Characterization of biofilms produced by *Escherichia coli* O157 isolated from cattle hides

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**Abstract.** This study aimed to investigate possibility *E. coli* O157 from cattle hides to produced biofilms. We had 28 suspect primoisolates and 17 were confirmed to be *E. coli* O157. Biofilm production test showed that more than 50% of this isolates did not produce biofilm. From the other half of the isolates, 5 of them were weakly adherent, 3 were moderately adherent. Since *E. coli* O157 are one of the main foodborne hazards in meat processing industry and the discovery that some of them can produce moderately adherent biofilms, request necessity of strict implementation of HACCP procedures to prevent further expansion this pathogen.

1. **Introduction**

*Escherichia coli* is a Gram-negative rod-shaped enteric bacterium, and their species are commonly classified by their virulence properties, mechanisms of pathogenicity, clinical syndromes, and O and H serotypes [1]. Following this, *E. coli* O157:H7 is classified by its serotype, O157:H7, because it expresses the 157th somatic (O) antigen and the 7th flagellar (H) antigen[2]. *E. coli* O157:H7 produces Shiga toxins 1 and 2 (Stx1 and Stx 2) as important virulence factors. This bacterium is significant for public health, it causes disorders that are characterized as with hemorrhagic colitis, bloody diarrhea and hemolytic uremic syndrome (HUS) with renal tubular damage [3]. This pathogen has a very low infective dose and it is sufficient low than 50 organisms to cause disease [4]. The most common mode of transmission *E. coli* O157:H7 infection is through food and water, but it is also possible to transfer from person to person [5], but the most important source of this pathogen is the cattle since *E. coli* O157:H7 naturally colonizes the intestinal tract of about 1% of healthy cattle [6]. Beef carcasses can become contaminated by fecal during slaughtering and processing [7].

*E. coli* is one of many bacteria that can switch between planktonic form and biofilm form. Several reasons can explain the need for bacteria to create biofilm, in this way bacteria can avoid being washed away by water flow or, cells in biofilms are about 1000 times more resistant than their planktonic [8]. *E. coli* O157:H7 has shown the ability to attach, colonize, and form biofilms on a variety of surfaces [9]. Biofilms that are attached to food contact surfaces, such as stainless steel, polyvinyl chloride, and polyurethane. To create a biofilm, the bacteria must be close enough to the surface. At about 10–20 nm distance from the surface, the negative charges on the bacterial surface are repelled by negative charges on most environmental surfaces. This reflection transcends van der Waals forces, and thanks to the fimbiae and flagella provide mechanical attachment to the surface [10]. Biofilm formation can be described in three stages: attachment, maturation and dispersion. The attachment step is categorize as a two-stage process: initial reversible attachment and irreversible
attachment. The irreversible attached biofilm can tolerate stronger physical or chemical forces [11]. During the maturation process, more biofilm scaffolds, such as proteins, DNA, polysaccharides, etc. are secreted into the biofilm by the entrapped bacteria. After maturation, the dispersion step is followed, which is also very important for the biofilm life cycle. Biofilms disperse because of myriads of factors, such as lack of nutrients, intense competition, outgrown population and dispersal could occur in the whole or just a part of it. Biofilm consists of the structure of the Extracellular Polymeric Substance (EPS). Matrix of biofilms is composed of one or more of extracellular polysaccharides, DNA and proteins. This exopolysaccharides are synthesized extracellularly or intracellularly and secreted into the outside, they serve as scaffolds for other carbohydrates, proteins, nucleic acids and lipids to adhere. Some of the most carbohydrates are mannose, galactose and glucose, followed by N-acetyl-glucosamine, galacturonic acid, arabinose, rhamnose and xylose and most of them are not biofilm specific, but their production can increase as a result of a stress response, as colonic acid production in *Escherichia coli* [12].

Since *E. coli* O157:H7 is significant for public health and the most important source of the pathogen are beef carcaseses, the goal of this study was to detect the prevalence *E. coli* O157 of these samples to create biofilms.

### 2. Material and Methods

#### DNA Extraction and *rfbE* gene amplification

The 1.5 ml microcentrifuge tubes containing 1 mL of cell suspension (3.1 × 10^9 CFU/mL) were centrifuged for 10 min at 10,000× g. Supernatant was then discarded and DNA extracted from cell pellet using the DNeasy Blood and Tissue kit (Qiagen, Germany) following manufacturer's instructions for Gram-negative bacteria.

DNA concentration was measured using a Biophotometer spectrophotometer (Eppendorf, Germany) and its integrity checked by visualization on 1.2% agarose gels. Then, samples were stored at −20°C before analyses.

A qPCR assay targeting the *E. coli* O157:H7 *rfbE* gene was performed using *rfbE* forward (5'-TTT CAC ACT TAT TGG ATG GTCTCAA-3') and *rfbE* reverse primer (5'-CGA TGA GTT TAT CTG CAA GGT GAT -3'). The 20 µL volume reaction mixture contained 2×Brilliance SYBR Green qPCR buffer (Agilent, USA), 500 nm of each PCR primer, and 2 µL of DNA template. After a 3 min denaturation at 95°C, the qPCR mixtures were subjected to 40 cycles of amplification at 95°C for 10 sec, 60°C for 20 sec, using a qPCR thermal cycler (MX3005P, Agilent, USA).

#### Biofilm characterization

Biofilm formation on microtiter plate was performed following the methodology of Christensen’s et al., (1985), by inoculating the microtiter wells with bacterial strains [13]. The strains were grown aerobically in blood-heart-infusion (BHI) broth at 37°C for 24 hours. After incubation, the OD of the cultures was adjusted to 0.5 at 600nm. In 96 well-microtiter plates, 200µL of cell suspension diluted to 1:100 was added. BHI broth, *E. coli* O157 biofilm-positive and biofilm-negative strains were used as controls. The plates were incubated at 37°C for 24 hours. After incubation, the growth medium was discarded and microtiter plate wells were washed twice with 200 µL of 0.85 % NaCl. The plates were vigorously shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were fixed by subsequent drying in incubator at 60°C for 60 minutes.

The bound cells were stained with 200 µL of 0.1 % (v/v) crystal violet (CV) solution for 15 minutes at room temperature. Excess dye was removed by washing each well (3×) with 200 µL of 0.85 % NaCl. The quantification of attached cells was performed by adding 200 µL of 33% (v/v) glacial acetic acid as a CV solvent. O.D at 595 nm of dissolved CV was measured in a microtiter plate reader. Biofilm assays were performed in triplicate.

All strains were classified into the following categories: non-adherent, weakly, moderately, or strongly adherent, based upon the ODs of bacterial biofilms. We defined the cut-off OD for the microtiter-plate test as three standard deviations above the mean OD of the negative control.
3. Results and Discussion

Out of 28 tested suspect primoisolates (using mTSB broth with novobiocin and cefixime-tellurite sorbitol MacConkey agar), 17 were confirmed to be \textit{E. coli} O157 (60.7\%) Figure 1.

These samples were subsequently tested on capability for biofilm production.

\textbf{Figure 1.} Dissociation curves of O157 positive strains

Biofilm production test indicated that more than 50\% of the isolates did not produce biofilm (Figure 2). However, within the other half of the isolates, 5 of them were weakly adherent, 3 were moderately adherent, while none of the isolates exhibited particularly high capability for biofilm production. In his research [14], Dourou confirmed the ability of \textit{E. coli} O157 to form biofilms, and showed that the formation of biofilms can depend on a number of factors such as temperature or surface. Narisawa [15] came to research results that 2,1\% and 0,5\% \textit{E. coli} form biofilms, which is approximately equal to the prevalence that we found in our study. Al-Shabib [16] found that of the 10 tested strains, four strains formed strong biofilm while other formed moderate to week biofilm which is much higher prevalence than ours. In contrast, Biscola [17] has published the results in which 5 of 18 \textit{E. coli} O157 formed biofilms, which is very similar to our results.
4. Conclusion

*E. coli* O157 which produce moderately adherent biofilms on cattle hides are one of the main foodborne hazards in meat processing industry. For sure, these findings require necessity of strict implementation of HACCP procedures during exenteration and dehiding of cattle in order to decrease the number of it and prevent further dissemination of this pathogen.

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