Acta Microbiologica et Immunologica Hungarica, 61 (2), pp. 121–130 (2014)
DOI: 10.1556/AMicr.61.2014.2.3

USE OF TAQMAN® REAL-TIME PCR FOR RAPID DETECTION OF *SALMONELLA ENTERICA* SEROVAR TYPHI

REZA RANJBAR[1], ALI NAGHONI[1]*, SHOHREH FARSHAD[2], HADI LASHINI[3], ALI NAJAFI[1], NOURKHODA SADEGHIFARD[4] and CATERINA MAMMINA[5]

[1]Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
[2]Prof. Alborzi Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[3]Applied Virology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
[4]Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran
[5]Department of Sciences for Health Promotion and Mother-Child Care 'G. D'Alessandro', University, Palermo, Italy

(Received: 8 October 2013; accepted: 5 February 2014)

We evaluated the performances of a newly designed real-time polymerase chain reaction (PCR) assay using TaqMan® probes to detect *Salmonella* Typhi. TaqMan® real-time PCR assays were performed by designed primers and probe based on the *staG* gene for detecting *S.* Typhi. The specificity of the assay was evaluated on 15 *Salmonella* serovars. The analytical specificity was evaluated on 20 non-*Salmonella* microorganisms. The analytical sensitivity was assessed using decreasing DNA quantities of *S.* Typhi ATCC 19430. Finally the detection capability of the TaqMan® real-time PCR assay on isolates recovered from patients with *Salmonella* infections was compared to the conventional PCR assay. Only *S.* Typhi strain had positive results when subjected to the assay using Typhi-specific real-time PCR. No amplification products were observed in real-time PCR with any of the non-*Salmonella* microorganisms tested. The TaqMan® real-time PCR was more sensitive than the conventional PCR. In conclusion, we found that the easy-to-use real-time PCR assays were faster than conventional PCR systems. The *staG*-based TaqMan® real-time PCR assay showed to be specific and sensitive method for the safe and rapid detection of the *S.* Typhi.

Keywords: polymerase chain reaction, *Salmonella* Typhi, TaqMan® real-time PCR assay

* Corresponding author; E-mail: alinaghoni@gmail.com

Introduction

Salmonella species, the causative agents of typhoid fever and diarrhoeal diseases in humans, are estimated to account for between 17 and 33 million cases of typhoid fever worldwide each year with 600,000 associated deaths and also, approximately ~1.4 million non-typhoidal *Salmonella* cases occur in the United States each year [1–3]. These diseases still pose a serious public health threat in various parts of the world, particularly in the developing countries [4].

The detection of *Salmonella* spp. is still primarily based on traditional microbiological culture and serological methods that may take several days to complete [5]. These practices, albeit effective, are costly and time-consuming [6]. *Salmonella* strains can be not easily detectable in clinical samples containing a small concentration of microorganisms [7]. Rapid and accurate detection of *Salmonella,* therefore, continues to be of considerable interest for clinical diagnosis [8, 9].

Recently, a number of rapid methods for detection of *Salmonella* including immunoassays, nucleic acid hybridization, and polymerase chain reaction (PCR) techniques have been developed [10]. Methods based on PCR have gained momentum in terms of use for rapid, specific, and sensitive detection of food-borne pathogens [7, 11]. A new fluorogenic PCR-based format has been recently developed, which utilizes an internal fluorogenic probe (probe with an attached fluorophore) that is specific to the target gene [12, 13]. During the PCR assay, the target gene is amplified and simultaneously recognized and monitored by the fluorescent probe moiety. The resulting increase in fluorescence can be computed to monitor the amplification of the target DNA [5, 14]. There are two types of fluorogenic PCR-based detection methods. One is based on utilizing a fluorogenic probe which has flanking GC-rich arm sequences complementary to one another (also known as molecular beacon), while the other based on a linear fluorogenic probe and requires the 5’ to 3’ exonuclease activity of the DNA polymerase (also known as TaqMan® assay or hydrolysis probe) [14–16]. In both types of real-time PCR probes, a fluorescent moiety is conjugated to one end of the sequence, and a quencher moiety is attached to the other end of the sequence [5]. TaqMan® real-time PCR assays are faster and more sensitive than conventional PCR, making them ideal for diagnostic purposes [17].

Unlike traditional PCR methods, which use agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction, real-time chemistries allow for a more timely detection of PCR amplification by measuring the kinetics of the reaction in the early phases of PCR [18]. Moreover, end-point detection is very time-consuming and results are generally based on size discrimination, which may not be very accurate [18, 19].

The objective of this study was to construct and validate a real-time PCR-based detection assay, based on the TaqMan® technology to detect *S.* Typhi, and to compare the specificity and sensitivity of TaqMan® assay with the conventional PCR assay.

Materials and Methods

The *Salmonella* strains used in this study are listed in Table I. Twenty non-*Salmonella* bacterial strains were also used for specificity checking (Table I). All the *Salmonella* and non-*Salmonella* strains were grown either on Brain Heart Infusion (BHI) (Difco Laboratories, Detroit, Michigan, USA) or Luria-Bertani (LB) broth (Merck, Darmstadt, Germany) at 37°C for 18–24 h.

Also, the study included 60 *Salmonella* isolates recovered from patients with *Salmonella* infections hospitalized in several hospitals in Tehran, Iran during 2011–2012. These isolates had been identified at the microbiology laboratory of the hospitals as *Salmonella,* based upon their morphology on selective media, biochemical and serological characteristics. All strains were stored at –20°C in trypticase soy broth (Merck, Darmstadt, Germany) with 10% of glycerol.

Bacterial strains were harvested, washed and resuspended in phosphate-buffered saline (PBS). The DNA of all micro-organisms was extracted using AccuPrep Genomic DNA Extraction kit (Bioneer, South Korea) according to the manufacturer's instructions. DNA concentration and purity were spectrophotometrically assessed by reading A_{260} and A_{280} and confirmed by visualization on 1% agarose gel. Then, DNA was diluted to 1 mg/mL in nuclease-free water and stored at –20°C until required for analysis.

The AlleleID software version 7.01 (Premier Biosoft Int., Palo Alto, CA, USA) was used for all the oligonucleotide primers and the fluorescent dye-labelled probe designed in this study. All primers were purchased from Bioneer (Bioneer, South Korea). The *in silico* specificity was analysed using the Basic Local Alignment Search Tool from the GenBank database. The characteristics of primers and probe used for TaqMan® real-time PCR assay are given in Table II.

Conventional PCR amplifications were carried out in final volume of 50 µL. The mixture contained 39.4 µL of RNAse free water, 4 µL of 10 × PCR buffer, 2 mM of $MgCl_2$, 200 µM of dNTP mix, 1 µM of each primer, 1 U of *Taq* polymerase, and 1 ng of DNA. PCR conditions were an initial denaturation at

Table I

Salmonella species and serovars and non-*Salmonella* microorganisms included in this study; and performance of the real-time PCR and conventional PCR assays for detecting *S.* Typhi

Strains	Reference	Typhi-specific PCR results	Typhi-specific real-time PCR results
Salmonella species			
Salmonella serovar Albany	ATCC[1] 51960	–	–
Salmonella serovar Enteritidis	ATCC 4931	–	–
Salmonella serovar Hadar	ATCC 51956	–	–
Salmonella serovar Haifa	Field strain	–	–
Salmonella serovar Havana	Field strain	–	–
Salmonella serovar Infantis	Field strain	–	–
Salmonella serovar Kentucky	ATCC 9263	–	–
Salmonella serovar Muenchen	ATCC 8388	–	–
Salmonella serovar Newport	ATCC 6962	–	–
Salmonella serovar Orion	Field strain	–	–
Salmonella serovar Paratyphi B	ATCC 8759	–	–
Salmonella serovar Reading	ATCC 6967	–	–
Salmonella serovar Richmond	Field strain	–	–
Salmonella serovar Typhi	ATCC 19430	+	+
Salmonella serovar Typhimurium	ATCC 14028	–	–
Non-*Salmonella* organisms			
Acinetobacter baumannii	ATCC 17978	–	–
Bacillus cereus	PTCC[2] 1154	–	–
Bacillus subtilis	PTCC 1254	–	–
Brucella abortus	ATCC 2344	–	–
Campylobacter jejuni	ATCC 33560	–	–
Enterobacter aerogenes	PTCC 1221	–	–
Enterococcus faecalis	PTCC 1393	–	–
Escherichia coli	ATCC 25922	–	–
Klebsiella oxytoca	ATCC 68831	–	–
Listeria monocytogenes	PTCC 1297	–	–
Micrococcus luteus	PTCC 1408	–	–
Proteus mirabilis	PTCC 1076	–	–
Pseudomonas aeruginosa	ATCC 27853	–	–
Serratia marcescens	ATCC 14223	–	–
Shigella flexneri	PTCC 1234	–	–
Shigella soneii	ATCC 9290	–	–
Staphylococcus aureus	PTCC 1189	–	–
Staphylococcus epidermidis	PTCC 1435	–	–
Staphylococcus haemolyticus	PTCC 1437	–	–
Vibrio cholerae	PTCC 1611	–	–

[1] ATCC, American Type Culture Collection (USA).
[2] PTCC, Persian Type Culture Collection (Iran).

Table II

Primers and probe sequences used for amplification by conventional PCR* and TaqMan® real-time PCR

Specific target	Primer/ probe name	Sequence (5' → 3')	Product (bp)	Target (location)	Refer ence
Salmonella serovar Typhi	staG-F staG-R staG-Pr	CAAGGTTGCTATAAACATTTG CGGTTTTTATTTCACCATTG FAM-CCTTCCTTCAGCC AGCAGAG-TAMRA	155	*sta*G (210264-211439)	This study

* For conventional PCR just forward and reverse primers were used.

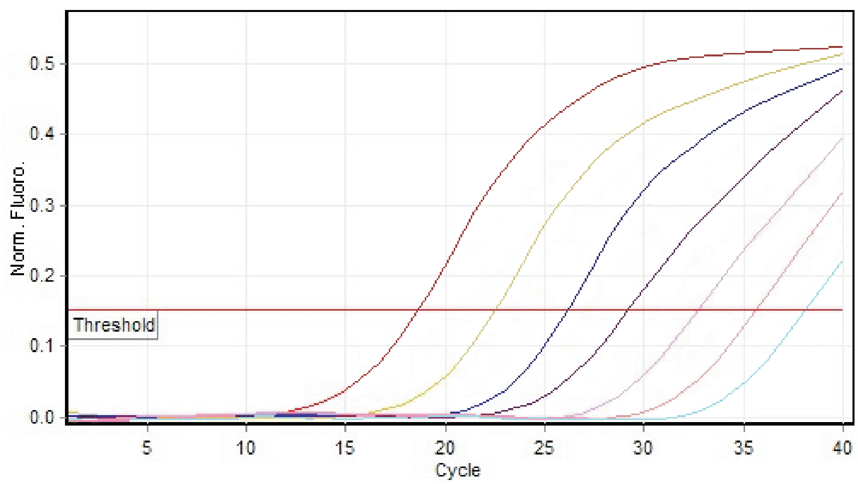

Figure 1. *Salmonella* Typhi sensitivity assay by TaqMan® real-time PCR. A 10-fold dilutions series (from 2 ng to 0.002 fg) was used as a template

95°C for 2 min; 40 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 40 s; and a final extension at 72°C for 5 min for detection of *S.* Typhi (Typhi-specific PCR).The reaction products were visualized on 1.5% agarose gels. To avoid any contamination, reaction mixture preparation, DNA amplification and gel migration were done in separate rooms.

TaqMan® real-time PCR assays were performed in a final volume of 25 µL, with 9.65 µL of RNAse free water, 12.5 µL of qPCR ProbesMaster (Jena Bioscience, Germany), each primer and TaqMan® probe at concentrations of 0.3 µM and 0.2 µM, respectively, and 2 ng of DNA template for all of samples. The reaction mixture was initially incubated for 2 min at 95°C. Amplification was performed for 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 55°C for 1 min for detection of *S.* Typhi (Typhi-specific real-time PCR). TaqMan®

real-time PCR reactions were performed on a Rotor-Gene^{TM} 6000 (Corbett Research Biosciences, Sydney, Australia).

Sensitivity was determined by testing decreasing DNA quantities of *S*. Typhi ATCC 19430 (10-fold dilutions from 10^{-6} to 10^{-15} g/mL).

The specificity of the different primers and probe was first assayed *in silico* by using BLAST software in order to avoid non-specific amplification. Analytical specificity was then evaluated on the 20 non-*Salmonella* microorganisms listed in Table I to rule out cross-reactivity. In conventional PCR assays, 1 ng of *S.* Typhi DNA was used as a positive control. For all PCR assays, the negative control consisted in sterile water instead of DNA template.

Thirty samples were amplified in the same run for the intra-assay repeatability and 10 samples were amplified in a single run on 4 consecutive days for the inter-assay reproducibility. The respective coefficients of variation (CVs) were then calculated.

Sixty *Salmonella* isolates recovered from patients with *Salmonella* infections hospitalized in several hospitals in Tehran, Iran during 2011–2012, were also analyzed by conventional PCR and TaqMan® real-time PCR methods for detection of *S.* Typhi.

Results

The results indicated that only *S.* Typhi showed positive results when subjected to the assay using Typhi-specific real-time PCR. No amplification products were observed in real-time PCR with any of the other *Salmonella* serovars or non-*Salmonella* microorganisms when using the specific primers and probe for detecting *S.* Typhi.

The respective lower limit of detections of conventional and real-time PCR assay observed with 10-fold serial dilutions of *Salmonella* serovar Typhi DNA were 100 fg and 2 fg, respectively. The real-time PCR was more sensitive than the conventional PCR. A 50-fold higher sensitivity for *S.* Typhi was observed in real-time PCR.

The C_t values intra-assay and inter-assay CVs were 1.11% and less than 4.0%, respectively, for detecting *S.* Typhi confirming the reproducibility and repeatability of the assay. Non specific reaction was PCR amplification at the final phase or end-point of the PCR reaction observed in conventional PCR (Table I).

Of the 60 clinical specimens positive by culture and serological methods for *Salmonella*, one tested positive for *S.* Typhi with both techniques (Table III).

Table III

TaqMan® real-time PCR and conventional PCR assays for *Salmonella* Typhi on *Salmonella* isolates from patients

Strains	Number	Conventional PCR		TaqMan® real-time PCR	
		Positive	Negative	Positive	Negavtive
Total *Salmonella* isolates	60	1	59	1	59
Salmonella serovar Typhi	1	1	0	1	0
Salmonella serovar Typhimurium	8	0	8	0	8
Salmonella serovar Enteritidis	25	0	25	0	25
Salmonella serovar Infantis	18	0	18	0	18
Salmonella serovar Muenchen	2	0	2	0	2
Salmonella serovar Albany	1	0	1	0	1
Salmonella serovar Newport	1	0	1	0	1
Salmonella serovar Richmond	1	0	1	0	1
Salmonella serovar Paratyphi B	1	0	1	0	1
Salmonella serovar Reading	1	0	1	0	1
Salmonella serovar Orion	1	0	1	0	1

Discussion

Molecular techniques, like real-time PCR, are proving useful in the detection of pathogens in a wide range of matrices [4, 6]. The use of real-time PCR greatly reduces the time and manpower required when compared with conventional methodologies [20]. The real-time PCR method could be of benefit when information on the presence of *Salmonella* in samples is required rapidly such as in outbreak and epidemiological studies [4, 21].

We designed and tested and now report a sensitive and specific TaqMan® real-time PCR assay for the detection of *S.* Typhi. We think this assay could be potentially useful as a molecular tool for screening suspected colonies. We proved this hypothesis, first by testing strains of *Salmonella*, including *S.* Typhi and commonly occurring serovars. Typhi-specific real-time PCR assay correctly identified *S.* Typhi.

Subsequently, we assessed the performance of TaqMan® real-time PCR (Typhi-specific real-time PCR) and conventional PCR (Typhi-specific PCR) assays on isolates recovered from patients with *Salmonella* infections hospitalized in Tehran. The results were similar for both assays (Table III).

In the past few years, several authors have evaluated sensitivity and specificity of real-time PCR assays for the detection of *S.* Typhi [6, 9, 20, 22]. Various loci have been used as target genes including *vexC*, *viaB*, and *tyv* [20, 22–25]. In this study, we used bioinformatics to identify a region specific to *S.* Typhi. To our knowledge, no previously published real-time PCR presented primers based on the *staG* (putative fimbrial protein) gene for detecting *Salmonella* serovar Typhi.

The detection limits of *staG* real-time PCR assay in this study were markedly improved in comparison to real-time PCR assays published previously. Chen et al. (2010) who evaluated the detection limit of the assay using a 5'-nuclease real-time PCR assay reported the detections limits of 41.2 fg for *S.* Typhimurium and 18.6 fg for *S. enteritidis* with their own specific primers [9]. Moreover, Anderson et al. (2011) described a detection limit of 100 fg for *Salmonella* species [26]. The detection limit of *staG* real-time PCR assay in this work was 2 fg. A higher sensitivity was observed for the detection of *S.* Typhi by real-time PCR: In comparison with the conventional PCR method, 50-fold higher sensitivity for *S.* Typhi was observed for its target in real-time PCR.

The real-time PCR assays were also evaluated with a variety of other bacterial species and were highlighted as being specific; neither phylogenetically related (i.e. belonging to the proteobacteria group) microorganisms nor other pathogens showed cross-reactivity. Interestingly, no amplification occurred with *Shigella flexneri* and *Shigella sonnei,* thus indicating the specificity of the selected target sequence (Table I).

In conclusion, we developed and successfully applied a TaqMan® real-time PCR for rapid identification of *S.* Typhi. Real-time PCR appears to offer several advantages over conventional PCR. Apart from being faster, this closed system obviates the need for post-PCR handling and prevents DNA contamination. This technology often is adopted to detect fastidious microbes or microorganisms that cannot be cultivated. As we observe, the specificity of TaqMan® real-time PCR and PCR assays were similar but, TaqMan® real-time PCR was more sensitive than conventional PCR. Therefore, the use of TaqMan® real-time PCR assay appears promising due to its high sensitivity for the safe, rapid and specific detection of the *Salmonella* serovar Typhi in clinical samples or this technique can be used in children and newborns, particularly those for whom the serovar Typhi detection is of great concern and importance from the therapeutic and prognostic point of view as well as for public health purposes.

Conflict of interest: The authors declare that there are no conflicts of interest.

References

1. Hald, T., Lo Fo Wong, D.M., Aarestrup, F.M.: The attribution of human infections with antimicrobial resistant *Salmonella* bacteria in Denmark to sources of animal origin. Food-Borne Patho Dis **4**, 313–326 (2007).
2. Naghoni, A., Ranjbar, R., Tabaraie, B., Farshad, S., Owlia, P., Safiri, Z., Mammina, C.: High prevalence of integron-mediated resistances among clinical isolates of *Salmonella enterica*. Jpn J of Infect Dis **63**, 417–421 (2010).
3. World Health Organization: World Health Organization antimicrobial resistance fact sheet no. 139. Available from http://www.who.int/mediacentre/factsheets/fs139 (2003).
4. Karami, A., Ranjbar, R., Ahmadi, Z., Safiri, Z.: Rapid detection of different serovares of *Salmonella enterica* by multiplex PCR. Iran J Public Health **36**, 38–42 (2007).
5. Liming, S.H., Bhagwat, A.A.: Application of a molecular beacon – real-time PCR technology to detect *Salmonella* species contaminating fruits and vegetables. Int J Food Microbiol **95**, 177–187 (2004).
6. Bohaychuk, V.M., Gensler, G.E., McFall, M.E., King, R.K., Renter, D.G.: A real-time PCR assay for the detection of *Salmonella* in a wide variety of food and food-animal matrices. J Food Prot **70**, 1080–1087 (2007).
7. Feng, P.: Impact of molecular biology on the detection of foodborne pathogens. Mol Biotechnol **7**, 267–278 (1997).
8. Kurowski, P.B., Traub-Dargatz, J.L., Morley, P.S., Gentry-Weeks, C.R.: Detection of *Salmonella* spp in fecal specimens by use of real-time polymerase chain reaction assay. Am J Vet Res **63**, 1265–1268 (2002).
9. Chen, J., Zhang, L., Paoli, G.C.: A real-time PCR method for the detection of *Salmonella enterica* from food using a target sequence identified by comparative genomic analysis. Int J Food Microbiol **137**, 168–174 (2010).
10. Li, X., Boudjellab, N., Zhao, X.: Combined PCR and slot blot assay for detection of *Salmonella* and *Listeria monocytogenes*. Int J Food Microbiol **56**, 167–177 (2000).
11. Fratamico, P., Strobaugh, T.P.: Simultaneous detection of *Salmonella* spp. and *Escherichia coli* O157:H7 by multiplex PCR. J Ind Microbiol Biotech **21**, 92–98 (1998).
12. Chen, W., Martinez, G., Mulchandani, A.: Molecular beacons: A real-time polymerase chain reaction assay for detecting *Salmonella*. Anal Biochem **280**, 166–172 (2000).
13. Hoorfar, J., Radstrem, P.: Automated 5' nuclease PCR assay for identification of *Salmonella enterica*. J Clin Microbiol **38**, 3429–3435 (2000).
14. Tyagi, S., Kramer, F.R.: Molecular beacon: Probes that fluoresce upon hybridization. Nat Biotechnol **14**, 303–308 (1996).
15. Chen, S., Yee, A., Griffiths, M., Larkin, C., Yamashiro, C.T., Behari, R., Paszko-Kolva, C., Rahn, K., De Grandis, S.A.: The evaluation of a fluorogenic polymerase chain reaction assay for the detection of *Salmonella* species in food commodities. Int J Food Microbiol **35**, 239–250 (1997).
16. McKllip, J.L., Drake, M.: Molecular beacon polymerase chain reaction detection of *Escherichia coli* O157:H7 in milk. J Food Prot **63**, 855–859 (2000).
17. Bounaadja, L., Albert, D., Chenais, B., Henault, S., Zygmunt, M.S., Poliak, S., Garin-Bastuji, B.: Real-time PCR for identification of *Brucella* spp.: A comparative study of IS711, bcsp31 and per target genes. Vet Microbiol **137**, 156–164 (2009).
18. Arya, M., Shergill, I.S., Williamson, M., Gommersall, L., Arya, N., Patel, H.R.: Basic principles of real-time quantitative PCR. Expert Rev Mol Diagn **5**, 209–219 (2005).

19. Smith, C.J., Osborn, A.M.: Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiol Ecol **67**, 6–20 (2009).
20. Farrell, J.J., Doyle, L.J., Addison, R.M., Reller, L.B., Hall, G.S., Procop, G.W.: Broad-range (Pan) *Salmonella* and *Salmonella* serotype Typhi–specific real-time PCR assays. Am J Clin Pathol **123**, 339–345 (2005).
21. Van Kessel, J.S., Karns, J.S., Perdue, M.L.: Using a portable real-time PCR assay to detect *Salmonella* in raw milk. J Food Prot **66**, 1762–1767 (2003).
22. Nga, T.V., Karkey, A., Dongol, S., Thuy, H.N., Dunstan, S., Holt, K., Tu le, T. P., Campbell, J.I., Chau, T.T., Chau, N.V., Arjyal, A., Koirala, S., Basnyat, B., Dolecek, C., Farrar, J., Baker, S.: The sensitivity of real-time PCR amplification targeting invasive *Salmonella* serovars in biological specimens. BMC Infect Dis **10**, 125 (2010).
23. Jordan, R., van Heerden, E., Hugo, C.J. Piater, L.A.: Using current molecular techniques for rapid differentiation of *Salmonella* Typhi and *Salmonella* Typhimurium. Afr J Biotechnol **8**, 1815–1818 (2009).
24. Hashimoto, Y., Itho, Y., Fujinaga, Y., Khan, A.Q., Sultana, F., Miyake, M., Hirose, K., Yamamoto, H., Ezaki, T.: Development of nested PCR based on the ViaB sequence to detect *Salmonella typhi.* J Clin Microbiol **33**, 775–777 (1995).
25. Moussa, I.M., Gassem, M.A., Al-Doss, A.A., Mahmoud, W.A., Sadik Abdel Mawgood, A.L.: Using molecular techniques for rapid detection of *Salmonella* serovars in frozen chicken and chicken products collected from Riyadh, Saudi Arabia. Afr J Biotechnol **9**, 612–619 (2010).
26. Anderson, A., Pietsch, K., Zucker, R., Mayr, A., Müller-Hohe, E., Messelhäusser, U., Sing, A., Busch, U., Huber, I.: Validation of a duplex real-time PCR for the detection of *Salmonella* spp. in different food products. Food Anal Methods **4**, 259–267 (2011).