# Evaluation of random amplified polymorphic DNA analysis and antibiotic susceptibility application in discrimination of salmonella typhimurium isolates in Iran

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**SUMMARY**

The purpose of this study was to determine the utility of RAPD-PCR and antibiotic susceptibility tests in differentiation of *S. typhimurium* isolates in Iran. Thirty isolates of *S. typhimurium*, selected based on animal source; place and time of the isolation and a reference strain of the bacterium were used in this study. Serotyping of the isolates was performed by reliable antisera and confirmed with a multiplex PCR. Genomic DNAs were extracted and subjected to an optimized RAPD-PCR, using two previously reported arbitrary primers (P1254 and 23L). Bacteria were also examined for resistance against 8 antibiotics, using a standard antibiotic susceptibility test. While the antibiotic susceptibility test resulted in the identification of 13 profiles of R-type among the bacterial isolates, application of primers P1254 and 23L in the RAPD-PCR could discriminate the isolates only in four and six profiles respectively. However, combination of the two methods could differentiate the 30 isolates in 20 different profiles. The results of this study indicate that the discriminatory power of RAPD-PCR for *S. typhimurium* is low but a combination of this method with antibiotic susceptibility test could be considered an easy and relatively reliable discriminatory approach in differentiation of *S. typhimurium* for epidemiologic purposes in Iran.

*KEY WORDS: S. typhimurium,* Serotyping, RAPD-PCR, Antibiotic susceptibility, Iran

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## INTRODUCTION

*Salmonella* serovars, classified in the *Enterobacteriaceae*, are divided into seven subspecies based on their biochemical characteristics (Olsen *et al.,* 1995). *Salmonella* serovars are among the most frequent causes of bacterial infections in animals and human. They are also the major causes of food born diseases (Anonymouse, 2004. Herikstad, 2002).

Identification and genotype characterization of the bacterial isolates are essential for epidemiological surveillance and outbreak investigations. The primary method used for characterizing members of the genus *Salmonella* is serotyping. According to the Kaufmann-White scheme, serotypes are defined by the combination of O, H and Vi surface antigens (Popoff, 2001; Le Monor and Rhod, 1974).

Over 2.668 different serotypes have been identified with the majority of them belonging to *Salmonella enterica* subspecies *enterica* (Popoff *et al.*, 2004). Among the most frequently isolated serotypes, *S. enterica* subspecies *enterica*,

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serotypes *Typhimurium* and *Enteritidis* (*S. typhimurium* and *S. enteritidis*) account for more than half of the isolates in most regions.
Therefore, serotyping is not sufficiently efficient to track the common source of outbreaks by these two serotypes, as well as other frequently isolated serotypes.
For further discrimination of isolates within the serotypes *S. typhimurium* and *S. enteritidis*, phage typing is the primary sub-typing method. However, phage typing frequently fails to discriminate between outbreak-related and unrelated isolates and usually applied by the reference laboratories (Maslow, 1993; Torpdahl *et al.*, 2005).
In this regard, a suitable technique should satisfy four criteria of rapidity, reproducibility, ease of use and the ability to differentiate strains with similar phenotypes which are genetically unrelated (Lim *et al.*, 2005).
So far, a variety of DNA-based typing methods such as plasmid profile, biotyping, ribotyping, IS*200* profile, PFGE and multilocus enzyme analysis have provided useful insights into identification and evolutionary and epidemiological relationships of several *Salmonella* serovars (Lin *et al.*, 1996). However, when several isolates are to be compared over the course of a few days, random amplified polymorphic DNA (RAPD) analysis might be a more suitable procedure (Del Tufo *et al.*, 1994). Based on the work of Welsh and McClelland (1990) and that of Williams *et al.* (1990), when the genomic DNA is subjected to PCR, primed by short (10 to 25 base) oligonucleotide primers of arbitrary sequences, RAPD analysis produces reproducible and often distinctive sets of DNA fragments. This approach has frequently been applied to detect genomic diversity among plants, animals, parasites, and microbial organisms. The attractiveness of this method is that no knowledge of the sequence of the target organism is required and a very large number of arbitrary primers can be tested to identify those that might be suited to a particular application (Lin *et al.*, 1996).
Several points must be considered, when optimizing random or arbitrary primed fingerprinting methods. Since the assay is amplification based and employ nonspecific primers, the principal limitations of the technique arise from the high sensitivity of RAPD-PCR to reaction conditions. Slight changes in the conditions can affect the reproducibility of the amplification of fragments. The technique is highly sensitive to temperatures, reaction times, source and concentration of polymerase, $MgCl_2$ concentration, quality and quantity of template DNA and sequence, length and concentration of primers (Persing *et al.*, 2004).
This study evaluated RAPD analysis and antibiotic susceptibility test for discrimination of 30 isolates of *S. typhimurium* selected with regard to differences in animal source, time and place of isolation.

## MATERIALS AND METHODS

### Bacterial isolates

Among 100 isolates of *S. typhimurium,* collected in our laboratory during the years 1975-2006, 30 isolates were selected for the study, based on differences in animal source, time and place of isolation. As a standard, a reference strain of the bacterium (ATCC 14028) was also used along with the local isolates.
Lyophilized and recently isolated strains, after overnight incubation in brain heart infusion, were streaked on LB (Luria-Bertani) agar and incubated overnight for isolation of single colonies.

### Serotyping

Serotyping of bacteria was performed by reliable antisera (Difco) and confirmed with multiplex PCR, using 4 pairs of primers, as described by Lim, Y. H *et al.*, 2003 and Rahn *et al.,* 1992. All of the isolates had virulence gene of *inv*A.

### DNA extraction

A single colony of each isolate on agar plate was picked and resuspended in 200 µl of distilled water. After vortexing, the suspension was boiled for 5 min, centrifuged for 10 min at 14000 rpm and the supernatant was transferred to a new microtube.
For comparison, bacterial DNAs were also prepared with a standard kit (QiaGene) and the related protocol.

### RAPD fingerprinting

PCR was conducted in a 25 µl volume containing 40 ng of total *S. typhimurium* DNA, 1.5 mM $MgCl_2$, 0.5 µM of primer, 1 U of *Smartaq* DNA

polymerase and 200 mM dNTPs mix in 1X PCR buffer (all reagents were from CinnaGene Inc, Iran).
The thermal program consisted of 4 cycles of 94°C for 4 min, 35°C for 4 min, and 72°C for 4 min; 30 cycles of 94°C for 30s, 35°C for 1 min, and 72°C for 2 min; and 1 cycle of 72°C for 10 min for final extension. After PCR, 10 µl of each amplified DNA product was loaded on 1.5% agarose gels *(CinnaGene Inc, Iran)* in 0.5 X TBE buffer. A 100bp DNA ladder (Gene Ruler plus, Fermentas) was included in each gel.
The samples were electrophoresed for 1 h at 100 V and the amplified DNA fragments were visualized by UV illuminator, after staining with ethidium bromide.

**Antibiotic susceptibility testing**
Antibiotic susceptibility test was performed by the standard disk diffusion method in Mueller-Hinton agar, and the results were interpreted in accordance to the criteria of the National Committee for Clinical Laboratory Standards. The strains were screened for resistance to the following antibiotics: cephalexin (30 µg), oxytetracycline (30 µg), nalidixic acid (30 µg), trimethoprim (5 µg), lincospectin (15/200), enrofloxacin (5 µg), trimethoprim-sulfamethoxazole and nitrofurantoin (300 µg).

## RESULTS

**Optimization of RAPD fingerprinting**
To optimize the discriminatory power of the RAPD fingerprinting technique, the optimal concentrations of arbitrary primer, DNA template, $MgCl_2$, *Taq* DNA polymerase and dNTPs were first determined (Lin *et al.*, 1996 and Meunier *et al.*, 1993).
To select suitable candidate primers for subtyping *S. typhimurium* isolates, 9 arbitrary primers were tested. The primers have previously been described by Lin *et al.*, 1996, Hyungkun Lim *et al.*, 2005 and Tekeli *et al.*, 2006. Consequently, P1254 (5´-CCGCAGCCAA-3´) and 23L (5´-CCGAAGCTGC-3´) primers with acceptable discrimination were selected. To obtain the optimal concentration of arbitrary primer (0.5 µM), 0.5, 1, 1.5, 2 and 2.5 µM concentrations were tested.
DNA template was tested at the concentrations of 20, 40, 60 and 100 ng per reaction, among which the 40 ng concentration was selected. The results obtained by boiled extract DNA were approximately the same as those produced by the kit extracted DNA.
The optimal concentration of $MgCl_2$ (1.5 mM) was obtained by testing the concentrations of 1.5, 2.5 and 3.5 mM.
*Taq* DNA polymerase was tested at the amounts of 0.5, 1 and 2 U and 1 U per reaction was found suitable.
Finally, it was found that a 200 mM concentration of dNTPs mix works better than the concentration of 400 mM.

**Reproducibility of RAPD fingerprinting**
The reproducibility of the RAPD fingerprinting technique was confirmed by comparing the fingerprint patterns obtained from duplicate strains and duplicate run of strains.

**Discrimination of *S. typhimurium* isolates by RAPD, antibiotic susceptibility test and combination of the two tests**
RAPD analysis by Primers P1254 and 23L revealed four and six polymorphic patterns of DNA from 30 isolates of *S. typhimurium* of Iran, respectively. The results obtained by the primer P1254 are shown in Figure 1.
With application of eight antibiotics, 30 isolates of the bacteria could be divided into 13 patterns of R-type (Resistance type) (Table 1).
The results showed that with the combination of

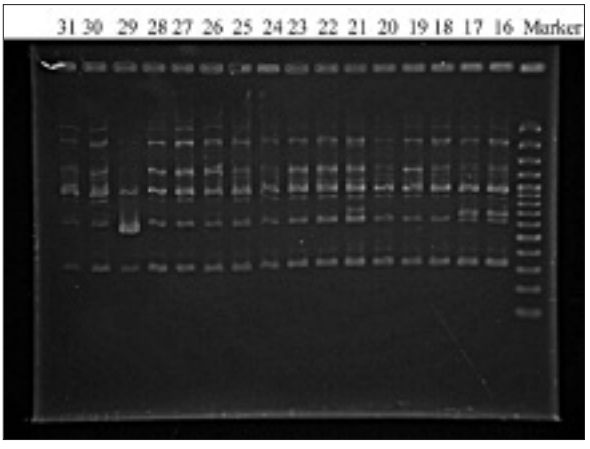


FIGURE 1 - *RAPD fingerprintings of S. typhimurium isolates with primer P1254: Marker and isolates of 16-31.*

Table 1 - *Results of RAPD fingerprinting and antibiotic susceptibility testing of S. typhimurium isolates by using of 2 primers and 8 antibiotics.*

| *Designition* | *Isolates* | | *RAPD fingerprinting* | | *Antibiotic Susceptibility Test** |
|---|---|---|---|---|---|
| | *Source* | *Year* | *P1254 type* | *23L type* | |
| 1 | Cats | 1976 | A | A | A |
| 2 | Cats | 1976 | A | A | G |
| 3 | Cats | 1976 | A | A | D |
| 4 | Cats | 1976 | A | A | D |
| 5 | Cats | 1976 | A | B | A |
| 6 | Chicken | 2001 | A | C | C |
| 7 | Chicken | 2001 | A | C | C |
| 8 | Chicken | 2001 | A | C | C |
| 9 | Cow | 2003 | B | A | E |
| 10 | Cow | 2003 | B | D | E |
| 11 | Cow | 2002 | B | A | L |
| 12 | Cow | 2002 | B | D | E |
| 13 | Cow | 2002 | A | A | D |
| 14 | Cow | 2003 | C | A | M |
| 15 | ATCC 14028 | - | A | A | B |
| 16 | Cow | 2003 | B | A | E |
| 17 | Cow | 2006 | B | A | J |
| 18 | Sheep | 2006 | A | A | C |
| 19 | Dove | 2005 | A | A | I |
| 20 | Parrot | 2005 | A | D | G |
| 21 | Chicken | 1998 | B | E | G |
| 22 | Chicken | 1998 | A | A | K |
| 23 | Canary | 2006 | A | A | D |
| 24 | Sparrow | 2005 | A | F | C |
| 25 | Sparrow | 2005 | A | F | D |
| 26 | Sparrow | 2005 | A | F | D |
| 27 | Sparrow | 2005 | A | A | H |
| 28 | Sparrow | 2005 | A | A | D |
| 29 | Dove | 2006 | D | A | F |
| 30 | Cats | 2006 | A | F | C |
| 31 | Pony | 2003 | A | A | B |

*Letters show resistance profiles that indicate to: A (sensitive to all antibiotics); B (CN); C (CN, TMP); D (TMP); E (TMP, T, LP); F (T, TMP); G (CN, TMP, SXT); H (TMP, SXT); I (CN, LP); J (CN, T, LP); K (CN, TMP, LP, SXT); L (T, TMP, LP, SXT) and M (T, NA, TMP, LP, FM, SXT).

RAPD (with two primers) and antibiotic susceptibility tests (with eight antibiotics), 30 isolates of *S. typhimurium* could be divided into 20 different profiles.

## DISCUSSION

The application of PCR-based techniques has had a revolutionary impact on the diagnosis of infectious diseases. Due to the ability for detection or allowing analysis of minute amounts of microbial DNA or RNA sequences, these techniques have emerged as highly sensitive and specific methods for identifying pathogens.

The PCR-based RAPD fingerprinting technique, utilizing arbitrary oligonucleotides to prime DNA synthesis at low annealing temperatures to divulge genomic diversity, is particularly a powerful typing method. Unlike the traditional PCR analysis, which requires specific knowledge of DNA sequences and the application of target-specific sequences, RAPD does not require any specific knowledge of the DNA sequences of the target organism. This makes it a tool of great power and general applicability (Lin *et al.*, 1996).

The random and arbitrary PCR fingerprinting methods have not proven to be especially suited to establishing genomic library database (Persing *et al.*, 2004).

Consequently we principally did not intend to compare DNA, for example, of animals and poultry by RAPD fingerprinting.

Comparative typing of relatively small sets of isolates by using fingerprints obtained in the same amplification can yield results relatively quickly and easily (Persing *et al.*, 2004).

MgCl2, dNTPs and *Taq* DNA polymerase concentrations proved very important in producing reliable and reproducible RAPD fingerprinting patterns.

Reproducibility of RAPD-PCR has been studied in different researches and was confirmed and denied frequently. However this issue has no effect on our study because our objective was only differentiation ability without any genomic library profiles for surveillance or other purposes.

By application of antibiotic susceptibility test as a phenotypic method, great diversity of resistant patterns (13 patterns in 31 isolated strains) were probably due to very different and widely varying consumption of antibiotics in different regions and times and also illegal trafficking of animals and food materials from neighbour countries without an optimum surveillance system. Consequently, according to non host adopting *S. typhimurium* serotype, transfer of these isolates, their plasmids and chromosomal antibiotic resistance genes has been seen very rapidly and probable and has made antibiotic susceptibility test a powerful phenotypic method for differentiation of *S. typhimurium* isolates in Iran.

Also other studies have indicated that RAPD-PCR with primer P1254 and 23L, separately, have acceptable discriminatory power between *S. entritidis* strains (Lin *et al.*, 1996 in the U.S.A and Tekeli *et al.*, 2006 in Turkey).

It seems that setting up and fixing of reagents and conditions, enough pipetting and also use of a single DNA template in producing instant patterns of the RAPD fingerprinting is very important and plays a critical role.

However, when one considers combination of discriminatory power and ease of application, RAPD PCR and antibiotic susceptibility in combination, emerge as a particularly attractive phenotypic and molecular technique. This is especially true if one employs the simplified RAPD technique which uses boiled bacterial DNA templates. This modification greatly reduces the amount of time and labor required for the performance of this molecular technique.

The results of this study show that while RAPD fingerprinting (with two primers) will differentiate 34% of isolates, this increases to 67% in combination with antibiotic susceptibility test. It is apparent, with application of more suitable primers and antibiotics, that we can easily increase the discriminatory power to upper limits (to likely optimum).

Results establish when an appropriately chosen set of primers are employed, RAPD analysis in combination with antibiotic susceptibility test (with an appropriate set of antibiotics chosen) provides an alternative rapid, reproducible and relatively powerful method for *S. typhimurium* diversity discrimination in Iran.

Finally, RAPD method is not a whole genome technique, and is not appropriate for comparison of the whole genome of *S. typhimurium* isolated from different sources. For this purpose we propose another molecular technique such as

PFGE, REP PCR or AFLP that we are planning to apply in a subsequent study.

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