than Tth DNA polymerase (Table 4). However, both enzymes gave similar results with the 324/326 primers and it is possible that a convenient 'one tube' RT-PCR for the detection of pestiviruses could be developed using these primers.

Differentiation of pestiviruses is an important veterinary problem from the view of control and eradication of hog cholera, BVD and BD in domestic animals. An attempt to use specific primers from the 5′ non-coding genomic region for differentiation of BVDV from other pestiviruses has been unsuccessful

[40], although primers selected from a genomic region specific for HCV were able to differentiate between HCV and ruminant pestiviruses [25, 49].

Computer-generated restriction maps of published sequences showed that within the 324/326 PCR product the AvaI restriction site is strongly conserved among NADL, Osloss, SD-1, Alfort and Brescia strains, while only the Alfort and Brescia strains of HCV contained a BglI site. On the basis of our findings there is evidence that pestiviruses can be divided into at least 3 genogroups. Group 1 (HCV) which contained 32 (not the UK 87/6 isolate) of 33 porcine viruses-cut by BglI and mostly by AvaI. Group 2 (BVDV) which contained all the bovine isolates tested plus the Weybridge group of 6 ovine isolates-cut only by AvaI. Group 3 (BDV) which contained the 11 ovine isolates of the Moredun group and the one porcine isolate (UK 87/6)-not cut by either enzyme. The UK 87/6 porcine isolates, has been shown to resemble sheep isolates by sequence analysis of the p20 gene and may have originated from sheep [42]. Sequence analysis of the 5′ end of the genome of American isolates of BVDV has confirmed that they too all possess the AvaI restriction site [41].

Interestingly, our results obtained at the genetic level are largely in agreement with the analyses of pestiviruses using panels of monoclonal antibodies. This immunological approach divided ovine isolates into three groups; one, which was BVDV-like, included the Weybridge strain, whilst the two other groups were considered distinct from BVDV and HCV and included five viruses from the Moredun group as well as the Aveyron strain (Paton et al., in press).

We hope that our most successful primers can be used for developing a routine method for detecting pestiviruses in biological fluids, such as semen, fetal bovine serum or vaccines. The variations detected in the amplified DNA show that this genomic region can be used to differentiate isolates by restriction endonuclease digestion and still more information should be obtainable using heteroduplex analysis or direct sequencing.

Acknowledgements

We thank the Scottish Office Agriculture and Fisheries Department for providing support for Š. Vilček under their fellowship scheme, and the many colleagues who kindly provided the pestivirus isolates and reference strains.

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Received November 17, 1993