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A corn-based delivery system for animal vaccines: an oral transmissible gastroenteritis virus vaccine boosts lactogenic immunity in swine

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Abstract

Recombinant plant expression systems offer a means to produce large quantities of selected antigens for subunit vaccines. Cereals are particularly well-suited expression vehicles since the expressed proteins can be stored at relatively high concentrations for extended periods of time without degradation and dry seed can be formulated into oral vaccines suitable for commercial applications. A subunit vaccine candidate directed against porcine transmissible gastroenteritis virus and expressed in corn seed has been developed for oral delivery to swine. Here, we show that this vaccine, when administered to previously sensitized gilts, can boost neutralizing antibody levels in the animals' serum, colostrum and milk. Thus, this vaccine candidate is effective at boosting lactogenic immunity and is appropriate to pursue through large-scale field trials preceding commercialization.

Keywords: Lactogenic immunity; Plant-based vaccines; Transmissible gastroenteritis virus

1. Introduction

Oral administration of vaccines has the potential to greatly cut the cost and increase the safety of vaccine delivery. In the case of human vaccines, avoiding the use of needles reduces equipment costs, removes the requirement for trained medical personnel to supervise delivery and eliminates safety concerns associated with needle disposal. The economic benefits of oral over parenteral delivery are also apparent with animal vaccines, where equipment and labor costs can be substantially reduced. Also, in the cases of farmed animals destined for meat markets, carcass quality may be compromised by repeated injections and oral vaccines overcome this concern. Subunit vaccines are generally considered to have a low safety risk since they are well defined and do not contain attenuated or inactivated pathogens with the potential for adverse affects. Thus, oral delivery of subunit vaccines is a particularly attractive option for safe, inexpensive vaccination programs.

However, oral delivery generally requires very high levels of specified antigens to be administered in order to attain efficacy. This is presumably because of degradation of the selected proteins in the digestive tract and only a small proportion of the relatively intact molecules being presented to the immune system in a manner favoring an immunogenic response. Several recombinant systems are being utilized to generate large amounts of subunit vaccines, including, for example, the use of yeast to produce the surface protein of hepatitis B. However, despite the development of such recombinant vaccines, the economic production of large quantities of desired antigens is severely limited.

Recently, certain recombinant plant expression systems have begun to offer a means to produce very large quantities of proteins in a sufficiently concentrated form to make oral delivery feasible for a wider array of antigens. Levels of expression have been achieved with various antigens in plants that allow practical oral delivery of a sufficient dose to elicit desired immune responses in humans and target animals (reviewed in [1]). The approaches followed to achieve these high levels of expression include the use of tissue-specific promoters to direct expression to tissues well suited to the stable storage of proteins [2] and the targeting

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of the expressed proteins to sub-cellular locations conducive to their accumulation [3].

Many plant-based oral vaccine candidates have been tested in animal studies and several responses have been noted, including the generation of serum and mucosal antibodies (reviewed in [1]) and raised cytokine levels [4]. Protective efficacy has also been recorded with some of these vaccine candidates in model species trials (reviewed in [1]). A few plant-based vaccine candidates have advanced into early phase human clinical trials or target animal trials. Among human vaccines, these include those directed against travelers' diarrhea and Norwalk virus delivered in potato tubers [5,6], against hepatitis B virus delivered in lettuce leaves [7] and against rabies virus delivered in spinach leaves, themselves infected with a recombinant plant virus [8]. Immune responses were observed during these trials, and although there were some reports of nausea, presumably resulting from the administration of up to 150 g of unprocessed, unpalatable plant material, the vaccine candidates were generally well tolerated. In the case of farmed animals, a corn-based vaccine directed against transmissible gastroenteritis virus (TGEV) can induce protective immunity in piglets [9,10].

A key issue in producing plant-based oral vaccines is the selection of plant material that both expresses high levels of a chosen subunit vaccine candidate and is also suitable for extensive storage and oral delivery. The chosen plant material must also be ready for direct administration or must be suitable for inexpensive processing into an appropriate form for oral delivery to the target species. Much of the work to date on plant expression systems has been conducted using tobacco leaf tissue (discussed in [11]). However, tobacco leaves are inedible, and therefore protein extraction is required prior to delivery. Several edible options have also been pursued, including the tubers and leaves of certain vegetable crops such as potato and lettuce, respectively [5–7]. Some fruits, such as bananas, are also being considered. However, perishable items are not practical for extended storage and expression levels can vary considerably between, for example, potato tubers taken from a single harvest [5,6].

Cereal seeds are particularly well-suited systems for the oral delivery of subunit vaccines. They have low water contents and naturally store proteins over long periods of time without degradation. Corn (*Zea mays*) is an especially attractive option because of its intensively studied genetics and the availability of established transformation procedures. Several vaccine candidate antigens have been expressed at high levels in corn seed and the proteins are stable when stored in this tissue for periods of at least a year and probably for much longer, obviating the requirement for a cold chain during distribution and storage [10]. Furthermore, the antigen concentration is uniform across a corn grain harvest, facilitating even dosing [3]. A wide range of processing alternatives have been developed by the food and feed industries to convert corn grain into readily edible forms and pilot-scale processes have been developed that ensure antigens are not degraded during processing [12]. In the case of farmed animals, such processing is unnecessary since the livestock can consume corn grain directly.

Here, we focus on the development of a corn seed-based subunit vaccine directed against swine TGEV. This virus causes a contagious enteric disease that is particularly severe for piglets. It results in severe diarrhea and vomiting and is associated with high mortality rates among piglets under 2 weeks of age [13]. TGEV is a coronavirus and has a large surface glycoprotein referred to as the spike (S) protein displayed on its surface [13]. The TGEV vaccine candidate assessed here comprises the S protein expressed in corn seed. Feeding studies have been conducted with this vaccine candidate delivered orally to piglets. The animals showed a priming of their immune system and were protected against infection [9,10]. Here, we extend these swine feeding studies to assess the potential for this oral TGEV vaccine candidate to boost immunogenic responses in gilts (young sows) previously sensitized with a commercially available modified live viral vaccine. We focus particularly on the level of antibodies in the colostrum and milk as a guide to whether immunity could be acquired passively by piglets through suckling.

2. Materials and methods

2.1. Vaccine candidate and placebo materials

The subunit vaccine candidate comprised milled yellow grain corn expressing the S protein of TGEV. A single dose corresponded to 2 kg of corn containing 26 mg of the antigen. The placebo for the study comprised 2 kg of non-transformed milled yellow grain corn. A commercially available modified live TGEV vaccine (Intervet Inc., Millsboro, DE) with a titer of $10^{6.9}$ TCID (tissue culture infectious doses)/50 ml was used to prime all animals and to provide booster treatments to a positive control group. This vaccine was administered according to label directions.

2.2. Test animals

A total of 39 specific pathogen free gilts of suitable age for breeding were included in the study. They were taken from a low disease incidence herd and were seronegative for TGEV at the outset of the study. The gilts were randomized into six treatment groups with from five to eight animals in each group (Table 1). Duplicate ear tags were used for identification purposes.

2.3. Immunization and housing protocol

All gilts in all groups were orally administered the modified live TGEV vaccine on the day of breeding (115 days before farrowing) and also 102 days before farrowing. They were then administered the TGEV modified live vaccine by intramuscular injection 88 days before farrowing. The

Table 1
Boosting regimen for gilts

Group	No. of gilts	35 Days before farrowing	14 Days before farrowing
A	8	Oral corn vaccine (days −35 to −29)	Oral corn vaccine (days −14 to −8)
B	7	Oral corn vaccine (days −35 to −33)	Oral corn vaccine (days −14 to −12)
C	7	Oral corn vaccine (day −35)	Oral corn vaccine (day −14)
D	5	No treatment	Oral corn placebo (days −14 to −8)
E	5	IM[a] modified live virus (day −35)	IM[a] modified live virus (day −14)
F	7	No treatment	Oral corn vaccine (days −14 to −8)

[a] IM: intramuscular.

subsequent immunization regimen for each group is outlined in Table 1. All animals were fasted overnight prior to oral administrations of the corn-based vaccine to test groups. Standard lactation gestation rations were administered to all gilts throughout the study. During the period comprising the three administrations of modified live virus to all gilts, the groups were housed together and allowed pen-to-pen contact. Prior to 35 days before farrowing gilts were separated into their separate groups and during subsequent vaccine administrations, animals were individually isolated.

2.4. Analysis of samples for the presence of neutralizing antibodies

Blood samples were collected from gilts on the day of breeding (115 days prior to farrowing), 35 and 14 days prior to farrowing and on the day of farrowing. Blood was allowed to clot and was sedimented by centrifugation, so allowing the serum to be collected. TGEV neutralizing titers were determined by incubating a specific dilution of TGEV with multiple serum dilutions for 1 h at 37 °C. These mixtures were then inoculated onto a swine testicular cell line and the capacity of the serum to interfere with the viral infection was assessed after 3 days. Sample titers were calculated using a Spearman–Karber 50% endpoint table. Colostrum samples of at least 25 ml were collected on the day of farrowing and at least 25 ml milk samples were collected 3, 7, 10 and 14 days after farrowing. The samples were sedimented by centrifugation, and the central region was collected. TGEV neutralizing titers were determined as with serum samples. For all TGEV neutralization data, geometric mean titers were compared and differences in excess of four-fold were considered to be significant.

3. Results and discussion

3.1. Preparation of a plant-based vaccine candidate for swine to combat TGEV

The generation of transgenic corn containing the S protein of TGEV has been previously described [9,10]. In brief, sequence encoding the S protein was synthesized with optimal codon usage for expression in *Z. mays*. An N-terminal cell surface targeting signal was included to direct accumulation of the protein to the cell wall. DNA encoding the S protein was introduced into immature zygotic *Z. mays* embryos by *Agrobacterium tumefaciens* mediated transformation and selection was imposed for transgenic callus. Transgenic plants with sequence encoding the S protein integrated into the nucleus were regenerated, and those expressing the highest levels of the S protein were taken through a plant-breeding scheme to increase and stabilize expression levels. This culminated in a large-scale grain harvest in which the S protein was present at 13 mg kg^{-1}, as determined using a sandwich enzyme linked immunosorbent assay [9]. At this concentration a practical antigen dose of 20–30 mg can be delivered in an amount of corn material easily consumed at a single feeding.

3.2. The oral plant-based TGEV vaccine induces serum immune responses in gilts

All animals were seronegative for TGEV at the time of breeding. Subsequent serum neutralization titers are summarized for each study group in Fig. 1. The modified live virus vaccine, which was administered twice orally and then once intramuscularly resulted in gilts in all groups having

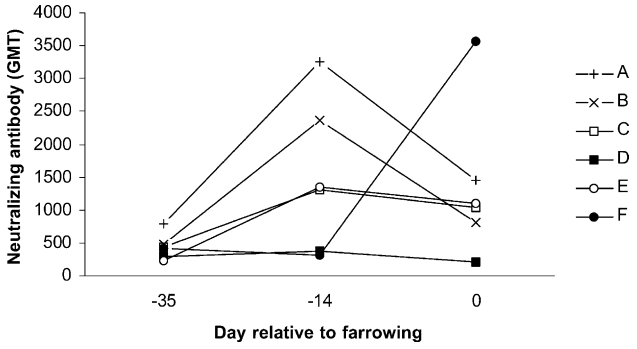

Fig. 1. Serum TGEV neutralization titers for gilts in the 5 weeks prior to farrowing. Geometric mean titer (GMT) values are shown. Animals received the following treatments: group A (oral corn vaccine on days −35 to −29 and −14 to −8); group B (oral corn vaccine on days −35 to −33 and −14 to −12); group C (oral corn vaccine on days −35 and −14); group D (oral corn placebo on days −14 to −8); group E (intramuscular live vaccine on days −35 and −14); group F (oral corn vaccine on days −14 to −8).

similar TGEV serum neutralizing titers 35 days prior to farrowing.

Analysis of serum samples taken from gilts at 14 days prior to farrowing showed that animals that had received the oral corn-based TGEV vaccine (groups A–C) had notably higher serum neutralization titers than those that had received no material at this stage (groups D and F). The difference between the test and control groups was significant in all cases except for that of a single administration of corn-based vaccine (day −35 to group C) over group D. Animals that had received the modified live virus vaccine as a single intramuscular boost (day −35 to group E) responded to an almost identical level to those that had received a single oral administration of the corn-based vaccine (group C). Although more oral administrations of the corn-based vaccine appeared to increase the neutralization titer, differences between the treatment groups (A–C) were not significant and none of the treatments induced a significantly stronger response than the intramuscular boost of modified live vaccine delivered to group E.

Similarly, at the time of farrowing the TGEV serum neutralization titers in gilts administered the corn-based TGEV vaccine as a boost (groups A–C and F) were raised over those observed with animals that had received the corn placebo (group D). This difference was significant in all but the case of gilts that had received six administrations of the corn-based vaccine (group B) compared to those that had received the placebo (group D). Animals given intramuscular administrations of the modified live virus vaccine as a boost (group E) responded similarly to those that received the oral corn-based vaccine, again with two administrations of either vaccine giving almost identical results (groups C and E). Gilts administered a boost of the corn-based TGEV vaccine only during the second week before farrowing (group F) showed the most marked increase in the serum neutralization titer at the time of farrowing, although differences between the groups that received the corn-based vaccine (groups A–C and F) were generally not significant.

Interestingly, for groups that received two blocks of booster administrations (A–C and E), in no case did the second set of treatments elevate the serum neutralization titers over those observed with the first set. Indeed, neutralization titers appeared to decline with the second set of administrations, although in no case was the drop statistically significant.

3.3. The oral plant-based TGEV vaccine induces lactogenic immunity

Colostrum and milk neutralization titers are summarized for each study group in Figs. 2 and 3, respectively. Each of the groups of gilts that were orally administered the corn-based TGEV vaccine as a booster (groups A–C and F) showed a greater level of neutralizing antibodies than did gilts administered two intramuscular injections of the TGEV modified live virus vaccine as a booster (group E).

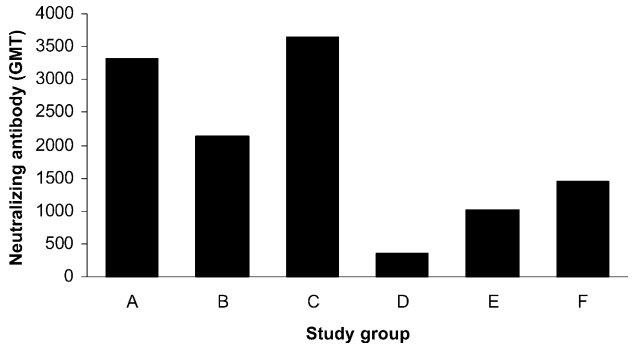

Fig. 2. Colostrum TGEV neutralization titers on the day of farrowing. Geometric mean titer (GMT) values are shown. Animals received the following treatments: group A (oral corn vaccine on days −35 to −29 and −14 to −8); group B (oral corn vaccine on days −35 to −33 and −14 to −12); group C (oral corn vaccine on days −35 and −14); group D (oral corn placebo on days −14 to −8); group E (intramuscular live vaccine on days −35 and −14); group F (oral corn vaccine on days −14 to −8).

However, these differences were not sufficient to be considered significant, and therefore all of the booster treatments with either the corn-based oral vaccine or with the modified live intramuscular vaccine are considered similarly effective. All of the booster regimens with the corn-based vaccine (groups A–C and F) resulted in significantly greater neutralizing antibody levels than those observed among animals that were administered the control corn placebo material (group D).

Gilts in all groups that received an oral corn-based TGEV vaccine boost (groups A–C and F) showed similar levels of neutralizing antibodies in their milk, with levels trailing off steeply between 3 and 7 days after farrowing and continuing to decline thereafter. These levels correspond closely to those observed with the modified live TGEV vaccine delivered intramuscularly (group E). With all groups that received a corn-based oral booster treatment (groups A–C and F) the neutralizing antibody titer in milk 3 days after

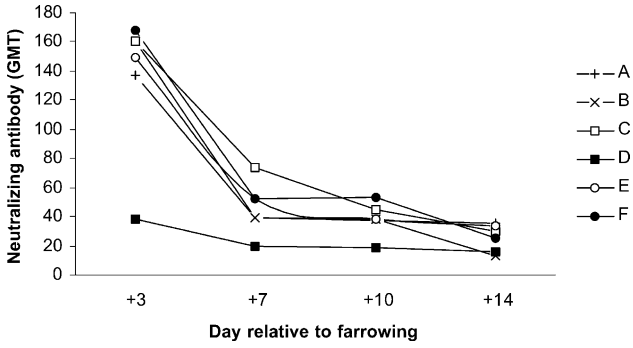

Fig. 3. Milk TGEV neutralization titers in the 2 weeks following farrowing. Geometric mean titer (GMT) values are shown. Animals received the following treatments: group A (oral corn vaccine on days −35 to −29 and −14 to −8); group B (oral corn vaccine on days −35 to −33 and −14 to −12); group C (oral corn vaccine on days −35 and −14); group D (oral corn placebo on days −14 to −8); group E (intramuscular live vaccine on days −35 and −14); group F (oral corn vaccine on days −14 to −8).

farrowing is considerably higher than for the group that received the control corn placebo (group D). This difference is significant in all cases except that of the group that received two blocks, each of seven consecutive days, of the corn-based vaccine (group A). By 7 days after farrowing differences in the neutralization titers between the placebo (group D) and other groups are not significant.

3.4. Plant-based vaccines hold great promise for the efficient vaccination of large animals

The corn-based TGEV vaccine candidate described here shows great potential for expediting the administration of an efficacious vaccine to large herds of swine. The current standard regimen for administrating a vaccine comprises both priming and boosting stages. During the priming phase of the regimen the modified live TGEV vaccine is often administered along with other swine vaccines. Thus, at this point no reduction in labor costs is achieved through delivering an oral corn-based TGEV vaccine separately. However, replacing subsequent injections of the modified live TGEV vaccine with oral corn vaccine boosters would clearly save considerable time and effort.

The orally administered corn-based TGEV vaccine is effective in boosting the serum neutralizing titer response in animals previously sensitized to TGEV using the modified live virus vaccine. When administered as a booster to gilts the corn-based vaccine also results in increased levels of neutralizing antibodies in the colostrum and early milk. Milk antibodies of the IgG class have typically disappeared within 48 h of farrowing, so the neutralizing antibody activities observed in milk samples collected 3 days after farrowing most likely reflect IgA levels. Protection against TGEV amongst nursing piglets has been linked to IgA levels [14], indicating that the corn-based TGEV vaccine is inducing an immune response with the potential to confer protection. In this regard, a potato-based vaccine candidate directed against rotavirus, and assessed in a mouse feeding study, has been shown to confer passive immunity to pups when administered orally to dams [4].

Protective efficacy has previously been demonstrated with a corn-based oral vaccine directed against TGEV and administered to piglets [9,10]. The neutralizing antibody levels achieved here in the colostrum and early milk of gilts extends the scope for how this vaccine candidate can be deployed. Future studies with this TGEV oral vaccine candidate will focus on optimizing the administration regimen for maximum responses and on conducting larger scale trials. These will include an assessment of whether the lactogenic immunity observed here results in protection being conferred to piglets. The results presented here successfully demonstrate a commercial application for a corn-based vaccine and indicate that there is great promise for plant-based vaccines that can be easily administered to large farmed animals by oral delivery.

References

[1] Streatfield SJ, Howard JA. Plant-based vaccines. Int J Parasitol 2003;33:479–93.
[2] Chikwamba R, McMurray J, Shou H, Frame B, Pegg SE, Scott P, et al. Expression of a synthetic *E. coli* heat-labile enterotoxin B sub-unit (LT-B) in maize. Mol Breed 2002;10:253–65.
[3] Streatfield SJ, Lane JR, Brooks CA, Barker DK, Poage ML, Mayor JM, et al. Corn as a production system for human and animal vaccines. Vaccine 2003;21:812–5.
[4] Yu J, Langridge WHR. A plant-based multicomponent vaccine protects mice from enteric diseases. Nat Biotechnol 2001;19:548–52.
[5] Tacket CO, Mason HS, Losonsky G, Clements JD, Levine MM, Arntzen CJ. Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. Nat Med 1998;4:607–9.
[6] Tacket CO, Mason HS, Losonsky G, Estes MK, Levine MM, Arntzen CJ. Human immune responses to a novel Norwalk virus vaccine delivered in transgenic potatoes. J Infect Dis 2000;182:302–5.
[7] Kapusta J, Modelska A, Pniewski T, Figlerowicz M, Cetelier M, Lisowa O, et al. A plant-derived edible vaccine against hepatitis B virus. FASEB J 1999;13:1796–9.
[8] Yusibov V, Hooper DC, Spitsin SV, Fleysh N, Kean RB, Mikheeva T, et al. Expression in plants and immunogenicity of plant virus-based experimental rabies vaccine. Vaccine 2002;20:3155–64.
[9] Streatfield SJ, Jilka JM, Hood EE, Turner DD, Bailey MR, Mayo JM, et al. Plant-based vaccines: unique advantages. Vaccine 2001;19:2742–8.
[10] Lamphear BJ, Streatfield SJ, Jilka JM, Brooks CA, Barker DK, Turner DD, et al. Delivery of subunit vaccines in maize seed. J Control Release 2002;85:169–80.
[11] Daniell H, Streatfield SJ, Wycoff K. Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci 2001;6:219–26.
[12] Streatfield SJ, Mayor JM, Barker DK, Brooks C, Lamphear BJ, Woodard SL, et al. 2000 Congress Symposium on Molecular Farming: development of an edible subunit vaccine in corn against enterotoxigenic strains of *Escherichia coli*. In Vitro Cell Dev Biol Plant 2002;38:11–7.
[13] Laude H, Rasschaert D, Delmas B, Godet M, Gelfi J, Charley B. Molecular biology of transmissible gastroenteritis virus. Vet Microbiol 1990;23:147–54.
[14] Saif LJ, van Cott JL, Brim TA. Immunity to transmissible gastroenteritis virus and porcine respiratory coronavirus infections in swine. Vet Immunol Immunopathol 1994;43:89–97.