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Development of 23 individual TaqMan® real-time PCR assays for identifying common foodborne pathogens using a single set of amplification conditions

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ABSTRACT

Most of the acute intestinal diseases are caused by foodborne pathogens with infants and elderly people being at major risk. The aim of this study was to develop a procedure to simultaneously detect 20 foodborne pathogens in complex alimentary matrices such as milk, cheese and meat. The list of targets include, among the others, *Listeria* spp., *Salmonella* spp., *Shigella* spp., *Escherichia coli* spp., *Campylobacter* spp., *Clostridium* spp. and *Staphylococcus aureus*. The accuracy of detection was determined by using ATCC strains as positive and negative controls. The achieved sensitivity of each of assays was 1 pg of genomic DNA, which was equivalent to ~1 cfu. The working ranges of the TaqMan® Real-time PCR assays, when used quantitatively on cheese and meat samples inoculated with serial dilution of *Listeria* spp., *Listeria monocytogenes*, *S. aureus*, *Salmonella enterica*, *Shigella boydii*, *E. coli* O157:H7, *Bacillus cereus*, *Campylobacter coli*, *Yersinia enterocolitica*, *Enterobacter sakazakii* and *Pseudomonas aeruginosa* was 10^8 cfu/g to 10^4 cfu/g. No matrix interferences were observed.

1. Introduction

Outbreaks of foodborne illness occur worldwide almost daily (Fleckenstein et al., 2010), and up to 30% of the population in industrialized nations suffers from foodborne illness each year (Severgnini et al., 2011). Over 320,000 new infections are reported each year in European Union only, but the real number is likely to be much higher.

Zoonoses are diseases that can be transmitted directly or indirectly from animals to humans through contaminated foodstuffs or contact with infected animals. The severity of human diseases varies from mild clinical signs to life-threatening conditions. Foodborne zoonotic diseases are caused by consuming food or drinking water contaminated with pathogenic bacteria, bacterial toxins, viruses, or parasites that invade the body via the gastrointestinal tract, where the first symptoms usually occur. Many of these microorganisms are commonly found in the intestines of healthy food-producing animals. The threat of foodborne pathogen contamination is present from farm to fork, requiring prevention and control throughout the food chain.

Microorganisms that are involved in foodborne illness include *Bacillus cereus*, *Clostridium botulinum*, and *Staphylococcus aureus* producing emetic toxin, botulinum toxin and enterotoxins, respectively (Balaban and Rasooly, 2000; Stevens et al., 2012; Kotiranta et al., 2000). Additionally, *Campylobacter* spp., *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7 are known to be responsible for the majority of foodborne illness outbreaks (Vijayalakshmi et al., 2010).

The traditional culturing techniques for the direct isolation and identification of foodborne pathogens are time-consuming and laborious. Conventional diagnostic methods mainly rely on specific biochemical and immunological identification. These methods are

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sensitive, inexpensive and in some cases quantitative. Culture-based methods also distinguish between viable and non-viable microorganisms. Major weak points include assay time, which can take 5–6 days for presumptive identification, and the presence of matrix-associated inhibitors that reduce assay sensitivity. Additionally, the levels of background microflora in the test sample may negatively affect the quality and quantity of DNA obtained (Leblanc-Maridor et al., 2011). Moreover, for most of foodborne pathogens an initial enrichment is needed to improve sensitivity since foodborne pathogens are typically present at low levels.

In vitro amplification of nucleic acid via PCR remains the most widely applied technique in both research and clinical laboratories for detection, identification, and enumeration of foodborne pathogens (Postollec et al., 2011). Compared with traditional plating methods, PCR is faster and more specific; PCR allows detection of sub-dominant species populations, directly in food test samples or following enrichment even in the absence of a species-differentiating medium. Real-time PCR has emerged as rapid diagnostic technique for foodborne pathogen detection (Fukushima et al., 2010). Quantitative PCR-based protocols are currently applied to enumerate a wide array of foodborne pathogens (Hoorfar, 2011). Moreover, the entire procedure, from DNA isolation to reaction preparation, is less expensive and final detection can be automated, this makes the technique suitable for routine analysis. For instance, detection of *L. monocytogenes* by real-time PCR methods, following enrichment requires 2 working days as opposed to 7 days by standard plating methods (O'Grady et al., 2009). Molecular detection of *Salmonella* in meat carcasses was performed in 26 h versus 5 days with the standard ISO method (McGuinness et al., 2009). The detection of *B. cereus* could be achieved within 2 h versus 2 days of the standard method, with comparable costs (Reekmans et al., 2009). The current trend is moving towards identification of several pathogens in the same reaction (Postollec et al., 2011; Garrido et al., 2012).

The objective of the present study was to develop a PCR protocol comprising 23 individual TaqMan reactions to simultaneously detect without selective enrichment, the most common foodborne pathogens present in products of animal origin such as milk, cheese and meat. The performance of this assay was assessed by using DNA purified from various ATCC strains. After determining the specificity and sensitivity of the PCR assay, the procedure was applied to complex matrices, including artificially contaminated cheese and meat.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Reference ATCC target and non-target bacterial strains as well as their genotype information relevant to this study are listed in Table 1. For *Mycobacterium* spp. (DSM 43990, DMS 44133^T, DSM 44156^T), only genomic DNA was available. The lyophilized strains of *Salmonella enterica* (DSM 17058), *Shigella* spp. (DSM 5570, 4782, 7532), *Listeria* spp. (DSM 20649, 20750, 20751, 20601, 20650, 20600, ATCC 19115, BAA679, 5178), *E. coli* spp. (DSM 19206^T, 30083^T, 4064, 9033^T, 10833, 10816, ED 324, EDL 933), *Staphylococcus* spp. (DSM 20231^T, 20459^T, 21284^T, 20373^T, 7068^T, 19048, 19041, 19040), *Bacillus* spp. (DSM 2048, 31, 2046, 4312^T, 4313^T), *Yersinia enterocolitica* (DSM 4780), *Aeromonas* spp. (DSM 30015, 30187, 7323, 7386), *Cronobacter malonaticus* (DSM 18702) and *Enterobacter* spp. (DSM 30053, 30054) were aerobically grown in Brain Heart Infusion Broth (Oxoid, Italy) at 37 °C for 24 h. *Campylobacter* strains (DSM 4689, 11375, 4688, 5365) were grown in Brain Heart Infusion Broth (Oxoid, Italy), under microaerophilic atmosphere (CO_2Gen, Oxoid, Italy) at 42 °C for 24–48 h. *Clostridum*

Table 1
Reference pathogenic and non-pathogenic bacterial strains used in this study.

Species	Strain	Toxins type
Listeria innocua	DSM 20649^T	
Listeria ivanovii	DSM 20750^T	
Listeria seeligeri	DSM 20751^T	
Listeria grayi	DSM 20601^T	
Listeria welshimeri	DSM 20650^T	
Listeria monocytogenes	DSM 20600^T	
Salmonella enterica typhimurium	DSM 17058^T	
Salmonella enterica heidelberg	DSM 9379	
Shigella sonnei	DSM 5570^T	
Shigella flexneri	DSM 4782^T	
Shigella boydii	DSM 7532^T	
Escherichia coli O157:H7	DSM 19206^T	*eae, e-hly*
Escherichia coli O1:K1:H7	DSM 30083^T	
Escherichia coli O157:H7	ED 324	*hly*A, *eae*, *rfbE*
Escherichia coli O157:H7	EDL 933	*fliC*
Escherichia coli	DSM 4064	
Escherichia coli O167:H5	DSM 9033^T	
Escherichia coli O18ac:K5:H-	DSM 10833	
Escherichia coli O18ac:K1:H7	DSM 10816	
Bacillus cereus	DSM 31^T	
Bacillus cereus	DSM 4312^T	serotype 1, emetic
Bacillus cereus	DSM 4313^T	serotype 2, diarrheal
Bacillus mycoides	DSM 2048^T	
Bacillus thuringiensis	DSM 2046^T	
Campylobacter coli	DSM 4689^T	
Campylobacter lari	DSM 11375^T	
Campylobacter jejuni	DSM 4688^T	
Campylobacter upsaliensis	DSM 5365^T	
Yersinia enterocolitica	DSM 4780^T	
Yersinia pseudotuberculosis	DSM 8992^T	
Aeromonas hydrophilia	DSM 30015	
Aeromonas hydrophilia subsp. *hydrophilia*	DSM 30187^T	
Aeromonas caviae	DSM 7323^T	
Aeromonas veronii subsp. *Sobria*	DSM 7386^T	
Mycobacterium bovis	DSM 43990	
Mycobacterium avium subsp. *Paratuberculosis*	DSM 44133^T	
Mycobacterium avium subsp. *Avium*	DSM 44156^T	
Enterobacter sakazakii	DSM 18702^T	
Enterobacter aerogenes	DSM 30053^T	
Enterobacter cloacae	DSM 30054^T	
Pseudomonas aeruginosa	DSM 50071^T	
Pseudomonas fluorescens	DSM 50090^T	
Pseudomonas fragi	DSM 3456^T	
Pseudomonas putida	DSM 291^T	
Streptococcus equi zooepidemicus	DSM 20727^T	
Clostridium perfringens	DSM 756^T	
Clostridium difficile	DSM 1296^T	
Clostridium tyrobutiricum	DSM 2637^T	
Clostridium sporogenes	DSM 795^T	
Clostridium butyricum	DSM 10702^T	
Clostridium baratii	DSM 601^T	
Clostridium beijerinckii	DSM 791^T	
Staphylococcus aureus	DSM 20231^T	
Staphylococcus hyicus	DSM 20459^T	
Staphylococcus pseudointermedius	DSM 21284^T	
Staphylococcus intermedius	DSM 20373^T	
Staphylococcus muscae	DSM 7068^T	
Staphylococcus aureus	DSM 19048	*seg, sei, eta, etb*
Staphylococcus aureus	DSM 19041	*sea, seb, sed, seg, sei, sej, pvl*
Staphylococcus aureus	DSM 19040	*sec, see, tsst*
Listeria monocytogenes	ATCC 19115	
Listeria monocytogenes	ATCC BAA679	
Listeria monocytogenes	ATCC 5178	

DSM – Strains obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig (Germany).
ATCC – Strains obtained from the American Type Culture Collection.