

Expression of vaccine antigens to edema disease in tobacco seeds and evaluation of immunogenicity on mouse model

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ABSTRACT

Plant-derived vaccines present many potential advantages related to the management in intensive livestock. They could be administered without restraint of the animals, with low stress and without labour costs related to multiple injections of traditional vaccines. The aim of this study was the construction and subsequent evaluation in mouse model of transgenic tobacco seeds as edible vaccines for swine Edema disease. We focused our attention on verocytotoxic *Escherichia coli* strains (O138, O139, O141), responsible of Edema disease, that occurs in pigs approximately one week after weaning and is characterized by edema in various sites and by damages to vascular endothelium. The adhesion of bacterial strains is related to different fimbriae and Shiga-like toxins (VT2e), that play an important role in the pathogenesis. Structural parts of F18 fimbriae and B-subunit of VT2e genes were inserted in expression vectors, under control of GLOB promoter to obtain specific seed accumulation of heterologous proteins, and transformed in tobacco by agroinfection. We obtained two stable lines of transformed tobacco expressing the proteins in the seed: one included F18 gene (F18+) and another one included B-subunit of VT2e gene (VT2e-B+). Tobacco lines were characterized by molecular and immunoenzymatic techniques for the expression of F18 and VT2e-B proteins. The amount of transgenic proteins was estimated at around 10µg/g of seeds. 14 Balb-c mice were divided randomly in two groups Control (CG) and Treatment (TG), with 7 mice each. Treatment diet, prepared as pellet to avoid different feed intakes in animals, contained 10% of tobacco seeds from F18+ and 10% of tobacco seeds from VT2e-B+. CG received a diet containing 20% of not-transgenic tobacco seeds. Treatments were administered on days 0,5,8,14,19,23. TG revealed an increment of fecal IgA at day 26, while CG at the same period decreased. The histometric data of the small intestine showed that TG crypts of the duodenum were significantly deeper than those of the CG. Immunostaining of the intestine showed that administration of transgenic tobacco seeds promotes a significant increase in the IgA-positive plasma cells production of the tonaca propria if compared to control group. In conclusion our findings suggest that tobacco seeds might be a potential source of oral vaccines.

INTRODUCTION



FIG. 1: Edema in mesentery of piglet dead for edema disease (Isolated *E.coli* F18+ VT2e+ strains)

- **Edema disease (ED)** is an enterotoxaemia that occurs in pigs during the weaning period and it is the result of an infection with certain serotypes of *Escherichia coli* (most frequently O138, O139, O141) F18+ able to produce verotoxins (VT2e). ED is responsible of important economic losses in pig livestock. The average morbidity is 30-40%, and the mortality among affected pigs is often as high as 90%, with typical lesions (Fig.1).



- **Shiga-like toxins (VT2e)** has been used to reproduce the clinical signs and pathological lesions of Edema disease. VT2e is composed of a single A subunit, that is responsible of enzymatic activity, in non covalent association with a pentamer of **B subunit**, that confers binding activity. Different studies confirm the antigenicity of B-subunit.

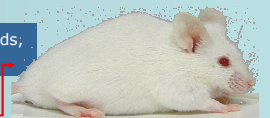
- **F18 fimbriae** is responsible of adhesion of *E.coli* serotypes, related to Edema Disease. The fimbrial antigenic factor f18 is likely to be responsible for the local immunity

- Parenteral route of vaccination is not effective in the development of local immunity (IgA), that can be stimulated through mucosal delivery of antigen vaccine. **Oral vaccination** is a prerequisite for stimulation of immunity against the majority of enteric and mucosal pathogens of both man and animals.

Plant based oral vaccines offer a new approach to vaccination strategies, especially in cases where local intestinal immune response is crucial in the prevention of infections.



OBJECTIVE
 The aim of this study was to evaluate the immunological effects related to oral administration of tobacco seeds, expressing F18 fimbriae and VT2e-B subunit, as a model of oral vaccine against Edema Disease in BALB-C mice.



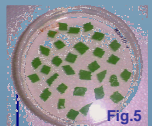
MATERIAL & METHODS

ISOLATION OF F18 AND VT2e B-SUBUNIT GENES

- Genomic DNA was extracted from O139 *E.coli* strains, isolated from different organs of swines dead for Edema Disease.
- F18 and B-subunit of VT2e genes were isolated by PCR analyses (Fig.2,3)
- Oligonucleotide primers included sites for specific endonucleases (BamHI-5'; SacI-3') to facilitate direct subcloning of the fragments.

PRIMERS FOR VT2e-B subunit:
F-ggatccatgaagaagatgttatagcgg
R-aacgggtccactcaatgattctcag

PRIMERS FOR F18:
F-ggatcc atgaaaagactgttatt tcttttg
R-cgaatgcgcaatgaatgtcatt ctcgag



TRANSFORMATION

- VT2e-B and f18 genes were cloned into binary plasmid, under control of Glob, seed specific promoter.
- The chimeric constructs (fig.4) were introduced in *Agrobacterium tumefaciens* EHA105 strains by electroporation.
- Leaf disks were infected with recombinant *Agrobacterium* (figg. 5,6,7) and plants or seeds were evaluated trough PCR, Northern Blotting, Western Blotting, agglutination on slides.

Fig.5,6,7: phases related to tobacco transformations and plants growth



MICE INVOLVED AND TREATMENTS

- 14 female Balb-c mice (4 weeks old) were allotted in cages with 7 mice (Treatment groups, TG) and 7 mice (Control group, CG).
- Mice, fasted for 12 hours, were fed one of four treatments on days 0, 5, 8, 14, 19, 23.
- Treatment diet, prepared as pellet to avoid different feed intakes in animals, contained 10% of tobacco seeds from f18+ and 10% of tobacco seeds from VT2e-B+.
- CG received a diet containing 20% of not-transgenic tobacco seeds.

ANALYSES AND MEASUREMENTS

- IgA and IgG amounts were evaluated in fecal samples collected on days 0,5,8,14,19,23,26
- Small intestine samples were evaluated through histometrical and immunistochemical analyses.

CONCLUSION

In conclusion our findings suggest that tobacco seeds, transformed for the expression of VT2e-B and F18, might be a potential source of oral vaccines to protect animals for Edema Disease. They could be administered without restraint of the animals, with low stress and without labour costs related to multiple injections of traditional vaccines.

RESULTS

EVALUATION OF *Escherichia coli* STRAINS

- E.coli* strains, analyzed by PCR, presented f18 and VT2-B genes (fig.2,3).



Fig.2: PCR for detection of VT2e B subunit (264 pb) using primers included sites for specific endonucleases



Fig.3: isolation of F18 gene (513 pb) from genomic DNA extracted from *E.coli* strains isolated from swines dead for edema disease.

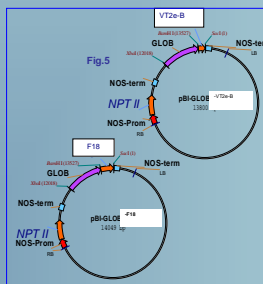


Fig.4: chimeric constructs used for *A. tumefaciens* transformations

EFFICIENCY OF TRANSFORMATION

- About 90% of tobacco plant presented f18 and VT2e-B genes.
- Northern blot analysis, carried out with a specific RNA probes on total RNA extracted from seeds of transformed plants, showed about 85% of positive samples for F18 fimbriae and about 45% of positive samples for VT2e B-subunit
- All samples, positive for the f18 mRNA and for VT2e B subunit mRNA, were positive for the protein Expression.
- The amount of transgenic proteins was estimated around 10µg/g of seeds.

TO vs T1

- We obtained stable lines of transformed tobacco expressing F18 and VT2e B-subunit.
- The second generation of seeds was able to maintain seed accumulation of transgenic proteins (fig.8).

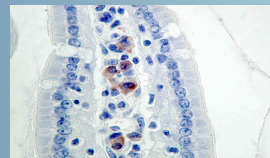


Fig.9: Intestinal villus (longitudinal section). IgA-immunopositive cells were observed in connective axis of villus of duodenum. TG samples presented also specific signal in tonaca propria.

IMMUNOLOGICAL EVALUATION

- TG showed an increment of fecal IgA at day 26, while CG at the same period decreased.
- The histometric data of the small intestine showed that TG crypts of the duodenum were significantly deeper than those of the CG (63.48µm vs 59.17µm; P<0.001).
- Immunostaining of the intestine (fig.9) showed that administration of transgenic tobacco seeds promotes a significant increase in the IgA-positive cells production of the tonaca propria if compared to control group (IgA ileum 6.22% vs 2.93%; P<0.001).

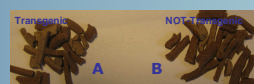


Fig.10: pellets administered to mice during experimental period. A: diet containing tobacco seeds F18+ and VT2e-B+; B: diet containing 20% of non-transgenic tobacco seeds