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Vaccines against human diarrheal pathogens: current status and perspectives

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Further important bacterial pathogens causing diarrhea

Enteropathogenic *Escherichia coli* (EPEC)

With more than one million estimated deaths of children every year, EPEC is the main cause of diarrhea in children [1]. By means of a type III secretion system (T3SS) encoding by the pathogenicity island LEE (locus of enterocyte effacement) [2], EPEC can attach to intestinal epithelial cells and initiate the formation of pedestal [3]. The T3SS translocates effector TIR molecule into host cells, that acts as a receptor for the non-fimbrial adhesin intimin expressed on the surface of EPEC [4]. Additionally, some EPEC strains harbor the adherence factor (EAF) plasmid, encoding for bundle-forming pili that contribute to formation of microcolonies and subsequent assembly of biofilms.

Campylobacter spp.

Campylobacter spp., a microaerophil ε-proteobacterium, currently causes more cases of diarrhea than foodborne *Salmonella* and is a major cause of acute gastroenteritis throughout the world. Campylobacteriosis caused by *Campylobacter* spp. is a zoonotic infection. The predominant natural host for this pathogen is poultry and the pathogen can be transmit by undercook meat and raw animal products from poultry and other livestock animals (WHO October 2011). Because of their high motility, *Campylobacter* easily penetrates the viscous intestinal mucus and then travel to the deeper intestinal crypts in which it comes to a ulcus inflammation and cryptabscess [5]. A further complication of the recurrent *C. jejuni* is a neuropathy, the Guillain-Barré syndrome [6]. It is initiated through the production of autoantibodies induced by the hyper-variable sequences in the genome, which encode the genes for the biosynthesis or modification of surface structures with lipopolysaccharides and capsule polysaccharides [7]. Most symptoms of Campylobacteriosis are treated with substitution of water and electrolytes and, in more complicated courses of disease, by antibiotic therapy. Currently there is no vaccine against *Campylobacter* infections available.

Non-typhoidal *Salmonella enterica*

Non-typhoidal Salmonellosis is a zoonosis and can be transmitted by oral ingestion of contaminated food and water or by direct contact to pets or livestock animals. The Gram-negative rod-shaped and motile γ-proteobacteria are armed with a large array of virulence factors [8]. Following the adhesion to enterocytes, *Salmonella* Pathogenicity Island 1 (SPI1)-encoded T3SS enables an active invasion by the trigger mechanism, similar to *Shigella* spp. SPI1-T3SS effector induce n actin remodeling leading to macropinocytosis and uptake of the pathogen. The intracellular lifestyle of *Salmonella* is characterized by formation of the *Salmonella-containing vacuole* (SCV), a modified phagosome with a maturation process manipulated by SPI2-T3SS, leading to intracellular replication of the pathogen [9]. Because of the self-limiting gastroenteritis in the intestine, where the stimulated inflammatory response contributes to diarrhea, an antibiotic treatment is unnecessary but the symptoms can be treated.

***Aeromonas* spp.**

Infections with *Aeromonas* spp. cause gastrointestinal diseases and nosocomial complication, for example the 'flesh-eating bacteria syndrome' as worst outcome of *A. hydrophila* infections. Although causing these kind of infections, the Gram-negative rods of the *Vibrionaceae* family belong to bacteria with a low pathogenicity and are classical opportunists like many Enterobacteriaceae. The *Aeromonas* cytotoxic enterotoxins result in degeneration of crypts and villi of the small intestine. This is supported by another virulence factor of some strains, secreted aerolysin, which permeabilizes susceptible membranes by channel formation, similar to the α-toxin of *Staphylococcus aureus* [1]. Gastroenteritis triggered by these toxins ranges from a mild, self-limiting watery diarrhea to a dysenteric form [10]. Because of the self-limiting course of disease, treatment will only address the resolution of symptoms without application of antibiotics.

***Proteus* spp.**

The genus *Proteus* describes Gram-negative rods, which occur as putrefactive agents in water, soil, on the surface of carcasses, in some food and in the natural intestinal flora. Typical characteristic for these opportunistic bacteria is the rapid motility with swarming growth and the production of the enzyme urease (reviewed in [11]), which hydrolyzes urea in ammonia and carbon dioxide. This leads to an increased pH value and the precipitation of magnesium and calcium salts. Because of this, *P. mirabelis* is the most frequent cause of infection-related kidney stones. The production of ammonia in close proximity to the gastric epithelium causes cell damage and inflammation reactions [12]. Further areas can be infected by *Proteus* bacteria, like the respiratory tract, skin and urinary tract. It is also a nosocomial pathogen like *Clostrid-*

ium difficile and can be treated with antibiotics and prevented with higher hygiene standards combined with other control methods for enteric pathogens.

Enterobacter spp.

Enterobacter spp. are Gram-negative, rod-shaped, motile and opportunistic members of Enterobacteriaceae and can cause various disease patterns such as urinary tract and abdominal infections. One of the four pathogenic strains, *E. sakazakii*, has been proposed for reclassification as new genus *Cronobacter* by Iversen *et al.* [13]. *Cronobacter* can contaminate powdered milk and infant formulas [14], leading to necrotizing enterocolitis (NEC) in infants [15]. In vitro experiments have shown that *C. sakazakii* disrupt tight junctions, induce monolayer permeability and cause apoptosis by binding to intestinal epithelial cells [16]. Due to the lack of knowledge on the action cause of NEC, the treatment involves different approaches like the administration of probiotics, antibiotics, surgical examination, Epidermal Growth Factor (EGF) and more [17].

Yersinia spp.

Yersinia enterocolitica, Y. pseudotuberculosis and *Y. pestis* are the three only human pathogen species of this genus of Gram-negative, rod-shaped, and zoonotic bacteria. The two first named species cause enterocolitis, mesenteric lypmphadenitis, septicemia and reactive arthritis. *Yersinia* spp. use for invading and colonization the Peyer's patches and the M cells for transport [18]. Furthermore, they can resist in phagocytic cells, which transfer the pathogens in the spleen. The virulence factors for adhesion to and invasion of host cells are encoded chromosomally (outer membrane proteins Inv and Ail) and the virulence plasmid (YadA). Further plasmid-encoded antiphagocytic factors (Yop proteins) and a T3SS (Ysc) lead to inactivation of phagocytes [19]. Because of the similarity in contamination of food between *Salmonella* and *Yersinia* the prevention is the same. Only in serious and chronic cases an antibiotic therapy is necessary.

Staphylococcus aureus

One of the most common and clinical important pathogen is *S. aureus.* It synthesizes an array of different virulence factors, for example the staphylococcal enterotoxins (SEs) that trigger food-mediated intoxications and lead to diarrhea and vomiting [20]. The SEs are often found in dairy products and meats, if improperly stored or processed [20]. The targets of these toxins are the major histocompatibility complex class II (MHCII) on antigen-presenting cells and the T-cell receptor (TCR) on T-cells, where they act as super antigens [21]. Characteristic SEs are high thermostability and low degradation in the stomach and intestinal tract [22]. Food poisoning

caused by SE is not lethal [20]. Depending on the toxin, rapamycin is effective after an SE B intoxication [20]. The generation of clinically efficacious vaccines has failed so far [23].

Clostridium difficile

Clostridium difficile are Gram-positive, spore-forming, thick rod-shaped bacteria and the major cause for diseases in hospitals associated with broad-spectrum antibiotic treatment. Antibiotic therapy causes alterations of the normal colonic microflora [24] and induces stress responses in bacteria such as *C. difficile*. This pathogen produces two toxins, the enterotoxin A (TcdA) [25] and the cytotoxin B (TcdB), which provoke diarrhea and pseudomembranous colitis [26]. Traditionally, the treatment consists of curing the symptoms and discontinuing the initial antibiotic therapy. Then, the administration of oral metronidazole or oral vancomycin is reasonable. Metronidazole is the first choice therapy in order to prevent proliferation of vancomycin-resistant nosocomial pathogens [24]. Several new approaches for treatment are under investigation, for example vaccines, toxin-binding agents, flora-sparing antibiotics and antibodies [27].

Bacillus cereus

The Gram-positive, spore-forming species *B. cereus* also produce a variety of toxins that causes gastrointestinal diseases [28]. It is present in nature, and in rice, dairy and meat products and vegetables [29]. Normally, the induced diarrhea is self-limiting and heals quickly [30]. There are three enterotoxins, i) the hemolytic enterotoxin hemolysin BL (HBL), ii) the non-hemolytic enterotoxin (Nhe) and iii) cytotoxin K (CytK), that play major roles in diarrheal infection, but there are others causing vomiting (emetic type) [31]. It is assumed that the three toxins have an destructive effect on epithelial cell membrane integrity in the small intestine [31]. Similar to *S. aureus*, *B. cereus* is resistant against many antibiotics [29]. A specific vaccine has not been reported.

Protozoans causing diarrheal diseases

Entamoeba histolytica

The protozoan parasite *Entamoeba histolytica* causes invasive amoebic dysentery and can lead to death if not treated [32, 33]. It is a cosmopolitan pathogen, which is most common in tropic regions [34], where poor sanitary standards prevail and water and food are often contaminated with feces [32]. Annually, 50 million people worldwide contract an amoebic dysentery and about 40,000 to 100,000 cases are fatal [35]. *E. histolytica* secretes proteases that degrade red

blood cells and tissues [33]. For treating amoebiasis, metronidazole and nitazoxanide are the most widely used drugs [36].

Giardia lamblia

Giardia lamblia is a flagellated human pathogenic protozoan and causative agent of giardiasis and noninvasive diarrhea [37, 38]. *G. lamblia* exist in two stages, an inactive form as cyst found in polluted water and food, and in vegetative form as trophozoites in human [38]. Inside the intestine, the parasite changes its surface molecules continuously, allowing to escape immune responses and repeating infections [39]. Worldwide, more than 280 million people suffer from giardiasis every year [40]. For treatment, metronidazole is prescribed, but there are already metronidazole-resistant strains. New drugs are required, one could be auranofin, which blocks the thioredoxin oxidoreductase activity of *G. lamblia* [41].

Cryptosporidia

The water-borne, obligate intracellular pathogen *Cryptosporidium* is part of the phylum Apicomplexa and causes diarrhea in humans [42-44]. Especially *C. parvum* invades gastrointestinal epithelial cells [45]. An infection with Cryptosporidia is self-limiting in healthy humans, but can be fatal for persons with reduced immune functions [46]. The infective form are oocysts and only a small amount is required [44]. In form of oocysts, the protozoans are tolerant against most of the chemicals used of water disinfection, such as chloride, and they survive for several months in nature [44]. Invasion is mediated via glycoproteins and circumsporozoite surface ligand (CSL). After infection, the microvilli are destroyed and loss of enzymes and ions is detected [44]. For treating a *Cryptosporidium* spp. infection, paromomycin and nitazoxanide were proved to be effective [47]. A vaccine against Cryptosporidia is pending.

Further viruses causing diarrheal diseases

Norovirus

Another pandemic virus is norovirus, which is also found in feces and contaminated water and food [48, 49]. The RNA virus is non-enveloped and contains a capsid [50]. GII4 is the most widespread human-specific *norovirus* strain and antigen and receptor variation lead to recurrent infections [51]. Additionally, it is not cultivable, most of the information are based on RT-PCR [48]. Mortality of estimated 200,000 children under 5 years was reported for developing countries [52]. Hitherto, there are no vaccines available, but there is a new approach based on blocking the histo-blood group antigens that norovirus uses as receptor**.**

Table S1. Important microbial pathogens causing diarrhea

Characteristics	**Main virulence factors**	**Route and target of infection**	**Infection rate, mortality**	**Treatment**
Gram-negative bacteria				
Vibrio cholerae				
Curved rod-shaped Clinically and epidemiologically serotypes: *V. cholerae* O1 and O139 (2 serotypes of 200) [53, 54]	Toxin co-regulated pilus (TCP), Cholera Toxin (CT), a secreted AB-toxin, triggers hypersecretion (see Fig. 1)	Oral-fecal transmission, CT affects the small intestine	3 - 5 million cases and 100,000 – 130,000 death p.a. [55]	Oral dehydration, improving sanitary facilities, antibiotic therapy, three licensed vaccines
Enteropathogenic *E. coli* (EPEC)				
Main cause of diarrhoea in infants, initiate pedestal formation by attaching intestinal epithelial cells [3]	T3SS, translocated TIR effector molecule, acting as a intimin receptor, adherence factor EAF [4]	Oral-fecal transmission	Ca. 1 million deaths of children p.a. [1]	Antimicrobial therapy, no licensed vaccines
***Shigella* spp.**				
Four species Only 10-100 bacteria are sufficient for triggering epidemic mucosal ulceration and bloody diarrhea, [56] self-imitated	characteristic invading mechanism (apical and basolateral), Shiga Toxin, actin-mediated intracellular motility [57]	Transmitted through person-to person contact (smear infection), ingested by contaminated water or food	165 million cases and 1.1 million death p.a. [58]	antibiotic therapy, no licensed vaccines
***Campylobacter* spp.**				
Microaerophil ε-proteobacterium, high motile, major cause of acute gastroenteritis worldwide, Guillain-Barré syndrome (*C. jejuni*) [6]	Invading process up to the deeper intestinal crypts causing in ulcus inflammation and crypt abscess [5]	Zoonotic infection, natural host are poultry and other livestock animals, foodborne transmission [59]	400 million cases of diarrhea p.a. [60]	Substitution of water and electrolytes, antibiotic therapy, no licensed vaccines
***Salmonella enterica, n*on-typhoidal**				
rod shaped γ-proteobacteria	Trigger invasion by SPI1-encoded T3SS with actin remodelling, SPI2-T3SS enables an intracellular lifestyle [8, 9]	Oral ingestion of contaminated food and water/ direct contact to pets or livestock animals	Annually tens of millions cases occurring worldwide	Substitution of water and electrolytes, antimicrobial therapy only for health risk groups
***Aeromonas* spp.**				
Cause nosocomial complications, like "flesh-eating bacteria syndrome" (*A. hydrophila*)[61], but actually with low pathogenicity, a classical opportunists	Produce enterotoxins, like the secreted aerolysin, which cause channel formation, similar to the α-toxin of *S. aureus* [1]	Immune-competent individuals are compromised, pathogen of fish and other ectotherms [62]	Not reported	Substitution of water and electrolytes, combat the symptoms

***Proteus* spp.**				
Rapid motile proteobacteria with swarming growth, putrefactive agents in water, soil, on the surface of carcasses and in the natural intestinal flora [63]	Production of the enzyme urease, increasing pH value and precipitation of magnesium and calcium salts [11]	Nosocomial infections	*P. mirabilis* raise approximately 3% of nosocomial infections in the United States [64]	antimicrobial therapy, higher hygiene standard
***Enterobacter* spp. (alias *Cronobacter sakazakii*)**				
Cause various disease patterns such as urinary tract and abdominal infections, reclassification as new genus *Cronobacter* by Iversen *et al*. [13]	disrupting tight junctions, induce monolayer permeability and cause apoptosis by binding to intestinal epithelial cells [16]	*Cronobacter* can contaminate powdered milk and infant formulas [14], leading to necrotizing enterocolitis (NEC) in infants [15]	Rare neonatal *Cronobacter* infections	Administration (against NEC) of probiotics, antibiotics, surgical examination and Epidermal Growth Factor (EGF) [17]
***Yersinia* spp.**				
Y. enterocolitica and *Y. pseudotuberculosis* causing human intestinal infections	plasmid-encoded antiphagocytic factors (Yop proteins),T3SS (Ysc) lead to inactivation of phagocytes [19] chromosomally encoded OMPs (Inv & Ail) and virulence plasmid (YadA)	Zoonosis, Invading process via colonization the Peyer's patches and M cells for transport [18]. Resting in phagocytic cells with transfer to the spleen	1,903 *Yersina* infections (1996-2007) 1,4717 with species information (92 % *Y. enterocolitica,* 1 % *Y. pseudotuberculosis*, FoodNeT, USA [65]	Substitution of water and electrolytes, antimicrobial therapy only for health risk groups
ETEC – Enterotoxic *E.coli*				
most frequent *E. coli* intestinal pathotypes, which cause infectious diarrhoea in infants and young children [66]	Unstable in heat enterotoxin (LT) similar to CT, heat-stable toxins (STa & STb) specific fimbriae (CFA) for adhesion to enterocytes of small intestine	Oral-fecal transmission	200 million diarrhea cases & 150,000 deaths p.a. [67]	Antimicrobial therapy, no licensed vaccines[68], but multivalent live-attenuated vaccine (ACE527) completed Phase IIb [69]
Gram-positive bacteria				
Staphylococcus aureus				
facultative, β-hemolytic, , halo-tolerant bacterium, colonizes skin, mucosal surfaces, soft tissues, bones and medical devices, causes among other lethal toxic shock, food poisoning [20]	Thermo stable staphylococcal enterotoxins (SEs) [20] Multi drug resistance (MRSA)	MHCII on antigen-presenting cells [21] nosocomial transmission, contaminated dairy products and meat [20]	Not reported for pure gastroenteritis cases	Rapamycin [20] efficacious vaccines failed [23]

Clostridium difficile				
Spore-forming bacteria, major cause for broad-spectrum-antibiotic-associated disease in hospitals[24]	Entero- and Cytotoxin (TcdA & TcdB) [25] *C. difficile* transferase (CDTa/CDTb) functions as a binary actin ADP-ribosylating toxin [70]	faecal-oral, TcdB provoke diarrhea and pseudo-membranous colitis in the colon [26], Lipolysis-stimulated lipoprotein receptor (LSR) on host cells [70] nosocomial transmission, faecal-oral	336,600 infections p.a. (2009) [71], 14,000 deaths p.a. (USA) (2006-2007) [72]	Combat the symptoms, discontinuing the initial antibiotic therapy plus oral metronidazole and vancomycin treatment [24] Vaccine based on toxin A and B in Phase I [73]
Bacillus cereus				
aerobic/ facultatively anaerobic, motile, spore-forming, rod-shaped bacterium, causes food poisoning [29], close relationship to *B. anthraces*	many antibiotics resistances [29] hemolytic enterotoxin hemolysin BL (HBL), non-hemolytic enterotoxin (Nhe) cytotoxin K (CytK) [31]	destructive effect on epithelial cell membrane integrity in the small intestine [31], transmission via nature, rice, dairy and meat products, vegetables [29]	unknown	A specific vaccine has not been reported
Protozoans				
Entamoeba histolytica				
infectious form: cyst (10–15 μm) amoeboid form: trophozoites are highly motile, pleomorphic shape (10 to 50 μm) [33], causes invasive amoebic dysentery/amoebic colitis [32, 33], no mitochondria	Secrets proteases (e.g. cysteine proteases) [33, 74] Pore-forming peptide amoebapore [75]	degrade mucosal barrier, tissues, epithelial cells and erythrocytes [33, 74] Transmission: faeces, food, water [32].	50 million infections, 40,000-100,000 deaths p.a. [35]	Potential vaccine target Gal/GalNAc lectin (involved in adhesion process) [76] metronidazole and nitazoxanide [36]
Giardia lamblia				
Flagellated, unicellular protozoan [37, 38], infectious form: cyst, amoeboid form: trophozoites [38], causes giardiasis	Non-invasive changes of surface molecules [39]	Cells of the small intestine [77] transmission via contaminated water	280 million infections p.a. [40]	New approach based on Auranofin (block thioredoxin oxidoreductase activity) [41] Other potential targets could be cyst wall proteins Metronidazole [41]

Cryptosporidia				
obligate, intracellular, protozoan, phylum Apicomplexa [78], infectious form: oocysts (10 to 40 µm) [44, 78], causes cryptosporidiosis, important species: *Cryptosporidium parvum* and *Cryptosporidium hominis*	tolerant against most chemicals used for water disinfection [44] Invasion via glycoproteins and circumsporozoite surface ligand (CSL) [79], Destruction of microvilli [44]	Gastrointestinal epithelial cells [45] Transmission: person-to-person, water, food, animal-to-human [44]	58 million infections p.a. (1992) (young children), high mortality rate in immunocompromised human [80]	Self-limiting in healthy human [44, 46] Potential target: mucin-like glycoproteins CpMuc4 and CpMuc5 (in vitro) [81] Further unlicensed approaches are listed in Table 3 paromomycin and nitazoxanide [47]
Viruses				
Rotavirus				
Part of the family *Reoviridae*, triple-layered viral particle, 11 dsRNA segments (6 structural viralproteins (VPs) and 5 or 6 non-structural proteins (NSPs)), vaccine targets are VP7 (12 variations) and VP4 (15 variations) located in the outer membrane [82]	Enterotoxin NSP4 (interaction with calcium-activated chloride channels) [83] Reduced effectiveness of digestive enzymes [84]	Gastrointestinal, polarized epithelial cells [83] Transmission: fecal-oral route, by close person-to-person contact	453,000 death p.a. in children (2008) [85]	Self-limiting via dehydration [86] licensed since 2006 Rotarix® and RotaTeq® [87], see further below
Norovirus				
Also Norwalk-Virus, Part of the Family *Caliciviridae*, five genogroups (GI–GV), only GI, GII and GIV are human specific, +ssRNA, three ORFs encoding polyprotein for non-structural proteins, major capsid protein (VP1) and minor capsid protein (VP2) [48]	Antigen and receptor variation [51] No cultivation [48]	Histo-blood group antigens on mucosal epithelial cells [88, 89] Transmission: feces, contaminated water and food [48, 49]	200,000 infections p.a., children (< 5 years)/year [52]	Self-limiting infections new approach based on blocking the histo-blood group antigens (receptor for norovirus) [50]

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