2016

Immunogenicity and protective efficacy against enterotoxigenic Escherichia coli colonization following intradermal, sublingual, or oral vaccination with EtpA adhesin

Qingwei Luo  
Washington University School of Medicine in St. Louis

Tim J. Vickers  
Washington University School of Medicine in St. Louis

James M. Fleckenstein  
Washington University School of Medicine in St. Louis

Follow this and additional works at: https://digitalcommons.wustl.edu/open_access_pubs

Recommended Citation
Luo, Qingwei; Vickers, Tim J.; and Fleckenstein, James M., "Immunogenicity and protective efficacy against enterotoxigenic Escherichia coli colonization following intradermal, sublingual, or oral vaccination with EtpA adhesin." Clinical and Vaccine Immunology. 23,7: 628-637. (2016).  
https://digitalcommons.wustl.edu/open_access_pubs/5064
Immunogenicity and Protective Efficacy against Enterotoxigenic Escherichia coli Colonization following Intradermal, Sublingual, or Oral Vaccination with EtpA Adhesin

Qingwei Luo, Tim J. Vickers, James M. Fleckenstein

Enterotoxigenic Escherichia coli (ETEC) strains are a common cause of diarrhea. Extraordinary antigenic diversity has prompted a search for conserved antigens to complement canonical approaches to ETEC vaccine development. EtpA, an immunogenic extracellular ETEC adhesin relatively conserved in the ETEC pathovar, has previously been shown to be a protective antigen following intranasal immunization. These studies were undertaken to explore alternative routes of EtpA vaccination that would permit use of a double mutant (R192G L211A) heat-labile toxin (dmLT) adjuvant. Here, oral vaccination with EtpA adjuvanted with dmLT afforded significant protection against small intestinal colonization, and the degree of protection correlated with fecal IgG, IgA, or total fecal antibody responses to EtpA. Sublingual vaccination yielded compartmentalized mucosal immune responses with significant increases in anti-EtpA fecal IgG and IgA, and mice vaccinated via this route were also protected against colonization. In contrast, while intradermal (i.d.) vaccination achieved high levels of both serum and fecal antibodies against both EtpA and dmLT, mice vaccinated via the i.d. route were not protected against subsequent colonization and the avidity of serum IgG and IgA EtpA-specific antibodies was significantly lower after i.d. immunization compared to other routes. Finally, we demonstrate that antiserum from vaccinated mice significantly impairs binding of LT to cognate GM1 receptors and shows near complete neutralization of toxin delivery by ETEC in vitro. Collectively, these data provide further evidence that EtpA could complement future vaccine strategies but also suggest that additional effort will be required to optimize its use as a protective immunogen.
TABLE 1 Bacterial strains and plasmids used in this study

| Strain or plasmid | Descriptiona | Source or reference(s) |
|-------------------|--------------|------------------------|
| H10407            | Wild-type ETEC strain O78:H11; CFA/1 LT- | 57, 58 |
| jf570             | eltAB LT deletion mutant of H10407 | 59 |
| jf1696            | top10(pJL017, pJL030) Amp' Cm' | 24 |
| TOP10             | F- mcrA Δ(mvr-hudRMS-merBC) Δospa2 Δ33ΔM15 invitrogen | |
|                   | lacX74 recA1 araD139 araE197 Δ(arab ena)7967 galU galK rpsL (Str') endA1 supG | |

Plasmids

pJL017  etpA cloned into pBAD/myc-His A, with etpA in frame with myc and 6His coding regions; Amp' 

pJL030  etpC gene cloned into pACYC184; Cm' 

a Kmr, kanamycin resistant; Cmr, chloramphenicol resistant; Ampr, ampicillin resistant; Str', streptomycin resistant.

of EtpA when delivered by other routes using a double mutant (R192G L211A) heat-labile toxin (dmLT) as the adjuvant.

MATERIALS AND METHODS

Adjuvant and immunogen preparation. The double mutant (R192G L211A) heat-labile toxin (dmLT) (23) used in these studies was manufactured by the Bioproduction Facility at Walter Reed Army Institute for Research, Silver Spring, MD (BPR-1037-00, lot no. 1735) and was stored lyophilized at −20°C prior to use. dmLT was reconstituted to 1 mg/ml in sterile phosphate-buffered saline (PBS) immediately before use and then diluted as needed with PBS. Recombinant histidine-tagged EtpA glycoprotein (rEtpA) was purified as previously described using metal affinity chromatography from culture supernatants of an E. coli mutant (24). Briefly, overnight cultures grown in LB medium, grown to an OD600 of 0.8, were diluted 1:100 into fresh LB medium and grown at 37°C to an optical density at 600 nm (OD600) of ~0.6. Arabinose (0.0002%) was then added to induce EtpA expression. After 3 h of incubation, cultures were harvested at 6,000 rpm at 4°C for 10 min, and supernatant was filtered and saved for subsequent purification. Supernatant was concentrated ~10-fold using a Pellicon concentrator with 30,000-molecular-weight (MW) cutoff (Millipore). After being loaded onto metal affinity columns (5 ml; HiTrap, GE), unbound protein was removed by washing with buffer containing 25 mM sodium phosphate, 300 mM sodium chloride (pH 7.4), and 0 to 100 mM imidazole. rEtpA was then eluted from the column in the same buffer over a gradient ranging from 100 to 600 mM imidazole. Eluate fractions containing rEtpA were pooled and then concentrated with an Amicon Ultra-15 Ultracel-100k centrifuge (Millipore), and the buffer was exchanged with PBS (pH 7.4) before being stored at −80°C. rEtpA and dmLT were combined immediately prior to vaccination.

Immunoassay protocols. Female CD-1 mice (5 to 8 weeks old) were purchased from Charles River Laboratories. Groups of 10 to 12 mice were vaccinated on days 1, 29, and 43 via the sublingual, intradermal (i.d.), or orogastric routes, with additional vaccination given on day 57 by the sublingual route only. Before vaccination, mice were lightly anesthetized with isoflurane. For orogastric vaccination, mice were first fasted for 2 h and then gavaged with 100 μl of NaHCO3 (7.5%) to neutralize stomach acid. After 5 min, mice were then gavaged with the vaccine or controls in a final volume of 300 μl of PBS. For sublingual vaccination, 10 μl of the preparation was pipetted under the tongue, and the head was maintained up-right until the mouse regained consciousness. For intradermal vaccination, an alcohol swab was used to wet and separate abdominal fur, after which 50 μl of the vaccine preparation or control solution was delivered intradermally using a 1/2-ml insulin syringe fitted with a 29-gauge needle. To confirm intradermal placement of the antigen, we performed test i.d. injections of tattoo ink in parallel sets of live CD-1 mice. Sections of skin were subsequently processed for hematoxylin and eosin staining and examination by light microscopy.

Orogastric vaccination produced 25 μg of dmLT in adjuvant-only controls and as the adjuvant with doses of either 200 or 400 μg of rEtpA. Sublingual vaccination used 5 μg of dmLT/mouse ± 10 μg of EtpA. Intradermal vaccination was done in two experiments with doses of either 100 ng of dmLT ± 250 ng of rEtpA or 1 μg of dmLT ± 2.5 μg of rEtpA.

Sample collection. Mouse fecal samples were collected 2 weeks after each boost. Six fecal pellets from each mouse were resuspended in 1.5 ml of fecal resuspension buffer (containing 10 mM Tris base, 100 mM NaCl, 0.05% Tween 20, and 5 mM sodium azide, pH 7.4) and stored at 4°C overnight. Mouse serum samples were collected either from abdominal aorta or from terminal cardiac bleeds using 25-gauge tuberculin syringes.

Bacterial strains and growth conditions. Strain jf876, a derivative of ETEC strain H10407 bearing a kanamycin resistance marker in the lacZYA locus (lacZYA:kan) was used in all colonization experiments as previously described (25). Briefly, jf876 maintained as a frozen glycerol stock at −80°C was used to inoculate sterile Luria broth (LB). After overnight growth at 37°C at 225 rpm, the culture was diluted 1:100 into fresh LB medium, grown to an OD600 of ~0.3, and serially diluted to an inoculum of ~106 to 108 for challenge.

Intestinal colonization studies in mice. Intestinal colonization experiments were performed as previously described (25). Briefly, prior to challenge, mice were pretreated with streptomycin (5 g/liter) in drinking water for 24 h, followed by regular water for 12 h, and famotidine (50 mg/kg body weight) was given 1 to 3 h prior to challenge to neutralize stomach acid. Mice were then challenged with ~1010 CFU of ETEC bacteria by oral gavage (25). Twenty-four hours after challenge, mice were sacrificed and two 3-cm sections of small intestine (ileum) were collected as previously described. Following incubation in saponin (5%) for 10 min, dilutions of intestinal lysates in PBS were plated onto Luria agar plates containing kanamycin (50 μg/ml). Following overnight incubation, bacteria were enumerated by counting kanamycin-resistant colonies. All experiments with mice were performed under protocols approved by the Animal Studies Committee at Washington University School of Medicine.

Immunologic assessment. A kinetic enzyme-linked immunosorbent assay (ELISA) was used to detect immune responses in both fecal and serum samples as previously described (15). Briefly, 96-well plates were coated with 0.1 μg/well of EtpA or GM1 gangliosides (Sigma; catalog no. G2375) in carbonate buffer (15 mM Na2CO3, 35 mM NaHCO3, 0.2 g/liter NaN3, pH 8.6) overnight at 4°C. After washing, dmLT (1 μg/ml in PBS) was added to wells containing GM1 gangliosides and incubated for 1 h at 37°C. Plates were washed and blocked with 1% bovine serum albumin (BSA) for 1 h at 37°C. Plates were again washed and then incubated with fetal extracts (undiluted) or with sera (diluted 1:100 in PBS containing 1% BSA). After incubation at 37°C for 1 h, plates were washed and incubated with horseradish peroxidase (HRP)-secondary antibody conjugates for 30 min at 37°C. After washing, TMB (3,3′,5′,5′-tetramethylbenzidine)-peroxidase substrate (KPL) was added, and plates were immediately read at 650 nm to collect kinetic ELISA data (26). Data were analyzed using Gen5 software (BioTek) and are reported as Vmax (milliliters per minute).

Serum EtpA antibody avidity determinations. Avidity indexes for EtpA-specific serum IgG and IgA antibodies were determined as previously described (27). Briefly, microtiter wells were coated with EtpA as described above. After being washed and blocked, plates were incubated with sera diluted 1:10,000 at 37°C for 1 h. Plates were washed and then incubated with either 6 M urea solution or PBS for 10 min at 37°C. After being washed, plates were incubated with either anti-mouse IgA or anti-
FIG 1 Immunogenicity and inhibition of intestinal colonization following oral immunization with EtpA adjuvanted with dmLT. (a to f) Kinetic ELISA data demonstrating fecal antibody responses to dmLT (a to c) and EtpA (d to f) following oral immunization with dmLT adjuvant alone (25 μg), 25 μg dmLT plus 200 μg rEtpA, or 25 μg dmLT plus 400 μg rEtpA. The antigen and antibody isotype tested are shown in the upper left-hand corner of each graph. Serum responses (IgG) at a dilution of 1:100 are shown for (g) dmLT and (h) EtpA, and panels i and j correspond to serum IgA responses. (k) Intestinal colonization in mice (grey symbols represent individual mice) following challenge with enterotoxigenic *E. coli*. Dashed horizontal lines in each figure panel represent geometric means. Comparisons to the PBS control group were made by Mann-Whitney nonparametric testing (*, P < 0.05; ***, P < 0.001; ****, P < 0.0001).
To examine the ability of antisera to prevent binding of heat-labile toxin to its receptor, plates were coated with GM1 gangliosides at a 1 μg/ml final concentration in PBS overnight at 4°C. Serum from vaccinated or control mice was diluted 1:128 in PBS and then incubated 1:1 with LT at a final concentration of 1 g/ml at 4°C overnight. After washing of plates with PBS, LT preincubated with serum was then allowed to bind to target gangliosides for 1 h at 37°C. Plates were then washed and blocked with 1% BSA for 1 h at 37°C. Bound LT was determined by anti-LT-B rabbit ELISA. Briefly, plates were incubated using primary anti-LT-B rabbit polyclonal antiserum diluted 1:1,000 in PBS containing 1% BSA at 37°C for 1 h and washed, followed by addition of goat anti-rabbit IgG-HRP conjugate diluted 1:5,000 in 1% BSA at 37°C for 30 min. After the final washing, plates were developed with TMB substrate and data collected kinetically as described above.

To examine the ability of antisera from immunized animals to prevent effective toxin delivery by ETEC, a 96-well tissue culture plate was first seeded with Caco-2 cells and the culture was grown at 37°C in 5% CO₂ until the cells formed a confluent monolayer. One microliter of sera from individual mice following vaccination and intestinal colonization. Because EtpA functions as an adhesion, we examined the correlation between the levels of fecal antibody levels achieved in individual mice following vaccination and intestinal colonization. Levels of intestinal IgG (Fig. 2a) and IgA (Fig. 2b) were both related to reductions in intestinal colonization, while the strongest correlation (P = 0.005) was observed with total fecal antibody levels (Fig. 2c).

Sublingual administration of EtpA with dmLT. Previous studies have shown that sublingual administration of antigens can induce both systemic and mucosal antibody responses with doses that are appreciably smaller than those required for oral administration (29). Similar to oral administration, sublingual vaccination with the combination of dmLT and EtpA also stimulated production of fecal IgG (Fig. 3a) and fecal IgA (Fig. 3b) as well as serum IgG (Fig. 3c) and serum IgA (Fig. 3d) antibodies to the dmLT adjuvant. Although sublingual vaccination resulted in modest increases in responses to EtpA in feces (Fig. 3e and f), there was no significant increase in serum antibodies (Fig. 3g and h), consistent with compartmentalization of the mucosal immune response. While vaccination of mice sublingually with EtpA adjuvanted with dmLT did afford some protection against colonization relative to unvaccinated mice (Fig. 3i), we did not observe a significant correlation between fecal antibody levels and the level of intestinal colonization (Fig. 3j).

Intradermal administration of EtpA with dmLT. The dose of
The vaccine required to achieve an immunologic response is typically several orders of magnitude smaller with i.d. vaccination than that required for other routes, offering significant dose sparing. Moreover, the emergence of needle-free technologies (30, 31) could make this route feasible for deployment to developing countries, where cost and ease of administration are important considerations. Therefore, we also examined intradermal vaccination with EtpA in mice. After verification of intradermal placement of potential immunogens (see Fig. S1 in the supplemental material), we were able to achieve high titers of both serum and fecal antibodies to both the adjuvant (Fig. 4a to d) and the EtpA immunogen (Fig. 4e to h) following intradermal administration. Curiously, however, we saw no significant protection against colonization when mice were vaccinated via the i.d. route (Fig. 4g), suggesting that the route of administration could have a substantial impact on vaccine efficacy.

Because intradermal vaccination resulted in high titers of antibody that did not protect against intestinal colonization, we questioned whether the quality of antibody from mice vaccinated intradermally differed from that of mice vaccinated orally. Antibody avidity, which examines the functional affinity of antibody-antigen interactions, has been used as a marker of B cell maturation and a surrogate of protective immunity to a number of important pathogens. Importantly, antibody avidity has been shown to correlate with the presence of antigen-specific memory B cells following Vibrio cholerae infection (27), potentially permitting efficient measurement of responses that might predict protection against a number of important diarrheal pathogens. Interestingly, the se-

**FIG 3** Immunogenicity and inhibition of intestinal colonization following sublingual immunization with EtpA adjuvanted with dmLT. (a and b) Fecal IgG (a) and fecal IgA (b) responses to the dmLT adjuvant. (c and d) Serum IgG (c) and serum IgA (d) responses to dmLT. (e and f) Fecal IgG (e) and fecal IgA (f) responses to rEtpA. (g and h) Serum IgG (g) and serum IgA (h) responses to rEtpA. (i) Colonization of mice following vaccination with rEtpA adjuvanted with dmLT versus dmLT alone or PBS controls. (j) Correlation between fecal IgA and intestinal colonization following sublingual vaccination. Comparisons between groups were made by Mann-Whitney nonparametric testing (*, P < 0.05; ***, P < 0.001; ****, P < 0.0001).
rum IgG antibody avidity in the intradermally vaccinated mice was considerably lower than that in the mice vaccinated orally with EtpA (P < 0.002) (Fig. 5a), as was the EtpA-specific IgA avidity index (AI) (Fig. 5b). Collectively, these data suggest that further assessment of immunization routes, timing of administration, and doses of antigen and adjuvant will likely be important considerations in optimizing the protective efficacy of more recently described antigens, including EtpA.

**Toxin neutralization by sera from vaccinated mice.** Because immunization using dmLT as the adjuvant resulted in significant production of anti-LT antibody, we also examined whether the antibodies were functionally relevant. As demonstrated in Fig. 6a, sera from mice immunized using dmLT prevented effective binding of wild-type heat-labile toxin to target GM1 gangliosides in vitro relative to sera from PBS control mice. Importantly, we found that antisera from either mice immunized with dmLT adjuvant only or from mice immunized with recombinant EtpA and dmLT yielded marked reduction in effective toxin delivery by the
ETEC strain H10407, as determined by activation of cAMP in target Caco-2 epithelial cell monolayers (Fig. 6b). These data provide further evidence vaccines combining novel adhesin and toxin approaches could offer a viable strategy to protect against ETEC.

DISCUSSION

Construction of ETEC vaccines has been hampered in part by the extraordinary genetic plasticity of E. coli confounding efforts to define the ideal group of antigens that will achieve broad-based protection. Vaccines currently under study attempt to confront this challenge by using multiple live attenuated strains (32) or whole-cell killed strains that each present different CF antigens and are combined with mutant forms or subunits of heat-labile toxin (28). Interestingly, recent studies of more than 800 isolates from ETEC diarrhea cases in Bangladesh demonstrated that only approximately half of the strains expressed colonization factors (CFs) that could be identified by immunoassays (33), and new CF antigens continue to be identified by genomic sequencing (34).

The potential complexity of vaccines based exclusively on classical ETEC targets has driven investigation of other antigens common to the ETEC pathovar. Theoretically, these antigens, not currently part of ETEC vaccine platforms, could be incorporated into future iterations of vaccines to expand antigenic valency and perhaps act in concert with canonical ETEC vaccine targets to enhance efficacy. One potential alternative target is the two-partner secretion system that encodes EtpA. This system, originally discovered by transposon mutagenesis of the prototype H10407 strain (12), appears to be relatively conserved within the ETEC pathovar (7, 15, 16). EtpA-specific antibodies are present in human convalescent-phase sera, suggesting that this protein is expressed during the course of infection and is immunogenic (15, 35). These features and the molecular and functional similarity of EtpA to filamentous hemagglutinin (36), a component of acellular pertussis vaccines (37), have prompted preclinical investigation of the utility of EtpA as a protective antigen. Multiple studies with mice have now demonstrated that EtpA vaccination protects against colonization of the small intestine (14, 19, 20), thought to be a critical determinant of ETEC diarrheal illness. Another consideration driving the present studies is that ETEC and Shigella are among the most common bacterial pathogens causing serious di-arrheal illness among young children in developing countries; therefore, we questioned whether subunit approaches presently being developed for Shigella effectors (38, 39) could be also applied to novel secreted ETEC antigens, including EtpA.

Because all prior studies of this target antigen to date have involved intranasal immunization, we examined whether we might be able to deliver EtpA by other means and elicit protective immune responses. These most recent studies provide further evidence that EtpA affords protection against intestinal colonization and that this antigen retains substantial immunogenicity when administered by a variety of different routes.

Perhaps not surprisingly, however, protective efficacy varied with the route of administration (40, 41), and despite substantial antibody responses following i.d. immunization, we did not achieve protection. One possible explanation for these seemingly discordant results is that the levels of quality of antibodies generated in different vaccination protocols could differ substantially. Antibody avidity is thought to represent the overall strength of antibody affinity maturation have previously been associated with vaccine failures (46–50). The current studies seem to support the idea that antibody avidity could be an important parameter in evaluating antigen-specific memory responses and vaccine performance following immunization with novel and classical ETEC antigens (51).

The present data suggest that additional studies will need to take place to optimize both the route of administration, the doses of EtpA required to achieve protection, and conditions for coformulation with other antigens. Nevertheless, our studies suggest that it is possible to generate protective immune responses with the recombinant adhesin. The current studies are an extension of

Figures 5 and 6 show the avidity and neutralization of EtpA-specific antibodies in vaccinated mice.
earlier work with *Shigella* subunit proteins, and no attempt was made here to optimize immune responses to EtpA. Clearly, the doses of antigen delivered orally in these experiments would be impractical for effective immunization on a large scale. However, these studies suggest that enhanced delivery through an oral vaccine strain could be of benefit (32) and that EtpA could be used to complement existing canonical approaches to development of live attenuated ETEC vaccines (32). Not unexpectedly (33, 34), some routes of immunization resulted in highly compartmentalized responses. While serum antibody responses to EtpA were low following sublingual administration of antigen, we did observe measurable increases in fecal antibody (IgG and IgA) and sublingual immunization was protective. Application of emerging methods that enhance sublingual or buccal antigen delivery (35) could accelerate development of subunit vaccines that incorporate ETEC novel antigens.

Interestingly, emerging data do suggest that intradermal vaccination with mutant LT and ETEC fimbrial tip adhesins can protect against ETEC diarrhea in a human experimental challenge model (56), and i.d. immunization with secreted *Shigella* effectors adjuvanted with dNLT was protective against experimental challenge in mice (39). Therefore, the i.d. route could conceivably be used as a platform for development of hybrid subunit vaccines against important enteric pathogens. Nevertheless, further effort will be required to optimize this approach and refine antigen formulations with sufficient valency to achieve broad protection against ETEC and *Shigella*.

Collectively, these early data with EtpA support the concept that this antigen could complement ongoing approaches to vaccine development and expand the valency of ETEC vaccines. Additional efforts will need to focus on first defining and subsequently optimizing parameters that define protection mediated by this and other novel immunogens.

**ACKNOWLEDGMENTS**

We thank Heather Wentzel at PATH for assistance in obtaining the double mutant LT used in these studies. We thank Suellen Greco, Director of the Division of Comparative Medicine Research Animal Diagnostic Laboratory, for kind assistance in processing and interpretation of patholog-

The contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIAID, NIH, PATH, or the VA.

**FUNDING INFORMATION**

This work, including the efforts of James M. Fleckenstein, was funded by Department of Veterans Affairs (5101BX001469-04), grant 2R01AI89894 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and PATH.

The contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIAID, NIH, PATH, or the VA.

**REFERENCES**

1. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Pan- chalingam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque AS, Zaidi AK, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieri JB, Omore R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hossain MJ, Akinsola A, Mandomando I, Nhampossa T, Acacio S, Biswas K, O’Reilly CE, Mintz ED, Berkeley LY, MuhSEN K, Sommerfelt H, Robins-Browne RM, Levine MM. 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382:209–222. http://dx.doi.org/10.1016/S0140-6736(13)60844-2.

2. Platts-Mills JA, Babji S, Bodhidatta L, Gratz J, Haque R, Havit A, McCormick BJ, McGrath M, Olortegui MP, Samie A, Shakoors S, Mondal D, Lima IF, Hariraju D, Rayamajhi BB, Qureshi S, Kabir F, Yori PP, Muhamad B, Amor A, Carreon JD, RICHA SA, Lang D, Bessong P, Mduma E, Ahmed T, Lima AA, Mason CJ, Zaidi AK, Bhutta ZA, Koskem G, Guer rant RL, Gottlieb M, Miller M, Kang G, Houpt ER, MAL-ED Network Investigators. 2015. Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). Lancet Glob Health 3:e564–e575. http://dx.doi.org/10.1016/S2214-109X(15)00151-5.

3. Shah N, DuPont HL, Ramsey DJ. 2009. Global etiology of travelers’ diarrhea: systematic review from 1973 to the present. Am J Trop Med Hyg 80:659–614.

4. Svennerholm AM, Lundgren A. 2012. Recent progress toward an enterotoxigenic Escherichia coli vaccine. Expert Rev Vaccines 11:495–507. http://dx.doi.org/10.1586/erv.12.12.

5. sack RB, Gorbach SL, Banwell JG, Jacobs B, Chatterjee BD, Mitra RC. 1971. Enterotoxigenic Escherichia coli isolated from patients with severe cholera-like disease. J Infect Dis 123:378–385. http://dx.doi.org/10.1093/infdis/123.A.378.

6. von Mentzer A, Connor TR, Wieler LH, Semmler T, Iuchi A, Thom- son NR, Rasko DA, Joffre E, Corander J, Pickard D, Wiklund G, Svennerholm AM, Sjolong A, Dowgan G. 2014. Identification of enterotoxigenic Escherichia coli (ETEC) clades with long-term global distribution. Nat Genet 46:1321–1326. http://dx.doi.org/10.1038/ng.3145.

7. Sahl JW, Steindl H, Redman JC, Angiulli SV, Johnson JM, Schuch K, Fleckenstein JM, Rasko DA. 2014. A comparative genomic analysis of di-

8. Rasko DA, Rosovitz MJ, Myers GS, Mongin EF, Fricke WF, Gajer P, Crabtree J, Sebaihia M, Thomson NR, Chaudhuri R, Henderson IR, Sperandio V, Raw J. 2008. The pangenome structure of Escherichia coli: comparative genomic analysis of E. coli commensal and pathogenic iso-

9. Isidean SD, Riddle MS, Savarino SJ, Porter CK. 2011. A systematic review of ETEC epidemiology focusing on colonization factor and toxin expression. Vaccine 29:6167–6178. http://dx.doi.org/10.1016/j.vaccine.2011.06.084.

10. Rasko DA, Pachter LF, Jr, Kay BA, El-Yazeed RA, El-Etr SH, Cravioto A, Wierzba TF, Rao M, El-Ghorab N, Shaheen H, Khalil SB, Kamal K, Wasfy MO, Svennerholm AM, Clemens JD, Savarino SJ. 1999. Phenotypic diversity of enterotoxigenic Escherichia coli strains from a community-based study of pediatric diarrhea in periurban Egypt. J Clin Microbiol 37:2974–2978.

11. Fleckenstein J, Sheikh A, Qadri F. 2014. Novel antigens for enterotoxig- enic Escherichia coli vaccines. Expert Rev Vaccines 13:631–639. http://dx.doi.org/10.1586/14760784.2014.905745.

12. Fleckenstein JM, Roy K, Fischer JF, Burkett M. 2006. Identification of a two-partner secretion locus of enterotoxigenic Escherichia coli. Infect Immun 74:2245–2258. http://dx.doi.org/10.1128/IAI.74.4.2245-2258.2006.

13. Roy K, Hilliard GM, Hamilton DJ, Luo J, Ostmann MM, Fleckenstein JM. 2009. Enterotoxigenic Escherichia coli EtpA mediates adhesion be-

14. Roy K, Hamilton D, Allen KP, Randolph MP, Fleckenstein JM. 2008. The EtpA exoprotein of enterotoxigenic Escherichia coli promotes intesti-

15. Luo Q, Qadri F, Kansal R, Rasko DA, Sheikh A, Fleckenstein JM. 2015. Conservation and immunogenicity of novel antigens in diverse isolates of enterotoxigenic Escherichia coli. PLoS Negl Trop Dis 9:e0003446. http://dx.doi.org/10.1371/journal.pntd.0003446.

16. Del Canto F, Valenzuela P, Canettor L, Bronstein J, Blanco JE, Blanco J, Prado V, Levine M, Nataro J, Sommerfelt H, Vidal R. 2011. Distribu-

17. Drummond JF, Duran JD, King TA, Mandarano E, Nataro JP, O’Brien SJ, Omer BL, Panlilio AV, Persing DH, Sack RB, Sartorius D, Sahl HG, Svennerholm AM, Lundgren A, Rakos DA. 2014. Pathogen-specific burdens of enterotoxigenic Escherichia coli isolates revealed pathovar-specific conservation. Infect Immun 82:79–89. http://dx.doi.org/10.1128/IAI.003741.

18. Isidean SD, Riddle MS, Savarino SJ, Porter CK. 2011. A systematic review of ETEC epidemiology focusing on colonization factor and toxin expression. Vaccine 29:6167–6178. http://dx.doi.org/10.1016/j.vaccine.2011.06.084.

19. Rasko DA, Rosovitz MJ, Meyers GS, Mongin EF, Frick LF, Gajer P, Crabtree J, Sebaihia M, Thomson NR, Chaudhuri R, Henderson IR, Sperandio V, Ravel J. 2008. The pangenome structure of Escherichia coli: comparative genomic analysis of E. coli commensal and pathogenic iso-

20. Svennerholm AM, Clemens JD, Savarino SJ. 1999. Phenotypic diversity of enterotoxigenic Escherichia coli strains from a community-based study of pediatric diarrhea in periurban Egypt. J Clin Microbiol 37:2974–2978.

21. Fleckenstein J, Sheikh A, Qadri F. 2014. Novel antigens for enterotoxigenic Escherichia coli vaccines. Expert Rev Vaccines 13:631–639. http://dx.doi.org/10.1586/14760784.2014.905745.

22. Fleckenstein JM, Roy K, Fischer JF, Burkett M. 2006. Identification of a two-partner secretion locus of enterotoxigenic Escherichia coli. Infect Immun 74:2245–2258. http://dx.doi.org/10.1128/IAI.74.4.2245-2258.2006.
Escherichia coli isolates from Chilian children and tRNA gene screening for putative insertion sites for genomic islands. J Clin Microbiol 49:3198–3203. http://dx.doi.org/10.1128/JCM.02473-10.

17. Jones T, Cyr S, Allard F, Bellerose N, Lowell GH, Burt DS. 2004. Protollin: a novel adjuvant for intranasal vaccines. Vaccine 22:3691–3697. http://dx.doi.org/10.1016/j.vaccine.2004.03.035.

18. Chabot S, Brewer A, Lowell L, Plante M, Cyr S, Burt DS, Ward BJ. 2005. A novel intranasal Protollin-based measles vaccine induces mucosal and systemic neutralizing antibody responses and cell-mediated immunity in mice. Vaccine 23:1374–1383. http://dx.doi.org/10.1016/j.vaccine.2004.09.010.

19. Roy K, Hamilton D, Ostmann MM, Fleckenstein JM. 2009. Vaccination with EtpA glycoprotein or flagellin protects against colonization with enterotoxigenic Escherichia coli in a murine model. Vaccine 27:4601–4608. http://dx.doi.org/10.1016/j.vaccine.2009.05.076.

20. Roy K, Hamilton DJ, Fleckenstein JM. 2012. Cooperative role of antibodies against heat-labile toxin and the EtpA adhesin in preventing toxin delivery and intestinal colonization by enterotoxigenic Escherichia coli. Clin Vaccine Immunol 19:1603–1608. http://dx.doi.org/10.1128/CVI.00351-12.

21. Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, Spyr C, Steffen R. 2004. Use of the inactivated intranasal influenza vaccine and the risk of Bell’s palsy in Switzerland. N Engl J Med 350:896–903. http://dx.doi.org/10.1056/NEJMoa030595.

22. Lewis DJ, Huo Z, Barnett S, Kromann I, Giemza R, Galiza E, Woodrow M, Thierry-Carstensen B, Andersen P, Novicki D, Del Giudice G, Rappuoli R. 2009. transient facial nerve paralysis (Bell’s palsy) following intranasal delivery of a genetically detoxified mutant of Escherichia coli heat labile toxin. PLoS One 4:e6699. http://dx.doi.org/10.1371/journal.pone.0006999.

23. Norton EB, Lawson LB, Freytag LG, Clements JD. 2011. Characterization of a mutant Escherichia coli heat-labile toxin, LT(R192G/L211A), as a safe and effective oral adjuvant. Clin Vaccine Immunol 18:546–551. http://dx.doi.org/10.1128/CVI.00358-10.

24. Fleckenstein JM, Roy K. 2009. Purification of recombinant high molecular weight two-partner secretion proteins from Escherichia coli. Nat Protoc 4:1083–1092. http://dx.doi.org/10.1038/nprot.2009.87.

25. Allen KP, Randolph MM, Fleckenstein JM. 2006. Importance of heat-labile enterotoxin in colonization of the adult mouse small intestine by human enterotoxigenic Escherichia coli strains. Infect Immun 74:869–875. http://dx.doi.org/10.1128/IAI.74.2.869-875.2006.

26. Tsang VC, Wilson BC, Maddison SE. 1980. Kinetic studies of a quantitative single-tube enzyme-linked immunosorbant assay. Clin Chem 26:1255–1260.

27. Alam MM, Arifuzzaman M, Ahmad SM, Hosen MI, Rahman MA, Rashu R, Sheikh A, Ryan ET, Calderwood SB, Qadri F. 2013. Study of avian-specific antigen antibody as a means of understanding development of long-term immunological memory after Vibrio cholerae O1 infection. Clin Vaccine Immunol 20:17–23. http://dx.doi.org/10.1128/CVI.00521-12.

28. Lundgren A, Bourgeois L, Carlin N, Clements J, Gustafsson B, Hartford YA, Baby NI, Faraque AS, Jahan N, Cravioto A, Svennerholm AM, Qadri F. 2014. Shift in phenotypic characteristics of enterotoxigenic Escherichia coli (ETEC) isolated from diarrheal patients in Bangladesh. PLoS Negl Trop Dis 8:e3031. http://dx.doi.org/10.1371/journal.pntd.0003031.

29. von Mentzer A, Sjoling A, Dougan G, Svennerholm A. 2016. Whole genome sequencing of enterotoxigenic Escherichia coli (ETEC)—search for novel colonization factors, p 115–118. In 50th US-Japan Cooperative Medical Sciences Program Joint Panel Conference on cholera and other bacterial enteric infections, Bethesda, MD.

30. Roy K, Bartels S, Qadri F, Fleckenstein JM. 2010. Enterotoxigenic Escherichia coli elicits immune responses to multiple surface proteins. Infect Immun 78:3027–3035. http://dx.doi.org/10.1128/IAI.00264-10.

31. Selman DA, Donnenhim M, Tuumanen E, Rappuoli R, Falkow S. 1989. filamentous hemagglutinin of Bordetella pertussis: nucleotide sequence and crucial role in adherence. Proc Natl Acad Sci U S A 86:2637–2641. http://dx.doi.org/10.1073/pnas.86.8.2637.

32. Ward J, Cherry JD, Chang SJ, Partridge S, Lee H, Treanor J, Greenberg DP, Keitel W, Barenkamp S, Bernstein DJ, Edelman R, Edwards K. 2005. Efficacy of an acellular pertussis vaccine among adolescents and adults. N Engl J Med 353:1555–1563. http://dx.doi.org/10.1056/NEJMoa050824.

33. Lee S, Pickering WL, Spiziri S. 2014. The immune response of two microbial antigens delivered intradermally, sublingually, or the combination thereof. Microbes Infect 16:796–803. http://dx.doi.org/10.1016/j.micinf.2014.07.013.

34. Heine SJ, Diaz-Mcaij N, Andar AU, Drachenberg CB, van de Verg L, Walker R, Pickering WL, Pasetti MF. 2014. Intradermal delivery of Shigella Ipab and Ipald type III secretion proteins: kinetics of cell recruitment and antigen uptake, mucosal and systemic immunity, and protection across serotypes. J Immunol 192:1630–1640. http://dx.doi.org/10.4049/jimmunol.1302743.

35. Belyakov IM, Ahlers JD. 2009. What role does the route of immunization play in the generation of protective immunity against mucosal pathogens? Immunol Lett 135:683–689. http://dx.doi.org/10.1016/j.imlet.2009.09.046.

36. Boyle JS, Silva A, Brady JL, Lew AM. 1997. DNA immunization: induction of higher avidity antibody and effect of route on T cell cytotoxicity. Proc Natl Acad Sci U S A 94:14626–14631. http://dx.doi.org/10.1073/pnas.94.26.14626.

37. Goodman CC, Vinuesa CG, Randall KL, Mackay F, Brink R. 2010. Control systems and decision making for antibody production. Nat Immunol 11:681–688. http://dx.doi.org/10.1038/ni.1900.

38. Goldblatt D, Vaz AR, Miller E. 1998. Antibody avidity as a surrogate marker of successful priming by Haemophilus influenzae type b conjugate vaccines following infant immunization. J Infect Dis 177:1112–1115. http://dx.doi.org/10.1086/517407.

39. Usami WR, Lucas AH. 1999. Avidity as a determinant of the protective efficacy of human antibodies to pneumococcal capsular polysaccharides. Infect Immun 67:2366–2370.

40. Khurana S, Wu J, Dimitrova M, Linchitz J, Graham BS, Ledgewood JE, Golding H. 2013. DNA priming prior to inactivated influenza A(H5N1) vaccination expands the antibody epitope repertoire and increases affinity maturation in a boost-interval-dependent manner in adults. J Infect Dis 208:413–417. http://dx.doi.org/10.1093/infdis/jit178.

41. Lee YC, Kelly DF, Yu LM, Slack MP, Booy R, Heath PT, Siegrist CA, Moxon RE, Pollard AJ. 2008. Haemophilus influenzae type b vaccine failure in children is associated with inadequate production of high-quality antibody. Clin Infect Dis 46:186–192. http://dx.doi.org/10.1086/524668.

42. Sanz-Moreno JC, Jimena-Leiva J, Garcia-Comas L, Mosquera-Gutierrez MM, Echevarria-Mayo JE, Castellanos-Nadal A, de Ory-Machon F. 2005. Detection of secondary mumps vaccine failure by means of avidity testing for specific immunoglobulin G. Vaccine 23:4921–4925. http://dx.doi.org/10.1016/j.vaccine.2005.05.018.

43. Narita M, Matsuomo Y, Takekoshi Y, Yamada S, Hikura O, Kubota M, Totaka H, Togashi T. 1998. Analysis of mumps vaccine failure by means of avidity testing for mumps virus-specific immunoglobulin G. Clin Diag Lab Immunol 5:799–803.

44. Delgado MF, Coviello S, Monsalvo AG, Melendi GA, Hernandez JD, Bataille JP, Diaz L, Trento A, Chang HY, Mitzner W, Ravetch J, Melero JA, Frtsma PF, Polack FP. 2009. Lack of antibody avidity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syn-
50. Polack FP, Hoffman SJ, Crujeiras G, Griffin DE. 2003. A role for nonprotective complement-fixing antibodies with low avidity for measles virus in atypical measles. Nat Med 9:1209–1213. http://dx.doi.org/10.1038/nm918.

51. Alam MM, Aftab A, Afrin S, Rahman MA, Akhter S, Uddin T, Rahman MA, Al Mahbuba D, Chowdhury F, Khan AI, Bhuiyan TR, Begum YA, Ryan ET, Calderwood SB, Svennerholm AM, Qadri F. 2014. Antigen-specific memory B-cell responses to enterotoxigenic Escherichia coli infection in Bangladeshi adults. PLoS Negl Trop Dis 8:e2822. http://dx.doi.org/10.1371/journal.pntd.0002822.

52. Czerkinsky C, Holmgren J. 2013. Secretory IgA: designed for anti-microbial defense. Front Immunol 4:222. http://dx.doi.org/10.3389/fimmu.2013.00222.

53. Brandzaeg P. 2013. Secretory IgA: designed for anti-microbial defense. Front Immunol 4:222. http://dx.doi.org/10.3389/fimmu.2013.00222.

54. Czerkinsky C, Holmgren J. 2012. Mucosal delivery routes for optimal immunization: targeting immunity to the right tissues. Curr Top Microbiol Immunol 354:1–18. http://dx.doi.org/10.1007/82_2010_112.

55. White JA, Blum JS, Hosken NA, Marshak JO, Duncan L, Zhu C, Norton EB, Clements JD, Koelle DM, Chen D, Weldon WC, Oberste MS, Lal M. 2014. Serum and mucosal antibody responses to inactivated polio vaccine after sublingual immunization using a thermoresponsive gel delivery system. Hum Vaccin Immunother 10:3611–3621. http://dx.doi.org/10.4161/hv.32253.

56. Harro C, Gutierrez R, Talata K, Porter C, Riddle M, Maciel M, Poole S, Laird R, Savarino S. 2016. Protective efficacy of an enterotoxigenic E. coli fimbrial tip adhesin vaccine given with LTR192G by intradermal vaccination against experimental challenge with CFA/I-ETEC in adult volunteers, p 126–130. In 50th US-Japan Cooperative Medical Sciences Program. Joint Panel Conference on Cholera and Other Bacterial Enteric Infections, Bethesda, MD.

57. Evans DJ, Jr, Evans DG. 1975. Plasmid-controlled colonization factor associated with virulence in Escherichia coli enterotoxigenic for humans. Infect Immun 12:656–667.

58. Evans DJ, Jr, Evans DG. 1973. Three characteristics associated with enterotoxigenic Escherichia coli isolated from man. Infect Immun 8:322–328.

59. Dorsey FC, Fischer JF, Fleckenstein JM. 2006. Directed delivery of heat-labile enterotoxin by enterotoxigenic Escherichia coli. Cell Microbiol 8:1516–1527. http://dx.doi.org/10.1111/j.1462-5822.2006.00736.x.