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## Genotypic Characterization of Avian *Escherichia coli* by Random Amplification of Polymorphic DNA

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**Abstract:** Fifteen serotypes of *E. coli* isolated from poultry were subjected to RAPD-PCR using eight oligonucleotide random primers. Amplification with each of the primers resulted in generation of different DNA fingerprinting profile with varied number of bands. Dendrogram based generation of clustering of *E. coli* serotypes showed two major clusters. The polymorphic amplicons served as markers for *E. coli* species where as the unique amplicons served as markers for *E. coli* serotypes. RAPD based fingerprinting provided a rapid means of discriminating *E. coli* serotypes and considered a valuable tool for molecular typing.

**Key words:** *E. coli*, RAPD-PCR, poultry industry, pathogenic strains

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### Introduction

*Escherichia coli* infections are of significant concern to the poultry industry. It is one of the most frequently encountered important bacterial avian pathogen causing a wide variety of disease syndrome in farmed birds causing 5-50 per cent of mortality in poultry industry. Several strategies have been adopted to characterize isolates of *E. coli* and to aid in the identification of pathogenic strains. But the so far available serological and bacteriological methods for typing *E. coli* are often limited to a few selected reference laboratories, and are not sensitive enough to further differentiate bacterial isolates. Bacterial isolates, including those of *E. coli* can be differentiated into genetically distinct isolates using procedures that identify differences in the genetic composition of a microbial population.

Random amplification of polymorphic DNA (RAPD) has received considerable attention in recent years as a molecular typing method due to its simplicity, sensitivity, flexibility and relatively low cost (Welsh and McClelland, 1990; Williams *et al.*, 1990; Belkum, 1994). The technique was successfully applied to the study of genetic diversity among groups of *E. coli* strains previously regarded as homogenous (Alos *et al.*, 1993; Brikun *et al.*, 1994). Small 10-bp oligonucleotides were chosen as primers due to greater probability that these short sequences are reiterated many times with in the bacterial genome. The genetic composition of each bacterial isolate ultimately dictates the position and number of times that the primer anneals to the chromosome. The distinctive DNA patterns generated by RAPD for each bacterial strain is a reflection of the

genetic diversity present in a species. In this study, we present the application of a RAPD typing method for characterization of avian *E. coli* serotypes.

### Materials and Methods

**Bacterial isolates:** A total of fifteen different *E. coli* serotypes isolated from poultry, predominantly from birds with colibacillosis in and around Bangalore, India, serotyped at Central Research Institute, Kasauli, India, were included in the study. The serotypes are as follows: O2, O78, O22, O25, O29, O41, O84, O119, O141, O156, O159, O161, Rough, O9 and O39.

**RAPD by PCR:** Chromosomal DNA was isolated from *E. coli* serotypes using phenol: chloroform extraction (Marshall *et al.*, 1981). Table 1 lists the primers and their nucleotide sequence for RAPD PCR. The amplification reactions were carried out in a 0.2 ml micro centrifuge tubes using a programmable thermal cycler (Corbett Research, Germany). Ten µl (750ng) of sample DNA template was denatured initially at 95°C for five minutes and then snap cooled in crushed ice pack for 10 minutes. 40 µl of reaction mix containing 25mM Tris HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.4 mM (each) dNTP (Biogene, USA), 100 pmol of primer, 1.5U of Taq polymerase (Bangalore Genei, Bangalore) was added to each tube. Temperature cycling was programmed as follows: 1) 94°C for 30sec, 35°C for 1 min and 72°C for 5 min for 40 cycles; 2) 72°C for 10 min and 3) 10°C for 3 min. reaction products were analyzed by electrophoresis on 1.5% agarose gel in 0.5X TBE (Tris borate EDTA) containing 0.5 µg of ethidium bromide per ml. A 100bp

Table 1: Nucleotide sequence of RAPD primers

Sl. No.	Primer code	Nucleotide sequence
1	OPAC 01	5 <sup>1</sup> -TCC CAG CAGT- 3 <sup>1</sup>
2	OPAC 02	5 <sup>1</sup> -GTC GTC GTCT- 3 <sup>1</sup>
3	OPAC 04	5 <sup>1</sup> -ACG GGA CCTG-3 <sup>1</sup>
4	OPAC 05	5 <sup>1</sup> -GTT AGT GCGG- 3 <sup>1</sup>
5	OPAC 07	5 <sup>1</sup> -GTG GCC GATG- 3 <sup>1</sup>
6	OPAC 09	5 <sup>1</sup> -AGA GCG TACC- 3 <sup>1</sup>
7	OPAC 11	5 <sup>1</sup> -CCT GGG TCAG- 3 <sup>1</sup>
8	OPAC 12	5 <sup>1</sup> -GGC GAG TGTG- 3 <sup>1</sup>

DNA ladder (Biogene, USA) was used as a molecular weight marker. Gels were imaged with the gel documentation system (Alphamager 2200, USA).

**Analysis of RAPD data:** The bands were manually scored '1' for the presence and '0' for the absence and the binary data were used for statistical analysis. The scored band data (Presence or absence) was subjected to cluster analysis using STATISTICA. The dendrogram was constructed by Ward's method of clustering using minimum variance algorithm. The dissimilarity matrix was developed using Squared Euclidean Distance (SED), which estimated all the pairwise differences in the amplification product.

**Results**

All eight RAPD primers yielded reproducible DNA profiles. Control assays in which distilled water was used yielded no amplified products. There was no correlation between the G + C content of the primer and their ability to detect polymorphisms. Analysis of fifteen *E. coli* serotypes by RAPD revealed distinct RAPD patterns with all eight primers.

A dendrogram or a phylogenetic tree was generated from RAPD patterns of *E. coli* serotypes (Fig. 1). Grouping of *E. coli* into each cluster or branch correlated with similarities in their RAPD DNA patterns. For example, the *E. coli* serotypes that produced the same DNA banding pattern were also recognized as being similar from phylogenetic analysis. These serotypes belonged to the same cluster in the resulting phylogenetic tree.

In the dendrogram the serotypes are grouped into two major clusters A and B. The whole dendrogram was distributed between O2 and O39. The maximum linkage distance between the two major clusters was 162 units. The cluster A consisted of twelve serotypes distributed between O2 and O29. This cluster was grouped into two subclusters A<sub>1</sub> and A<sub>2</sub>. The subcluster A<sub>1</sub> was further grouped into two subgroups A<sub>1.1</sub> and A<sub>1.2</sub>. Serotypes O2, O78, O22, O25, O41, O141, O119 and O161 were placed under subgroup A<sub>1.1</sub>. Subgroup A<sub>1.2</sub> consisted of O84, O156 and O159. Subcluster A<sub>2</sub> consisted of only one serotype, O29.

The cluster B consisted of three serotypes, which

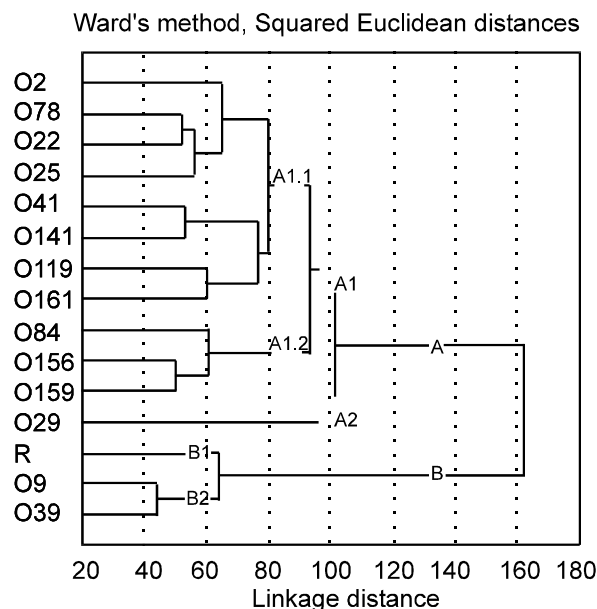


Fig. 1: Dendrogram based on eight RAPD primers

showed less variation than cluster A. It was again grouped into two subclusters B<sub>1</sub> and B<sub>2</sub>. B<sub>1</sub> consisted of rough and B<sub>2</sub> consisted of O9 and O39. The maximum dissimilarity of 75 per cent was found between O78 and O9. Minimum of 33 per cent dissimilarity was observed between O39 and O9.

**Discussion**

The application of PCR based techniques has a revolutionary impact on the diagnosis of infectious diseases. The most commonly used molecular genetic fingerprinting technique is a restriction fragment length polymorphism (RFLP) analysis. Maurer *et al.*, (1998) claimed that fingerprinting by RAPD revealed more genetic differences among avian *E. coli* strains than RFLP analysis. Akopyanz *et al.* (1992); Makino *et al.* (1994) and Pacheco *et al.* (1996) observed that oligos with high GC content (>60%) resulted in a greater and better reproducible number of strain specific bands in enterotoxigenic *E. coli*. On the contrary, the size and GC content of the arbitrary primers did not seem to be an essential requirements for the generation of informative RAPD profiles in *E. coli* as opined by Alos *et al.* (1993); Brikun *et al.* (1994); Cave *et al.* (1994). Similarly in our RAPD studies, we did not observe influence of GC content of the primer on the number of polymorphic bands.

The most challenging aspect of a protocol for bacterial subtyping by RAPD analysis was the selection of suitable primers. We found that primers OPAC 04, OPAC 07, OPAC 09, OPAC 11 and OPAC 12 generated entirely different banding pattern for each serotype and could differentiate all the serotypes from one another. Hence,

these primers are suggested for molecular typing of *E. coli* isolates as they disclosed inter serotype variations. A phylogenetic tree was generated from the diverse *E. coli* RAPD patterns obtained in this study. These dendrograms generated from different primers were highly branched, suggestive of a genetically diverse population. Similar findings have been reported from avian *E. coli* in general using multilocus enzyme electrophoresis (MLEE) to measure genetic diversity (Selander *et al.*, 1986). However, Wang *et al.* (1993) claimed that RAPD-PCR was more sensitive than MLEE. Phylogenetic analysis on the DNA sequences amplified from 15 different *E. coli* serotypes indicated the close genetic relatedness among the serotypes O9, O39 and rough type as these three were included in the root cluster in the dendrogram plotted based on combination of all eight random primers. Moreover, serotype O9 is not a primary pathogen of poultry, but is predominantly responsible for diarrhoea in calves and post weaning diarrhoea in pigs (Gyles, 1993). The birds, however appear to acquire *E. coli* from a common source, which could be either from feed or hatchery. Additional studies would be necessary to identify the source of avian *E. coli* on farms.

In the recent years with the advent of molecular DNA techniques, several arbitrary primer based RAPD-PCR technique has been used for delineating the bacteria according to their genetic relatedness (Muzurier and Wernas, 1992; Eisen *et al.*, 1995; Lin *et al.*, 1996). The earlier workers opined that RAPD was the best method used for detecting genetic differences with respect to its speed and ability to type a wide variety of bacterial species and suggested that it would be an increasingly useful molecular epidemiologic tool. In the past, dendrogram based analysis of the RAPD profiles of various bacteria allowed in understanding the genetic relationship between isolates grouped into several clusters. These phylogenetic studies successfully showed the predominance of a single epidemic strain that was transmitted between hosts and its persistence over a period of time.

In this study, employing this novel technique we have shown the delineation of *E. coli* serotypes in the dendrograms, which are suggestive of an evolutionary pattern among *E. coli* serotypes. Dendrogram branch lengths were proportional to genetic distance between serotypes. This allowed easy identification of lineage groups. Furthermore, phylogenetic comparisons of *E. coli* from different geographical regions provide taxonomical and epidemiological investigations. Thus, we recommend the application of this valuable new alternative approach in molecular characterization of *E. coli* infections to avail the results in a short duration, hence dramatically increasing its clinical relevance over existing molecular methods.

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