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Development of a Capture ELISA for Rapid Detection of *Salmonella enterica* in Food Samples

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Abstract
In this study, an immunology-based assay that employed specific monoclonal antibodies binding with somatic or flagella antigens of *Salmonella enterica* subsp. *enterica* was performed. As this pathogen is one of the most important bacterial species responsible for foodborne outbreaks, its detection in food by rapid and easy methods is properly suitable. After a first screening by indirect ELISA, three monoclonal antibodies (1B6D9, 1B6C11, 1D12F11) *versus S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 (whole antigen) and another one (4E6F11) *versus S. enterica* flagellin were further characterized by immunoblotting and mass spectrometry analysis. Then, a total of 84 food samples (dairy products, meat, pasta and flour, eggs, and animal feed) were analyzed by both the official method ISO 6579:2002 and *S. enterica* capture ELISA. For the standardization of the last method, the specific monoclonal antibody 4E6F11 was selected. The developed *Salmonella* capture ELISA showed a significant agreement with the official method (ISO 6579:2002). Relative sensitivity, specificity, and accuracy were 100%, 81.0%, and 90.5%, respectively. Therefore, this assay could represent a valid alternative to conventional methods able to detect this pathogen in food.

Keywords *Salmonella enterica* · Monoclonal antibodies · Capture ELISA · Food

Introduction

The microorganisms of genus *Salmonella* are Gram-negative, non-spore forming, and facultative anaerobic bacilli belonging to the family *Enterobacteriaceae*. This nomenclature has been an issue of debate for many years until in 2005, the Judicial Commission of the International Committee on the Systematics of Prokaryotes officially approved for this designation (Tindall et al. 2005). The genus involves 2 species, *Salmonella enterica* and *Salmonella bongori*, and over 2,500 serotypes that are distinguished on the basis of O- and H-antigen present in lipopolysaccharide and flagellar complex, respectively (Bell et al. 2016). The flagellin protein containing the H-antigenic determinants constitutes the main structural component of flagella (Ronholm et al. 2011).

Salmonella-related foodborne infections are mostly caused by the two main serovars of *S. enterica*, i.e., *S.* Enteritidis and *S.* Typhimurium, which are found above all in poultry meat and eggs, but also beef, milk, seafood, sprouted seeds, and fruits (Abdelhaseib et al. 2016; Kang et al. 2017). *Salmonella* spp. are generally present in the intestinal tract of humans and animals, and therefore, the fecal matter can be considered the main source of contamination (Andino and Hanning 2015). Some species, especially poultry and pigs, are relevant font of the microorganism, and their carcasses can be contaminated at abattoirs by carrier animal excreting/harboring *Salmonella* spp. or by equipment and utensils (Kore et al. 2017). Also, the water can be polluted by this pathogen causing the infection (Ho et al. 2018).

Salmonella enterica is the second most frequently reported zoonotic agent in the EU after thermotolerant *Campylobacter* spp., and more precisely, 45.7% and 15.8% of all reported serovars in human cases were represented by *S.* Enteritidis and *S.* Typhimurium, respectively. A total of 94,530 confirmed outbreaks of salmonellosis (128 fatal cases) were reported in the Member States of European Union (EU) in 2016, even if during the last 5 years (2012–2016) the trend did not show any statistically significant increase or decrease

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(EFSA and ECDC 2017). The human infection is characterized by symptoms of gastroenteritis (i.e., fever, nausea, vomiting, and diarrhea), generally self-limited but sometimes involving in invasive or recurrent forms (Bonardi et al. 2016; Fardsanei et al. 2017). Some authors (Andino and Hanning 2015) reported other chronic conditions including aseptic reactive arthritis and Reiter's syndrome. Moreover, severe systemic diseases can occur in infants, the elderly, and immune-compromised persons (Luo et al. 2018). Some infected animals and/or humans can become carriers of the microorganism releasing it in their feces for long periods of time. For this reason, they represent a reservoir and spread the pathogen among all the other farmed animals in the first case, or they constitute a source of contamination when healthy carrier personnel are working in a food company (Gopinath et al. 2012). Another concern linked to this microorganism is the potential antibiotic resistance to many antimicrobials such as penicillin, tetracyclines, fluoroquinolones, sulfonamides, aminoglycosides, and cephalosporins (Lamas et al. 2018). Moreover, in recent years, a wide range of outbreaks due to this microorganism has been reported in literature (García-Fierro et al. 2016; Brown et al. 2017).

Different methods able to detect, identify, and subtype *Salmonella* spp. in food have been developed. They can be distinguished in conventional culture-dependent methods, which require 3–7 days including selective enrichment steps for positive results to be confirmed by biochemical assays such as fermentation of glucose, urease reaction, and lysine decarboxylase, and molecular methods which allow a faster detection with high sensitivity and specificity, even if they involve expensive equipment and highly trained personnel (Bell et al. 2016). The latter often requires sample enrichment to increase *Salmonella* spp. numbers, improve sensitivity, and dilute compounds that may interfere with the assay. Moreover, several sets of primers are designed to detect genes specific for *Salmonella* spp. (Zaki et al. 2009). In recent years, immunology-based methods have been performed for the detection of foodborne pathogens (Wang et al. 2016; Luciani et al. 2018). These assays are often preferred for the screening of large number of food samples (Karoonuthaisiri et al. 2015). Moreover, these methods allow the detection of viable non-culturable *Salmonella* cells (Maciorowski et al. 2006). Nevertheless, they show some disadvantages, such as the potential of cross-reaction with closely related antigens, antigen variations, and limits in sensitivity for some sample matrices (Lee et al. 2015). The aim of the present study was the development of a rapid and easy method for the detection of *S. enterica* in food samples by use of mono- and polyclonal antibodies, in comparison with the official assay of ISO 6579:2002 (ISO 2002).

Materials and Methods

Bacterial Strains

Two strains of *Salmonella enterica* subsp. *enterica* (serovar Enteritidis and serovar Typhimurium) and other 12 strains belonging to different bacteria species (Table 1) were obtained from the American Type Culture Collection (ATCC). Further, 4 strains of *S. enterica* subsp. *enterica* (different serovars) were obtained from the National Collection of Type Cultures (NCTC). One strain of *Escherichia coli* O14 was provided from the Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin (BGVV), and one strain of *E. coli* O157:H7 was obtained from the Istituto Superiore di Sanità (ISS). Two strains of *Brucella* genus were provided from the Central Veterinary Laboratory (CVL, Weybridge), while one strain of *Vibrio cholerae* El Tor was obtained from the Istituto Sieroterapico of Milano. The remaining used strains ($N = 31$) of different microorganisms were taken from the collection of the IZSAM.

Preparation of Bacterial Antigens and *Salmonella enterica* Flagellin

The selected strains were cultured at 37 °C in Brain Heart Infusion (BHI) broth (Oxoid Ltd., London, UK) for 14–16 h. The final bacterial concentration was 2×10^8 CFU/mL. The bacterial cells were inactivated at 60 °C for 1 h and centrifuged at 5500 *g* for 30 min. The pellet was washed for three times with 0.01 M phosphate-buffered saline (PBS) pH 7.2 and resuspended in PBS. These antigens were used to characterize monoclonal antibodies (MAbs) by indirect ELISA (i-ELISA) and to evaluate the selectivity of capture ELISA.

Salmonella enterica subsp. *enterica* serovar Typhimurium ATCC 14028 was used for mice and rabbit immunization and hybridoma screening. It was sonicated in ice bath for 2 cycles of 2.5 s, and the sample was put on ice for 5 min between the two rounds of sonication. The bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA) was used to determine the total protein concentration of the sonicated antigen.

Flagellin was prepared as described by Ibrahim et al. (1985) from a culture of *S. enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 grown for 16 h at 37 °C on Luria-Bertani (LB) agar with agitation (Hiriart et al. 2013).

Cell suspensions and flagellin were stored at − 20 °C until use.

Immunization of Mice

Six 8-week-old female BALB/c mice were injected intraperitoneally with heat inactivated and sonicated *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 as described in

Table 1 Bacterial strains used in this study

Bacterial strains	*n*	Bacterial strains	*n*
Bacillus cereus ATCC 11778[b]	1	*Listeria ivanovii* ATCC 19119[b]	1
B. cereus (IZSAM)[b]	1	*Listeria monocytogenes* ATCC 7644[ab]	1
Bacillus subtilis (IZSAM)[b]	1	*L. monocytogenes* (IZSAM)[ab]	2
Bordetella bronchiseptica (IZSAM)[b]	2	*Proteus vulgaris* ATCC 49132[b]	1
Brucella abortus S99 (CVL Weybridge)[ab]	1	*Pseudomonas aeruginosa* (IZSAM)[b]	1
Brucella melitensis biovar 1 (CVL Weybridge)[ab]	1	*Salmonella enterica* subsp. *enterica* serovar Bredeney NCTC 5731[ab]	1
Campylobacter jejuni ATCC 33291[ab]	1	*S. enterica* subsp. *enterica* serovar Derby NCTC 5722[ab]	1
Citrobacter freundii (IZSAM)[b]	1	*S. enterica* subsp. *enterica* serovar Enteritidis ATCC 13076[ab]	1
Enterobacter agglomerans (IZSAM)[b]	1	*S. enterica* subsp. *enterica* serovar Enteritidis (IZSAM)[ab]	6
Enterobacter amnigenus (IZSAM)[b]	1	*S. enterica* subsp. *enterica* serovar Hadar NCTC 9877[ab]	1
Enterobacter cloacae (IZSAM)[b]	2	*S. enterica* subsp. *enterica* serovar Saintpaul NCTC 6022[ab]	1
Enterococcus faecium (IZSAM)[b]	2	*S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028[ab]	1
Escherichia coli O14 (BGVV)[ab]	1	*Shigella flexneri* ATCC 12022[b]	1
E. coli O6 ATCC 25922[ab]	1	*Staphylococcus aureus* ATCC 25923[b]	1
E. coli O157:H7 (ISS)[ab]	1	*Staphylococcus epidermidis* (IZSAM)[b]	2
E. coli O157:H7 (IZSAM)[ab]	1	*Staphylococcus faecalis* ATCC 29212[b]	1
Escherichia fergusonii (IZSAM)[b]	1	*Vibrio cholerae* El Tor (Sieroterapico Milano)[ab]	1
Klebsiella oxytoca ATCC 49131[b]	1	*Yersinia enterocolitica* O:8 ATCC 23715[ab]	1
Klebsiella pneumoniae (IZSAM)[b]	1	*Y. enterocolitica* O:9 (IZSAM)[ab]	3
Listeria innocua ATCC 33090[b]	1	*Y. enterocolitica* O:3 (IZSAM)[ab]	3

[a] Strains used for MAbs characterization; [b] Strains used for cross-reactivity determination of *Salmonella enterica* capture ELISA

Luciani et al. (2018). For the production of MAbs *versus S. enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 flagellin, female BALB/c mice were inoculated intraperitoneally with flagellin diluted in sterile PBS to a protein concentration of 50 μg/mL and emulsified with complete Freund adjuvant (CFA, Sigma, St. Louis, MO, USA). After 14 days, a second immunization was performed using the same concentration of antigen emulsified with incomplete Freund adjuvant (IFA, Sigma). On 20th and 50th days, 25 and 40 μg/mL of flagellin, respectively, diluted only in sterile PBS were injected.

The protocol for the animal experimentation was approved by the Italian Ministry of Health (number 5146 of 26/04/2012).

Characterization of Monoclonal Antibodies

The MAbs were characterized by i-ELISA *versus S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 (whole antigen), *S. enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 flagellin, and all the other strains (Table 1) as reported in Luciani et al. (2018). Then, the MAbs were isotyped using the Mouse-Typer Isotyping Panel (Bio-Rad, Hercules, CA, USA). The MAbs showing IgG isotype were further characterized by immunoblotting.

Five μg/well of *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028, *S. enterica* subsp. *enterica* serovar Enteritidis ATCC 13076, and flagellin were separated by SDS-PAGE at 200 V using NuPAGE 12% Bis-Tris Gels Mini (Life Technologies, Carlsbad, CA, USA). Then, they were transferred onto nitrocellulose membrane with iBlot Dry Blotting System (Life Technologies). The membranes were blocked with 5% skim milk in PBS containing 0.05% Tween 20 (PBS-T) for 2 h at room temperature (RT). After washing with PBS-T, they were incubated overnight at 4 °C with MAbs supernatants diluted 1:2 in PBS-T containing 2.5% skim milk. ECL anti-mouse IgG HRP-conjugated (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and a chemiluminescent substrate (ECL Select Western Blotting Detection Reagent, GE Healthcare) were used to detect immune-complexes. The results were analyzed by ChemiDoc MP (Bio-Rad) and Image Lab Software, version 4.0 (Bio-Rad).

The affinity chromatography by a HiTrap rProtein A FF column, 5 mL (GE Healthcare), was used to purify the MAbs with IgG isotype specific for *S. enterica*, produced *in vitro* on a large scale.

Mass Spectrometry Analysis (nLC-ESI-MS/MS)

Salmonella enterica subsp. *enterica* serovar Enteritidis ATCC 13076 proteins were separated using a NuPage® 12% Bis-Tris pre-cast gel (Life Technologies) (9.0 μg per well) at 200 V. The proteins were stained overnight with SimplyBlue SafeStain (Life Technologies). The stained gel was stored in deionized water containing 0.1% acetic acid at + 4 °C until protein analysis.

Two bands with an apparent molecular weight of respectively 88 and 80 kDa (Fig. 1) were cut out; the proteins were then subjected to standard in-gel destaining, reduction with 10 mM DTT, alkylation with 55 mM IAA, and trypsin digestion as previously reported by Shevchenko et al. (1996, 2006). After acidification with 0.1% formic acid, the peptide mixtures were concentrated and desalted on home-made StageTips C18 (Rappsilber et al. 2007; Soffientini and Bachi 2016). The peptides were injected on an UPLC EASY-nLC 1000 (Thermo Scientific) and separated on a home-made fused silica capillary column (75 μm i.d., length 25 cm), packed in house with ReproSil-Pur C18-AQ 1.9 μm beads (Dr. Maisch, Ammerbuch-Entringen, Germany). A gradient of eluents A (2% acetonitrile, 0.1% formic acid) and B (80% acetonitrile with 0.1% formic acid) was used to achieve the separation, from 5 to 100% B (in 30 min, 250 nL/min flow rate). The nLC system was connected to a quadrupole Orbitrap QExactive-HF mass spectrometer (Thermo Fisher) equipped with a nanoelectrospray ion source (Proxeon Biosystems). The MS data were acquired using a data-dependent top 15 method for HCD fragmentation. Survey full scan MS spectra (300–1750 Th) were acquired in the Orbitrap with 60,000 resolution, AGC target 1^{e6}, IT 120 ms. For HCD spectra, resolution was set to 15,000 at *m*/*z* 200, AGC target 1^{e5}, IT 120 ms; NCE, 28%; and isolation width, 3.0 *m*/*z*. The raw data were processed with Proteome Discoverer (version 1.4.1.14, Thermo Scientific) and Mascot (version 2.6.0, Matrix Science) searching against *S. enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 protein database, assuming a fragment ion mass tolerance of 20 mg/Kg and a parent ion tolerance of 10 mg/Kg; the specified enzyme was trypsin, the carbamidomethylation of cysteine was set as a fixed modification, the oxidation of methionine and acetylation of the N-terminus of proteins were set as variable modifications. Scaffold (version 4.4.3, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS-based peptide and protein identifications. The peptide identifications were accepted if they could be established at greater than 95% probability by the Scaffold Local FDR algorithm. The protein identifications were accepted if they could be established at greater than 99% probability and contained at least three identified peptides. The protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Proteins that contained similar peptides could not be differentiated based on MS/MS analysis alone, and they were grouped to satisfy the principles of parsimony.

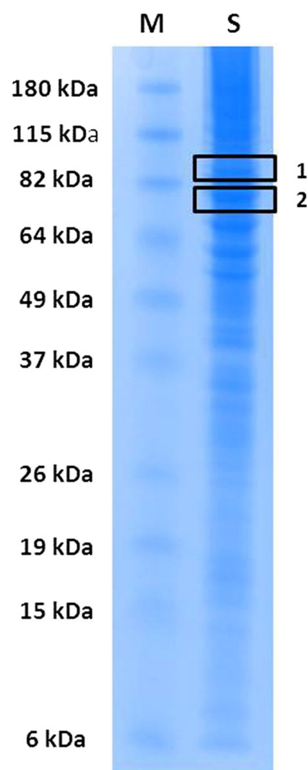

Fig. 1 SDS-PAGE protein pattern of *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 (Lane S) obtained using a 12% polyacrylamide gel. The two bands analyzed by mass spectrometry are shown. Lane M: molecular weight marker (BenchMark Prestained Protein Ladder, Life Technologies)

Polyclonal Antibodies Production and Characterization

Female New Zealand rabbits were inoculated subcutaneously six times over 50 days with heat inactivated and sonicated *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 (whole antigen) diluted with sterile PBS to a protein concentration of 200 μg/mL and emulsified with CFA (Sigma). Serum titers were checked by i-ELISA. Two months after the first immunization, antisera with adequate titer, affinity, and specificity were obtained. The rabbit IgG were purified using the same protocol described for MAbs purification. Rabbit IgG protein concentration was measured spectrophotometrically at 280 nm. Purified polyclonal antibodies were labeled with horseradish peroxidase (HRP) (Sigma) as reported in the literature (Nakane and Kawaoi 1974).

The protocol of the animal experimentation was approved by the Italian Ministry of Health (number 11520 of 24/10/2013).

Salmonella enterica Capture ELISA

One hundred microliters per well of a MAb *versus S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 at a concentration of 20 μg/mL in 50 mM carbonate/bicarbonate buffer, pH 9.6, was put onto 96 well microplates (High Binding Costar, Corning, New York, NY, USA). The microplates were incubated overnight at RT. After washing with PBS-T for three times, 100 μL/well of culture positive, negative controls, and samples was added. The microplates were incubated under gentle shaking for 1 h at 37 °C and then washed. Further 100 μL/well of rabbit IgG HRP-conjugated was dispensed into all wells, and the microplates were incubated for 1 h at 37 °C. After further washing, they were again incubated with 100 μL/well of 3,3′,5,5′-tetramethylbenzidine (TMB Sigma) for 30 min at RT; the enzyme reaction was stopped by adding 50 μL/well 0.5 N sulfuric acid. The OD_{450nm} was measured with a microplate reader. The samples were considered positive or negative for the presence of *S. enterica* based on the ratio S/N between the OD_{450nm} of artificially contaminated samples (S) and the OD_{450nm} produced by the negative controls (N). *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 in PBS was used as positive control, and Rappaport-Vassiliadis (RV) Enrichment Broth (Oxoid) was used as negative control.

The cutoff was determined by analyzing serial dilutions in RV broth of *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 culture with a known bacterial concentration (1×10^5 CFU/mL). The number of CFU/mL in *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 culture was determined by plate count on xylose lysine deoxycholate (XLD) agar (Oxoid). The cutoff value, expressed as S/N, and the limit of detection were calculated by interpolating the mean absorbance of the negative control plus three times the standard deviation on the calibration curve obtained by plotting the bacterial concentration of the serially diluted *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 *versus* OD_{450nm} and S/N values.

The repeatability of this test was evaluated by analyzing the positive and the negative controls for 40 repetitions each; the reproducibility was assessed by testing the positive and the negative controls for 120 repetitions each. The selectivity was determined by analyzing the bacterial strains listed in Table 1.

Salmonella enterica Capture ELISA: Analysis of Food Samples

A total of 84 samples belonging to different categories, i.e., dairy product, meat, pasta and flour, egg, and animal feed (Table 2) were analyzed by both the official method ISO 6579:2002 and *S. enterica* capture ELISA. Among samples, 42 were artificially contaminated with strains of *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028. More in detail, 25 g of each sample was inoculated with *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 suspensions at a final concentration of 10^2 CFU/g. The inoculated food matrices were diluted with RV broth, homogenized in a stomacher, and then incubated at 25 °C for 48 h. The remaining 42 food samples were negative for *Salmonella* spp.

Table 2 Matrix of different food used in this study

Category	Matrix	P	N
Dairy product	Caciotta	4	8
	Gorgonzola	3	8
Meat	Rabbit flesh	2	0
	Chicken flesh	20	9
	Salami	4	0
	Sausage	0	2
Pasta and flour	Wheat flour	2	2
	Dried pasta	5	9
Egg	Egg yolk	0	1
	Egg shell	0	2
Animal feed	Chicken feed	2	1
Total		42	42

P, positive samples artificially contaminated; *N*, negative samples

Statistical Analysis

The comparison between results of the *S. enterica* capture ELISA and the official method ISO 6579:2002 was assessed using Cohen's kappa statistical test (Microsoft Office Excel 2010).

Results

Production and Characterization of Monoclonal Antibodies

The MAbs *versus S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 and *S. enterica* flagellin were first tested by i-ELISA *versus Yersinia enterocolitica* O:8 ATCC 23715, *E. coli* O157:H7 (ISS), and *Listeria monocytogenes* ATCC 7644. The MAbs that resulted specific for *S. enterica* were then screened with the other strains reported in Table 1. Upon screening, three MAbs (1B6D9, 1B6C11, 1D12F11) *versus S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 and one MAb (4E6F11) *versus S. enterica* flagellin were selected for further analysis and characterization.

The MAbs 1B6D9, 1B6C11, and 1D12F11 reacted with the following serovars of *S. enterica* subsp. *enterica*: Typhimurium, Bredeney, Derby, Hadar, and Saintpaul with a strong reaction ($OD_{450nm} > 0.600$); regarding cross-reactions

versus serovar Enteritidis ATCC 13076, MAb 1B6D9 did not react with it, and MAbs 1B6C11 and 1D12F11 showed a weak reaction ($0.300 < OD_{450nm} < 0.600$). The MAb 4E6F11 reacted with all the six serovars of *S. enterica* with a strong reaction ($OD_{450nm} > 0.600$). All the mentioned MAbs reacted also with *S. aureus* ATCC 25923 ($OD_{450nm} > 0.600$); the MAb 1B6D9 revealed a weak cross-reaction with *Pseudomonas aeruginosa* and *V. cholerae* El Tor, and the MAb 1B6C11 reacted with *V. cholerae* El Tor ($0.300 < OD_{450nm} < 0.600$).

The MAbs characterization performed using immunoblotting showed that MAbs 1B6D9, 1B6C11, and 1D12F11 reacted with low molecular weight proteins (MW < 6 kDa) of the serovars Typhimurium, Bredeney, Derby, Hadar, and Saintpaul. The MAb 1D12F11 showed also a weak reaction with a 52 kDa band of all the six serovars (data not shown). The MAb 4E6F11 reacted with an 88 kDa band and with low molecular weight proteins (MW < 6 kDa) of the six serovars; it also reacted with an 80 kDa band of serovar Enteritidis ATCC 13076 and with two bands (75 and 64 kDa) of serovar Derby NCTC 5722 (Fig. 2). The MAb 4E6F11, tested by immunoblotting *versus* the purified flagellin, reacted with bands showing molecular weight ranging from 47 to 59 kDa, according to a previously published study (Ibrahim et al. 1985). The other three MAbs (1B6D9, 1B6C11, and 1D12F11) did not react with the purified flagellin.

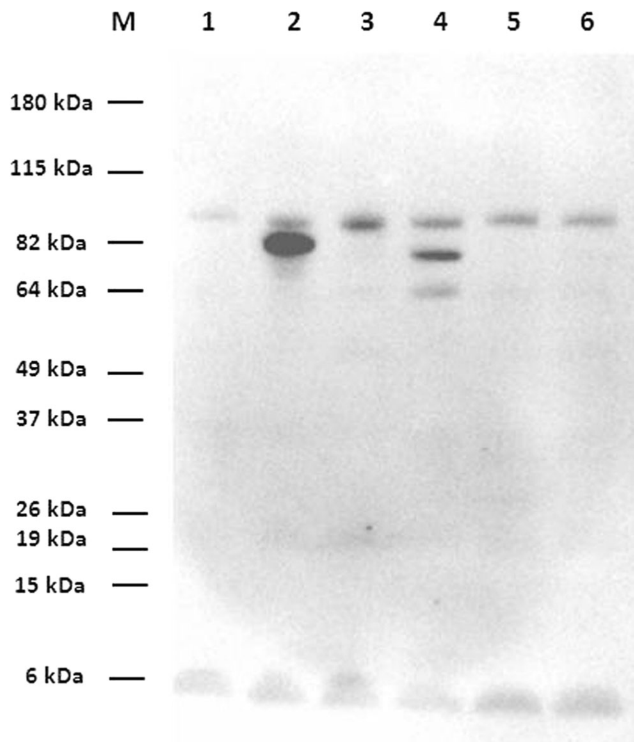

Fig. 2 Characterization of the monoclonal antibody 4E6F11 by western blottting. Lane 1: *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028; lane 2: *S. enterica* subsp. *enterica* serovar Enteritidis ATCC 13076; lane 3: *S. enterica* subsp. *enterica* serovar Bredeney NCTC 5731; lane 4: *S. enterica* subsp. *enterica* serovar Derby NCTC 5722; lane 5: *S. enterica* subsp. *enterica* serovar Hadar NCTC 9877; lane 6: *S. enterica* subsp. *enterica* serovar Saintpaul NCTC 6022; lane M: molecular weight marker (BenchMark Prestained Protein Ladder, Life Technologies)

Table 3 Repeatability (40 repetitions) and reproducibility (120 repetitions) of *Salmonella enterica* capture ELISA

Samples	Repeatability			Reproducibility		
	S/N	SD	CV %	S/N	SD	CV %
Positive	2.4	0.191	8.1	2.6	0.377	14.5
Negative	1.0	0.120	12.6	1.0	0.171	17.4

S/N, OD 450 nm sample/OD 450 nm negative control; *SD*, standard deviation; *CV*, coefficient of variation

Mass Spectrometry Analysis (nLC-ESI-MS/MS)

The SDS-PAGE bands with MW 88 (band 1) and 80 kDa (band 2) of *S. enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 (Fig. 1) were analyzed by mass spectrometry in order to identify proteins recognized by the MAb 4E6F11. The band 1 was recognized by this MAb in all the six serovars of *S. enterica* subsp. *enterica* tested in this study; the band 2 resulted to be specific for the serovar Enteritidis.

A total of 261 proteins were identified, according to selected validation criteria (protein threshold, 99.0%; peptide threshold, 95.0%; minimum three peptides/protein). Two hundred eleven proteins were found in the band 1, and 154 proteins were found in the band 2; 104 proteins were found in both the analyzed bands.

Among the proteins resulted common to the two bands, flagellin (accession number FLIC_SALEN) was identified. According to UniProt data, flagellin contains 505 amino acids and has a molecular weight of 53 kDa.

Salmonella enterica Capture ELISA

For the standardization of the *S. enterica* capture ELISA, the MAb 4E6F11 was selected as capture antibody coated to ELISA microplates. The cutoff value of the capture ELISA, expressed as S/N, was found to be 1.70. Food samples producing a S/N ratio greater than or equal to the cutoff value were considered as positive for *S. enterica*, whereas samples producing an S/N ratio lower than 1.70 were considered as negative. The limit of detection was 7.8×10^2 CFU/mL.

The repeatability of the capture ELISA for the positive and the negative controls was 8.1% and 12.6%, respectively; the reproducibility for the positive and the negative controls was 14.5% and 17.4%, respectively (Table 3).

Among bacterial strains used to evaluate selectivity of *S. enterica* capture ELISA, only *Salmonella* strains and *S. aureus* ATCC 25923 resulted to be cross-reactive.

Table 4 Cross-reactions of *Salmonella enterica* capture ELISA

Bacterial strains	S/N*	Bacterial strains	S/N
Bacillus cereus ATCC 11778	0.6	*Listeria ivanovii* ATCC 19119	0.6
B. cereus (IZSAM)	0.7	*Listeria monocytogenes* ATCC 7644	0.5
Bacillus subtilis (IZSAM)	0.8	*L. monocytogenes* (IZSAM)	0.6
Bordetella bronchiseptica (IZSAM)	1.5	*Proteus vulgaris* ATCC 49132	0.9
Brucella abortus S99 (CVL Weybridge)	0.9	*Pseudomonas aeruginosa* (IZSAM)	0.9
Brucella melitensis biovar 1 (CVL Weybridge)	0.9	*S. enterica* subsp. *enterica* serovar Bredeney NCTC 5731	2.1
Campylobacter jejuni ATCC 33291	0.8	*S. enterica* subsp. *enterica* serovar Derby NCTC 5722	2.4
Citrobacter freundii (IZSAM)	0.8	*S. enterica* subsp. *enterica* serovar Enteritidis ATCC 13076	2.7
Enterobacter agglomerans (IZSAM)	0.8	*S. enterica* subsp. *enterica* serovar Enteritidis (IZSAM)	2.5
Enterobacter amnigenus (IZSAM)	1.4	*S. enterica* subsp. *enterica* serovar Hadar NCTC 9877	2.1
Enterobacter cloacae (IZSAM)	0.9	*S. enterica* subsp. *enterica* serovar Saintpaul NCTC 6022	3.6
Enterococcus faecium (IZSAM)	0.7	*S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028	4.4
Escherichia coli O14 (BGVV)	1.1	*Shigella flexneri* ATCC 12022	0.9
E. coli O6 ATCC 25922	0.8	*Staphylococcus aureus* ATCC 25923	2.5
E. coli O157:H7 (ISS)	1.2	*Staphylococcus epidermidis* (IZSAM)	1.0
E. coli O157:H7 (IZSAM)	1.0	*Staphylococcus faecalis* ATCC 29212	0.6
Escherichia fergusonii (IZSAM)	0.9	*Vibrio cholerae* El Tor (Sieroterapico Milano)	0.9
Klebsiella oxytoca ATCC 49131	0.9	*Yersinia enterocolitica* O:8 ATCC 23715	1.2
Klebsiella pneumoniae (IZSAM)	0.6	*Y. enterocolitica* O:9 (IZSAM)	0.9
Listeria innocua ATCC 33090	0.6	*Y. enterocolitica* O:3 (IZSAM)	1.1

* S/N ≥ 1.70, positive; S/N < 1.70, negative

Salmonella strains showed S/N ratios ranging from 2.1 to 4.4; *S. aureus* ATCC 25923 gave an S/N ratio of 2.5. Other tested bacterial strains provided S/N ratios lower than the cutoff value (Table 4).

The relative sensitivity, specificity, and accuracy for the 84 food samples analyzed in this study were 100%, 81.0%, and 90.5%, respectively (Table 5). All the positive food samples were positive with *S. enterica* capture ELISA; 8 out of the 42 negative samples resulted to be false positive. They were as follows: 2 samples of egg shell, 1 sample of dried pasta, and 5 samples of chicken flesh. The microbiological analysis revealed the presence of *Proteus* spp., *Serratia* spp., and *Staphylococcus xylosus* in all the false positive samples. However, the agreement index of *Salmonella* capture ELISA against the ISO 6579:2002 was significant (Cohen's kappa value, 0.81).

Table 5 Relative sensitivity, specificity, and accuracy (expressed as percentage, %) of *Salmonella enterica* capture ELISA compared to ISO 6579:2002

Parameters	Observed value	95% LCL	95% UCL
Relative sensitivity	100.0	93.3	100.0
Relative specificity	81.0	66.6	90.0
Relative accuracy	90.5	82.3	95.0
Positive predictive value	84.0	71.4	91.6
Negative predictive value	100.0	91.8	100.0
Cohen's kappa value	0.81		

LCL, lower confidence limit; *UCL*, upper confidence limit

Discussion

In the present study, monoclonal and polyclonal antibodies were produced in order to develop a rapid immunoenzymatic method for the detection of *Salmonella* spp. in food. The MAb 4E6F11 *versus* flagellin of *S. enterica* subsp. *enterica* serovar Enteritidis was selected because it showed the best selectivity in comparison with other MAbs. When tested by immunoblotting *versus* purified flagellin, the MAb 4E6F11 reacted with bands ranging from 47 to 59 kDa, according to literature data (Ibrahim et al. 1985), whereas when it was tested *versus* the whole antigen of *Salmonella* serovar Enteritidis, it recognized

two bands of 88 and 80 kDa. The mass spectrometry analyses confirmed the presence of flagellin (MW 53 kDa) in both the bands. Probably, in the whole antigen, flagellin could be bound to other proteins that caused its migration in bands showing a molecular weight higher than 53 kDa.

The developed *S. enterica* capture ELISA showed a significant agreement with the official method ISO 6579:2002. The MAb 4E6F11 revealed a cross-reaction with *S. aureus* ATCC 25923; it could cross-react with other species of the genus *Staphylococcus* or other bacteria not tested in this study. In fact, 8 food samples negative for *Salmonella* resulted as positive when tested by capture ELISA: in these samples, *Proteus* spp., *Serratia* spp., and *S. xylosus* were found.

As *S. enterica* is a microorganism causing one of the most common foodborne infections, its occurrence in food can be a serious risk for the public health. Therefore, its rapid and easy detection is of basic importance. The production of MAbs against structural antigens of *Salmonella* spp. has been described in other studies (Schneid et al. 2005; Ronholm et al. 2011; Hiriart et al. 2013; Aribam et al. 2015), and the use of MAbs in ELISA tests has also improved the detection of *Salmonella* spp. in food. In particular, Schneid et al. (2005) reported a highly specific MAb for *Salmonella* spp., not reacting with heat-extracted antigens from other bacteria, whereas Ronholm et al. (2011) described new MAbs against lipopolysaccharide antigens of *S. enterica* serotype Typhimurium DT104.

The capture ELISA reported in this study could be considered a screening assay allowing to obtain results in shorter time than the official ISO 6579:2002. This last method requires multiple identification steps taking about 5 days for complete isolation and confirmation of the pathogen. In addition, it can give false positive results and is not properly addressed for the screening of large number of samples (Lee et al. 2015). *Salmonella enterica* capture ELISA could be suitable for the surveillance and monitoring of this microorganism because it required less time than traditional culture techniques and showed high specificity toward the target pathogen.

Conclusions

A rapid and alternative assay for the detection of *S. enterica* was developed and validated to apply to different food matrices. The investigated parameters (sensitivity, specificity, and accuracy) showed good values, and therefore, a large number of samples could be analyzed by this approach. The capture ELISA used in this study could represent a screening method able to obtain similar results to the official ISO 6579:2002, but in a shorter time and easy application.

Compliance with Ethical Standards

Conflict of Interest Tiziana Di Febo declares that she has no conflict of interest. Maria Schirone declares that she has no conflict of interest. Pierina Visciano declares that she has no conflict of interest. Ottavio Portanti declares that he has no conflict of interest. Gisella Armillotta declares that she has no conflict of interest. Tiziana Persiani declares that she has no conflict of interest. Elisabetta Di Giannatale declares that she has no conflict of interest. Manuela Tittarelli declares that she has no conflict of interest. Mirella Luciani declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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