In Situ Localization of Escherichia coli O157:H7 in Food by Confocal Scanning Laser Microscopy

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ABSTRACT

The aim of this study was to use confocal scanning laser microscopy to examine the in situ localization of Escherichia coli O157:H7 on beef (knuckle or brisket) and carrots and in semisoft cheese made from pasteurized milk. Using a combination of specific immunolabeling and dual-excitation confocal scanning laser microscopy, it was possible to clearly demonstrate the localization of E. coli O157:H7 within various food types. In carrots, bacteria were found mainly at cell junctions and in intracellular spaces up to 50 μm deep. In beef, bacteria were located primarily between muscle fibers and within connective tissue (at a depth of 25 μm), whereas in cheese the bacteria occurred singly or in small clumps of up to 10 cells and were observed within the protein matrix of the cheese. These results revealed how E. coli O157:H7 can penetrate beef and carrot surfaces where it is protected from decontamination processes.

Escherichia coli O157:H7 is among the foodborne pathogens that are of most concern to the food industry. Its low infective dose of <10 CFU ingested (3, 24) and high risk of complications such as hemolytic uremic syndrome (14) or death are cause for concern. An integral part of the cycle of infection of E. coli O157:H7 is retention on food surfaces by attachment to or entrapment in the food. Typically, bacteria are removed from food samples before analysis; therefore, conventional microbiological methods such as plate counts do not allow a study of the localization of bacteria in food. Although analytical methods can provide data on the overall number of cells retained, no information can be obtained on the depth of penetration or the attachment or entrapment of the bacterial cells. This information is important for understanding the mechanisms by which pathogens resist decontamination processes and removal by washing (20).

Confocal scanning laser microscopy (CSLM) can be used to study in situ localization of bacteria in food and has been used successfully to examine various bacteria on apples, alfalfa sprouts, beef muscle, and roots of Lotus japonicus (6, 13, 19, 23). Food samples can be studied directly in the fully hydrated form without sample preparation, such as homogenization or extraction. CSLM also has been used in conjunction with cyanogen-2,3-ditolyl tetrazolium chloride staining to distinguish between live and dead Campylobacter jejuni cells on chicken skins (9). Genetically modified strains producing green fluorescent protein are suitable markers for in situ localization studies because they do not require the addition of exogenous substrate for fluorescence (8, 18, 22). Alternatively, the use of fluorescent isothiocyanate (FITC)–labeled antibodies makes it possible to study specific bacteria, and many studies have been undertaken on food pathogens such as E. coli O157:H7 and Listeria monocytogenes (for review, see (10)).

Attachment and penetration of E. coli O157:H7 varies depending on the food type and the cut surface. No E. coli O157:H7 cells were found below 6 μm on the surface of washed apples (15), but cells were found at 73 μm below the cut surface of lettuce (20), and E. coli O157:H7 was essentially removed from alfalfa sprouts by repeated washing (4). This variation in attachment to different foods and the different depths of penetration indicate that data obtained for one food cannot be extrapolated to another.

Using conventional methods, researchers have found that when bacterial suspensions contact meat tissue, most attachment occurs in the first minute (4, 12), although in some instances continued attachment occurs over a 30-min period (4). Differences in attachment of various bacteria to lettuce have also been observed (21, 23). Bacteria can attach loosely to surfaces and can be easily removed by washing, or they can attach firmly and remain attached even after washing (2, 17).

The purpose of this study was to examine E. coli O157: H7 retention on and penetration of meat and carrot surfaces and localization in cheese to improve our understanding of the importance of food surfaces as a microenvironment for pathogens.

MATERIALS AND METHODS

Indicator organism. A nonpathogenic strain of E. coli O157:H7 (B6-914) expressing green fluorescent protein was obtained from Dr. Pina Fratamico (Eastern Regional Research Center, U.S. Department of Agriculture, Agricultural Research Service, Wyndmoor, Pa.). Although this strain does not have the toxin...
genes, it does have eae genes (11), indicating that its attachment properties would be similar to those of pathogenic strains. Even though the strain used was transformed with green fluorescent protein, fluorescence was not detectable by CSLM under the conditions used in our experiments. Therefore, any fluorescence detected resulted from the FITC antibodies used.

**Preparation of inoculum.** An *E. coli* O157:H7 suspension was prepared by centrifugation of a freshly grown culture and resuspension of the cells in sterile distilled water at a concentration of $10^7$ CFU/ml (carrot samples) or in maximum recovery diluent (MRD; Oxoid, Basingstoke, UK) at a concentration of $10^8$ CFU/ml (beef samples).

**Inoculation of food samples.** Carrots (cultivar ‘Nantaise’) that had been imported from France were purchased from a local supplier and stored overnight at 8°C until use. Carrots (5 to 12 kg) were peeled and sliced using a sharp knife and then hand peeled. The end slices were discarded. Transverse sections (6 mm thick) were placed in a cooling incubator with 5.0, containing 200 mg/liter calcium) for 75 min, removed, and resuspended of the cells in sterile distilled water at a concentration of $10^7$ CFU/ml (carrot samples).

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The inoculum was preequilibrated to the temperature of the experiment prior to inoculation. After inoculation, samples were washed in 50 ml of MRD for 15 s. Beef samples were then frozen and examined by CSLM after thawing.

A semisoft brine-salted cheese was made from pasteurized milk (16) free of *E. coli* O157:H7. Twenty liters of pasteurized whole milk was gradually heated to 32°C with constant gentle agitation and inoculated with *E. coli* O157:H7 strain B6-914 so as to contain about $10^3$ CFU/ml. The starter culture (freeze-dried CHN-19, Chr. Hansen, Cork, Ireland) was added when the temperature of the milk reached 25°C. The culture was ripened until the pH reached 6.55 (50 to 55 min). Rennet (3.3 ml Chymogen, Chr. Hansen) was added, and the culture was agitated and left to set for 75 min. The curd was carefully cut (2 cm cubes) using curd knives, left to settle for 5 min, and stirred for 5 min before being heated gradually to 37°C at a rate of 1°C every 5 min. The curd and whey were stirred continuously and maintained at 37°C until the pH decreased to 6.4. The curd was then used to filter sterile cheese meal (10 cm diameter). The cheeses were inverted at regular intervals until brining, which occurred once the pH reached 5.1. The cheeses were immersed in brine (23% NaCl, pH 5.0, containing 200 mg/liter calcium) for 75 min, removed, and placed in a cooling incubator with $\pm$95% relative humidity at 15°C until examined.

**Determination of *E. coli* O157:H7 numbers.** Background numbers of *E. coli* O157:H7 in beef and carrot were determined using sorbitol MacConkey agar (Difco, Becton Dickinson, Sparks, Md.). Samples (approximately 4 g of beef and 25 g of carrot) were diluted 1:10 with MRD, macerated in a stomacher (Seward Medical Co., Thetford, UK) for 2 min, and serially diluted. Plates were incubated at 37°C for 18 h. Cheese samples (approximately 10 g) were suspended (1:10 dilution) in 2% (wt/vol) trisodium citrate, homogenized, diluted, and plated on *E. coli* O157 ID medium (bioMérieux, Marcy l’Etoile, France), which were incubated at 37°C for 18 h.

**FITC-labeled antibodies.** FITC-labeled antibodies specific for *E. coli* O157:H7 (FITC-labeled goat anti-*E. coli* O157:H7 polyclonal antibody, KPL, Gaithersburg, Md.) were used to label the *E. coli* O157:H7 cells. The antibodies attach to the organism and fluoresce, showing the outline of the organism. The antibodies are specific for *E. coli* O157:H7 and do not react with other organisms.

Sample preparation for CSLM. The FITC solution was prepared at a concentration of 5 μmol/ml in phosphate-buffered saline containing 1% bovine serum albumin. A sample of carrot or beef (approximately 20 by 20 by 5 mm) was immersed in 20 ml of the FITC solution for 30 min at room temperature (approximately 20°C). For cheese samples, 60 min of incubation in a 10-μmol/ml solution was used for a sample of similar size. Excess FITC was removed by rinsing with sterile distilled water in a 10-ml volume (contained in a 50-ml beaker) for 1 min at room temperature. The sample was then examined by CSLM.

CSLM. Thick sections (approximately 10 by 10 by 3 mm) of pre-FITC-labeled meat, carrot, or cheese were aseptically cut and placed on a microscope slide. Fifty microliters of low-melting-point agar (Sigma-Aldrich, St. Louis, Mo.) was added to the top of the sample before the coverslip was put in place. One drop of 0.01% (wt/vol) Nile blue (a protein stain) was added to the agar, and a coverslip was placed on top. The agar, from which the Nile blue could easily diffuse, solidified at room temperature and helped to stabilize the sample during microscopy. Labeled and stained samples were examined both by conventional epifluorescence microscopy and by CSLM. For epifluorescence microscopy, samples were illuminated by blue light (excitation filter, 450 to 490 nm; long-pass emission filter, 520 nm). For CSLM, dual excitation was used: 633 nm (red) to reveal proteins (cheese, meat) and cell wall material (carrot) and 488 nm to reveal FITC-labeled bacteria. This protocol allowed simultaneous visualization of immunolabeled bacteria and surrounding food microstructures. Optical sections approximately 1 μm thick were digitally acquired and processed. The background fluorescence from the FITC-labeled antibodies was negligible and did not interfere with sample analysis.

**RESULTS**

**Carrot samples.** After inoculation and washing, the number of *E. coli* O157:H7 on carrot samples was estimated at $10^7$ CFU/g. Figure 1 is the CSLM image of *E. coli* O157:H7 cells on carrot. The *E. coli* cells did not penetrate the carrot cells (none were seen intercellularly) but were found surrounding carrot cells and in intracellular spaces. Even after a 5-day storage period at 8°C, *E. coli* O157:H7 cells did not penetrate the carrot cells (data not shown). The Nile blue stain allowed clear differentiation between the cellular structure of the carrot and that of the bacteria. FITC-labeled *E. coli* O157:H7 cells were detected at 60 to 80 μm below the carrot tissue surface.

**Beef samples.** Beef (knuckle or brisket) samples were immersed in the *E. coli* O157:H7 solution at 3, 12, and 25°C for 1 to 30 min. There was no significant difference ($P > 0.05$) in retention of *E. coli* O157:H7 by sample type, temperature, or time (Table 1). The retention that occurred...
after 1 min of immersion in the *E. coli* O157:H7 solution was similar to that after 30 min of immersion, indicating that there was a rapid, strong retention of the *E. coli* O157:H7 cells to the beef surface.

The striated muscle fibers and connective tissue (elastin fibers) of the beef could be seen with differential staining. *E. coli* O157:H7 cells were located primarily between muscle fibers and around the elastin fibers (Fig. 2). Cells were also located within the connective tissue (Fig. 3) at a depth of 25 μm. Nile blue, which was used to stain the protein, does not easily distinguish between connective tissue and collagen fibers.

**Cheese samples.** At the beginning of cheese manufacture, *E. coli* O157:H7 was added at a concentration of about $10^7$ CFU/ml. The numbers increased during manufacture to a maximum of $10^7$ CFU/g and decreased during ripening to $10^3$ CFU/g on day 4. On days 1 and 4, the cheese was examined by CSLM using FITC with Nile blue. *E. coli* O157:H7 was visible as green cells against a background of red protein. The cells appeared primarily in small clumps of about 10 cells (Fig. 4), although a few single cells were also seen.

**DISCUSSION**

Using specific FITC-labeled antibodies, it was possible to use CSLM to visualize *E. coli* O157:H7 in food samples. With standard fluorescence microscopy, it is also possible to detect FITC-labeled organisms. However, one of the advantages of CSLM in conjunction with fluorescence microscopy is that it is possible to visualize the structure of the food sample in addition to the bacterial cells and therefore study localization to a greater extent (15, 21). Applications of CSLM in food research have illustrated its use for characterizing bacterium-tissue interactions, demonstrating that attachment and penetration of food pathogens differ with the food type and the nature of the surface (15). The

![Figure 1](image1.png)

**FIGURE 1.** Confocal scanning laser micrograph of a transverse section of carrot showing *E. coli* O157:H7 after FITC staining (green). Nile blue was used to stain the protein red.

![Figure 2](image2.png)

**FIGURE 2.** Confocal scanning laser micrograph of *E. coli* O157:H7 cells in the interspacial region between muscle fibers of beef knuckle.

![Figure 3](image3.png)

**FIGURE 3.** Confocal scanning laser micrograph of beef connective tissue. Single optical section is approximately 25 μm deep. Nile blue was used to stain the protein red. Note the bacterium (green) embedded between connective tissue fibers.

<table>
<thead>
<tr>
<th>Beef Sample</th>
<th>Temperature Immersion (°C)</th>
<th>1 min</th>
<th>5 min</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
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<tbody>
<tr>
<td>Knuckle</td>
<td>25</td>
<td>6.31</td>
<td>6.69</td>
<td>6.55</td>
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<tr>
<td>Brisket</td>
<td>25</td>
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<td>6.53</td>
<td>6.53</td>
<td>7.08</td>
<td>6.30</td>
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<tr>
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<td>6.33</td>
<td>6.41</td>
<td>6.42</td>
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<tr>
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<td>6.29</td>
<td>6.87</td>
<td>6.87</td>
<td>6.28</td>
</tr>
<tr>
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<td>6.45</td>
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<tr>
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<td>6.83</td>
<td>7.04</td>
<td>7.15</td>
<td>6.78</td>
</tr>
</tbody>
</table>

*Standard deviation of the mean, 0.51; degrees of freedom, 14.
depth of penetration of the bacteria into the sample can also be studied (20). A potential problem for this method is high numbers of background organisms. However, the high specificity of the antibodies and the high concentration of the inoculum used in these experiments overcome this problem.

Conventional plate count methods were used to determine retention of *E. coli* O157:H7 on beef (either knuckle or brisket) after 1 min of immersion in a solution of bacterial cells (Table 1). Factors such as time and temperature of immersion solution had little influence on the degree of retention. Previous results (7) also indicated that temperature had no effect on attachment of organisms to beef. Repeated washing of the sample with sterile distilled water had a negligible effect on the number of cells, indicating the rapid and firm retention of the *E. coli* O157:H7 cells to the beef surface. Figures 2 and 3 clearly illustrate this retention. *E. coli* O157:H7 cells are visible along the beef fibers and embedded in the connective tissue at a depth of 25 μm. Experiments with green fluorescent protein have revealed penetration of *E. coli* O157:H7 to a depth of 45 μm (19). This depth of penetration explains why repeated washing was not effective for reducing cell numbers.

Very little research has been undertaken to determine why bacteria attach to food, whether attachment is site specific, or whether they actually attach or are just trapped in the food. Attachment of *E. coli* O157:H7 to collagen fibers has been observed by scanning electron microscopy (12). In the present study, the stain used was a protein stain, so collagen fibers could not be distinguished. Similar to previous results (12), there was no difference between lean and fat tissue in retention of bacteria, indicating that if there are receptors for attachment they are similar on all types of tissue.

Nile blue staining clearly distinguished the cell wall material of carrot (red in Fig. 1) and allowed localization of the *E. coli* O157:H7 cells in relation to the cell wall material. The *E. coli* O157:H7 cells did not penetrate into the carrot cells but remained outside the cell walls and in the intracellular spaces, even after a 5-day storage period. Similar results were obtained in a study of *E. coli* O157: H7 penetration of lettuce leaves, where bacterial cells penetrated into leaf tissues through the cut edges and were located at cell junctions but not within lettuce cells (20).

Information on the depth of *E. coli* O157:H7 cells also was obtained using CSLM. FITC-labeled bacterial cells were found at depths of 60 to 80 μm below the carrot tissue surface. *E. coli* O157:H7 cells located at greater depths were difficult to detect because the microscope could not scan accurately below these depths owing to the nature of the carrot tissue. In previous work, *E. coli* O157:H7 cells were detected at an average of 101 μm below the cut surfaces of lettuce tissue and up to 70 μm below the surface of damaged apple tissue (6, 21).

Carrot cellular fluid contains sugar and other nutrients that can be metabolized by *E. coli* O157:H7. However, the juice from raw carrots has a lethal effect on *L. monocytogenes* (5) and a damaging effect on *E. coli* O157:H7 (1). *E. coli* O157:H7 cells that have penetrated the surface, where carrot juice and nutrient concentrations are highest, will be protected from these influences but as a result may suffer from nutrient stress, thus limiting the survival potential of cells penetrating into the cut tissue during storage.

In this study, we have demonstrated the potential for food surfaces to provide a microenvironment for *E. coli* O157:H7, where they are protected from removal by decontamination processes such as washing and sanitizing. Within this microenvironment, the potential for growth exists if nutrients are available (1). Therefore, there is a need for new methods of inactivation and/or removal of bacteria from food surfaces, taking the deep penetration below the surface into account. The penetration issue is particularly important for *E. coli* O157:H7, because a small number of surviving cells can be potentially lethal.

These applications of CSLM in food research have illustrated the usefulness of this procedure for characterizing bacterium-tissue interactions and demonstrating retention and penetration of *E. coli* O157:H7 below the surface of different food types.

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