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Real-time PCR detection of *Salmonella* spp. and *Listeria monocytogenes* in ready-to-eat foods: a comparison between the biomolecolar method and traditional microbiology

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Abstract

Objective: The aim of the present study is to evaluate the presence of *Salmonella* spp. and *Listeria monocytogenes* in ready-to-eat foods by comparing the performance and sensitivity of BIO-RAD commercial Kits based on real-time PCR detection with traditional culture (ISO) procedures.

Materials and methods: Sixty-five samples of ready-to-eat foods were analysed as described above. In order to verify the validity of both culture and biomolecolar methods and to compare the sensitivity of real-time PCR versus conventional culture (ISO) procedures, five food samples were artificially contaminated with the *Salmonella enteritidis* ATCC strain by using scalar concentration from 10^3 to 10^{-1} cfu/g while one food sample was artificially contaminated with the *Listeria monocytogenes* ATCC strain. Finally, statistical analyses of the results were performed using the statistics "K" to confirm the agreement between the compared methods.

Results: Both procedures showed the absence of *Salmonella* spp. and *Listeria monocytogenes* in the processed samples; results in agreement appeared both for the five food samples artificially contaminated with *Salmonella enteritidis* ATCC strain and for the food sample artificially contaminated with *Listeria monocytogenes* ATCC strain. The sensitivity of the biomolecolar test was 1 cfu/g. Therefore full agreement between the two methods was detected, with a K value of 1.

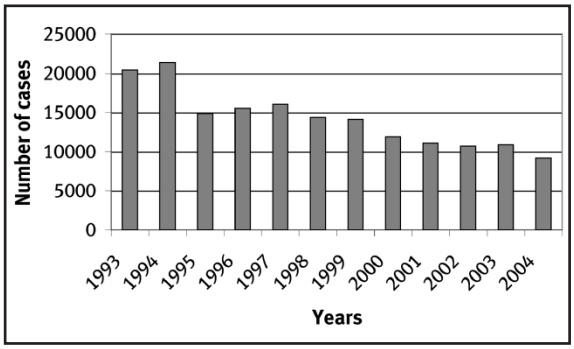
Conclusions: The real-time PCR system appears to be extremely useful in the rapid screening of food samples, allowing for the rapid identification of *Salmonella* spp. and *L. monocytogenes*.

Key words: Salmonella, Listeria monocytogenes, ready-to-eat foods, real-time PCR, conventional culture methods.

Introduction

In recent years the diffusion of mass catering has increased in the industrialised countries. In Italy it is estimated that around 10 million people each day consume one of the daily meals in a collective restaurant. [1] Moreover, fully cooked, modified atmosphere or vacuum packaged foods, including savoury fully prepared meals, fully cooked side dishes, dairy and dessert items, deli meats and sandwiches are becoming increasingly common. [2] The spread of ready-to-eat products is an important issue for Public Health since no further cooking with high, bactericidal temperatures affect them before their distribution. Food-borne infections caused by members of the genus *Salmonella* still continue to be a problem all over the world (Table 1). These infections cause considerable mortality, morbidity and economic burden and are especially severe in the immunocompromised, the very young and the elderly.[4] Despite the use of good manufacturing practices (GMPs), the introduction of the Hazard Analysis and Critical Control Point (H.A.C.C.P.) and a decreasing trend for these food-borne infections, in Italy an average of 14,200 cases of Salmonellosis were notified each year during the time-period 1993-2004, with a minimum of 9,180 cases in 2004 and a maximum of 21,350 in 1994 (Figure 1). [5]

Figure 1. Notified cases of Salmonellosis in Italy from 1993 to 2004 (Data source: Ministry of Health)



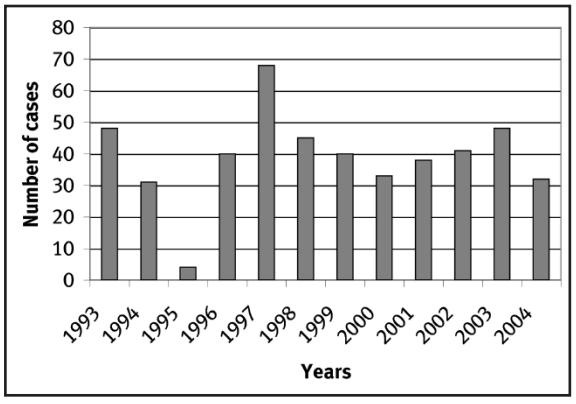
In addition to the *Salmonella* genus, *Listeria monocytogenes* represents a significant cause of

Table 1. Reported foodborne outbreaks of Salmonellosis and Listeriosis

Year	Country	Food	Cases	Outbreak
1979	USA	Sedano, tomatoes, lettuce	20	Listeriosis
1980	New Zealand	Seafoods, raw fish	29	Listeriosis
1980-1981	Canada	Coleslaw	41	Listeriosis
1981	Maritime (CDN)	Cabbages salad	41	Listeriosis
1982	Great Britain	Chocolate	245	Salmonellosis
1982	Norway	Black pepper	126	Salmonellosis
1983	USA	Pasteurized milk	49	Listeriosis
1983-1987	Switzerland	Vacherin cheese	122	Listeriosis
1984	Great Britain	Gelatine meat	1,000	Salmonellosis
1985	California (USA)	Cheese	142	Listeriosis
1986	Austria	Crude milk, vegetables	28	Listeriosis
1986-87	Philadelphia	Ice-creams, salted pork meats	36	Listeriosis
1987	China	Ice cream	1,113	Salmonellosis
1987-89	United Kingdom (UK)	Belgian paté	› 350	Listeriosis
1988	Great Britain	Raw shoot	143	Salmonellosis
1989	Connecticut	Prawns	9	Listeriosis
1990	Perth (Austria)	Paté	20	Listeriosis
1990	USA	Melon	245	Salmonellosis
1991	Etiopia	Eggs	79	Salmonellosis
1992	Japan	Water	680	Salmonellosis
1992	Sri Lanka	Monkey meat	9	Salmonellosis
1992	New Zealand	Smoked mussels	4	Listeriosis
1992	France	Pork tongue in aspic	279	Listeriosis
1993	Germany	Fried potatoes with paprika	1,000	Salmonellosis
1993	France	Poultry	276	Salmonellosis
1994	Austria	Eggs	219	Salmonellosis
1994	USA	Milk chocolate	45	Listeriosis
1995	France	Raw-milk soft cheese	20	Listeriosis
1995	Great Britain	Curry rice	6	Salmonellosis
1998-1999	Finland	Butter	11	Listeriosis
1998-1999	US	Frankfurters	101	Listeriosis
2000	US	Turkes deli meat	29	Listeriosis

(Source: Boccia A, Ricciardi G, De Giusti M, La Torre G. Igiene generale, della scuola e dello sport, 2002 [3], Harris LJ. Foodborne disease [2], modified).

Figure 2. Notified cases of Listeriosis in Italy from 1993 to 2004 (Data source: Ministry of Health)



food-borne disease in the USA and in other developed countries. [2] It has been responsible for a relatively small number of food-borne outbreaks (Table 1) but is of concern because of its high case-fatality rate. [2] In Italy, during the period 1993-2004 an average of 39 [2], modified cases of Listeriosis were notified annually [5], however the underreporting of infectious diseases and the increasing trend during the years 2000-2003 (Figure 2) must be taken into consideration.

Traditional detection procedures for the mentioned bacteria use a selective enrichment in broth followed by the isolation of colonies on selective agar. *Salmonella* cells can take three to four days to provide a negative result and up to seven days in order to confirm a positive one. [4] Conventional bacteriological methods for the detection of *Listeria monocytogenes* are also time consuming, taking up to 13 days to provide a definitive serological confirmation. The lengthy nature of all these procedures has led to the development of diagnostic systems for the rapid detection of *Salmonella* and *Listeria* in agricultural products and foods, among them enzyme-linked immunosorbent assays (ELISA),

DNA probe hybridisation and polymerase chain reaction (PCR) techniques. [6] These methods, in some instances, can eliminate or reduce the time required for enrichment, by quickly providing a positive or negative sample evaluation. [2]

The aim of the present study is to evaluate the presence of *Salmonella* spp. and *Listeria monocytogenes* in ready-to-eat foods by comparing the BIO-RAD commercial Kits based on a real-time PCR detection with traditional culture procedures.

Materials and methods

Sixty-five samples of ready-to-eat foods – pasta, rice, meat, chicken, turkey, cooked mozzarella, fish, omelette, cooked and raw vegetables as well as potatoes - collected from a variety of refectories from February to December 2006 were analysed for the evaluation of the presence of *Salmonella* spp. and *Listeria* spp. using ISO methods.

The same samples were also screened using the biomolecolar methods iQ-Check *Salmonella* and *Listeria* kits for real-time PCR detection by BIORAD.

The ISO method for *Salmonella* spp. detection [7] in foods is based on five distinct steps. Firstly, a non selective pre-enrichment is performed in Buffered Peptone Water (BPW) with 25 grams of the food sample; a second selective enrichment can be achieved by using a variety of different selective broths, those that are generally recommended are Rappaport-Vassiliadis medium with soya broth (RVS) and Muller-Kauffmann tetrathionate novobiocin broth (MKTTn). After incubation, the cultures obtained are inoculated by a sterile loop on Petri dishes containing two selective agars: Xylose Lysine Desoxycholate Agar (XLD) and Brilliant Green Agar Modified (BGA).

The ISO method for *Listeria spp*. detection [8] in food combines primary enrichment in Half Fraser Broth with 25 grams of sample and secondary enrichment in Fraser Broth. The cultures obtained in both enrichment broths are inoculated on two selective plating-out media, Palcam Agar and Oxford Agar.

These methods can take an average of four days before a negative result is known and up to 13 days for biochemical screening and serological confirmation.

The iQ-Check *Salmonella* and *Listeria* Kits are simple and rapid qualitative tests, with results obtainable within three hours following overnight pre-enrichment of sample. Using real-time PCR *Salmonella* or *Listeria* specific DNA sequences are amplified and detected simultaneously by means of fluorescent probes.

In real-time PCR, specific oligonucleotide probes, called Molecular Beacons, are used to detect DNA during the amplification, by hybridizing to the amplicons. These probes are linked to a fluoresces only when hybridized to the target sequence; in the iQ-Check *Salmonella* Kit, FAM is the fluorophore linked to the probe hybridizing to the *Salmonella* specific DNA sequence; in the iQ-Check *Listeria* Kit, TEXAS RED is the fluorophore linked to the probe hybridizing to the *Listeria* specific DNA sequence. In the absence of target DNA, no fluorescence will be detected and the sample determined to be negative. As the amount of amplicons increases with each round of amplification, fluorescence intensity also increases, whereas the associated software plots the fluorescence intensity versus number of cycles. To monitor for a successful reaction, a synthetic DNA "internal control" is included in the reaction mix; this control is amplified with a specific probe at the same time as the target DNA sequence and detected by second fluorophore. The results of the method can be analysed directly at the end of the PCR run by the iCycler software showing the graph for the fluorophore to analyse. Results are interpreted by analysing the Ct value of each sample (the cycle at which the amplification curve crossed the threshold). A positive sample must have a Ct value $\geq$ 10 for the specific fluorophore, with the internal control always being positive. In addition, the kits provide an external positive control, with a Ct value from 26 to 36 being considered as positive and a negative control that must provide a Ct value = N/A (not applicable, when the fluorescence of a sample does not rise significantly above the background noise, and hence does not cross the threshold).

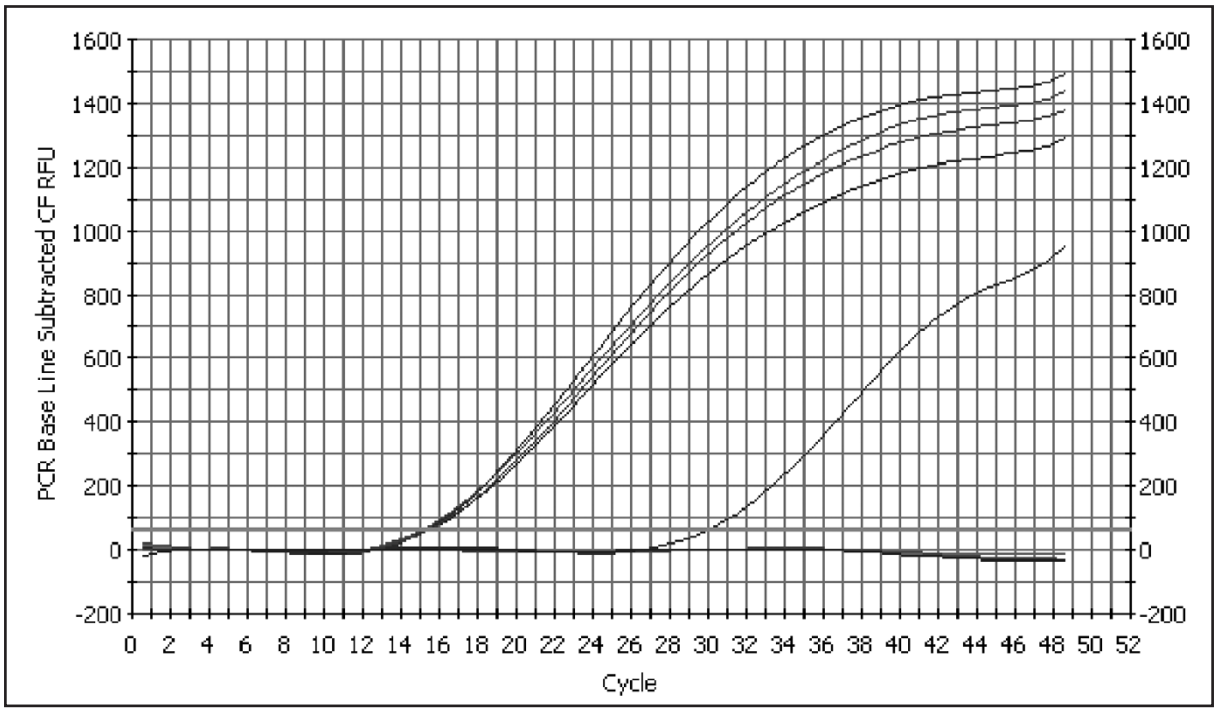
Lastly, in order to verify the validity of both culture methods and the biomolecolar ones and to compare the sensitivity of the real-time PCR versus conventional culture (ISO) procedures, five food samples were artificially contaminated with *Salmonella enteritidis* ATCC 13076 strain by using scalar concentrations from 10^3 to 10^{-1} cfu/g, while one food sample was artificially contaminated with *Listeria monocytogenes* ATCC 7644 strain.

Finally, statistical analyses of the results were performed using the statistics "K" to confirm the agreement between the compared methods.

Results

Both standard culture methods and biomolecolar methods showed the total absence of *Salmonella* spp. and *Listeria monocytogenes* in all of the analysed samples. Results that are in agreement appeared for the artificially contaminated samples,

Figure 3. PCR amplification cycle graph (FAM-490) for a food sample contaminated with different concentrations of *Salmonella enteritidis* ATCC strain (ranging from 10^3-1 CFU/g). (The positive internal control of the kit appears at the 29.9 cycle).



both for *Salmonella enteritidis* and for *Listeria monocytogenes*. Thus we had a 100% correlation between biomolecolar method and ISO methods, with the sensitivity of the biomolecolar test of 1 cfu/g. For the food samples artificially contaminated with *Salmonella enteritidis* ATCC strain with different concentration from 10^3 to 1 cfu/g., the Ct value was > 10, indicating a positive result; negative result appeared when the concentration was 10^{-1} cfu/g (Figure 3). The Ct value of the positive control was 29.9, while for the negative control it was N/A (Figure 3). Identical results were obtained for the conventional microbiological cultures.

The full agreement between the two methods was statistically confirmed, with a K value of 1.

Discussion

Food-borne infections caused by members of the genus *Salmonella* and *Listeria* continue to be a public health problem in Italy, in developed countries and in the industrialized world. Furthermore, the emergence and persistence of highly virulent and antibiotic-resistant *Salmonella* strains in recent years are major public health concerns. The increasing prevalence of the pentavalent antibiotic-resistant *S. typhimurium* DT 104 in humans and in animal meats and the relentless human pandemic of *S. enteritidis* from the consumption of eggshells and poultry are disquieting. [6] *Salmonella* spp. still remains the main cause of notified food-borne infections in Italy. [5]

In addition to the importance of raw and undercooked eggs, meat, poultry and dairy products as potential vehicles of human Salmonellosis, there are increasing reports of outbreaks associated with vegetables and fresh fruit. [9] In recent years new foods such as fruit juices and vegetable sprouts have been incriminated as vehicles of human *Salmonella* infections. [6]

Moreover, the survival of *Salmonella* spp. on dry stainless steel surfaces reported in a study published four years ago has pointed out the risk of cross-contamination of ready-to-eat foods. [10]

Outbreaks of Listeriosis have been associated with vegetable, dairy, seafood, meat products, deli meats, paté and hot dogs [2]. It is estimated that 80-90% of listeriosis cases are linked to ingestion of contaminated food [2]. *L. monocytogenes* continue to be a major public health problem because it is capable of multiplying at temperatures between approximately 0° and 45-50°C (this means that *L. monocytogenes* can multiply under refrigerated conditions) [2]; growth at refrigerator temperatures on a wide variety of fresh-cut fruits and vegetables has been reported [2]. It is relatively resistant to NaCl and low pH, it is not inhibited significantly by carbon dioxide and can survive many processing techniques such as drying and freezing (*L. monocytogenes* can survive for long periods of time in frozen foods). [2]

The risk of infection by *Salmonella* spp. and *L. monocytogenes* is high if contaminated foods are "ready-to-eat", because they are not subjected to further cooking at high temperatures.

Moreover, the Regulation CE 2073/2005 points out the importance of the absence of *Salmonella* spp. and *L. monocytogenes* in ready-to-eat foods, in order to ensure food safety and consumer health. [11]

Conventional detection methods for *Salmonella* cells can take 3 to 4 days to produce a negative result and up to 7 days for a confirmed positive result. [4] Conventional bacteriological methods for *Listeria monocytogenes* detection can take up to 13 days for a definitive serological confirmation.

For all of these reasons, it is evident why the necessity for quick methods to evaluate the presence of *Salmonella* spp. and *L. monocytogenes* in food samples exists, especially if they are "ready-to-eat products".

In recent years the lengthy nature of the culture procedures available has led to the development of many different diagnostic systems for the rapid detection of *Salmonella* and *Listeria* in foods and agricultural products. [6]

Among them, the development of chromogenic media such as agar *Listeria* according to Ottaviani and Agosti (ALOA) has allowed for the more rapid detection of *Listeria monocytogenes*, with presumptive identification of this pathogenic species after only 24 hours of incubation. [12]

Biomolecolar methods available for detection and identification of *L. monocytogenes* have increased in the past decade, as well. Enzyme-linked immunosorbent assays and assays based on DNA probes or polymerase chain reaction (PCR) have been developed, tested and are readily available. [13,14] These methods can, in some cases, reduce the time required for enrichment or, after enrichment, quickly provide a positive or negative sample evaluation.

The DNA assay is able to detect values as low as 1 colony-forming unit of *Listeria* and *Salmonella* in 25 g of food sample, with results available as early as 48 hours after the start of sample enrichment process. [15]

Among all of these procedures, the biomolecolar methods tested (iQ-Check™) by using a PCR based method, amplify *Salmonella* spp. and *Listeria monocytogenes* specific sequences by means of fluorescent probes. Up to 96 samples can be processed using the same kit, with a minimized risk of contamination as well as being easy to use. One of the most important advantages of the kit experienced is that the use of this test allows results to be obtained within a few hours following the pre-enrichment of a sample. [16,17]

In the present study, no *Salmonella* and *L. monocytogenes* were found in any of the samples examined, perhaps because almost all the collected samples were cooked, heat-treated ready-to-eat foods.

Prevalence data for *Salmonella* and *Listeria monocytogenes* in nine different categories of ready-to-eat meat and poultry products produced at approximately 1,800 federally inspected establishments were presented and discussed in a study published six years ago [18]; some of cumulative 10-year *Salmonella* prevalence figures were as follows: cooked, uncured poultry products, 0.10%; large-diameter cooked sausages, 0.07%; small-diameter cooked sausages, 0.20%; cooked beef, roast beef, and cooked corned beef, 0.22%; while some cumulative 10-year *L. monocytogenes* prevalences were: cooked, uncured poultry products, 2.12%; large-diameter cooked sausages, 1.31%; small-diameter cooked sausages, 3.56%; cooked beef, roast beef, and cooked corned beef, 3.09%. The prevalence data presented show the general low contamination rate of ready-to-eat food products, when they are heat treated.

Furthermore, the results of the BIO-RAD iQ-Check™ in our study were found to be in agreement with the conventional plating results. The correspondence both of negative results for the samples analysed and of positive controls for the samples artificially contaminated by well characterized strains of *Salmonella* and *Listeria* demonstrates the full agreement between the real-time PCR method tested and the conventional culture procedures based on ISO methods. Moreover, statistical analyses of these results indicate that this biomolecolar procedure performs equally well to cultural reference methods.

Furthermore, iQ-Check™ *Salmonella* and *Listeria* are validated by AFNOR as alternative methods to the reference method NF EN ISO 6579 (2002) and ISO 11290-1: 2004 (Appendix A) for the detection of *Salmonella* spp. and *Listeria monocytogenes* in all products for human consumption, as well as environmental samples. [16,17]

However, it is always important to underline that a positive test shows the presence of the DNA of the bacteria, but it is not able to demonstrate the presence of viable bacterial cells.

For this reason, the biomolecolar method should not be used as the sole basis for determining product safety, nor should it be used as the sole test for the release of a product.

Considering that one of the most important potential applications of PCR is identification testing, the real-time PCR is useful in supplementing, rather than replacing, existing tests for the final identification of, often problematic, rough presumptive *Salmonella* and *Listeria* isolates, for the rapid screening of samples, to ensure the early arrest of the productive cycle and for the quick identification of *Salmonella* and *Listeria* in food products, in order to ensure food safety and to protect consumers from infections.

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