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Random Amplification of Polymorphic DNA (RAPD) of *Salmonella enteritidis* Isolated from Chicken Samples

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Abstract: An attempt was made to study RAPD using 30 isolates of *S. enteritidis* obtained from chicken samples for the purpose of strain discrimination. The primer used was P1254, (5'CCGCAGCCAA3') to amplify the genomic fragment of *S. enteritidis* isolates, which resulted in production of six clones. Six isolates each in clone 1, 2 and 5 and another four isolates each in clone 2, 4 and 6, respectively. In clone 1, having an additional 900 bp amplified products but it was lacking in clones 2 and shared a common 500 bp fragments. Clone 3 had additional amplified fragment at 450 bp. It was lacking in clone 4 and clone 5 had the additional amplified products at 950, 900, 750 and 560 bp but it was lacking in clone 6. The final cluster 6 had three fragments with molecular weight 1550, 1300 and 400 bp, respectively. To sum up, there were 6 distinct clones of *S. enteritidis* serovars identified based on RAPD profiling.

Key words: *Salmonella enteritidis*, chromosomal DNA, RAPD-PCR, clone, cluster

INTRODUCTION

The district of Namakkal, Tamilnadu, South India is an important global poultry center and the poultry industry is one of the major sources of economy. The export of poultry product from this region is increasingly on the rise. The incidence of *Salmonella* in poultry product may pose health hazard to many a population world over. Due to their occurrence in common place foods especially poultry, milk and eggs, the opportunity for the consumption of contaminated food is significant (Herikstad *et al*., 2002).

Due to the ubiquitous nature of the organisms, typing scheme capable of detailed strain identification is essential for epidemiological studies (Betancor *et al*., 2004). Epidemiological studies based on the available taxonomical tool and classification system are done for the prevention and treatment of infection. The ability to distinguish isolates of *Salmonella* may be very important to trace the source of infection or outbreaks. The relationship among the isolates, the comparison between and within serotypes may be significant. The methods that have been used for deciphering the relatedness among the isolates are biotyping, antibiograms, plasmid typing, phage typing serotyping, ribotyping and PCR fingerprinting. Some of the above techniques have low discriminating potential, where as others demand considerable amount of expertise, time and equipment. Randomly amplified polymorphic DNA analysis has been widely used in characterization and diagnosis of *Salmonella* infection (Tikoo *et al*., 2001).

RAPD has been described as a simple and rapid method able to offer detailed fingerprinting of the genomic composition of the organism (Welsch and McClelland, 1990). The success of this method is due to the fact that no prior sequence information about the target is needed and a single short universal 10-mer oligonucleotide primer work in any genome and hence can be used in the reaction. The amplification happens at low stringency, allowing the primers to anneal to several locations on the two strands of the DNA. These primers detect polymorphisms in the absence of specific sequence information and the DNA sequence variations may work as genetic markers that can be used in epidemiologic studies. In this study RAPD is used as a molecular typing tool to distinguish serotypes within strains of *Salmonella* isolated from chicken samples of Namakkal and preliminary data on the nature of selected RAPD fragments is reported. This study is first of its kind from this part of India and may have serious implications

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considering the quantum of poultry product exported from this part of India. Hence an attempt has been made to characterize *Salmonella* of chicken origin by RAPD which may aid in the rapid diagnosis and prevention of Salmonellosis.

Earlier Betancor *et al.* (2004) have applied molecular and phenotyping techniques to study *S. enterica* servovar *enteritidis* strains both from human cases of infection and of avian origin isolated in Uruguay from 1995 to 2002. Comparative examination of the results obtained by RAPD analysis and phenotypic analysis and by strain source provided evidence of the reliable discriminatory power of RAPD analysis. Overall, the results showed the existence of a predominant genetic type that is present in poultry and that is transmitted to humans.

MATERIALS AND METHODS

Bacterial isolates: Five hundred seventy eight chicken samples were collected from different retail chicken outlet in Namakkal, Tamilnadu, India, from which 92 strains were isolated between 2002 and 2004. All the isolated strains were identified based on morphological, physiological and biochemical tests as described by Cox and Williams (1976). Of these, 30 isolates were randomly selected and subjected to RAPD analysis.

Preparation of chromosomal DNA: *Salmonella enteritidis* was grown in 5 mL of Luria Bertani broth at 37°C for 18-24 h. 1.5 mL volume of culture was transferred to an eppendorf tube and centrifuged and the pellets resuspended in Tris EDTA (TE) buffer (10 mmol L^{-1} Tris, 1 mmol L^{-1} EDTA, pH 8). DNA were extracted from the resuspended cells using a standard Sodium Dodecyl Sulphate (SDS) lysis protocol and the lysate treated with RNAse and proteinase. The DNA sample was then extracted with phenol: chloroform: isoamyl alcohol (25:24:1), the aqueous phase recovered and the DNA was precipitated in ethanol. The DNA solutions were stored at -20° C until required (Sambrook and Russel, 2001).

RAPD reactions: PCR including cycling conditions viz., $MgCl_2$ and KCI concentration and pH were optimized. A suitable primer P1254 (5'CCGCAGCCAA 3') (Geni, Bangalore, India) was used (Akopyanz *et al.*, 1992). The PCR was performed in a 40 μL volume containing 4 μL of 10 x PCR buffer (100 mmol L^{-1} Tris-HCl, 35 mmol L^{-1} $MgCl_2$, 750 mmol L^{-1} KCI, pH 8.8), 3 μL of 10 mmol L^{-1} dNTPs (2.5 mmol L^{-1} each of dATP, dCTP, dGTP and dTTP), 1 μL of 100 μmol L^{-1} primer, 1.25 units of Taq DNA polymerase, 29 μL of H_2O and 2 μL of DNA template (5 ng μL^{-1}). Amplification was performed in a PCR (Eppendorf Netheler-Hinz GmbH, Hamburg, Germany) with maximal ramping as follows one cycle of 4 min 30 s at 94°C followed by five low stringency cycles comprising 30 s at 94°C, 2 min at 20°C, 2 min at 72°C and 30 high stridency cycles of 30 s at 94°C, 1 min at 32°C and 2 min at 72°C. Total of 35 cycles were performed with final extension at 72°C for 5 min and the amplified products were stored at 4°C until required.

DNA analysis: A 15 μL portion of the RAPD reaction product was loaded on to a 1.5% agarose gel containing 0.5 μg mL^{-1} ethidium bromide and electrophoresed in TBE buffer. The DNA fragments were visualized by placing the gel on a UV (300 nm) transilluminator and the gel recorded using the Alfa Digital documentation imaging system (Alfa Innotech Corportation, USA).

RESULTS

The present study concerns with the characterization of *S. enteritidis* strains isolated from chicken samples by RAPD analysis. The study period extended between July 2002 and April 2004. During this period 92 isolates belonging to *S. enteritidis* were made, of which 30 isolates were randomly selected and subjected to RAPD analysis. The results of the RAPD analysis of the isolates, the similarities and variation among the strains are reported in chronological order of year.

In order to use RAPD for the differentiation of *S. enteritidis* strains certain parameters of PCR needed to the optimized. The optimal concentration of arbitrary oligonucllеotide, DNA template and $MgCl_2$ used in PCR were first determined. $MgCl_2$ concentration of 3-5 mM produced no variation in banding pattern. Higher concentration of $MgCl_2$ yielded some artificial background and lower concentration of $MgCl_2$ resulted in poor amplification. The optimum concentration of $MgCl_2$ for amplification was found to be 15 mM. The level of DNA template less than 50 ng was found to result in relatively poor amplification. The RAPD patterns however did not vary when the amount of 50-180 ng of DNA were used. Primer at a final concentration of 5 μm was used in most of the reaction. RAPD patterns remain the same when the concentration of primer was raised to 10 μm, while lower concentration of primers resulted in relatively inefficient amplification. The reproducibility of RAPD finger printing technique was confirmed by comparing the reproducibility of the fingerprint pattern obtained form duplicate run of RAPD analysis of different isolates of *S. enterica* serovars.

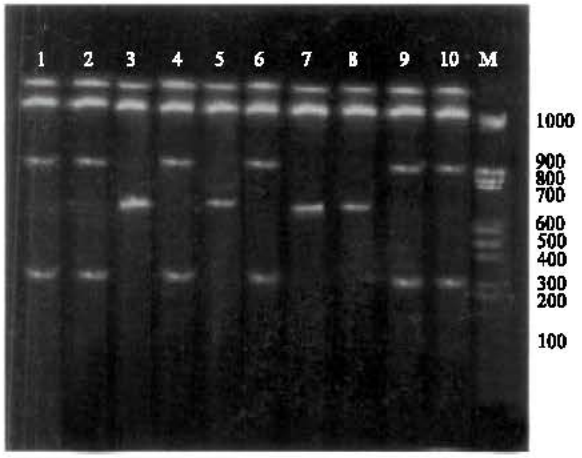

Fig. 1: RAPD Profile of *S. Enteritidis* isolated in the year (2002), Lane 1, 2, 4, 6, 9, 10: *S. enteritidis* Isolates 1, 5, 12, 14, 17 and 20 show identical PAPD PATTERNS with amplified fragment size 1500, 1200, 900 and 500 bp. Lane 3, 5, 7 and 8: *S. enteritidis* isolates 2, 9, 18 and 22 showing the amplified fragments size 1500, 1200 and 700 bp. Lane M: 1Kb DNA marker

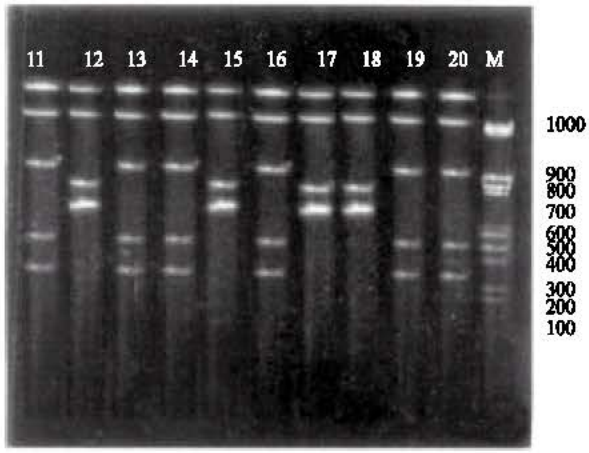

Fig. 2: RAPD profile of *S. enteritidis* isolated in the year (2003), Lane 11, 13, 14, 16, 19 and 20: *S. entritidis* isolates 24, 30, 41, 49, 53 and 59 showing indentical RAPD patterns with amplified fragments size 1600, 1350, 950 560 and 450 bp. Lame 12, 15, 17 and 18: *S. entritidis* isolates 26, 33, 45 and 57 showing the amplified fragments size 1600, 1350, 800 and 700 bp. Lane M: 1Kb DNA marker

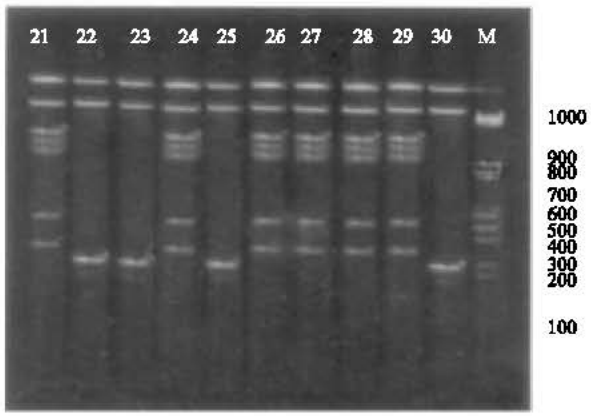

Fig. 3: RAPD profile of *S. enteritidis* isolated in the year (2004), Lane 21, 24, 26, 27, 28 and 29: *S. entritidis* isolates 64, 66, 74, 79, 84 and 90 showing indentical RAPD patterns with amplified fragments size 1550, 1300, 950 900, 750, 560 and 450 bp. Lame 22, 23, 25 and 30: *S. entritidis* isolates 65, 68, 77 and 917 showing the amplified fragments size 1550, 130 and 400 bp. Lane M: 1Kb DNA marker

In the period between July 2002 and December 2002, 21 strains of *S. enteritidis* were isolated of which 10 were subjected to RAPD analysis. Out of 10 isolates, 6 isolate (SE 1, SE 5, SE 12, SE 14, SE 17 and SE 20) had an amplified product size ranging from approximately from 1500 to 500 bp. In these 6 isolates, 4 amplified products were obtained with the molecular weight 1500, 1200, 900 and 500 bp. The RAPD banding pattern of the remaining 4 isolates approximately ranged from 1450 base pairs to 650 base pairs. Of these 4 isolates strain SE 2, SE 9, SE 18 and SE 22 had unique patterns of RAPD profile. The size of the amplified product ranged between 1300 to 500 bp. It is important here to note that above 10 isolates of *S. enterica* serovars gave similar biochemical results and hence are difficult to characterize and differentiate on the basis of biochemical test alone. But by RAPD profiling, discrimination and differentiation of *S. enterica* serovars into different clones or cluster sub species was possible (Fig. 1).

In the year 2003, a total of 45 strains were isolated from chicken sample. Of this, 10 isolates were randomly selected and subjected to RAPD analysis using the primer P1254. In all the 10 isolates 2 different RAPD profile have been obtained. The molecular size of one of the amplified product ranged between 1600 to 450 bp. A total of 5 fragments were generated during the amplification of RAPD PCR. The strains exhibiting such banding patterns were SE 24, SE 30, SE 41, SE 49, SE 53 and SE 59. The second type of RAPD profile was exhibited by the strains SE 26, SE 33, SE 45 and SE 57. Total of 4 amplified fragments were generated and the molecular size was between 1600 to 700 bp (Fig. 2).

In the year 2004, 26 isolates were obtained, out of which, 10 isolates were randomly selected and subjected to RAPD profile. All the 10 isolates produced at least 2 unique bands at 1550 and 1300 bp. Distinguishing each isolate on the basis of RAPD profile is possible, although similarities exist between the profiles generated for the different serotypes especially the common fragment of 600 bp and in some cases another of 400 bp. Although this ampiliconappearsto be of same molecular weight they are not necessarily identical fragments (Fig. 3).

A total of the 30 isolates were subjected to RAPD finger printing during the period of study. When collectively considered 6 RAPD clusters were observed. Cluster 1 was formed by amplified fragments of strain SE 1, SE 5, SE 12, SE 14, SE 17 and SE 20, Cluster 2 by fragments of strain SE 2, SE 9, SE 18 and SE 22, Cluster 3 by fragments of strain SE 24, SE 30, SE 41, SE 49, SE 53 and SE 59 Cluster 4 by fragment of strain SE 26, SE 33, SE 45 and SE 57, cluster 5 by fragments of strain SE 64, SE 66, SE 74, SE 79, SE 84 and SE 90. Cluster 6 by fragments of strain SE 65, SE 68, SE 77 and SE 91.

There were four fragments generated in the amplification of gene in cluster 1, the molecular weight of which were 1500, 1200, 900 and 500 bp. Three fragments were apparent in cluster, with molecular weight at 1500, 1200 and 750 bp. In the cluster 3, five fragments were observed, the molecular weight of which were 1600, 1350, 950, 560 and 450 bp. The cluster 4 had four fragments with molecular weight 1600, 1350, 800 and 700 bp. Cluster 5 had seven fragments with molecular weight 1550, 1300, 950, 900, 750, 560 and 450 bp. The final cluster 6 had three fragments with molecular weight 1550, 1300 and 400 bp, respectively.

DISCUSSION

The available phenotypic and serotyping methods limits the *Salmonella* to the sub species levels but it is known from the earlier reports Nair *et al*. (2002) that there is exist over 2400 serovars and many a strains within them. The identification these strains is based on cumbersome, time consuming phenotypic and serological tests (Aarts *et al*., 1998). Inspite of application of wide range of taxonomical tools, the classification of *Salmonella* is not yet resolved. In the absence of a single reliable and rapid identification tool the salmonellosis of human and animal canot is traced back to their origin. This will be an impediment in the epidemiological survey and hence in the treatment of salmonellosis. As an alternative genotypic methods can be employed for the consenses of *Salmonella* identification. This holds significance in detecting the incidence of *Salmonella* in chicken samples which can be potential source of outbreak in the country of origin and in the country where it is imported. The findings of the study indicate that inspite of biochemial similarities there exist six genotypic clones of *S. enterititis* based on RAPD profiles.

As per Liu *et al*. (2003) microhetergenecity of sequences among strains arises due to continuous point mutation and other variation in the genome. Every change in the genome gave rise to polymorphic fragment detectable in an excessively discretionary RAPD analysis. It is not expected that there should be any significant correlation between the results gained from a phenotypic method such as biotyping and RAPD, as biotyping relies on the expression of a member of genes compared to the whole genome interrogated by RAPD. The use of the AP-PCR DNA analysis recognizes polymorphism in nucleotide sequences among isolates of *S. enteritidis* (Lin *et al*., 1996). The different amplified DNA fragment in *Salmonella* isolate found in the present study confirms this fact.

In the present study P 1254 primer was used to amplify the genomic fragment of *S. enteritidis* isolates which resuted in production of six clones. Clone 1, 3 and 5 was formed by amplified fragments of 6 isolates each, clone 2, 4 and 6 by fragments of 4 isolates. Hilton *et al*. (1996) have observed that *S. enteritidis* isolates appeared to fall into four RAPD groupings indicating the existence of four clones. Four isolates were in clone one and two isolates each in clone 2, 3 and 4 respectively. All the *S. enteritidis* isolates shared a basic profile that is thus unique to the type, again with two common fragments of 300 and 600 bp and polymorphisms giving rise to discrete changes in the basic profile. The different RAPD profiles highlight the potential additional extra subtype differentiation obtainable by RAPD.

In another study, Kantama and Javanetra (1996) have reported that *S. enteritidis* which caused country wide outbreak in Thailand were found in chicken and human, 125 isolates of *S. enteritidis* which were isolated between 1990-1993 were clonally identified by the technique of RAPD analysis. They have found eight RAPD profiles in 125 isolates indicating presence of 8 clones designated as no 1-8. The predominant clone was profile No. 4 while the rest of the profile comprised only 0.8-1.6%. The predominant clone was distributed mainly in isolates from chickens and humans. The major clone involved in this outbreak and that chicken were the source of *S. enteritidis* infection. In our study the predominant clones were 1, 3 and 5 indicating genetic diversity among the isolates which potentially may cause an outbreak.

Betancor *et al.* (2004) while analyzing RAPD fingerprinting with five different primers in *S. enteridis,* discriminated 10 different genetic profiles. Their contention is genetic diversity is greater among *S. enteritidis* strains of animal origin than among isolates of human origin. The presence of 6 different clones in the present study emphasizes this fact.

The findings in present study indicate that the genetic diversity was greater among strains of *S. enteritidis* in chicken origin. Comparative examination of the results obtained by RAPD and phenotypic analysis and by strain source provided evidence of the reliable discriminatory power of RAPD analysis. Overall the results show the existence of predominant genetic types present in poultry and that maybe transmitted to humans. This profiling will be an aid in the diagnosis and characterization of *S. enteritidis.*

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