Letters in Applied Microbiology 2002, **34**, 37–41

Implementation of real-time PCR to tetrathionate broth enrichment step of *Salmonella* detection in poultry

A. Eyigor[1], K.T. Carli[2] and C.B. Unal[3]

[1]Department of Food Hygiene and Technology, [2]Department of Microbiology, Faculty of Veterinary Medicine, Uludag University, Gorukle Kampusu, 16043 Bursa, Turkey, [3]Iontek Ltd, Bursa, Turkey

2001/185: received 21 June 2001, revised 24 September 2001 and accepted 5 October 2001

A. EYIGOR, K.TAYFUN CARLI AND C.B. UNAL. 2002.

Aims: The present study describes the implementation of real-time PCR to tetrathionate broth enrichment step of *Salmonella* detection in poultry.

Methods and Results: Real-time PCR with *Salmonella invA*-specific primers and a standard bacteriological method was applied to detect *Salmonella* in tetrathionate enrichment cultures of 492 intestinal homogenates and 27 drag swabs from 47 poultry flocks. The number of positive individual samples by real-time PCR and culture method was 65 (12·5%) and 35 (6·8%), respectively. The number of *Salmonella*-positive flocks was 13 (27·7%) by both methods. PCR detection required 25 min for up to 32 samples. Melting curve analysis revealed the T_m for *Salmonella*-specific PCR product as 87 ± 1°C.

Conclusions: Implementation of real-time PCR to tetrathionate broth enrichment step reduces the *Salmonella* detection time to 18 h and 25 min. Isolation of *Salmonella* should be carried out with PCR to determine the serovar.

Significance and Impact of the Study: Real-time PCR is a powerful tool in rapid and accurate *Salmonella* monitoring in poultry companies, together with standard bacteriology.

INTRODUCTION

Detection of *Salmonella* by bacteriological methods is time-consuming (Wallace *et al*. 1999; Anonymous 1996). Also, false-negative results can be obtained by bacteriology, when the initial number of salmonellae is low in the sample (Fricker 1987). Therefore, a rapid and sensitive primary screening method with a proper sampling plan is required to detect *Salmonella* in the flock or in the food industry. For this purpose, polymerase chain reaction (PCR) has been applied to various types of samples after incubation in an enrichment broth (Stone *et al*. 1994; Aabo *et al*. 1995; Chiu and Ou 1996; Cohen *et al*. 1996; Luk *et al*. 1997; Bennett *et al*. 1998; Lin and Tsen 1999; Soumet *et al*. 1999). We have detected salmonellae in chicken faeces by a tetrathionate broth (TTB) enrichment, capillary PCR and capillary gel electrophoresis, and obtained positive results with high sensitivity (Carli *et al*. 2001a).

Correspondence to: K. Tayfun Carli, Department of Microbiology, Faculty of Veterinary Medicine, Uludag University, Gorukle Kampusu, 16384, Bursa, Turkey (e-mail: tayfun@uludag.edu.tr).

© 2002 The Society for Applied Microbiology

Recent advancements in the PCR technology combined capillary thermocycling and real-time fluorescence detection of the PCR product (Wittwer *et al*. 1997; Ke *et al*. 2000; Loeffler *et al*. 2000; Pietila *et al*. 2000). The LightCycler (Roche Diagnostics, Mannheim, Germany) real-time PCR system reduces the detection time, allows the process of up to 32 samples simultaneously, and determines the specificity of the PCR product by melting curve analysis.

We applied this real-time PCR technology to TTB enrichment culture of a standard *Salmonella* isolation method from poultry. Thus, we were able to monitor and determine *Salmonella*-infected flocks by a reliable and a rapid primary screeening procedure.

MATERIALS AND METHODS

Bacterium

Salmonella enterica serovar Enteritidis 64K (obtained from Institut Pasteur, Paris, France) was propagated and used as a positive control in PCR.

Samples and application of standard bacteriology for *Salmonella*

Four hundred and ninety-two ileocecal part of the intestines were collected from 24 chicken flocks, which were brought to our laboratory. Twenty-seven drag swabs from breeder chicks of 27 flocks were transferred into 50 ml TTB, and shipped to the laboratory within 1 h. Both sample types were supplied from five major breeder companies in Turkey. Ten grams of the ileocecal parts were homogenized in 250 ml TTB with ultraturrax, and 1 g of the homogenate was inoculated into 10 ml TTB. Each drag swab sample in 50 ml was added into 200 ml TTB.

The National Poultry Improvement Plan (NPIP) and Auxillary Provisions, United States Department of Agriculture (Anonymous 1996) procedure was applied to isolate and identify salmonellae (Fig. 1), with a determined detection limit of 10 cfu ml^{-1} (Carli *et al*. 2001b).

Template preparation for real-time PCR

Crude DNA was extracted by a method described by Carli *et al*. (Fig. 1) (2001a).

Primers

We used a *Salmonella inv*A gene-based primer pair with a previously determined specificity and sensitivity (Rahn *et al*. 1992). These primers, invA1 and invA2, have the following nucleotide sequences, respectively: 5′-GTG AAA TTA TCG CCA CGT TCG GGC AA-3′ and 5′-TCA TCG CAC CGT CAA AGG AAC C-3′. Both primers were synthesized in Expedite DNA synthesizer (Perseptive Biosystems, CA, USA) and were purified using reverse phase-High Pressure Liquid Chromatography (Bio-CAD700E, Perseptive Biosystems, USA).

Real-time PCR

We used LightCycler PCR methodology for this purpose. Two microlitres from the sample template, *Salmonella enteritidis* 64K culture template, or deionized water was added into 18 μl of PCR mixture as unknown, positive control, or negative (no-target) control, respectively. The reaction mixture optimized for LightCycler PCR contained the following: 2 μl of 10 × concentration Light Cycler-DNA Master SYBR Green I ready to use reaction mix for PCR (*Taq* DNA polymerase, reaction buffer, dNTP mix [with dUTP instead of dTTP], SYBR Green I dye, and 10 mmol l^{-1} $MgCl_2$), 2·4 μl of 25 mmol l^{-1} $MgCl_2$, 1 μl of each 100 μmol l^{-1} primers, 2 μl of template and 11·6 μl of deionized water. The amplification protocol included the initial denaturation step at 95°C for 30 s and 40 cycles of denaturation at 95°C for 5 s, annealing at 55°C, and 10 s of primer extension at 72°C. Temperature transition rate was 20°C s^{-1}. Fluorescence was measured automatically at the end of each extension step.

Melting curve analysis

After PCR amplification, all the DNA in the capillary was denaturated by rapid heating to 95°C. Then the reaction was cooled to 65°C for 15 s and slowly heated to 95°C (with a rate of 0·1°C s^{-1}). Fluorescence was measured continously during the slow temperature rise to monitor dissociation of double stranded DNA as it melted at a characteristic temperature (87 ± 1°C). Fluorescence signals were first plotted automatically in real-time *vs.* temperature (*T*) to produce melting curves for specific PCR products from salmonellae, and then were converted to melting peaks by plotting the negative derivative of fluorescence *vs. T* (*–d*F1/*dT vs. T*).

Sensitivity and detection-limit determination of real-time PCR

In order to determine the detection sensitivity of real-time PCR, we used primary TTB enrichment cultures of artificially spiked samples with *Salm. enterica* serovar Enteritidis 64K. Briefly, 1 g of *Salmonella*-free chicken iliocecal samples (determined by a bacteriological method and PCR) were minced and added into 10 ml of TTB. Ten fold dilutions from 6×10^3 cfu ml^{-1} to 6 cfu ml^{-1} of serovar Enteritidis 64K was inoculated into TTB with intestinal homogenate and were incubated at 37°C for 18 h. Real-time PCR was performed by using the crude DNA extracted (Carli *et al*. 2001a) from 1 ml of these cultures. *Salmonella* growth was confirmed by plating from each dilution.

RESULTS AND DISCUSSION

In this study, we combined LightCycler real-time PCR and the bacteriological enrichment step of a standard *Salmonella* isolation method, in order to rapidly and reliably determine *Salmonella*-infected chicken flocks (Fig. 1).

We were able to detect salmonellae in 25 min from up to 32 18-h primary enrichment sample cultures from subclinically infected chickens. The LightCycler real-time PCR, with the SYBR Green I Dye format, is about 10 times faster than previously reported conventional PCR detection methods (Stone *et al*. 1994; Aabo *et al*. 1995; Cohen *et al*. 1996) and gives more rapid results than the capillary PCR method combined with capillary gel electrophoresis described by Carli *et al*. (2001a).

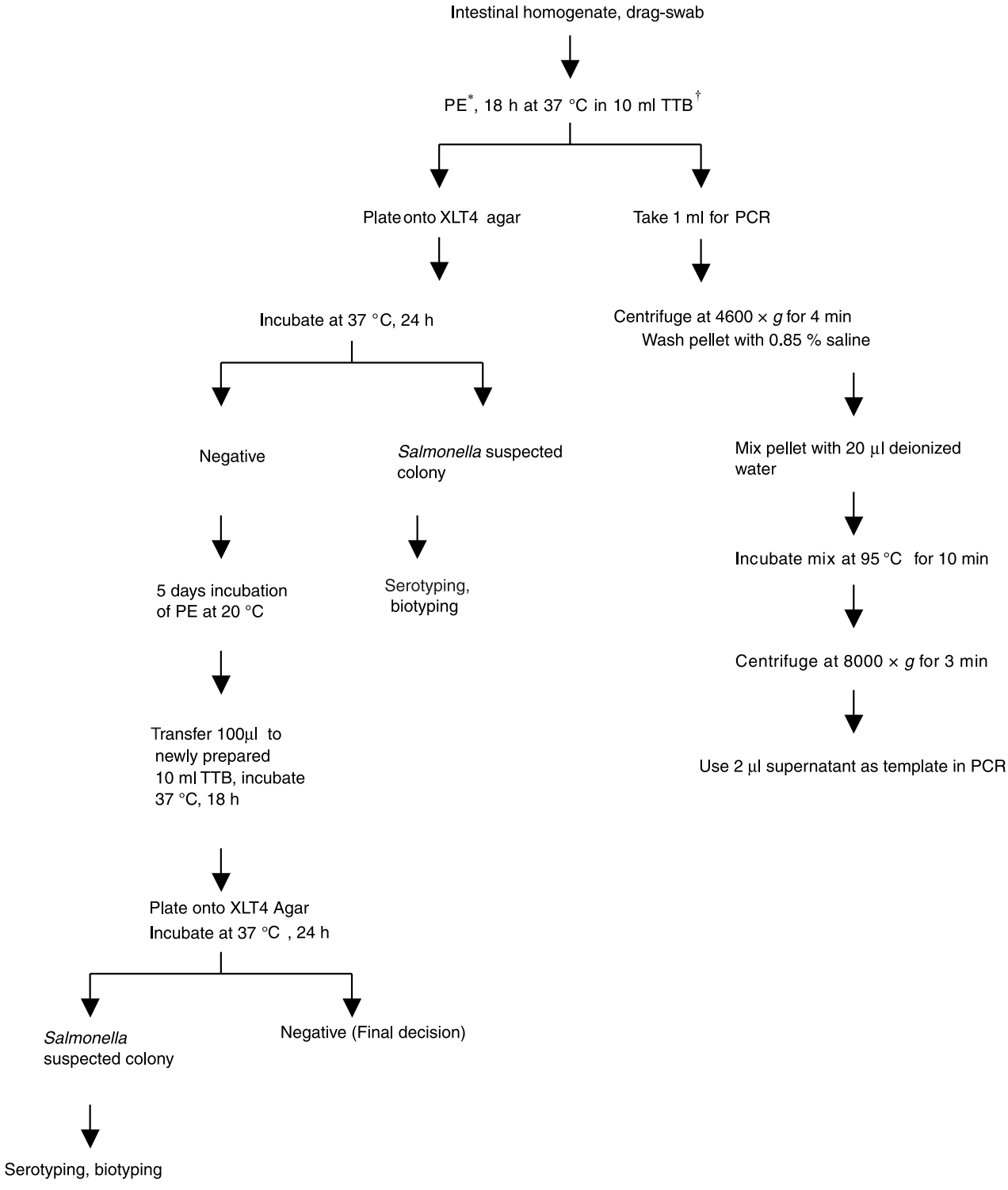

Fig. 1 Diagram of the procedures for bacteriological isolation and template preparation for real-time PCR. *PE: Primary Enrichment; †TTB: Tetrathionate Broth

In this system, PCR amplification and analysis occurred simultaneously. During each PCR cycle, the amount of newly synthesized *Salmonella*-specific PCR product was monitored by using the double-stranded DNA binding dye SYBR Green I, as a proportionally increasing fluorescent signal. Then, S*almonella* PCR products with specific melting temperatures (T_m) were identified by DNA melting curve analysis and melting peaks were plotted (Fig. 2). This allowed us to identify the amplified product within 5 min without time-consuming gel electrophoresis and gel staining used after conventional PCR. We have further confirmed that the sizes of the PCR products from selected *Salmonella* strains were 281 bp by capillary gel electrophoresis (Carli *et al*. 2001a).

The detection sensitivity of this real-time PCR was determined as 6 cfu ml^{-1} from artificially spiked intestinal samples. This high detection sensitivity allowed us to determine the presence of *Salmonella* even in heavily contaminated samples, such as intestinal samples and drag swabs.

In this study, we found that real-time PCR detection sensitivity from natural samples was higher than bacteriology both in intestinal samples and in total, when the samples taken from each flock were considered individually (Table 1). Possible reasons for this can be as follows: salmonellae found in natural samples may show atypical biochemical profiles and may not be detected in bacteriology (Bennet *et al*. 1998), and *Salmonella* cells may be present in a viable but non-culturable status (Knight *et al*. 1990). The number of *Salmonella*-positive flocks was 13 (27·7%) both by PCR and culture method. Since *Salmonella* screening programs in poultry production is based on the diagnosis of *Salmonella*-infected flocks, not the individuals, when a sufficient sample size is used, the presence of PCR-positive and culture-negative individual samples will not affect the final diagnosis for the flock.

In conclusion, this study indicates that the use of real-time PCR with samples from tetrathionate primary enrichment step is a valuable method to dramatically decrease the diagnosis time of *Salmonella*-infected chicken flocks, and

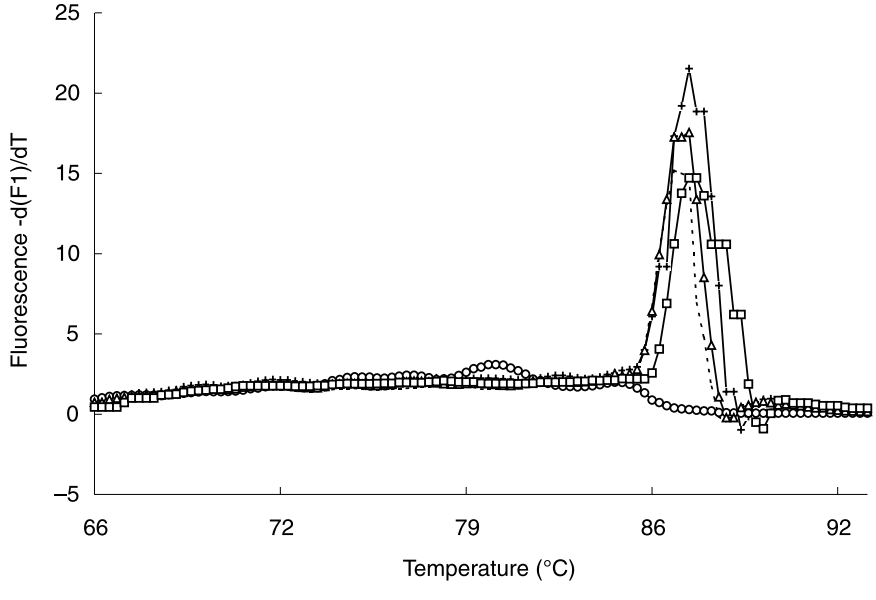

Fig. 2 Melting peaks of selected *Salmonella* isolates determined by real-time PCR. Strains: (+) positive control – *Salmonella enteritidis* 64K; (O) negative control – deionized water; (–) *Salm*. serovar Enteritidis (△) *Salm*. serovar Thompson; (□) *Salm*. serovar Agona

Table 1 Comparison of the results from intestinal homogenate and drag swab samples by real-time PCR and bacteriological culture method

Sample type	No. of flocks	No. of samples	No. of positive individual samples		No. of positive flocks	
			PCR (%)	Culture method (%)	PCR (%)	Culture method (%)
Intestinal homogenate	24	492	62 (12·6)	32* (6·5)	10 (41·6)	10 (41·6)
Drag swab	27	27	3 (11·1)	3† (11·1)	3 (11·1)	3 (11·1)
Total	47	519	65 (12·5)	35 (6·8)	13 (27·7)	13 (27·7)

*All of the isolates were *Salm*. serovar Enteritidis.

†Isolates were identified as *Salm*. serovar Agona, Thompson, and serogroup C2.

that bacteriology should be continued for confirmatory and strain identification purposes.

ACKNOWLEDGEMENTS

This work was supported by the Scientific and Technical Research Council of Turkey (TUBITAK) grant TOG-TAG/TARP-2203. We thank Sevket Gunay and Sevil Sener for laboratory assistance.

REFERENCES

Aabo, S., Andersen, J.K. and Olsen, J.E. (1995) Research note: detection of *Salmonella* in minced meat by the polymerase chain reaction method. *Letters in Applied Microbiology* **21**, 180–182.

Anonymous. (1996) *Laboratory Procedure Recommended for the Bacteriological Examination of Salmonella APHIS 91-55-031*. US Department of Agriculture, Animal and Plant Health. Inspection Service. National Poultry Improvement Plan and Auxiliary Provisions, Subpart B- Bacteriological Examination Procedure 147–11, pp. 14–19, Washington DC: Government Printing Office.

Bennett, A.R., Greenwood, D., Tennant, C., Banks, J.G. and Betts, R.P. (1998) Rapid and definitive detection of *Salmonella* in foods by PCR. *Letters in Applied Microbiology* **26**, 437–441.

Carli, K.T., Eyigor, A. and Caner, V. (2001b) Prevalence of *Salmonella* serovars in chickens in Turkey. *Journal of Food Protection* **64**, 1832–1835.

Carli, K.T., Unal, C.B., Caner, V. and Eyigor, A. (2001a) Detection of *Salmonella* in chicken faeces by a combination of tetrathionate broth enrichment, capillary PCR and capillary gel electrophoresis. *Journal of Clinical Microbiology* **39**, 1871–1876.

Chiu, C.H. and Ou, J.T. (1996) Rapid identification of *Salmonella* serovars in faeces by specific detection of virulence genes, *invA* and *spvC*, by an enrichment broth culture-multiplex PCR combination assay. *Journal of Clinical Microbiology* **34**, 2619–2622.

Cohen, N.D., Martin, L.J., Simpson, B., Wallis, D.E. and Neibergs, H.E. (1996) Comparison of polymerase chain reaction and microbiological culture for detection of *Salmonella* in equine faeces and environmental samples. *American Journal of Veterinary Research* **57**, 780–786.

Fricker, C.R. (1987) The isolation of salmonellas and campylobacters. *Journal of Applied Bacteriology* **63**, 99–116.

Ke, D., Menard, C., Picard, F.J., Boissinot, M., Ouelette, M., Roy, P.H. and Bergeron, M.G. (2000) Development of conventional and real-time PCR asssay for the rapid detection of group B Streptococci. *Clinical Chemistry* **46**, 324–331.

Knight, I.T., Shults, S., Kaspar, C.W. and Colwell, R.R. (1990) Direct detection of *Salmonella* spp. in estuaries by using a DNA probe. *Applied and Environmental Microbiology* **56**, 1059–1061.

Lin, J.S. and Tsen, H.Y. (1999) Development and use of polymerase chain reaction for the specific detection of *Salmonella typhimurium* in stool and food samples. *Journal of Food Protection* **62**, 1103–1110.

Loeffler, J., Henke, N., Hebart, H., Schmidt, D., Hagmeyer, L., Schumacher, U. and Einsele, H. (2000) Quantification of fungal DNA by using fluorescence resonance energy transfer and the light cycler system. *Journal of Clinical Microbiology* **38**, 586–590.

Luk, J.M., Kongmuang, U., Tsang, R.S.W. and Lindberg, A.A. (1997) An enzyme-linked immunosorbent assay to detect PCR products of the *rfbS* gene from serogroup D salmonellae: a rapid screening prototype. *Journal of Clinical Microbiology* **35**, 714–718.

Pietila, J., He, Q., Oksi, J. and Viljanen, M.K. (2000) Rapid Differentiation of *Borrellia garinii* from *Borrelia afzelii* and *Borrelia burgdorferi* sensu stricto by LightCycler Flourescence melting curve analysis of a PCR product of the *recA* gene. *Journal of Clinical Microbiology* **38**, 2756–2759.

Rahn, K., De Grandis, S.A., Clarke, R.C., McEwen, S.A., Galan, J.E., Ginocchio, C., Curtis,R. and IIIGyles, C.L. (1992) Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Molecular and Cellular Probes* **6**, 271–279.

Soumet, C., Ermel, G., Rose, V., Rose, N., Droin, P., Salvat, G. and Colin, P. (1999) Identification by a multiplex PCR-based assay of *Salmonella* Typhimurium and *Salmonella* Enteritidis strains from environmental swabs of poultry houses. *Letters in Applied Microbiology* **29**, 1–6.

Stone, G.G., Oberst, R.D., Hays, M.P., McVey, S. and Chengappa, M.M. (1994) Detection of *Salmonella* serovars from clinical samples by enrichment broth cultivation-PCR procedure. *Journal of Clinical Microbiology* **32**, 1742–1749.

Wallace, H.A., June, G., Sherrod, P., Hammack, T.S. and Amaguana, R.M. (1999) *Salmonella*. In: *Food and Drug Administration Bacteriological Analytical Manual* (Ed. by Tomlison, L.A.). Hypertext Source*: http://vm.cfsan.fda.gov/~comm/bam-5.html#Intro.*

Wittwer, C.T., Ririe, K.M., Andrew, R.V., David, D.A., Gundry, R.A. and Balis, U.J. (1997) The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. *Biotechniques* **22**, 176–181.