Vaccine Against Entheropathogenic *E. coli*: A Systematic Review

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Abstract

Enteric diseases are a major cause of childhood death in the developing world, ranking as the second cause of death in children. Enteropathogenic *Escherichia coli*(EPEC) are important diarrheal pathogens of children under 5 years of age. Due to high mortality, several international organizations such as the WHO and UNICEF have dedicated preventive and control programs for diarrheal diseases. Among the suggested initiatives aiming to prevent infectious diarrhea include vaccine development and improvement in sanitation and water and food supplies.

Upon contact with host cells, EPEC delivers an array of virulence protein factors, which integrated actions interferes with the normal adjusting the targets molecular cell functions leading to diarrhea. The locus of enterocyte effacement (LEE)pathogenicity island contains genes encoding synthesis of the EPEC virulence factors membrane adhesin intimin, T3SS (Esc and Sep proteins), chaperones (Ces proteins), translocators (EspA, EspB, and EspD), effector proteins (EspF, EspG, EspH, Map and EspZ), the translocated intimin receptor (Tir), and the regulatory protein Ler (LEE-encoded regulator).Bundle-forming pilus (BfpA) is another virulence factor that mediates the initial contact between EPEC and the host cell. BfpA is encoded by a gene localized on a 50–70 MDa plasmid and is designated as EPEC adherence factor (EAF). BfpA, intimin and translocated Tir initiate EPEC infection. This repertoire of virulence factors offers strategic epitopes as vaccine candidates.

Employing the proposed technologies for modern vaccines, recombinant *Mycobacterium smegmatis* (Smeg) and *Mycobacterium bovis* BCG strains were constructed to express BfpA, intimin and EspA. Recombinant clones were selected based on kanamycin resistance. Recombinant proteins are immunogenic, and the resultant antibodies recognize and block EPEC adhesion onto HEp-2 target cells. In attention to regulatory agency recommendations, kanamycin should not be used to produce rBCG expressing the *E. coli* EPEC virulence factors BfpA, intimin and EspA. Accordingly, an expression system with a Pasteur auxotrophic BCG mutant for leucine (BCG Pasteur ΔleuD) and a replicative vector (pUP410)should be used. Obtained recombinants will be grown in large scale, and their immunogenic effectivity and human safety submitted to WHO indicated quality controls.

Keywords: Diarrhea; *Escherichia coli*; Virulence factors; Immune response; Immunoglobulins recombinants.

Introduction

Vaccines – Immunization

Vaccines are modified infectious agents that, upon injection into susceptible hosts, induce specific protection but not infection. These agents are prepared as either killed, inactivated, or attenuated entire pathogens, maybe: as toxins or certain strategic molecules involved in pathogen survival and multiplication in infected hosts. Therefore, vaccination is a non-natural procedure to induce an effective immune response.

The history of vaccines began with the observation that some humans and animals who recovered from infections become partially or even completely resistant to infection with the same or related infectious agents. The explosion of infectious diseases such as plague caused by *Yersinia pestis*, tuberculosis caused by *Mycobacterium tuberculosis*, infantile paralysis caused by poliovirus, and influenza caused by the influenza virus, which cause disability and mortality, accelerated the development of new vaccines. Since earlier times, three general qualities should be expressed by any vaccine candidate: safety, efficacy and feasibility.

In 1798, Edward Jenner, observing that milkmaids who contact edcowpox-virus-infected cows after having had local mild infections became protected against the smallpox virus responsible for one of the gravest human infections, decided to introduce systematic immunization using person-to-person inoculation with cowpox virus. Although the cowpox-derived vaccine reduced smallpox transmission in Europe and North

America, the infection transmission persisted in developing countries. The introduction of a stable, freeze-dried smallpox virus vaccine was the solution. Consequently, the vaccinology became established [1]. In 1885, Louis Pasteur attenuated the rabies virus and developed the rabies vaccine [2]. In 1927, a *Mycobacterium bovis* strain was attenuated, and the vaccine Bacillus Calmette-Guérin (BCG) against *Mycobacterium tuberculosis* infection was created [3]. Next, samples of *Vibrio cholera*, *Salmonellae typhi* and *Yersinia pestis* were killed, and vaccines against cholera, typhoid fever and plague diseases became available [4,5]. This still small vaccine repertoire was rapidly increased with the production of some inactivated toxins, the toxoids, resulting in the diphtheria and tetanus vaccines [6-8]. Data on Table 1 indicates the actual vaccines stage.

With the introduction of cell culture in biological research allowing large-scale virus growth, the Salk and Sabin polio vaccines were developed [9]. Improvements in vaccine preparations aiming to increase their immunogenicity and to reduce the remaining pathogenicity were stimulated by the accumulative, solid knowledge regarding the cellular and molecular mechanisms involved in the immune response. Remarkable was the demonstration that memory instead of naïve T and B cells require 10-50 fewer antigens to respond to immunization; their receptors have a high affinity to the specific epitope and appear to have a longer life span [10-12]. B cells expressing somatically VL(J):VH(D)J molecular rearrangements, upon contact with the specific epitopes, differentiate into long-lived memory and plasma cells [13].

The yellow fever vaccine, comprising an attenuated virus, was empirically developed [14]. A single injection induces cytotoxic T lymphocytes and T helper Th1-Th2 differentiation, aside from the production of neutralization antibody. Effective immunity persists for up to 30 years. To investigate the refined mechanisms involved in immune response induction by the yellow fever vaccine, the multiplex analysis of cytokines-chemokines and multi-parameter flow cytometry combined with computational modelling were applied to identify the yellow fever virus response signature in immunized humans. This possibly demonstrates that systems biology approaches not only permit the observation of a global picture of vaccine-induced innate immune responses but also can be used to predict the magnitude of the subsequent adaptive immune response and uncover new correlates of vaccine efficacy [15]. A method for obtaining high-affinity anti-HIV monoclonal antibodies was developed by cloning human B cells [16]. The obtained data may permit the identification of correct epitopes in vaccines that induce high specific and affinity antibodies. Current vaccine states are in Table1.

Table -1

Needed vaccines:

- Campylobacter; Chlamydia; Cytomegalovirus; Dengue; Epstein-Barr (Mononucleosis); Helicobacter pylori- Gastrointestinal ulcers; Hepatitis C; Herpes Simplex; HIV; Influenza (Universal flu vaccine to replace need for annual flu vaccine); Leishmaniasis; Malaria; Respiratory syncytial virus; Rhinovirus, and Schistosomiasis.

Developed vaccines

- **Bacterial vaccines**: Cholera, Diphytheria, *Haemophilus influenza*, Meningococcal Meningitis; Plague, *Pneumococcal pneumonia*, Tetanus, Tuberculosis, and Typhoid Fever.
- **Virus vaccines**: Adenovirus Based Diseases; Diphtheria Hepatitis A; Hepatitis B; Human papillomavirus; Influenza; Japanese Encephalitis; Mumps; Polio; Rabies; Rotavirus; Diarrhea; Rubella; Smallpox; Tick Borne Encephalitis; Varicella-Zoster, and Yellow fever.

Effective Vaccines Work by Eliciting Effector and Memory Immune Responses that Confer Protection Against Infection and Disease.

T cells are actively involved in both humoral and cellular immune responses. They are derived from hematopoietic stem cells (HSCs) first in the foetal liver during embryogenesis and later postnatally in the bone marrow as pluripotent common lymphoid progenitors (CLPs). HSCs, after sequential differentiation stages, yield immature T lymphocytes that migrate and mature in the thymus. In parallel, the T lymphocyte cell surface TCRs alternatively express CD8 glycoprotein CD8+T cells (T Cytotoxic) or CD4 glycoprotein CD4+T cells (Helper T Cells). CD4+T cells further differentiate into subsets: Th1, Th2, Th9, Th17, Th22, Treg (Regulatory T Cells), and Tfg (Follicular Helper T Cells). Each subset is characterized by different cytokine profiles: Th1, IFN-γ and TNF; Th2, IL-4, IL-5, IL-13;Th9, Il-9; Th17, IL-17, IL-21, IL-22, IL-25, IL-26; Th22, IL-22; Treg (Regulatory T Cells), IL-10, TGF-β; Tfg, IL-21 (Follicular Helper T Cells) [17]. Upon contact with pathogen antigens, naïve CD4+ T cells multiply and differentiate into effector cells and migrate to the infected tissue sites [18].

In contrast withnaïve short-lifeCD4+ T, memory sub-setsCD4+ T sub-sets are long-lived cells. Re-exposure to the pathogen antigens causes memory CD4+ T sub-sets to undergo rapid expansion and to exhibit a potent capacity to eliminate the infectious pathogens. The previous expansion and activation persist even in the absence of the antigen and are potentiated upon antigen re-exposure [19,20].

B cells are also derived from HSCs, initially residing in the embryo, then in the foetal liver and spleen, and finally in the bone marrow (which, after birth, is the preferential residence) as mature naïve B cells. Ten developmental stages initiates from HSPs and maturate to naïve B cells (MNBs) in where are identified by the presence of cell surface markers: HSP → common lymphoid progenitor (CLPs) →pro-B cell (ProB)→pre-B-1-cell (pre-B-1) →pre-B-2-cell (pre-B-II-1)→ pre-B-II-2→ pre-B-II-3→immature naïve B cell (ImnB) →mature naïve B cell (MNB) that leaves the bone marrow and migrates to secondary lymphoid

tissues, including the lymph nodes, tonsils, Peyer`s patches, and spleen [20-22].

Naïve B cell Life Span is Approximately 1-4 Days.

In secondary lymphoid tissues, germinative centre B cells (GCBs) recall a robust secondary immune response in a cohort of switched-memory B cells upon new contact with the antigen. BCRs are structurally remodelled after a transcriptional four-stage program, resulting in switched-memory B cells. The resultant BCRs express a higher specificity and affinity to the epitopes and enhance durable immune protection in comparison with those previously expressed by naïve BCRs [22]. The Figure 1 highlights distinctions among naïve and memory B cells.
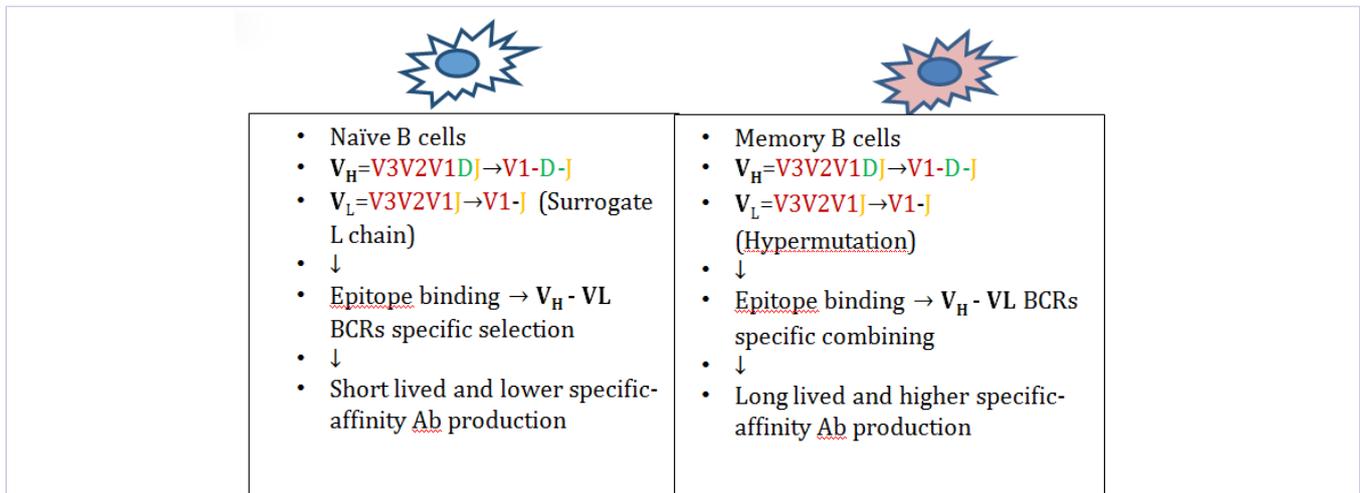

- Naïve B cells
- V_H=V3V2V1DJ→V1-D-J
- V_L=V3V2V1J→V1-J (Surrogate L chain)
- ↓
- Epitope binding → V_H - VL BCRs specific selection
- ↓
- Short lived and lower specific-affinity Ab production

- Memory B cells
- V_H=V3V2V1DJ→V1-D-J
- V_L=V3V2V1J→V1-J (Hypermutation)
- ↓
- Epitope binding → V_H - VL BCRs specific combining
- ↓
- Long lived and higher specific-affinity Ab production

Figure 1: This figure highlight distinctions among naïve and memory B cells

Cellular and molecular knowledge regarding the basic immune response allowing the deciphering of the human immune response mechanisms governing the differentiation of T and B cells expressing high specific-affinity cellular receptors must be observed in vaccinology. In association with emerging data from related technologies such as the identification of new high immunogenic epitope vaccine antigens, discovery of new adjuvants, development of high-throughput technologies for identifying the best epitope combining paired VH:VL and epitope signatures on mRNA and DNA sequences after vaccine antigen exposures are fuelling a revolution in vaccinology [23,24]. Accumulate capacity to sequence whole genomes of microorganisms and to utilize bioinformatics for designing vaccines is a relatively recent approach to antigen discovery and has been termed "reverse vaccinology" [25]. Proteins and peptides are the building blocks of life and are now evolving as a very promising brand of therapeutic entities. The generation of stable vectors expressing the desired epitopes is the goal of modern vaccine technology. The inclusion of encoding genes of relevant epitopes into living, non-infective vectors that constitutively express immunological adjuvant components would be ideal. Attenuated bacteria have been used as vectors to express and deliver heterologous antigens. This type of vaccine vector is an attractive system because it can elicit mucosal, humoral and cellular host immune responses to foreign antigens [26]. These live vectors have been used extensively to express antigens of different types of pathogens, including viruses, bacteria and parasites, some of which have demonstrated positive results [27]. However, each vector has its unique features that should be considered before being used.

Diarrhea Epidemiology and Pathogenesis.

The "Bill & Melinda Gates Foundation" (BMGF) in collaboration with the "Center for Vaccine Development of the University of Maryland School of Medicine" CVDUMSM) has retrieved worldwide epidemiologic pediatric diarrhea data [28].

Sub-Saharan Africa, Pakistan, and South Asian countries' populations were selected. Groups of children 0-11, 12-23 and 24-59 months totalling 47,000 from Kenia, Mali, Mozambique, Bangladesh, India and Pakistan were followed up for 36 months. Morbidity and mortality were evaluated. The clinical follow-up used typical diarrhea symptoms such as simple gastroenteritis, enteritis, persistent liquid diarrhea vomiting and acute crisis. EPEC and ETEC cause simple gastroenteritis. Shigella, *Campylobacter jejuni, Entamoeba histolytica* and *Non-thyroid Salmonella,* cause enteritis. *Vibrio cholerae* and ETEC cause profuse diarrhea. The pediatric lethality index caused by diarrhea in Brazil is higher [29]. Gastrointestinal Infections (GIs) are among the leading causes of childhood mortality worldwide and are responsible for millions of deaths every year; India has surpassed Brazil (200.0000) [30-32]. Among the causative agents are several pathotypes of non-invasive *Escherichia coli* that cause diarrhea but do not produce heat-labile or heat-stable enterotoxins [33].

In Brazil, the most prevalent diarrhea-associated pathotypes among children are the typical entero aggregative and atypical enteropathogenic types of *Escherichia coli* [34]. Typical (tEPEC) and atypical (aEPEC) enteropathogenic Escherichia coli strains are the main diarrhea infectious agents [35-37]. The complex mechanisms involved in EPEC-induced infection initiate with bacterial virulence factor synthesis by EPEC once approaching enterocyte target cells [38]. The EPEC adheres to the external

membrane of enterocytes, causing the typical "attaching and effacing" (A/E) lesion [39].

As indicated, diarrhea remains one of the top causes of death in low-and middle-income countries, in children under 5 years of age [35,36]. A wide range conditions can be responsible for this illness. EPEC strains are among the main bacterial causes of this disease [35,36]. EPEC adheres to the host cells and induces attaching and effacing (A/E) lesions, culminating in the induction of diarrhea [39]. The formation of A/E lesions involves a type III secretion system encoded on a pathogenicity island locus of enterocyte effacement (LEE), which is responsible for delivering several pathogenic factors into host cells [38]. Intimin is a 94–97 kDa protein expressed on the EPEC surface that mediates EPEC adhesion to epithelial gut cells, which in turn mediate intimate contact with the bacterial translocated intimin receptor (Tir) [40]. The integration of enteropathogenic *E. coli* virulence factors on epithelial cell surface and subsequent pedestal formation is schematically represented on (Figure 2).
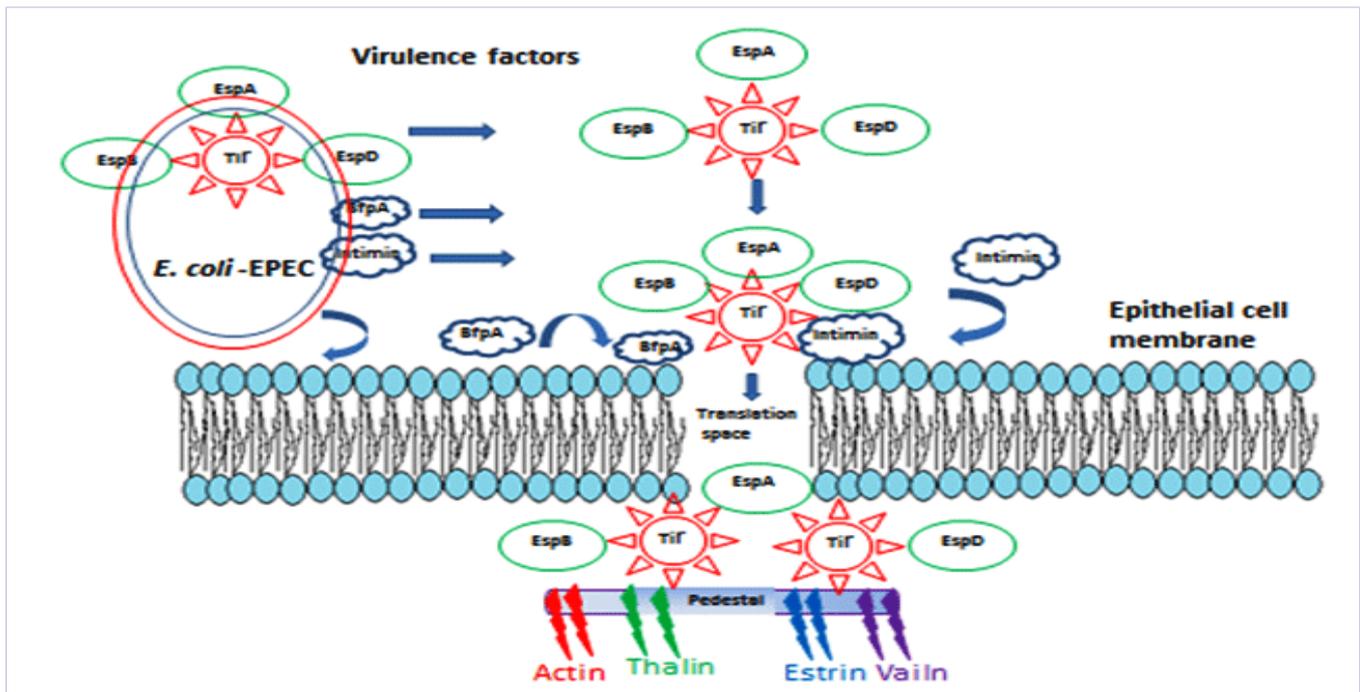

Figure 2: EPEC adheres to the target cells, induces attaching and effacing (A/E) lesions, culminating with diarrhea. This figure depicts the sequential association of the EPEC virulence factors BfpA, Intimin, Tir, EspA, EspB and EspD encoded by plasmids specific genes and expressed by the bacteria *(top of the figure)*. Once bacteria approaching epithelial cell surface BfpA stablishes weak contact but sufficient of allowing transfer the Tir-EspA-EspB-EspD complex and Intimin to the external target cell surface. Along the Tir-EspA-EspB-EspD complex migration to cell cytoplasm an intra membrane tube is created and fixed by EspA. Once internalized the resulting Tir-EspB-EspD complex mediates pedestal formation *(bottom of the figure).*

The N-terminal region is conserved among the different intimin subtypes, while the C-terminal regions are highly variable. The 29 intimin subtypes are identified according to their C-terminal amino acid sequences [40-46]. Intimin-β is the most common subtype expressed in EPEC isolates [44-46,56]. Bundle-Forming Pilus (BfpA) is another virulence factor, which mediates the initial contact between EPEC and the host cell [47]. BfpA is encoded by a gene localized on a plasmid 50–70 MDa in size and is designated as EPEC adherence factor (EAF) [48,49]. Within adherent micro-colonies of EPEC, BfpA organizes a meshwork that allows bacteria to attach to each other and to tether themselves to the host cell surface [48]. The EspA (*E. coli* secreted protein A) filament, a hollow tube that acts like a molecular syringe for delivery of the Tir (translocated intimin receptor) protein and other effector molecules into the host cell, it is an excellent immunodiagnostic target for infant diarrhoea caused by EPEC. It is a key virulence factor present in all EPEC strains, it can be induced to high levels in culture and its structure makes it accessible from all sides to antibodies [50]. Therefore, BfpA, Intimin and EspA are three important virulence factors and are considered strategic target candidates for designing a new vaccine against EPEC. The generation of stable vectors expressing the desired immunogens is the goal of modern vaccine technology. The inclusion of genes encoding relevant epitopes into living, non-infective vectors that constitutively express immunological adjuvant components would be ideal. Attenuated bacteria have been used as vectors to express and deliver heterologous antigens. This type of vaccine vector is an attractive system because it can elicit mucosal, humoral and cellular host immune responses to foreign antigens [51].

These live vectors have been used extensively to express antigens of different types of pathogens, including viruses, bacteria and parasites, some of which have demonstrated positive results [52]. However, each vector has its unique features that should be considered before it is used.

Gene Vectors.

Bacillus Calmette-Guerin (BCG) is a strain of *Mycobacterium bovis* that was empirically attenuated along 1906 to 1920 by repeated cultivation on a glycerinated bile-potato medium. Bacillus samples recovered after the last cultivation were inoculated in mice, guinea pigs, calves, rhesus monkeys, chimpanzees, and virulence and immunogenicity evaluated by available standard methods. Although virulence was completely abrogated the immunogenicity was preserved. Recent analysis using refined molecular analyses it was verified that BCG samples underwent the loss and/or rearrangement of several gene complexes [52]. In 1928, after experimental evaluations, BCG was recommended by the League of Nations as the official vaccine against human tuberculosis (TB). Since then, it remains the only official and commercially available vaccine against TB [56].

BCG offers unique advantages as a vaccine: (1) it is unaffected by maternal antibodies, and therefore, it can be given at any time after birth; (2) BCG is usually given as a single dose eliciting a long-lasting immunity; (3) it is stable and safe; (4) BCG can be administrated orally; and (5) it is inexpensive in comparison with other live vaccines [53]. In addition, the extraordinary adjuvant properties of some bacterial intrinsic mycobacterial cell components make them an attractive vector for the development of recombinant vaccines [54].

The interest in BCG increased considerably in the 1990s as a result of the development of different genetic systems for the expression of foreign antigens in mycobacteria. These systems include the development of different shuttle vectors, and systems to express and secrete heterologous antigens. Moreover, technological advancements in the genomics of mycobacteria have improved our understanding of the biology of this slow-growing pathogen and have helped the conception of strategies to evaluate BCG as a vaccine delivery vector [57]. Consequently, antigens of bacteria, parasites, and viruses have been expressed in BCG and it has been shown that recombinant BCG (rBCG) elicits both cellular and humoral immune responses against heterologous antigens [54-56]. However, it was only in recent years that rBCG has attracted more attention as consequences stimulated by initiatives to develop new vaccines against TB. It has been demonstrated that rBCG over expressing antigens of M. tuberculosis is more efficient in conferring protection against tuberculosis than the wild type BCG strain [55].

Vaccine Anti - *E. coli*

E. coli EPEC recombinants

Enteropathogenics *Escherichia coli* (EPEC) are an important cause of diarrhea in children. EPEC adheres to the intestinal epithelium and causes attaching and effacing (A/E) lesions. Recombinant *Mycobacterium smegmatis* (Smeg) and *Mycobacterium bovis* BCG strains were constructed to express either BfpA or intimin. The entire *bfpA* gene and a portion of the *Intimin* gene were amplified by PCR from EPEC genomic DNA and inserted into the pMIP12 vector at the BamHI/KpnI sites. The pMIP *bfpA* and pMIP Intimin vectors were introduced separately into Smeg and BCG. Recombinant clones were selected based on kanamycin resistance and designated rSmeg pMIP *(bfpA or intimin)* and rBCG pMIP *(bfpA or intimin)*. The expression of *bfpA* and *intimin* was detected by Immunoblotting using polyclonal anti-BfpA and anti-Intimin antibodies. The immunogenicity of these proteins was assessed in C57BL/6 mice by assaying the feces and serum for the presence of anti-BfpA and anti-Intimin IgA and IgG antibodies. TNF-α and INF-y were produced in vitro by spleen cells from mice immunized with recombinant BfpA, whereas TNF-α was produced in mice immunized with recombinant Intimin. The adhesion of EPEC (E2348/69) to HEp-2 target cells was blocked by IgA or IgG antibodies from mice immunized with recombinant BfpA or Intimin but not by antibodies from non-immunized mice. Immunogenic non-infectious vectors containing relevant EPEC virulence genes may be promising vaccine candidates [58].

Other vaccine candidates proved to be effective against EPEC. Immunization of cattle with a combination of recombinant EspA, Intimin and Tir have been demonstrated to be protective against *E. coli* O157 challenge [59]. *Lactobacillus casei* expressing intimin-β fragments and containing the immune dominant epitopes of Int280 induced both humoral and cellular immune responses in mice. The antibodies were able to bind to EPEC and inhibit bacterial adhesion to the epithelial cell surface in vitro [60]. The mixed vaccine including ETEC, EHEC, EIEC, EAEC and EPEC, induces protection against the five *E. coli* pathotypes [61].

Clinical Assay

The experimental data covering *E. coli* EPEC virulence factors deeply involved in the bacterial attachment onto target cells, the proper plasmids encoding them, the feasible vectors, and reliable molecular methods for detecting the presence of the genes inside vectors, up-to-date assays for identifying developed recombinant proteins and quantifying their activities justify attempts to use the obtained BCG-BfpA-Intimin recombinants for developing a new anti-EPEC vaccine.

The clinical development of a new vaccine begins with a plan [62]. The plan must comprise three phases: Phase 1, Phase 2 and Phase 3.

A pre-clinical assay using experimental animals must anticipate trials in human subjects. Local and systemic undesirable reactions such as inflammation, pain, discomfort, tumour markers, and specific signals to the proposed vaccine epitopes are the focus.

Phase 1, comprises submission of the proposed vaccine previously approved in the pre-clinical assay to a first human assay enrolling 50 subjects. This involves detecting human reactogenicity by evaluating local and general inflammation and immunogenicity measurement by the emergence of specific antibodies and T cells to vaccine epitopes.

In Phase 2, several hundred subjects and multiple variable clinical centres must be enrolled. In this phase, blinding, minimum and maximal vaccine doses and vaccine-cost must also be considered.

Phase 3, extends observations on the proposed vaccine that has been previously considered safe and protective after Phases 1 and 2 [63]. For additional information on the proposed clinical vaccine, several items, such as ethical principles, risks and

inconveniences, subjects' rights, scientific and technology availability of the product under test, and medical care for the selected subjects, must be observed (Table 1). Principles of Good Clinical (practicity) as proposed [64]. The randomized, controlled clinical trial (RCT) is considered a gold-standard design to follow-up vaccine performance [65]. The formula used to determine the sample sizes in two-group trials is available [66].

Pre-Clinical Assay rBCG-EPEC Vaccine.

In attention to the regulatory agency recommendations, the rBCG expressing the *E. coli* EPEC virulence factors BfpA and Intimin by our described method using kanamycin was modified using an expressing system with a Pasteur auxotrophic BCG mutant for leucine (BCG Pasteur ΔleuD) and a replicative vector (pUP410) that supplement constructions instead of leucine [58,67]. With this expression of BfpA, Intimin and EspA recombinants using specific primers, the normal development and maintenance of memory cells during vaccination will be preserved [68]. This strategy is based on the BCG ΔleuD ability to multiply inside macrophages only in the presence of complementation [69].

Encompassing modern vaccine proposed technologies recombinant *Mycobacterium smegmatis* (Smeg) and *Mycobacterium bovis* BCG strains were initially constructed to express either BfpA or Intimin. The *bfpA* and Intimin (eae) genes were amplified by polymerase chain reaction (PCR). The EPEC E2348/69 prototype genomic DNA was used as a template, and the constructed oligonucleotide primers were as follows:
bfpA (FP 5-TAG GGA TCC CTG TCT TTG ATT GAA TCT GCA ATG GTG CTT-3- and RP 5-TAG GGT ACC TTA CTT CAT AAA ATA TGT AAC TTT ATT GGT-3 -.

intimin fp 5-TAG GGA TCC GGG ATC GAT TAC C-3 -and RP 5-TAG GGT ACC TTT ATC AGC CTT AAT CTC A-3-.

espa: 14: PF: GCTCTAGAGCATACATCAACTACAGCAT; P15PR: CCC AAGCTTTTATTTACCAAGGGA;P16:RP: GGGTACCTTATTTACCAAG GGATA;P17:PF: GCGGATCCCATACATCAACT -.

The underlined regions indicate *kpnI* and *BamHI* sites. Briefly, the amplified BfpA and Intimin (eae) PCR products were purified and sub-cloned into the pGEM-T Easy vector (Promega, USA). Both genes were digested with BamHI and Kpnl and sub-cloned into the mycobacterial vector pMIP12 (kindly provided by Brigitte Gicquel, Pasteur Institute, France). The resulting plasmids were identified as pMH12-*bfpA* and pMH12-*intimin.* The plasmids were validated by successive analyses with restriction endonucleases and DNA sequencing using the primer 5-TTC AAA CTA TCG CCG GCT GA-3-[58].

Concluding Remarks

Childhood diarrhea continues to cause suffering in developing countries. Viruses and bacteria are included among the causative agents. Poor living conditions such as low quality of water and poor food and healthcare systems are perversely associated with aggravation of the problem. Safe, low-cost and efficient vaccines that are already available may be the solution. Important details on infectious agent biology and molecular constitution are also available. A BCG bacillus expressing *E. coli* EPEC strategic virulence factors, BfpA and Intimin was constructed associating genetic and immunologic properties rescued from the original pathogenic bacteria with modern molecular biology methods following scientific requirements. The construction is prepared for clinical assay using as the expression system a Pasteur auxotrophic BCG mutant for leucine (BCG Pasteur δleuD) and a replicative vector (pUP410) that supplements constructions instead of kanamycin. The unavailability of public or private resources is, however, the next difficulty.

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