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ORAL IMMUNOGENICITY OF A PLANT VIRUS VECTOR BASED PORCINE CIRCOVIRUS ANTIGEN – SHORT COMMUNICATION

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A recombinant cucumber mosaic virus based expression system has been developed for the production of an immunogenic porcine circovirus epitope. The resulting nanoparticle was shown to elicit specific immune response in mice and pigs, when administered parenterally. To evaluate the oral applicability of this vaccine candidate, two experiments were performed. In the first one, the resistance of the vector itself to mucosal environment was tested in mice. Cucumber mosaic virus particles fed to mice were able to elicit specific mucosal and serum antibody production. In the second experiment, recombinant cucumber mosaic virus fed to piglets resulted in the appearance of porcine circovirus specific serum antibodies. The vector proved to be able to survive in the gastrointestinal tract, so that an epitope expressed on its surface could induce specific immune response. These results indicate that the developed plant virus based expression system offers an effective method for mucosal vaccine production.

Key words: Cucumber mosaic virus, porcine circovirus, subunit vaccine, oral immunisation

The expression of antigens using plant virus vectors is an inexpensive, effective and promising method of subunit vaccine production. Many antigens of public health and veterinary importance have already been expressed in this way, including viral antigens (e.g. human immunodeficiency virus, hepatitis B virus, rabies virus, foot and mouth disease virus, canine parvovirus epitopes), antigens of bacterial origin (e.g. *Staphylococcus aureus*, *Yersinia pestis*, *Mycobacterium tuberculosis*, *Bacillus anthracis*) and other vaccine candidates (e.g. *Plasmodium falciparum*, B cell lymphoma antigens) (Tiwari et al., 2009). Unlike protein production in transgenic plants, this method does not involve genetic modification of

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plants, the genetic code of the protein or peptide to be expressed is inserted into the genome of a virus infecting a host plant. These viruses are easier to manipulate than eukaryotic cell genomes, and production yields can be much higher (Hefferon, 2012), although the size of the insertable nucleic acids may be limited. Vaccines produced in plants are suitable to be used as oral vaccines, offering the advantage of simple delivery and induction of mucosal immunity (Yusibov et al., 2011).

One of the vector candidates is cucumber mosaic virus (CMV), a plant pathogen of the genus *Cucumovirus* in the family *Bromoviridae*, infecting more than 1200 species (Mochizuki and Ohki, 2012) including many agriculturally important, edible vegetables and ornamental plants. CMV particles are isometric, approximately 28–30 nm in diameter, with messenger sense single-stranded RNA genomes. The properties of the virus (wide host range, extensively studied genome, modes of virus replication, cell-to-cell and systemic movement in plants, mechanical transmission between plants) made CMV a promising vector candidate for protein production. CMV-based vectors have already been developed for expression of peptides of interest in human health, namely the R9 hepatitis C virus envelope derived epitope (Nuzzaci et al., 2009; Nuzzaci et al., 2010) and amyloid β protein fragments (Vitti et al., 2010).

Porcine circovirus type 2 (PCV2) is a well-known pathogen of wild boars and domestic swine, causing major economic losses in the pig industry worldwide, due to PCV2-associated diseases facilitating the development of secondary infections and lowering responses to vaccinations by immune suppression (Segalés, 2012). Today, inactivated and recombinant baculovirus-based subunit vaccines are commercially available to reduce the losses caused by PCV2. Beside these, other experimental vaccines are under development to strengthen PCV2-specific immune responses, including modified live attenuated, DNA, vector and marker vaccines (Beach and Meng, 2012).

For the production of a cheap, safe and potentially edible PCV2 subunit vaccine, a CMV vector based expression system has been developed, resulting in recombinant CMV nanoparticles carrying an immunogenic PCV2 epitope (amino acids 224–233 of the PCV2 capsid protein) on the surface of the coat proteins. In a previous study, the recombinant CMV was able to induce specific immune response in mice and pigs when injected parenterally, causing a rise in PCV2-specific serum antibody levels comparable to those elicited by an inactivated commercial PCV2 vaccine. The protection induced by the single circovirus epitope expressed in CMV was able to protect piglets from PCV2 challenge infection, although at a lower rate than the inactivated vaccine which contained full PCV2 virions (Gellért et al., 2012). To see if immunogenicity could also be achieved when recombinant CMV was given orally, two *in vivo* experiments were performed. The animal experiments were carried out in accordance with the Guidelines for Animal Experiments of Szent István University and with EU Directive 2010/63/EU (Permit Number: 22.1/1020/3/2010).

In the first experiment, our purpose was to determine if the CMV virions were resistant to physicochemical and biological conditions specific to the gastrointestinal tract, and if intact antigens were able to reach lymphoid tissues in the mucosa where immunological priming was possible. In this pilot study we used 40 female, 8-week-old SPF mice (CRL: NMRI BR, Charles River, USA) divided into 6 groups. Group 1 (n = 5) served as negative control. Every mouse in Group 2 (n = 5) received purified wild-type CMV, extracted (as described by Lot et al., 1972) from 1 g *Nicotiana benthamiana* leaf material (containing approximately 0.7 mg of the virus) intraperitoneally. Each mouse in Group 3 (n = 5) and Group 4 (n = 10) was fed the same amount of the extracted virus. Mice in Groups 5 (n = 5) and 6 (n = 10) were given CMV purified from 4 g infected leaf material (equivalent to approximately 2.8 mg CMV per mouse) orally. Feedings were carried out using gavage tubes to ensure full ingestion of the exact doses. Injections and feedings were repeated after 14 days. Serum samples were collected in Groups 1, 2, 3 and 5 every week. Five mice in Groups 4 and 6 each were euthanised on day 14. All remaining animals were euthanised on day 28. In Groups 4 and 6, mucosal fluid was collected after homogenisation of the ilea in 0.5 ml PBS. To detect CMV-specific antibodies, indirect enzyme-linked immunosorbent assay (ELISA) was used, performed on wild-type CMV-coated ELISA plates, using a standard ELISA protocol (Gellért et al., 2012), with the following modifications: twofold dilutions starting with 1:50 were applied in the case of sera, and CMV-specific IgG antibodies were detected by horseradish peroxidase-(HRPO-) labelled anti-mouse IgG conjugates (1:10,000). In the case of intestinal fluid samples, twofold dilutions started at 1:2, and CMV-specific IgA antibodies were detected using HRPO-labelled anti-mouse IgA conjugate (1:4,000).

CMV-specific antibody levels could be measured in all immunised animals. In Group 1 (negative control), CMV-specific antibodies could not be detected throughout the whole experiment. All mice were negative for CMV-specific antibodies at the time of the first immunisation (day 0). Detectable serum IgG levels appeared after one week in the positive control group, which was injected parenterally with the antigen. Mice in Groups 3, 4, 5 and 6 received CMV orally, in Groups 5 and 6 four times more virions than in Groups 3 and 4. This difference in the doses caused an earlier appearance of IgGs in the group which ingested more CMV, but had little effect on the final serum antibody titres. Mean IgG titres are shown in Fig. 1.

In Groups 4 and 6, CMV-specific IgA levels were measured from intestinal mucosal fluids on days 14 and 28. On day 14, IgA could only be detected in Group 6 (the mean titre was 8), where mice were immunised with antigen doses four times higher than mice in Group 4, where specific IgA could not be detected at that time. After the second immunisation, samples from both groups were positive, the mean levels peaking around 16 in Group 4 and 256 in Group 6. The higher antigen dose resulted in the earlier appearance of secretory antibodies, and

it even had a notable effect on the final IgA levels. These findings indicate that orally administered CMV antigens survive the conditions present in the digestive tract, and immunogenic epitopes reach immune effector cells in a form that is suitable for the induction of specific immune responses, which confirms the observations of Nuzzaci et al. (2010).

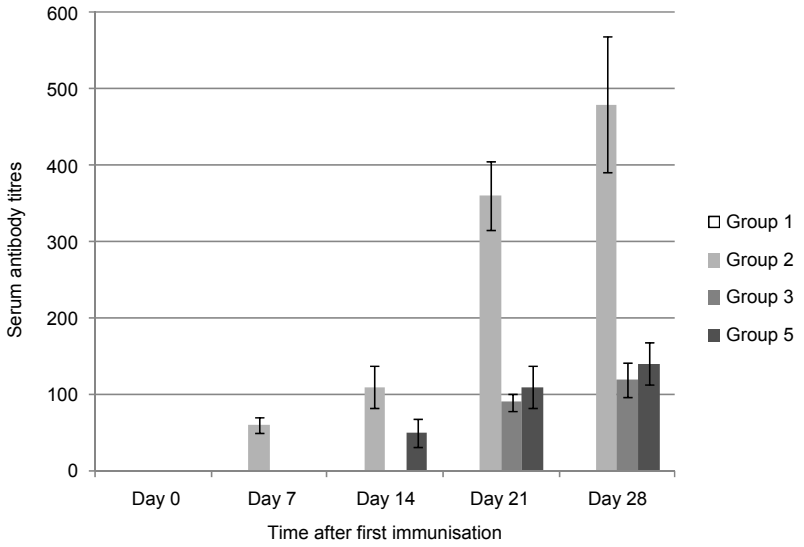

Fig. 1. Mean serum antibody titres in immunised mice. Animals in Group 1 were used as negative control. Mice in Group 2 were immunised with 0.7 mg cucumber mosaic virus (CMV) intraperitoneally. Each mouse in Groups 3 and 5 was fed 0.7 and 2.8 mg CMV, respectively. Injections and feedings were carried out on days 0 and 14

Based on the results of the mouse immunisation trial described above and the pig immunisation and challenge experiment mentioned earlier (Gellért et al., 2012), a second experiment was conducted to evaluate the oral immunogenicity of the recombinant CMV vaccine candidate in the target species. Two groups of conventional littermate piglets, from a PCV2-free herd, weaned at 4 weeks of age were used, five piglets in each group. Animals in Group 1 (n = 5) were fed 50 mg purified recombinant CMV every week from the time of weaning for four weeks (a total of 200 mg recombinant CMV per animal). Virus preparations were mixed with small amounts of the regular feed given individually. Five piglets in Group 2 served as negative control. Serum samples were collected weekly. Indirect immune fluorescence (IF) was used for PCV2-specific IgG detection with FITC-conjugated antibodies (Sigma Aldrich, St. Louis, USA) diluted 1:400, as described by Gellért et al. (2012).

PCV2-specific antibodies were not detected at the time of weaning, when the feeding experiment started. All sera from piglets in Group 2 (negative control group) remained negative throughout the trial, while antibody levels in two piglets in Group 1 started to rise by the end of the second week, followed by the seroconversion of the other three piglets in this group by week 3. The PCV2-specific serum IgG titres are shown in Fig. 2.

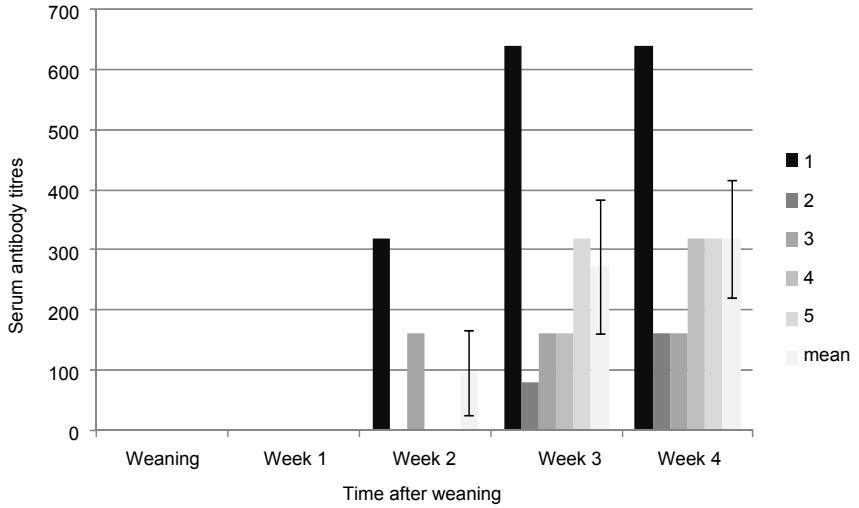

Fig. 2. Individual (numbering of the bars refers to the number of each piglet) and mean PCV2-specific IgG levels in the sera of piglets orally immunised with recombinant cucumber mosaic virus (CMV). Piglets numbered 1 to 5 were fed 50 mg recombinant CMV each at weaning and three more times for 3 consecutive weeks. Piglets numbered 6 to 10 were used as negative control and remained seronegative for PCV2; therefore, they are not indicated

Similar antibody levels in piglets immunised parenterally with the same recombinant CMV were shown to be able to lower PCV2 virus replication, when piglets were challenged both orally and parenterally (Gellért et al., 2012). The average virus production rate in tobacco leaves is 70 mg virus per 100 g leaf material. In this experiment, each piglet was fed a total of 200 mg recombinant virions, extracted from roughly 300 g of leaves. The feeding of this quantity of the infected plant mixed with regular feed should be feasible, and leaves open the possibility of administering higher doses if needed. The immunogenicity of virions contained by intact plant tissues needs to be studied further, but plants are believed to protect antigen integrity in the gastrointestinal tract (Streatfield, 2006). The relatively inexpensive development and production of this nanoparticle and its demonstrated ability to be applied orally make it a potential vaccine candidate of choice against PCV2.

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References

Beach, N. M. and Meng, X. J. (2012): Efficacy and future prospects of commercially available and experimental vaccines against porcine circovirus type 2 (PCV2). Virus Res. **164,** 33–42.

Gellért, Á., Salánki, K., Tombácz, K., Tuboly, T. and Balázs, E. (2012): A cucumber mosaic virus based expression system for the production of porcine circovirus specific vaccines. PLoS ONE **7,** e52688.

Hefferon, K. L. (2012): Plant virus expression vectors set the stage as production platforms for biopharmaceutical proteins. Virology **433,** 1–6.

Lot, H., Marrou, J., Quiot, J. B. and Esvan, C. (1972): Contribution á l'étude du virus de la mosaique du concombre (CMV). I. Méthode de purification rapide du virus [in French]. Ann. Phytopath. **4,** 25–38.

Mochizuki, T. and Ohki, S. T. (2012): Cucumber mosaic virus: viral genes as virulence determinants. Mol. Plant Pathol. **13,** 217–225.

Nuzzaci, M., Bochicchio, I., De Stradis, A., Vitti, A., Natilla, A., Piazzolla, P. and Tamburro, A. M. (2009): Structural and biological properties of Cucumber mosaic virus particles carrying hepatitis C virus-derived epitopes. J. Virol. Methods **155,** 118–121.

Nuzzaci, M., Vitti, A., Condelli, V., Lanorte, M. T., Tortorella, C., Boscia, D., Piazzolla, P. and Piazzolla, G. (2010): *In vitro* stability of Cucumber mosaic virus nanoparticles carrying a hepatitis C virus-derived epitope under simulated gastrointestinal conditions and *in vivo* efficacy of an edible vaccine. J. Virol. Methods **165,** 211–215.

Segalés, J. (2012): Porcine circovirus type 2 (PCV2) infections: clinical signs, pathology and laboratory diagnosis. Virus Res. **164,** 10–19.

Streatfield, S. J. (2006): Mucosal immunization using recombinant plant-based oral vaccines. Methods **38,** 150–157.

Tiwari, S., Verma, P. C., Singh, P. K. and Tuli, R. (2009): Plants as bioreactors for the production of vaccine antigens. Biotechnol. Adv. **27,** 449–467.

Vitti, A., Piazzolla, G., Condelli, V., Nuzzaci, M., Lanorte, M. T., Boscia, D., De Stradis, A., Antonaci, S., Piazzolla, P. and Tortorella, C. (2010): Cucumber mosaic virus as the expression system for a potential vaccine against Alzheimer's disease. J. Virol. Methods **169,** 332–340.

Yusibov, V., Streatfield, S. J. and Kushnir, N. (2011): Clinical development of plant-produced recombinant pharmaceuticals: vaccines, antibodies and beyond. Hum. Vaccin. **7,** 313–321.