# Development and Application of Latex Agglutination Assays for Non-O157 Shiga Toxin-Producing *Escherichia coli*

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## ABSTRACT

Latex agglutination assays to detect the United States (US) top six non-O157 Shiga toxin-producing *Escherichia coli* (STEC) serogroups: O26, O45, O103, O111, O121 and O145 were previously developed in our laboratory. In this research, new latex-antibody reagents were developed with rabbit polyclonal antibodies prepared using inactivated bacterial cells as the immunogen. The avidity and specificity of the antisera were evaluated with ELISA assays. The antisera were affinity purified and covalently linked to polystyrene latex microbeads. In the latex assay, buffer was applied to the glass slides followed by transfer of *E. coli* colonies and dispersion in the buffer; instant agglutination indicated positive strains. Thirty-nine STEC strains showed positive results while the non-STEC *E. coli* yielded negative results. These reagents are being used for the identification of presumptive positive non-O157:H7 STEC colonies cultured on agar media. The latex reagents will be used by the USDA Food Safety Inspection Service for implementation of the USDA regulation for testing the top six non-O157 STEC.

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## INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are foodborne pathogens responsible for numerous outbreaks of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). The STECs produce virulence factors, including the Shiga toxins (Stx1 and Stx2) with roles in pathogenicity (*1-3*). *E. coli* O157:H7 is the most commonly recognized STEC in the United States (US); however, many other STEC serogroups isolated from animals and food have caused human illness. US Centers for Disease Control and Prevention (CDC) data show that non-O157 STEC infections are more common than illnesses caused by *E. coli* O157:H7 (*4*). In the period of 1983-2002, CDC's reference laboratory examined non-O157 isolates provided from the state public health laboratories, and results showed that six serogroups, O26, O45, O103, O111, O121 and O145 of the 61 serogroups identified accounted for 71% of the isolates recovered in the United States (*5*).

In 1994, the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) declared *E. coli* O157:H7 as an adulterant in beef products, and on September 2011, the US top six *E. coli* serogroups: O26, O45, O103, O111, O121, and O145 were declared as adulterants in raw, non-intact beef products or components in the same manner as *E. coli* O157:H7. In June 4, 2012, the zero tolerance policy was implemented on manufacturing beef trim. In a prevention-based food safety approach, FSIS declared these STECs as adulterants to protect consumers against a potential public health emergency. The USDA-FSIS performs a verification sampling program to test for these pathogens in samples collected from federally inspected establishments and retail stores. The testing protocol for the non-O157 STEC utilized and mandated by the USDA-FSIS is described in the USDA-FSIS Microbiology Laboratory Guidebook (MLG) Chapter 5B.02 (6).

Most diagnostic methods for STEC have been designed to detect only serogroup O157 in food and water. With the recent classification by USDA-FSIS of serotypes O26, O45, O103, O111, O121, and O145 as adulterants in beef products, there is a need to develop a simple and rapid method to detect and confirm the non-

O157 STEC serogroups after screening beef enrichments by PCR methods. The latex agglutination assay was developed (7) and has been used by USDA-FSIS (6). The successful development of these tests and reagents at the USDA Agricultural Research Service (ARS), allowed USDA-FSIS to declare the top six STECs as adulterants in 'manufacturing beef trim' with an implementation date for regulatory testing of June 4, 2012.

Methods for detection and isolation of important non-O157 STEC serogroups are needed to determine their prevalence in food and their incidence in causing human infections. Culture- and PCR-based methods have been used to detect and isolate non-O157 STEC in beef (6, *8-10*); however, isolation can be problematic due to the lack of suitable selective/differential agar media to distinguish the genetically diverse non-O157serogroups. After enrichment and plating, presumptive positive STEC colonies were often selected from plates and retested by the PCR and/or other methods to confirm their identity, and this process is costly and time consuming. Therefore, an alternative approach to identify the colonies by latex agglutination tests was developed (6, 7).

Latex agglutination reagents for *E. coli* O157:H7 using O157-specific antisera were commercially available; however, there was a lack of such reagents for the top six non-O157 STEC serogroups that are prevalent in the US and are of interest to USDA-FSIS. The latex agglutination reagents specific for the top six non-O157 STEC serogroups were prepared using rabbit polyclonal antisera. The polyclonal immunoglobulin (IgG) fractions were isolated through Protein A/G affinity columns and covalently immobilized to polystyrene latex particles to provide more uniformly coated beads. Agglutination assays were performed for the identification of the top six non-O157 STEC strains in USDA-ARS and USDA-FSIS laboratories (7). The objective of the USDA-ARS and Abraxis joint research was to develop antibodies leading to the commercialization of test kits for the detection and identification of the top six non-O157 STEC serogroups: O26, O45, O103, O104, O111, O145.

## MATERIALS AND METHODS

**Reagents and materials**. Polystyrene carboxylated latex micro-particles were obtained from Bangs Laboratories (Fisher, IN, USA); N–hydroxysuccinimide (NHS), ethyl-N'-(dimethylaminopropyl) carbodiimide (EDC), Hepes (free acid), ethylenediamine tetraacetic acid (EDTA), sodium phosphate, sodium azide, glycine, citric acid, sodium citrate, glycine, *tris* (hydroxymethyl) aminomethane (TRIS), Tween 20 and Brij surfactants, ethanolamine, and protease-free bovine serum albumin (BSA) were obtained from Sigma (St. Louis, Missouri, USA). Protein A/G Ultralink columns (3 ml) was from Pierce/Thermo Scientific (Rockford, Illinois, USA). Sonicator 3000 was from Misonix (Farmingdale, New York, USA).

**Preparation and inactivation of non-O157 STEC cultures for Abraxis.** Three strains of each of the top-six non-O157 Shiga toxin-producing *E. coli* serogroups (O26, O45, O103, O111, O121, and O145) were streaked onto 18 tryptic soy agar plates. One colony from the three plates of different strains from a single serogroup was picked and inoculated into 1.5 ml of tryptic soy broth and grown at 37°C for about 18 h. Approximately 0.5 ml of the cultured cells was lysed at 99°C for DNA extraction. The DNA was tested by the polymerase chain reaction (PCR) for the *stx*1, *stx*2, and *eae* virulence genes and for an O serogroup-specific gene to verify the serogroup. The remaining portion of the cultures (ca. 1 ml) was stored at -80°C in 20% glycerol.

The frozen cultures were plated onto TSB agar, and one colony of each of the three strains of the same serogroup was inoculated into the same flask with 2 liters of TSB, which was then incubated at 37°C for 18 h. The 2-liter culture for each serogroup was centrifuged at 10,000 rpm (16,300 x g) for 15 min at 10°C. The supernatant was discarded, and the pellet was washed twice with 100 ml of PBS, pH 7.4 and centrifuged at 10,000 rpm for 10 min at 10°C. The supernatant was discarded, and the pellets were re-suspended in 8-10 ml of PBS, pH 7.4 in centrifuge bottles for each serogroup. The bottles were washed with an additional 15 ml of PBS, and this was added to the bacterial suspension yielding a total cell suspension of ca. 65 ml for each serogroup. The concentration of the cells was ca. $10^9$ CFU/ml determined by plating onto TSA. The cultures were irradiated to inactivate the cells and the irradiated cells (100 µl of the suspensions) were plated onto TSA agar and incubated overnight at 37°C to determine if all of the cells were inactivated.

**Antibody production and screening**

New Zealand rabbits were immunized with the inactivated bacterial cells followed by testing of their serum to monitor the production of antibodies. The antisera were produced in a commercial facility. The antibody production was screened with an indirect ELISA utilizing microtiter plates with the specific bacterial serogroup

attached covalently to the wells, followed by the addition of test rabbit antisera. A second antibody (anti-rabbit) labeled with the enzyme horseradish peroxidase (HRP) was added to determine binding of the specific antibodies to the STEC serogroup.

**Immobilization of anti-*E.coli* IgG to latex particles:** The rabbit antisera were purified according to the procedure described in Medina et al (7), and the covalent attachment also followed the procedures as described. Briefly, the affinity purified anti-*E. coli* immunoglobulins were solubilized in 10 mM sodium acetate buffer, pH 4.5 at 0.3 mg/mL. The carboxy latex particles were sonicated, 0.2 mL transferred into 12 ml conical polypropylene centrifuge tubes, and were activated with N–hydroxysuccinimide (NHS, 11.5 mg/mL) and ethyl-N'-(dimethylaminopropyl) carbodiimide (EDC, 75 mg/mL). The EDC/NHS mixture (50 µl) was added to the latex particles and mixed for 30 min at room temperature. Anti-STEC IgG was added to each tube and mixed for 60 min at room temperature. The derivatized latex- anti-*E. coli* IgG mixtures were separated by centrifugation, and the supernatants were analyzed for protein content and compared with the original pre-immobilized concentrations (7). The derivatized latex particles were resuspended 10 mM Hepes buffered saline wash buffer (pH 7) and sonicated. The unreacted active carboxyls were neutralized with 1 M ethanolamine and further mixed for 45 min at room temperature. The derivatized latex-IgG was separated by centrifugation, and the pellet was reconstituted in 2 ml HBS storage buffer, pH 7, containing 0.015% Brij surfactant and 0.1 % BSA. The latex derivatives were sonicated and used in subsequent agglutination assays.

**Latex agglutination assay reagents**: A. TBST assay buffer: 50 mM TRIS, pH 8 (1.51 g); 0.1% BSA (protease free) (0.25g); 5 mM EDTA (0..4 g); 0.2% sodium azide (0.5 g) in 250 mL, adjusted to pH 8 + 0.5% Tween 20 (0.5 g/100 ml). B. Latex~antibody conjugate: latex~IgG sonicated before use. The latex anti-STEC preparation was diluted 1:1 with TBST buffer resulting to 50% latex~IgG concentration (0.5% solids).

**Bacteria:** Heat inactivated *E. coli* strains used for antibody production were tested with the latex-anti-STEC reagents. One mL of a suspension of irradiated cells were diluted with 9 mL of sterile water and heated for 2 hr in boiling water. Heated cells were cooled down and divided into 2 portions. One portion was treated with 0.5 mL formalin per 5 mL per serogroup cell suspension. To both heated and formalin treated cells, 1 mL of 10% NaCl (1% final concentration) and 100 µL of Thimerosal (0.5% final concentration) were added. *E. coli* K12 was also used as a negative control. STEC *E. coli* strains were also grown overnight (16 -18 hr) for testing of the latex agglutination reagents and kits.

**Assay Procedure:** Three circles were drawn on the glass slides. The latex-STEC IgG reagents were vortexed gently and 20 µL of TBST were transferred to each circle. Colonies (about 2 – 3) of the *E. coli* strains were transferred to the circles and mixed with TBST using sterilized toothpicks. Latex-antibody conjugate (20 µL) in TBST was transferred to the circles, followed by mixing with the cells using the toothpicks and further mixed by rotating the slides. Agglutination was observed in 3–10 sec. Negative results showed latex dispersion, and positive results showed agglutination/flocculation or network formation.

## RESULTS AND DISCUSSION

**Antibody production and latex-IgG preparation.** The ELISA assays indicated the antibody titers and cross-reactions of the rabbit antiserum. The antiserum from the animals producing the highest titers and least cross-reactions with non-target STEC strains were selected for the preparation of the latex reagents as described in previous paragraphs.

**Latex agglutination assays (LAT).** The latex agglutination reagents and procedures were tested at the USDA-ARS-ERRC (Wyndmoor, PA). The select antisera and the white latex reagents showed specific reactions against the 39 strains and negative results with *E. coli* K12 as shown in Table 1. Anti-O111 latex reagent had a very slow agglutinating reaction with O111:NM SJ15 strain.

Table 1. Latex agglutination assay of anti-STEC latex (white) on 40 *E. coli* cultures tested at ERRC-ARS-USDA

| Number | Serogroup/Strains | Anti-O26 | Anti-O103 | Anti-O111 | Anti-O121 | Anti-O145 |
|---|---|---|---|---|---|---|
| 1 | K12 ATCC 29425 | — | — | — | — | — |
| 2 | O26:NM TB352 | +++, +++ | — | — | — | |
| 3 | O26:H11 05-6544 | +++, +++ | — | — | — | |
| 4 | O26:H11 SJ1 | +++, +++ | — | — | — | |
| 5 | O26:H11 SJ2 | +++, +++ | — | — | — | |
| 6 | O26:H11 SJ3 | +++, +++ | — | — | — | |
| 7 | O26:TW 00971 | +++, +++ | — | — | — | |
| 8 | O45:H210.2360 | — | — | — | — | — |
| 9 | O45:H2 96-3285 | — | — | — | — | — |
| 10 | O45:H2 05-6545 | — | — | — | — | — |
| 11 | O45:H2 SJ7 | — | — | — | — | — |
| 12 | O45:H2 SJ8 | — | — | — | — | — |
| 13 | O45:H2 SJ9 | — | — | — | — | — |
| 14 | O103 H11 SJ12 | — | +++, +++ | — | — | — |
| 15 | O103:H11-04-3973 | — | +++, +++ | — | — | — |
| 16 | O103:H2 04-2446 | — | +++, +++ | — | — | — |
| 17 | O103:H2 99-2076 | — | +++, +++ | — | — | — |
| 18 | O103:H2 SJ10 | — | +++, +++ | — | — | — |
| 19 | O103:H25 SJ11 | — | +++, +++ | — | — | — |
| 20 | O103:H25 03-244 | — | +++, +++ | — | — | — |
| 21 | O103:TW 04162 | — | +++, +++ | — | — | — |
| 22 | O103:H6 04162 | — | +++, +++ | — | — | — |
| 23 | O111:H8 01387 | — | — | +++, +++ | — | — |
| 24 | O111:H8 01387 | — | — | +++, +++ | — | — |
| 25 | O111:H8 SJ14 | — | — | +++, +++ | — | — |
| 26 | O111:NM 00-4748 | — | — | +++, +++ | — | — |
| 27 | O111:NM 98-8338 | — | — | +++, +++ | — | — |
| 28 | O111:NM SJ13 | — | — | +++, +++ | — | — |
| 29 | O111:NM SJ15 | — | — | +, +<br>very slow | — | — |
| 30 | O121:H19 SJ18 | — | — | — | +++, +++ | — |
| 31 | O121:H19 03-2832 | — | — | — | +++, +++ | — |
| 32 | O121:H19 SJ16 | — | — | — | +++, +++ | — |
| 33 | O121:NM 03-4064 | — | — | — | +++, +++ | — |
| 34 | O121:H19 08023 | — | — | — | +++, +++ | — |
| 35 | O145:NM SJ23 | — | — | — | — | +++, +++ |
| 36 | O145:NM 8235 | — | — | — | — | +++, +++ |
| 37 | O145:H8 07865 | — | — | — | — | +++, +++ |
| 38 | O145:NM 03-4699 | — | — | — | — | +++, +++ |
| 39 | O145:NM 83-75 | — | — | — | — | +++, +++ |
| 40 | O145:H-94-0941 | — | — | — | — | +++, +++ |

Testing results of the heated cells and formalin stabilized cells are shown in Tables 2A and 2B where red latex particles were used for the preparation of the latex – anti-STEC. "Heated only" cells had smaller and finer aggregates than the heated and formalin treated cells. Treatment of the bacterial cells with formalin resulted in inter-cellular complex of the STEC cells resulting to larger agglutination or flocculation with the latex-antibody

reagents. Colored latex reagents such as the red latex in Tables 2A and 2B were also prepared to improve the visual observation of the agglutination of bacterial cells and latex-IgG reagents compared with the white latex. The mixed strains used for antibody production was heated and treated with formalin were used for testing the red latex-IgG reagents. In Table 2A, latex-anti-O103 and latex-anti-O121 showed very fine aggregates, and latex-anti-O121 showed fine and slower agglutination (Table 2B). In Table 3, dark pink latex-IgG were prepared for the Abraxis kits and were tested with single strains from each of the six serogroups. The kit's positive and negative controls showed accurate results. The six serogroups yielded strong agglutination results (+++) with the commercial kits.

Table 2A. Latex agglutination of the mixed STEC strains heated at 100°C for 2 hr using red latex reagents immobilized with anti-STEC IgG.

| Serogroup | Anti-O26 | Anti-O45 | Anti-O103 | Anti-O111 | Anti-O121 | Anti-O145 |
|---|---|---|---|---|---|---|
| **K 12** | – | | | | | |
| **O26** | ++ | | | | | |
| **O45** | | ++ | | | | |
| **O103** | | | + | | | |
| **O111** | | | | ++ | | |
| **O121** | | | | | + | |
| **O145** | | | | | | ++ |

Table 2B. Latex agglutination of the mixed STEC strains heated at 100°C for 2 hr and stabilized with formalin using red latex reagents immobilized with anti-STEC IgG.

| Serogroup | Anti-O26 | Anti-O45 | Anti-O103 | Anti-O111 | Anti-O121 | Anti-O145 |
|---|---|---|---|---|---|---|
| **K 12** | – | | | | | |
| **O26** | +++ | | | | | |
| **O45** | | +++ | | | | |
| **O103** | | | ++ | | | |
| **O111** | | | | +++ | | |
| **O121** | | | | | + | |
| **O145** | | | | | | +++ |

Table 3. Latex agglutination test of cultured cells using Abraxis kits.

| **Strains** | **Anti-O26** | **Anti-O45** | **Anti-O103** | **Anti-O111** | **Anti-O121** | **Anti-O145** |
|---|---|---|---|---|---|---|
| **Negative control** | - | - | - | - | - | - |
| **Positive control** | ++ | ++ | ++ | ++ | ++ | ++ |
| **K12 ATCC 29425** | - | - | - | - | - | - |
| **O26:TW 00971** | +++ | | | | | |
| **O45:H2 SJ8** | | +++ | | | | |
| **O103:H2 04-2446** | | | +++ | | | |
| **O111:H8 SJ14** | | | | +++ | | |
| **O111:NM SJ15** | | | | +++ | | |
| **O121:H19 SJ18** | | | | | +++ | |
| **O145:NM 8235** | | | | | | +++ |

## CONCLUSIONS

Simple and rapid latex agglutination assays developed at the USDA-ARS-ERRC for the detection of non-O157 Shiga toxin-producing *E. coli* belonging to serogroups O26, O45, O103, O111, O121, O145 were successfully adapted in a commercial facility, Abraxis. The latex agglutination assay reagents were prepared

with ease, and results indicate reliable recognition/identification of target STECs. The latex reagents are being used by the USDA Food Safety Inspection Service for the identification of presumptive positive non-O157 STEC colonies picked from various types of agar media after PCR beef enrichment screening assays and plating and before PCR confirmatory assays. This method is rapid and less costly than testing numerous colonies by PCR assays.

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