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Rapid diagnosis of pseudorabies virus infection in swine tissues using the polymerase chain reaction (PCR)

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SUMMARY

In Argentina pseudorabies is an endemic disease. Routine diagnosis is made by virus isolation. It is a very long procedure to carry out and gives variable results depending on the quality of sample, hence the need for effective techniques, which are rapid and not dependent on the isolation of infectious virus. The polymerase chain reaction (PCR) technique has provided a sensitive, specific and rapid mean to detect DNA sequences. This study describes a PCR method for detection of pseudorabies virus sequences in swine tissues. In order to determine the presence of suid herpesvirus-1 DNA and antigens, 36 tissue samples collected from 19 dead pigs, whith signs of pseudorabies infection, were examined by PCR, virus isolation and indirect immunofluorescence, respectively. Fifteen out of 19 pigs were positive at least for one tissue by PCR (15/19) while only three pseudorabies virus strains were isolated (3/19). All the amplified products were identified by digestion with *SalI* and hybridization. The method described herein circumvents tedious viral isolation and DNA purification and would be a valuable tool for rapid diagnosis, since it would take less than 5 h to reach an accurate result even in poorly preserved tissue samples.

Key words: diagnosis, pseudorabies virus, polymerase chain reaction

RESUMEN

Diagnóstico rápido del virus de la pseudorrabia en tejidos mediante el método de reacción en cadena de la polimerasa. La pseudorrabia (enfermedad de Aujeszky) es endémica en la Argentina. El diagnóstico de rutina se realiza por aislamiento del virus, procedimiento lento y de resultados variables que dependen de la calidad de la muestra, por lo que es necesario una técnica efectiva y rápida como alternativa. La reacción en cadena de la polimerasa (PCR) permite detectar secuencias de ADN de forma rápida, específica y sensible. En este trabajo se describe un método de PCR para detectar secuencias de ADN del virus de la pseudorrabia a partir de tejidos. Se analizaron 36 órganos provenientes de 19 animales con sintomatología compatible con la enfermedad de Aujeszky, por PCR, aislamiento viral e inmunofluorescencia indirecta. Quince del total de casos analizados resultaron positivos por PCR, al menos para un tejido (15/19) mientras que, sólo tres cepas virales fueron aisladas (3/19). Todos los productos obtenidos resultaron específicos ya que fueron digeridos con *SalI* y reaccionaron frente a una sonda biotinilada. Consideramos que la técnica descripta es de mucha utilidad en el diagnóstico de enfermedad de Aujeszky, dado que no requiere purificar el ADN y permite obtener resultados en muestras aún no aptas para el aislamiento viral y en menos de 5 horas.

Palabras claves: diagnóstico, pseudorrabia, reacción en cadena de la polimerasa

INTRODUCTION

Pseudorabies virus (PRV) is the etiologic agent of a major disease that has a substantial economic impact on the swine industry. The disease is fatal for young pigs but in adults the infection is less severe, manifested by various degrees of respiratory distress, nervous and genital disorders.

Pigs surviving PRV infection remain latently infected for life. PRV is classified as suid herpesvirus-1, a member of subfamily *Alphaherpesvirinae*, and has a linear double stranded DNA genome of approximately 90 megadaltons. The essential glycoprotein gD is one of the major PRV glycoproteins. It is an important immunogen that elicits the production of neutralizing antibodies and is highly conserved. Virus isolation has been used during many years for diagnosis of PRV infection. The virus can be effectively isolated in cell culture although it is a time consuming process and false-negative results may be caused by post-mortem changes with subsequent virus inactivation. Moreover, viral isolation is not suitable for use on decomposed or poorly preserved field samples submitted for laboratory diagnosis. The polymerase chain reaction (PCR) has provided a rapid and sensitive method applied to the detection of low amounts of viral DNA in latently infected tissues and productive infections in other biological materials (1, 4, 7, 9, 10, 13, 15, 16). Among the several advantages it presents, PCR is a specific method less affected by extreme pH values than tissue culture techniques usually employed to recover the virus (14). The objective of this work was to establish a PCR method for detection of PRV sequences in swine tissues, which are often the only source of material available for analysis.

MATERIALS AND METHODS

Virus isolation

Virus isolation was attempted from 36 organs belonging to 19 animals arrived to our laboratory. Several different samples from cases where processed: 1 case from 1994, 2 cases from 1996, 5 cases from 1997, 9 cases from 1998 and 2 cases from 1999. RK13 cell monolayers grown in 24-well plates were inoculated with 0.2 ml of the clarified organ homogenates (10% weight per volume in Eagle's MEM) and observed daily. Cell culture without inoculum was used as control. Observations were carried out over a period of 3 weeks (1 blind passage per week), during which the cytopathic effect (CPE) was checked every day.

Indirect immunofluorescence (IF) assay

To identify and confirm the presence of PRV antigen on cell cultures, the positive results, expressed as CPE, were checked by an indirect IF assay with monoclonal antibodies produced by us (11).

Isolation of total DNA for PCR

Viral DNA from Indiana-S PRV strain (provided by Dr. K. Sekikawa, NIAH, Tsukuba, Japan) was extracted from the culture fluid by the procedure described previously (6). This DNA was used to optimize the PCR reaction.

Organ sample preparation for PCR

In order to destroy the cell membranes, approximately 1 cm^3 of tissue from different organs were boiled for 15 min in 1 ml of phosphate buffer (3, 9). Subsequently, the samples were chilled on ice 5 min followed by centrifugation (10,000 rpm for 5 min at 4 °C). A 5 ml aliquot of the supernatant (pure and 1:10 dilution in TE buffer - 10 mM Tris-ClH pH 8.0 and 1 mM EDTA pH 8.0-) was used for PCR in each case. To obtain total DNA as negative control for PCR, the same procedure was carried out in organs from a four week old swine purchased from a free PRV commercial herd.

PCR

A single PCR was used for the amplification of a 217 base pairs (bp) sequence of the gene encoding the essential glycoprotein gD. Primer A (5'-CAC GGA GGA CGA GCT GGG GCT-3') and primer B (5'-GTC CAC GCC CCG CTT GAA GCT-3') amplified the sequence which lies between bases 433 and 651 of the gD sequence. These contain a *SalI* site, which after cleavage produces 2 fragments of 140 and 77 bp long (16). The PCR was performed in a volume of 50 ml containing 10 % of sample DNA. The reaction mixture contained 5 ml 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatine), 4 ml dNTP mixture (10 mM each), 1 ml of each of the primers (20 pM) and 2.5 U of TaqDNA polymerase (0.5ml). To prevent false positives, PCR mixtures and DNA solutions were manipulated in separated

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rooms. The aqueous phase was overlaid with a drop of mineral oil. The amplifications were performed in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). Two amplification conditions were carried out:

a) 40 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min and extension at 72°C for 3 min, according with Wheeler and Osorio (16).

b) 45 cycles of denaturation at 94 °C for 1 min, and annealing and extension at 72 °C for 1.30 min.

Ten microliters of the PCR product were run in 2% agarose gels at 100V for 30 min. The bands were stained with ethidium bromide and identified according to their size. Positive samples were retested. To investigate the specificity of the PCR amplification, the semi-purified DNA of the following viruses was tested: bovine herpesvirus 1, equine herpesvirus 1, canine herpesvirus 1 and feline herpesvirus 1. The chemicals and the conditions of amplification were first optimized on purified viral DNA of Indiana-S strain. Total DNA from the corresponding organs of non-infected animals and reaction mixture without DNA ("no template reaction") (8) provided negative controls.

Analysis of amplified products

The amplified products generated by the PCR were further characterized by restriction analysis. The DNA was recovered from PCR mixtures by phenol-chloroform extraction and ethanol precipitation (5). The DNA was resuspended, digested by using *Sal*I and electrophoresed in a 3% agarose gel.

Southern blotting

Amplification products were purified and analyzed on a 1% agarose gel. After ethidium bromide staining, the gel was further blotted to nylon membrane in 20X SSC (3 M sodium chloride, 300 mM sodium citrate, pH 7.0) and then prehybridized at 42 °C for 4 h in a solution containing 50% formamide, 6X SSC (0.9 M sodium chloride and 0.09 M sodium citrate), 5X Denhardt's (0.1% bovine serumalbumin –BSA-, 0.1% polyvinyl-pyrrolidone, 0.1% Ficoll), 0.2 % BSA, 1% SDS, 10 mM EDTA and 100 mg/ml denatured salmon sperm DNA. Hybridization was carried out overnight at 42 °C with the *Bam*HI 7 fragment of the Indiana-S (cloned in pUC19) biotin labelled as a probe (Bio Prime DNA kit labelling system BRL Life Technology) in hybridization buffer (40mM Tris, 1 mM EDTA, 5X Denhardt's, 6X SSC, 0.1% BSA, 0.1% SDS).

RESULTS

Viral isolation

The detail of the processed organs is presented in Table 1. Of the 19 animals tested for PRV infection by this method, the virus was recovered from brain samples of 3 animals showing nervous clinical signs (cases N° 3, 16 and 17). The three strains were identified as PRV by indirect IF assay using monoclonal antibodies.

PCR

In initial experiments, we run the amplification with 40 cycles of three different temperatures (94 °C 1 min, 65 °C 1 min and 72 °C 3 min). Under these conditions, in addition to the expected size fragment, a non-specific band was obtained from total DNA of the tissue samples (Figure 1). In order to enhance the specificity of the PCR, we arbitrarily increased the temperature until 72 °C directly after the denaturation step. Under these conditions, the amplified DNA fragment corresponded to the predicted fragment size of 217 bp, in positive control DNA and in positive DNA of tissue samples (Figure 2). Neither negative controls (non-infected tissues) nor no template reactions (waterblank) yielded a DNA fragment. Fifteen out of 19 cases from which organs were collected, at least one tissue sample was positive, corresponding to 13 brain samples (cases N° 2, 3, 6, 8, 9, 10, 12, 13, 15, 16, 17, 18 and 19) and 2 liver samples were positive (case N° 11, the only organ processed) and pool of foetal tissues (case N° 14). In the cases where more than one organ was processed, when the brain samples were positive, also resulted positive other tissue samples -liver, kidney, lung, medulla, ganglion and tonsil- (cases N° 3, 12, 15, 16, 18 and 19). The PCR assay has proven to be specific to PRV since none of the other alphaherpesvirinae included as negative controls, gave any amplification product at the described conditions. Diluting the samples at 10-fold in TE buffer overcome inhibitory effects of sample components on TaqDNA polymerase.

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Table 1. Results of viral isolation, PCR, hybridization and *SalI* digestion in various organs from clinical nervous signs or reproductive disorders affecting swine.

Case N°	Origin	Signs	Tissue	VI [1]	PCR	SBH [2]	RE [3]
1	Piglet	NS [4]	Kidney	-[7]	-	ND [9]	ND
			Spleen	-	-	ND	ND
			Brain	-	-	ND	ND
2	Piglet	NS	Brain	-	+[8]	+	+
3	Piglet	NS – S [5]	Liver	-	+	ND	ND
			Kidney	-	+	ND	ND
			Lung	-	+	ND	ND
			Brain	+	+	+	+
			Medulla	-	+	ND	ND
4	Sow	RS	Lung	-	-	ND	ND
5	Sow	RS	Lung	-	-	ND	ND
6	Piglet	NS	Brain	-	+	+	+
7	Piglet	NS	Brain	-	-	ND	ND
8	Piglet	NS	Brain	-	+	+	+
9	Piglet	NS – S	Brain	-	+	+	+
10	Piglet	NS – S	Brain	-	+	+	+
11	Piglet	NS	Liver	-	+	+	+
12	Stillbirth		Liver	-	+	ND	ND
			Brain	-	+	+	+
13	Piglet	NS	Brain	-	+	+	+
14	Stillbirth		Pool	-	+	+	+
15	Sow	NS+RS [6]	Ganglion	-	+	ND	ND
			Kidney	-	-	ND	ND
			Brain	-	+	+	+
16	Piglet	NS	Tonsil	-	+	ND	ND
			Lung		+	ND	ND
			Brain	+	+	+	+
17	Piglet	NS	Brain	+	+	+	+
18	Stillbirth		Lung	-	+	ND	ND
			Liver	-	+	ND	ND
			Brain	-	+	+	+
			Kidney	-	+	ND	ND
19	Stillbirth		Lung	-	+	ND	ND
			Tonsil	-	+	ND	ND
			Brain	-	+	+	+
			Kidney	-	+	ND	ND

[1] VI = virus isolation; [2] SBH = Southern blot hybridization; [3] RE = *SalI* restriction enzyme; [4] NS = nervous signs; [5] S = sacrificed; [6] RS = respiratory signs; [7] (-) negative result; [8] (+) positive result; [9] ND = not done

Using this PCR system, the overall time for preparation and detection by agarose gel was 5 h.

The specificity of the PCR amplified products was confirmed by hybridization with a probe and by *SalI* digestion. Hybridization signals were obtained with all amplicons (Figure 3). The digestion patterns of the amplified DNA products corresponded to the predicted fragment sites calculated from the native gene sequences. Digestion of the PCR product with *SalI* cleaved the 217 bp product into 2 fragments that migrated in

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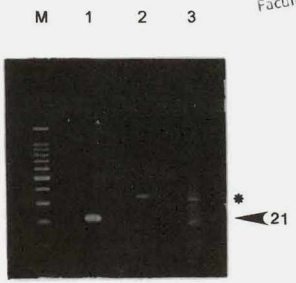

Figure 1. Bands obtained after amplification using 3 different temperatures of denaturation, annealing and extension (94 °C 1 min, 65 °C 1 min and 72 °C 3 min respectively) using semi-purified DNA and DNA from supernatants of boiled organs (1% agarose). Lane M = molecular size marker; lane 1 = known positive sample (viral DNA from Indiana-S strain; lane 2 = DNA obtained from boiled organ (pure); lane 3 = DNA obtained from boiled organ (1:10 dilution). Arrow indicates the specific bands (expressed in bp) and asterisk denotes the non-specific bands.

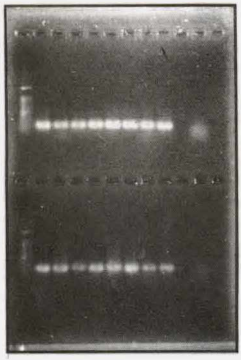

Figure 2. Electrophoretic analysis of sixteen 217 bp PCR products obtained from supernatants of boiled organs (1% agarose). Lanes M = molecular size marker; lanes 1 to 15 = cases N° 2, 3, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19, respectively; lane P = known positive sample (viral DNA from Indiana-S strain; lane N = known negative sample (DNA from non-infected tissue); lane W = waterblank.

Figure 3. Southern blot hybridization of the sixteen 217-bp PCR products obtained from supernatants of boiled organs. Lanes 1 to 15 = cases N° 2, 3, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19, respectively; lane P = known positive sample (viral DNA from Indiana-S strain); lane N = known negative sample (DNA from non-infected tissue); lane W = waterblank.

a pattern consistent with the calculated sizes of 141 and 77 bp corroborating their specificity (Figure 4). In all cases, specific sequences were not detected in any of the non-infected pigs DNA nor in the no-template controls (Figures 2 and 3).

DISCUSSION

PRV can be effectively isolated in cell culture, although it is a time consuming process, and is dependent on finding viable virus in field samples. Moreover, the conditions of the sampling method, preservation of the organs and distances from the swine producing areas to the laboratory, might contribute to obtain low efficiency of isolation.

In this study, a rapid PCR protocol that specifically amplifies coding sequences for the gD was applied. In the initial experiments using 3 different temperatures for denaturation, annealing and extension and working on DNA from tissue samples, we obtained more than one band. This might be due to the high G-C content of the region

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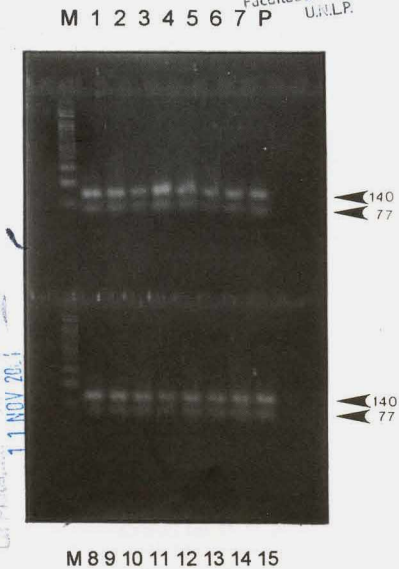

Figure 4. Electrophoretic analysis of the sixteen 217 bp PCR products obtained from supernatants of boiled organs digested with *SalI* restriction enzyme (3% agarose). Lanes M = molecular size marker; lanes 1 to 15 = cases N° 2, 3, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19, respectively; lane P = known positive sample (viral DNA from Indiana-S strain). Arrows indicate the fragments size (expressed in bp).

to be amplified or the presence of reaction inhibition. High percentage of G-C could be responsible of extensive secondary structure formation, which would prevent correct primer hybridization at lower annealing temperature and could lead to incorrect amplification. Furthermore, although the tissue preparations were heated at 100°C for 15 min, there appeared to be heat stable tissue factors that interfere with the reaction. We removed inhibitors by diluting the template, thus eliminating interference as was demonstrated by Scherba *et al* (13) and Ashbaugh *et al* (2). The possibility of cross-reaction with other alphaherpesvirinae was ruled out since none of the heterologous herpesviruses was amplified by the PCR assays. The specificity of the amplified products was maintained even without purification of total DNA, as was also demonstrated by Osterreider *et al* (12). Restriction analysis of the amplicons corroborated their specificity when were digested with *SalI* and subjected to electrophoresis in agarose gel, yielding fragments 144 and 77 bp.

Our results confirm that PCR is a valuable technique for detection of PRV particularly when it is present in such biological materials which otherwise posses a problem for conventional diagnostic techniques. DNA purification for PCR was not necessary since heated homogenates were specific and sensitive enough. Besides, it is remarkable that the material costs were lower than the expenses of our routine virus isolation test.

We conclude that this PCR would be a valuable tool for rapid, sensitive and specific diagnosis. The PCR method described herein, which uses DNA directly obtained from boiling organ tissues, circumvents the need of conventional DNA extraction using phenol/chloroform and thus manipulation of the samples is minimized, reducing the risk of cross-contamination. Results can be obtained in 5 h instead of the weeks required by cell culture techniques. Likewise, the material costs and time-consuming of the virus isolation test would enhance the use of PCR assays in routine laboratories.

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