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Rapid Identification of Avian *Salmonella* Isolates by Air Thermal Cycler Amplification and Capillary Gel Electrophoresis

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Abstract : The possible use of a genus-specific polymerase chain reaction (PCR) for the identification of *Salmonella* colonies was tested on 14 reference strains and 38 clinical *Salmonella* strains isolated from chickens by the standard method of the National Poultry Improvement Plan and Auxiliary Provisions (NPIP), USDA, U.S.A. All the PCRs using primer pairs InvA1 and InvA2 (5) gave 281 bp amplification product specific for the *Salmonella* genus and produced results identical (100%) to those obtained with biochemical and serological methods. Thus, the genus-specific capillary PCR for the identification of a colony of *Salmonella*e from a selective agar plate was performed within approximately 2 h and the total time required for definitive diagnosis of infection was 2 days using primary enrichment (PE) and 7 days using delayed secondary enrichment (DSE).

Key Words: *Salmonella*, poultry, polymerase chain reaction

Kanatlı *Salmonella* İzolatlarının Air Thermal Cycler Amplifikasyonu ve Kapiller Jel Elektroforezi ile Hızlı İdentifikasyonu

Özet : On dört referens suş ve NPIP standart metoduyla tavuklardan izole edilen 38 klinik *Salmonella* izolatı kullanılarak kolonilerin identifikasyonu için cinse-özgün polimeraz zincir reaksiyonunun (PZR) olası kullanımı test edildi. InvA1 ve InvA2 primer çiftinin kullanımıyla yapılan tüm PZR'lar *Salmonella* cinsine özgün 281 bp büyüklüğünde PZR ürünleri verdi, ve biyokimyasal ve serolojik identifikasyon bulgularına eşdeğer sonuçlar oluşturdu. Böylece, bir seçici agardan alınan *Salmonella* kolonisinin identifikasyonu için cinse-özgün kapillar PZR yaklaşık 2 saat içinde yapıldı ve infeksiyonun tam tanısı için gerekli süre ise birincil zenginleştirme kullanımıyla 2 gün, ikincil gecikmiş zenginleştirme kullanımıyla 7 gün olarak saptandı.

Anahtar Sözcükler: *Salmonella*, kanatlı, polimeraz zincir reaksiyonu

Introduction

Salmonellae are important pathogens in chicken, and are transferred to humans through poultry food products (1, 2). Because of this, detection of *Salmonellae* in poultry may be considered the primary step in the prevention of salmonellosis. Several detection methods have been described for the diagnosis of *Salmonella*-infected chicken and definitive diagnosis has been based on isolation of the bacteria. The isolation and identification of *Salmonella* requires 5 to 11 days under optimal conditions (3). This is too long to prevent the spread of *Salmonella* within a flock and too long effective measures to be taken against salmonellosis if there is an infection in a flock. In order to shorten the identification period, confirmation of *Salmonella* colonies was obtained by PCR with food isolates using primers ST11 and ST15 (4). Using a similar approach, this paper describes genus-specific PCR using the *Inv*A primer pairs reported by Rahn et al. (5) in order to identify *Salmonella* colonies from chicken intestine. The PCR results are compared with those of bacteriological procedure described by the NPIP (3).

Materials and Methods

Clinical Isolates. A total of 38 isolates, of which 24 were *Salmonella enterica* serovar Enteritidis, 13 were *Salmonella enterica* serovar Agona, and one was *Salmonella enterica* serovar Thompson, were isolated from chicken in a previous study (6) according to the bacteriological procedures described in the NPIP, U.S.A. (3), and then serotyped.

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Control Strains. S. serovar Enteritidis 64K (SE64K), S. serovar Typhimurium LT2-CIP60-62 (ST LT2-CIP60-62) and *Salmonella enterica* serovar Gallinarum 64K (SG64K) were obtained from Dr. M. Y. Popoff, Institute Pasteur, 28 rue du Doctor Roux, 7572 Paris Cedex 15, France. Five S. serovar Enteritidis, 4 S. serovar Typhimurium, 2 S. serovar Gallinarum, and non-*Salmonella* strains (*Citrobacter* sp., *Escherichia coli, Klebsiella* sp., *Pseudomonas aeriginosa* and *Streptococcus* sp.) were obtained from the Department of Microbiology, Schools of Medicine and Veterinary Medicine, University of Uludağ, Bursa, Turkey. The colonies of these strains were also tested with the PCR procedure used for the clinical isolates.

Polymerase Chain Reaction (PCR). Crude DNA was prepared by boiling a suspension of colonies from the control and clinical isolates with minor modifications to the method described by Rahn et al. (5). Eppendorf tubes containing 100 µl suspension of suspected colonies in 0.85% NaCl were washed twice with 0.85% saline and the pellet was resuspended in 20 µl deionized water. This suspension was boiled for 10 min, centrifuged for 3 min at 14,000 rpm so as to pellet the particulate matter, and 5 µl of the supernatant was used as the template in the PCR mixture. *Salmonella* genus-specific primers, InvA1 (5' -GTG AAA TTA TCG CCA CGT TCG GGC AA-3') and InvA2 (5' –TCA TCG CAC CGT CAA AGG AAC C-3'), which are based on the S. serovar Typhimurium *invA* gene sequence (5), were synthesized in an Expetide DNA synthesizer (PerSeptive Biosystems, USA) and were purified using reverse-phase High Pressure Liquid Chromatography (BioCAD700E, PerSeptive Biosystems, U.S.A.). InvA1 was synthesized FAM-labeled. The 25 µl PCR mixture, which contained 0.3 µl (5U/µl) Taq DNA polymerase (Iontaq), 2.5 µl of 10XPCR buffer (3.5mM $MgCl_2$), 2.5 µl dNTP mixture (2mM), 1 µl of each primer (5 pmol/ml), 5 µl of template, 2 µl of BSA (Bovine Serum Albumin, 2.5mg/ml) and 10.2 µl of deionized water, was taken into Borosilicate capillary tubes (Idaho Technology, U.S.A.) by capillary action.

The PCR incubations were performed using a DNA Air Thermal Cycler Model 1605 (Idaho Technologies, U.S.A.). The cycle times were as follows: an initial incubation at 94°C for 15 sec; 30 cycles of denaturation at 94°C for 0 sec; primer annealing at 50°C for 0 sec; and primer extension at 72°C for 15 sec. Following the last cycle, there was a 5-min incubation at 72°C. A 2-µl aliquot of the reaction mixture was added to 12 µl formamide (denaturating agent) /TAMRA (Molecular Weight Marker) (12v/0.5v) mixture, and the final mixture was incubated at 95°C for 5 min. Straight after this incubation, the mixture was put on wet ice for 3 min and spun briefly. Bands (peaks) forming after PCR were visualized by capillary gel electrophoresis using ABI PRISM 310 (PE Applied Biosystems, GB).

Results

Thirty-eight *Salmonella* isolates obtained from the iliocecal junctions of chickens of clinically healthy appearance and 14 reference *Salmonella* strains (Table) produced a specific 281 bp amplicon. No amplification was observed in PCRs with non-*Salmonella* strains. The amplification peaks of the selected isolates of S. serovar Enteritidis, S. serovar Agona, S. serovar Thompson, and of the reference strains SE64K, ST LT2-CIP60-62 and SG64K, are shown in the electropherograms in Figures 1 and 2, respectively.

Table 1. Serogroup/ Serotype and PCR results of the clinical *Salmonella* isolates and the reference strains used in this study.

Serogroup /Serotype	Positive PCR Results with	
	PE[a]	DSE[b]
Clinical Isolates		
B/ S. serovar Agona	4	13
D/ S. serovar Enteritidis	18	24
C1/ S. serovar Thompson	1	1
TOTAL	23	38
Reference Strains		
D/ S. serovar Enteritidis 64K	1	
B/ S.serovar Typhimurium LT2-CIP60-62	1	
D/ S. serovar Gallinarum 64K	1	
D/ S. serovar Enteritidis[c]	5	
B/ S.serovar Typhimurium[c]	4	
D/ S. serovar Gallinarum[c]	2	
TOTAL	14	

[a]Primary Enrichment, [b]Delayed Secondary Enrichment, [c]Department of Microbiology, Uludağ University, Bursa, Turkey.

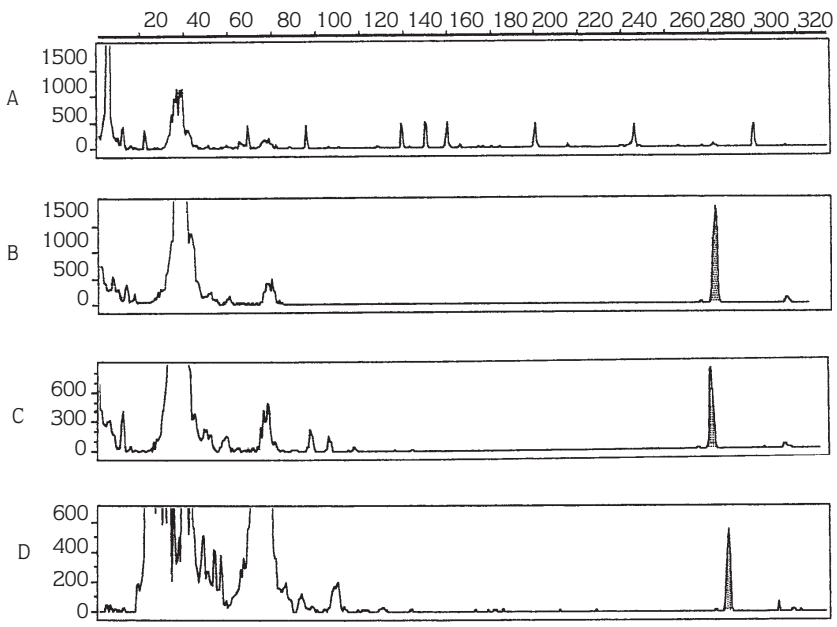

Figure 1. *Salmonella* genus-specific PCR amplification peaks from selected isolates. Electropherograms: A, peak patterns of GeneScan 500-size standard fragments run under denaturing conditions; B, C, and D, 281 bp PCR product detected from S. serovar Enteritidis, S. serovar Agona and S. serovar Thompson strains, respectively.

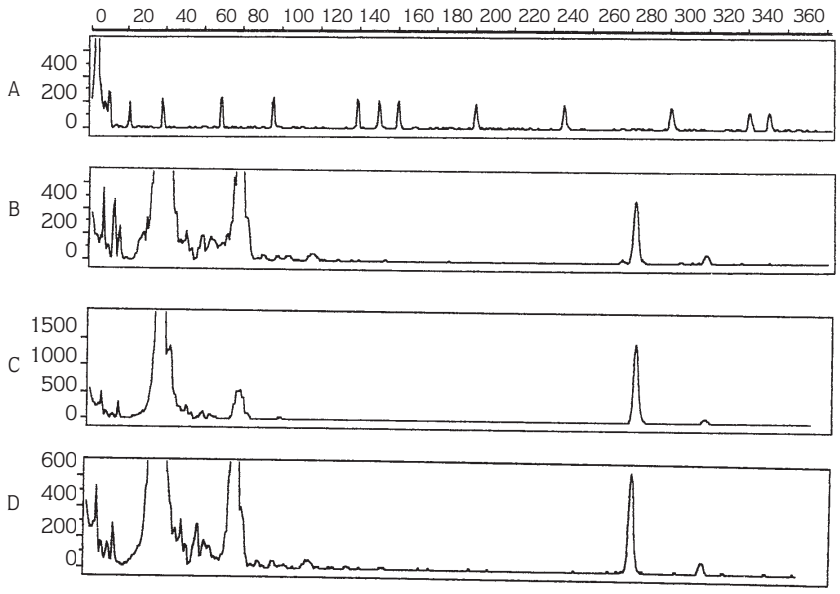

Figure 2. *Salmonella* genus specific PCR amplification peaks from reference strains. Electropherograms: A, peak patterns of GeneScan 500-size standard fragments run under denaturing conditions; B, C, and D, 281 bp PCR product detected from SE64K, ST LT2-CIP60-62 and SG64K, respectively.

Discussion

The isolation and identification of *Salmonellae* to genus level from poultry generally requires 5 to 11 days with standard methods and involves biochemical confirmation (3). This time-consuming procedure does not seem to be economically feasible for poultry producers in the field, as they need a precise diagnosis as soon as possible in order to take action against a possible salmonellosis problem in their flocks.

In order to shorten the identification time, we used PCR as part of the isolation procedure. Using genus-specific primers, which were applied to presumptive *Salmonella* colonies which appeared on Xylose Lysine Tergitol 4 (XLT4) agar, the time required for

identification was reduced to 2 days for PE and 7 days for DSE. If the capillary PCR together with capillary gel electrophoresis is considered alone, it required 70-80 min (7). This was a significantly shortened time when compared with conventional biochemical and serological tests.

The results of this study show that the genus-specific PCR using *invA* gene-based primers can be reliably used for the identification of presumptive colonies from the PE and DSE steps of the NPIP method for the detection of *Salmonella*. These capillary PCR and gel electrophoresis methods correlate 100% with identification by biochemical and serological tests. This is also the fastest procedure, requiring a maximum of 70-80 min, and allows the diagnostic poultry laboratories to give a quick and definitive response to poultry producers.

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