

## Molecular epidemiology of systemic *Salmonella enterica* serovar Typhimurium outbreak in canaries

Zahraei Salehi T<sup>1\*</sup>, Madadgar O<sup>1</sup>, Ghafari M M<sup>1</sup>, Ashrafi Tamai I<sup>1</sup>, Madani S A<sup>2</sup>

<sup>1</sup>Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. <sup>2</sup>Department of Poultry Science, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

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### ABSTRACT

**Background and Objectives:** In May 2007, high mortality with severe septicemia was reported in 17 flocks of canaries in different regions of Tehran province. This study was designed to follow up and study a great outbreak of salmonellosis in these canaries.

**Materials and Methods:** Two carcasses from every flock, environment, food and water resources were examined. After isolating the bacteria, serotyping and multiplex PCR were performed to confirm the bacteria identified. The isolates within the same serovars were investigated by R-typing using 33 antibiotics and then subjected to RAPD-PCR with three primers. The genomic DNA from these isolates were digested with *XbaI* and the macro restriction fragment were separated by PFGE.

**Results:** *S. Typhimurium* was isolated from dead carcasses, visceral organs, stools and feed. Thirty-six isolated strains (35 isolates from canary carcasses and one isolate from feed) showed similar results in all of the tests, confirming the occurrence of an outbreak.

**Conclusion:** Canaries seem to be very susceptible to infection with *S. Typhimurium*. The clonality of isolated organisms and its characteristics is significantly important due to the severe septicemia and high mortalities in this outbreak and its public health threats. Environmental contamination within the cages, and food contaminated with stools of other canaries were the sources of infection. Inspection for food hygiene, daily cleaning of canary's cage from stool and carrier insects and rodents are necessary to prevent such outbreaks. Combination of R-typing, RAPD-PCR and PFGE increase the differentiation power of isolates, however, they showed clonality of *S. Typhimurium* involved in this outbreak.

**Keywords:** *Salmonella Typhimurium*, PFGE, RAPD-PCR, R-typing, canary, Iran.

### INTRODUCTION

*Salmonella* serovars are among the most frequent causes of bacterial infections in animals and human (1). *S. enterica* subsp *enterica* serovar Typhimurium is the most frequently isolated serovars and is one of the top three serotypes isolated worldwide over many decades from human, domestic and wild animals, feed, and the environment, with cattle and poultry being important source of infection for humans (2, 3). *Salmonella* can also be isolated from domestic birds, captive raptors, and other wild birds, especially gulls and corvids (4).

Effective public health monitoring of *Salmonella* infections by serotyping and/or other typing methods does interrupt transmission and reduce number of infections (4).

In May 2007, high mortality with severe septicemia was reported in 17 flocks of adult canaries in different regions of Tehran province. This study was performed to follow the infection, confirm an epidemic, test antibiotic susceptibility and fingerprinting characteristics of this highly virulent strain and finally interrupt transmission to humans.

### MATERIALS AND METHODS

**Samples.** Carcasses of canaries were referred to the Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran. Carcasses belonged to the seventeen flocks around the Molavi Street in downtown of Tehran. Number of canaries, number of deaths and mortality rate of every flock is shown in

\* Corresponding Author: Taghi Zahraei Salehi; DVM, Ph.D.  
Address: Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran.  
P.O. Box: 14155-6453, Tehran, Iran.  
Tel: +98-21-66427517  
Fax: +98-21-66431105  
E-mail: tsalehi@ut.ac.ir

Table 1.

**Pathology.** Macroscopic and microscopic necropsy examinations were performed by H&E staining of liver and proventriculus sections as soon as possible.

**Bacterial isolates.** For outbreak follow-up, two samples from every flock were worked up for isolation of bacteria. Food and water sources were also examined.

**Biotyping.** Biotyping was performed by the conventional methods and biochemical tests.

**Serotyping.** Serotyping of isolated *Salmonella* was performed by antisera (Difco, Detroit, MI, USA).

**M-PCR.** Serotyping was confirmed with multiplex PCR, using 4 pairs of primers, as described by Lim, Y. H *et al.*, 2003 and Rahn *et al.*, 1992 (5, 6).

**Antibiotic susceptibility testing.** Antibiotic susceptibility testing was performed by the standard disk diffusion method in Mueller- Hinton agar, and the results were interpreted according to the criteria of the Clinical & Laboratory Standards Institute (CLSI) (7, 8). The isolates were screened for resistance to the following antibiotics: tiamulin (30µg), tylosin (30µg), lincospectine (15/200), flumequine (30µg), difloxacin (25µg), neomycin (30µg), sulphamethoxazole (100µg), florfenicol (30µg), trimethoprim (5µg), enrofloxacin (5µg), cloxacillin (1µg), oxytetracycline (30µg), furazolidone (100µg), chloramphenicol (30µg), ampicillin (10µg), ceftriaxone (30µg), co-amoxiclav (30µg), imipenem (10µg), difloxacin (25µg), clarithromycin (15µg), ceftazidime (30µg), cefotaxime (30µg), kanamycin (30µg), cephalosin (30µg), ciprofloxacin (5µg), cefixime (5µg), piperacillin (100µg), norfloxacin (10µg), carbenicillin (100µg), ofloxacin (5µg), cefuraxime (30µg), vancomycin (30µg) and ticarcillin (75µg).

**RAPD fingerprinting.** A single colony of each isolate was picked from agar plate and suspended in 200 µl of distilled H<sub>2</sub>O. After vortexing, the suspension was boiled for 5 min, and 50 µl of the supernatant was collected after spinning for 10 min at 14,000 rpm in a microcentrifuge. The DNA concentration of boiled extracts was determined with spectrophotometer.

PCR was conducted in a 25 µl volume containing 40 ng of total *S. Typhimurium* DNA (extracted by boiling), 1.5 mM MgCl<sub>2</sub>, 0.5 µM of primer, 1 U of *Smartaq* DNA polymerase and 200 mM dNTP mix in 1× PCR buffer. Thermal program and electrophoresis condition was as previously described by Lin *et al.*, (9).

**Table 1.** Number of canaries and mortality rates in each flock in the outbreaks of salmonellosis.

Number of flock	Number of canary in flock	Number of death in flock	Mortality rate (%)
1	16	15	94
2	40	9	23
3	20	10	50
4	15	8	53
5	26	16	62
6	50	10	20
7	25	10	40
8	4	2	50
9	100	50	50
10	40	20	50
11	15	8	54
12	100	25	25
13	32	18	56
14	60	25	42
15	44	20	45
16	80	60	75
17	25	20	80
			<b>Average</b>
			<b>50.11%</b>

Primers selected for this study were P1254 5'-CCGCAGCCAA-3', 23L 5'-CCGAAGCTGC-3' and OPA-4 5'-AATCGGGCTG-3'.

**PFGE.** Pulsed-field gel electrophoresis was performed according to the procedures developed by the CDC for molecular subtyping of *Escherichia coli* O157:H7, non-typhoidal *Salmonella* serovars and *Shigella sonnei* as previously described (10). Briefly, agarose-embedded DNA was digested with 50 U of *Xba*I (Fermentas, Vilnius, Lithuania) overnight in a water bath at 37°C. The restriction fragments were separated by electrophoresis in 0.5×X TBE buffer at 14°C for 20 h in 6 V/Cm using a CHEFF DR II electrophoresis system (Gene Navigator, Pharmacia, Sweden) with pulse times of 2.2 to 63.8 seconds. The gels were stained with ethidium bromide (1 µg/ml) and destained with the buffer remained in the electrophoresis apparatus for 60- 90 min. A Gel Doc 2000 equipped with quantity one software (Bio- Rad, Hercules, CA) was used for image capture and conversion of gel images to Tiff file. Also isolates with DNA smear patterns were rechecked. The molecular weight standard used for all gels was *Xba*I-digested DNA from *Salmonella* Braenderup strain

H9812 (American Type Culture Collection catalog no. BAA- 664).

## RESULTS

**Pathology.** Microscopic inspection revealed disseminated granulocyte and mononuclear leukocyte infiltration in liver and proventriculus. Macroscopic and microscopic examination indicated septicemia and focal necrosis with hepatitis in liver and proventriculitis. Mean mortality rate was calculated to be about 51% in the flocks.

**Bacterial isolates and biotyping.** *Salmonella* were isolated in biotyping by the conventional method in all of the 35 dead canaries aside from the feed. No bacterium was isolated from water sources.

**Serotyping.** *Salmonella* Typhimurium (4,5,12: i: 1, 2) resulted in serotyping while were tested by O:4, 5, 12; H<sub>1</sub>: i and H<sub>2</sub>: 1, 2 antisera (Difco, Detroit, MI, USA) in all of 35 dead canaries.

**Multiplex PCR.** Multiplex PCR confirmed the serotype of isolated *Salmonella* Typhimurium in all of 35 dead canaries. Results of multiplex PCR of some isolates are presented in fig. 1. Sizes of 183, 284, 526 and 663 bp indicate *fliC*, *invA*, *fljB* and *rfbJ* genes that represent H<sub>1</sub>: i, invasion that indicates genus of *Salmonella* and virulence type, H<sub>2</sub>:1, 2 and O<sub>4</sub>, respectively.

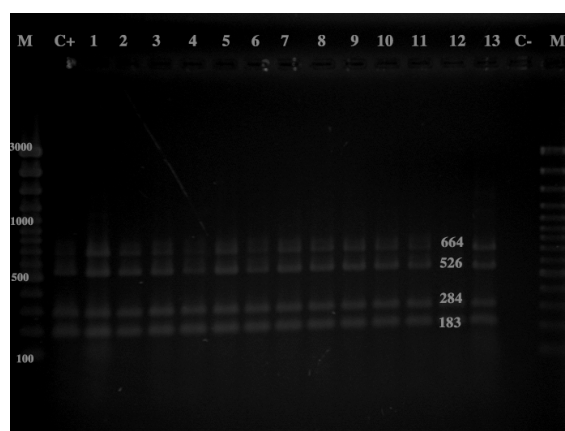
**Antibiotic susceptibility testing.** All of the isolated *S. Typhimurium* revealed the same R-type pattern. With the exceptions of sulphamethoxazole, tylosin, and cloxacillin, the isolates were susceptible to all antibiotics.

**RAPD-PCR.** RAPD- PCR with P1254, 23L and OPA- 4 primers revealed identical patterns in all of the 36 *S. Typhimurium* (35 carcass isolates and one feed isolate) isolates. RAPD fingerprints of some isolates with OPA- 4 primer are presented in figure 2 The similarity between *S. Typhimurium* ATCC 14028 and the outbreak strain was noticeable.

**PFGE.** Using *Xba*I endonuclease, PFGE revealed identical patterns in all of the 36 strains of isolated *S. Typhimurium* (figure 3). Interestingly, the type strain *S. Typhimurium* ATCC 14028 and the outbreak strain produce similar patterns in PFGE. They were different from an isolate cultured from pony in this study.

## DISCUSSION

Diseases caused by non-host adapted *Salmonella* infection is uncommon and is usually seen in chicks,

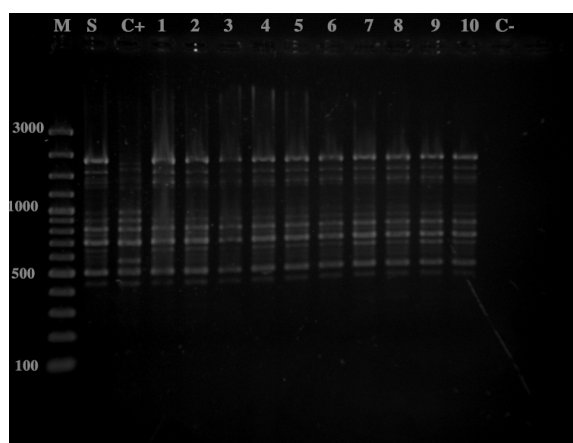


**Fig. 1.** Results of Multiplex PCR of some *S. Typhimurium* isolates of this study. Sizes of 183, 284, 526 and 663 bp indicate to *fliC*, *invA*, *fljB* and *rfbJ* genes, respectively. (M: 100 bp Ladder; C+: *S. Typhimurium* ATCC 14028; 1- 13: *S. Typhimurium* isolates of flocks of 1- 13 in the table 1).

poultrys or ducklings less than 2 weeks of age and rarely in birds over 4 weeks of age. The morbidity and mortality varies considerably and deaths usually occur in less than 20% of the affected group (11). In very susceptible young chicks and poultry, paratyphoid infection can sometimes lead to illness and death at high frequencies. Older birds are considerably less susceptible to the lethal effects of paratyphoid *Salmonella* and may experience intestinal colonization and even systemic dissemination without significant morbidity or mortality (12). Paratyphoid infection seldom causes acute systemic disease except in high subjected to stressful conditions (12).

This outbreak occurred in adult canaries with all of the systemic symptoms. Mortality rate varied from 20% to 94% with average of 51.1%. This indicates the high virulence of *S. Typhimurium* for a very susceptible host (canary) living in a stressful condition (cage). Keyvanfar *et al.* (1968) reported lethal systemic outbreak of *S. Typhimurium* in a flock in Tehran with 100 canaries with a mortality rate of more than 50%. Food contamination with other canary stool was the cause of that outbreak (13). Due to the low immune defense of canaries against *S. Typhimurium*, this organism is the causative agent of lethal infection in adult canaries. Moreover, breeding and environment conditions in the cage trigger the situation.

Death caused by non typhoid *Salmonella* occurs primarily in human adults with serious underlying disease such as HIV infection, cancer, leukemia and cirrhosis (4) Importance of infections such as salmonellosis of pet animals like canaries have



**Fig. 2.** Results of RAPD- PCR of some *S. Typhimurium* isolates of this study with OPA- 4 primer. Notice to the similarity between *S. Typhimurium* ATCC 14028 and outbreak strains (M: 100bp ladder; S: *S. Typhimurium* ATCC 14028; C+: *S. Typhimurium* isolate of the cow; 1- 10: *S. Typhimurium* isolates of flocks of 1-10 in the table).

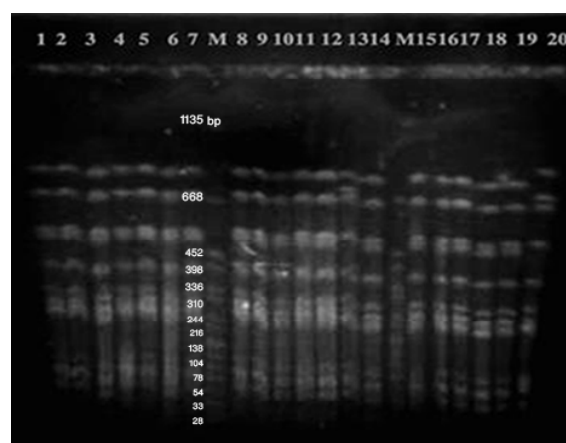
increased because of crowding and increasing rates of underlying type of infections in humans living in cities. It is obvious that following identification and reporting of these lethal strains for human health surveillance systems are very important .

Contamination of cages and sources of food with *S. Typhimurium* observed during inspections in this epidemic and food contamination with other canary stools in the study of Keyvanfar *et al.*, indicates importance of inspection of food stuff and daily cleaning of canary cages from stool and carrier insects and rodents (13). However, distinguishing the source of infection and regions such as what is performed in this research is very important in campaign against these infections.

Madadgar *et al.*, revealed that a combination of RAPD- PCR and R- typing is the method with high discriminatory ability for *S. Typhimurium* strain differentiation (14). Practicality of this idea and discovery of identical isolates in an outbreak has been revealed in this study. In addition, similarity between *S. Typhimurium* ATCC 14028 and this outbreak strain was observed in RAPD- PCR with all primers and PFGE with *Xba*I. This is the first study in which molecular epidemiological methods were used to trace the source of outbreak with *Salmonella* Typhimurium in canaries. This also showed the high susceptibility of canary to this serovar of *Salmonella*.

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**Fig. 3.** Results of PFGE with *Xba*I enzyme digestion of *S. typhimurium* isolates of this study. Notice to the similarity between *S. Typhimurium* ATCC14028 and outbreak strains (M: *S. Braenderup* H9812; 1- 17: *S. Typhimurium* isolates of flocks of 1- 17 in the table; 18: the *S. Typhimurium* isolated from contaminated food stuff of this study; 19: *S. Typhimurium* ATCC 14028; 20: *S. Typhimurium* isolate of the pony)

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