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STUDYING THE PATHOGENICITY AND RAPD- PCR ANALYSIS OF DIFFERENT *ESCHERICHIA COLI* SEROTYPES ISOLATED FROM BROILERS AND LAYER CHICKENS

(With 2 Tables and 3 Figures)

By

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دراسة مدى ضراوة وتحليل البلمرة المتسلسل العشوائي لحامض الدي إن إية الغير متشابه للإنماط السيرولوجية المختلفة من الميكروب القولوني المعزول من قطعان البدارى والدجاج البياض

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في هذه الدراسة تم عزل الميكروب القولوني من حالات النفوق والحالات المرضية للطيور وتم تصنيف الميكروب المعزول بيوكيميائيا وسيرولوجيا (الأنتيجين الجسمي) إلى ثمانية أنماط مختلفة (٢، ٨ ، ١١، ١٢، ٥٥ ، ٨٧ ، ١٢٧، ١٥٧) بالإضافة إلى ستة عترات لم يتم تصنيفها سيرولوجيا. تم إختبار الضراوة المعزولة في الكتاكيت الصغيرة بواسطة الحقن تحت الجلد بجرعة تحتوي على 10^8 ميكروب لكل مللي. تم تحليل الفروقات الجينية لثلاثون عترة باستخدام تفاعل البلمرة المتسلسل العشوائي لحامض الدي إن إية الغير متشابه والذي أدى إلى تقسيم الميكروبات المعزولة إلى ستة مجموعات تمثل الشجرة الجينية واشتملت كل مجموعة على مجموعات صغيرة تمثل عترات مختلفة من الميكروبات المصنفة والغير مصنفة. أثبتت النتائج وجود تقارب جيني بين جميع العترات المختبرة بالرغم من وجود اختلافات بينها وأن استخدام البصمة الوراثية لدراسة العلاقة الوراثية بين السلالات البكتيرية تؤدي إلى إعادة تقسيم هذه العترات على أسس جينية مختلفة عن تصنيفها بيوكيميائيا وسيرولوجيا.

SUMMARY

In the present study successful isolation of *Escherichia coli (E.coli)* was carried out from freshly dead and diseased birds. The isolates were biochemically and serologically identified using O- antigen including

eight different serotypes (O2, O8, O11, O22, O55, O87, O127 and O157) beside six untypable strains. Pathogenicity of all the isolated strains was tested in 7 days - old chicks by subcutaneous inoculation of 10^8 CFU/ml. Genetic variation of 30 isolates was analysed using the random amplified polmorphic DNA (RAPD) technique. A dendograph generated using a random primer of arbitrary sequence demonstrated 6 major clusters. Each cluster encompassed small clusters corresponding to different serotypes including the untypable strains. The results showed genetic relatedness of all strains, however, inter- and intraserotype variations were also evident. It also highlighted the importance of RAPD fingerprinting in studying population genetics and epidemiology of different micro-organisms, rather than biochemical and serological identification.

Key words: *Pathogenicity, RAPD-PCR, E.coli, chickens*

INTRODUCTION

Escherichia coli is one of the most important infections among all species of poultry. It is incriminated in production of severe infections such as serositis, arthritis, gastrointestinal colibacillosis and respiratory infections (Gross *et al.*, 1991). Acute septicemia is the most prevalent problem in young birds but the subacute infection is the common classical form in the field (Nakamura *et al.*, 1990 ; Frommer *et al.*, 1990). In Egypt, *E. coli* infection in broilers is causing increased mortality and condemnation rates (Farid *et al.*, 1983; Khaled, 1990; Ibrahim *et al.*, 1997). Random amplification of polymorphic DNA (RAPD) has received considerable attention as a molecular typing method due to its simplicity, sensitivity and flexibility (Williams *et al.*, 1990). The technique was successfully applied to the study of genetic diversity among groups of *E.coli* strains previously regarded as homogeneous (Alos *et al.*, 1993; Brikun *et al.*, 1994).

In this article, we aimed to isolate and identify E. coli from the diseased birds and study their individual pathogenicity. Furthermore, inter- and intraserotype specific genotypic variations of the isolated strains were investigated using the RAPD fingerprinting technique.

MATERIALS and METHODS

Clinical findings:

A total of 350 diseased chickens (299 broilers of 28 to 45 days - old and 51 layers aged 180 - to 220 - days) were collected from different localities in Assiut governorate, Egypt. Clinical signs and necropsy findings of the examined birds were recorded.

Bacterial isolation and identification:

Trials for isolation and identification of the microorganisms were carried out from the heart, liver and air sacs in nutrient broth (Oxoid) for 24 hours at 37 °C, subcultured on nutrient agar (Oxoid), MacConkey agar (Oxoid) and 5-10 % sheep blood agar (Oxoid) and incubated at 37 °C for 24 hours.

The cellular morphology was examined by Gram staining and the colonies produced on different agar media were studied for colonial morphology. Motility and haemolysis were determined on semi-solid agar and sheep blood agar. Sugar fermentation of glucose, sucrose, mannitol, sorbitol and lactose were carried out using phenol red broth base. The biochemical tests included catalase, oxidase, indol production, methyl red, Voges Proskauer, citrate utilization, urease and H_2S production.

Serology:

The polyvalent and monovalent *E. coli* O- antisera were prepared at the Department of Poultry Research and Disease Diagnosis of Animal Health Research Institute, Egypt, according to Sojka (1965) against standard *E. coli* serogroups provided from Veterinary Academy of Moscow. The adopted technique was the tube agglutination test and the antisera used were as follows: Polyvalent I (O1, O11, O20,O157); Polyvalent II (O2, O10, O55, O142); Polyvalent III (O8, O22, O12, O127); Polyvalent VI (O87, O86, O 114, O 109); and Polyvalent V (O139, O153, O107, O143).

***In vivo* Pathogenicity:**

One hundred and twenty chicks (7 days- old) of Hyline breed (El-Menia governmental farm, Egypt) were raised on wooden slat floors. Ten randomly selected chicks were clinically examined and subjected to bacteriological examination to prove their freedom from infections. The remaining chicks were divided into 11 groups of ten birds each. Each group was inoculated subcutaneously with 0.2 ml of broth culture

containing about 10^8 CFU/ml of one of the isolated serotypes including the untypable strains. Ten birds were inoculated with sterile broth and kept as control. All birds were kept under clinical observation for 2 weeks. Sacrificed as well as dead birds were subjected to clinical, PM and bacteriological examinations. The degree of pathogenicity based on morbidity, mortality and scored lesions was interpreted according to Rosenberg *et al.* (1985).

Extraction of bacterial chromosomal DNA:

Thirty *E. coli* isolates representing 8 different serotypes (O2, O8, O11, O22, O55, O87, O127, O157) and one untypable serotype (UNT) were grown overnight at 37°C in Luria - Bertani medium (LB medium) with shaking. Genomic DNA was extracted from the bacteria using the lysing and nuclease- inactivating properties of the chaotropic agent goanidinium thiocyanate together with the nucleic acid – binding properties of diatoms in the presence of this agent Boom *et al.* (1990). The concentration and purity of the extracted DNA was determined by spectrophotometry (Gene Quant II, Pharmacia Biotech.).

RAPD- PCR technique :

RAPD reaction was carried out using RAPD Analysis Beads (Amersham Pharmacia Biotech). The RAPD analysis primer set contained 2.5 nmol of each of the following six primers of 10 mers of arbitrary sequence:

Primer 1-(5`- d [GGTGCGGGAA]- 3`).

Primer 2- (5`- d [GTTTCGCTCC]- 3`).

Primer 3-(5`- d [GTAGACCCGT ]-3`).

Primer 4- (5`- d [AAGAGCCCGT]- 3`).

Primer 5-(5`- d [AACGCGCAAC]- 3`).

Primer 6- (5`- d[CCCGTCAGCA]- 3`).

In addition 2 control *E. coli* DNAs (BL21 and C1a) were also provided. PCRs were performed in 25 ul reaction volumes containing 25 pmol of a single RAPD primer, 40-60 ng template DNA (2 ul) and 18 ul distilled deionised water added to the lyophilized beads. Temperature cycling was controlled in thermal cycler (Biometra) programmed as follows: 1 cycle at 94°C for 1 minutes followed by 45 cycles at 94°C for 1 minute, 36°C for 1 minute and 72°C for 2 minutes followed by 1 cycle

at 72°C for 3 minutes. Reaction products were analyzed by electrophoresis on 2% agarose gels at 150 volts for 1.5 hour and stained with 0.5 ug/ml of ethidium. Gels were photographed under UV transilluminator (Biometra). A 100- bp ladder (Amersham Pharmacia) was used as a molecular weight marker. The 6 primers were tested on one randomly- selected DNA sample to demonstrate the banding pattern of each primer. Primer 5 gave the best RAPD markers, hence it was used with all the extracted DNAs from the tested isolates.

Analysis of RAPD data:

Analysis of RAPD patterns derived from the DNA amplification of all bacterial isolates with primer 5 was performed by using computer software Scan Pack-3 from Biometra for phylogenetic analysis. Calculation of the similarity matrix was performed with the Pearson product moment correlation coefficient and the UPGMA method for clustering (unweighted pair- group method using arithmetic averages) according to Sneath and Sokal (1973).

RESULTS

Clinical and necropsy findings:

The clinical symptoms appeared as feather ruffling, huddling, white pasty diarrhea, loss of body weight, recumbency of some birds while others showed lameness and gasping. Gross lesions of fibrinous pericarditis, perihepatitis, air saculitis and colisepticaemia were also found.

Isolation and identification:

The results of isolation revealed recovery of 44 isolates from broilers and 17 isolates from layers. All the obtained biochemical data were quite identical to those of genus *E. coli* and the isolated strains were non-haemolytic strains except serotype O157.

Serotyping:

The results of serological identification are demonstrated in Table. 1 Flagellar serotyping of serotype O157 was carried out due to its zoonotic potential and proved to be negative for flagellar antigen H7.

Pathogenicity to 7 days- old chicks:

Some chicks in the groups infected with serotypes O2, O8 and O87 died at the first day post-inoculation. All the infected groups

showed the clinical signs mentioned which became more severe by time. In addition, serotype O87 caused closure of the eyes. The morbidity was considered from the appearance of clinical signs as shown from Table 2.

Table 1: Serological identification of *E.coli* isolates

Birds	No.of Isolates	Serogroups								Untypable serogroup
		O2	O8	O11	O22	O55	O87	O127	O157	
Broiler	44	7	4	4	3	8	3	-	6	8
Layer	17	-	-	4	3	3	-	7	-	-
Total	61	7	4	8	6	11	3	7	6	8
%		11.48	6.56	13.11	9.84	18.03	4.92	11.48	9.84	13.11

Table 2: Morbidity and mortality rates of experimentally-infected chicks

Group number	Source	O-Serogroup	Number of inoculated chicks	Morbidity	Mortality
1	Broiler	O2	10	9 (90%)	1 (10%)
2	Broiler	O8	10	5 (50%)	5 (50%)
3	Layer	O11	10	9(90%)	1 (10%)
4	Layer	O55	10	8 (80%)	2 (20%)
5	Layer	O22	10	10 (100%)	-
6	Broiler	O87	10	7 (70%)	3 (30%)
7	Layer	O127	10	10 (100%)	-
8	Broiler	O157	10	10 (100%)	-
9	Broiler	Untypable	10	9 (90%)	1 (10%)
10	Control		10	-	-

N.B. The infecting dose is o.2 ml broth culture containing 10^8 CFU/ ml

The pathogenicity varied according to the infecting serotype. Five serotypes (O2, O8, O11, O55, O87) were classified as highly pathogenic in addition to the untypable strains while three serotypes (O22, O127, O157) were regarded as intermediate pathogens.

Agarose gel electrophoresis:

Agarose gels visualized by ethidium bromide staining products using primer 5 yielded arrays of amplified DNA products ranging in sizes from 100 to 1500 bp (Figures 1&2). The strains of the same serotype shared identical or almost identical banding patterns suggesting a similar genetic composition. However, these band profiles differed among different serotypes which indicated interserotype variations.

Phylogenetic analysis:

We compared the genetic relatedness of the isolated strains using a dendogram constructed with Scan pack- 3.0 software. The resulting matrix of pairwise distances was used to generate a phenogram based on

the unweighted pair- group method with arithmetic mean (UPGMA) method.

A phenogram based on the proportion of identical bands in RAPD profiles was constructed (Figure. 3). Six major clonal clusters were identified where each cluster group included strains of different serotypes which referred to the interserotype variation. These clonal clusters were rather homogenous with an overall band similarity of at least 57%. Some serotypes in different clusters showed 91% similarity and most of the amplified bands were identical among them.

Concerning the untypable strains, they were genotyped into 3 different clusters. In the first cluster, two strains were genetically related to serotypes O127 and O2 with 46% similarity. In the second cluster, the degree of similarity between the two untypable strains and serotype O22 was 56% and similar to other *E. coli* serotypes of the same cluster at 67%. In the same cluster, O55 *E. coli* strain was identical to O22 strain as well. In the third cluster, one untypable strain was closely related to serotype O55 at 66% and the other at 39% similarity. However, these two untypable strains and O55 were genetically related to serotype O22 in the same cluster at 23% relatedness.

DISCUSSION

In the present work, we could isolate different *E. coli* serotypes with high recovery rates (72.9% in broilers and 27.9% in layers) from clinical cases showing signs and lesions suggestive for the disease in different localities of Assiut province (Cloud *et al.*, 1985; Filali *et al.*, 1988). Some of the clinical signs and necropsy findings recorded in this study were previously described by (Vidotto *et al.*, 1990; Moltarana *et al.*, 1993; Gowda *et al.*, 1996).

Physical, chemical, sugar fermentation and biochemical reactions were quite similar to species of *E. coli* (Catrer, 1986). All the isolated strains were non- haemolytic except serogroup O157. Eight somatic serotypes O2 (11.5 %), O8 (6.6 %), O11 (13.1 %), O22 (9.8 %), O55 (18.0 %), O87 (4.9 %) and O157 (9.8 %) as well as the untypable strains (13.1 %) were recovered from broiler chickens. In cases of layer chickens, serotypes O11, O22, O55 and O127 (11.5 %) were isolated. Similar *E. coli* serotypes were reported by (Awad *et al.*, 1973; Youssef *et al.*, 1983; Karmy *et al.*, 1987). In our work, we could isolate *E. coli* serotype O157 from the affected birds. This serotype has previously been isolated in Egypt (Abd El- Gafar , 1979; Farid *et al.*,1983). Of particular interest, Schoeni and Doyles (1994) mentioned that serotype

O157 can readily colonize the caeca of young chicks and they continue to shed the organism for several months.

The pathogenicity of the isolated *E. coli* serotypes was evaluated in this study. The results revealed that serotypes O8, O87, O55, O2, O11 and the untypable strains were highly pathogenic while serotypes O157, O127 and O22 were intermediate. Frommer, *et al.* (1990) reported that the high rate of mortality among chicks was attributed to colisepticaemia.

In our study, RAPD profiles of the tested *E. coli* serotypes demonstrated polymorphic bands among the different isolates, however a more or less similarity could also be detected. Similarity among some isolates had an average 66% of the amplified bands in common inside the cluster (minimum value 57% & maximum 91%). These observations proved that all the tested strains had the same clonal nature and agreed with the findings of Pacheco *et al.* (1998).

Regarding the 6 untypable strains , they were equally distributed into 3 different clusters and genetically related to serotypes O127, O2, O22 and O55 with variable degrees of similarities. This relatedness clarified the genetic inheritance of the isolated serotypes despite of their serologic variations. These results are in agreement with (Arbeit *et al.*, 1990; Wang *et al.*, 1993; Pacheco *et al.*, 1997) who mentioned that RAPD analysis can readily distinguish genetically distant strains and also disclose variations among phenotypically related strains. Moreover, Schmidt *et al.* (1999) reported on similar data.

In conclusion, this study proved that beside the importance of serological typing of *E. coli,* PCR- based fingerprinting methods are simple powerful tools for genetic characterization of the organism and may also contribute to a better understanding of the epidemiology of this pathogen.

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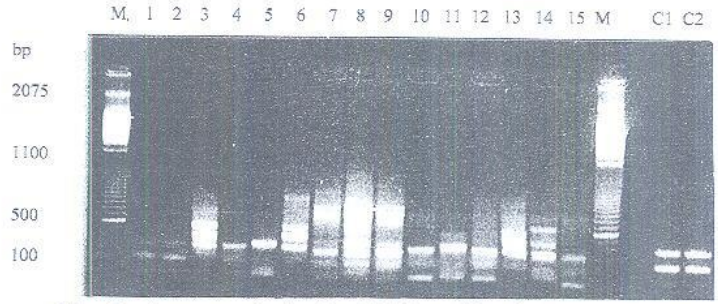

Fig. 1: Agarose gel showing RAPD profiles that are polymorphic between 15 *E.coli* isolates using RAPD primer 5. Fragment sizes are illustrated as base pairs and read from left to right as follows: Lanes M, 100 base pair ladder marker; Lanes 1,2,3, serotype O157; Lanes 4,5,6, serotype O87; Lanes 7,8,9,serotype O11; Lanes 10,11,12, serotype O55; Lanes 13,14,15, serotype O22; Lanes C1 & C2, control *E.coli* DNA.

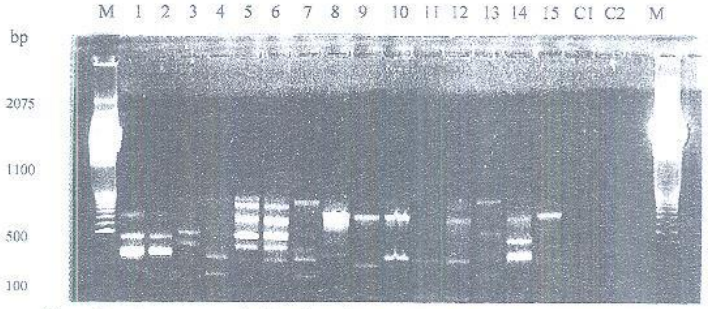

Fig. 2: Agarose gel showing RAPD profiles that are polymorphic between 15 *E.coli* isolates using RAPD primer 5. Fragment sizes are illustrated as base pairs and read from left to right as follows: Lanes M, 100 base pair ladder marker; Lanes 1,2,3,4,5,6, untypable strains; Lanes 7,8,9, serotype O127; Lanes 10,11,12,serotype O8; Lanes 13,14,15,serotype O2; Lanes C1 & C2, control *E.coli* DNA.

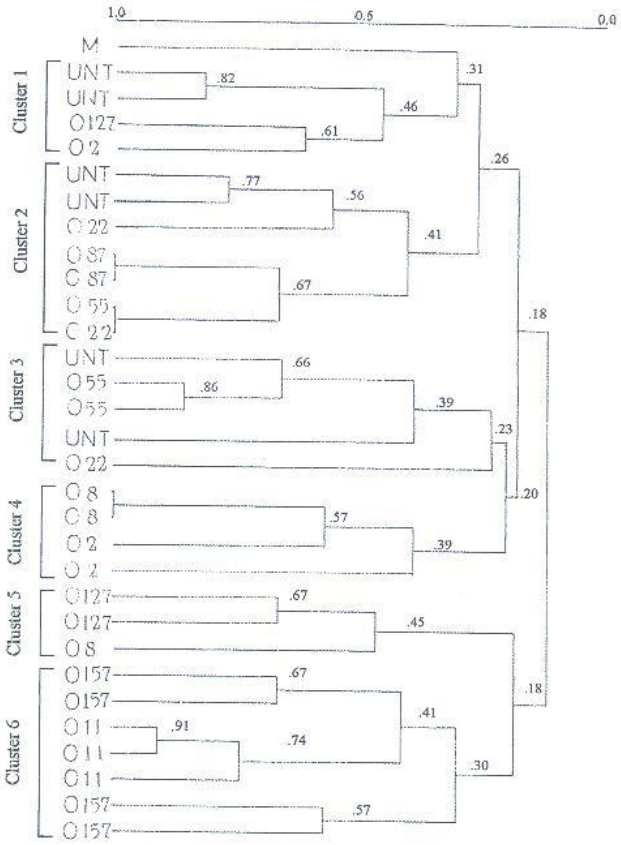

Figure 3. Phenogram of thirty E. coli strains used in this study based on RAPD analysis- PCR pattern similarity. The phylogenetic tree was constructed applying UPGMA to matrix of similarity established from the combined RAPD data obtained by using RAPD primer (5). The numbers indicate the percentages of similarity among RAPD profiles based on pairwise band sharing. Matching peaks based on Rf- values and Match-Precision: 0.0050. M is the molecular weight marker.