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Veterinary Microbiology 80 (2001) 75–83

veterinary microbiology

www.elsevier.com/locate/vetmic

Differentiation of avian pathogenic *Escherichia coli* (APEC) strains by random amplified polymorphic DNA (RAPD) analysis

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Received 6 March 2000; received in revised form 13 October 2000; accepted 4 November 2000

Abstract

Here we describe the application of a random amplified polymorphic DNA (RAPD) analysis for molecular genetic typing avian pathogenic *Escherichia coli* (APEC) strains. The RAPD technique was shown to be highly reproducible. Stable banding patterns with a high discriminatory capacity were obtained using two different primers. Overall, 55 *E. coli* strains were analyzed with a RAPD technique. The RAPD analysis showed that the *E. coli* strains isolated from poultry in Thailand and Sweden could be grouped into 50 of RAPD types by using these two different primer sets. Most of these different *E. coli* RAPD types were not geographically restricted. There was, as expected, a tendency of higher genetic relationship among *E. coli* strains isolated from the same farm. It is suggested that the RAPD technique may provide a rapid, low cost, simple and powerful tool to study the clonal epidemiology of avian *E. coli* infections.

Keywords: RAPD; Avian pathogenic *Escherichia coli* (APEC); Molecular typing

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0378-1135/01/$ – see front matter
PII: S0378-1135(00)00380-1

1. Introduction

Escherichia coli is one of the most common and important bacterial avian pathogens. Likewise there are some of specific *E. coli* clones that are highly pathogenic for birds. Such avian pathogenic *E. coli* strains (APEC) cause several severe disease syndromes in farmed birds such as peritonitis (Gross and Siegel, 1959), enteritis (Nagi and Mathey, 1972), air sac disease, pericarditis, perihepatitis, salpingitis, synovitis, panophthalmitis (Gross and Siegel, 1959; Nagi and Mathey, 1972; Piercy and West, 1976; Gross and Domermuth, 1980; Shane, 1981; Filali et al., 1988; Gross, 1991; Chansiripornchai et al., 1995) and swollen head syndrome (Morley and Thomson, 1984; Fernandez et al., 1986).

The clonal identification of different of *E. coli* strains may aid in defining those that are specifically pathogenic for a certain host, and give guidance for epidemiological studies of sources of infection, and disease transmission. The rapid development in modern molecular genetics has given rise to many new nucleic acid fingerprinting techniques. Arbitrary amplification of polymorphic DNA sequences, termed random amplification of polymorphic DNA (RAPD) analysis or Arbitrarily Primed PCR (AP-PCR) typing (Welsh and McClelland, 1990; Williams et al., 1990), is one such new technique that is being used in many epidemiological studies mainly of human pathogens (van Belkum, 1994). In this study, we present the application of a RAPD typing method for identification and typing of avian pathogenic *E. coli* strains.

2. Materials and methods

2.1. Isolation and identification of avian E. coli

Fifteen avian *E. coli* strains were isolated from various avian species and sources in Sweden. Thirty-five strains were isolated from poultry outbreaks of APEC infections occurring during 1998–1999, in intensive farming units in the central plain and northeastern part of Thailand. Five control strains were isolated from 1-day-old healthy chicks in Thai hatcheries. Bacteria from diseased animals were isolated from necropsy specimens and cultured on 5% horse blood and MacConkey agar. *E. coli* strains were stored in tryptone soy broth (Oxoid, Hampshire, UK) with 15% glycerol at -70°C. Information on the *E. coli* strains examined in this study is given in Table 1.

2.2. Preparation of DNA

All *E. coli* strains were cultured on 5% horse blood agar. Five randomly picked bacterial colonies, approximately 2.5×10^8 cfu/ml, were transferred to conical tubes containing 0.5 ml of sterile deionized water, vortexed for 10 s, sonicated for 5 min and then centrifuged (13,000 rpm) for 5 min at 4°C. The DNA in the supernatant was purified by an equal volume of phenol:chloroform:isoamyl alcohol in the ratio 25:24:1 and then by chloroform:isoamyl alcohol (24:1). The DNA was precipitated with 1/10 volume of 3 M NaCl and two volumes of 95% cold ethanol and dried at room temperature before being dissolved in 100 μl sterile distilled water. The concentration and purity of DNA was

Table 1
Avian *E. coli* strains and RAPD profiles

Strain	Location	Source	RAPD profiles		Cumulative RAPD profiles based on two primers
			Primer 4	Primer 6	
1	Sweden	Lung, ostrich	A1	B1	E1
2	Sweden	N/A, hen	A2	B2	E2
3	Sweden	Intestine, ostrich	A3	B3	E3
4	Sweden	Bone marrow, hen	A4	B4	E4
5	Sweden	Spleen, hen	A4	B5	E5
6	Sweden	Spleen, hen	A5	B6	E6
7	Sweden	Epicardial fluid, quail	A6	B7	E7
8	Sweden	N/A, ostrich	A7	B8	E8
9	Sweden	Egg-box	A8	B9	E9
10	Sweden	Egg-box	A9	B10	E10
11	Sweden	Egg follicle, hen	A10	B11	E11
12	Sweden	3-day-old chick, layer	A11	B11	E12
13	Sweden	Egg follicle, layer	A11	B11	E12
14	Sweden	2-day-old chick, chick	A12	B12	E13
15	Sweden	Egg follicle, hen	A13	B13	E14
16	Ratchaburi[a]	Liver, broiler	A14	B14	E15
17	Ratchaburi[a]	Liver, broiler	A14	B14	E15
18	Ratchaburi[b]	Pericardial fluid, broiler	A15	B15	E16
19	Ratchaburi[b]	Liver, broiler	A15	B16	E17
20	Singburi[c]	Liver, broiler	A16	B17	E18
21	Singburi[c]	Liver, broiler	A17	B18	E19
22	Singburi[d]	Heart, broiler	A18	B18	E20
23	Nakonprathom[e]	Liver, duck	A19	B19	E21
24	Nakonprathom[f]	Liver, broiler	A20	B20	E22
25	Nakonprathom[f]	Liver, broiler	A20	B20	E22
26	Chonburi[g]	Liver, broiler	A21	B21	E23
27	Chonburi[g]	Liver, broiler	A22	B22	E24
28	Chonburi[g]	Liver, broiler	A23	B23	E25
29	Chonburi[h]	Abdomen, layer	A24	B24	E26
30	Chonburi[g]	Liver, broiler	A25	B25	E27
31	Chonburi[g]	Liver, broiler	A26	B26	E28
32	Chonburi[g]	Liver, broiler	A27	B27	E29
33	Ratchaburi[i]	Liver, broiler	A28	B27	E30
34	Ratchaburi[i]	Liver, broiler	A28	B27	E30
35	Supanburi[j]	Liver, broiler	A29	B28	E31
36	Chachoensao[k]	Oviduct, layer	A30	B29	E32
37	Supanburi[l]	Liver, broiler	A31	B30	E33
38	Supanburi[m]	Liver, broiler	A32	B31	E34
39	Supanburi[n]	Liver, broiler	A33	B32	E35
40	Nongbualumpu[o]	Abdomen, broiler	A34	B33	E36
41	Phichit[p]	Lung, breeder	A35	B34	E37
42	Nakornratchasima[q]	Yolk sac, broiler	A36	B32	E38
43	Nakornratchasima[q]	Yolk sac, broiler	A37	B32	E39
44	Nakornratchasima[q]	Yolk sac, broiler	A38	B35	E40
45	Nakornratchasima[q]	Yolk sac, broiler	A39	B36	E41
46	Nakornratchasima[q]	Yolk sac, broiler	A39	B36	E41
47	Lopburi[r]	Lesion, broiler	A40	B37	E42

Table 1 (*Continued*)

Strain	Location	Source	RAPD profiles		Cumulative RAPD profiles based on two primers
			Primer 4	Primer 6	
48	Lopburi[s]	Lesion, broiler	A41	B38	E43
49	Lopburi[t]	Lesion, broiler	A42	B39	E44
50	Lopburi[u]	Lesion, broiler	A43	B40	E45
51	Nakornratchasima[v]	Healthy, chick	A44	B41	E46
52	Nakornratchasima[w]	Healthy, chick	A45	B41	E47
53	Lopburi[x]	Healthy, chick	A46	B42	E48
54	Lopburi[y]	Healthy, chick	A47	B43	E49
55	Lopburi[z]	Healthy, chick	A48	B44	E50

[a] Different superscripts represent different farms.

[b] Letters, A, B, and E represent RAPD fragment product shown as a single band on the gel.

[c] A1–A48, and B1–B44 represent the different RAPD fragment patterns with respect to each primer. E1–E50 represent the different RAPD fragment patterns with respect to primer numbers 4 and 6.

determined by spectrophotometer at optical densities of 260 and 280 nm using Gene Quant Calculator (Pharmacia Biotech, Uppsala, Sweden).

2.3. Selection of RAPD primers

Extracted DNA from 25 strains of *E. coli* was first amplified with six different RAPD primers (Amersham Pharmacia Biotech, Uppsala, Sweden). The results were analyzed using the Simpson's index of diversity:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1)$$

where D is the numerical index of discrimination, N the total number of strains, s the total number of types described, and n_j the number of strains belonging to the j type (Hunter and Gaston, 1988). We continued the experiments for the 55 strains using primers 4 and 6.

2.4. RAPD-polymerase chain reaction conditions

The RAPD kit (Amersham Pharmacia Biotech) containing room-temperature stable dried Ready-to-Go beads was used throughout the study. The kit was used as described by the supplier. The kit contains thermostable polymerase (Ampli*Taq* DNA polymerase and Stoffel fragment), dNTPs (0.4 mM each dNTP in a 25 μl reaction volume), Bovine serum albumin (2.5 μg) and buffer (3 mM $MgCl_2$, 30 mM KCl and 10 mM Tris (pH 8.3), in a 25 μl reaction volume). A solution 10 μl of 2.5 pM/μl of RAPD analysis reagent was added to each primer, 8 μl of Template DNA (10 ng/μl) and 7 μl sterile deionized water. The total volume of reaction mixture was thus 25 μl which was processed in a Gene Amp

PCR system 2400 thermocycler (Perkin-Elmer) for 45 cycles at 30 s, 94°C; 15 s, 36°C; and 30 s, 72°C. In negative control reactions, the DNA template or the primer were replaced by sterile deionized water. RAPD products were visualized by means of ethidium bromide staining after electrophoresis in a 2% Nusieve agarose gel. The RAPD electrophoresis patterns were recorded as Tiff files. The bacterial strains were analyzed with each of the two primers in at least two independent reactions and the banding patterns obtained by at least two runs were considered as a fingerprint for that particular isolate. Different banding profiles were designated by letter codes.

2.5. Interpretation of RAPD data

The RAPD patterns of individual strains were scored based on band presence or absence. The index of similarity (F) between samples was calculated using the formula (Nei and Li, 1979)

$$F_{xy} = \frac{2n_{xy}}{n_x + n_y}$$

where n_{xy} is the number of RAPD bands shared by the two samples, and n_x and n_y the number of RAPD bands scored in each sample. The genetic distance (d) was calculated using the formula of Hillis and Moritz (1990):

$$d = 1 - F$$

3. Results

The reproducibility of the RAPD technique was tested by repeated testing. There was no loss or shift in the position of banding patterns when the RAPD procedures were repeated, and no amplification products were seen when the reactions were performed in the absence of primer or in the absence of template DNA. Of the six different primers tested for 25 strains (Table 2), primer numbers 4 and 6 both showed high discrimination indices — slightly higher for primer 4 (Figs. 1 and 2). We continued the experiments for the 55 strains using primers 4 and 6. The discrimination indices obtained after amplification 55 *E. coli* strains with primers 4, 6 and combined primer numbers 4 and 6

Table 2
Random amplified polymorphic DNA primers used in this study for 25 strains

Primer	Sequence (5′ to 3′)	Discrimination index
RAPD analysis primer 1	5′-d (GGTGCGGGAA)-3′	0.977
RAPD analysis primer 2	5′-d (GTTTCGCTCC)-3′	0.973
RAPD analysis primer 3	5′-d (GTAGACCCGT)-3′	0.983
RAPD analysis primer 4	5′-d (AAGAGCCCGT)-3′	0.983
RAPD analysis primer 5	5′-d (AACGCGCAAC)-3′	0.983
RAPD analysis primer 6	5′-d (CCCGTCAGCA)-3’	0.980
RAPD analysis primer 1–6	All sequences	1.0

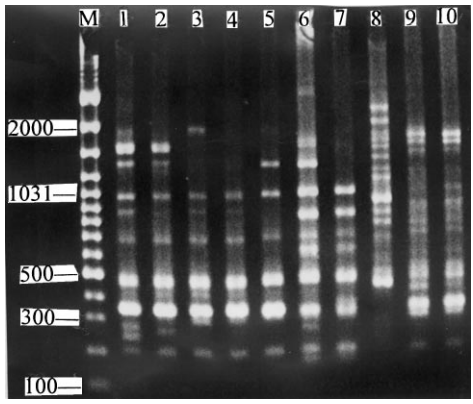

Fig. 1. RAPD profiles of 10 avian *E. coli* strains using primer 4 (sequence = 5′-d (AAGAGCCCGT)-3′). Lanes 1–10 show the patterns obtained with strain 16–25, respectively. Lane *M* = 100 bps ladder.

were 0.995, 0.991 and 0.997, respectively. The Nei and Li (1979) method of analysis was chosen to identify similarities in *E. coli* RAPD patterns. The primer numbers 4 and 6 (Table 2) gave the high rate of discrimination and both gave rise to high numbers (7–14) of amplicons distributed over a wide molecular size range. Repeated testing of samples showed that the RAPD assay was stable and reproducible. The discriminatory capacity, calculated as Simpson's index of diversity, was 0.95 for both primers 4 and 6.

Three main clusters A (six strains), B (nine strains) and C (12 strains) and a large number of minor clusters could be discerned (Fig. 3). Primer numbers 4 and 6 generated a total of 48 and 44 RAPD sub-patterns, respectively, from the 55 strains. These two combined primers revealed 44 distinct RAPD sub-patterns from 48 *E. coli* strains cultured from chickens, three *E. coli* strains from ostriches, one *E. coli* strain from one quail (RAPD cluster B), two *E. coli* strains from chicken egg-boxes (one in cluster B and one in

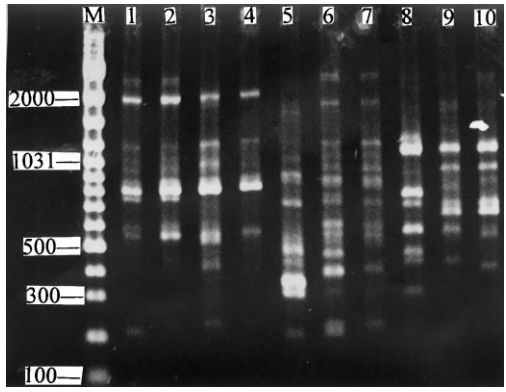

Fig. 2. RAPD profiles of 10 avian *E. coli* strains using primer 6 (sequence = 5′-d (CCCGTCAGCA)-3′). Lanes 1–10 show the patterns obtained with strain 16–25, respectively. Lane *M* = 100 bps ladder.

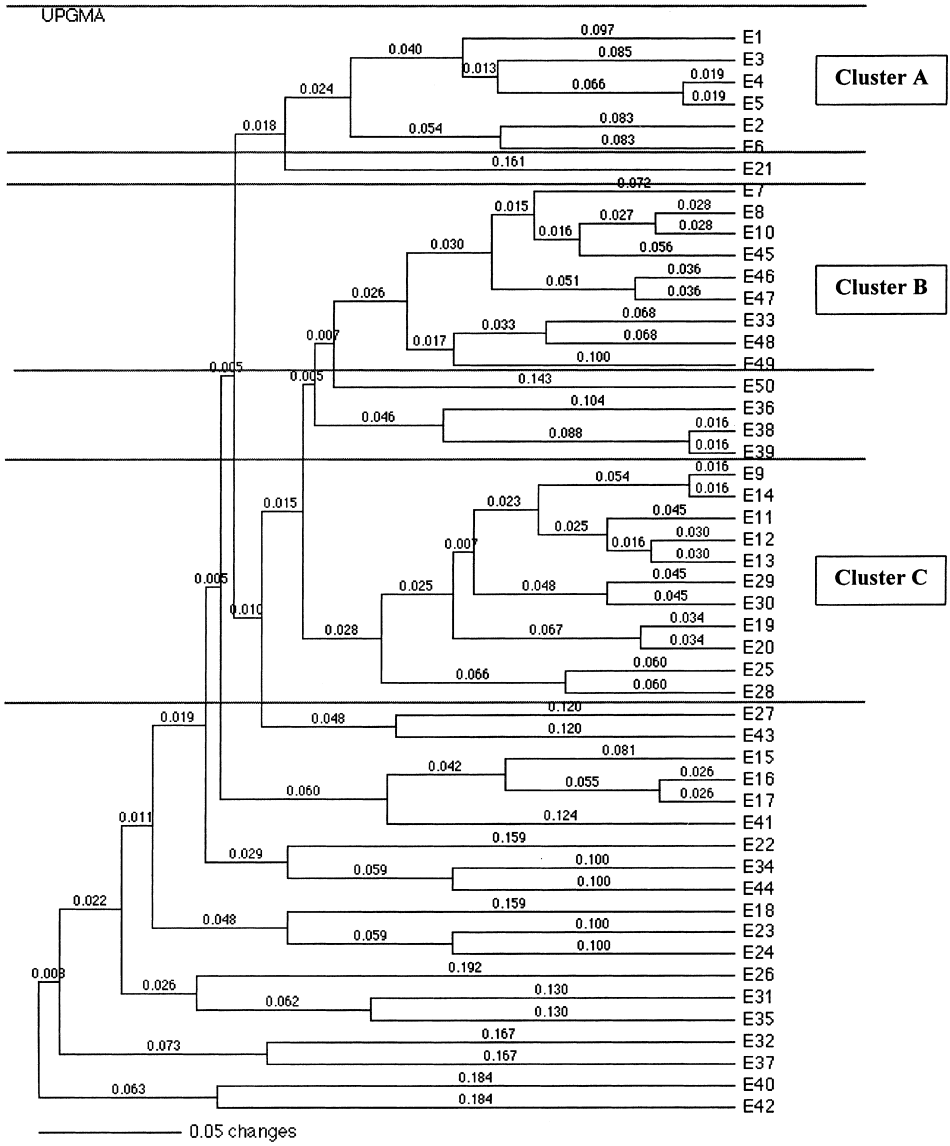

Fig. 3. Phenogram showing the relationships among 55 *E. coli* isolates of 50 RAPD types generated for primers 4 and 6.

cluster C) (Table 1), and a single isolate from a duck. The latter strain fell into none of the three major clusters (Table 1).

Identical RAPD patterns were obtained from four of five pairs of *E. coli* strains (strain numbers 16 and 17, 24 and 25, 33 and 34, 45 and 46) isolated from the same chicken farm in Thailand. One pair of *E. coli* (strain numbers 12 and 13) isolated from chickens from

Sweden also gave the identical RAPD sub-pattern (Table 1). *E. coli* strains isolated from the same flock, although yielding different RAPD patterns, tended to show greater similarities as compared to *E. coli* strains isolated from different flocks. There appears to be no geographically restricted *E. coli* RAPD patterns of avian samples from Sweden and Thailand. We could not identify clear difference in the genetic similarities between avian pathogenic *E. coli* and avian non-pathogenic *E. coli* (control) strains.

4. Discussion

The most commonly used molecular genetic finger-printing technique is restriction fragment length polymorphism (RFLP) analysis. However, RFLP analysis requires relatively large amounts of DNA, expensive equipment and it often takes days to obtain results. By contrast, RAPD results are generated within 4 h and hence are time and cost saving. Maurer et al. (1998) claimed that fingerprinting by RAPD revealed more genetic differences among avian *E. coli* strains than RFLP analysis.

The avian *E. coli* strains analyzed here (Table 1, Fig. 3) showed rather high polymorphism upon RAPD analysis. This is in accordance with the results performed by other techniques, such as multilocus enzyme electrophoresis (Selander et al., 1986). However, Wang et al. (1993) earlier claimed that RAPD PCR was more sensitive than multilocus enzyme electrophoresis. The RAPD analysis results presented here show that the 55 avian *E. coli* strains collected from Sweden and Thailand could be differentiated into 50 different RAPD sub-types with three major clusters — designed A (six strains), B (nine strains) and C (12 strains). We could not clearly see the difference in the genetic similarities between avian pathogenic *E. coli* and avian non-pathogenic *E. coli* (control) strains. It means RAPD cannot differentiate between the pathogenic and non-pathogenic strains.

Obviously, more work is needed before RAPD typing can replace already long established and recognized typing techniques but it is suggested that method in the near future may contribute to a better epidemiological control of APEC infections due to its simplicity, rapidity and low cost. Studies will aid in the development of more effective control measures and also, by identification of particularly virulent APEC clones, aid in the development of new efficient and affordable vaccines to control these infections.

Acknowledgements

We thank Dr. Carles Vilà from the Department of Evolutionary Biology, Uppsala University for his help in phenogram representation of groups and Dr. Ruud Jansen from the Department of Veterinary Bacteriology, Utrecht University, for suggestions the manuscript.

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