Exposure of Piglets to Enteroviruses Investigated by an Immunoassay Based on the EV-G1 VP4 Peptide

Mehdi A. Benkahla^a Famara Sane^a Rachel Desailoud^b Didier Hober^a

^aUniversité de Lille, CHU de Lille, Laboratoire de Virologie/EA3610, Lille, and ^bUPJV, CHU d'Amiens, Service d'Endocrinologie-Diabétologie-Nutrition, Amiens, France

Key Words

Pig · Serum · Enterovirus · ELISA · VP4

Abstract

Objective: The aim of this study was to investigate the exposure of piglets to enteroviruses-G (EV-G) through the presence of antibodies in their serum. **Methods:** Serum samples were obtained from the vena cava of 10 piglets at 9 weeks of age and again 39 days later (day 39). They were tested using an immunoassay based on the EV-G1 VP4 peptide, since VP4 is highly conserved among the four *Enterovirus* capsid proteins, and by using a seroneutralization assay. **Results:** For each serum collected on day 39 the optical density was high compared to the value obtained in serum collected earlier ($p = 0.002$). However, the titers of anti-EV-G1 serum neutralizing activity were not different in paired samples ($p > 0.999$). The sequence alignment of the EV-G1 VP4 peptide, encompassing 50 amino acids, used in the immunoassay showed 88% homology with EV-G, suggesting that antibodies directed toward other EV-G than EV-G1 may be detected. **Conclusion:** An immunoassay based on EV-G1 VP4 can detect an increased level of EV-G antibodies in piglet serum samples. Further studies are needed to determine whether this immunoassay may be useful for diagnosis and/or epidemiological studies and to monitor EV-G infection in pigs to evaluate strategies aimed to prevent enterovirus infections.

Introduction

The genus *Enterovirus* of the *Picornaviridae* family encompasses a wide range of human and animal viruses divided into 12 species [1]. The porcine enteroviruses were segregated into 13 serotypes on the basis of a virus neutralization assay [2, 3]. These serotypes were classified into 3 groups on the basis of the nature of the cytopathic effect: CPE group I (serotype 1–7 and 11–13), CPE group II (serotype 8) and CPE group III (serotypes 9 and 10) [2]. This classification has been revised based on a new genetic analysis [4] and the porcine enteroviruses have been split into three genera among the *Picornaviridae* family, the genus *Teschovirus* that consists of 10 serotypes, porcine teschovirus 1–7 (previously referred to as porcine enterovirus 1–7) and porcine teschovirus 8–10 (previously referred to as porcine enterovirus 11–13). The porcine enterovirus 8 classified as porcine enterovirus A has been reclassified as a new genus, the *Sapelovirus*, which currently consists of 3 species. The last serotypes, PEV-9 and PEV-10, belong to the species porcine enterovirus B, which has been renamed enteroviruses-G (EV-G) [1].

The EV-G are small nonenveloped viruses with a positive-sense ssRNA protected by an icosahedral capsid. EV-G share the same genetic organization as human enteroviruses [5]. The genome is approximately 7,400 nucleotides in length, with a single open reading frame,

© 2016 S. Karger AG, Basel

KARGER

© 2016 S. Karger AG, Basel
0300-5526/16/0592-0069\$39.50/0

E-Mail karger@karger.com
www.karger.com/int

D. Hober
Université de Lille, CHU de Lille
Laboratoire de Virologie/EA3610
FR-59000 Lille (France)
E-Mail didier.hober@chru-lille.fr

flanked by 5' and 3' UTRs, translated in 7 nonstructural proteins and 4 structural proteins, VP1, VP2, VP3 and VP4 [6]. EV-G VP4 is a highly conserved protein [7, 8].

EV-G are ubiquitous and natural infection occurs through the ingestion of infected feces. Infections are usually asymptomatic but some strains have been associated with diseases such as polioencephalomyelitis [9].

Pigs are characterized by a typical epitheliochorial placenta, where maternal and fetal blood are separated by six tissue layers [10] forming a thick barrier preventing even maternal antibodies from passing to the fetus during gestation [11]. Thus, the antibodies detected in newborn piglet blood are de novo synthesized following an in utero infection.

Newborn piglets are protected by active immunity as a result of an in utero infection or passive immunity acquired from the colostrum or milk until 4–9 weeks of age [12]. After weaning the piglets are exposed to EV-G.

The exposure to pathogens is reflected by the increase in antibody levels. Our team has previously reported the detection of anti-VP4 antibodies directed toward coxsackievirus B4, a human pathogen belonging to EV-B species of the *Enterovirus* genus. An immunosorbent assay based on CVB4 VP4 and peptides has been used to detect anti-VP4 antibodies in the serum of individuals [13, 14].

Based on our previous studies regarding the detection of anti-VP4 antibodies in human EV-B infection, we decided to grasp the opportunity of access to serum samples of piglets in order to evaluate whether they have been in contact with enteroviruses through the detection of antibodies by using an immunoassay based on EV-G1 VP4.

Materials and Methods

Cells

PSEK cells were kindly provided by Dr. Antonio Lavazza (IZSLER, Brescia, Italy). The cell line was grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% inactivated fetal calf serum, penicillin (100 U/ml)-streptomycin (100 mg/ml) and 1% L-glutamine (Invitrogen, Saint Aubin, France).

Virus

The EV-G1 kindly provided by Dr. Antonio Lavazza was grown in PSEK cells. Virus titration was determined on PSEK cells using end-point dilutions, and the titer was determined by the Spearman-Kärber statistical method.

Serum Samples

All experiments were conducted following the 2010 EU directive guidelines and were approved by the ethical committee for animal experimentation. A group of 10 female Danish Landrace ×

Pietrain pigs were supplied to the test facility at CODA-CERVA, Machelen, Belgium at 9 weeks of age. The animals underwent blood sampling from the vena cava with dry tubes on day 1 and day 39. Sera obtained following centrifugation were stored at −20°C.

Virus Neutralization Assay

The antiviral activity of porcine serum samples was assessed by a plaque neutralization assay. Serial 3-fold dilutions of 50 µl of each sample were mixed with equal volumes of DMEM medium in 96-well microtiter plates, then 25 µl of viral suspension containing 10⁴ TCID₅₀/ml of EV-G1 were added to each well. After 2 h at 37°C in a humid atmosphere containing 5% CO₂, 100 µl of DMEM containing 1.10⁵ PSEK cells were added to each well, and the plates were incubated for 48 h. The results were expressed as the inverse of the final dilution (titer) that inhibited the viral cytopathic effect.

ELISA for Anti-VP4 Antibody Detection

To detect anti-VP4 antibodies in piglet serum samples, 96-well microtiter plates (Nunc-Immuno Module F8 MaxiSorp; Thermo Scientific, Roskilde, Denmark) were coated overnight at room temperature with a VP4 peptide from EV-G1 encompassing 50 amino acids (Bachem, Bubendorf, Switzerland) at 10 µg/ml in PBS, pH 7.4. Wells were then washed three times with washing solution (PBS 1×, pH 7.4, 0.05% Tween 20), saturated for 1 h at 37°C with saturation buffer (PBS, pH 7.4, 1% BSA, 0.0375% Tween 20) and washed again four times with the washing solution. Afterwards, 0.1 ml of serum sample diluted in buffer (PBS 1×, pH 7.4, 2% BSA, 0.0375% Tween 20, 0.3 M NaCl) at 1/50 was added to the micro-wells. After 1 h of incubation at 37°C, the wells were washed four times and 0.1 ml of biotin-linked rabbit anti-wild boar IgG (USCN Life Science Inc.) diluted to 1/100 was added for 1 h at 37°C. After six washing cycles, 0.1 ml of Extravidin peroxidase (Sigma-Aldrich) was added for 1 h at 37°C. After another six washing cycles, 0.1 ml of the substrate solution (0.4 mg/ml o-phenylenediamine, 0.012% H₂O₂ in 0.05 M phosphate citrate buffer, pH 5.0; Sigma-Aldrich, Saint Quentin Fallavier, France) was added for 30 min at room temperature. The reaction was stopped by the addition of 25 µl of 3 M sulfuric acid (Sigma-Aldrich). Absorbance readings were measured at 490 nm in a Dynex MRX microplate reader (Thermo Life Science, Cergy Pontoise, France). The results are expressed as the optical density (OD).

Sequences

The sequence of the VP4 peptide used in the ELISA and in the in silico study was selected from the polyprotein sequence of EV-G1 (GenBank Protein accession No. AIA21703). Amino acid sequences of the VP4 and, if not available, of the polyprotein of EV-G1, EV-G2, EV-G3, EV-G5, EV-G6, EV-G and of *Teschovirus* and *Sapelovirus* used for the alignment were collected from GenBank.

Alignments

The protein sequences were aligned using the web version of Clustal Omega.

Statistical Analysis

Statistical analysis of the serology results was performed by the Wilcoxon paired t test (GraphPad quickCalcs). Differences were considered to be statistically significant at $p < 0.05$.

Fig. 1. Individual representation of anti-EV-G1 VP4 IgG detected by ELISA (a) and anti-EV-G1 neutralizing activity by seroneutralization assay (b) in serum samples from 10 pigs collected at 9 weeks of age (day 1) and 39 days later (day 39).

Results

The blood from 10 pigs was collected successively at 9 weeks of age (day 1) and 39 days later (day 39). Serum was obtained from these samples and the levels of anti-VP4 antibodies were determined by ELISA. A moderate increase in OD was observed in the paired serum samples of pig number 2 (0.70 vs. 0.53 on day 1 and day 39, respectively), whereas a higher increase in OD was observed in the paired serum samples of pig number 5 (1.11 vs. 0.33). Overall, in each pig the OD values were higher in the second serum samples than in the first (fig. 1a). The difference in OD values obtained between day 1 and day 39 in the paired samples of 10 pigs in our study was statistically significant ($p = 0.002$).

The detection of increased OD values in serum obtained on day 39 compared with day 1 by using our assay based on EV-G1 VP4 peptide-coated plates prompted us to investigate the neutralizing activity of these serum samples against EV-G1. In 7 out of 10 serum samples obtained on day 1 the anti-EV-G1 neutralizing activity titers were 8, while in the 3 others the titers were 32 or 16 (pigs 5, 6 and 10). In paired serum samples obtained on day 39, the titer values were almost unchanged (one dilution only; fig. 1b). The difference in titer values obtained between day 1 and day 39 in the paired samples of 10 pigs in our study was not statistically significant ($p > 0.9$).

There was no relationship between the pattern of values obtained by immunoassay based on EV-G1 VP4 and by neutralizing assay based on EV-G1 in our experiments.

Since VP4 is the most conserved protein among the four enteroviral capsid proteins, it cannot be excluded that an immunoassay based on EV-G1 VP4 allows the detection of antibodies directed toward the VP4 protein of other porcine enteroviruses. Thus, to investigate this hypothesis further, available sequences of EV-G1-4 and EV-G6 were collected from GenBank and the VP4 sequences were compared using Clustal Omega software.

The alignment of sequences revealed that 44 residues of the VP4 sequence of various EV-G and of the VP4 sequence of EV-G1 were similar (88% homology), encompassing 37 identical residues (74% identity; fig. 2a). In contrast the level of homology between the VP4 sequence of EV-G1 and those of pig viruses belonging to the *Teschovirus* and *Sapelovirus* genus was much lower. There were only 15 similar residues (30% homology) encompassing 6 identical residues only (12% identity; fig. 2b).

Discussion

The current study is different in many respects from those of other investigators. It is reported for the first time that an ELISA based on EV-G VP4 can detect an increased level of IgG antibodies in serum samples from consecutive piglets collected at 9 weeks of age and then 39 days later.

Several considerations in the present report are noteworthy. The immunoassay displayed a difference between the levels of anti-EV-G1 VP4 antibodies in paired

VP4 peptide	MGMQMSKNVAGSHTTVTKASGGSNIIYYTNVNYNHSASASQNKQDVTQDP
EV-G1	T.....Q.....H.N...I...S.....I....
EV-G2	..A.V...T.....G.Y.T....H...I...S.....LA...
EV-G3	Q.T....H.....IQ...
EV-G4	Q.....T.K...I...S.....I....
EV-G6	Q.TN..K.H...I.....I....
Enterovirus G	T.....Q.....H.N...I...S.....I....
a	** *:***.***** * *: ** * ***:***.*****: ***
VP4 peptide	MGMQMSKNVAGSHTTVTKASGGSNIIYYTNVNYNHSASASQNKQ-----DVTQDP
<i>Sapelovirus</i>	LGQVHSSNT--GNKPQGAYNHG--SGSITQVNYYGSDYSQAWNPT-----QQQMDP
<i>Teschovirus</i>	---SSRMENG---NTNNSG--NTGVINYNFYSNSYTDVLDLGMSSQESNAENAASGP
b	* * : * . : * * . : : : : *

Fig. 2. Bioinformatics analysis of the variability in amino acid residues of VP4 capsid proteins of EV-G1–4 and EV-G6 (**a**) and *Teschovirus* and *Sapelovirus* (**b**) compared with the VP4 peptide sequence of EV-G1, encompassing 50 amino acids, used as an antigen in ELISA. * = residue fully conserved; : = residue with strongly similar properties; . = residue with weakly similar properties.

serum samples, whereas in contrast the titers of the anti-EV-G1 neutralizing activity of paired serum samples were not different. It can be assumed that anti-VP4 antibodies had no neutralizing activity or that the levels were too low to be detected in our biological assay. Furthermore, this pattern of results is in agreement with the statement that neutralizing anti-EV antibodies are directed towards VP1 rather than VP4 [15].

The increased levels of anti-VP4 antibodies in serum samples in our studies suggest that the piglets were exposed to EV-G after weaning. The negative detection of anti-EV-G1 neutralizing activity of serum does not exclude a possible infection with this serotype accompanied by a low level of neutralizing antibodies. Nevertheless, it can be hypothesized that anti-VP4 antibodies detected by ELISA are directed toward other EV-G than EV-G1 since the VP4 sequence is strongly conserved within the EV-G species. Indeed, as shown by sequence alignment, EV-G VP4 shares 88% homology (74% identity) of amino acids. In contrast, the detection of antibodies directed against the VP4 of viruses belonging to other genera of the Picornaviridae family, such as *Sapelovirus* and *Teschovirus*, is unlikely since the VP4 of these viruses that can infect pigs shares only 30% homology (12% identity) with the VP4 peptide used in our assay.

The exposure of piglets to enteroviruses can be reflected by an increased level of antibodies in serum samples. The detection of antibodies by ELISA is rapid and requires a small volume of blood only. Further studies are needed to determine whether an ELISA based on EV-G

VP4 peptide may be used for diagnosis purposes and/or epidemiological studies. Interestingly, EV-G infection in pigs is a relevant model with which to investigate strategies aimed to prevent natural enterovirus infections. The detection of anti-EV-G VP4 antibodies by ELISA could be useful to monitor enterovirus infections in such protocols. Future studies will be directed along this line in our laboratory.

Acknowledgements

This study was supported by Ministère de l'Education Nationale, de l'Enseignement supérieure et de la Recherche, Université Lille 2 (Equipe d'Accueil 3610), France, and Centre Hospitalier Regional et Universitaire de Lille.

M.A.B. is supported by an Erasmus Mundus Action 2 Fatima Al Fihri 2012–2602 scholarship. The authors thank Geert Vandenbossche and Eddy Rommel (Univac, Belgium) for providing pig serum samples, and Dr. Antonio Lavazza (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, IZSLER, Brescia, Italy) for providing PSEK cells and EV-G1.

References

- 1 King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ: Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Amsterdam, Elsevier, 2012, pp 850–880.
- 2 Knowles NJ, Buckley LS, Pereira HG: Classification of porcine enteroviruses by antigenic analysis and cytopathic effects in tissue culture: description of 3 new serotypes. Arch Virol 1979;62:201–208.

- 3 Auerbach J, Prager D, Neuhaus S, Loss U, Witte KH: Grouping of porcine enteroviruses by indirect immunofluorescence and description of two new serotypes. *Zentralbl Veterinarmed B* 1994;41:277–282.
- 4 Kaku Y, Sarai A, Murakami Y: Genetic reclassification of porcine enteroviruses. *J Gen Virol* 2001;82:417–424.
- 5 Boros Á, Nemes C, Pankovics P, Bíró H, Kapusinszky B, Delwart E, et al: Characterization of a novel porcine enterovirus in wild boars in Hungary. *Arch Virol* 2012;157:981–986.
- 6 Pallansch MA, Oberste MS, Whitton JL: Enteroviruses: Polioviruses, Coxsackieviruses, Echoviruses and newer Enteroviruses; in Knipe DM, Howley PM (eds): *Fields Virology*, ed 6. Philadelphia, Lippincott Williams & Wilkins, 2013, pp 490–531.
- 7 Oberste MS: Comparative genomics of the coxsackie B viruses and related enteroviruses. *Curr Top Microbiol Immunol* 2008;323:33–47.
- 8 van Dung N, Anh PH, van Cuong N, Hoa NT, Carrique-Mas J, Hien VB, et al: Prevalence, genetic diversity and recombination of species G enteroviruses infecting pigs in Vietnam. *J Gen Virol* 2014;95:549–556.
- 9 Yang S, Wang Y, Shen Q, Zhang W, Hua X: Prevalence of porcine enterovirus 9 in pigs in Middle and Eastern China. *Virol J* 2013;10:99.
- 10 Karniychuk UU, Nauwynck HJ: Pathogenesis and prevention of placental and transplacental porcine reproductive and respiratory syndrome virus infection. *Vet Res* 2013;44:95.
- 11 Šterzl J, Rejnek J, Trávníček J: Impermeability of pig placenta for antibodies. *Folia Microbiol* 1966;11:7–10.
- 12 Singh KV, Bohl EH: The pattern of enteroviral infections in a herd of swine. *Can J Comp Med* 1972;243–248.
- 13 Chehadeh W, Lobert PE, Sauter P, Goffard A, Lucas B, Weill J, et al: Viral protein VP4 is a target of human antibodies enhancing coxsackievirus B4- and B3-induced synthesis of alpha interferon. *J Virol* 2005;79:13882–13891.
- 14 Sauter P, Chehadeh W, Lobert PE, Lazrek M, Goffard A, Soumillon M, et al: A part of the VP4 capsid protein exhibited by coxsackievirus B4 E2 is the target of antibodies contained in plasma from patients with type 1 diabetes. *J Med Virol* 2008;80:866–878.
- 15 Li Q, Yafal AG, Lee YM, Hogle J, Chow M: Poliovirus neutralization by antibodies to internal epitopes of VP4 and VP1 results from reversible exposure of these sequences at physiological temperature. *J Virol* 1994;68:3965–3970.