Detection, isolation and assessment of *Salmonella entiritidis* in milk by conventional culture methods and real-time PCR in Iran

Hossein Rastegar[1,2,3], Hamidreza Ahmadi Ashtiani[4], kaveh Afraz[3*], Mina Andalibi[1,2], Shima Hallaj Nehshabouri[1,2], Minoo Akbari[1,2], Hedieyeh Rassam[1,2],Sadolla Parvizi[1,2], Soghra Anjarani[5]

[1]Food and Drug Control Labs and Research Center, Ministry of health and Medical Education
[2]Cosmetic Products Research Center, Ministry of health and Medical Education
[3]Science & Research Branch of Islamic Azad University (SRBIAU), Tehran, Iran
[4]Islamic Azad University, pharmaceutical sciences branch, Tehran, Iran
[5]Health Reference Labs, Ministry of health and Medical Education
Corresponding authors: kaveh Afraz
Science & Research Branch of Islamic Azad University (SRBIAU), Tehran, Iran
E-mail: sina_saadatabad@yahoo.com

Abstract

Salmonellas are wide-range organisms which can cause disease in human and other organisms. They can invade the intestinal and extra-intestinal tissues. The Salmonellosis is one of the main world health challenges. Salmonellas are usually detected in milk by conventional methods of bacterial culture. However, this method is time consuming and lacks the desired specificity. So, the objective of this study was to develop and evaluate a SYBR Green real-time PCR method for the specific detection of Salmonella Entiritidis in cow milk farm samples. In this article, we present a real-time PCR assay for quantitative detection of Salmonella Entiritidis in100 samples milk. The standard curve correlation coefficient for the threshold cycle versus the copy number of initial Salmonella Entiritidis cells was 0.996. To test the PCR system, a set of 100 milk samples were screened for the presence of Salmonella Entiritidis. 17% (17/100) of milk samples, tested positive for Salmonella Entiritidis. This result indicated that the real-time PCR assay provides a specific, sensitive and rapid method for quantitative detection of Salmonella Entiritidis. Moreover, it is concluded cow milk is commonly contaminated with Salmonella Entiritidis and could serve as a potential risk for consumers in Iran, especially if proper hygienic and cooking conditions are not maintained.

Running title: Assessment of *Salmonella Entiritidis* in milk

{**Citation:** Hossein Rastegar, Hamidreza Ahmadi Ashtiani, kaveh Afraz, Mina Andalibi, Shima Hallaj Nehshabouri, Minoo Akbari, Hedieyeh Rassam,Sadolla Parvizi, Soghra Anjarani. Detection, isolation and assessment of *Salmonella entiritidis* in milk by conventional culture methods and real-time PCR in Iran. American Journal of Research Communication, 2013: 1(8): 81-97} www.usa-journals.com, ISSN: 2325-4076.

Introduction

Salmonellosis is common cause mortality due to food and water borne diseases in developed and developing countries that causes gastroenteritis and typhoid in humans [1]. Foods of animal origin are frequently implicated in human salmonellosis owing to the high prevalence of Salmonella strains in animals [2]. Dairy cows and their environment harbor pathogens that pose a potential human health hazard. Salmonellosis is commonly diagnosed in dairy cows and calves, and the presence of Salmonella on dairy farms has been well documented [3]. Recently, it has been demonstrated that countries in Asia exhibit high burden of Salmonellosis [4]. Most Salmonella serovars are potential pathogens and standard methods for detection of these pathogens in complex biological samples are time consuming. Moreover, Low numbers of Salmonella in food may pose a public health risk given that their infective dose can be as low as 15-100 CFU [5, 6]. Only higher levels of Salmonella (10^2 - 10^3 $CFUg^{-1}$ or 10^2-10^3 $CFUml^{-1}$) are detectable by conventional culturable methods [1]. Further, conventional bacteriological detection and quantitative enumeration of Salmonella in foods is costly, laborious, and time-consuming, requiring up to 5 days obtaining a confirmed result 1, 7]. Thus, PCR-based and real-time PCR (RT-PCR)-based diagnostic methods have been developed in the last two decades to detect Salmonella DNA in samples [8-17]. The main benefits of real-time PCR are high speed, high sensitivity, high specificity, reduced amplicon

size and no post-PCR steps that reduce risks of cross-contamination [18]. Many researchers use simple and less expensive SYBR Green dye for real-time PCR studies. A number of SYBR Green real-time PCR assays for detection of microbial pathogens such as Escherichia coli, Campylobacter and Vibrio have been reported [19-21]. Four SYBR Green real-time PCR methods for detection of Salmonella from various types of samples have been described. Medici et al. and Bhagwat et al. reported SYBR Green based PCR assays with poultry samples and vegetable rinse-water, respectively [22, 23]. Additionally, multiplex real-time PCR using SYBR Green for simultaneous detection of Salmonella in milk samples have been reported [24]. Although a few real-time PCR assays in are available for quantification of Salmonella in food, vegetables and clinical samples in developed countries [25-27], but also a simple and fast method for detection of Salmonella spp was not elucidated in developing countries such as Iran. Therefore, the objective of the present study was to establish a simple real-time PCR method using SYBR Green that would be suitable for routine analysis of Salmonella spp. in cow milk samples. To determine the method's effectiveness, experiments were conducted with different types of both artificially and naturally contaminated milk samples.

Material and Methods

Sample collection

100 cow milk farm samples were collected from two dairy farm following methods described previously [28, 29]. Samples were collected using aseptic techniques, transported to the laboratory on ice, and stored overnight at 58C before analysis.

Microscopic analysis of cell morphology and biochemical tests for isolation and identification of Salmonella Entiritidis

Cow milk samples were tested at the collection site. Aliquots of 50 g were centrifuged at 20,000×*g* for 40 min. The supernatant was discarded and the pellet (not fat layer) suspended in 10 ml enrichment broth. It was then transferred to 90 ml enrichment broth. This was followed by a 24-h enrichment step in which the cultures were transferred to a 42°C microaerobic incubator and incubated statically. All the enriched samples were linearly cultured in plates containing S.S and B.G.A culture media and incubated at 37±1°C for 24 hours. Absence of colony in S.S and B.G.A media was the result of lack of Salmonella contamination but in the presence of contamination the suspicious colonies in S.S agar media (Salmonella Shigela) were transparent and colorless with a dark center and in B.G.A media (Brilliant green agar) were pink. To prove the Salmonella contamination, the colonies were transferred to a test tube containing 0.5 ml distilled water and OPNG disk and were incubated at 37±1°C for 2-6 hours. Any change in the color of OPNG solution was due to Salmonella contamination and lack of color change was the proof for the lack of Salmonella contamination. Biochemical and serological tests such as; TSI tests (triple sugar iron agar), LIA (lysine decarboxylase), Simon citrate, Urea an SIM (Solphite Endol Motility) and VIP test (Voges-Proskave) can also be used to detect Salmonella Entiritidisin primary milk samples or milks undergoing test.

Sensitivity studies with pure cultures

Sensitivity studies were performed with Salmonella Enteritidis ATCC 13076 (American Type Culture Collection, Manassas, VA) pure cultures in broth and in sterilized milk sample to determine the lower detection limit of the real-time PCR assay. DNA was prepared from a dilution series with a concentration range of 10^0 to 10^8 CFU/ml in 10-fold dilutions of

Salmonella Enteritidis grown overnight in Trypticsoy broth (TSB; Difco, Detroit, MI) and in ultra high temperature (UHT) sterilized whole milk. The number of CFU for each dilution was obtained by the standard plate count method using Xylose Lysine Deoxycholate (XLD; Difco) agar plates. To rule out false positives, one uninoculated aliquot was used as a negative control in every experiment. These experi- ments were conducted in triplicate. Standard curves were constructed by plotting CFU versus threshold cycle (C_T) produced for the target gene. For a comparison of PCR amplification efficiencies and detection sensitivities among different experiments, slopes of standard curves were calculated by performing a linear regression analysis with iCycle iQ software (version 3.0; Bio-Rad).

Detection studies with milk farm samples

Twenty-five grams or 25 ml of each sample were homogenized in 225 ml of UPB in a Stomacher filter bag. Filtrates were transferred to sterile bottles and incubated at 37 8C for 18-24 h. After incubation, enrichment cultures were subdivided into two aliquots: one aliquot was analyzed by the standard culture method (FDA/BAM, 2001), and the other aliquot was subjected to DNA extraction and purification for SYBR Green real-time PCR and melting curve analysis. For isolation of Salmonella, one ml of pre-enriched broth was transferred into 9 ml of Tetrathionate broth (TTB, Difco) and incubated at 37 8C for 18-24 h. Enriched cultures were sub-cultured onto XLD and novobiocin (Sigma, St. Louis, MO) supplemented Brilliant Green (BG, Difco) agar medium, and culture plates were incubated as described previously.

Identification of Salmonella isolates

More than 5 presumptive Salmonella colonies per duplicate plates were used for biochemical and serological reactions, and the identities of Salmonella isolates were

confirmed by the API 20E system (bioMerieux, Hazelwood, MO).

Extraction of DNA from pure culture and enrichment broth cultures

Total bacterial DNA was extracted from pure cultured strains using InstaGene Matrix (Bio-Rad Laboratories) according to manufacturer's instruc tions. For extraction and purification of bacterial DNA from broth cultures of samples, PrepMank Ultra (Applied Biosystems, Foster City, CA) sample preparation reagent was used according to manufacturer's directions.

SYBR Green real time PCR assay

Real-time PCR and data analysis were performed in the ABI 7500 Sequence Detection System (Applied Biosystems) using SYBR Green PCR Master Mix (Qiagen, Valencia, CA). The specific Salmonella primer sequences were: left-5' TCGTCATTCCATTACCTACC-3' and right 5'AAACGTTGAAAAACTGAGGA3' that were chosen to amplify a 119-base pair fragment of the invasion (invA) gene that previously described by Hoorfar et al. [30]. The PCR mixture contained 25 µl QuantiTectk SYBR Green PCR Master Mix (SYBR Green PCR Buffer, Hot Start Taq DNA Polymerase, dNTP mix including dUTP, SYBR Green 1, passive reference dye ROX, and 5mM $MgCl_2$); 0.3 µl of each primer; 1.5 µl of DNA template; and 0.1 µl of dilute fluorescein solution for the collection of dynamic well factors as specified in the iCycler iQ manual. To reach a total volume of 50 µl per well, RNase-free distilled water was added. All runs included a negative control without target DNA and Salmonella Enteritidis ATCC 13076 as the positive control. Thermal cycling conditions were as follows: 95 8C for 15 min, followed by 40 cycles of 95 8C for 15 s, 55 8C for 15 s, and 72 8C for 30 s. A final extension of 72 8C for 5 min was employed. All PCR reactions were performed in triplicate. Reproducibility of SYBR Green real-time PCR was assessed by running samples

independently on different days.

Results

Sensitivity of real-time PCR

Sensitivity and detection limits in pure cultures Serial dilutions of purified DNA were prepared and a CFU standard curve was constructed. DNA isolated Salmonella Entiritidis cells were serially 10-fold diluted in 1×TE buffer and subjected to PCR. As shown in Fig. 1, there were ranging from 10^0 to 10^5 CFU/ml in the application curve for these serial dilutions of Salmonella Entiritidis DNA. Standard curves were constructed using mean C_T and resulted in a linear relationship between C_T and log input DNA. Correlation coefficients of standard curves were R^2=0.996 (y = 2.178x + 36.17) and the minimum levels of detection were 10^2 CFU/ml, respectively (Fig. 1). Real-time PCR amplification of Salmonella Entiritidis DNA and melting curve analysis of SYBR Green real-time PCR product of Salmonella Enteritidis after 40 cycles were shown in figure 2.

Detection of Salmonella from milk farm samples by real-time PCR and conventional culture methods

As an application of the real-time PCR system and conventional culture methods , a set of 100 milk samples were screened for the presence of Salmonella Entiritidis. As shown in Table 1, for real-time PCR detection, 17% (17/100) of milk samples were found to be positive for Salmonella Entiritidis. Using the conventional culture methods 11% (11/100) of milk samples was found to be positive for Salmonella Entiritidis. The results for Salmonella Entiritidis detection in milk by real-time PCR and culture methods showed significant statistical differences (r=0.62, P<0.05). All culture-positive samples were real-time PCR-positive as well; however, 6 samples positive by PCR were culture-negative. Quantitative

results for Salmonella Entiritidis in milk by real-time PCR are presented in Table 2 and 3.

Table 1: Detection for Salmonella Entiritidis in milk by real-time PCR and culture method

Number of sample	Number of positive		Positive ratio (percent)	
	Real-Time PCR method	Culture method	Real-Time PCR method	Culture method
Milk (100)	17	11	17%	11%

Figure 1 A

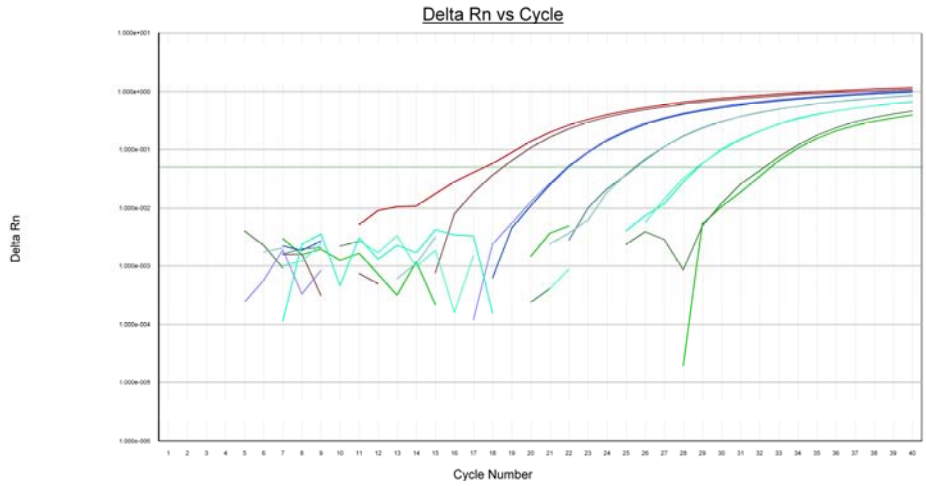

Figure 1 B

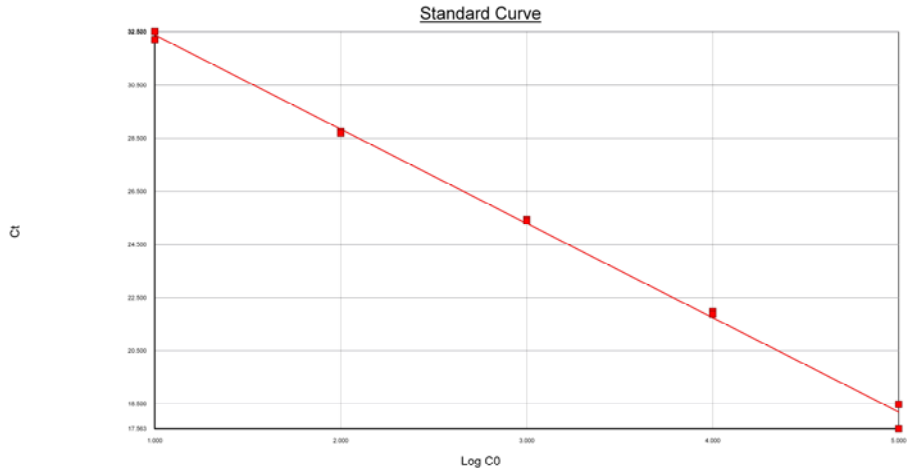

Fig 1: A: Application curve for serial 10-fold dilutions of Salmonella Entiritidis DNA in LightCycler. Reactions contained ranging from 10^0 to 10^5 CFU/ml. B: Standard curve for serial 10-fold dilutions of Salmonella Entiritidis DNA. Quantification was performed by determining the threshold cycle (Ct). Linear regression coefficients were R^2=0.996 (y = 2.178x + 36.17).

Figure 2A

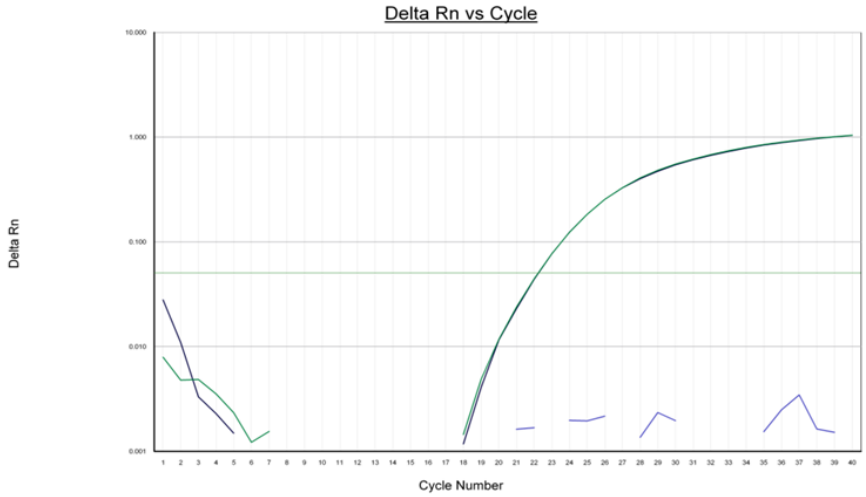

Figure 2B

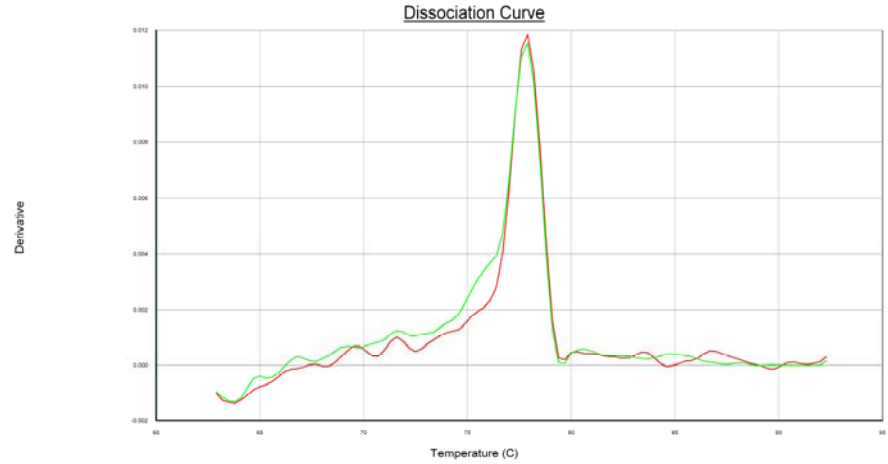

Fig 2: (A) Real-time PCR amplification of Salmonella Entiritidis DNA. (B) Melting curve analysis of SYBR Green real-time PCR product of Salmonella Enteritidis after 40 cycles.

Table 2: Detection of Salmonella Entiritidis in milk Samples by real-time PCR

sample	Number of positive	Amount (CFU ml $^{-1}$)
Milk	17	$5.8 \times 10^{6} \pm 4.1 \times 10^{4}$

Table 3: Comparisons between culture-negative and culture-positive samples among real-time PCR-positive samples for detection of Salmonella Entiritidis

sample	Amount of detected using real-time PCR (CFU ml $^{-1}$)	
	Culture-negative sample	Culture-positive sample
Milk	$4.9 \times 10^3 \pm 2.3 \times 10^2$	$6.1 \times 10^6 \pm 3.1 \times 10^5$

Discussion

Salmonella is one of the main causes of food toxicity. Salmonella enters the intestine of animals and human through mouth and result in food toxicities such as; Septisemy and intestinal fever (Tiphoid). So, controlling germs in foods has a great importance [31, 32]. The milk of animals such as cow may be full of Salmonella which can be infectious for human [33]. One of the most common species of Salmonella that can contaminate milk is entiritidis [34]. Therefore, early detection of Salmonella contamination is critically important.

In the present study, a simple and reproducible real-time PCR method based on SYBR Green fluorescence for detection of Salmonella from milk farm samples is reported. Experiments carried out on milk farm samples showed that real-time PCR could detect Salmonella in <10 CFU/ml of Salmonella contaminated samples. These results agree with those of other reports, which described samples had improved detection limits of the real-time PCR assay [24, 35]. Fukushima et al. also recommended duplex real-time SYBR Green PCR assays to achieve adequate sensitivity in samples that contained low levels of target pathogens [36].

The R^2 after the linear regression indicated a good correlation between the amount of template (log CFU) and C_t in the standard curves (R^2=0.996). The linearity of the standard curves and the fact that the PCR operates with constant efficiency confirmed that the assay was well suited to quantitative measurements of Salmonella Entiritidis. The detection limit of the present PCR assay was estimated to be approximately 1 CFU per PCR from bacterial

culture. However, the sensitivity for detecting Salmonella Entiritidis in milk was 6–15 CFU per PCR. These indicated the possible presence of inhibitory substances in these samples. Our limit of detection is similar to those reported by others using a fluorogenic 5′-nuclease PCR assay for endpoint detection. Bassler et al. obtained a detection level for *Listeria monocytogenes* of approximately 50 CFU per PCR [37], while Chen et al. showed a detection limit of as low as 2 CFU per PCR from a pure culture of *Salmonella enterica* serovar Typhimurium [38].

Real-time PCR and culture methods were screened for the presence of Salmonella Entiritidis in 100 milk samples. The results from the two methods of study supported the fact that cow milk is commonly contaminated with Salmonella Entiritidis in IRAN. Our reported positive ratio of detection was similar to those of other reports in China [39, 40], But the positive ratio of real-time PCR detection was higher than for the culture method. All culture-positive samples were real-time PCR-positive; however, 7 samples that were PCR-positive were culture-negative. DNA extracted from samples was used as template in the PCR assay. These samples may have contained dead or sublethally damaged cells, which were present at a high enough concentration for the Salmonella Entiritidis DNA to be detected in the real-time PCR assay, although they could not be recovered by enrichment culture methods.

As shown in Table 2, an apparently high number of Salmonella Entiritidis was detected by the real-time PCR method in the samples examined. These numbers refer to the concentrated suspensions obtained after centrifugation. As shown in Table 3, comparison of the numbers of Salmonella Entiritidis detected by PCR in the culture-negative compared to the cultural-positive samples indicates that the very high numbers of Salmonella Entiritidis detected by real-time PCR were probably either non-viable or VNC cells. These dead cells could not pose a risk to human health, but do indicate that this milk was previously contaminated with Salmonella Entiritidis. Therefore, quantitative detection systems are crucial to estimate

the possibility of Salmonella Entiritidis contamination in milk samples, and such risk assessments are important for future legislative work.

The real-time quantitative PCR assay demonstrates several advantages over other conventional PCR approaches. The fluorogenic assay is a convenient, self-contained process. The only necessary steps are the reaction set-up and the tube sealing. Unlike other quantitative PCR methods, real-time PCR does not require post-PCR sample handling, thus avoiding potential PCR product carryover contamination. This enables the assays to be conducted much faster and in a high-throughput format. The real-time PCR method has a very large dynamic range of starting target molecule determination. Moreover, this real-time method is extremely accurate and is less labor-intensive than methods such as the immunocapture PCR assay [41].

Adaptation of real-time PCR for quantification of Salmonella Entiritidis in milk is feasible [42]. Choosing proper protocols for the isolation of bacterial DNA/cells is an important factor. A possible approach may be the use of magnetic beads for specific isolation of the bacteria, followed by DNA isolation while the bacteria are still attached to the beads [43]. Real-time PCR may be particularly useful both for routine screening for Salmonella Entiritidis contamination and for large-scale screening during outbreaks.

In summary, the data presented here showed that the real-time PCR assay was suitable for identification of Salmonella Entiritidis. A set of 100 milk samples were screened for the presence of Salmonella Entiritidis. The results indicated that cow milk is commonly contaminated with Salmonella Entiritidis and could serve as a potential risk for consumers in Iran. The real-time PCR assay provides a specific, sensitive and rapid method for quantitative detection of Salmonella Entiritidis in milk samples.

References

1. Malorny B, Löfström C, Wagner M, Krämer N, Hoorfar J. Enumeration of Salmonella bacteria in food and feed samples by real-time PCR for quantitative microbial risk assessment. Appl Environ Microbiol 2008;74:1299-304.

2. Oosterom, J. Epidemiological studies and proposed preventive measures in the fight against human salmonellosis. Int. J. Food Microbiol. 1991;12: 41 - 51.

3. Wells, S.J., Fedorka-Cray, P.J., Dargatz, D.A., Ferris, K., Green, A. Fecal shedding of Salmonella spp. by dairy cows on farm and at cull cow markets. J. Food Prot. 2001; 64: 3 - 11.

4. Ochiai RL, Acosta CJ, Danovaro -Holliday MC, Baiqing D, Bhattacharya SK, Agtini MD, et al. Domi Typhoid Study Group. A study of typhoid fever in five Asian countries: disease burden and implications for controls. Bull World Health Organ 2008;86: 260-8.

5. Cobbold RN, Rice DH, Davis MA, Besser TE, Hancock DD. Long-term persistence of multidrug-resistant Salmonella enterica serovar Newport in two dairy herds. J Amer Vet Med Assoc 2006;228:585-91.

6. Seo KH, Valentin-Bon IE, Brackett RE. Detection and enumeration of Salmonella enteritidis in homemade ice cream associated with an outbreak: com parison of conventional and real-time PCR methods. J Food Prot 2006;69: 639-43.

7. Josefsen MH, Krause M, Hansen F, Hoorfar J. Optimization of a 12-Hour TaqMan PCR-based method for detection of Salmonella bacteria in meat. Appl Environ Microbiol 2007;73:3040-8.

8. Casanas MC, Queipo-Ortuno MI, Rodriguez-Torres A, Orduna A, Colmenero JD, Morata P. Specificity of a polymerase chain reaction assay of a target sequence on the 31-kilodalton Brucella antigen DNA used to diagnose human brucellosis. Eur J Clin Microbiol Infect Dis 2001; 20:127–131

9. Matar GM, Khneisser IA, Abdelnoor AM. Rapid laboratory confirmation of human brucellosis by PCR analysis of a target sequence on the 31-kilodalton Brucella antigen DNA. J Clin Microbiol 1996;34:477–478.

10. Queipo-Ortuno MI, Morata P, Ocon P, Manchado P, Colmenero JD. Rapid diagnosis of human brucellosis by peripheral-blood PCR assay. J Clin Microbiol 1997;35:2927 –2930.

11. Morata P, Queipo-Ortuno MI, Reguera JM, Miralles F, Lopez-Gonzales JJ, Colmenero JD. Diagnostic yield of a PCR assay in focal complications of brucellosis. J Clin Microbiol 2001;39:3743–3746.

12. Morata P, Queipo-Ortuno MI, Reguera JM, Garcia-Ordonez MA, Gardenas A, Colmenero JD. Development and evaluation of a PCR-enzyme-linked immunosorbent assay for diagnosis of human brucellosis. J Clin Microbiol 2003; 41:144 –148.

13. Baily GG, Krahn JB, Drasar BS, Stoker NG. Detection of Brucella melitensis and Brucella abortus by DNA amplifi-cation. J Trop Med Hyg 1992; 95:271 – 275.

14. Newby DT, Hadfield TL, Roberto FF. Real-time PCR detection of Brucellaabortus: a comparative study of SYBR green I, 5 ’ -exonuclease, and hybridization probe assays. Applied Environ Microbiol 2003;69:4753–4759.

15. Probert WS, Schrader KN, Khuong NY, Bystrom SL, Graves MH. Real-time multiplex PCR assay for detection of Brucella spp., B. abortus, and B. melitensis. J Clin Microbiol 2004;42:1290–1293.

16. Queipo-Ortuno MI, Colmenero JD, Baeza G, Morata P.Comparison between lightCycler real-time polymerase chain reaction (PCR) assay with serum and PCR-enzyme-linked immunosorbent assay with whole blood samples for the diagnosis of human brucellosis. Clin Infect Dis 2005; 40:260–264.

17. Redkar R, Rosa S, Bricker B, DelVecchio V. Real-time detection of Brucella abortus , Brucella melitensis and Brucella suis . Mol Cell Probes 2001;15:43 –52.

18. Lazaro, D.R., Hernandez, M., Esteve, T., Hoorfar, J., Pla, M.A rapid and direct real time PCR-based method for identifica- tion of Salmonella spp. J. Microbiol. Methods 2003; 54:381- 390.

19. Jothikumar, N., Griffiths, M.W. Rapid detection of Escherichia coli O157:H7 with multiplex real-time PCR assays. Appl. Environ. Microbiol 2002;68: 3169 - 3171.

20. Inglis, G.D., Kalischuk, L.D. Direct quantification of Campylobacter jejuni and Campylobacter lanienae in feces of cattle by real-time quantitative PCR. Appl. Environ. Microbiol 2004;70: 2296 - 2306.

21. Panicker, G., Myers, M.L., Bej, A.K. Rapid detection of Vibrio vulnificus in shellfish and Gulf of Mexico water by real- time PCR. Appl. Environ. Microbiol 2004;70: 498 - 507.

22. Medici, D.D., Croci, L., Delibato, E., Di Pasquale, S., Filetici, E.,Toti, T. Evaluation of DNA extraction methods for use in combination with SYBR Green I real-time PCR to detect Salmonella enterica serotype enteritidis in poultry. Appl. Environ. Microbiol 2003;69: 3456 - 3461.

23. Bhagwat, A.A. Rapid detection of Salmonella from vegetable rinse-water using real-time PCR. Food Microbiol 2004;21: 73 - 78.

24. Jothikumar, N., Wang, X., Griffiths, M.W. Real-time multiplex SYBR Green 1-based PCR assay for simultaneous detection of Salmonella serovars and Listeria monocytogenes. J. Food Prot 2003;6: 2141 - 2145.

25. Daum LT, Barnes WJ, McAvin JC, Neidert MS, Cooper LA, Huff WB, et al. Real-time PCR detection of Salmonella in suspect foods from a gastroenteritis outbreak in Kerr County, Texas. J Clin Microbiol 2002;40:3050-2.

26. Malorny B, Bunge C, Helmuth R. A real-time PCR for the detection of Salmonella Enteritidis in poultry meat and consumption eggs. J Microbiol Meth 2007;70: 245-51.

27. Hadjinicolaou AV, Demetriou VL, Emmanuel MA, Kakoyiannis CK, Kostrikis LG.

Molecular beacon-based real-time PCR detection of primary isolates of Salmonella Typhimurium and Salmonella Enteritidis in environmental and clinical samples. BMC Microbiology 2009; doi:10.1186/1471-2180-9-97.

28. Murinda, S.E., Nguyen, L.T., Nam, H.M., Almeida, R.A., Headrick, S.J., Oliver, S.P. Detection of Shiga toxin-producing Escherichia coli, Listeria monocytogenes, Campylobacter jejuni, and Salmonella spp. in dairy farm environmental samples. Foodborne Pathog. Dis 2004;1: 97 - 104.

29. van Elsas, J.D., Smalla, K., Lilley, A.K., Bailey, M.J. Methods for sampling soil microorganisms. In: Hurst, C.J., Knudson, G.R., McInerney, M.J., Stetzenbach, L.D., Walter, M.V. (Eds.), Manual of Environmental Microbiology. ASM, Washington, 2004;pp. 505 - 515.

30. Hoorfar, J., Ahrens, P., R3dstrfm, P. Automated 5V nuclease PCR assay for identification of Salmonella enterica. J. Clin. Microbiol 2000;38: 3429 - 3435.

31. Coburn B, Grassl GA, Finlay BB. Immunol Cell Biol. 2007;85(2):112-8.

32. Pui, C. F., Wong, W. C., Chai, L. C., Tunung, R., Jeyaletchumi, P., et al..Review Article Salmonella: A foodborne pathogen. International Food Research Journal 2001;18: 465-473.

33. Reilly WJ, Sharp JCM, Forbes GI, Paterson GM. Milkborne salmonellosis in Scotland 1980 to 1982. Vet Rec 1983; 112:578-580.

34. James D. Wood, Gordon A. Chalmers, Raymond A. Fenton, Jane Pritchard, Matthew Schoonderwoerd, William L. Lichtenberger. Persistent shedding of Salmonella enteritidis from the udder of a cow. Can Vet J 1991; 32: 738-741.

35. Wang, X., Jothikumar, N., Griffiths, M.W. Enrichment and DNA extraction protocols for the simultaneous detection of Salmonella and Listeria monocytogenes in raw sausage meat with multiplex real-time PCR. J. Food Prot 2004;67: 189 - 192.

36. Fukushima, H., Tsunomori, Y., Seki, R. Duplex real-time SYBR Green PCR assays for detection of 17 species of food- or waterborne pathogens in stools. J. Clin. Microbiol

2003;41:5134 - 5146.

37. Bassler, H.A., Flood, S.J.A., Livak, K.J., Marmaro, J., Knorr, R. and Batt, C.A. Use of a £uorogenic probe in a PCR-based assay for the detection of Listeria monocytogenes. Appl. Environ. Microbiol 1995;61: 3724-28

38. Chen, S., Yee, A., Gri√ths, M., Larkin, C., Yamashiro, C.T., Behari,R., Paszko-Kolva, C., Rahn, K. and De Grandis, S.A. The evaluation of a £uorogenic polymerase chain reaction assay for the detection of Salmonella species in food commodities. Int. J. Food Microbiol 1997;35: 239-250.

39. Wu, R., Liu, G. and She, S.H. Carriage states, isolation and cultivation of Campylobacter jejunifrom human, domestic animals and poultry. Chin. J. Vet. Sci. Tech 2000;30: 13-15.

40. Wang, Z.G., Qin, X., Zheng, X.M., Liu, H.P. and Wu, J. Isolation and identi¢cation of Campylobacterspecies on frozen chick-en meat. Chin. J. Zoonoses 2001;17: 63-65.

41. David, F. and Steven, A. Quantitative immunocapture PCR assay for detection of Campylobacter jejuni in foods. Appl. Environ. Microbiol 2000;66: 4115-4118.

42. Padungtod, P., Hanson, R., Wilson, D.L., Bell, J., Linz, J.E. and Kaneene, J.B. Identi¢cation of Campylobacter jejuniisolates from cloacal and carcass swabs of chickens in Thailand by a 5 P nuclease £uorogenic polymerase chain reaction assay. J. Food Prot 2002;65: 1712-1716.

43. Hege, K.N., Anette, B., Askild, H. and Knut, R. Application of the 5P -nuclease PCR assay in evaluation and development of meth-ods for quantitative detection of Campylobacter jejuni. Appl. Envi-ron. Microbiol 2000;66: 4029-4036.