

A POLYCLONAL -ANTIBODY-IMMUNOPEROXIDASE-CONJUGATE FOR THE SPECIFIC DETECTION OF PORCINE CIRCOVIRUS TYPE 2

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ABSTRACT: Porcine circovirus 2 is nowadays accepted as the essential infectious agent of postweaning multisystemic wasting syndrome which causes severe economic losses in porcine production worldwide. The diagnosis of PMWS is a difficult task and must follow three criteria: (i) the presence of compatible clinical signs, (ii) the presence of characteristic microscopic histopathological lesions, and (iii) the presence of PCV2 within these lesions in moderate or high. The presence of PCV2 in lymphoid tissues must be demonstrated by in situ hybridization or immunohistochemical methods. The in situ hybridization is a more complex and expensive compared to other diagnostic tools, on the other hand, one problem concerning the immunohistochemical methods for PMWS diagnostic is the lack of a commercial anti-PCV2 peroxidase conjugate; therefore, the aim of this work was to obtain a polyclonal-antibody-immunoperoxidase-conjugate for the PCV2 specific detection. An anti-PCV2-peroxidase conjugated for the PCV2 specific detection was obtained based on the use of the available commercial vaccine against PCV2 as immunogenic inoculation for producing a polyclonal antibody in rabbits. The conjugate obtained was able to discriminate between PCV2 and PCV1 infections and a high sensitivity and specificity of the conjugate were observed.

(Keywords: porcine circovirus 2; immunohistochemical; conjugate; polyclonal antibody)

ANTICUERPO POLICLONAL CONJUGADO CON PEROXIDASA PARA LA DETECCIÓN ESPECÍFICA DE CIRCOVIRUS PORCINO TIPO 2

RESUMEN: El circovirus porcino 2 (PCV2) se considera actualmente como el agente infeccioso esencial del síndrome del desmedro post-destete (PMWS), el cual causa grandes pérdidas económicas en la producción porcina de todo el mundo. El diagnóstico del PMWS es difícil y debe seguir tres criterios: (i) la presencia de signos clínicos compatibles, (ii) la presencia de lesiones histopatológicas microscópicas características y (iii) la presencia de cantidades moderadas o altas del PCV2 dentro de las lesiones. La presencia de PCV2 en los tejidos linfoides se debe demostrar por hibridación *in situ* o métodos inmunohistoquímicos. La hibridación *in situ* es un método más complejo y costoso comparado con otras herramientas diagnósticas, por otra parte, un problema con relación a los métodos inmunohistoquímicos para el diagnóstico de PMWS es la carencia de un conjugado con peroxidasa comercial específico contra PCV2, por lo tanto el objetivo de este trabajo fue obtener un anticuerpo policlonal conjugado con peroxidasa para la detección específica de PCV2. Se obtuvo un conjugado con peroxidasa para la detección específica de PCV2 con el uso de una vacuna contra PCV2 disponible comercialmente como inmunógeno en conejos con el fin de obtener el anticuerpo policlonal contra PCV2. El conjugado obtenido fue capaz de discriminar la infección por PCV2 de la infección por PCV1, y mostró elevadas sensibilidad y especificidad.

(Palabras clave: circovirus porcino 2; inmunohistoquímica; conjugado; anticuerpo policlonal)

INTRODUCTION

Porcine circoviruses are classified into two species included into *Circoviridae* family (1). PCV1 was discovered as a noncytopathic contaminant of the continuous porcine kidney cell line PK-15 (ATCC CCL-33 and is not regarded as a pathogen for pigs (2,3). PCV2 is nowadays accepted as the essential infectious agent of postweaning multisystemic wasting syndrome (PMWS) (4) porcine dermatitis and nephropathy syndrome (PDNS), (5,6), and has been also associated to reproductive failure (7,8,9). All these syndrome were collectively grouped as porcine circovirus diseases (PCVD) (10).

The PMWS is the most important disease within PCVD due to this syndrome disease causes severe economic losses in consequences of an increased mortality rates and reduced feed conversion efficiency in weaning and fattening pigs at the worldwide (10). In addition, PMWS is considered an immunosuppressant disease, thence facilitates the infection with opportunist pathogens affecting the swine herd health status (11).

The diagnosis of PMWS is difficult and must follow three criteria: (i) the presence of compatible clinical signs, (ii) the presence of characteristic microscopic histopathological lesions, and (iii) the presence of PCV2 within these lesions in moderate or high amounts (10,12). The presence of PCV2 DNA or antigen in lymphoid tissues must be demonstrated by *in situ* hybridization and immunohistochemical methods (10,12,13).

In spite of that *in situ* hybridization has recently become increasingly important in diagnostic procedures (14,15,16), the use of this technique remains restricted to a few diagnostic laboratories because of its greater technical complexity and expense compared to other diagnostic tools. In the case of the immunohistochemical although a certain expertise is required, this is a cost effective and easy method that could be implemented in several laboratories for the PMWS diagnostic. Nevertheless, one problem concerning the immunohistochemistry methods for the PMWS diagnostic is the lack of a commercial anti-PCV2 peroxidase conjugate, for this reason, several laboratories has developed its own anti-PCV2 conjugate for this purpose (17,18). Therefore, the aim of this work was to obtain a polyclonal-antibody-immunoperoxidase-conjugate for the PCV2 specific detection.

MATERIAL AND METHOD

Virus and cell lines

The Cuban PCV2 field isolate identified as PNE1 (genome sequence deposited in GenBank under the accession number FM999737) from CENSA viral collection (19) was propagated as follows: the PK15A cell line monolayer was trypsinised and resuspended to contain 5x10⁴ cells/mL in growth medium (Dulbecco's modified Eagle's medium, DMEM) supplemented with 10% foetal bovine serum (Gibco, free from virus) and 100 µg/ml gentamicin. Three-millilitres of the virus propagated was added to 20 mL of this cell suspension and seeded in 10 mL volumes into two 25 cm² cell culture flasks. After 18 h incubation at 37°C the resulting semi-confluent monolayers were treated with 300mM D-glucosamine in Hanks basal salt solution for 30 min and the virus/cells were passaged.

Nucleic acid isolation and polymerase chain reaction

Total DNA was extracted from 100 µl of each sample (10% tissue homogenate), with Wizard® Genomic DNA Purification Kit, (*Promega, Madison, WI, USA*) following manufacturer instructions. DNA was diluted in 10 µl of nuclease free water (*Promega, Madison, WI, USA*). To detect PCV2 DNA in order to evaluate the status of PCV2 infections in pigs, a polymerase chain reaction (PCR) assay was carry out as described by Sandvik et al., (2001) (20), using the primers PCV2-2A: 5'-CACCTTCGGATATACTGTCAA-3' and PCV2-2B: 5'-TACATGGTTACACGGATA TTGTA-3'. The primer pair targeted an amplicon of 501 bp of the ORF2. Briefly, the amplification reaction was carried out in a volume of 50 µl comprised of 2 µl of DNA sample (extracted as described above), 1x GoTaq® Flexi DNA Polymerase (*Promega, Madison, WI, USA*) [200uM of each dNTP, 1.5mM MgCl₂ (pH 8.5)] and 1 µM of each primer. The PCR reaction was done under the conditions described by Sandvik *et al.* (20) in a thermal cycler (*Eppendorf Mastercycler*). The amplicons (10 µl) were visualized by electrophoresis on 2.0% agarose gel in TBE buffer (90mM Tris-borate, 2 mM EDTA) ethidium bromide stained.

Polyclonal antiserum

A rabbit polyclonal antiserum was produced at the Institute, briefly: two *Chinchilla* rabbits were intradermic immunized with a total 8ml of a killed PCV1-2 chimeric vaccine (*Suvaxyn® PCV2 One Dose™; Fort Dodge Animal Health, Inc., Fort Dodge, IA, USA; Lot number 1861133A*) delivered in 10 doses on the back

in multiple sites close to the neck and groin. A booster inoculation was carried out 3 weeks later at same inoculum and via. In order to improve affinity of the antiserum a third inoculation at 4 weeks after a second inoculation was performed. The animals were monitored; blood samples were taken before each immunization and evaluated on PK15 monolayer infected with PCV2 by an immunoperoxidase monolayer assay described by Fort *et al.* (21). Rabbits were euthanized, and serum was harvested 2 weeks after the final immunization and anti-PCV2 antiserum was assessed.

IgG purification and conjugated

The immunoglobulin using a protein A-Sepharose (Amersham, Pharmacia) column chromatography were purified following standard protocols for this method (22). The concentration of the immunoglobulin purified was calculated using the expression [1]. A total of 10 mg/mL of immunoglobulin purified was conjugated with peroxidase (Peroxidase from horseradish Type XII, Sigma) using the oxidation with periodate method (23) and the conjugation ratio (IgG/peroxidase) was calculated.

$Conc(mg/mL) = O.D_{280} \cdot \text{dilution factor} \cdot \text{extinction coef}$
 $IgG(\text{rabbit}) \text{ expression [1]}$

Specificity of the anti-PCV2 conjugate

The specificity of the anti-PCV2 conjugated was evaluated using PK15A cell line infected with the PCV2 isolated E1 and the PK15 cell line persistently infected with PCV1. A immunoperoxidase monolayer assay as described by Fort *et al.*, 2007 (21) was carry out and the presences of PCV2 and PCV1 in PK15A and PK15 cell lines was checked thorough a PCR assay described by Sandvik *et al.* (20) and Fenaux *et al.* (24) respectively.

RESULTS

Obtaining the anti-PCV2-peroxidase conjugate

Two fraction of IgG total corresponding to a concentration of 24mg/mL each were collected. The anti-PCV2-peroxidase conjugated was obtained and the IgG/peroxidase ratio showed a value of 0.4. The obtained conjugated was mixed with 1% of bovine serum albumin (BSA) and a final mix (v/v) glycerol/anti-PCV2-peroxidase conjugated was conserved at -20°C.

Test of specificity of the anti-PCV2 conjugate

The presence of PCV2 E1 isolated in PK15A infected cell lines was verified. Likewise the absence

of PCV2 in PK15 persistently infected with PCV1 was tested, (figure1). (In this same sense the presence of PCV1 in persistently infected with PCV1 was make sure (data not shown)). The anti-PCV2-peroxidase conjugate obtained when a dilution 1/40 was used revealed a specific reaction in PCV2 infected cell and a lack of color in PK15 PCV1 infected cell was observed, figure 2. Hence, the anti-PCV2-peroxidase conjugate obtained showed to be specific in the PCV2 detection.

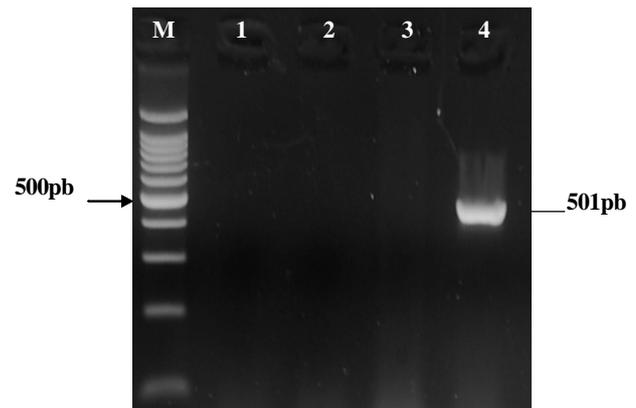


FIGURE 1. PCR for PCV2 specific detection in PK15 and PK15A cell lines culture. M- Molecular weight marker 100pb (Promega). Lane 1- PK15 cell line persistently infected with PCV1 and PCV2 non-inoculated; lane 2- PK15A cells free to PCV1 and PCV2 non-inoculated; lane 3- PCV2 first passage in PK15 cells; lane 4- PCV2 second passage in PK15 cells./ *Marcador peso molecular (Promega) Línea 1 línea celular PK15 persistentemente infectada con PCV1 y PCV2 no inoculada, Línea 2 células PK15A libres de PCV1 y no inoculadas con PCV2 ; Línea 3 primer pase de PCV2 en PK15; Línea 4 segundo pase de PCV2 en células PK15.*

DISCUSSION

In this work an anti-PCV2-peroxidase conjugated for the PCV2 specific detection was obtained.

The PCV2 is considered an emerging pathogen, causing economically important diseases worldwide (25,26). This virus reported by Meehan *et al.*, 1998 as a novel circovirus (27) has nucleotide identity of 66% concerning to the proteins encoded by ORF2 to be compared with PCV1 (28), thence these two viral agent can be differentiated using polyclonal antibodies against the capsid protein (18).

The fact that for the PMWS diagnosis the presence of the PCV2 within these lesions in moderate or high

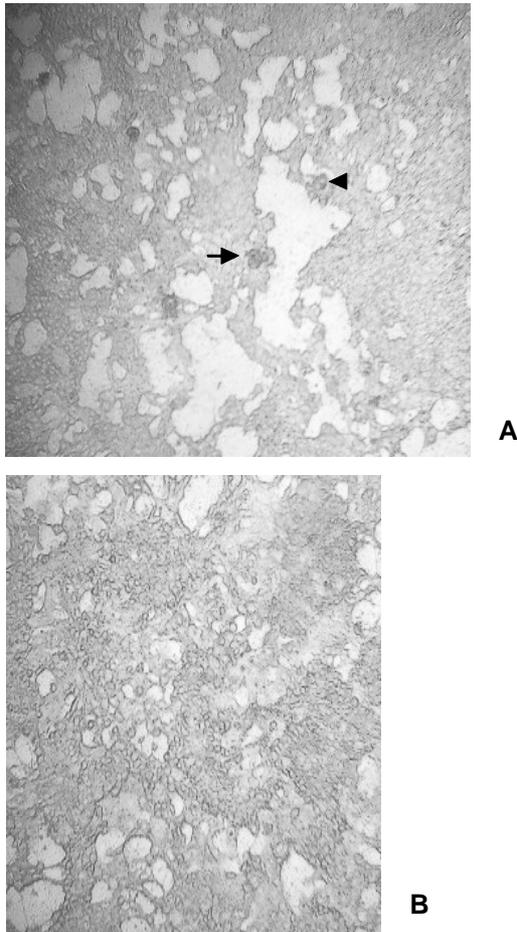


FIGURE 2. Specificity of anti-PCV2-peroxidase conjugate obtained. A) PK15A cell line infected with PCV2 E1 isolated. B) PK15 persistently infected with PCV1 and PCV2 non-inoculated. Black arrows indicate cell infected with PCV2 E1 isolated. /A) célula PK15A infectada con aislado PCV2 E1. B) PK15 persistentemente infectada com PCV1 y no inoculada con PCV2. Las flechas negras indican las células infectadas con el aislado PCV2 E1.

amounts of the virus must be demonstrated by immunohistochemical methods (10,12) as well as the lack of commercial anti-PCV2-peroxidase conjugate imply the requirement by the laboratories involved in the PMWS diagnosis to develop their own anti-PCV2-peroxidase conjugate.

The use of the killed PCV1-2 chimeric vaccine for obtaining polyclonal antibodies against PCV2 protein capsid described here is a useful strategy that could be assumed for those laboratories with problems in cell cultures technologies or because of the complicated PCV2 isolation and multiplication, hence to obtain enough viral amount to be used as immunogenic inoculation will be a very difficult task.

The PCV2 infection and replication in cell culture occur to very low titer of the virus (29), thus with the purpose of increasing the viral amount different strategies have been described (29,30). Nevertheless, the fact that the PCV2 multiplication in cell culture is carried out to very low amount of the virus suggests that the anti-PCV2-peroxidase conjugate obtained has a high sensitivity and could be able to distinguish between subclinical PCV2 infection and PMWS condition.

The high sensitivity and specificity of the anti-PCV2-peroxidase conjugate obtained in this work will allow perform the PMWS diagnosis in the future. Nevertheless a further evaluation of this conjugate in tissues samples will be needed to carry out.

In summary, in this paper we described the anti-PCV2-peroxidase conjugate obtainment based on the use of the available commercial vaccine against PCV2 as immunogenic inoculation to produce a polyclonal antibody in rabbits. The obtained conjugate was able to discriminate between PCV2 and PCV1 infections and a high sensitivity of the conjugate was observed.

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