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# A Rapid Multiplexed Chemiluminescent Immunoassay for the Detection of *Escherichia coli* O157:H7, *Yersinia enterocolitica*, *Salmonella typhimurium*, and *Listeria monocytogenes* Pathogen Bacteria

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A simple and rapid multiplexed sandwich chemiluminescent enzyme immunoassay has been developed for the simultaneous detection of *Escherichia coli* O157:H7, *Yersinia enterocolitica*, *Salmonella typhimurium*, and *Listeria monocytogenes*. To achieve the multiplexed detection of the four pathogens, a new polystyrene 96 well microtiter plate format has been designed, in which each main well contains four subwells in the bottom. The monoclonal antibodies specific for each bacteria were separately immobilized in each subwell. When the samples were added to the main wells, the bacteria able to specifically bind to the corresponding monoclonal antibody were captured in one of the four subwells. Subsequently, a mixture of peroxidase-labeled polyclonal antibodies against the four bacteria was added and the peroxidase activity of the bound polyclonal labeled antibodies in each well was measured by an enhanced luminol-based chemiluminescent cocktail using a low-light charge-coupled imaging device. The assay was simple and fast, and the limit of quantification was in the order of $10^4-10^5$ CFU/mL for all bacterial species. The accuracy of the method, evaluated by comparison of the results with a conventional culturing methodology, was satisfactory, with recovery values ranging from 90 to 120%. This method can be used as a screening test to evaluate the presence of these pathogen bacteria in different foodstuffs.

**KEYWORDS: Pathogen bacteria; multiplexed method; chemiluminescence immunoassay**

## INTRODUCTION

Bacterial foodborne pathogens are a serious health threat worldwide. The World Health Organization estimates that diarrhea-related diseases caused by the consumption of contaminated food or water are the third leading cause of death in developing countries (*1*, *2*), and the socioeconomic implications of gastroenteritis are a major concern. *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Clostridium perfringens*, *Campylobacter jejuni*, *Yersinia enterocolitica*, and *Listeria monocytogenes* are the major bacterial pathogens commonly involved in foodborne illnesses (*3*). Therefore, the production of high-quality, pathogen-free food and the assessment of bacterial growth when foods and other biological materials are stored under inappropriate conditions represent priorities for the agrofood industry. In addition, bacterial contamination is a matter of concern related to terrorist attack.

Despite the continuous improvements, conventional microbiological methods for the detection and identification of pathogenic bacteria in food are still labor-intensive and time-consuming (*4*); therefore, the search for fast, accurate, and sensitive alternative methods is an active research task (*5*−*7*).

Several molecular methods for the detection of foodborne pathogens based on polymerase chain reaction (PCR) have been developed. Such methods are of great diagnostic importance because they offer unique detection specificity and sensitivity (*8*, *9*). Immunological methods for pathogenic microrganisms have also been extensively studied because they potentially combine detection specificity and sensitivity with simple and economical assay formats. Most of the commercially available immunoassays for bacteria detection are based on standard sandwich immunoassay (*10*), which involves the formation of an immunocomplex between an immobilized antibody, the target bacteria, and a secondary labeled antibody. Generally, the primary antibody is immobilized on beads (magnetic, silica, or

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gold particles), polystyrene microtiter plates, filter membranes, or directly on the surface of transducers, and the secondary antibody is labeled with enzymes or fluorescent molecules. The detection limits of immunoassays ranged between $10^3$ and $10^6$ colony-forming units (CFU)/mL, with assay times from 10 min to several hours (*11*, *12*).

More recently, many interesting approaches have been reported to improve the immunoassay detectability. For example, Zhao and co-workers (*13*) used antibody-conjugated silica fluorescent nanoparticles to develop a fast and ultrasensitive immunoassay for in situ pathogen quantification down to a single bacterium without amplification or enrichment. Unfortunately, most of these tests are expensive, laborious, and often require specialized personnel and instrumentation. As an alternative, DNA microarray technology has been exploited, which allows simultaneous detection of several bacteria, but the construction of DNA arrays is still limited to a restricted number of specialized centers (*14*).

"Multiplexed" format immunoassays suitable for the simultaneous evaluation of different bacteria in a sample can be developed to increase the analytical productivity and drastically reduce analysis costs and sample and reagent consumption (*15*, *16*). Feasibility studies of multiplexed immunometric methods in microarray format, either employing "sandwich" or, more recently, competitive type assay formats (*17*, *18*) have already been reported, but none of them has still been applied for detection of bacteria.

In this work, we report the development of a simple, multiplexed sandwich chemiluminescent enzyme immunoassay (CL-EIA) for the simultaneous detection of four of the major foodborne pathogens: *E. coli* O157:H7, *Y. enterocolitica*, *S. typhimurium*, and *L. monocytogenes*.

Polyclonal and monoclonal antibodies (MAbs) were produced by immunizing rabbit or balb/c mice, respectively, with heat-inactivated and sonicated ATCC bacteria strains of the four pathogens. To achieve the simultaneous detection of the four pathogens, a new polystyrene 96 × four well microtiter plate format was designed. The microtiter plate, which has a conventional 12.8 cm × 8.6 cm frame size, consists of 96 main wells, each of them containing four internal subwells.

The developed method involves the immobilization of four MAbs, each specific for one bacterium, in the bottom of the different subwells of the main well. When the samples were added to the main well and incubated, the bacterial cells were captured in one of the four subwells by the corresponding MAb. Subsequently, a mixture of horseradish peroxidase (HRP)-labeled polyclonal antibodies was added and the peroxidase activity was determined by means of an enhanced luminol-based CL substrate. The light emitted was measured with an ultrasensitive, cooled low-light charge-coupled imaging device (CCD). Chemiluminescence is particularly suited for these applications since the CL signal is characterized by high detectability and specificity, fast kinetics, and low background. In addition, CL detection techniques allow us to localize and quantify the light emission on each subwell down to single-photon level (*19*).

The analytical performances of the method in term of specificity and its preliminary validation will be discussed by comparison with conventional microbiological procedures. For method application and validation, a total of 32 naturally contaminated fecal and meat samples have been analyzed.

## MATERIALS AND METHODS

**Bacterial Strains.** *E. coli* O157:H7 ATCC 35150 (American Type Culture Collection, Rockville, MD), *S. typhimurium* ATCC 14028, *Y. enterocolitica* ATCC 23715, and *L. monocytogenes* 13M ATCC 7644 were used for polyclonal antibodies and MAbs production and as a positive control for antibodies screening. The specificity of each antibody was evaluated against both different bacterial species and other wild-type strains of the target bacteria. The bacteria used were either obtained by ATCC or kindly provided by the Istituto Zooprofilattico of Teramo. Stock cultures in 30% glycerol were maintained frozen at −80 °C.

**MAb Production.** MAbs were produced by immunizing Balb/c mice using a slightly modified published protocol (*20*). Briefly, heat-inactivated and sonicated ATCC reference strains were diluted in Freud's incomplete adjuvant (IFA) (Sigma Chemical Co., St. Louis, MO) to obtain a protein concentration of 50 μg/mL and inoculated by intraperitoneal injection. The mice received their second injection at 2 weeks, and two inoculations of 25 μg/mL were then administered. A booster injection of 50 μg/mL was given 31 days after immunization, then the mice were sacrificed, and the spleen cells were fused with myeloma cells. Cell fusion and hybridoma cloning were performed using Galfre's method (*21*) with some modifications. Briefly, splenocytes from the immunized mice were subjected to cell fusion with myeloma cells from Sp2/O-Ag-14 mice (American Type Culture Collection) using 50% polyethylene glycol 1550 solution. Hybridomas were cultured for 2 weeks in Dulbecco's modified Eagle's medium (Sigma Chemical Co.) containing 20% fetal bovine serum (Euroclone, United Kingdom), 2 mM L-glutamine (HybriMax, Sigma Chemical Co.), amphotericinpenicillin−streptomycin 100× (APS, Sigma Chemical Co.), 50 mg/mL gentamicin (gentamicin sulfate solution, Sigma Chemical Co.), 10000 UI/mL nystatin (nystatin suspension HybriMax, Sigma Chemical Co.), and HAT 50x (HAT media supplement HybriMax, Sigma Chemical Co.). The hybridomas of interest were cloned by limiting dilution method (*22*, *23*). MAbs were produced in vitro on a large scale by serial culturing of antibody-secreting hybridomas and collection of their supernatants.

The cell culture fluids from actively growing hybridomas were screened using an indirect enzyme-linked immunosorbent assay (ELISA) procedure against other isolated strains of the target bacteria and different bacteria species. Briefly, the hybridoma culture medium, properly diluted, was added to a well plate previously coated with the immunizing antigen. The plate was then incubated for 30 min at 37 °C, and, after washes, HRP-labeled anti-mouse antibody (purchased from Sigma Chemical Co.) was added. The HRP activity was measured by adding a chromogenic substrate (TMB, KPL, Gaithersburg, MD). After incubation, the enzymatic reaction was stopped with 2 N $H_2SO_4$ and the absorbance at 450 nm was recorded using a Multiskan EX spectrophotometer microtiter reader (Labsystems, Helsinki, Finland).

MAbs were screened in Immuno Western-blotting (*24*), and the MAb isotype was determined with an isotyping kit (Pierce, Rockford, IL). IgG MAbs were purified by affinity chromatography using a protein A column (HiTrap rProtein A FF, 5 mL, Amersham Biosciences, Arlington Heights, IL) according to the manufacturer's instructions. The protein concentration was determined by using a Bradford assay.

**Polyclonal Antibody Production.** Polyclonal antibodies against the target bacteria were produced in rabbit following a previously described protocol (*25*). Briefly, heat inactivated and sonicated ATCC reference strains were diluted in Freud's complete adjuvant (FCA) (Sigma Chemical Co.) to a protein concentration of 200 μg/mL and subcutaneously injected six times over 50 days. Antiserum with adequate titer, affinity, and specificity was obtained 3−4 months after the first immunization. The IgG-rich fraction was purified using the same procedure for IgG MAb purification. Polyclonal antibodies were coupled to HRP (Sigma Chemical Co.) using the periodate method (*26*).

**Multiplexed CL Enzyme Immunoassay**. *Microtiter Plate Format.* To achieve the simultaneous detection of the four pathogens in the same sample, a new polystyrene 96 × four well microtiter plate format was developed (**Figure 1A**). The prototype microtiter plate was fabricated in our lab by modifying a commercial 384 well polystyrene microtiter plate (Labsystems). The 96 main wells were obtained by drilling the plate with a cylindrical milling cutter (Ø = 6 mm) to obtain a main well among each of the four wells. While the depth of the wells of the microtiter plate was 8 mm, the drilling depth was only 7 mm; thus, four small independent subwells remained in the bottom of the

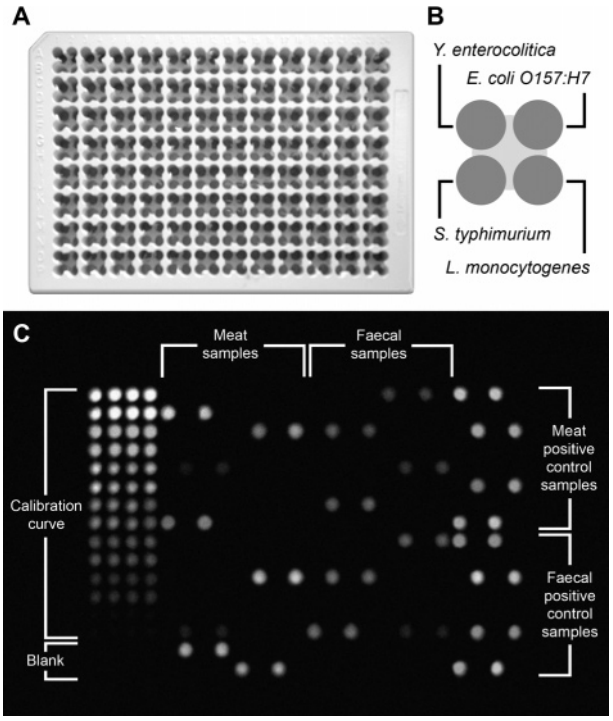


**Figure 1.** (**A**) Live image of the new 96 × four well microtiter plate. The plate contains 96 main wells, each of them divided into four subwells. (**B**) Position of the immobilized antibodies within each subwell. (**C**) CL image of the microtiter plate obtained after a 40 s exposure time (the position of standards and samples within the plate is also shown).

**Table 1.** MAb CRs with Various Bacterial Antigens Evaluated by Indirect ELISA[a]

| | | CR% | | | |
|---|---|---|---|---|---|
| bacterium | source | MAb 8B8C3 | MAb 54B11 | MAb 1B6D9 | MAb 6F12C8 |
| | | *Escherichia* | | | |
| *E. coli O14* | BGVV | 2.1 | 1.1 | 2.2 | 1.1 |
| *E. coli* | ATCC 25922 | 1.2 | 2.1 | 2.2 | 1.2 |
| *E. coli* O157:H7 | ATCC 35150 | **100** | 1.1 | 2.3 | 1.3 |
| *E. coli* O157:H7 | wild-type strain | 97 | 1.1 | 2.4 | 1.4 |
| *E. fergussoni* | wild-type strain | 1.1 | 2.4 | 2.1 | 1.2 |
| | | *Listeria* | | | |
| *L. innocua* | ATCC 33090 | 3.1 | 1.3 | 3.1 | 4.3 |
| *L. ivanovii* | ATCC 19119 | 1.3 | 2.3 | 1.1 | 3.5 |
| *L. monocytogenes* | ATCC 7644 | 1.2 | 1.2 | 1.1 | **100** |
| *L. monocytogenes* | wild-type strain | 1.3 | 1.3 | 1.2 | 97 |
| | | *Salmonella* | | | |
| *S. bredeney* | wild-type strain | 2.1 | 1.1 | 3.4 | 2.1 |
| *S. derby* | wild-type strain | 1.2 | 3.4 | 3.3 | 2.1 |
| *S. enteritidis* | ATCC 13076 | 1.1 | 2.2 | 2.3 | 2.2 |
| *S. enteritidis* | wild-type strain | 2.1 | 3.2 | 1.2 | 2.2 |
| *S. hadar* | wild-type strain | 1.2 | 2.3 | 1.1 | 2.3 |
| *S. muenchen* | wild-type strain | 1.2 | 2.3 | 1.5 | 2.3 |
| *S. panama* | wild-type strain | 1.2 | 2.2 | 1.2 | 2.3 |
| *S. saint-paul* | wild-type strain | 0 | 1.2 | 1.2 | 2.1 |
| *S. typhimurium* | ATCC 14028 | 0 | 1.1 | **100** | 2.1 |
| *S. typhimurium* | wild-type strain | 1.5 | 1.1 | 99 | 2.1 |
| | | *Yersinia* | | | |
| *Y. enterocolitica* | ATCC 23715 | 3.1 | **100** | 1.1 | 3.2 |
| *Y. enterocolitica* | wild-type strain | 3.1 | 98 | 1.1 | 3.4 |

[a] The percentage of CR (%) was calculated by dividing the $OD_{450}$ value of each bacteria by the $OD_{450}$ for the target bacteria.

main well, each of them with a volume of about 15 $\mu$L. Such subwells allowed the immobilization of different monoclonal antibodies in separate positions within the main well (**Figure 1B**). The overall volume of each main well was about 400 $\mu$L, even if 100 $\mu$L solution was sufficient to completely fill the subwells and cover the bottom of the main well. In addition, because the plate has a standard frame size (12.8 cm × 8.6 cm), it can be used with commercially available automation systems and robotics for reagent dispensing, incubation, and washing steps. The prototypes were produced using either black or white polystyrene microtiter plates: the white plates gave the best results in terms of signal-to-noise ratio and were used for the immunoassay. A microtiter plate based on the same principle but with seven subwells for each main well has been already used by us to develop a PCR-CL immunoassay method for the detection of the seven most frequent high-risk papillomavirus DNAs (*27*).

*Calibration Curves*. Calibration curves for the CL-EIA assay were obtained using a mixture of the four bacteria. In particular, universal peptone broth (UPB) (*28, 29*) (Difco Laboratories, Detroit, MI) was inoculated with a mixture of the four bacteria and then incubated for 12−18 h at 37 °C. An aliquot (2 mL) of cells suspension in UPB was centrifuged at 3000*g* for 10 min, the supernatant was carefully removed, and the pellet was washed twice with phosphate-buffered saline (PBS). Washed bacteria were diluted in PBS to give a $10^9$ CFU/mL cell concentration (corresponding to an $OD_{600}$ approximately of 0.8). The bacteria were then serially diluted in PBS to obtain suspensions in the range from $10^8$ to $10^2$ CFU/mL (total cell concentration), which were used as standards in the CL-EIA method. For each dilution, the actual concentration (CFU/mL) of each bacterium was determined by colonies counting on selective media agar plates. The obtained CFU/mL values were used for calibration curve plots. Each calibration curve consisted of seven points in the range from $10^3$ to $10^7$ CFU/mL.

*Samples*. Spiked human fecal and bovine meat samples were used to evaluate the analytical performance and the applicability of the multianalyte CL-EIA method for the detection of bacteria. Fecal (obtained from healthy volunteers) and bovine meat (purchased from local food stores) samples were previously assayed with conventional procedures to verify the absence of the four bacteria. Then, they were divided in two portions: one portion was used as a negative control, and one was artificially contaminated with the four pathogens, alone or in combination, by using previously reported methods (*30, 31*) to achieve final bacteria concentrations ranging from 1 to 100 CFU/g. For each sample, the bacterial concentration was verified by using a conventional microbiological reference method (see below). After contamination, the samples were subjected to a preanalytical enrichment step. Briefly, a 25 g aliquot of each sample was homogenized in a stomacher bag and incubated with 225 mL of buffered peptone water (BPW) for 3 h at 37 °C. Subsequently, 1 mL of suspension was removed and incubated with 9 mL of UPB for 6 h at 37 °C with shaking, and then the resulting solution was assayed for the four pathogen bacteria using the CL-EIA assay. The initial concentration of pathogens in the spiked samples was assessed by interpolating the measured CL signals on a calibration curve obtained from standard bacteria suspensions (concentrations ranging from 1 to 200 CFU/mL) that were subjected to the same enrichment procedure.

For validation of the newly developed CL-EIA method, we tested a total of 32 fecal and meat samples for the presence of the four bacteria using the procedure described above, and the results were compared with those obtained using the following reference microbiological methods: "Microbiology of food and animal feeding stuffs−Horizontal method for the detection of *Escherichia coli* O:157", ISO 16654:2001; "Microbiology−General guidance for the detection of presumptive pathogen *Y. enterocolitica*," ISO 10273:2003; "Microbiology of food and animal feeding stuffs−Horizontal method for the detection of *Salmonella spp*", ISO 6579:2002; and "Microbiology of food and animal feedingstuffs−Horizontal method for the detection and enumeration of *L. monocytogenes*", ISO 11290−1:1996.

*Multiplexed CL Enzyme Immunoassay*. The four MAbs, specific for each bacteria species, were immobilized in the different subwells of the main well. In particular, 10 $\mu$L of a 300 ng/mL antibody solution in coating buffer (0.05 M carbonate/bicarbonate buffer solution, pH

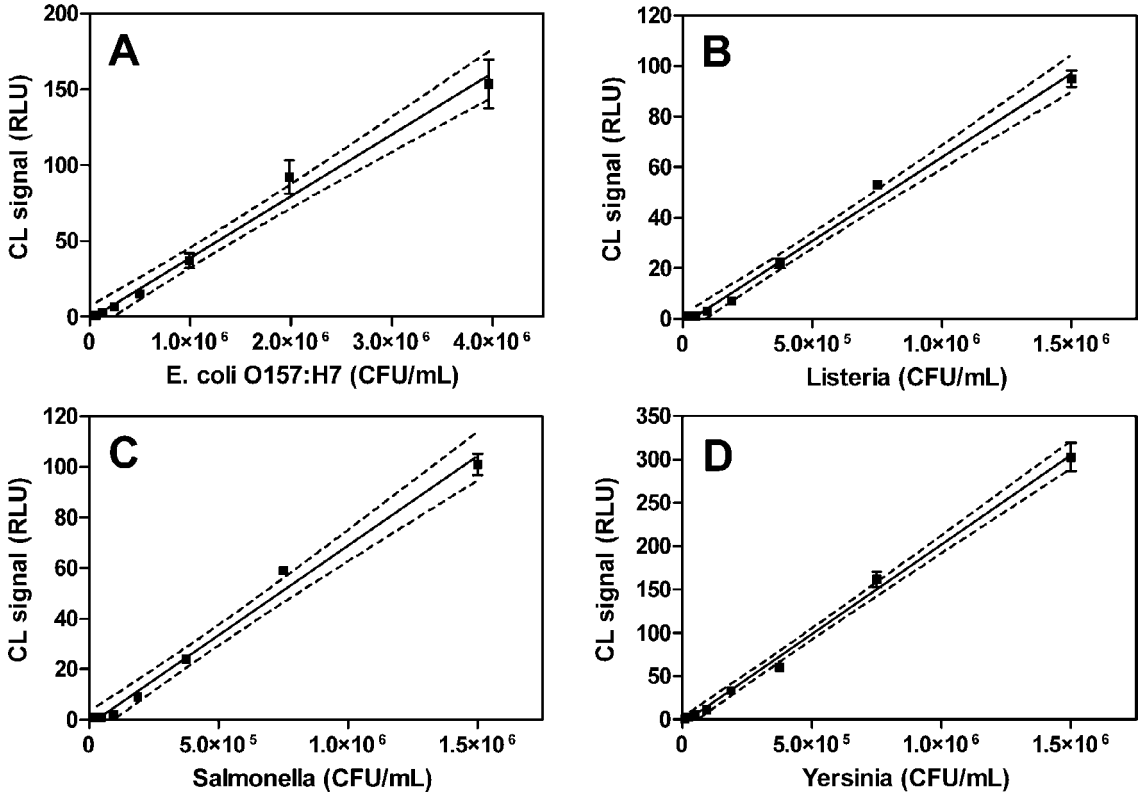


**Figure 2.** Representative calibration curves, obtained by averaging 10 standard curves obtained in different days for the determination of (**A**) *E. coli* O157:H7, (**B**) *L. monocytogenes*, (**C**) *S. typhimurium*, and (**D**) *Y. enterocolitica* using the CL-EIA method. The curves are expressed as relative light units (RLU) against the bacteria concentration (CFU/mL).

9.6) was dispensed in each individual subwell and the plates were incubated overnight at 4 °C. After the wells were washed with washing buffer (PBS containing 0.05% Tween 20), 100 $\mu$L of blocking solution (PBS containing 1% bovine serum albumin) was added to each main well, and the plates were further incubated at 4 °C for 2 h. The solution was discarded, and the plates were washed three times with washing buffer and vacuum-dried. The plates were either used immediately or sealed in plastic bags together with a 5 g package of silica gel desiccant (Sigma) and stored at 4 °C until use.

To perform the assay, 100 $\mu$L of sample suspensions (or bacteria standard solutions) subjected to the preanalytical enrichment step was added to each main well of the microtiter plate and incubated for 30 min at 37 °C. During the incubation, the bacterial cells in the sample were captured by the corresponding MAb in one of the four subwells. After three washings, 100 $\mu$L of a mixture of the HRP-labeled polyclonal antibodies specific for the four bacteria species was added and incubated for 30 min at 37 °C. The HRP activity of the bound labeled antibodies was measured, after washing, by means of an enhanced luminol-based CL substrate (SuperSignal ELISA Femto, Pierce) and an imaging device (LB 981 Night Owl, EG&G Berthold, Pforzheim, Germany), which employs a highly sensitive, back-illuminated, cooled CCD camera. Images were acquired using a 40 s exposure time, and the CL signal of each subwell was measured by integrating the photon emission over the subwell area. Before data analysis, the blank signal was subtracted from all of the sample and calibration curve signals. Then, the initial concentration of each bacterium in the samples was determined by interpolation of the CL signals on the calibration curves obtained from the bacteria standard solutions.

## RESULTS AND DISCUSSION

**Monoclonal Antibodies Screening**. The peculiarity of the monoclonal antibodies used in this study was their ability to recognize all of the strains of the four target bacteria without any cross-reactivity with the other bacteria species tested. To evaluate the MAbs specificity, all of the strains correlated with the four target bacteria (i.e., with the same antigenic characteristics) were tested. In addition, some other common food-related bacterial species were examined (see **Table 1** and the Supporting Information). Approximately 3600 hybridoma supernatants were obtained, and among these, 200−400 clones showed positive reaction to the four target bacteria by an indirect ELISA test. Subsequently, a further ELISA screening with other bacterial antigens led to the identification of four MAbs (8B8C3, 54B11, 1B6D9, and 6F12C8) specifically reacting with *E. coli* O157:H7, *Y. enterocolitica*, *S. typhimurium*, and *L. monocytogenes*, respectively, and showing no cross-reaction (CR) to the other bacterial strains tested. These MAbs, which were identified as IgG1 isotypes, were used for the development of the CL-EIA method. The anti-*Y. enterocolitica* MAb was also used in a previously published work (*32*).

**Table 2.** Precision of the CL-EIA Method[a]

| sample concentration | found concentration mean ± SD | RSD (%) |
|---|---|---|
| | within-assay ($n = 6$) | |
| 1 | 1.10 ± 0.09 | 8.2 |
| 10 | 9.72 ± 0.82 | 8.4 |
| 100 | 99.2 ± 7.5 | 7.6 |
| | between-assay ($n = 6$) | |
| 1 | 1.22 ± 0.16 | 13.1 |
| 10 | 9.33 ± 1.24 | 13.3 |
| 100 | 99.5 ± 11.2 | 11.2 |

[a] The table reports the results obtained for *E. coli* O157:H7 spiked meat samples. The values are expressed as CFU/g.

**Table 3.** Results Obtained after the Analysis of Fecal and Meat Samples with Both the Developed CL-EIA Method and the Reference Microbiological Method[a]

| meat samples | | | | fecal samples | | | |
|---|---|---|---|---|---|---|---|
| Cl-EIA method | | reference method | | Cl-EIA method | | reference method | |
| *S. typhimurium* | 150 | *S. typhimurium* | 148 | negative | | negative | |
| negative | | negative | | *Y. enterocolitica* | 50 | *Y. enterocolitica* | 44 |
| negative | | negative | | *E. coli* O157:H7 | 75 | *E. coli* O157:H7 | 77 |
| *E. coli* O157:H7 | 102 | *E. coli* O157:H7 | 107 | negative | | negative | |
| *E. coli* O157:H7 | 16 | *E. coli* O157:H7 | 18 | negative | | negative | |
| negative | | negative | | *E. coli* O157:H7 | 64 | *E .coli* O157:H7 | 57 |
| *S. typhimurium* | 112 | *S. typhimurium* | 121 | *E. coli* O157:H7 | 94 | *E. coli* O157:H7 | 96 |
| negative | | negative | | negative | | negative | |
| negative | | negative | | negative | | negative | |
| negative | | negative | | *E. coli* O157:H7 | 97 | *E. coli* O157:H7 | 95 |
| negative | | negative | | *E. coli* O157:H7 | 102 | *E. coli* O157:H7 | 105 |
| *E. coli* O157:H7 | 134 | *E. coli* O157:H7 | 126 | negative | | negative | |
| *L. monocytogenes* | 32 | *L. monocytogenes* | 36 | S. *typhimurium* | 110 | *S. typhimurium* | 98 |
| negative | | negative | | *L. monocytogenes* | 28 | *L. monocytogenes* | 23 |
| *E. coli* O157:H7 | 140 | *E. coli* O157:H7 | 147 | negative | | negative | |
| *S. typhimurium* | 120 | *S. typhimurium* | 118 | negative | | negative | |

[a] The values are expressed as CFU/g.

**CL-EIA Method.** *MAbs and HRP-Labeled Polyclonal Antibodies Concentrations.* Preliminary experiments were performed to evaluate the optimal concentration of the four MAbs and the HRP-labeled polyclonal antibodies. Antibody concentrations were optimized by comparing dose−response curves obtained using different initial concentrations of MAbs (300, 400, and 600 ng/mL) and different dilutions of HRP-labeled polyclonal antibodies (1:40000, 1:60000, and 1:80000 v/v), according to an optimized experimental design. A satisfying compromise in terms of linearity of the dose−response curve and limit of detection was obtained by using a MAb concentration of 300 ng/mL in the immobilization step and a 1:60000 (v/v) HRP-policlonal antibody dilution in the detection step.

*Detection System.* The developed CL-EIA assay allows us to simultaneously recognize the four target bacteria thanks to the specificity of each antibody pair and the use of an imaging detection technique based on an ultrasensitive CCD camera. A typical CL signal obtained using the new microtiter plate format is shown in **Figure 1C**: the CL signal is well-localized in the subwells, thus allowing its quantification by integrating the CL emission over the entire subwell area (*33*).

Thanks to the spatial resolution of the imaging system and the high detectability of the HRP label enzyme by CL, the sensitive and rapid quantification of each bacterial species was performed in low reaction volumes without any interference of the CL signal among adjacent subwells even in presence of signals with different intensities. This assay format thus prevents cross-talk phenomena, which have been observed, for example, in microarray systems (*34*, *35*). In addition, because of the standard size of the plate, the measurement could also be performed in conventional microtiter plate luminometers for 384 well microtiter plates. However, the sequential reading of all of the subwells could require as long as 2−5 min, depending on the signal integration time for each well, whereas an imaging device allows the simultaneous measurement of the whole plate. Therefore, one would have to check the constancy of the light output over the entire period of measurement, especially for high CL signals. The instrumental possibility to discriminate between the signal from a subwell and those from the other subwells in the same main well should be also verified.

*Calibration Curves.* The calibration curves of the CL-EIA assay obtained for the four bacteria (**Figure 2**) were linear in the range between $10^4$ and $10^6$ CFU/mL, and the relative standard deviation (RSD) of individual points ranged from 3 to 9%. The quantification limit (LOQ), calculated as the bacteria concentration giving a signal equal to the mean signal of negative controls plus 10 times their standard deviations (SDs), were in the range of $10^4-10^5$ CFU/mL.

Different incubation times and temperatures were also examined, and the best performance was obtained when each incubation step was performed for 30 min at 37 °C. Longer incubation times did not produce any significant improvement in the performance of the immunoassay.

*Within-Assay and Between-Assay Precision*. The precision of the method was determined by analyzing replicates of meat and fecal samples spiked with the four target bacteria at three different concentrations: 1 (low level), 10 (medium level), and 100 (high level) CFU/g of each bacteria. The bacteria-fortified samples were analyzed in six replicates and on four different days to evaluate the within-assay and the between-assay precision. The results obtained for *E. coli* O157:H7 (mean and relative SD calculated at each theoretical concentration level) are summarized in **Table 2**. Both the within- and the between-assay RSDs were below 15%. Similar results were obtained for all of the other bacteria species tested.

*Stability of the Coated Plates*. The stability of the antibody-coated plates (vacuum-dried and stored at 4 °C) was investigated by comparing five calibration curves obtained using plates stored at either 0, 1, 3, 6, or 8 months. Experimental data show that storage of the antibody-coated plates for up to 6 months did not significantly alter the performance of the assay in terms of the limit of detection.

**Method Validation.** A total of 32 samples (16 fecal and 16 meat samples) were analyzed for the presence of the four target bacteria with both the developed CL-EIA method and the reference microbiological methods. No false negative or false positive results were observed, and for all samples the bacteria strains detected using the CL-EIA method corresponded to those revealed by the microbiological reference methods (**Table 3**). In addition, there was a good agreement between the bacteria concentrations determined with the CL-EIA method and those measured with the microbiological ones, as demonstrated by the recovery values ranging between 90 and 120%.

The obtained results prove that the newly developed CL-EIA method allows the simultaneous quantification of four pathogen bacteria in a given sample with accuracy and precision

comparable to those achievable with the conventional microbiological methods. In addition, the multiplexed assay format allows a reduction of assay time and sample and reagents consumption with respect to conventional single-analyte immunoassays and classical culture microbiology procedures. Other multiplexed assays could also be developed using the same approach, that is, by customizing the plate layout to divide each main well in the required number of subwells.

## ABBREVIATIONS USED

LOQ, limit of quantification; CL, chemiluminescent; HRP, horseradish peroxidase; CL-EIA, chemiluminescent enzyme immunoassay; FCA, Freud's complete adjuvant; IFA, Freud's incomplete adjuvant; MAb, monoclonal antibody; UPB, universal peptone broth; PBS, phosphate-buffered saline; BPW, buffered peptone water; SD, standard deviation; RSD, relative standard deviation; CFU, colony-forming units; RLU, relative light units; CR, cross-reaction.

**Supporting Information Available:** List of other bacterial strains screened by indirect ELISA. This material is available free of charge via the Internet at http://pubs.acs.org.

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