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Mini review

Phage display and Shiga toxin neutralizers

Robert Alvin Bernedo-Navarro*, Tomomasa Yano

Department of Genetics, Evolution and Bioagents, University of Campinas (UNICAMP), SP, Brazil

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ABSTRACT

The current work presents an overview of the use of phage display technology for the identification and characterization of potential neutralizing agents for Shiga toxins. The last major Shiga toxin-associated disease outbreak, which took place in Germany in 2011, showed the international community that Shiga toxins remain a serious threat to public health. This is also demonstrated by the lack of specific therapies against Shiga toxin-induced Hemolytic Uremic Syndrome (HUS). Since its inception, phage display technology has played a key role in the development of antigen-specific (poly)-peptides or antibody fragments with specific biological properties. Herein, we review the current literature regarding the application of phage display to identify novel neutralizing agents against Shiga toxins. We also briefly highlight reported discoveries of peptides and heavy chain antibodies (VHH fragments or nanobodies) that can neutralize the cellular damage caused by these potent toxins.

1. Pathogen

Shiga toxin-producing *Escherichia coli* (STEC) are a heterogeneous and potentially fatal group of microorganisms that produce potent cytotoxins called Shiga toxins (Stxs). These toxins are similar to those produced by *Shigella dysenteriae* type 1.

In addition to asymptomatic infections, STEC can cause the following clinical manifestations: acute watery diarrhea; bloody diarrhea; hemorrhagic colitis; hemolytic uremic syndrome (HUS), a life-threatening thrombotic microangiopathy leading to acute renal dysfunction approximately one week after onset of diarrhea; and death (Pennington, 2010).

E. coli serotype O157:H7 is the most common member of the STEC pathotype and the leading cause of pediatric HUS (Banatvala et al., 2001; Verweyen et al., 1999). However, over 400 non-O157 serotypes isolated from different sources have reported involvement in human disease (Bettelheim, 2007; Mora et al., 2011).

It has previously been reported that children, the elderly and immunocompromised individuals are generally more susceptible to STEC infections; however, a recent *E. coli* O104 outbreak in Germany showed that infected healthy adults can also present with severe complications.

The Shiga toxins encoded by the *stx1* and *stx2* genes, which are carried by lysogenic lambdoid phages, are the main virulence factors associated with STEC. Strains carrying the *stx*2 gene (mainly the $stx2_{EDL933}$ subtype) are not only potentially more virulent but also are more frequently related to HUS than those harboring only *stx1* and those carrying both genes (Friedrich et al., 2002; Schmidt et al., 1995).

Among the STEC strains, seven serogroups have been more frequently associated with severe human illnesses, such as hemorrhagic colitis (HC) and HUS. These strains are also referred as enterohemorrhagic *E. coli* (EHEC) (Delannoy et al., 2013; Levine, 1987; Nataro and Kaper, 1998). In developed countries in the Northern Hemisphere, serotypes O26:H11, O45:H2, O103:H2, O111:H8, O121:H19, O145:H28, and O157:H7, as well as their non-motile derivatives, are considered the seven "priority" STEC serotypes (also referred as the "top 7" EHEC serotypes). These seven EHEC serotypes can be subdivided into seropathotypes (SPTs) A and B based on their phenotypic and molecular characteristics and the clinical features of their associated diseases. There are a total of five SPTs, which are denoted A, B, C, D, and E according to decreasing rank of pathogenicity (Karmali et al., 2003).

In addition to Stx production, the top 7 serotypes harbor the locus of enterocyte effacement (LEE), a genomic island encoding intimin, which participates in bacterial colonization of the gut and in attaching-and-effacing (A/E) lesions of the intestinal mucosa (Nataro and Kaper, 1998), in addition to regulatory elements, a type III secretion system, and secreted effector proteins, as well as their cognate chaperones (Elliott et al., 1998; Perna et al., 1998).

Other reported virulence-associated markers include a plasmid-

* Corresponding author.
E-mail address: alvinbn@gmail.com (R.A. Bernedo-Navarro).

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encoded enterohemolysin and, in strains lacking *eae*, an autoagglutinating adhesin (Saa) potentially involved in adhesion (Paton et al., 2001). This plasmid is present in STEC O157 and in non-O157 strains. Furthermore, STEC strains belonging to identical pulsed-field gel electrophoresis types can be further discriminated based on their plasmid-encoded genes, including hemolysin (*ehxA*), a catalase-peroxidase (*katP*), an extracellular serine protease (*espP*), a zinc metalloprotease (*stcE*, also called *tagA*), and a subtilase cytotoxin (*subAB*), among others (Etcheverría and Padola, 2013).

STEC strains are innocuous to ruminants and are transient members of their gastrointestinal flora. Cattle are the main STEC reservoir implicated in human disease (Nastasijevic et al., 2008), and STEC strains have also been found in the gastrointestinal tracts of sheep, pigs, goats, dogs, and cats (Paton and Paton, 1998). Fecal contamination of meat during slaughter is the most common route for transmission to humans (Chase-Topping et al., 2008; Kudva et al., 1999). In this way, foods of bovine origin, especially undercooked ground beef and unpasteurized milk, constitute important sources of human infection (Griffin and Tauxe, 1991; Rangel et al., 2005). Other sources linked to sporadic infection and outbreaks of illness in humans include lettuce, alfalfa sprouts, radish sprouts, spinach, fenugreek sprouts, and apple cider, as well as consumption or recreational use of water, direct contact with cattle or animal excreta, attendance at agricultural fairs, and recreational use of pastures (King et al., 2012; Rangel et al., 2005). Secondary person-to-person transmission, such as within families, day care centers, and healthcare institutions, has also been reported (Carter et al., 1987; Rangel et al., 2005; Rowe et al., 1993; Spika et al., 1986). A remarkable feature of STEC is its low infectious dose; approximately 50–100 bacteria are sufficient to cause disease in healthy individuals (Tilden et al., 1996).

Worldwide, STEC infections cause an estimated three-million acute illnesses annually (Majowicz et al., 2014). The average cost per STEC O157 case varies greatly according to disease severity: for patients not requiring medical care, the cost is approximately $26, whereas those who develop and die from HUS may cost up to $6.2 million per case (Frenzen et al., 2005).

2. Shiga toxins

The term “Shiga” originated in 1898, when Kiyoshi Shiga first described the infectious agent of epidemic bacterial dysentery, *S. dysenteriae* type 1 (Shiga's bacillus) (Trofa et al., 1999). Following this, Keusch et al. (1972) determined that a toxin produced by this bacterium caused fluid accumulation and enteritis in ligated intestinal segments from rabbits. In 1977, Konowalchuk et al. reported the discovery of a novel cytotoxin in cell-free culture filtrates of some *E. coli* strains. This cytotoxin differed from known heat-stable and heat-labile enterotoxins from *E. coli*. The compound had cytotoxic activity against Vero cells and produced markedly different effects from those of heat-labile enterotoxin.

Ten years after the discovery of Stx, which is produced by *S. dysenteriae*, O'Brien et al. (1983) reported that certain strains of *E. coli* produce a toxin that can be neutralized by anti-Stx serum. As a result, *E. coli* strains that produce Shiga-like toxins were named Shiga toxin-producing *E. coli*, as the Stx1 produced by *E. coli* is essentially identical at the genetic and protein levels to the Stx produced by *S. dysenteriae* 1. The ability of STEC strains to cause severe disease in humans is related mainly to their capacity to produce Stx toxins.

Stxs belong to the AB5 family of protein toxins (Fig. 1). These toxins are composed of an enzymatically active A moiety and a nontoxic B moiety, which is responsible for binding to cellular receptors. The StxB moiety is ring-shaped and pentameric, consisting of five identical B subunits (7.7 kDa) surrounding a central pore through which the C-terminus of the A moiety is anchored (Fraser et al., 1994; Stein et al., 1992). Each B subunit harbors three different binding sites (sites 1–3) that interact with a trisaccharide moiety on the glycosphingolipid receptor globotriaosylceramide (Gb3) (Jacewicz et al., 1986; Lindberg et al., 1987; Ling et al., 1998; Lingwood et al., 1987). In this way, each B moiety can potentially interact with up to 15 Gb3 molecules, resulting in high-affinity binding between StxB and Gb3 (Bergan et al., 2012). All Stxs, with the exception of one Stx2 variant known as Stx2e, bind to Gb3; Stx2e preferentially binds to the glycolipid globotetraosylceramide (Gb4) (Matise et al., 2003).

To exert its enzymatic activity on target cells, the A subunit of Stx (32.2 kDa) must becleaved into an enzymatically active A1 fragment (27.5 kDa) and a small A2 fragment (4.5 kDa) (Garred et al., 1995). After cleavage, the A1 fragment remains attached to the A2 fragment via a disulfide bond until the toxin is exposed to the reducing environment of the endoplasmic reticulum lumen (Garred et al., 1997). Then, the A1 fragment is released and translocates into the cytosol, where it exerts its cytotoxic action, leading to cellular death and apoptosis.

Within the ER, the Stx A1 fragment dissociates from the A2 fragment and the B subunits following furin-mediated proteolysis and disulfide bond reduction (Garred et al., 1995; Sandvig et al., 2010). From the ER, the proteolytically processed A1 enters the host cell cytosol and removes one adenine from the adenosine at position 4324 in the 28S ribosomal RNA. This removal inhibits the binding of aminoacyl-tRNA to the 60S ribosomal subunit and therefore inhibits cellular protein synthesis (Paton and Paton, 1998).

The precise mechanism(s) by which the different Stxs activate apoptosis remain to be clarified. Stx1 and Stx2 induce apoptosis and activate stress response pathways in endothelial cells. After internalization, Stx2 activates various intracellular stress pathways, such as the endoplasmic stress response pathway and the ribotoxic stress response pathway (Tesh, 2012). This may lead to apoptosis via the activation of various cell death signals, such as the down-regulation of the antiapoptotic Bcl2 protein (McCullough et al., 2001), the activation of the mitogen-activated protein kinases (MAPKs) p38α and c-Jun N-terminal kinase (JNK) (Kitamura, 2008; Smith et al., 2003), and the induction of caspase 3-dependent apoptosis (Orrenius et al., 2003; Zong et al., 2003).

The Stx family is divided into two immunologically non-cross reactive groups known as Stx1 and Stx2 (O'Brien and Holmes, 1987). STEC strains can express only Stx1, only Stx2 or both. The AB5 holotoxin structure is conserved among all Stx family members (Nataro and Kaper, 1998; Paton and Paton, 1998).

Although the Stx family members all share the same holotoxin structure and biological activity, differences exist among toxin variants. Stx produced by *S. dysenteriae* and Stx1 from *E. coli* have identical B moieties (De Grandis et al., 1987) and differ at only one residue in the A moiety (Strockbine et al., 1988). However, Stx2 is immunologically distinct from Stx1, and they share only approximately 56% amino acid sequence identity (Calderwood et al., 1987; Jackson et al., 1987).

There are three Stx1 subtypes (Stx1a, Stx1c, and Stx1d) and seven Stx2 subtypes (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g) (Scheutz et al., 2012).

Epidemiologically, Stx2 is considered the most important Stx subtype, as the probability of HUS development following infections with STEC strains producing only Stx2 is higher than that following infections with STEC strains synthesizing only Stx1 or both Stx1 and Stx2 (Friedrich et al., 2002). As such, Stx2a, Stx2c and Stx2d are the toxin variants most commonly associated with severe outcomes in STEC-infected humans (Bielaszewska et al., 2006; Persson et al., 2007). Stx2e is associated with high mortality rates