

AN ATTENUATED 2+/O132 Δ *tir* ENTEROPATHOGENIC *ESCHERICHIA COLI* (EPEC) OFFERS CROSS PROTECTION AGAINST A 3-/O15 CHALLENGE AND PARTIAL PROTECTION AGAINST AN 8+/O103 CHALLENGE

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ABSTRACT

Enteropathogenic *Escherichia coli* (EPEC) induce severe diarrhoea in rabbits and cause important losses after weaning. In Belgium and the Netherlands the bio/serotype 3-/O15 is the most prevalent, while in France, Spain and Italy EPEC of the 8+/O103 and less frequently 4+/O26 types are detected. An effective vaccine would protect rabbits against EPEC-associated diarrhoea, and limit antibiotic use. One of the virulence characteristics of EPEC is the mechanism of attachment and effacement (A/E). The adhesion of the bacterial outer membrane protein intimin to the translocated receptor of bacterial origin in the enterocyte's cell membrane (Tir), results in intimate attachment and is followed by effacement of the enterocyte's microvilli. We have created an attenuated mutant, as shown by experimental infection, by deleting the *tir* gene of EPEC strain 82/90 (2+/O132 Δ *tir*). The rabbits vaccinated *per os* with this live attenuated strain were protected against a heterologous challenge with a 3-/O15 strain and partially protected against a heterologous challenge with an 8+/O103 strain. Previous experiments with a 3-/O15 EPEC strain deleted in the *eae* gene, thus unable to produce intimin, showed that this Δ *eae* mutant induced insufficient cross protection against other pathotypes. This led to the conclusion that none of the LEE gene products, other than intimin, offered a sufficient cross protection in vaccination-challenge trials with heterologous EPEC strains. In the experiments presented here, it was shown that an attenuated strain still producing the highly immunogenic intimin was not capable either of offering sufficient cross protection. A study identifying immunoprotective antigens of EPEC should be performed. Its results might allow the construction of one or more attenuated mutants that could be used as vaccine strains, and offer perspectives towards the control of colibacillosis in meat rabbits.

Key words: EPEC, vaccine, Tir, heterologous challenge.

INTRODUCTION

EPEC (enteropathogenic *Escherichia coli*) are an important cause of rabbit enteritis, involving a virulence mechanism encoded by genes situated in the locus for enterocyte effacement (LEE) (ELLIOTT *et al.*, 1998). After initial adhesion, the bacterial protein Tir is inserted into the enterocyte membrane, allowing interaction with intimin, an outer

membrane adhesin of EPEC. This induces the effacement of the microvilli and the formation of pedestals (DE VINNEY *et al.*, 1999). In diarrhoeic weaned rabbits, the pathotypes 3-/O15, 4+/O26 and 8+/O103 are the most important, whereas pathotypes 2+/O128 and 2+/O132 are of variable virulence (PEETERS *et al.*, 1988). In Belgium and the Netherlands the bio-/serotype 3-/O15 is the most prevalent, while in France, Spain and Italy EPEC of the 8+/O103 and 4+/O26 types are detected. In view of the important economic losses caused by EPEC and the development of antibiotic resistance in the field strains, there is a need for an efficacious vaccine. Experiments with inactivated strains and with a non-pathogenic strain possessing the colonisation factor of 8+/O103 strains did not yield applicable results (CAMGUILHEM *et al.*, 1990; MILON *et al.*, 1990; CAMGUILHEM and MILON, 1991; MILON *et al.*, 1992). The factors inducing protection against a reinfection with EPEC have not been identified yet. Intimin, the *eae* gene product, and its receptor, Tir, encoded by the *tir* gene, are important virulence factors. Mutants deleted in *eae* no longer induce the typical attachment and effacement lesions (DONNENBERG *et al.*, 1993; TZIPORI *et al.*, 1995). We have previously created an attenuated strain by deletion of the *eae* gene of a 3-/O15 strain (97/241.6 Δeae) (STAKENBORG *et al.*, 2001). The rabbits vaccinated *per os* with this live attenuated strain were protected against a homologous infection, and the strain persisted long enough to induce an immunological response. Protection against heterologous challenge strains, however, was limited, showing that antibodies induced by LEE-encoded antigens other than intimin do not offer an efficacious protection. Intimin on the other hand is a strongly immunogenic protein (MANJARREZ-HERNANDEZ *et al.*, 2000). We constructed a mutant deleted in the *tir* gene, incapable of reverse mutation, and tested its remaining virulence and protective potential against other pathotypes.

MATERIAL AND METHODS

E. coli strains

The *E. coli* strains were kept at -80°C in Luria Bertani broth (LB) containing 50% glycerol, and cultured in Penassay Broth (PAB) at 37°C . Strain 97/223.10 (3-/O15) and 82/90 (2+/O132) were Belgian isolates. Strain 87/N6651 (8+/O103) was a kind gift of L. Renault (Laboratoires Vétérinaires Sanders, Athis-Mons, France). The 2+/O132 Δtir strain, deleted by 422 bp in the *tir* gene, no longer expressed Tir. A PCR test covering the deleted *tir* fragment was available to differentiate the wild-type (WT) EPEC strain from the mutant.

Experimental infections

Animals

For all experiments, minimum disease level (MDL) rabbits (Cunistar, Verlabreed, Nevele, Belgium), aged 4 weeks, were used. When they arrived, they were examined for possible presence of EPEC, *Clostridium spiroforme*, *Eimeria* and rotavirus, as described by PEETERS *et al.* (1986) and VAN OPDENBOSCH *et al.* (1981). The rabbits were individually housed. Water was available in individual bottles and they received a non-

supplemented, pelleted feed (Konix, Belgium). Water and feed were given *ad libitum*. *E. coli* excretion was examined three times per week using rectal swabbings, as described by PEETERS *et al.* (1988).

Assessment of remaining virulence (experiment n°1)

To examine the remaining virulence of the deleted strain *in vivo*, two groups of 6 rabbits were inoculated *per os* with 1 ml of PAB containing respectively 0 (control group) and 10^9 colony forming units (CFU) of the 2+/O132 Δtir strain. For 4 weeks the animals were assessed three times per week for feed intake, body weight and diarrhoea score (from 0=no diarrhoea, to 3=confluent fecal material).

Heterologous challenge experiments with 3-/O15 and 8+/O103 strains (experiment n°2)

Forty-three rabbits were distributed homogenously in five groups: a control group (group 1) of 8 rabbits, and four groups of 9, 9, 8 and 9 rabbits, respectively designated as groups 2 to 5. The day after their arrival (day {d} 0), groups 2 and 4 were vaccinated *per os* with 10^9 CFU of the deleted 2+/O132 Δtir strain. Four weeks later (d30) groups 2 and 3 were challenged *per os* with 10^8 CFU of the WT 3-/O15 EPEC strain. At the same time, groups 4 and 5 were challenged *per os* with 10^7 CFU of the WT 8+/O103 EPEC strain. For 8 weeks the animals were assessed three times per week for feed intake, body weight and diarrhoea score.

Statistical analysis

The groups were compared using a generalised linear repeated measures model (SAS 8.02, U.S.A.).

RESULTS AND DISCUSSION

All rabbits were free of EPEC, rotavirus and *Eimeria spp.* at the start of the experiment. *C. spiroforme* was not detected in the rabbits of experiment n°1, but was present in 5 rabbits at the start of experiment n°2. No treatment was given to avoid interference with the attenuated vaccine strain.

During experiment n°1, none of the rabbits showed symptoms of enteritis and no significant difference was observed between the control and the vaccinated group for feed intake or body weight. The vaccine strain was isolated in low numbers from the vaccinated animals for more than 20 days after vaccination. These results show that EPEC strain 2+/O132 Δtir is no longer virulent, and that the attachment of intimin to Tir is necessary for induction of diarrhoea. This corresponds with the results obtained by MARCHES *et al.* (2000).

During experiment n°2, one rabbit of group 2 (d14) and two rabbits of group 4 (d1 and 5) died as a result of enteritis caused by *C. spiroforme*. It is not probable that this enterotoxaemia was secondary to the vaccination with 2+/O132 Δtir , since the latter could not be isolated from the caecal contents of any of the three rabbits. If the vaccine strain had induced the enteritis, it would have been present in high numbers. These rabbits were excluded from the statistical analyses. No significant difference was observed between the five groups during the 4 weeks following vaccination.

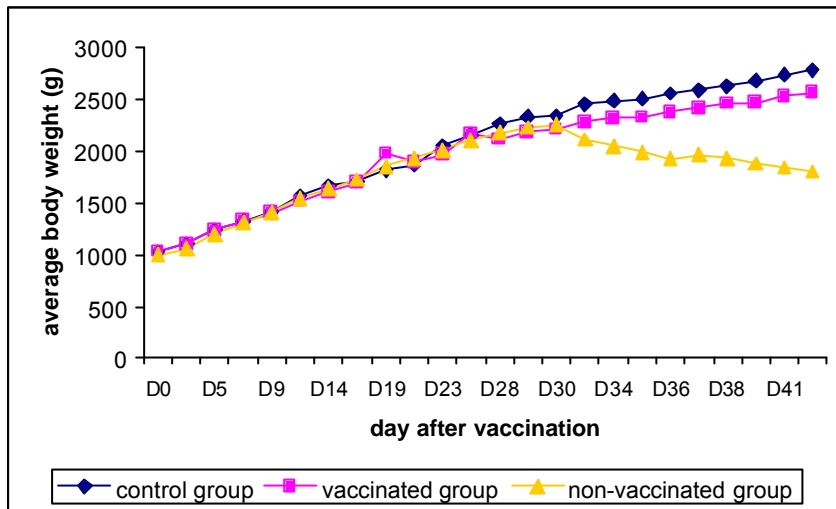


Figure 1. Challenge with pathotype 3-/O15 (d0: vaccination, d30: challenge). Average body weight per group. Starting at d32, the difference between the non-vaccinated group and the two other groups is significant ($P<0.0001$).

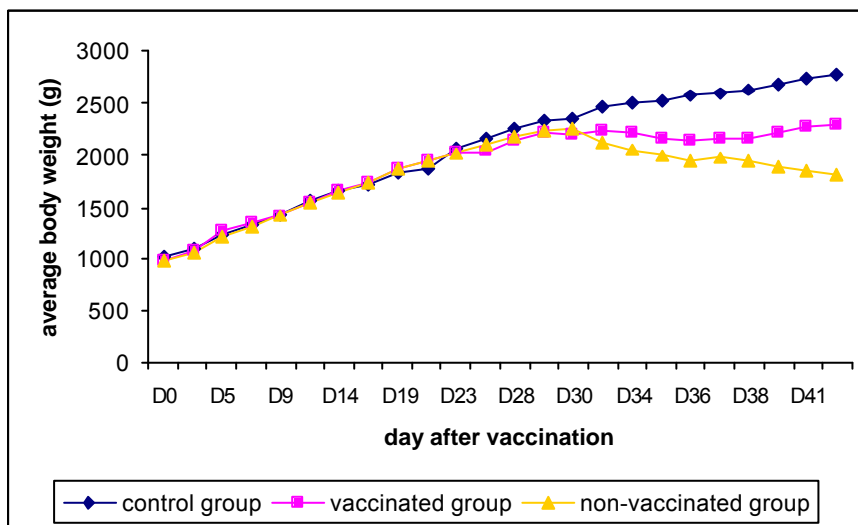


Figure 2. Challenge with pathotype 8+/O103 (d0: vaccination, d30: challenge). Average body weight per group. Starting at d32, the difference between the vaccinated challenged group and the control group is significant ($P=0.0017$), as well as the difference between the non-vaccinated and the vaccinated group ($P=0.0010$), and the non-vaccinated and the control group ($P<0.0001$).

After challenge with WT strain 3-/O15 in groups 2 and 3, no significant difference was detected between the vaccinated challenged group and the control group. In the non-vaccinated challenged group we observed diarrhoea as well as a significant difference

($P < 0.0001$) with the control and vaccinated groups for body weight (Figure 1) and feed intake. After challenge with WT strain 8+/O103, a significant difference was detected between the vaccinated challenged group and the control group for body weight ($P = 0.0017$) (Figure 2) and feed intake ($P = 0.0002$). In the non-vaccinated challenged group, diarrhoea and a significant difference with the vaccinated group ($P = 0.0010$) and the control group ($P < 0.0001$) were observed for body weight and feed intake. We may therefore conclude that the 2+/O132 Δtir strain offers good cross protection against the 3/O15 challenge strain, but only partial cross protection against the 8+/O103 challenge strain.

Vaccination with strain 3-/O15 Δeae offers a complete protection against infection with a homologous virulent strain (STAKENBORG *et al.*, 2001), but cross protection against other pathotypes was limited (results not shown). This indicates that the LEE-encoded antigens other than intimin are not sufficiently immunogenic to induce a protective immunological response against other pathotypes. The 2+/O132 Δtir strain produces the highly immunogenic intimin. Nevertheless, it does not offer a complete cross protection against the tested challenge strains. Consequently, a protective immunological response against intimin is not likely.

CONCLUSION

It was shown previously that none of the LEE gene products, other than intimin, offered a sufficient cross protection in vaccination-challenge trials with other pathotypes. In this study, it was shown that an attenuated strain producing the highly immunogenic intimin was not capable either of offering sufficient cross protection. A study identifying immunoprotective antigens of REPEC should be performed. Its results may allow the construction of one or more mutants that could be useful as vaccine strains.

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