Biologicals 41 (2013) 430–434

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

The serological evidence in humans supports a negligible risk of zoonotic infection from porcine circovirus type 2

Peter D. Burbelo [a,*], Jack A. Ragheb [b], Amit Kapoor [c], Yanjin Zhang [d]

[a] Clinical Dental Research Core, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892, USA
[b] Division of Therapeutic Proteins, OBP/OPS, CDER, FDA, Bethesda, MD 20892, USA
[c] Center for Infection and Immunity, Columbia University, New York, NY 10032, USA
[d] Department of Veterinary Medicine, University of Maryland, College Park, MD 20742, USA

ARTICLE INFO

Article history:
Received 28 June 2013
Accepted 17 September 2013

Keywords:
Antibodies
Porcine circovirus
PCV-2
Seroepidemiology
Porcine-derived supplements

ABSTRACT

There are two porcine circovirus (PCV) genotypes, PCV-1 and PCV-2. In pigs, PCV-1 infection is asymptomatic but PCV-2 infection can cause severe respiratory disease and other pathology. Although humans ingest PCV-contaminated foods and are exposed to PCV through other sources, the potential of PCV-2 as a zoonotic agent in humans and other species has not been fully explored. Here, four recombinant proteins derived from the PCV-2 capsid gene were examined as antigens using the Luciferase Immunoprecipitation System (LIPS) assay for serological analysis of PCV-2 infection. PCV-2-CAP-Δ1 was the optimum recombinant protein in the LIPS assay with a sensitivity of 93% and specificity of 100% using porcine samples. Testing of healthy human blood donors, equine and bovine serum samples failed to demonstrate the presence of anti-PCV-2 antibodies. Additionally, analysis of two high-risk human groups, cystic fibrosis patients taking porcine derived oral supplements and type I diabetes patients who had undergone porcine islet cell transplantation, showed no evidence of anti-PCV-2 antibodies. These results extend the extensively demonstrated use of LIPS as a robust approach for identifying humoral responses and provide evidence that PCV-2 is likely not infectious in humans.

Published by Elsevier Ltd on behalf of The International Alliance for Biological Standardization.

1. Introduction

Porcine circoviruses consist of two distinct genotypes known as PCV-1 and PCV-2, which are small, circular, single-stranded DNA viruses of less than 2 kb in size [1]. Both the genomes of PCV-1 and PCV-2 encode only two major open-reading frames, the capsid and the RNA-dependent RNA polymerase. Comparison of the PCV-1 and PCV-2 capsids demonstrates that they have approximately 65% nucleotide identity and 63–68% amino acid identity [2]. PCV-1 was originally discovered as a non-pathogenic contaminant of a porcine kidney cell line [3]. In pigs, PCV-1 infection is asymptomatic, but PCV-2 is pathogenic, causing respiratory disease, nephropathy syndromes, and enteric disease that can lead to death [4]. The exact reason for the differences in pathogenicity between PCV-1 and PCV-2 are not fully understood [1].

Besides pigs, the potential host range of porcine circoviruses is not known. Metagenomic DNA analysis has detected PCV-1 and PCV-2 in a variety of human food products including pig and beef meat [5]. PCV DNA has also been detected in human feces [5] and is found in raw sewage [6]. Using deep sequencing, Delwart and colleagues recently identified PCV-1 contamination in several batches of Rotarix, a childhood vaccine for Rotavirus [7]. PCV-1 and PCV-2 contamination in Rotarix has been confirmed by other research groups [8–10] and in one study the vaccine associated PCV-1 was shown to infect cultured porcine kidney cells [10]. Serologic studies examining potential PCV infection in humans have yielded conflicting results. In one report, low levels of PCV antibody immunoreactivity was detected in humans by immunofluorescence, Western blotting and ELISA [11]. However, two other studies found no serologic evidence of PCV-2 infection in humans [12,13].

While antibodies against infectious agents are routinely detected using ELISA and Western blotting, these technologies suffer from high backgrounds, low signals, and often miss conformational epitopes. The Luciferase Immunoprecipitation Systems (LIPS), a highly robust technology that employs recombinant light-emitting proteins for detecting antibodies in a liquid phase assay, has been developed to address these deficiencies [14]. LIPS offers several advantages over ELISA, including the production of antigens in mammalian cells with low backgrounds and a large dynamic range

* Corresponding author. 10 Center Drive, Bldg. 10, Rm. 1N102, National Institutes of Health, Bethesda, MD 20892, USA.
E-mail address: burbelop@nidcr.nih.gov (P.D. Burbelo).

1045-1056/$36.00 Published by Elsevier Ltd on behalf of The International Alliance for Biological Standardization.
http://dx.doi.org/10.1016/j.biologicals.2013.09.005

of detection that often spans several orders of magnitude [14,15]. Here we report the development of a LIPS test for detecting antibodies against the PCV-2 capsid protein and its qualification using porcine serum samples. Using this serological assay, detection of antibodies against the PCV-2 capsid was also explored in other animal and human serum samples.

2. Material and methods

2.1. Serum samples

Porcine serum samples were provided by Synbiotics (Exton, PA). These porcine serum samples ($n = 46$) were previously tested by SERELISA® PCV2 Ab Mono Blocking ELISA (Synbiotics). Additional horse ($n = 20$) and bovine samples ($n = 20$) were obtained as residual samples collected for diagnostic or commercial use. No other sample identifiers, except that all animals were living in the New York State area, were provided for these samples. All serum samples were stored at −80 °C, thawed, and then left at 4 °C for less than a two week prior to processing by LIPS.

Three types of human samples were also analyzed for anti-PCV-2 antibodies. All were anonymized samples obtained under IRB approved protocols at the National Institutes of Health (#99-CC-0168) or the Food and Drug Administration (#08-0868D), Bethesda, Maryland. One group of human serum samples was from adult healthy blood donors ($n = 40$), and another from cystic fibrosis patients ($n = 37$) who chronically ingested porcine derived pancreatic supplements. The third group consisted of nine patients who had been transplanted with porcine β-cell islets as part of a therapeutic clinical trial [16].

2.2. Generation of Renilla luciferase PCV-2 capsid fragments

Based on the PCV-2 capsid sequence, several different constructs were generated by PCR. The primer adapter sequences used to clone each protein coding region are as follows: Full length PCV-2-CAP-FL, 5′-GAGGGATCCACGTATCCAAGGAGGCGT-3′ and 5′-GAGC TCGAGCATTTAGGGTTTAAGTGG-3′; PCV-2-CAP-Δ1 5′-GAGGGATC-CAGGAAAAAT GGCA TCTTC-3′ and 5′-GAGCTCGAGCATTTAGGGTT-TAAGTGG-3′; PCV-2-CAP-Δ2, 5′-GAGGGATCCACCCGCCTCT CCCGCACC-3′ and 5′-GAGCTCGAGCATTTAGGGT TTAAGTGG-3′; and PCV-2-CAP-Δ3, 5′-GAGGGATCCCAGCTTTGGCTGAGGCTA-3′ and 5′-GAGCTCGAGCATTTAGGGTTTAAGTGG-3′. The four PCV capsid fragments were subcloned downstream of *Renilla* luciferase using the pREN2 vector [15] and the endogenous stop codon was included at the end of the capsid coding sequence. The plasmid DNA was then prepared using a Qiagen Midi preparation kit. DNA sequencing was used to confirm the integrity of the four different fragments.

Cos-1 cells were cultured at 5% CO_2, 37 °C with DMEM supplemented with 10% FCS. FuGene-6 or XtremeGene was used for transfection of the different *Renilla* luciferase PCV-2 capsid fusion constructs into Cos-1 cells according to the manufacturer's instructions (Roche, Indianapolis, IN). Cell extracts were obtained 48 h post-transfection in 1.0 ml of lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 50% glycerol and protease inhibitors). The lysates were centrifuged twice at 12,500 g, supernatants collected and used at the time of preparation. The activities of the lysates in light units (LU)/μl were determined using a tube luminometer (20/20 from Turner Scientific) with a coelenterazine substrate mix (Promega, Madison, WI).

2.3. LIPS assay

A standard LIPS assay protocol in a 96-well format at room temperature was used to test all the serum samples [17]. Briefly, serum samples were first diluted 1:10 in assay buffer A (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100) using a 96-well polypropylene microtiter plate. Antibody levels were measured by adding 40 μl of buffer A, 10 μl of diluted sera (1 μl equivalent), and 1×10^7 LU of each of the Ruc-PCV-2 capsid antigens containing crude Cos-1 cell extract to wells of a polypropylene plate and incubated for 60 min at room temperature on a rotary shaker. Next, 5 μl of a 30% suspension of Ultralink protein A/G beads (Pierce Biotechnology, Rockford, IL) in PBS were added to the bottom of each well of a 96-well filter HTS plate (Millipore, Bedford, MA). To this filter plate, the 100 μl antigen–antibody reaction mixture was transferred and incubated for 60 min at room temperature on a rotary shaker. The washing steps of the retained protein A/G beads were performed on a Biomek Workstation or Tecan plate washer with a vacuum manifold. After the final wash, LU were measured in a Berthold LB 960 Centro microplate luminometer (Berthold Technologies, Bad Wilbad, Germany) using coelenterazine substrate mix. All LU data were obtained from the average of at least two separate experiments. For the porcine and human samples, the raw LU values were directly used for analysis. For the bovine and equine samples, which were all below the cut-off, the presented values were normalized using the buffer blanks.

2.4. Data analysis

GraphPad Prism software (San Diego, CA) was used for analysis and plotting of the data as well as for statistical analysis. For the calculation of sensitivity and specificity, the results obtained with the anti-PCV-2 ELISA from Synbiotics was used as the gold-standard comparator. The cut-off values for calculating seropositivity for both capsid fragments was calculated using the mean plus 2 standard deviation of the PCV-2 seronegative samples and matched that of a cut-off determined by receiver operator characteristics (ROC) analysis. The Mann–Whitney *U* test was used to test the statistical significance of the difference in antibody levels between PCV-2 positive and PCV-2 negative porcine samples.

3. Results

3.1. Expression of Renilla luciferase-PCV-2 capsid fusion proteins

Alignment of a representative PCV-1 capsid sequence with the sequence of the PCV-2 capsid template used in this study demonstrates that they show approximately 66% identity and 77% amino acid similarity (Fig. 1). In order to potentially detect antibodies against the capsid of PCV-2 by LIPS, a full length and three progressive N-terminal deletion mutants of the capsid were generated and fused with the C-terminus of *Renilla* luciferase (Fig. 1). Following transfection of each of these constructs into Cos-1 cells, cell extracts were prepared and tested for *Renilla* luciferase enzymatic activity, which is a surrogate marker for production of the different recombinant proteins, as described in the Material and Methods. Only the two largest capsid constructs, PCV-2-CAP-FL and PCV-2-CAP-Δ1 (missing the first 39 amino acids), demonstrated high levels of luciferase activity with values of 3 million LU/μl and 500,000 LU/μl, respectively. The two smaller fusion proteins, PCV-2-CAP-Δ2 and PCV-2-CAP-Δ3 showed low levels of activity having values of approximately 60,000/μl and were not studied further.

3.2. Identification of anti-PCV-2 capsid-specific antibodies in porcine serum samples

Using extracts of the PCV-2-CAP-FL and PCV-2-CAP-Δ1 *Renilla* luciferase fusion proteins, porcine serum samples were tested by LIPS and compared to the results obtained using the gold standard.