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Polymerase Chain Reaction for Diagnosis of Aujeszky Disease in Mexico

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Abstract: The objective of this study was to establish the optimal conditions for carrying out the PCR technique to detect the Aujeszky's Disease (AD) virus in tissues. For this purpose, researchers used different rabbit and pig tissue inoculated with the Shope strain of the AD virus as well as uninoculated animal tissues. Cell culture of the same strain was used as positive control. A commercial kit was employed for DNA extraction. DNA amplification was performed with a pair of primers flanking a 334 bp fragment of the *gB (gII)* gene. It was possible to obtain the expected product in 14 of 16 samples of tissues from infected animals. These results were consistent with those obtained by virus isolation. The assay sensitivity showed that the PCR can amplify DNA up to 65 fg. The specificity of the test was confirmed by failure to observe any amplification product from the 16 samples neither from negative animals nor from bovine herpesvirus DNA. It is a molecular method that may be useful to complement the diagnosis of the disease in Mexico.

Key words: Pigs, Aujeszky's disease, pseudorabies virus, PCR diagnosis, DNA, optimal condition

INTRODUCTION

Aujeszky's Disease (AD) may be fatal in pigs and affects the economy of the pig industry when animals have the infection in its acute phase (Yoon *et al*., 2005). The disease is caused by a herpes virus called porcine Herpesvirus type I (HVP-1) (Kluge *et al*., 1999). This herpesvirus contains five glycoproteins (gp) essential for its multiplication (gB, gD, gH, gK and gL) and five non-essential (gC, gE, gI and gM).

AD affects many animal species mainly domestic mammals. However, swine is the most important for being the primary host and the source of infection for other species. This becomes important from the health point of view when the disease occurs subclinically and inapparent. When it occurs in clinical form, signs are associated to the central nervous system and respiratory tract; it causes impaired reproduction in pregnant females. Piglets are highly susceptible to the infection whereas adults are more resistant (Kluge *et al*., 1999).

The pathogenesis of the disease depends on the virus strain, the age of the pigs, the amount of infective dose and the route of inoculation. Eradication of AD is difficult because of the ability of the virus to establish latent infections since vaccines do not prevent this type of infection so that animals can be carriers (Kluge *et al*., 1999).

Each country adopts control and eradication strategies according to several factors, one is the use of vaccines with deletion in conjunction with differential ELISA tests that can distinguish infected from vaccinated animals. If the disease is present in countries with high prevalence and high density in pig population, eradication is carried out with vaccination-eradication programs.

Diagnostic methods for AD are varied. These are based on viral isolation (VA) and identification of the virus, detection of antigens by immunofluorescence and detection of specific antibodies by immunoenzymatic techniques (ELISA) and its variants (Gutekunst, 1979; Afshar *et al*., 1986; Dorett, 1986; Sorensen and Lei, 1986; Qvist *et al*., 1990). Moreover, detection of viral nucleic acid is carried out by the Polymerase Chain Reaction technique (PCR) (Jestin *et al*., 1990; Wheeler and Osorio, 1991).

PCR is a highly sensitive and specific technique that can be done in short time. Allows detection of the virus in early stages or even when the infection is already established. This technique also offers the possibility of detecting the virus even if it is no longer viable or in

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samples where isolation is difficult to perform as in the case of semen. Using the PCR technique also offers many advantages since it can detect the dormant virus in which viral antigens are not expressed.

In Mexico, there is a campaign for control and eradication of AD which seeks to eradicate the disease from the country. Therefore, it is necessary to have more sensitive and specific diagnostic techniques to support this campaign. So, the aim of this study was to establish the PCR technique for detection of AD virus.

MATERIALS AND METHODS

Production of the viral strain: A batch of the virus shope strain was prepared in MDBK cells. The final titer was determined by serial dilutions of the virus from 10^{-1}-10^{-9}. Each dilution was inoculated into the cells and incubated until the Cytopathic Effect (CPE) was observed (3-4 days). Reading was taken from each dilution and the final titer was calculated by the Read and Muench Method (Burleson *et al*., 1992). The lot thus obtained had a titer of $10^{6.4}$ $DICC_{50}$ mL^{-1}.

Preparation of positive and negative tissues: Two healthy, 3 weeks old pigs, free of antibodies against AD by ELISA were used. Pigs were installed in isolation units. The pig 1 (C1) was used as negative control so that it was maintained for 1 week observation period. After this period it was sacrificed to collect spleen, brain, liver, bone marrow, lymph node, lung, kidney, tonsil and trigeminal ganglion. Samples were frozen at -70°C until use. Pig 2 (C2) was Intramuscularly (IM) inoculated with 2 mL of virus. Daily observations were made; rectal temperature and clinical signs were recorded. Signs clearly showed on day 4 postinoculation so that the pig was sacrificed. The same tissues were collected as from C1. In parallel to corroborate viral lethality, a rabbit was inoculated with 1 mL of virus Subcutaneously (SC). A healthy uninoculated and antibody free rabbit was used as negative control. When the inoculated rabbit died, the healthy rabbit was sacrificed and samples were collected from both: spleen, brain, lymph node, muscle, lung, kidney and liver which were frozen at -70°C until use.

Viral isolation (VA): All tissue samples recovered from animals were processed for VA by cell culture. A macerated was performed from each tissue at a 1:5 dilution in commercial Eagle MEM culture medium. This medium was added 250 UI μg mL^{-1} of a mixture of commercial penicillin and streptomycin. The macerated were centrifuged at 5000 g for 15 min and the supernatant was recovered. Twelve well plates were seeded with MDBK cells. When 100% cell confluence was achieved, the culture medium was removed and each well was added 200 μL of each supernatant in duplicate. The plate was incubated 1 h at 37°C. The inoculum was discarded and each well was added commercial Eagle MEM medium supplemented with 2% fetal bovine serum and 250 UI/μg/mL of a mixture of commercial penicillin and streptomycin and then was incubated again at 37°C with 5% CO_2. Wells were left with uninoculated cells as negative control and wells of cells were inoculated with the virus of Aujeszky at 1:10 dilution as positive control. The prepared samples were observed daily for 5 days or until the presence of CPE was observe.

DNA extraction: For total extraction of DNA from virus culture and tissues, a commercial kit (High Pure PCR template, Roche® was used. About 1 g of each tissue was macerated in 5 mL phosphate buffered saline and centrifuged at 8000 g for 5 min. Then, 200 μL of the supernatant was taken to carry out the extraction under the protocol described by the manufacturer. In the case of virus, 200 μL of culture were taken and processed in the same way.

DNA amplification: For the single PCR, a pair of primers that amplify a 334 bp fragment of the glycoprotein *B* gene (gII) of the Aujeszky virus was used whose sequence is: 5'-ATG GCC ATC TCG CGG TGC-3' y 5'-ACT CGC GGT CCT CCA GCA-3' (Yoon *et al*., 2005). The conditions of the amplification mix included: 1X buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTP's, 20 pmol of each primer, 1.25 U of Taq polymerase and 4 μL of DNA to a final volume of 25 μL. The amplification program consisted of one cycle at 95°C for 5 min, 35 cycles at 95°C for 1 min, 62°C for 1 min and 72°C for 1 min and a final extension cycle at 72°C for 7 min. As positive control, DNA from the viral culture was used. Viral DNA was quantified in order to determine the sensitivity of the test. Tenfold dilutions were made from 10^{-1}-10^{-8} and PCR was assayed. To confirm the specificity of the primers, all the samples from negative animals were analyzed by the technique. Likewise, a DNA extracted from a bovine herpesvirus (IBR) with a titer of 10^3 $TCID_{50}$ was tested.

RESULTS AND DISCUSSION

Virus isolation: Uninoculated cells remained unchanged during the period of observation of the plates. In contrast, the cells inoculated with AD virus showed a clear CPE, characteristic of the virus 48 h after inoculation. ADV isolation was possible in 14 of 16 samples from inoculated

animals. Virus isolation was negative in 16 of 16 samples from uninoculated animals. The inoculated rabbit tissues where ADV was detected were: spleen, brain, liver, lymph node, lung, kidney and muscle. In inoculated pigs positive tissues were: spleen, brain, liver, spinal cord, lymph node, lung and kidney.

DNA amplification: The expected amplification product of 334 bp was possible to obtain in 14 of the 16 samples from inoculated animals through PCR (Fig. 1). The inoculated rabbit tissues where the amplification product was detected were: spleen, brain, liver, lymph node, lung, kidney and muscle. In inoculated pigs positive tissues were: spleen, brain, liver, spinal cord, lymph node, lung and kidney. The assay was able to detect up to 65 fg of viral DNA in the sensitivity test (Fig. 2). Moreover, the test proved to be highly specific since no amplification was observed from any of the samples from uninoculated animals, nor from IBR virus DNA.

Losses caused by AD can be explained by the pathogenesis of the disease itself since the virus first replicates in the respiratory tract and subsequently spreads to different organs (Ma *et al*., 2008). As described, the methodology for diagnosis is varied but specific and is based on the identification of the virus by different techniques. PCR has been recently started to be used to detect viral nucleic acid (Jestin *et al*., 1990;

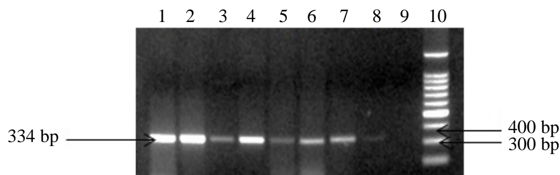

Fig. 1: Aujeszky' s disease virus detection by PCR from tissues of inoculated rabbit and pig. Rabbit: Lane 1: spleen; Lane 2: liver; Lane 3: muscle; Lane 4: lung pig; Lane 5: spleen; Lane 6: lymph node; Lane 7: lung; Lane 8: spinal cord; Lane 9: negative control; Lane 10: size marker

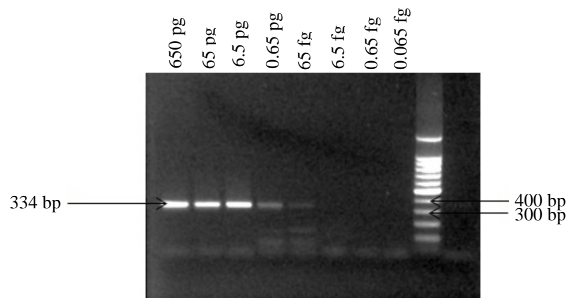

Fig. 2: Sensivity of the PCR test for detection of DNA isolated from Aujeszky's disease virus

Wheeler and Osorio, 1991). This is a useful method for detection of the virus in various tissues in infected animals as for detection of the virus in latent form and in the samples that are not suitable for VA (Jestin *et al*., 1990; Wheeler and Osorio, 1991; Perez and Diaz de Arce, 2009). For the establishment of this technique, the IBE mentions that primers must be designed to amplify the virus sequence that is common to all strains of the AD virus as those used from the *gB* or *gD* gene encoding essential viral glycoproteins. It has also been experimentally demonstrated that the disease can be transmitted by different routes (Kluge *et al*., 1999) and the presence of the virus has been detected in various tissues and fluids using PCR (Yoon *et al*., 2005). In this study, it was possible to reproduce the disease in the rabbit and pig inoculated with the virus, using the SC and IM routes, respectively corroborated by the detection of the virus by VA in cell cultures from the tissues studied. The primers used in this study corresponded to a 334 bp fragment of gene *B* of the virus genome which encodes for the glycoprotein gB (gII) which is involved in the formation of neutralizing antibodies, virus penetration and cell-cell dissemination (Kluge *et al*., 1999). The results obtained with the test showed that the primers were able to detect the AD virus under the amplification conditions research, achieving the expected product in 14 of 16 tissue samples from the inoculated animals (rabbit and pig) as described in different studies, setting the PCR technique (Yoon *et al*., 2005; Perez and Diaz de Arce, 2009). It should be mentioned that the PCR technique was as sensitive and specific as the VA test because both tests detected the virus in the same tissues studied. With these results it can be said that the positive tissues from the inoculated pig (spleen, brain, liver, spinal cord, lymph node, lung and kidney) can be considered to carry out a diagnosis of the disease. The specificity assay was demonstrated by the absence of amplification of DNA from the 16 negative tissues and bovine herpesvirus DNA studied. Assay sensitivity was also demonstrated by detecting DNA up to 65 fg.

CONCLUSION

This study managed to obtain a molecular methodology that can be used as an adjunct in the diagnosis of AD in Mexico without removing the techniques established by the OIE.

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