



Published in final edited form as:

Microb Pathog. 2010 January ; 48(1): 1. doi:10.1016/j.micpath.2009.10.005.

Development of a Live Oral Attaching and Effacing *Escherichia coli* Vaccine Candidate Using *Vibrio cholerae* CVD 103-HgR as Antigen Vector

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Abstract

Attaching and effacing *Escherichia coli* (AEEC) share the ability to induce pedestal formation and intimate adherence of the bacteria to the intestinal epithelial cell and effacement of microvilli of epithelial tissue. The Locus of Enterocyte Effacement (LEE) pathogenicity island encodes the ability to induce attaching and effacing (A/E) lesions and contains the gene *eae*, which encodes intimin, an outer membrane protein that is an adhesin for A/E lesion formation. Here we show the utility of using intimin as a vaccine to protect rabbits from challenge with rabbit Enteropathogenic *E. coli* (REPEC), a member of the AEEC family. The C-terminal portion of intimin was delivered by the attenuated *Vibrio cholerae* vaccine strain CVD 103-HgR. To export intimin, a fusion was engineered with ClyA, a secreted protein from *Salmonella enterica* serovar Typhi. After immunization, antibodies specific to intimin from serum and bile samples were detected and moderate protection against challenge with a virulent REPEC strain was observed. Compared to animals immunized with vector alone, intimin-immunized rabbits exhibited reduced fecal bacterial shedding, milder diarrheal symptoms, lower weight loss, and reduced colonization of REPEC in the cecum. *V. cholerae* CVD 103-HgR shows promise as a vector to deliver antigens and confer protection against AEEC pathogens.

Keywords

EHEC; EPEC; AEEC; REPEC; intimin; vaccine

1. Introduction

Attaching and effacing *Escherichia coli* (AEEC) represent a group of human and animal pathogens that produce a characteristic intestinal histopathology defined by attaching and effacing (A/E) lesions on epithelial cells in culture and in the intestine of experimentally inoculated animals [1,2]. A/E histopathology results from intimate attachment of the bacteria

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to the epithelial cells, effacement of the microvilli, and rearrangement of the host cell actin cytoskeleton [3,2]. Among AEEC are pathotypes such as Enterohemorrhagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC), atypical EPEC, rabbit EPEC (REPEC), and *Citrobacter rodentium* [1,4,5,6,7]. Together, these pathotypes form a family of pathogens able to cause disease in humans and a variety of animal hosts. The genes encoding the A/E phenotype are contained on a pathogenicity island called the Locus of Enterocyte Effacement (LEE) [8]. The LEE encodes proteins with a range of functions, including a type III secretion system (TTSS), various secreted effectors proteins, and their chaperones [8,9]. The central region of the LEE contains the *eae* (*E. coli* attachment and effacement) gene encoding the 94 to 97 kDa outer membrane protein known as intimin [10]. This protein mediates close contact between the bacteria and the target cell upon interaction with its translocated receptor Tir (Translocated intimin receptor) [11]. The TTSS is responsible for secreting effector proteins into epithelial cells and modulating eukaryotic pathways to produce pedestal-like structures and effaced microvilli [2].

All *eae* alleles described to date demonstrate high similarity to each other in their N-terminal regions [12-15], but great diversity in their C-terminal regions, the region essential for Tir recognition and binding. Different intimin proteins are designated by using a Greek letter. Intimins of AEEC, including EPEC O127:H6 (α intimin), EHEC O157:H7 (γ intimin), and REPEC O15:H- (β intimin), show greater than 94% protein identity over two-thirds of the molecule on the N-terminal end, while demonstrating only 55% protein identity over the remaining C terminus, the region responsible for subtype classification [15,16,17]

To date, no other adhesin have been implicated as strongly as intimin in both epidemiological surveys and animal models against AEEC strains. Further evidence of the crucial role of intimin in immunogenicity is provided by studies in mice that show the last 280 amino acids of the C-terminal fragment (Int₂₈₀) of EHEC can protect against homologous challenge [18,12,19,20]. Passive immunization of neonatal piglets from dams immunized with an intact intimin molecule exhibited protection against EHEC O157:H7 colonization and intestinal damage [19]. All of these studies used vaccines made from purified C terminus of intimin, demonstrating that this domain is sufficient for protective immunity.

One approach to induce mucosal immunity is to deliver antigens via attenuated enteric vaccine strains such as the attenuated *Salmonella enterica* serovar Typhi live-vector vaccine strain CVD 908-*htrA*. Such an approach was successfully utilized to induce antibodies to *Bacillus anthracis* protective antigen (PA) [21]. This study used a new antigen export system engineered from an endogenous cryptic hemolysin (ClyA), of *Salmonella enterica* serovar Typhi, fused to the domain 4 (D4) moiety of (PA).

Vibrio cholerae has previously been used as a vector to deliver EHEC antigens. Butterton *et al* [22,23] used the attenuated Peru-2 *Vibrio cholerae* strain carrying a chromosomal copy of the complete *eae* gene and found that one of two rabbits immunized with this strain developed antibodies to intimin. However, Peru2 transformed with a plasmid carrying *stxB*₁ and *eae* under the transcriptional control of the *V. cholerae* heat shock promoter *PhpG* was unstable and produced low levels of StxB1 *in vitro*. Animals immunized with this construct did not develop antibodies against intimin following oral immunization.

Here we report the use of an alternative attenuated *V. cholerae* vaccine strain, CVD 103-HgR, as a vector to deliver intimin derived from rabbit-specific *E. coli* strain RDEC-1. A relevant animal challenge model was used to demonstrate immunogenicity and partial protection in rabbits immunized with this construct.

2. Results

2.1 Construction and expression of ClyA fused to intimin

To export intimin across the bacterial cellular membrane, we employed the plasmid vector pSEC91 which contains the cytolysin A hemolysin of *Salmonella enterica* serovar Typhi (ClyA). Wai *et al* demonstrated that the ClyA is exported from the bacterial cell in outer-membrane vesicles and that this vesicle-mediated transport mechanism may contribute to the activation and delivery of pathogenic effector proteins [24]. An antigen fusion to ClyA has been shown to effectively deliver the domain 4 (D4) of the protective antigen (PA) from *Bacillus anthracis* to mice [21].

The 772 bp fragment encoding the C-terminus of the β -*eae* gene from REPEC strain RDEC-1- (GenBank accession no. [U59503](#)) was amplified using PCR and ligated into the *Nhe I* site of pSEC91 (~15 copies/chromosomal equivalent) immediately downstream of the *ompC* promoter (Fig. 1A). This region contains the immunodominant region of intimin and the resulting construct was named pInt₂₄₈ as it contains first 248 residues of the Int₂₈₀ C-terminal region (Fig. 1B). Plasmid pInt₂₄₈ was electroporated into *V. cholerae* CVD 103-HgR, and the resulting strain was examined for β -intimin expression. Whole-cell lysates from both *E. coli* DH5 α pInt₂₄₈ and *V. cholerae* CVD 103-HgR pInt₂₄₈ were analyzed by western blot with anti- β intimin antisera and expression of β -intimin was observed from both hosts (Fig. 1C). ClyA:: β intimin fusion protein was also detected in the supernatant from both strains, although in lower levels in comparison to the whole-cell extract (Fig. 1C). No protein was recognized by antiserum in whole-cell lysates from CVD 103-HgR (pSEC91) or DH5 α (pSEC91) controls containing only the plasmid vector without *eae* sequences (Fig. 1C).

2.2. Assessment of immunogenicity of intimin in rabbits using CVD 103-HgR (pInt₂₄₈)

To determine the ability of intimin to confer immunity in rabbits, experiments were performed examining the IgA levels in serum after oral vaccination with CVD 103-HgR (pInt₂₄₈). Three groups of six rabbits were primed on day 1 with one of three inocula: PBS, CVD 103-HgR (pSEC91), or CVD 103-HgR (pInt₂₄₈) and boosted on day 15 with the identical inoculum. Serum collected after inoculation (day 28) exhibited significantly higher levels of specific anti-intimin IgA antibodies than serum collected before exposure (day 0) in the rabbits who received CVD 103-HgR (pInt₂₄₈) ($p = 0.0046$) (Fig. 2). There was no difference in the day 0 and day 28 responses in the rabbits who received CVD 103-HgR containing the vector only [CVD 103-HgR (pSEC91)]. Fecal shedding was examined for seven consecutive days for both strains after inoculation and booster to confirm delivery of bacteria to the rabbit intestinal track (data not shown).

2.3. Protection against REPEC challenge after oral immunization

To ascertain whether intimin offers protection to rabbits immunized using our antigen delivery system, rabbits were challenged on day 29 with wild type REPEC strain E22 (6×10^7 CFU). Fecal characteristics, bacterial shedding in feces and weight loss were examined over a course of 6 consecutive days after challenge. All rabbits immunized with control construct CVD 103-HgR (pSEC91) developed diarrhea by the third day and two developed symptoms as early as day 1. Three rabbits were euthanized on day 3 and one on day 4 due to severity of the diarrhea, with only two surviving to the sixth day. Conversely, the subset of rabbits immunized with CVD 103-HgR (pInt₂₄₈) did not develop diarrhea until the third day; one rabbit was euthanized at day 3 and one on day 4. The four remaining rabbits survived with diarrhea symptoms throughout the course of the study (6 days). Overall, throughout the course of infection, animals immunized with intimin before the challenge exhibited milder diarrheal symptoms than the group receiving the vector alone (Table 2), with the major difference being the number of

animals having to be euthanized because of severe diarrhea being lower in the intimin-immunized group (2 of 6) compared to the sham-immunized group (4 of 6).

After challenge with REPEC E22, the feces were collected over a period of 5 days and bacterial shedding was assessed (Figure 3A). Shedding of REPEC E22 was lower in the rabbits immunized with intimin, and the difference was statistically significant at day 3 ($p=0.03$). The overall trend of REPEC E22 fecal shedding was consistently lower in the group who received intimin compared to vector alone, although a statistically significant difference was seen only on day 3.

The percentage of weight loss was studied over a period of seven days post REPEC infection among animals primed with PBS (data not shown), CVD 103-HgR (pSEC91), and CVD 103-HgR (pInt₂₄₈). Although the population immunized with intimin lost less weight than the other groups, the differences were not statistically significant using t-test analysis (Fig. 3B).

To test for antibody levels against intimin, IgA levels in the bile were evaluated after euthanization (Fig. 3C). Rabbits immunized with intimin had IgA levels eight times higher than those seen in animals immunized with the vector alone ($p = 0.032$) or PBS ($p = 0.033$) following challenge.

The amount of non-adherent REPEC E22 was assessed from the cecal contents post-mortem. Colony forming units were counted, and no statistically significant difference was found between rabbits exposed to CVD 103-HgR (pInt₂₄₈) and CVD 103-HgR (pSEC91) ($p=0.126$) (data not shown).

A powerful way to assess REPEC diarrheal disease is the evaluation of REPEC adherence, particularly in the cecum of the rabbits after challenge, in a quantitative manner. Bacterial adherence of the challenge organism was determined post-mortem by histological examination of intestinal sections and graded as the percentage of surface area in the microscopic field that was covered by closely adherent bacteria. The level of bacterial adherence in the cecum was lower (9%) in the group who received CVD 103-HgR expressing intimin than in the group who received the vector-only control (23%) (Fig. 3D). These results correlated with the level of microvilli effacement seen post mortem in the ileum, proximal cecum and distal cecum (Fig. 4). Microvilli effacement beneath adherent bacteria and irregular mucosal surfaces were seen in rabbits immunized with the vector control and then challenged with REPEC E22 (Fig. 4A and 4B) while normal mucosal surfaces were seen in animals vaccinated with intimin and then challenged (Fig. 4C and 4D).

3. Discussion and Conclusion

Antibodies against intimin are induced after both human and animal infections and are related to protection as well as inhibition of bacterial adhesion to epithelial cells *in vitro* [25,26]. The carboxyl end of intimin (Int₂₈₀) can be divided into two immunoglobulin-like domains (D1 and D2) and one lectin-like domain (D3) [27,28]. Despite the variability of these regions among the intimin subtypes, amino acid residues C₈₆₀, W₈₉₉ and C₉₃₇, located in D3, are highly conserved and important for protein stability, Tir binding, and A/E lesion induction [29,28]. A minimal intimin fragment capable of binding Tir is composed of the last 190 amino acids in the Int₂₈₀ sequence, but this smaller fragment binds with less affinity than the larger 280-residue fragment of Int₂₈₀ [29]. Although the Tir-binding site is located at the C-terminal end of Int₂₈₀, analysis of the immunodominant regions show that antibodies are directed towards two regions in the N-terminal of Int₂₈₀ (residues 1 – 80 and 80 – 130) [12]. Human colostrum samples specifically recognize these fragments, but not the C-terminal region of Int₂₈₀ [12]. Thus, natural exposure to intimin-bearing pathogens seems to elicit antibodies that are reactive only to the N-terminal region of Int₂₈₀. In the present work, the intimin fragments expressed

in *V. cholerae* CVD 103-HgR include the first 248 residues of Int₂₈₀, and therefore the immunodominant region.

V. cholerae has been used to delivery a variety of foreign antigens including Shiga toxin (Stx) B subunit, fragment C of tetanus toxin, tracheal colonization factor of *B. pertussis*, *Shigella sonnei* O antigen, the immunogenic portion of *Clostridium difficile* toxin A, the intimin colonization factor of enterohemorrhagic *E. coli*, hepatitis B middle surface antigen, gp120 from HIV, and the serine-rich *Entamoeba histolytica* protein (reviewed in [30]). Although no human studies have yet been reported with any of these constructs, protective immunity has been demonstrated in a rabbit model against challenge with *C. difficile* toxin A and Stx holotoxin (reviewed in [30]).

CVD 103-HgR is a live, oral attenuated *V. cholerae* vaccine strain that is immunogenic, safe, and protective in humans and was licensed in several countries including Switzerland, Canada, and Australia [31,32]. Previous research utilized this strain to induce protective antibodies in rabbits with a vector containing Shiga-like toxin 1B subunit (Stx-1B) [33]. Butterton *et al* used attenuated *V. cholerae* strain Peru-2 to express a variety of heterologous antigens such as fragment C of tetanus toxin and *C. difficile* toxin A and intimin of EHEC [22] [23]. In the present study, we utilized an antigen delivery system comprised of the cryptic hemolysin ClyA from *Salmonella* serovar Typhi Ty2 to express *V. cholerae* CVD 103-HgR. This system was previously reported by Galen *et al* to provide excellent secretion from *Salmonella* serovar Typhi of a Clya::D4 fusion of 50.2kDA encoding Protective Antigen (PA) of *Bacillus anthracis*, and the exported Clya::D4 induced an immune response in mice [21]. Our data demonstrates Int₂₄₈ was highly expressed in *V. cholerae* CVD 103-HgR using the pSEC91 vector but was minimally secreted into the surrounding medium (Fig. 1C). The reason for the poor extracellular secretion seen is not known but may possibly be due to the hydrophobicity of the intimin fragment.

Although poorly secreted, the intimin fusion construct in *V. cholerae* was found to be immunogenic in rabbits as noted by bile and serum IgA response against intimin orally inoculated with this construct. When the immunized animals were challenged with the wild type REPEC only partial protection against REPEC challenge was demonstrated as all rabbits developed diarrhea during the course of study. However, IgA levels were significantly increased, death and severity of illness much reduced, and histological samples indicated less tissue damage in the animals inoculated with CVD103-HgR (pInt₂₄₈) compared to the CVD103-HgR (pSEC91) vector control. Considering the high mortality associated with the REPEC E22 strain, the challenge of 10⁷ CFU may have been too high to observe full protection as it has a lethal dose of 6.7 × 10³ CFU [34].

Although conclusive demonstration of protective efficacy was not demonstrated in this sturdy, our results suggest that attenuated *V. cholerae* expressing Clya::intimin merits further study as a tool to confer protection against AEEC strains. The demonstration of protective efficacy may be improved by some changes in the experimental design. For example, we administered only two doses of the vaccine but three doses might prove to be more protective. A lower challenge dose more reflective of the natural infectious dose may also show increased protection. In the future additional proteins should be considered as potentially protective antigens such as EspA, EspB, or EspD to induce a broader immune response. In conclusion, *V. cholerae* CVD 103-HgR (pInt₂₄₈) has potential as a delivery vehicle for a new generation of live oral attenuated heterologous AEEC vaccine candidates for animals and humans but further work is required to optimize the antigen delivery system.

4. Materials and Methods

4.1. Strains

Strains and plasmids used in this study are detailed in Table 1. The antibiotics Ampicillin (Ap) and Kanamycin (Km) were used at concentrations of 100 and 50 µg/ml respectively in Luria-Bertani (LB) medium or agar plates.

4.2. Cloning of the beta-intimin fragment into pSEC91

The intimin fragment (Int₂₄₈) was PCR amplified using primers K3894 (5'-ctagctagcagcattactgagattaaggc) and K3895 (5'-ctagctagctcatatgtgcttgatacacc) that span the C terminal- end (D1, D2 and part of D3) of β intimin (residues 652 – 908 of RDEC-1 strain; GenBank accession no. [U59503](#)). The PCR protocol included the following cycles: denaturation at 94°C for 2 minutes followed by 34 cycles of 94°C for 30 seconds; 50°C for 30 seconds, 72°C for 1 minute, and final extension cycle at 72°C for 8 minutes. The 772 bp amplicon was purified using the Qiaquick PCR purification Kit (Qiagen). The β-intimin fragment and pSEC91 vector were digested with *Nhe* I and ligated using T4 DNA ligase (BioLabs). The ligation was transformed into competent *E. coli* DH-5α (Invitrogen) and *V. cholerae* CVD 103-HgR.

4.3. Screening of transformants by PCR

Both *E. coli* DH5α and *V. cholerae* CVD 103-HgR transformants were screened for the presence of *clyA::int₂₄₈* fusion by PCR using the *clyA* forward primer from pSEC91 (K 3901: 5'-tactagcttatcagctacag) and K3895. The PCR cycles remained the same as above for Int₂₄₈ amplification. DNA sequence analysis was performed to confirm the constructs.

4.4. Cloning the beta-intimin fragment into pBAD102

The intimin fragment (Int₂₄₈) was PCR amplified using primers K4096 (5'-caccgccagyattactgagattaaggct) and K4095 (5'-atcatatgtgcttgatacacc) of β intimin (residues 658 – 911 of RDEC-1 strain; GenBank accession no. [U59503](#)). PCR included the following cycles: denaturation at 94°C for 2 minutes followed by 34 cycles of 94°C for 15 seconds; 55°C for 30 seconds, 68°C for 1 minute, and final extension cycle at 68°C for 8 minutes. The 732 bp amplicon was purified using the Qiaquick PCR purification Kit (Qiagen). The β-intimin fragment was cloned into the pBAD102 vector following the manufacturer's instruction for the pBAD Directional TOPO Expression Kits (Invitrogen).

4.5 Expression and Purification of His-tagged beta-intimin from pCVD473

The expression and purification of His-tagged beta-intimin protein was performed using Ni-NTA Purification System (Invitrogen).

4.6 Secreted protein by Western Blot analysis

CVD 103-HgR (pInt₂₄₈), was grown overnight in 2ml LB and centrifuged at 3500 rpm for 10 minutes at 4° C (in a microcentrifuge). (The pellet was retained for the whole cell lysate analysis). Deoxycholic acid (5%) was added to the supernatant (10 µl) and mixed. Trichloroacetic acid solution 10% (Sigma Diagnostic, St. Louis, 200 µl) was added and the preparation was kept on ice for 15 min. The sample was centrifuged at 15,000 rpm for 10 min. at 4° C and the pellet was suspended in 2x SDS sample buffer and 2M Tris base (4:1). After boiling for 5 min., the samples were subjected to SDS-12% PAGE and transferred to Hybond-ECL™ Nitrocellulose membranes (GE Healthcare) following the standard western blot procedure [35]. After the transfer, the membranes were blocked with 3% bovine serum albumin [BSA] and incubated with β-intimin antiserum (1:5000) (kindly provided by Flavia A. Lima from Institute Butantan, São Paulo, Brazil) diluted in PBS-0.1% Tween-20 for 1 hr with

constant shaking at room temperature. The membranes were then incubated with secondary antibody (Goat α -rabbit IgG [diluted 1:10 000]) conjugated with horse-radish peroxidase [HRP] (Sigma). Membranes were washed three times consecutively after primary and secondary antibody incubations with PBS-0.1% Tween-20 for 5 min. each. The blots were developed with ECL plus Western Blotting Detection System (Amersham Biosciences) followed by X-ray film exposure.

4.7. Rabbit inoculation and immunization

The protocols for rabbit inoculation and immunization were done according to Zhu *et al* with some modifications [36]. Briefly, twelve male New Zealand White rabbits (approximately 7 weeks old) were anesthetized with xylazine and ketamine after a 12 hr fasting period. The rabbits were then given 50 mg cimetidine intravenously. After 15 minutes, a stomach tube was inserted in each rabbit and 10 ml of a 10% sodium bicarbonate solution was administered, followed by 3 ml of the bacterial inoculation (2×10^9 CFU) and 5 ml of PBS; 30 minutes later, each animal was given 1 ml of tincture of opium intraperitoneally. Three groups of six rabbits received one of the following: CVD 103-HgR (pSEC91), CVD 103-HgR (pInt₂₄₈), or PBS. Rabbits were given a second oral inoculation of the same inoculum (1×10^{10} CFU) 15 days after the first inoculation. Blood was drawn from the rabbits prior to the inoculation at days 0, 14, and 28.

4.8. Challenge studies

Rabbits were challenged with REPEC E22 (6×10^7 CFU) 29 days after the first inoculation. Rabbits were weighed daily and observed for clinical signs of illness and stool characteristics six consecutive days post challenge, and euthanized at day 6. Stool characteristics were classified as: soft stools (SS), diarrheic (D), diarrhea with mucus (DM), bloody diarrhea (BD), and severe diarrhea (SD). Rabbits that lost greater than 20% body weight or experienced severe or bloody diarrhea were euthanized. Bile was aspirated from the rabbit gall bladder at euthanization, and bacterial counts of REPEC in cecal contents were determined by plating serial 10-fold dilutions of weighed cecal contents on LB agar supplemented with appropriate antibiotics.

4.9. Evaluation of IgA serum and bile antibody titers

Total immunoglobulin A (IgA) antibodies specific for β -intimin were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, microtiter plates were coated with purified recombinant protein β -intimin at 5 μ g/ml (purified from pCVD473) in 100 μ l of PBS (pH=7.4 for 3 hrs. at 37° C). The plates were then blocked with 10% skim milk (Nestle USA, Inc., Glendale, Calif) in PBS overnight at 4° C. After each incubation, plates were washed six times with PBS containing 0.05% Tween-20 (PBST). To determine endpoint titers, eight two-fold dilutions of sera (or bile) in 10% milk PBST were tested. After washing, plates were incubated with 100 μ l of peroxidase-conjugated goat serum specific for rabbit IgA (Zymed, San Francisco, Calif.) and diluted 1/1,000 in the same diluent (1 hr incubation at room temperature). The substrate solution used was TMB Microwell Peroxidase (KPL Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). After a 15 min. incubation at room temperature, the reaction was stopped by the addition of 100 μ l of 1 M H₂PO₄, and the OD₄₅₀ nm was determined in an ELISA microplate reader (Multiskan Ascent; Thermo Labsystems, Helsinki, Finland). Serum samples were run in duplicate with negative and positive control sera included in each assay. Linear regression curves were plotted for each serum sample, and titers were calculated through equation parameters as the inverse of the serum dilution that produces an OD of 0.2 above the blank and expressed as ELISA units (EU)/ml.

4.10. Histological preparations

Transmural sections from the ileum, cecum, and colon were excised and fixed in 10% buffered formalin for sectioning. Tissues were stained with hematoxylin and eosin or with Giemsa stain. Microscopic examination of histopathology was based on 10 sequential, well-oriented, 400× fields from each sample. Bacterial enteroadherence was graded as the percentage of surface area in the field that is covered by closely adherent bacteria.

4.11. Statistical Analysis

The Student t-test was used as statistical analysis and a *P* value of <0.05 was considered to be statistically significant.

Acknowledgments

The authors would like to thank Flavia A. Lima for supplying the β-intimin antisera. Additionally, we would like to thank Jane Michalski for editorial assistance on the manuscript. This study was supported by NIH grants DK58957 and AI19716 (to JBK), DK 059012 and VA Merit Award to ECB, and the Mid-Atlantic Regional Center for Excellence for Biodefense and Emerging Infectious Diseases Research grant U54 AI57168 (to M. M. Levine).

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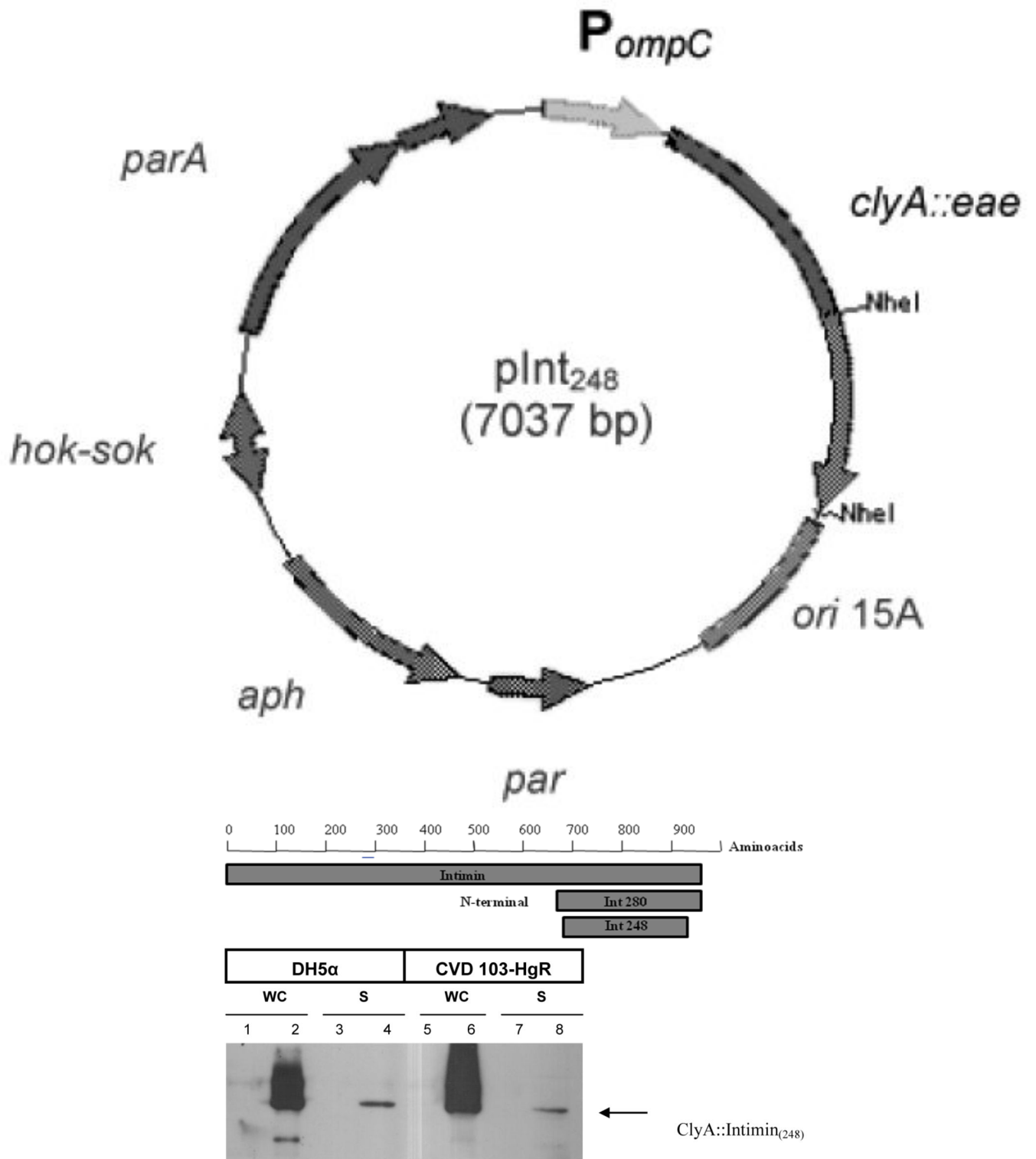


Figure 1. Intimin constructs (A) Genetic map of expression plasmid pInt₂₄₈ derived from pSEC91 encoding ClyA::Int₂₄₈ fusion protein. Abbreviations: *P_{ompC}*, modified osmotically controlled *ompC* promoter from *E. coli*; *clyA::eae₍₂₄₈₎*, gene encoding intimin₍₂₄₈₎ of RDEC-1

fused to the carboxyl terminus of ClyA from *Salmonella* serovar Typhi; *ori15A*, origin of replication from p15A providing an expected copy number of ~15 per chromosomal equivalent; *par*, passive partitioning system from pSC101; *aph*, gene encoding the aminoglycoside 3'-phosphotransferase conferring resistance to kanamycin; *hok-sok*, postsegregational killing locus from the antibiotic resistance R plasmid pR1; and *parA*, gene encoding the active partitioning system from pR1. **(B)** Diagram showing the localization of Intimin₂₄₈ in the complete intimin protein. **(C)** Western immunoblot analysis of bacterial whole cell fraction (WC) and supernatants(S). Whole cell fractions (WC) and supernatants (S) from either DH5 α (pSEC91) (lanes 1 and 3), DH5 α (pInt₂₄₈) (lanes 2 and 4), CVD 103-HgR(pSEC91) (lanes 5 and 7), CVD 103-HgR (pInt₂₄₈) (lanes 6 and 8) were transferred to a nitrocellulose membrane. After the transfer, the membranes were blocked and incubated with β -intimin antiserum.. ClyA::Intimin fusion protein was detected in the supernatant from both strains, although in lower levels in comparison to the whole-cell extract.

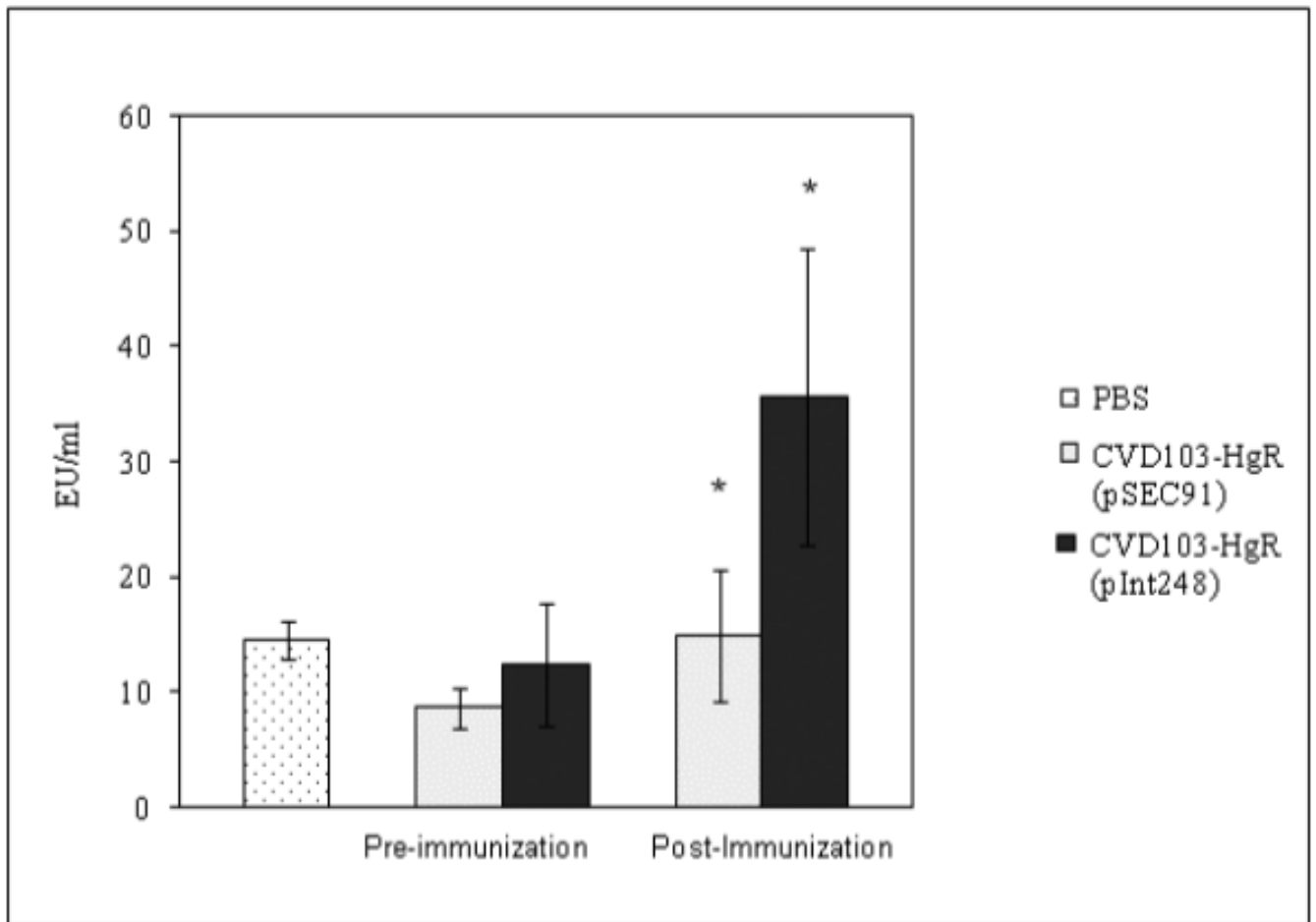


Figure 2.

Serum anti-intimin IgA titers pre-immunization and post-immunization. Immunoglobulin A (IgA) antibodies specific for β -intimin measured by enzyme-linked immunosorbent assay (ELISA). Serum collected before exposure (day 0) and post-immunization (day 28th) exhibited significantly higher levels of anti-intimin IgA specific antibodies in the rabbits who received CVD 103-HgR (pInt₂₄₈) compared to CVD 103-HgR alone (* p = 0.0046).

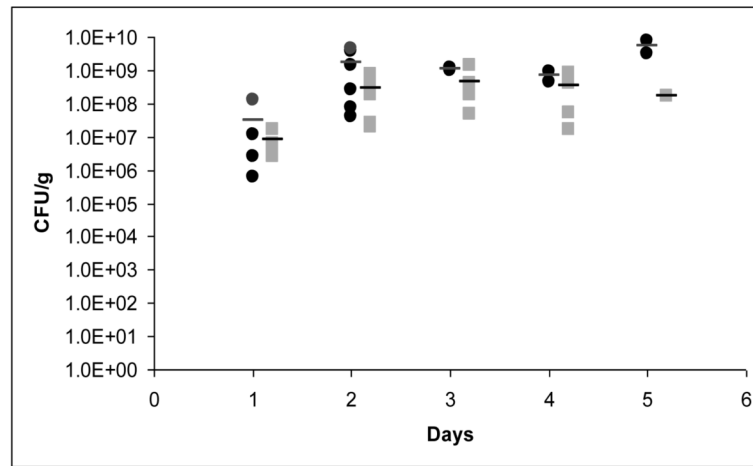


Figure 3A.

REPEC shedding from rabbits. After challenge with the wild-type REPEC E22 feces were collected and plated to determine bacterial colony forming units (CFUs) per gram of feces. Rabbits inoculated with CVD 103-HgR (pSEC91) are depicted in black and those inoculated with CVD 103-HgR (pInt₂₄₈) depicted in grey. The symbols indicate individual animals; the average in each group of rabbits is indicated by horizontal bars. Shedding of REPEC E22 was lower in the rabbits immunized with intimin, and was statistically significant at day 3 ($p=0.03$).

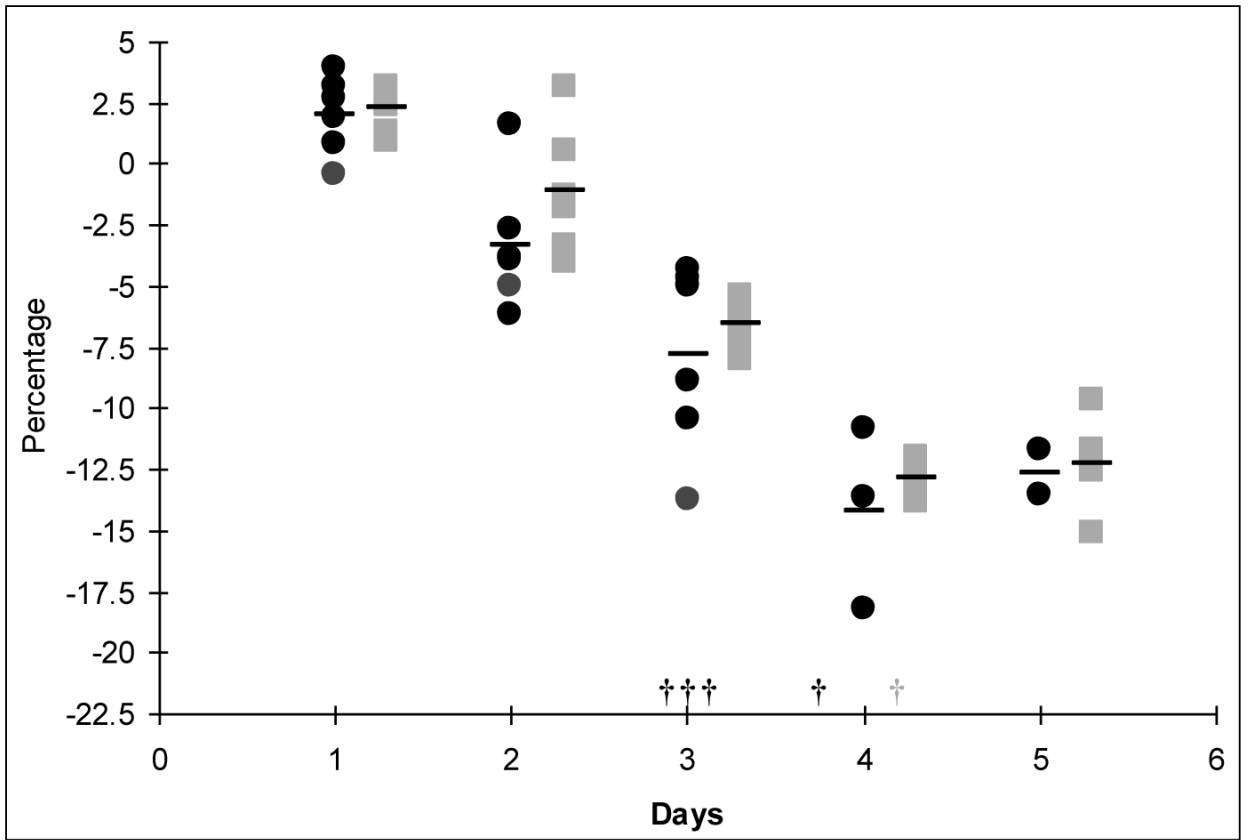


Figure 3B.

Percentage of weight loss in rabbits after challenge with REPEC E22. The rabbits were weighed every day after challenge with REPEC E22 and the weight loss or gain was represented as a percentage of their original weight. The group of rabbits receiving CVD 103-HgR(pSEC91) is presented in black and the group of rabbits receiving CVD 103-Hg (pInt₂₄₈) is in grey. Each symbol indicates an individual animal; the average of each group is indicated by horizontal bars. Mortality is represented by crosses. Although the population immunized with intimin lost less weight than the other groups, the differences were not statistically significant.

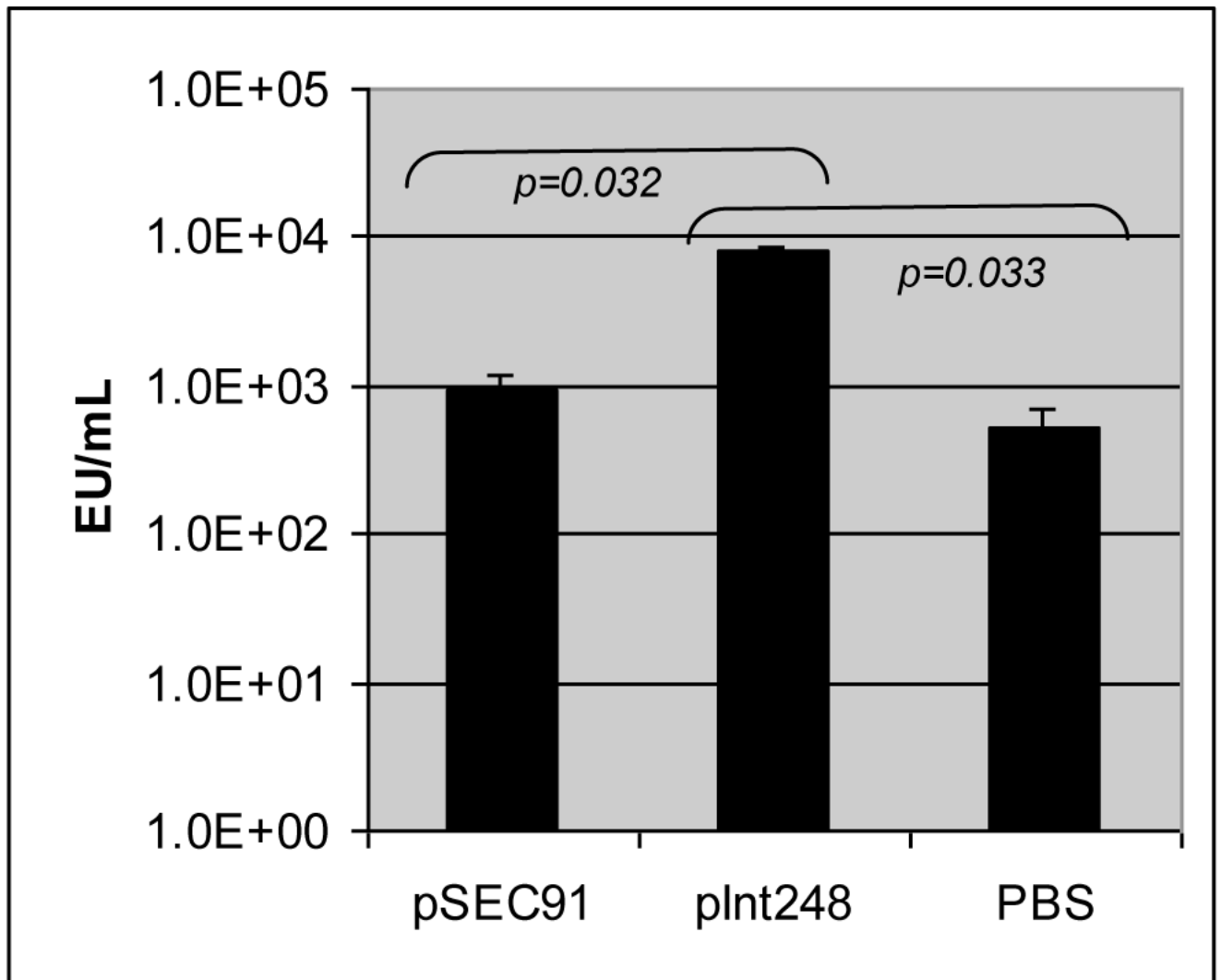


Figure 3C.

Bile anti-intimin IgA titers after REPEC challenge. Animals were immunized with CVD 103-HgR (pSEC91), CVD 103-Hg (pInt₂₄₈) or PBS. Significant differences were found between animals immunized with CVD 103-Hg (pInt₂₄₈) and those immunized with CVD 103-HgR (pSEC91) or PBS. The results shown are means of each with standard deviation indicated. Bile samples were collected either on day 6 or on earlier days, if the animals required euthanasia. Animals treated with PBS were not challenged with REPEC and bile samples were collected on day 6.

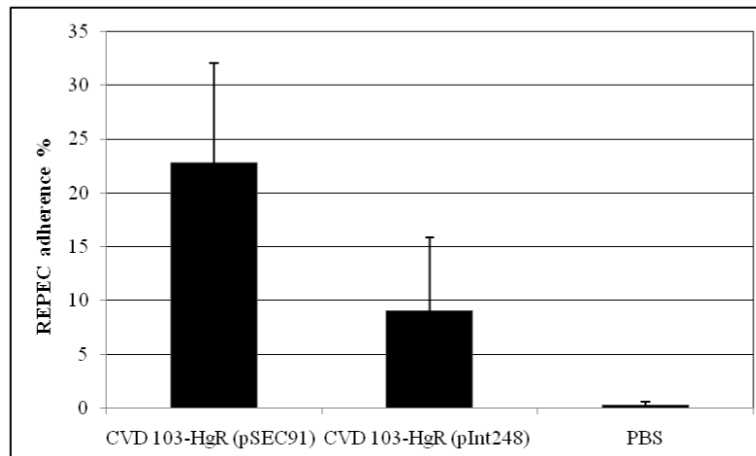


Figure 3D.

REPEC adherence in the cecum measured post mortem. Although the population immunized with intimin had less REPEC adhered than the other group the differences were not statistically significant. The results shown are means of each with standard deviation indicated. Bile samples were collected either on day 6 or on earlier days, if the animals required euthanasia. Animals treated with PBS were not challenged with REPEC and bile samples were collected on day 6.

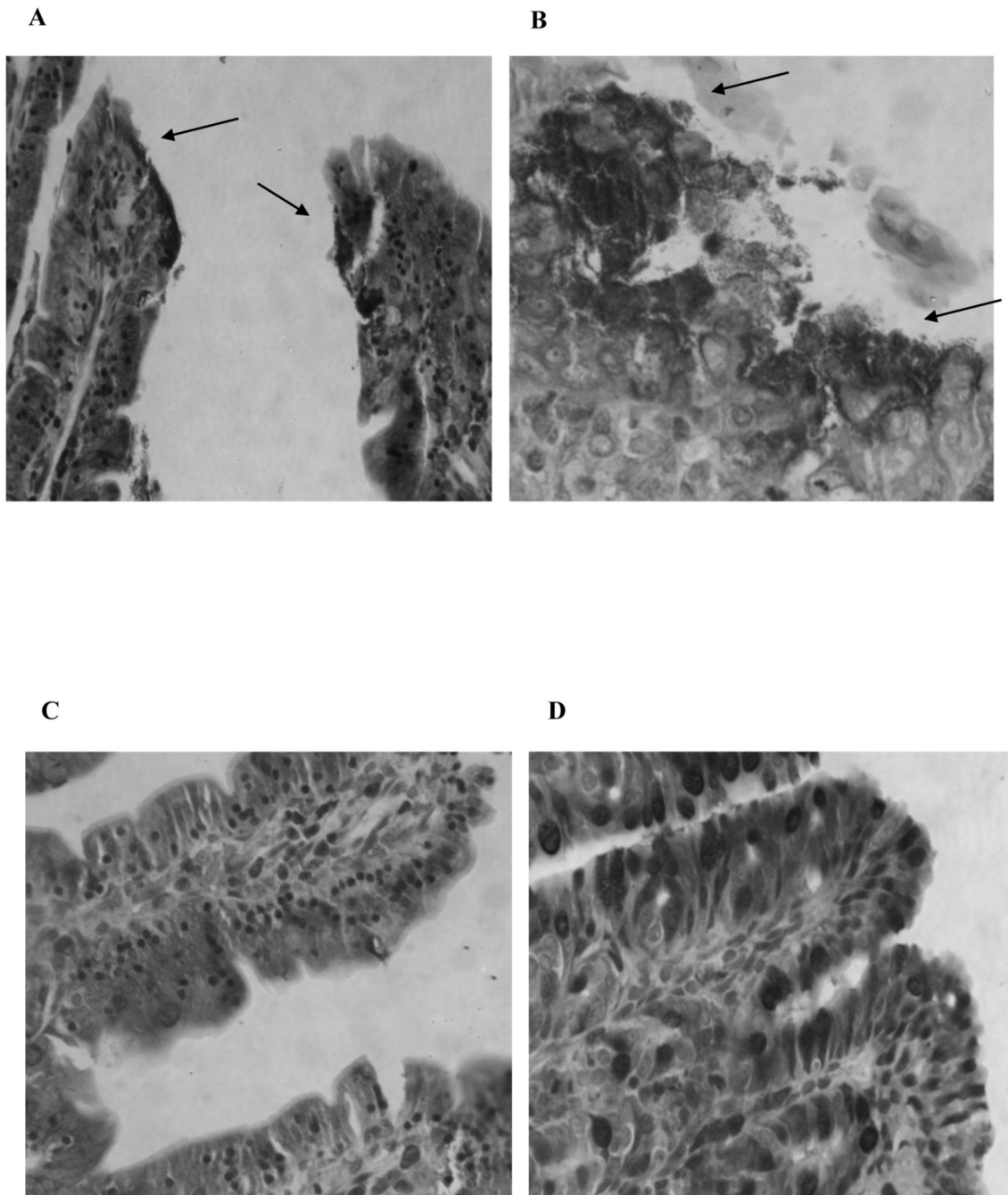


Figure 4.

Representative histological sections of ileum (A) and proximal cecum in (B) of a rabbit from the control group immunized with CVD 103-HgR (pSEC91) and challenged with REPEC E22 showing effacement of microvilli (arrows). Note that the mucosal surface has become irregular. In contrast, sections from the ileum (C) and from the proximal cecum (D) of a rabbit from the group who received CVD 103-HgR (pInt₂₄₈) and challenged with REPEC E22 strain showed normal histology of the mucosa. Giemsa staining; magnification X 400

Table 1

Strains and plasmids utilized in this study

Name	Reference or source
<i>E. coli</i> DH5 α	(Gibco-BRL Life Technologies)
<i>V. cholerae</i> CVD 103-HgR	[32]
<i>E. coli</i> RDEC-1 strain E43806	[5]
<i>E. coli</i> REPEC E22	[37]
pSEC91	[21]
<i>V. cholerae</i> CVD 103-HgR (pInt ₂₄₈)	Present study
<i>V. cholerae</i> CVD 103-HgR (pSEC91)	Present study
pCVD473	Present study

Table 2Characteristics of feces after vaccination with CVD 103-HgR (pSEC91) and CVD103-HgR (pInt₂₄₈)^{*}

Rabbit Number	Plasmid utilized	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
228		N	N	BD †			
229		SS	N	DM	DM	D	D
230	pSEC91	DM	D	SD	SD †		
231		N	SS	SD †			
232		N	SS	SD †			
233		N	N	D	DM	D	D
234		N	SS	SD †			
235		N	SS	DM	DM	SS	D
236	pInt ₂₄₈	N	N	DM	SD †		
237		N	N	SS	DM	SS	D
238		N	N	DM	DM	D	D
239		N	N	DM	DM	D	D

* Abbreviations: soft stools (SS), diarrhea (D), diarrhea with mucus (DM), bloody diarrhea (BD), and severe diarrhea (SD).

† indicate that the rabbit had to be euthanized