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Pro-inflammatory capacity of *Escherichia coli* O104:H4 outbreak strain during colonization of intestinal epithelial cells from human and cattle

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ABSTRACT

In 2011, Germany was struck by the largest outbreak of hemolytic uremic syndrome. The highly virulent *E. coli* O104:H4 outbreak strain LB226692 possesses a blended virulence profile combining genetic patterns of human adapted enteroaggregative *E. coli* (EAEC), rarely detected in animal hosts before, and enterohemorrhagic *E. coli* (EHEC), a subpopulation of Shiga toxin (Stx)-producing *E. coli* (STEC) basically adapted to the ruminant host. This study aimed at appraising the relative level of adaptation of the EAEC/EHEC hybrid strain LB226692 to humans and cattle. Adherence and invasion of the hybrid strain to intestinal (jejunal and colonic) epithelial cells (IEC) of human and bovine origin was compared to that of *E. coli* strains representative of different pathovars and commensal *E. coli* by means of light and electron microscopy and culture. Strain-specific host gene transcription profiles of selected cytokines and chemokines as well as host-induced transcription of bacterial virulence genes were assessed. The release of Stx upon host cell contact was quantified. The outbreak strain's immunomodulation was assessed by cultivating primary bovine macrophages with conditioned supernatants from IEC infection studies with *E. coli*, serving as model for the innate immunity of the bovine gut. The outbreak strain adhered to IEC of both, human and bovine origin. Electron microscopy of infected cells revealed the strain's particular affinity to human small IEC, in contrast to few interactions with bovine small IEC. The outbreak strain possessed a high-level of adhesive power, similar to human-associated *E. coli* strains and in contrast to bovine-associated STEC strains. The outbreak strain displayed a non-invasive phenotype, in contrast to some bovine-associated *E. coli* strains, which were invasive. The outbreak strain provoked some pro-inflammatory activity in human cells, but to a lower extent as compared to other pathotypes. In contrasts to bovine-associated *E. coli* strains, the outbreak strain induced marked pro-inflammatory activity when interacting with bovine host cells directly (IEC) and indirectly (macrophages). Among *stx2*-positive strains, the human-pathogenic strains (LB226692 and EHEC strain 86-24) released higher amounts of Stx compared to bovine-associated STEC. The findings imply that the outbreak strain is rather adapted to humans than to cattle. However, the outbreak strain's potential to colonize IEC of both host species and the rather mixed reaction patterns observed for all strains under study indicate, that even STEC strains with an unusual genotype as the EHEC O104:H4 outbreak strain, i.e. with an EAEC genetic background, may be able to conquer other reservoir hosts.

1. Introduction

In 2011, Germany was struck by the largest outbreak of hemolytic uremic syndrome (HUS) (Frank et al., 2011), caused by a novel and unusual enterohemorrhagic *Escherichia coli* (EHEC) O104:H4 strain. The highly virulent outbreak strain harbors major virulence genes of both, enteroaggregative *E. coli* (EAEC) and Shiga toxin (Stx)-producing *E. coli* (STEC) (Bielaszewska et al., 2011). Cattle represent the primary reservoir of STEC, which are capable of modulating the bovine immune system to promote a persistent and asymptomatic lifestyle and limit the intestinal inflammation in cattle by leaving the innate response unaffected but retarding the host's adaptive immune response by Stx (Beutin et al., 2013; Menge et al., 2015, 2003; Menge et al., 2004, 2001; Menge et al., 1999; Moussay et al., 2006; Paddock et al., 2013; Wieler et al., 2011). Stx, an extremely potent cytotoxin targets globotriaosyl ceramide receptors (Gb3), enters host cells and blocks protein synthesis by irreversibly damaging ribosomal RNA (Endo et al., 1988). The very same factors which account for the ability to persistently colonize cattle contribute to pathogenesis of human disease, as seen in EHEC O157:H7 (Smith et al., 2002; Stamm et al., 2002). EHEC are a subset of STEC

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which are capable of causing disease in humans with potentially life-threatening complications such as HUS. EHEC induce attaching and effacing lesions in the colon and secrete Stx, leading to intense intestinal inflammation and systemic absorption of Stx (Kaper et al., 2004). So far, only humans are known as natural reservoir of EAEC, since strains of this pathovar have rarely been detected in animal hosts before (Cabal et al., 2015; Cassar et al., 2004; Karch et al., 2012; Okeke, 2009; Uber et al., 2006; Veilleux and Dubreuil, 2006). EAEC are emerging diarrheal pathogens (Harrington et al., 2005) defined by a characteristic "stacked-brick" aggregative adherence pattern to epithelial cells (Nataro et al., 1987). EAEC pathogenesis involves abundant adherence to small and predominantly large bowel epithelium (ileal and colonic tissue) in a thick biofilm, elaboration of enterotoxins and cytotoxins as well as the induction of mucosal inflammation and damage (Harrington et al., 2006; Kaper et al., 2004).

Virulence profiling and rapid whole genome sequencing revealed that the outbreak strain is rather closely related to EAEC than to classical EHEC, in particular to strain 55989 (99.8% similarity), an isolate from Central Africa causing chronic diarrhea (Bielaszewska et al., 2011; Brzuszkiewicz et al., 2011; Mellmann et al., 2011; Mossoro et al., 2002; Rohde et al., 2011). The outbreak strain and O104:H4 strains isolated earlier from sporadic cases of HUS (2001–2011) revealed high similarity, suggesting that EHEC O104:H4 has become endemically established in Europe and very likely in the human population (Miko et al., 2013; Monecke et al., 2011; Navarro-Garcia, 2014). However strains possessing an O104 serogroup have also been detected in feces of feedlot cattle (Paddock et al., 2013) and sporadically in contaminated meat products and foodstuff, including minced red meat, wild boar, sheep, bovine carcasses, (sheep) meat and milk (ECDC, 2011). Although the outbreak strain was not isolated from cattle during the outbreak in Northern Germany (Wieler et al., 2011) and France (Auvray et al., 2012), its virulence-associated genes (*stx2*, *aggR*, *wzxO104*, *fliCH4*) have been detected concomitant in bovine feces from one German abattoir, located near the outbreak epicenter from 2011 at a later time (Cabal et al., 2015). In fact, the outbreak strain was able to transiently colonize calves after experimental infections (Hamm et al., 2016). New combinations of chromosomal and extrachromosomal elements continually emerge and propagate in the environment and susceptible hosts. Profound chromosomal changes can even occur during the brief period that EHEC passes through the host's gastrointestinal tract (Mellmann et al., 2011). The outbreak strain carried a *stx2a* encoding bacteriophage (P13803) (Bielaszewska et al., 2011; Brzuszkiewicz et al., 2011) which originates from the bovine STEC reservoir (Beutin et al., 2013) and can be either transferred in the intestine of mammals or in the environment. Besides *stx*, the outbreak strain possessed further STEC virulence factor genes, such as for adhesins (*iha*, *lpf*), and the EAEC-specific virulence plasmid (pAA), carrying genes for type I aggregative adherence fimbriae (AAF), that mediate colonization and biofilm formation (Bielaszewska et al., 2011; Nataro, 2005; Sheikh et al., 2001; Tarr et al., 2000). AAF expression is regulated by the AggR transcriptional factor (Nataro et al., 1994), which is essential for adhesion and elicit both cytokine release and opening of epithelial tight junctions in a polarized epithelial model (Harrington et al., 2005; Strauman et al., 2010). Among others O104:H4 also produces Pic, a serine protease (Bielaszewska et al., 2011), which facilitates intestinal colonization by enabling the bacterium to utilize mucus as a growth substrate (Harrington et al., 2009). The outbreak strain's ESBL-plasmid has also been identified in several *E. coli* strains of human and animal origin before (Brzuszkiewicz et al., 2011; Smet et al., 2010). Based upon the outbreak strain's isolation in the respective host species, a human reservoir was suggested, however other possible reservoirs could not be fully excluded (Karch et al., 2012). Due to the hybrid virulence profile, the natural reservoir of the outbreak is unknown (Karch et al., 2012). It needs to be unveiled if cattle, humans or both may act as potential carriers and unsuspected source to further spread (Karch et al., 2012; Wieler et al., 2011).

In the natural reservoir, virulence factors of bacterial pathogens counteract elements of the immune control generating a balance between pathogen and host (Sansonetti, 2004). In different hosts the outcome of infection depends on varying ability to colonize epithelial cells (Sonntag et al., 2005), specific differences in cellular receptors distribution (Mundy et al., 2007; Stamm et al., 2008), altered expression of bacterial factors (Rashid et al., 2006) as well as upon the respective host cell response. This study aimed at appraising the relative level of host adaption of *E. coli* O104:H4 outbreak strain to possible reservoir hosts, by comparing its interaction with host-specific cells in vitro and by linking its reaction profile to that of bovine- and human-associated *E. coli* strains. In addition, we addressed the potential of the outbreak strain to establish itself long-term in infected cattle, based on the Stx-mediated modulation of the bovine immunity (Menge et al., 2015). Elucidation of these strain-specific interactions may provide new insights in the outbreak strain's host adaptation in order to raise the level of preparedness against future outbreaks implicating unusual EHEC strains.

2. Materials and methods

2.1. Bacterial strains used in this study

Prior to use for this study, bacterial strains were tested in HEp-2 cell assay for extent of adhesion, pattern of adhesion (Nataro et al., 1987) and fluorescent actin staining (Knutton et al., 1989) (summarized in Table 1). Their pathotype was confirmed by detection of characteristic virulence genes as published elsewhere (Muller et al., 2007). For functional assays bacteria were routinely grown overnight in lysogeny broth (LB) (+1% D-mannose, 37 °C, 180 rpm) and then diluted (1:20) in the respective tissue culture medium without antibiotics for 2 h (37 °C, 180 rpm). Each bacterial suspension was adjusted to an optical density (OD_{600nm}) of 0.6 to have a final inoculum corresponding to $4 \times 10^8 \pm 1 \times 10^8$ cfu/ml.

2.2. Generation and maintenance of epithelial cell lines and primary cell cultures

The human intestinal epithelial cell (IEC) line CaCo-2 (colon adenocarcinoma; ATCC CCl-169; American Type Culture Collection, U.S.A.) was cultured in DMEM (4.5 g/l glucose) supplemented with 1% NEAS, 10% FCS and 1% penicillin/streptomycin. The human fetal epithelial cell line INT 407 (jejunal/ileum; ECACC 85,051,004; European Collection of Authenticated Cell Cultures, Public Health England, United Kingdom) was cultured in MEM (2.2 mg/l $NaHCO_3$) supplemented with 1% NEAS, 10% FCS, 1% penicillin/streptomycin. The bovine fetal intestinal jejunal epithelial cell line FKD-R 971 (jejunal/ileum; CCLV RIE 971, Collection of Cell Lines in Veterinary Medicine; Friedrich-Loeffler-Institut, Germany) was maintained in Ham's F12 and IMDM (1:1) supplemented with 1% NEAS and 10% FCS. As control African Green Monkey Vero cells (ATCC CRL-1587) and HEp-2 cells (CCLV RIE-0141) were used, that were cultured in RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin. Permanent IEC cultures were maintained under standardized conditions (37 °C, 5% CO_2). For functional assays cells were seeded (2×10^5 cells/well) in 24-well-culture plates (Corning®-Costar®, Sigma-Aldrich Chemie, Germany) using antibiotic-free medium, until they reached confluence (INT 407, FKD-R 971: 48 h; CaCo-2: 10–14 days). For transepithelial electric resistance (TEER) measurements (EVOM resistance meter, World Precision Instruments, U.S.A.) cells were grown onto 12 mm diameter transwell permeable culture supports (0.4 µm pore size, Corning® Costar®, Sigma-Aldrich Chemie), for microscopic onto glass cover slips.

Primary colonocytes (PC) were isolated from colon specimens which were derived freshly from the ascending colon of slaughtered cattle (approx. 9 month old, Frisian-Holstein) by mechanical preparation