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Detection of *Escherichia coli* O157:H7 bacteria by a combination of immunofluorescent staining and capillary electrophoresis

As the number of incidents of bacterial infections continues to rise around the globe, simpler, faster, and more sensitive diagnostic techniques are required to improve the safety of the food supply and to screen for potential bacterial infections in humans. We present here direct and indirect approaches for the detection of bacteria, which are based upon a combination of immunofluorescent staining and capillary electrophoresis. In the direct approach, *Escherichia coli* O157:H7 bacteria stained with fluorescein-tagged specific antibodies are detected by CE, while in the indirect approach fluorescein-tagged specific antibodies to *E. coli* are first captured by *E. coli* O157:H7 bacteria and then released and detected by CE. We have identified suitable bacteria staining and CE protocols, which involved a 10 mM Tris-borate-EDTA (TBE) buffer, 0.25 μg antibody/1 million bacteria, and capillaries dynamically coated with poly-*N*-hydroxyethylacrylamide (polyDuramide). We have also successfully detected the presence of *E. coli* O157:H7 in contaminated meat. The total time required for analysis was 6–8 h, which is less than that realized in most commercial assays presently available.

Keywords: Bacteria detection / Capillary electrophoresis / Immunofluorescent staining EL 5285

1 Introduction

Bacterial contamination of food and water is on the rise around the globe. The Center for Disease Control and Prevention (CDC) has estimated that microbial pathogens in food cause 76 million cases of human illness, 325 000 hospitalizations, and up to 5000 deaths in the United States each year [1]. These illnesses involve over 40 different food-borne microbial pathogens, including fungi, viruses, parasites, and bacteria. Although prevention efforts are reducing the number of outbreaks, their severity is continuing to rise. Food-borne microbial infections are of particular concern due to rising antibiotic resistance, strain variations, and the increased movement of people and food products across the globe. The cost of human illness in the USA for the top six bacterial pathogens is estimated to be $ 9–13 billion annually, with $ 3–7 billion attributed to food-borne bacteria.

Culturing methodology remains the gold standard for detection of food-borne pathogens, but it is time-consuming (24–48 h of enrichment prior to analysis). Moreover, the identification of certain types of pathogens (*e.g.*, *Escherichia coli* O157:H7) by culturing often results in false negatives due to a high background level of competing microorganisms. Combined culturing/immunoassay approaches typically offer either improved sensitivity of *E. coli* detection (*e.g.*, immunocapture kit, TECRA, French's Forest, Australia) or reduced time of the analysis (*e.g.*, visual immunoassay kit, TECRA), but not the two together. DNA-based approaches for bacteria detection are also being developed (*e.g.*, BAX-Qualicon, Dynabeads). While these approaches have superb sensitivity, their major drawbacks include the necessity for time-consuming DNA extraction and PCR amplification, which may be compromised by contaminants and broth components.

Another technique for bacteria detection is capillary electrophoresis (CE). CE equipped with UV detection (CE-UV) was first utilized for bacteria separation by Ebersloe *et al.* in 1993 [2]. They showed that a mixture of *Enterococcus faecalis, Streptococcus pyogenes, Streptococcus agalactiae*, and *Staphylococcus aureus* could be separated by CE into four discrete but very broad bands (1–8 min wide at half-heights). Four years later, Pfetsch *et al.* [3] achieved sharper CE-UV peaks (1–2 min wide at half-heights) associated with bacteria and reproducible peak migration times by employing a low-salt buffer (1–3 mM). More recently, Armstrong *et al.* [4–9] separated, identified, and quantitated a variety of culture-derived unlabeled and fluorescently stained bacteria and obtained remarkably sharp CE peaks (several s wide at half-height) and high separation efficiencies (up to 1.6 M theoretical plates/m). Armstrong *et al.* attributed their success to the presence

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Abbreviations: FAbs, fluorescein-tagged antibodies; **TBE**, Tris-borate-EDTA buffer

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of poly(ethylene oxide) (PEO) in the CE running buffer, which acted as a focusing agent, as well as to "meticulous sample preparation and exact separation conditions." CE protocols employed in the aforesaid studies were suitable for the separation of a relatively small number of carefully prepared, homogeneous, culture-derived bacteria. However, these protocols are much less applicable to the identification of bacteria derived from food containing multiple unknown interfering bacteria and residual fats, proteins, and carbohydrates, which may result in an excessive number of possibly overlapping CE-UV peaks.

In this study, we present direct and indirect approaches for detection of *E. coli* O157:H7 derived from food, which are based on a combination of immunofluorescent staining and CE-LIF. The direct approach involves the staining of target bacteria with fluorescein-tagged antibodies (FAbs), washing, and detection of bacteria by CE-LIF. The indirect approach involves the staining of target bacteria with FAbs, washing, release of the bound FAbs by sonication, and detection of the FAbs (not the bacteria) by CE-LIF. In both approaches, the high specificity of FAb binding ensures that only target bacteria are immunofluorescently stained and generate peaks in CE-LIF traces. Due to much sharper and more intense CE-LIF peaks associated with FAbs than those associated with stained bacteria, the indirect method has higher sensitivity of detection than the direct method, but it is less quantitative. We have also optimized sample preparation protocols for culture-derived bacteria and shown their utility for the detection of food-derived bacteria by analyzing *E. coli* derived from contaminated meat. To our knowledge, there are currently no reports in the literature of a successful application of CE methodology to the detection of bacteria derived from food. There are, however, reports of the application of CE to the analysis of bacteria in bodily fluids (urine) [6] and simulated food samples [10].

The viability of *E. coli* samples during and after our experiments was not evaluated. From a consumer viewpoint, it seems that the mere fact that food is contaminated with *E. coli* O157:H7 (either dead or viable) should be reason enough not to offer it for consumption. Furthermore, seemingly dead cells have been shown to eventually recover and proliferate during plating experiments, further complicating a precise definition of "dead" and "viable" [11].

2 Materials and methods

2.1 Materials

Non-enterohaemorrhagic *Escherichia coli* O157:H7 *(E. coli)* strain ACP201 and *Salmonella choleraesius* subsp. *choleraesius* Weldin serotype Typhimurium ATCC 14028 *(Salmonella)* were the bacterial strains used throughout this study. FAbs to *E. coli* and *Salmonella* (500 μg each) were purchased from KPL (Gaithersburg, MD, USA) in lyophilized form, rehydrated with 1 mL of H_2O, and stored frozen in aliquots of 50 μL. Culture media, agar and Bacto Peptone for bacteria growth were purchased from Beckton Dickinson Microbiology Systems (Franklin Lakes, NJ, USA). Fused bare silica capillary (75 μm ID) was purchased from Polymicro Technologies (Phoenix, AZ, USA). 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris), boric acid, and ethylenediaminetetraacetate (EDTA) were purchased from Amresco (Solon, OH, USA). Polyoxyethylenesorbitan monolaureate (Tween 20R) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Poly-*N*-hydroxyethylacrylamide (polyDuramide) was prepared according to a procedure reported by Albarghouthi *et al.* [12] from the monomer supplied by BioWhittaker Molecular Applications (Walkersville, MD, USA). A sample of bacteria derived from meat contaminated with ACP201 cells and a control sample derived from noncontaminated meat were obtained from the Department of Biology, University of Wisconsin, Parkside (Kenosha, WI, USA) in the form of bacterial suspensions in a PBS buffer also containing residual food matrix.

2.2 Equipment

Bacteria were grown in a 4200 Innova incubator shaker (200 W, 44 kHz; New Brunswick Scientific, Edison, NJ, USA). UV-Vis readings were done in a Cary 50 spectrophotometer (Varian, Walnut Creek, CA, USA). Bacteria were vortexed in a Vortex Genie (Fisher, Hanover Park, Il, USA) and sonicated in a Branson 8210 sonicator bath (200 W, 44 kV; Branson Ultrasonic Corporation, Danbury, CT, USA). Blender used in bacterial preparation from meat was a Warning commercial blender (Warning Products, Torrington, CT, USA). Bare fused capillaries were dynamically coated with polyDuramide using a syringe pump (kdScientific, New Hope, PA, USA). CE was performed on a BioRad single-capillary instrument equipped with dual UV/LIF detectors (BioRad Laboratories, Hercules, CA, USA). Bacteria were imaged on a Zeiss microscope (Carl Zeiss, Jena, Germany) with epifluorescent detection (ex. 470 ± 40 nm, em. 530 ± 25).

2.3 Methods

2.3.1 Bacteria growth, purification, and labeling

E. coli and *Salmonella* cultures were transferred from solid agar to Luria-Bertani (LB) broth (3–5 mL) and were grown for 3–6 h at 37°C. To purify bacteria, an aliquot of *E. coli* or *Salmonella* (1–1.5 mL) in LB broth was centrifuged at

1300 × *g* for 3–4 min to afford a loose pellet. The supernatant was carefully removed, and the pellet was resuspended by vortexing for 30 s in a CE buffer, which was comprised of 4.5 mM Tris, 4.5 mM boric acid, and 0.1 mM EDTA. These steps were repeated 2–3 times. To the purified suspension of bacteria (0.5 mL, OD_{600} ~ 0.5) were added FAbs (50 μL, 500 μg/mL), and the resulting mixture was incubated in the dark at room temperature for 1 h. After this time, the samples were purified for CE according to the purification procedure outlined above.

2.3.2 Preparation of samples derived from meat

Briefly, a sample of meat (25 g) was inoculated with a suspension of ACP201 cells (CFU ~ 100–200) and blended in a Warning blender for 5 min. The meat and bacterial cell suspension was then incubated for 5–6 h at 37°C and for 30 min at 4°C. The resulting sample was centrifuged at 2000 rpm for 20 min at 4°C and filtered through sterile gauze once and through 10 μm filters twice. The filtrate was centrifuged for 10 min at 7000 rpm, and the supernatant was discarded. The pellet was washed once with a PBS buffer and resuspended in a PBS buffer (10 mL). The control sample was prepared in the same fashion except that it was not inoculated with ACP201 cells. Both samples were purified and stained with FAbs as described in the previous section.

2.3.3 Capillary electrophoresis

Prior to CE, bare fused-silica capillaries were dynamically coated with polyDuramide by pushing an aqueous solution of polyDuramide (0.5% w/v, 50 μL, 15 min) through the capillary without any additional capillary conditioning. For CE, the capillary (75 μm ID, 22 cm effective length, 27 cm total length) was filled with the CE buffer (4.5 mM Tris, 4.5 mM boric acid, and 0.1 mM EDTA) containing a small amount of polyDuramide for coating purposes (0.1% w/v). Sample solutions containing bacteria were injected into the capillary by pressure (5 psi) and separated in less than 10 min at 370 V/cm with an associated electrical current of 2.4 μA. Each CE result was reproduced at least 3 times. The buffer solution in the capillary was replaced between the runs, without any further capillary conditioning.

2.3.4 Epifluorescence microscopy

Epifluorescence microscopy was performed using a Zeiss AxioPhot epifluorescence microscope (Carl Zeiss) equipped with fluorescein isothiocyanate and tetramethyl rhodamine isocyanate filter sets and a Zeiss AxioCam camera for image acquisition. Images were processed in a PC with Zeiss software and saved as jpg files.

3 Results and discussion

3.1 Control experiments

Prior to attempting CE-LIF detection of FAb-stained ACP201 *E. coli*, a series of control experiments was done to assess the quality of the polyDuramide coating as well as the quality of the samples. These experiments involved (i) CE-LIF analyses of FAbs alone, (ii) CE-UV analysis of unlabeled culture-derived *E. coli*, (iii) and optical and epifluorescence microscopy of culture-derived FAb-stained *E. coli*. CE-LIF traces of FAbs analyzed in the capillaries dynamically coated with polyDuramide [12] exhibited sharp peaks with minor tailing (data not shown). In a series of sequential analyses in the same capillary, migration times of pressure-injected FAbs varied somewhat in the first two runs but stabilized thereafter. This result is most likely due to additional passivation of the inner walls of the capillary with adsorbed FAbs. Electrokinetic injection of FAbs yielded even sharper CE-LIF peaks, which is attributed to efficient sample stacking. CE-LIF analysis of FAbs in uncoated bare fused-silica capillaries under identical conditions yielded much broader peaks (1–2 min wide at half-width, data not shown) confirming the need for a polyDuramide coating to prevent protein adsorption. In contrast to a sharp peak associated with FAbs, a CE-UV trace of unlabeled *E. coli* showed a broad peak (1–2 min wide at half-height, data not shown), on par with those reported by Pfetsch *et al.* [3]. Electrokinetic injection of bacteria was attempted but proved inefficient, presumably because of the large size of bacteria. Interestingly, pressure injections of bacteria at > 5 psi led to irreproducible peak shapes and migration times.

The impact of salt, bacteria, and antibody concentrations on the efficiency of immunofluorescent staining and bacteria aggregation was systematically investigated by epifluorescence microscopy. Stained, brightly fluorescent bacteria, which did not photobleach for several seconds, were observed when a ratio of 0.25 μg of the antibody per 1 000 000 cells was used. This ratio was also recommended as an optimum in at least one other immunofluorescent staining protocol [13]. In contrast, lower antibody/bacteria ratios yielded only dimly fluorescent bacteria, which photobleached in 1–2 s. Attempts to use lower antibody/bacteria ratios in conjunction with longer incubation times (2–3 h) did not increase the intensity of the fluorescent signal. It is noteworthy that detection of bac-

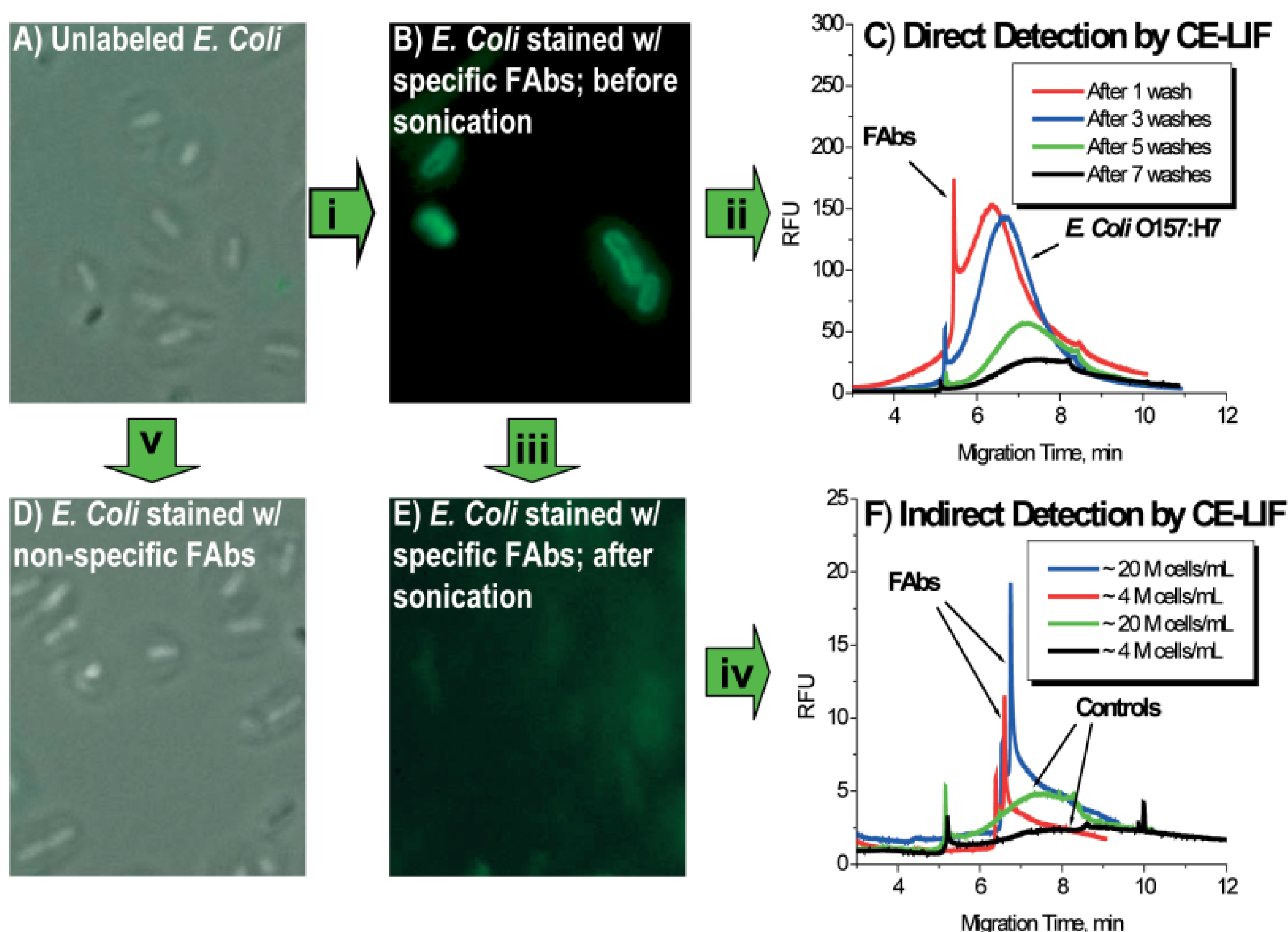

Figure 1. Direct and indirect approaches for the detection of bacteria by a combination of immunofluorescent staining and CE-LIF. (i) 0.25 μg Fabs/ 1M cells is added to *E. coli* and the mixture is incubated for 30 min in the dark; (ii) excess FAbs are removed by washing, and *E. coli* is directly detected by CE-LIF. Conditions: fused-silica capillary dynamically coated with polyDuramide (75 μm ID; total length, 27 cm; effective length, 22 cm), filled with 0.1% w/v solution of polyDuramide in a 10 mM TBE buffer; injection, 5 psi; separation, 370 V/cm with an associated current of ~2.4 μA; (iii) excess FAbs are removed, and *E. coli* is sonicated for 30 min with 1% v/v Tween 20; (iv) *E. coli* is indirectly detected by CE-LIF, with the same CE conditions as above; (v) a control experiment, in which *E. coli* is treated with FAbs to *Salmonella* and then washed, shows no fluorescent bacteria.

teria by epifluorescence microscopy required concentrations of at least 10^9 cells/mL, similar to those necessary for CE-UV. No fluorescent bacteria were visible in either a control sample of unlabeled *E. coli* (Fig. 1A), a control sample of *E. coli*, which was treated with FAbs specific to *Salmonella* (Fig. 1D), or a control sample of *Salmonella*, which was treated with FAbs specific to *E. coli*, thus confirming the high specificity of antibody binding.

The concentration of salt in the buffer, in which bacteria were suspended during immunofluorescent staining, had a profound impact on the degree of bacterial aggregation. Low salt content (10 mM) resulted in essentially no aggregation (Fig. 1B), while higher salt content ($>$ 50 mM) afforded significantly aggregated samples (data not shown). In the absence of FAbs, bacteria did not visibly aggregate even in high-salt buffers. This result is explained on the basis that higher salt concentrations allow for more efficient screening of surface charges and promote bacterial aggregation, much like they promote the aggregation of charged colloidal particles in aqueous solutions. While no aggregation was observed in a 10 mM Tris-borate-EDTA (TBE) buffer, the population of bacteria was not entirely homogeneous. It contained a significant number of bacterial doublets (two cells fused together), which may have resulted from arrested cell division. Additionally, bacterial debris were also observed by epifluorescence microscopy, as numerous fine particulates. The latter observation had important implications for CE-LIF with regard to peak shapes and migration times (*vide infra*).

3.2 Direct detection of bacteria derived from culture by CE-LIF

Samples of FAb-stained ACP201 *E. coli* of a quality such as shown in Fig. 1B were analyzed by CE-LIF. Prior to CE, a large excess of FAbs, which was used for staining, was removed by a 1–7 cycles of pelleting by centrifugation, removal of the supernatant, and resuspension in a clean buffer. In the absence of these washing steps, the only peak observed in CE-LIF traces was the one due to free antibodies. The identity of this peak was confirmed by a spiking experiment. The amount of free antibody in the sample decreased dramatically after 1–3 cycles of washing, with no further reduction observed after additional cycles of washing, as evident by CE-LIF (Fig. 1C). The washing also allowed for the observation of a broad peak, which was assigned to FAb-stained bacterial fragments. Initially, the excessive width of this peak was attributed to increased aggregation of intact *E. coli*, cross-linked by FAbs. However, later results showed that even samples with minimal aggregation, such as the one shown in Fig. 1B, also showed similarly broad CE-LIF peaks. The assignment of the broad CE-LIF peak was confirmed by the analysis of bacterial samples generated by sonication (no surfactant present), which did not contain intact cells, as evidenced by epifluorescence microscopy (Fig. 1E). A highly concentrated control sample of *Salmonella* labeled with FAbs specific to *E. coli*, analyzed using the direct detection protocol, showed no fluorescent bacteria in epifluorescence microscopy, and no CE-LIF peaks.

After repeated washing steps, the intensities of the broad CE-LIF peaks due to bacterial debris decreased from ~150 RFU to ~30 RFU (Fig. 1C), which is attributed to incomplete sedimentation of the bacterial debris during centrifugation and their loss during removal of the supernatant. The maxima of the CE-LIF peaks shifted from ~6.3 min after 1 washing to ~7.3 min after 7 washings (Fig. 1C). This result is explained by preferential sedimentation of larger bacterial fragments, which comprise the tail of the broad CE-LIF peak, over smaller bacterial fragments, which comprise the front of the broad CE-LIF peak. Interestingly, bacterial fragments were also observed by UV as broad peaks, but they were well separated from the sharp peaks due to intact bacteria and showed a much lower signal intensity. In CE-LIF, FAbs bound to bacterial fragments yielded peaks with a more prominent signal and altered migration times. Since a separate sharp peak for FAb-stained intact bacteria, the presence of which is confirmed by epifluorescence microscopy, is not detected by CE-LIF, we assume that it is obscured by the broad peak of the bacterial debris. The inability to completely remove free FAbs from the bacterial sample, even with repeated washing cycles, is explained as following. During washing cycles, the pellet is dispersed in a clean buffer by vigorous vortexing, which may result in the breakage of some cells accompanied by the release of a small amount of free FAbs.

3.3 Indirect detection of *E. coli* derived from culture by CE-LIF

As it was mentioned above, ACP201 *E. coli* stained with FAbs in a high-salt TBE buffer were substantially aggregated. Previously, Armstrong *et al.* [6] used brief sonication to disrupt bacterial aggregation and produce more homogenous samples. Nebe-von-Caron *et al.* [11], however, noted that the results of sonication of bacteria varied depending on the geometry and the material of the sample container, the water level in the sonicator, temperature, dissolved gas, *etc*. In their experience, some types of cells are fragile and cannot sustain even a 10 s sonication without being ruptured. We found that sonication of FAb-stained purified aggregated *E. coli* for 15–30 s disaggregated them but also resulted in an increased amount of bacterial debris. Sonication for a longer time ($>$ 5 min) lysed all of the intact cells (Fig. 1E) but did not have any impact on the width of the broad CE-LIF peaks assigned to bacterial debris. If, however, sonication of FAb-stained *E. coli* was done in the presence of a surfactant (Tween 20R) for more than 30 min, sharp peaks appeared in the CE-LIF traces (Fig. 1F, red and blue traces). These sharp peaks were not due to increased homogeneity of the debris, but rather they were due to a partial release of the bound FAbs as a result of the breaking of some intact bacteria or further fragmentation of bacterial fragments. This assignment was confirmed by a spiking experiment with free FAbs. A control sample consisting of a high concentration of *Salmonella* stained with FAbs specific to *E. coli* under the exact same conditions showed no CE-LIF peaks. Thus, these observations provide the foundation for an indirect method of detection of *E. coli*, in which the presence of bacteria is determined by the presence of free FAbs released during sonication of prewashed FAb-stained target bacteria.

The indirect method for *E. coli* detection by CE-LIF has higher sensitivity (higher CE-LIF peak intensities) than the direct method for *E. coli* detection by either CE-LIF or CE-UV. To quantitate this, we determined the CFU values for two *E. coli* samples grown to ~ OD_{600} = 0.5. Standard plate counts gave values of ~ 200 M CFU/mL, which were also confirmed by using a counting chamber and a microscope. At this concentration, *E. coli* was not detected by CE-UV, which required at least 50 × more concentrated bacterial suspensions (CFU ~ 1 B/mL). For CE-LIF analyses, the suspensions of *E. coli* with the OD_{600} ~ 0.5 were

diluted 10 × and 50 ×, and the CFU were assumed to be $2 \times 10^8/10 = 20$ M cells /mL and $2 \times 10^8/50 = 4$ M cells/mL, respectively. The resulting electropherograms are shown in Fig. 1F. The sample of *E. coli* with a concentration of 20 M cells/mL yielded the blue trace in the indirect detection mode, and the green trace in the direct detection mode. The sample of *E. coli* with a concentration of 4 M cells/mL gave the red trace in the indirect detection and the black trace in the direct detection mode.

The indirect method of bacteria detection offers two additional advantages: the potential for multiplexing and the possibility of analysis on microchips. During the control experiments, FAbs were successfully injected by both pressure and electrokinetically. Their CE-LIF peaks were sharp, well separated, and had reproducible migration times of ~ 3 min for *E. coli*-specific FAbs and ~ 2 min for *Salmonella*-specific antibodies. The direct detection method does not offer these options, due to the large CE-LIF peak widths and the need for pressure injection, which is less amenable to implementation on microchips.

3.4 Direct detection of *E. coli* derived from meat

The analysis of bacteria derived from food using any technique is generally much more difficult than the analysis of culture-derived bacteria. It involves extraction, isolation, and enrichment of the target bacteria, potentially in the presence of a large number of interfering bacteria. CE analyses of this type of samples are further complicated by the presence of residual fats, proteins, and carbohydrates that can compromise the protective capillary wall coating or even clog the capillary, which can result in poor peak shapes, low reproducibility of the peak migration times, adventitious CE peaks or the absence of any CE peaks.

In a proof-of-concept experiment, we analyzed a sample derived from meat inoculated with ACP201 *E. coli* cells using the direct protocol developed in this study. Its CE trace had a strong resemblance to the ones obtained during the analysis of *E. coli* derived from culture (Fig. 2). It shows a sharp peak due to residual free antibodies, and a broad peak due to FAb-stained bacteria. The reason for the faster electrophoretic mobility of *E. coli*, relative to the separation shown in Fig. 1C, is not entirely clear but could be attributed to the changes of bacteria surface charge due to the presence of ionic species derived from the meat matrix. The CE traces for a control sample, which was derived from noninoculated meat, showed only a very minor sharp peak due to some nonspecific FAb binding. No broad peak associated with bacteria was observed. The two samples were enriched (incubated) for only a brief time before the analysis (5–6 h), so

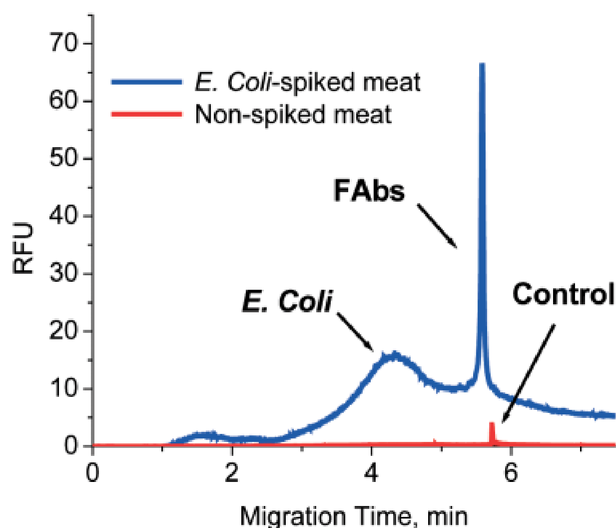

Figure 2. Direct detection of *E. coli* derived from a sample of contaminated meat by a combination of immunofluorescent staining and CE-LIF. Staining and CE protocols are analogous to those used to prepare samples shown in Fig. 1C.

that the total time required for the detection of *E. coli* derived from meat was 6–8 h. This timeframe compares favorably with other immunoassays that are presently commercially available.

4 Concluding remarks

Two approaches for the detection of bacteria by a combination of immunofluorescent staining and CE-LIF have been described. The direct approach involves CE-LIF detection of the FAb-stained bacteria, while the indirect approach involves CE-LIF detection of FAbs captured and then released by the target bacteria. While the direct approach allows for a better quantitation of bacteria, it is plagued with problems related to the complexities of the system, in particular bacterial lysing during the sample preparation steps. The indirect approach affords a higher sensitivity of detection due to much sharper and more intense CE peaks associated with FAbs, a possibility for multiplexing, and an opportunity to carry out the analysis on microfluidic chips, but it is less quantitative than the direct approach due to the variability in the amount of antibodies released upon sonication. While in this study we have successfully used commercially available FAbs, future experiments with other types of bacteria or more complex bacterial mixtures may require engineering of more specific antibodies, which could be time-consuming. In a proof of concept experiment, rapid analysis (6–8 h) of *E. coli* O157:H7 derived from contaminated

meat was successfully performed. We are presently attempting to optimize our protocols further to reduce the total time required for the analysis, improve the peak shapes obtained in the direct detection, and run the analyses on microfluidic chips.

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