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Production and characterisation of monoclonal antibodies specific for *Escherichia coli* O157:H7

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Summary

Seven monoclonal antibodies (MAbs) specific for *Escherichia coli* O157:H7, one of the major causes of haemorrhagic colitis in humans, were produced by immunising Balb/c mice with the strain *E. coli* O157:H7. These monoclonal antibodies do not cross-react with other bacteria such as *Salmonella enterica* serovar Typhimurium, *E. coli* O14, *E. coli* JM109, *S. enterica* serovar Enteritidis, *S. panama*, *S. saintpaul*, *S. derby*, *S. muenchen*, *S. bredeney*, *S. hadar*, *Yersinia enterocolitica*, *Proteus vulgaris*, *Shigella flexneri*, *Listeria ivanovii*, *L. monocytogenes* 13M, *L. innocua*, *Enterobacter cloacae*, *E. agglomerans*, *E. amnigenus*, *Citrobacter freundii*, *Escherichia fergussoni* or *Klebsiella pneumoniae*. Of the seven MAbs obtained, MAb 8B8C3 was selected to prepare a high-sensitivity sandwich ELISA method specific for O157:H7.

Keywords

Escherichia coli O157:H7, Monoclonal antibodies, Sandwich ELISA.

Introduction

Escherichia coli is a bacteria commonly present in both the animal and human intestinal tract, and is also widespread in the environment (3). There are numerous types and strains, some of which are potential pathogens which can cause disease in humans through various infectious and toxinogenic mechanisms. Symptoms vary, depending on the strain involved and the resistance of the individual to infection.

E. coli serotype O157:H7 falls in the verocytotoxin producing *E. coli* (VTEC) category (4, 12). It produces two toxin groups that are fundamental to the pathogenic effect, namely: verocytotoxins (verocytotoxin 1 and 2 rRNA-N-glycosidase) and intimin (bacterial Tir ligand). In 1982, it was identified for the first time as one of the main pathogens causing haemorrhagic diarrhoea and haemorrhagic colitis, accompanied by a series of complications such as haemolytic uraemic syndrome (HUS) and thrombocytopenic purpura (TTP) (21). It is the third most common microorganism isolated from faecal cultures, after *Salmonella* and *Campylobacter*.

Routes of infection include poorly cooked ground beef and pork, raw, semi-cured sausages, unpasteurised fruit juices, vegetables, drinking water contaminated with non-chlorinated water (22) and dairy products, especially soft cheese and unpasteurised milk. Furthermore, some studies have shown that transmission may occur through direct person to person contact (8) as well as

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through contaminated food.

The *E. coli* serotype O157:H7 is traditionally identified through agglutination and immunofluorescence testing using polyclonal antibodies (1). However, use of polyclonal hyperimmune sera may give diagnostic results not specific for *E. coli* O157:H7 (15, 20). It was therefore decided to produce highly specific monoclonal antibodies (MAbs) for use in the development of fast, highly sensitive and specific methods for the determination of O157:H7 in foods, given that the procedures currently available are lengthy and are not highly specific.

Materials and methods

Bacterial strains

E. coli O157:H7 strain ATCC 35150 (American Type Culture Collection, Rockville, Maryland), was used to produce monoclonal and polyclonal antibodies. The following bacterial strains were used to characterise the MAbs: *Salmonella enterica* serovar Typhimurium (ATCC 14028), *E. coli* O14 (BGVV), *E. coli* (ATCC 25922), *E. coli* JM109, *S. enterica* serovar Enteritidis (ATCC 13076), *S. panama* field strain, *S. saintpaul* field strain, *S. derby* field strain, *S. muenchen* field strain, *S. bredeney* field strain, *S. hadar* field strain, *Yersinia enterocolitica* (ATCC 23715), *Proteus vulgaris* (ATCC 49132), *Shigella flexneri* (ATCC 120222), *Listeria ivanovii* (ATCC 19119), *L. monocytogenes* 13M (ATCC 7644), *L. innocua* (ATCC 33090), *Enterobacter cloacae* field strain, *E. agglomerans* field strain, *E. amnigenus* field strain, *Citrobacter freundii* field strain, *Escherichia fergussoni* field strain and *Klebsiella pneumoniae* field strain. Seven field strains of *E. coli* O157:H7 were also used.

Antigen preparation

The bacterial strains were cultured in brain heart infusion broth (CM225, Oxoid Ltd, London) at 37°C for 14-16 h. The suspension was then subjected to heat treatment at 60°C for 1 h to obtain cell lysis, before being used at a concentration of 2×10^8 colony-forming units cfu/ml. Bacteria were then harvested by centrifugation at 5 468 x g for 30 min and washed 3 times in 0.01 M phosphate buffered saline (PBS) (pH 7.2) by centrifugation at 5 468 x g for 30 min. The pellet was then suspended in 1 xPBS. This antigen was used in MAb cross-reactivity tests in indirect ELISA. For mouse immunisation and subsequent ELISA screening of hybridomas and Western blotting, the suspension of *E. coli* O157:H7 strain ATCC 35150 was sonicated for 2.5 min x2 at five minute intervals in an ice bath. The protein concentration of all bacterial suspensions was determined using the BCA assay reagent kit (Pierce, Rockford).

Mouse immunisation

Anti *E. coli* O157:H7 MAbs were produced by immunising Balb/c mice with heat-deactivated, sonicated *E. coli* O157:H7 strain ATCC 35150 (10). The cell lysate was diluted in incomplete Freund's adjuvant (IFA) (Sigma, Missouri), to obtain a protein concentration of 50 µg/ml, and was then inoculated intraperitoneally. After 14 days, a second inoculation of 50 µg/ml cell lysate in IFA was performed. Two inoculations of 25 µg/ml cell lysate in PBS were then administered. A booster of 50 µg/ml cell lysate in PBS was given 31 days after immunisation. The mice were sacrificed on day 34.

Cell fusion and hybridoma cloning

Cell fusion and hybridoma cloning were performed using Galfre's method (6) with some modifications. Briefly, splenocytes from the immunised mice were subjected to cell fusion with myeloma cells from Sp2/O-Ag-14 mice (ATCC, Rockville, Maryland) using 50% polyethylene glycol (PEG) 1 550 solution. Hybridomas were cultured for two weeks in Dulbecco's modified Eagle's medium (DMEM) (Sigma, Missouri) containing 20% foetal bovine serum (Euroclone, UK), 2 mM L-glutamine (HybriMax®, Sigma, Missouri), amphotericin-penicillin-streptomycin 100x (APS) (Sigma, Missouri),

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gentamicin 50 mg/ml (gentamicin sulphate solution, Sigma, Missouri), nystatin 10 000 UI/ml (HybriMax® Nystatin suspension, Sigma, Missouri) and HAT 50x (HybriMax® HAT Media supplement, Sigma, Missouri). Antibody-secreting hybridomas were cloned by the limited dilution method (2, 7, 17). Monoclonal antibodies were produced *in vitro* on a large scale by serial culturing of antibody-secreting hybridomas and collection of their supernatants.

Indirect ELISA

Hybridomas secreting anti *E. coli* O157:H7 strain ATCC 35150 MAbs were screened by indirect ELISA (18). The same method was used to verify the cross-reaction with other bacterial strains and the various *E. coli* O157:H7 field strains (13). Briefly, the 96-well ELISA microtitre plates were coated with 10 µg/ml heat-deactivated, sonicated *E. coli* O157:H7 diluted in carbonate-bicarbonate buffer (50 mM, pH 9.6) for hybridoma screening and with 10 µg/ml of other heat-deactivated bacterial strains for MAb cross-reactivity tests. Plates were incubated overnight at 4°C, washed with PBS containing 0.05% Tween 20 (PBS-T) and saturated with 100 µl per well of PBS-T containing 1% yeast extract for 1 h at 37°C. Then, 100 µl of positive control (immunised mouse serum 1:5 000) and negative control (mouse serum 1:5 000) were added to the wells and the microtitre dish was incubated under agitation for 1 h at 37°C. After washing, 100 µl of an anti-mouse IgG polyvalent antibody conjugated with horseradish peroxidase (HRP) (Sigma, Missouri, USA) diluted 1:2 000 in PBS-T was added to each well. The microtitre dish was incubated for 30 min at 37°C. After further washing, 100 µl of chromogen substrate (3,3´, 5,5´-tetramethylbenzidine liquid substrate system for ELISA) was added to each well and the plate was incubated at room temperature for 30 min. The colorimetric reaction was then interrupted with 50 µl 1 N sulphure acid and the optical density (OD) was measured at 450 nm with a microtitre plate reader. Samples above the threshold value were considered positive. The threshold value was calculated as follows: 12 sera were taken from mice negative for *E. coli* O157:H7, tested in indirect ELISA against heat deactivated, sonicated *E. coli* O157:H7. Their OD_{450} was measured, with the mean plus three times the standard deviation being considered as the cut-off (0.3). The sensitivity of the method for hybridoma screening was assessed through the activation of plates containing decreasing concentrations of heat-deactivated, sonicated *E. coli* O157:H7 strain ATCC 35150. The specificity of the MAbs was evaluated using the following bacterial strains as indirect ELISA antigens: *S.* Typhimurium (ATCC 14028), *L. monocytogenes* (ATCC 7644) and *Y. enterocolitica* (ATCC 23715), prepared as described above.

Polyclonal antibodies

An anti-*E. coli* O157:H7 strain ATCC 35150 hyperimmune serum was produced by intradermally immunising New Zealand rabbits with an antigen quantity corresponding to 200 µg/ml of *E. coli* O157:H7 cell lysate in Freund's adjuvant complete (FAC) (Sigma, Missouri) six times over 50 days (9). The immunoglobulin G (IgG) serum fraction was purified by affinity chromatography with protein A using the Äkta-purifier chromatographic system (Amersham Pharmacia Biotech) and a column prepacked with recombinant protein A (HiTrap rProtein A FF, 5 ml, Amersham Biosciences), 0.1 M sodium phosphate binding buffer, pH 7.0, 0.1 M Glycine-HCl elution buffer, pH 3.0 and 1.0 M Tris-HCl neutralisation buffer, pH 9.0. IgGs were used as anti-*E. coli* capture antibodies. The IgG-isotype MAbs were purified using the same method and conjugated with peroxidase (19).

Monoclonal antibody characterisation

MAb isotypes were determined using the ImmunoPure® Monoclonal Antibody Isotyping Kit I (Pierce, Rockford). Sonicated, deactivated

E. coli O157:H7 was electrophoretically separated at 20 mA/gel constant current using a Mini-Protean 3 electrophoresis cell and 12% Tris-HCl polyacrylamide gel (Bio-Rad laboratories, California) (14). It was then transferred to 0.45 μm nitrocellulose membrane at a constant current of 35 mA for 55 min with the Mini Trans-blot® Electrophoretic transfer cell (Bio-Rad Laboratories, California). The nitrocellulose membrane was saturated with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 3% skimmed milk powder (Fluka, Missouri) for 1 h at 37°C. MAbs 8B8C3, 13A12G9, 1D6C8 and 5D4G5B7, purified and conjugated with peroxidase, were diluted in TBS-T and strips were incubated overnight at 4°C. After washing with TBS-T, the nitrocellulose strips were treated with Opti-4 Chloro-1-Naphthol substrate kit (Bio-Rad Laboratories, California) until the immunocomplexes were revealed (23).

Antigen preparation for sandwich ELISA

E. coli O157:H7 ATCC 35150 colonies were transferred from a Gram-negative bacteria-selective solid medium (Herellea Agar; Biolife Srl, Milan) to 5 ml of Luria-Bertani (LB) broth (Bacto® LB Broth, Lennox; Difco Laboratories; Detroit) and left to grow for 12-18 h at 37°C. The washed bacteria were diluted in isotonic phosphate-buffered saline (iPBS) until a spectrophotometric reading of 0.6 at OD_{600} was achieved, corresponding to approximately 7×10^8 cfu/ml. Heat-treated bacteria were used at various serial dilutions in iPBS to obtain suspensions in the range 6×10^5 –2×10^8 cfu/ml. These were used as standards for the ELISA calibration curve.

The other bacterial strains tested (*E. coli* strain JM109, *S.* Typhimurium ATCC 14028, *Y. enterocolitica* ATCC2371, *L. monocytogenes* ATCC7644) were cultured in brain heart infusion broth (CM225, Oxoid Ltd, London) at 37°C under static incubation conditions for 14-16 h. The suspension was then subjected to heat treatment to obtain cell lysis, before being used at a concentration of 2×10^8 cfu/ml.

Sandwich ELISA

The 96-well ELISA microtitre plates were coated with polyclonal anti *E. coli* O157:H7 antibodies (100 μl/well), at a concentration of 5 μg/ml in carbonate/bicarbonate buffer (50 mM, pH 9.6), incubated overnight at 4°C, and then saturated with iPBS containing 1% BSA at 4°C for 2 ions (from 6×10^5 to 2×10^8 cfu/ml), negative control (PBS 1x) and negative culture broth were dispensed in the microtitre plates, which were then incubated for 1 h at 37°C. After washing, 100 μl of the purified MAbs 8B8C3, 13A12G9, 1D6C8 and 5D4G5B7, diluted 1:250 v/v in iPBS with 0.05% Tween 20 (iPBS-T) were added and the plates were incubated for 1 h at 37°C. After a further wash, 100 μl/well of anti-mouse antibody conjugated with horseradish peroxidase (Miles Scientific, Naperville) diluted in iPBS was added, and incubated for 30 min at 25°C in the dark. For colorimetric measurement, 100 μl of the horseradish peroxidase chromogen substrate (1,2-phenylenediamine [0.5 mg/ml] and H_2O_2 [2.25 mmol/l] in citrate buffer [0.05 mol/l, pH 5.0]) were added and the plate was incubated in the dark for 20 min at room temperature. The colorimetric reaction was then blocked with 100 μl 4 N sulphuric acid and the OD was measured at 492 nm with a microtitre plate reader (Multiskan EX, Labsystems) (11). For chemiluminescence measurement, 100 μl of HRP chemiluminescent substrate was added, consisting of luminol/H_2O_2/enhancer (Pierce, Rockford) and the signal was immediately measured with a microtitre plate luminescence reader (Luminoskan Ascent, Labsystem). The calibration curve was obtained from the plot of the recorded signal (absorbance or chemiluminescent emission) against bacterial concentration. To evaluate the sandwich ELISA specificity, bacterial suspensions of *E. coli* strain JM109, *S.* Typhimurium (ATCC14028), *Y. enterocolitica* (ATCC23715) and *L. monocytogenes* (ATCC7644) were analysed.

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Results

Hybridoma screening

Six different fusions of murine splenocytes with the tumour cell line Sp2/O-Ag-14 were effected, from which 3 612 cell hybridoma supernatants were obtained and tested. A total of 101 hybridomas were positive against *E. coli* O157:H7 on indirect ELISA testing ($OD_{450}\geq0.3$). An initial cross-reactivity test was performed using the following as antigens: *E. coli* O157:H7 (ATCC 35150), *S.* Typhimurium (ATCC 14028), *L. monocytogenes* (ATCC 7644), *Y. enterocolitica* (ATCC 23715); and 34 MAbs were found positive for *E. coli* O157:H7 only and negative against the other 3 antigens tested. A further ELISA screening of these 34 MAbs with 25 bacterial antigens led to the identification of 7 MAbs reacting with *E. coli* O157:H7 only and not with the other bacterial antigens (Table I). These were subjected to further cross-reactivity testing against seven field strains of *E. coli* O157:H7. Results are reported in Table II.

Monoclonal antibody characterisation

The isotype was determined for seven MAbs found positive against *E. coli* O157:H7 on indirect ELISA (Table III). Subsequent characterisation tests using Western blotting were then performed on 4 IgG isotype MAbs. MAb 8B8C3 reacted with a single 37 kDa band, corresponding to a polysaccharide O chain component (5). This band was not present in immunoblotting of the other 3 MAbs; MAb 5D4G5B7 reacted with 4 bands of differing molecular weights (73, 78, 115, 140 kDa), MAb 13A12G9 produced two bands (73, 181 kDa) and MAb 1D6C8 produced one band (73 kDa) (Fig. 1).

Sandwich ELISA

Of the MAbs tested, the best result was obtained with MAb 8B8C3. MAb 13A12G9 did not produce any signal significantly greater than that of the blank even at high bacterial concentrations, while MAb 1D6C8 and MAb 5D4G5B7 produced a calibration curve characterised by a very high, aspecific signal, resulting in a significant increase in the limit of detection (LOD) of the method and a reduction in the linear response range.

The sandwich ELISA was refined using MAb 8B8C3. The LOD was calculated by extrapolating the mean absorbance/emission value of the blank plus three times its standard deviation from the standard curve. The LOD was found to be 1.8×10^6 cfu/ml for both the colorimetric and the chemiluminescence methods. The calibration curve ranged from 1.8×10^6 to 8×10^7 cfu/ml. The method is highly specific in comparison with both the *E. coli* strain JM109 and the other bacterial strains tested, which did not produce a signal significantly greater than that of the blank even at high concentrations (1×10^{10} cfu/ml).

Discussion

European Union (EU) laws on food hygiene control implemented in Italy have introduced a number of specifications that aim to produce hygienic, safe food products with long-term stability. There is currently a great need for methods that can rapidly detect the presence of potential pathogens and residues of unwanted substances in foods. In the first group, the identification of *E. coli* O157:H7 is of primary importance, and must also be distinguished from non-O157:H7 *E. coli*.

This approach has numerous advantages, as any immunoenzymatic methods developed on the basis of quality criteria enable the analysis of large numbers of samples in a very short time. In consequence, they can be used for rapid screening of foods potentially contaminated with bacteria – considerably reducing costs – as well as for the supply chain controls required by current EU standards, such as hazard analysis and critical control point (HACCP). Other possible benefits include greater specificity and a broad dynamic range.

Table I
Monoclonal antibody cross-reactions with various bacterial antigens at OD_{450} in indirect ELISA

Monoclonal antibodies	8B8C3*	13A12G9	5D4G5B7	1D6C8	13E4B10	13E4D6	13A12F10
Salmonella Enteritidis (ATCC13076)	0.02	0.00	0.00	0.00	0.00	0.01	0.00
Escherichia coli 014 (BGVV)	0.06	0.01	0.01	0.00	0.05	0.02	0.05
E. coli JM109 (field strain)	0.02	0.03	0.03	0.01	0.02	0.02	0.03
E. coli (ATCC25922)	0.02	0.00	0.00	0.00	0.00	0.00	0.00
Yersinia enterocolitica (ATCC23715)	0.01	0.00	0.02	0.00	0.00	0.00	0.00
Klebsiella oxitoca (ATCC49131)	0.00	0.00	0.00	0.00	0.00	0.00	0.02
Proteus vulgaris (ATCC48132)	0.00	0.00	0.00	0.00	0.03	0.00	0.00
Shigella flexneri (ATCC12022)	0.02	0.00	0.02	0.00	0.00	0.02	0.00
Listeria ivanovii (ATCC19119)	0.01	0.00	0.00	0.01	0.00	0.00	0.00
L. monocytogenes (ATCC7644)	0.04	0.00	0.00	0.01	0.00	0.00	0.01
L. innocua (ATCC33090)	0.02	0.00	0.05	0.01	0.04	0.00	0.00
Enterobacter cloacae (field strain)	0.01	0.00	0.00	0.00	0.00	0.03	0.00
E. cloacae (field strain)	0.01	0.00	0.00	0.00	0.00	0.00	0.00
E. agglomerans (field strain)	0.00	0.02	0.04	0.00	0.00	0.00	0.01
E. amnigenus (field strain)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Citrobacter freundii (field strain)	0.00	0.00	0.00	0.02	0.01	0.02	0.00
Escherichia fergussoni (field strain)	0.02	0.00	0.00	0.00	0.00	0.00	0.00
Klebsiella pneumoniae (field strain)	0.03	0.00	0.05	0.00	0.00	0.00	0.00
Salmonella panama (field strain)	0.03	0.00	0.00	0.02	0.00	0.02	0.04
S. saintpaul (field strain)	0.02	0.00	0.05	0.00	0.00	0.00	0.00
S. derby (field strain)	0.02	0.00	0.00	0.00	0.00	0.00	0.00
S. derby (field strain)	0.01	0.00	0.03	0.01	0.00	0.02	0.00
S. muenchen (field strain)	0.01	0.00	0.02	0.00	0.00	0.00	0.00
S. bredeney (field strain)	0.00	0.00	0.00	0.00	0.02	0.00	0.00
S. hadar (field strain)	0.00	0.00	0.01	0.00	0.01	0.02	0.01
E. coli 0157:H7 strain ATCC35150	3.00	0.40	1.80	0.30	2.60	1.00	0.30

* monoclonal antibody used for sandwich ELISA development

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Table II
Monocolonal antibody cross-reactions with various *Escherichia coli* O157:H7 field strains at OD_{450} in indirect ELISA

Monoclonal antibodies	E-D 393	E-D 412	E-D 506	E-D 390	E-D 268	E-D 416	E-D 521	*E. coli* O157:H7 strain ATCC 35150
8B8C3*	3.00	3.10	2.80	2.60	2.80	3.30	3.20	3.00
13A12G9	0.30	0.60	2.40	1.10	2.50	0.30	2.00	0.40
5D4G5B7	2.50	2.80	1.10	1.50	1.30	0.90	2.30	1.80
1D6C8	0.60	1.10	0.30	0.20	0.50	1.50	1.80	0.30
13E4B10	2.80	3.20	3.20	3.50	3.90	3.50	3.00	2.60
13E4D6	1.20	2.50	2.30	0.90	1.10	2.30	1.50	1.00
13A12F10	0.50	1.50	0.60	2.30	1.80	0.30	0.80	0.30

* monoclonal antibody used for sandwich ELISA development

A highly specific 'sandwich' immunoenzymatic method was developed to satisfy these requirements. The availability of reagents whose properties enable their successful use in the refinement of such methods, including MAbs specific for a given antigen, is of fundamental importance. MAb 8B8C3 has these properties; in fact, it does not react with the other bacteria tested – and, above all, does not recognise non-O157 *E. coli* strains – in either indirect or sandwich ELISA. At 1.8 x 10^6 cfu/ml, the sensitivity of the method is comparable with that of other commercially

Table III
Monoclonal antibody isotype

Monoclonal antibody	Isotype
8B8C3	IgG1 anti k
13A12G9	IgG1 anti k
5D4G5B7	IgG1 anti k
1D6C8	IgG1 anti k
13E4B10	IgA anti k
13E4D6	IgA anti k
13A12F10	IgA anti k

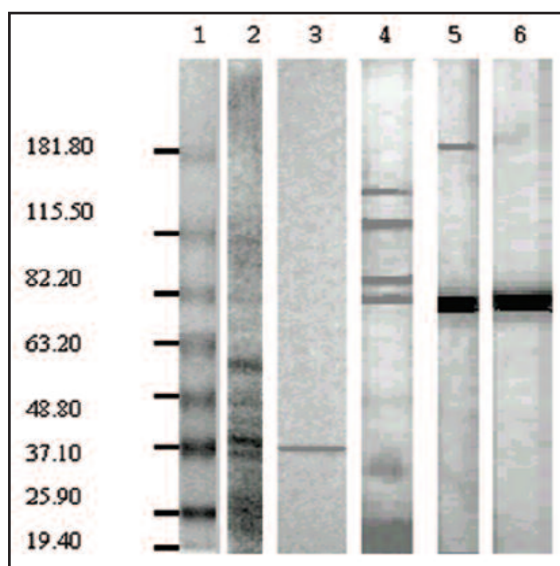

Figure 1
SDS PAGE electrophoresis and Western blotting of MAbs vs *Escherichia coli* O157:H7

1 Markers
2 SDS PAGE electrophoresis heat-deactivated, sonicated *E. coli* O157:H7
3 Western blotting MAb 8B8C3 vs heat-deactivated, sonicated *E. coli* O157:H7
4 Western blotting MAb 5D4G5B7 vs. heat-deactivated, sonicated *E. coli* O157:H7
5 Western blotting MAb 13A12G9 vs. heat-deactivated, sonicated *E. coli* O157:H7
6 Western blotting MAb 1D6C8 vs. heat-deactivated, sonicated *E. coli* O157:H7

available methods. In this study, the chemiluminescence detection system did not give a lower LOD than the colorimetric method, but is nonetheless advantageous as the signal can be read immediately after the addition of the substrate, thus reducing analysis times. Sandwich ELISA, combined with enrichment in culture broth, can be used as an alternative method for the detection of *E. coli* O157:H7 in food products. The procedure is easy to perform, cheaper and faster than traditional microbiological methods involving the isolation and subsequent typing of bacterial strains. Diagnostic screening of *E. coli* O157 in foods is conducted according to ISO/DIS 16654, which specifies pre-enrichment, subsequent concentration through magnetic immunoseparation and isolation on selective CT-SMAC culture medium (16). Confirmation is provided through biochemical and immunological tests (12). Other antigen or nucleic acid detection procedures, such as immunoblotting, PCR of virulence genes or nucleic acid probes, are expensive to conduct and, in contrast with sandwich ELISA, are less adapted to screening of high numbers of samples.

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