Research Article

Aptamer "Western" Blotting for *E. coli* Outer Membrane Proteins and Key Virulence Factors in Pathogenic *E. coli* Serotypes

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Abstract | Western blot-like analyses using DNA aptamers raised against the surfaces of general non-pathogenic *Escherichia coli* (ATCC No. 8739) as well as alpha and gamma intimins and Shiga-like toxin 2 (SLT-2) B subunit verified that the aptamers bound proteins of the correct molecular weights for intact outer membrane proteins (OMPs), intimins and SLT-2. Some bands on the aptamer Western blots were shared between the "Big 6" non-O157 Shiga toxin-producing *E. coli* (STEC), *E. coli* O157 and related Gram negative bacteria. However, unrelated Gram positive bacteria exhibited very few, if any, bands in common with those identified on the *E. coli* Western aptamer blots, thus attesting to the specificity of these aptamers. In general, the use of aptamers in Western blot formats is advantageous for validation of aptamer binding and specificity based on molecular weight matching of detected electrophoretic bands across a spectrum of related and unrelated bacteria.

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Introduction

Characterization of candidate aptamers often involves ELISA-like (also known as ELASA or ELONA) microplate screening to rank aptamer affinities, surface plasmon resonance (SPR) or other techniques to determine aptamer affinity constants and validation of binding to whole cells, if desired, by fluorescence microscopy or other cytochemical staining techniques (Bruno et al., 2008). Western blot techniques adapted to the use of aptamers, sometimes referred to as "Southwestern" blotting due to the use of DNA aptamers as probes to detect proteins, have the advantage over ELASA, SPR and some other techniques of elucidating the molecular weight of bands that react with various aptamers which can further validate aptamer binding and specificity. While aptamer Western blots have been used to validate binding to individual pure proteins (*e.g.*, Martín et al., 2013; Shin et al., 2010), the technique has also been used to identify specific bands among a multitude of bacterial proteins such as in the work of Joshi et al. (2009), who validated their *Salmonella enterica* aptamers by Western blotting or Bruno et al. (2016a) who validated their *Rickettsia typhi* aptamer by Western blotting of whole cell lysate. Herein, we present similar verification data by aptamer-based Western blotting for our general and pathogenic *E. coli*-reactive aptamers and aptamers developed against Shiga-like toxin 2 which is also called Verotoxin 2 (MacLeod and Gyles, 1990). Although, there are at least 70 different serotypes of *E. coli* known to cause disease in humans (Bosilevac and Koohmaraie, 2012), the *E. coli* serotypes chosen for our aptamer Western blot anal-

yses focused on the pathogenic O157: H7 serotype and the "Big 6" non-O157 Shiga toxin-producing *E. coli* (STEC; *i.e.*, O26, O45, O103, O111, O121 and O145) now officially recognized as testing targets by the United States Department of Agriculture's Food Safety Inspection Service (USDA FSIS). In addition, we included several related Gram negative species (*Salmonella enterica*, *Shigella dysenteriae*, and *Yersinia enterocolitica*) and nonrelated Gram positive species (nonpathogenic *Bacillus anthracis* Sterne strain and *Listeria monocytogenes*) for comparison in our aptamer Western blots.

Materials and Methods

Aptamers, bacteria and Shiga-like toxins (SLTs)

The aptamers designated EcO 3R and Eco 4F were developed against whole live *E. coli* ATCC 8739 as previously described by Bruno et al. (2010). These two aptamers have been validated in competitive fluorescence resonance energy transfer (FRET) assays (Bruno et al., 2010), lateral flow assays (Bruno, 2014), electrochemical assays (Queirós et al., 2013) and evanescent wave assays (Queirós et al., 2014). The EcO 3R and 4F sequences were lengthened to 100 bases with poly T spacers on the 5' ends and renamed poly T-EcO 3R and poly T-EcO 4F as shown below primarily for use in enhanced lateral flow assays (Bruno and Richarte, 2016b). However, these lengthened aptamers were also used for aptamer-based Western blotting, because the 27 or 28 base poly T spacers were thought to possibly aid detection on nitrocellulose membranes. All aptamers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) with a 5'-biotin linker as shown below:

Poly T-EcO 3R: 5'-Biotin-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTATCCGTCACACCTGCTCTGTCTGCGAGCGGGGCGCGGGCCCGGCGGGGGATGCGTGGTGTTGGCTCCCGTAT-3'

Poly T-EcO 4F: 5'-Biotin-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTATACGGGAGCCAACACCATAATATGCCGTAAGGAGAGGCCTGTTGGGAGCGCCGTAGAGCAGGTGTGACGGAT-3'

The remaining intimin and SLT aptamer DNA sequences cannot be divulged here because they are patent-pending and may have significant commercial value. A description of aptamer development for the intimin and SLT aptamers can be found in Bruno and Richarte (2016b). Recombinant SLT-1 and SLT-2 B subunits were purchased from Prospec-Tany Techno-Gene Ltd. (Rehovot, Israel).

Most bacteria were obtained from American Type Culture Collection (ATCC, Manassas, VA). In particular, the bacterial species were: *Escherichia coli* ATCC No. 8739 as well as *E. coli* serotypes O26, O45, O103, O111, O121, O145, O157:H7, *Salmonella enterica* serovar Typhimurium ATCC No. 13311, *Shigella dysenteriae*, and *Yersinia enterocolitica*, and *Listeria monocytogenes* ATCC No. 19115. *Bacillus anthracis* nonpathogenic Sterne strain was cultured from a live nonencapsulated and commercially available cattle vaccine (Colorado Serum Co., Denver, CO). All bacterial species were cultured on blood agar plates at 35°C overnight. Bacteria were washed from the blood agar plates in 2 ml of PBS and equalized to an absorbance reading of approximately 1.0 at 600 nm in a spectrophotometer. Bacteria were then pelleted by centrifugation at 14,000 x G for 5 min and the supernatant fluid was removed by gentle suction. Bacterial pellets were then processed for electrophoresis as described in succeeding paragraphs.

Sample Preparation, Polyacrylamide Gel Electrophoresis (PAGE) and Aptamer "Western" Blotting

Bacterial pellet samples (75 µl) were added to 25 µl of 4X Bolt® SDS-PAGE loading buffer containing beta-mercaptoethanol, mixed for 1 min and boiled for 5 minutes at 100°C. Samples were then centrifuged at 14,000 X G for 5 min and 30 µL samples of supernatant fluid were loaded into the wells of 12% Bolt® Bis-Tris Plus polyacrylamide gels. Gels were also loaded with either ColorBurst™ (8 kD – 220 kD markers) from Sigma-Aldrich (Cat. No. C1992) or Thermo-Fisher EZ-Run™ markers (10 kD -170 kD with an orange reference band at 72 kD). Gels were electrophoresed at 125V for approximately 1 h until the dye front reached the bottom of the gel. Some gels were Coomassie blue stained using 50 ml of Simply Blue™ (Invitrogen, Thermo-Fisher Corp.) for 1 h followed by destaining in 100 ml of deionized water for 1 h with gentle mixing.

Gels intended for aptamer Western blotting were overlaid with a pre-wetted nitrocellulose membrane in Tris transfer buffer containing 20% methanol and

bands were transferred at 4 mA overnight in a cold room. Membranes were blocked with 10 ml of SuperBlock® (Thermo Scientific) for 1 h with gentle mixing followed by addition of 5 µl of 100 µM DNA aptamers having 5'-biotin labels in SuperBlock® with an additional 1 h of gentle mixing at RT. Membranes were washed five times for 5 min per wash in 10 ml of Tris buffered saline plus 0.1% Tween 20 (TBST). Next, a 1:10,0000 dilution of Streptavidin-Alkaline Phosphatase (Sav-AP, ~ 2 mg/ml stock) in 10 ml of TBST was added and the membrane was incubated for 1h at RT with gentle mixing. Membranes were washed an additional five times for 5 min per wash in 10 ml of TBST and 3 times for 5 min per wash in 10 ml of TBS. Membranes were then transferred to 10 ml of AP substrate (Immunostar-AP®, BioRad Laboratories) and incubated 5 min at RT. Wet membranes were placed in an X-ray film cassette and developed for 10 sec or 1 min as indicated in the figures or figure legends.

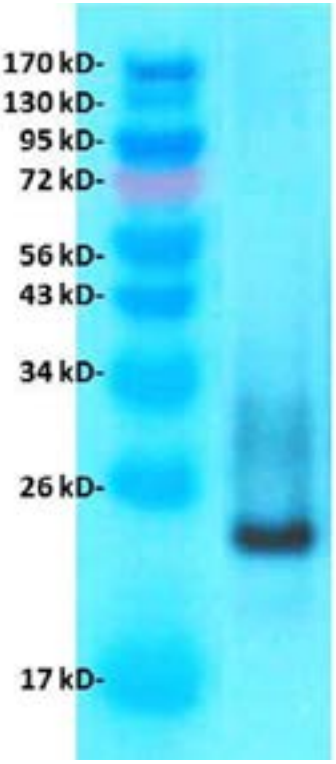

Figure 1: *Aptamer Western blot for Shiga-like toxin 2 (SLT-2) from a 12% nonreducing SDS polyacrylamide gel showing a dominant band at ~ 22 kD which correlates well with the reported molecular weight from the manufacturer of the SLT-2*

Results

Figure 1 presents results of an aptamer blot from a nonreducing SDS polyacrylamide gel in which SLT-2 was clearly detected below the 26 kD marker. MacLeod and Gyles (1990) defined bands at 7.5 kD, 27.5 kD and 33 kD for SLT-2 which does not match well with the results observed in Figure 1. However, the recombinant protein manufacturer of the SLT-2 (Prospec-Tany TechnoGene Ltd.) reported a molecular weight of 22 kD for its product which is consistent with the strongly detected band's weight in Figure 1. The 22 kD weight may be the result of multiple subunits remaining combined in the nonreducing SDS gel plus the addition of the 6X histidine tag added by the manufacturer. The aptamer used to detect SLT-2 did not detect SLT-1 (data not shown and DNA sequence not divulged due to its potentially high commercial market value), but this aptamer has detected Shiga toxin 1 in lateral flow test strips developed and tested by Bruno and Richarte (2016b), suggesting a common epitope between these toxins which is detected by the aptamer.

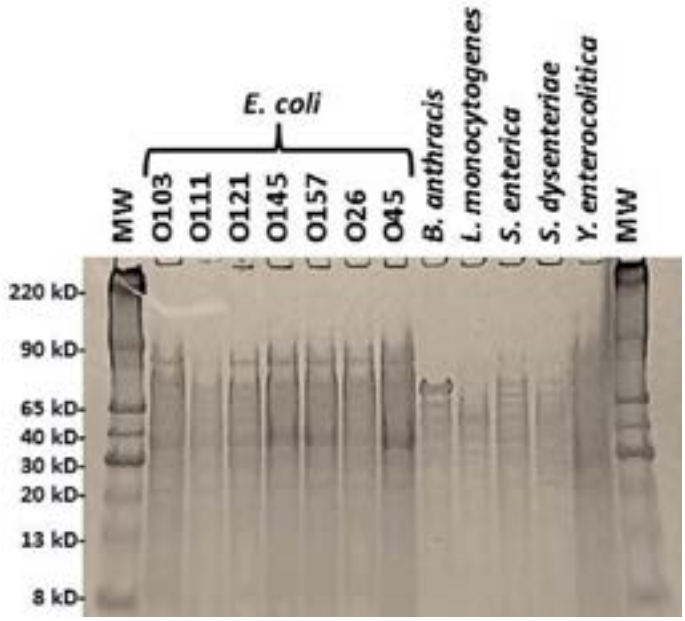

Figure 2: *Coomassie blue-stained 12% reducing SDS-polyacrylamide gel electrophoresis (PAGE) of the E. coli serotypes and other bacterial lysates used in the investigation. The molecular weight (MW) standards are shown in the far left lane*

Figure 2 shows results of a Coomassie blue-stained 12% reducing SDS polyacrylamide gel in which whole cell bacterial lysates were boiled in SDS loading buffer with beta-mercaptoethanol to liberate protein subunits from larger complexes. This gel illustrates the large number of proteins in each lysate of the "Big 6" and O157 serotypes of *E. coli* as well as several related Gram negative and unrelated Gram positive bacterial species. While these lysate samples represent all available surface and cytoplasmic proteins, it is noteworthy that at least 150 outer membrane proteins (OMPs) are known to exist on the surface of *E. coli* (Molloy et al., 2000) against which Bruno et al. (2010) developed

aptamers used in Figure 3 and 4 and with which Querós et al. (2013) and (2014) developed two successful biosensors for detection of general *E. coli*.

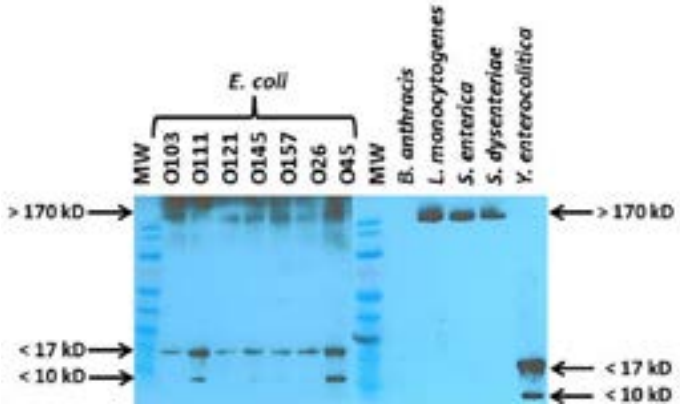

Figure 3: *Western blot using the 100 base poly T-EcO 3R-biotinylated aptamer. Bands at ~ 17 kD may represent detection of OmpX*

Figure 3 shows results of an aptamer blot with the extended 100 base poly T-EcO 3R aptamer generated against *E. coli* 8739 (Bruno et al., 2010) which appeared to detect more bands among the *E. coli* serotypes (left) than the related and unrelated bacterial species on the right. There were some shared high molecular weight bands across all species except *B. anthracis* and *Y. enterocolitica*. Indeed, the Gram positive *B. anthracis* showed no reactivity with the poly T-EcO 3R aptamer at all while another Gram positive, *L. monocytogenes* did share a high molecular weight band. In the lower molecular region, common bands at ~ 17 kD and 10 kD were noted among most of the pathogenic *E. coli* serotypes. The authors speculate that the 17 kD band may represent detection of OmpX which has a reported molecular weight of 17-19 kD (Vogt and Schulz, 1999) and is common among the Enterobacteriacae. This observation could explain detection of the 17 kD band in *Y. enterocolitica*, but the absence of a 17 kD band for *S. enterica* and *S. dysenteriae* is not explicable, if the band represents OmpX.

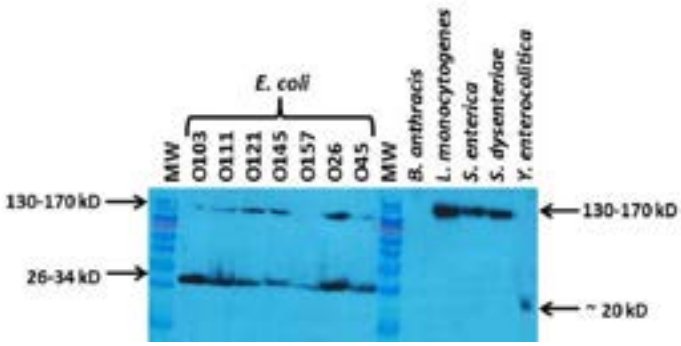

Figure 4: *Western blot using the 100 base poly T-EcO 4F-biotinylated aptamer. Bands at ~ 34 kD may represent detection of OmpA*

Figure 4 demonstrates detection of a different subset of bands by the 100 base poly T-EcO 4F aptamer, generated against *E. coli* 8739 (Bruno et al., 2010), versus those bands detected by poly T-EcO 3R in Figure 3. Poly T-EcO 4F appears to detect some high molecular weight bands across many of the bacterial species except *E. coli* O157, *B. anthracis* and *Y. enterocolitica*. Interestingly, common bands are detected between 26 kD and 34 kD only for the *E. coli* serotypes (left), but not for any of the other species. Özkanca and Flint (2002) report that OmpA of *E. coli* has a molecular weight of 34 kD, while OmpF weighs at 35 kD and OmpC weighs 36 kD. Thus, the detected bands most likely represent OmpA, but given inaccuracies in molecular weight determination by PAGE and the close proximity in weight of OmpC and OmpF, it is possible that the detected bands at ~ 34 kD represent another Omp besides OmpA. An odd, but noteworthy band at ~ 20 kD was also detected in *Y. enterocolitica* by the poly T-EcO 4F aptamer in Figure 4.

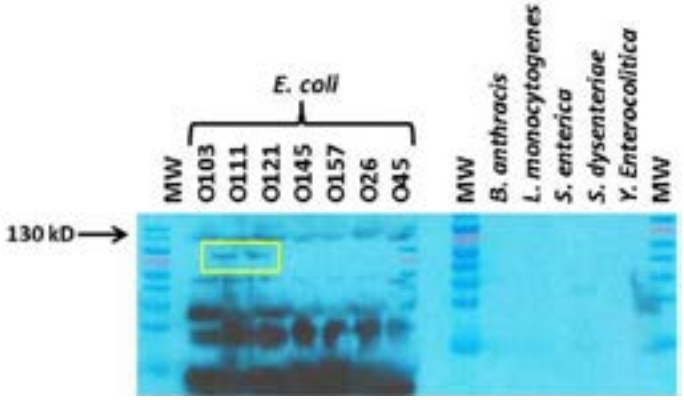

Figure 5: *Western blot using an intimin-alpha-biotinylated aptamer. Yellow boxed bands may represent detection of intimin-alpha*

Figures 5 and 6 illustrate similar detection patterns for two different intimin alpha and gamma aptamers (DNA sequences not divulged due to their potentially high commercial value) which detect the attaching and effacing (*eae*) gene products of pathogenic *E. coli* (Adu-Bobie et al., 1998; Gansheroff et al., 1999). The intimin family of virulence proteins is reported to weigh between 80-97 kD with 94 kD being the most commonly cited weight for intimins (Jerse and Kaper, 1991; McKee et al., 1995). Those molecular weights are consistent with some bands detected in the yellow boxed regions of Figure 5 and 6 (left sides) for the pathogenic *E. coli* serotypes. These same intimin aptamers were also used in lateral flow assays to detect pathogenic *E. coli* (Bruno and Richarte, 2016b). Bands detected on the left side of Figure 5 and 6 for the pathogenic *E. coli* serotypes were not detected

among the related and unrelated bacterial samples on the right side of Figure 5 and 6 unless the X-ray film exposure times were increased from 10 sec to 1 min (Figure 6, bottom vs. top). However, even with the increased exposure time in Figure 6 (top), only a few common lower molecular weight bands appeared to be shared between the *E. coli* samples and those samples from *S. enterica* and *Y. enterocolitica*, which attests to the high specificity of these intimin aptamers. Because intimins are known to weigh 80-97 kD, the lower molecular weight bands they detect may represent detection of intimin fragments. It is actually common to observe multiple bands of molecular weights far below 94-97 kD in classic Western blots especially when using polyclonal antisera (Adu-Bobie et al., 1998; Gansheroff et al., 1999).

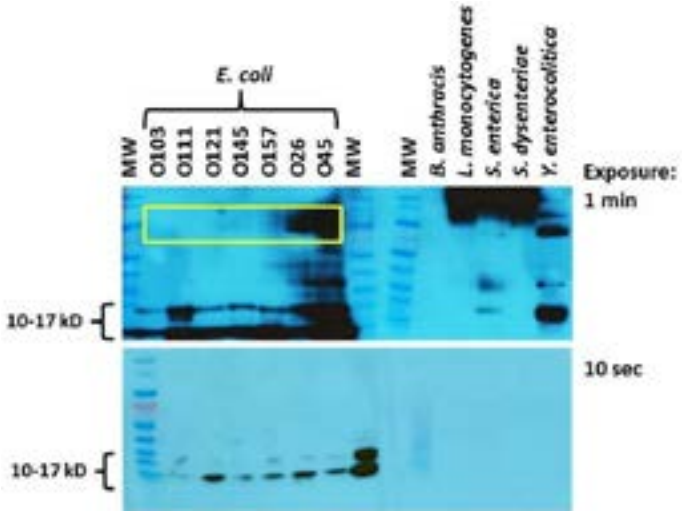

Figure 6: *Western blots using an intimin-gamma-biotinylated aptamer with exposure times of 10 sec (bottom) and 1 min (top). Yellow boxed bands may represent detection of intimin-gamma*

Discussions

The presently reported work illustrates the specificity of DNA aptamers which bind Shiga-like toxin 2 or Verotoxin 2 and surface virulence factors of *E. coli*. In general, the aptamers preferred to bind electrophoretically separated bands emanating from the pathogenic "Big 6" and O157 *E. coli* serotypes with limited or no binding to bands from related Gram negative or unrelated Gram positive bacterial lysates. In a number of cases, the approximate molecular weights of the detected bands from *E. coli* enables investigator to at least narrow the field of possible proteins being detected and to hypothesize about the identity of some of the detected bands which could eventually be validated by mass spectrometry of excised and eluted gel bands.

However, even without mass spectral analyses, there appeared to be good agreement with the molecular weights of the 22 kD recombinant SLT-2 (Figure 1) and some Omps (Figure 3 and 4), especially OmpA (~ 34 kD; Özkanca and Flint, 2002) and OmpX (Vogt and Schulz, 1999), and the intimins (80-97 kD; Jerse and Kaper, 1991; McKee et al., 1995) in Figure 5 and 6. Since the Omps and intimins are host cell attachment and invasion virulence factors, it is interesting to speculate too that some of the aptamers utilized in this study might also prevent or inhibit attachment and invasion of pathogenic *E. coli* in the same way that similar antibodies have inhibited or prevented attachment and invasion of host cells *in vitro* (Adu-Bobie et al., 1998; Gansheroff et al., 1999).

Finally, Figure 6 illustrates the need to optimize exposure times for Western aptamer blots conducted according to our method so that background signals do not dominate detection and the less abundant bands are given an opportunity to be detected. Molloy et al. (2000) reported that there are ~ 100,000 copies of OmpA on the surface of each *E. coli* cell making it the dominant target in whole cell DNA aptamer development and possibly explaining the ease with which the ~ 34 kD bands are detected with poly T-EcO 4F across the *E. coli* serotypes on the left side of Figure 4. However, less abundantly expressed proteins may require somewhat longer exposure times to become visible on blots (Figure 6 top versus bottom).

Conclusions

The preceding study clearly demonstrates that DNA aptamers developed against bacterial toxins and surface virulence factors can be used in Western blot formats to detect these targets with high specificity even in complex cell lysates. The ability to validate aptamer binding to targets of the correct putative molecular weight in complex environments is an advantage over simpler ELISA-like, dot blot, or lateral flow test formats which yield no information on the molecular weights of detected target molecules. In the present work, some of the molecular weight information from detected bands correlated well with known molecular weights for the cognate aptamers. Detected bands with unexpected molecular weights may represent detection of the same epitope in subunits or fragments of the targets or in other proteins or molecules which happen to share a common epitope with the target and such information could be potentially quite useful

in other Western blotting systems.

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Conflicts of Interest

The authors declare that no conflicts of interest exist.

Authors' Contributions

JGB conceived and directed the project, conducted some electrophoresis and gel staining and wrote the draft manuscript. JCS performed much of the electrophoresis and performed all of the aptamer Western blots.

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