Epidemiol. Infect. (1995), **114**, 113–121
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Clonal diffusion of EPEC-like *Escherichia coli* from rabbits as detected by ribotyping and random amplified polymorphic DNA assays

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(*Accepted 12 September 1994*)

SUMMARY

The genetic diversity and clonal relationships among 77 *Escherichia coli* strains isolated in France from diarrhoeic rabbits and that belonged to seven O serogroups including the predominant O103 serogroup, were estimated by ribotyping and random amplified polymorphic DNA (RAPD) assays. Fifteen ribotypes were defined. Most of the highly pathogenic O103 strains could be assigned to two major groups. Non-pathogenic strains were clearly distinguished. RAPD assays generally matched ribotyping, or gave more precision for subdividing strains from the two main O103 groups. The results on strains isolated from different areas and over a 9-year period showed the relevance of the association of these two methods for the survey of the spread of strains in breeding flocks and illustrated clonal diffusion in rabbit production structures.

INTRODUCTION

In industrial rabbit-fattening farms, enteritis caused by *Escherichia coli* is the main cause of morbidity and mortality in weaned rabbits. The disease affects 5–7 week-old rabbits, produces severe diarrhoea associated with colonization of the distal ileum and caecum by *E. coli* strains that belong to different serogroups [1, 2]. Since the isolation of the first highly pathogenic O15:H- strain designated RDEC-1, an enteropathogenic *E. coli* analog [3], many other *E. coli* strains that show the same features have been isolated from rabbits with enteric disease. In France, the most commonly isolated *E. coli* strains enteropathogenic for weaned rabbits belong to O103 serogroup. Like the RDEC-1 strain, strains of serogroups O26, O103, O128, and O132 isolated from diarrhoeic weaned rabbits have sequences homologous to the *eaeA* gene [4–6] which is essential for the production of intimin and the attaching and effacing lesions characteristic of enteropathogenic *E. coli* strains [4, 7].

In France, rabbit production is organized in a vertical and closed structure. Most production farms fatten animals derived from a few breeding flocks. Exchange between fattening farms are non-existent or very limited. These

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https://doi.org/10.1017/S0950268800051967 Published online by Cambridge University Press

(Boehringer) was used as a probe and labelled by random oligopriming using a mixture of hexanucleotides (Pharmacia) and M-MLV reverse transcriptase (BRL, Gaithersburg, Maryland) in the presence of [α-^{32}P]dCTP (Amersham) according to the method of Picard-Pasquier and colleagues [15]. Hybridization and washes were done at 65 °C under stringent conditions according to the membrane manufacturer's instructions in a hybridization oven. Ribosomal banding patterns were confirmed by repeated runs and hybridizations. Two strains were considered as belonging to the same pattern if all the bands were common. Raoul™ (Appligène, Illkirch, France) was used as molecular weight marker after hybridization with pUC18. Fragment sizes in the hybridization patterns were calculated from migration distances with the BIOIMAGE Analytical Imaging Instrument (Millipore, St-Quentin, France).

RAPD assays

Ten oligonucleotides (kit G, Operon technologies, Alameda, USA) were arbitrarily selected and used: OPG-01 (5′-CTACGGAGGA-3′), OPG-02 (5′-GGCACTGAGG-3′), OPG-03 (5′-GAGCCCTCCA-3′), OPG-04 (5′-AGCGTGTCTG-3′), OPG-05 (5′-CTGAGACGGA-3′), OPG-07 (5′-GAACCTGCGG-3′), OPG-08 (5′-TCACGTCCAC-3′), OPG-09 (5′-CTGACGTCAC-3′), OPG-11 (5′-TGCCCGTCGT-3′), OPG-12 (5′-CAGCTCACGA-3′). RAPD reaction was performed in total volumes of 25 μl containing 10 ng of purified genomic DNA; 0·2 μM appropriate primer; 200 μM (each) dATP, dCTP, dGTP and dTTP (Boehringer); and 0·5 U of Taq DNA polymerase (Appligène) in the buffer provided by the manufacturer. The reaction mixtures were overlaid with mineral oil to prevent evaporation and placed in an automatic thermal cycler (PTC-100™, TechGen, Les Ulis, France) for 40 cycles. The amplification cycles were as follows: 94 °C for 1 min, 35 °C for 30 s, and 72 °C for 1 min 30 s for the first cycle; 92 °C for 40 s, 35 °C for 30 s, and 72 °C for 1 min 30 s for the other 39 cycles. The final cycle included extension at 72 °C for 10 min. Each reaction included a control tube without template DNA. The amplified DNA products were resolved by electrophoresis on 1·5% agarose gel with 1 × TBE buffer. The molecular sizes of DNA fragments, relative to molecular weight marker Raoul™ were determined as described above. The reproducibility of the RAPD profiles was assessed by comparing profiles obtained with the same primer with two separate thermocyclers of the same type over two or three different experiments.

RESULTS

Ribotyping analysis

Chromosomal DNA of all *E. coli* strains was digested with *Bam*HI, *Eco*RI and *Hin*dIII restriction endonucleases. After digestion by *Bam*HI and *Eco*RI, the majority of the strains belonged to a common ribosomal banding pattern or ribotype (data not shown). In contrast, *Hin*dIII was able to discriminate between these strains and was then used to define groups.

After *Hin*dIII digestion, 7–12 rDNA fragments were obtained; sizes ranged from 0·6–18·5 kb. Among the 78 *E. coli* strains, 15 ribotypes were observed (H1–H15) and were assumed to be distinct patterns (Fig. 1). The differences between patterns were established on the presence or absence of one or several bands

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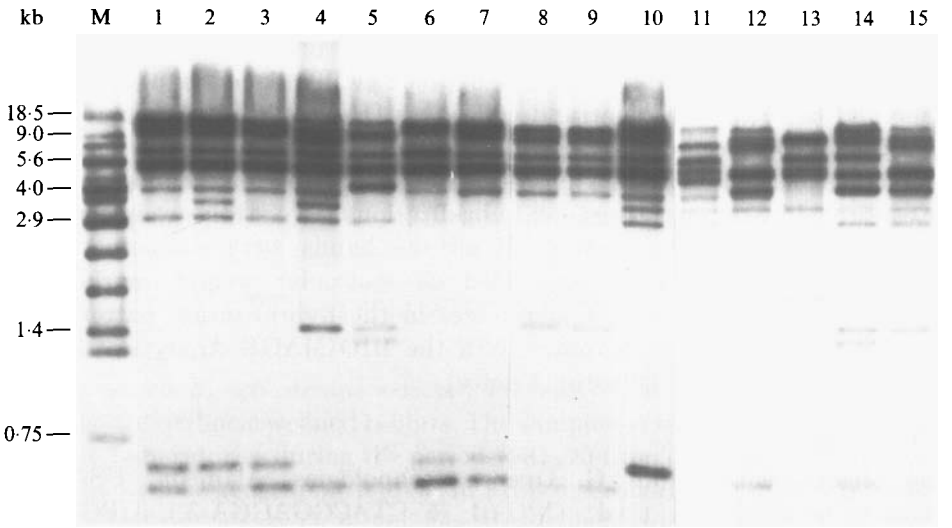

Fig. 1. Representative ribotypes of rabbit *E. coli* strains after *Hin*dIII digestion. Ribotypes H1–H15 (Table 1), lanes 1–15 respectively. Lane M, molecular weight marker (Raoul® (Appligène)).

without taking into account any difference of intensity. The ribotypes obtained were fully reproducible with no sign of partial digestion. For each serogroup, one or several specific patterns were found. Table 1 shows the distribution of the 78 *E. coli* isolates into different ribotypes and O serogroups. Four ribotypes (H1, H2, H3, H4) were distinguished within the 58 *E. coli* strains of serogroup O103, but 47 strains belonged to the major ribotype, H1. *E. coli* strains of other serogroups yielded 11 different ribotypes (H5–H15) which were distinct from those of serogroup O103. The O26 and rough strains had the same ribotype, H5. The highly pathogenic RDEC-1 strain showed a particular ribotype, distinct from the other non-pathogenic O15 strain.

In order to improve the detection of genomic diversity among the O103 *E. coli* strains, DNA of ten strains belonging to the ribotype H1 was digested with eight different restriction endonucleases (*Bgl*II, *Cla*I, *Hae*II, *Kpn*I, P*st*I, *Pvu*II, *Sal*I and *Sma*I). Digestion by *Pst*I provided different patterns between these ten strains and was subsequently used with all O103 strains. Ribotypes for these isolates are shown in Fig. 2. *Pst*I digestion of DNA samples produced 4–6 rDNA fragments from 4–29 kb in size. Four ribotypes (P1, P2, P3, P4) were determined within the 47 O103 *E. coli* strains of ribotype H1 (Table 1). The O103 strains of ribotypes H2, H3, H4 had a common *Pst*I pattern, ribotype P1. Forty-four of 58 *E. coli* strains of serogroup O103 were classified in ribotypes H1P1 or H1P2. As already reported [12] the three highly pathogenic strains, B10, E1, and E31 had the same ribotype and the two non-pathogenic rhamnose-positive O103 strains, C124 and C127, had the same original ribotype.

RAPD analysis

The ten primers were first tested for their ability to generate a useful selection of PCR DNA fragments. The RAPD analysis was performed with five O103 *E. coli* strains that belonged to five distinct ribotypes, H1P1, H1P2, H2P1, H3P1, and

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Table 1. *Results of ribotyping of the 78 rabbit* E. coli *strains tested, including RDEC-1 strain*

Ribotype			Nos. of strains (year of collection)	Representative strain(s)
*Hin*dIII	*Pst*I	O serogroup		
H1	P1	O103	25 (81–89)	BN9010, BN9014, B10, E1, E31
	P2	—	19 (86–90)	BN9012, BN9015, BN9031, D94, E13
	P3	-	1 (87)	BN9045
	P4	—	2 (86–87)	BN9028, BN9044
H2	P1	O103	8 (82–86)	BN9017, BN9039
H3	P1	O103	1 (88)	BN9041
H4	P1	O103	2 (85)	C124, C127
H5	ND*	O26	3 (85–86)	C102, C230, D145
		rough	1 (85)	C110
H6	ND	O128	1 (87)	BN9080
H7	ND	O128	2 (85)	C6, C104
H8	ND	O132	3 (86–87)	BN9081, D46, E82
H9	ND	O132	2 (86–87)	D136, E40
H10	ND	O132	1 (87)	E70
H11	ND	O2	3 (84–86)	B72, C157, D100
H12	ND	O2	1 (85)	C178
H13	ND	O15	1 (86)	D28
H14	ND	O15	1 (76)	RDEC-1
H15	ND	O85	1 (83)	A155

* ND, not done.

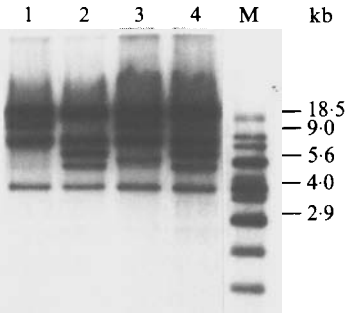

Fig. 2. Representative ribotypes of O103 *E. coli* strains after *Pst*I digestion. Ribotypes P1–P4 (Table 1), lanes 1–4 respectively. Lane M, molecular weight marker (Raoul® (Appligène)).

H4P1. Primer OPG-01 failed to produce amplification products with any of the *E. coli* strains. With primers OPG-02, OPG-03, and OPG-08, the obtained patterns were complex and difficult to analyse. Using primers OPG-05, OPG-07, OPG-09, OPG-11, OPG-12 the patterns of RAPD products from the five O103 *E. coli* DNAs were very similar. Only with the OPG-04 primer distinct profiles were obtained among the five O103 *E. coli* strains. This primer was used to study the 78 *E. coli* strains. To avoid misinterpretation due to lack of reproducibility [16] RAPD experiments were repeated two or three times and results were identical when making use of two similar thermal cyclers located in two different buildings. Figure

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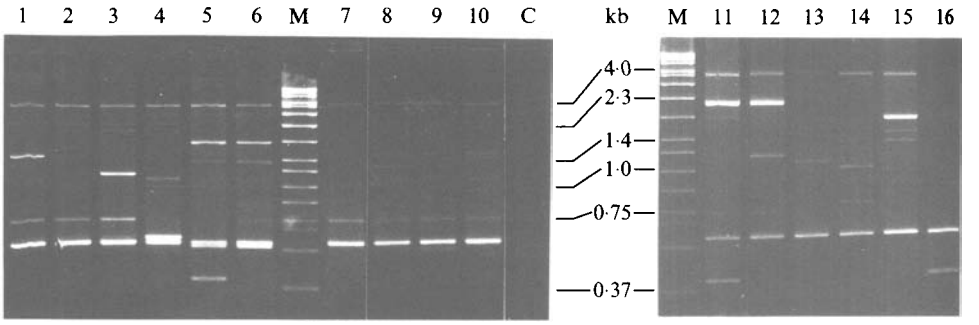

Fig. 3. Representative results of RAPD analysis of *E. coli* strains of rabbit origin. RAPD types I to XVI (Table 2), lanes 1–16 respectively. Lanes C, negative control and M, molecular weight marker (Raoul® (Appligène)).

Table 2. *Comparison of the results obtained by ribotyping and RAPD of the 78 rabbit* E. coli *strains studied, including RDEC-1 strain*

O serogroup	Ribotype (no. of strains)	RAPD type (no. of strains)
O103	H1P1 (25)	I (4), II (19), III (2)
	H1P2 (19)	I (15), II (4)
	H1P3 (1)	I (1)
	H1P4 (2)	I (1), II (1)
	H2P1 (8)	II (1), III (7)
	H3P1 (1)	II (1)
	H4P1 (2)	IV (2)
O26	H5 (3)	V (3)
rough	H5 (1)	VI (1)
O128	H6 (1)	VII (1)
	H7 (2)	I (1), VIII (1)
O132	H8 (3)	III (2), IX (1)
	H9 (2)	X (2)
	H10 (1)	III (1)
O2	H11 (3)	XI (1), XII (1), XIII (1)
	H12 (1)	XIII (1)
O15	H13 (1)	XIV (1)
	H14 (1)	XV (1)
O85	H15 (1)	XVI (1)

3 shows the different RAPD profiles obtained with the OPG-04 primer. Among the 78 *E. coli* strains, 16 different and reproducible RAPD profiles were observed. The size of amplified fragments ranged from 0·4–3·8 kb and two major bands of 0·6 and 3·8 kb were shared in all the preparations. Clear differences were seen between and within different serogroups (Table 2). However, one O128 strain and three O132 strains produced the same pattern of amplification as O103 strains (profiles I and III respectively), indicating that RAPD results did not match O serotyping.

RAPD assays and ribotyping yielded concordant results for about 70% of the strain tested (Table 2). However, RAPD discriminated within a particular ribotype. Several O103 strains and three O2 strains gave different RAPD profiles although they appeared homologous by ribotyping. The rough strain had a unique RAPD profile although it was similar to the profile of O26 strains by ribotyping after *Hin*dIII digestion. However we also observed a converse case where other

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strains of serogroup O103 or O2 had distinct ribotypes and were found within the same RAPD profile.

DISCUSSION

The 77 *E. coli* strains of diarrhoeic weaned rabbit origin tested in this study belonged to different serogroups and were isolated from diverse geographical locations in France over a 9-years period. In our rabbit production structures, a survey of the possible persistance and diffusion of potentially pathogenic *E. coli* is of interest. Recently, new methods have been developed and are now available for use in diagnostic bacteriology. We compared ribotyping which refers to highly conserved sequences, to RAPD assays which use primers of arbitrary nucleotide sequence to amplify at random segments of the genome.

From our results it appeared that *Hin*dIII was the most appropriate restriction endonuclease for ribotyping O103 *E. coli* strains and strains of other serogroups, including the RDEC-1 strain. The O103 strains that demonstrated identical *Hin*dIII ribotypes may be further subdivided by using a second enzyme, such as *Pst*I. The O103 strains were considered as belonging to the same ribotype when their both *Hin*dIII and *Pst*I patterns were identical. It was convenient to use the *Hin*dIII pattern for primary typing of the strains and the *Pst*I pattern for further discrimination. However this is rather arbitrary and *Pst*I could have been used for the first discriminating step of O103 strains. It should be noted here that *Hin*dIII pattern H1 could be subdivided into four *Pst*I subtypes (P1–P4) whereas patterns H2, H3 and H4 belonged to pattern P1. This demonstrated that although they can be assigned to different H and P ribotypes, most of the O103 strains are related. This interpretation was corroborated by the apparent absence of discrimination when other restriction enzymes were tested.

A correlation was observed between pathogenicity and ribotypes of O103 strains. The majority of the pathogenic O103 strains, already studied in an experimental model of infection [1, 6], were classified in the two predominant ribotypes H1P1 and H1P2, different from the H4P1 ribotype observed in the non-pathogenic strains.

A consistent correlation between serogroups and *Hin*dIII ribotypes was seen. Within a given serogroup, a number of different restriction patterns was observed. For instance, strains of serogroup O103 could be assigned to only four *Hin*dIII ribotypes and a given pattern did not appear to be repeated in different serotypes. However, four strains represented marginal cases, the three O26 and the rough strains exhibited an identical ribotype, but these rhamnose-negative strains had the same H11 flagellar antigen and similar pathogenic properties (9), and it is probable that the rough isolate was derived from an O26 parent strain.

Ribotyping analysis demonstrated the existence of limited diversity among the O103 strains associated with rabbit post-weaning diarrhoea. Forty-seven of 58 O103 strains belonged to the same *Hin*dIII ribotype H1 and 44 of these strains could be assigned to only two ribotypes after additional analysis with *Pst*I. More variability was seen among the strains of other serogroups (in particular O2, O128 and O132) and this diversity could not be explained by differences in origin of these strains. The O103 strains indistinguishable on the basis of their ribotypes were isolated in different fattening farms located in distant geographical areas.

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This result is in agreement with a clonal relationship between these strains and suggests a diffusion of a limited number of pathogenic O103 strains in different flocks which may be partly due to the vertical and closed organization of the rabbit production. With phenotypic methods, Peeters and colleagues [2] and Camguilhem and Milon [1] had also suggested such a clonal diffusion of rabbit *E. coli* strains.

Some O103 strains collected in 1981 had a ribotype similar to those observed in 1989. This result suggests a wide dissemination and a stable persistence of a few clones during a period of time of several years in the rabbit-fattening farms. These strains were isolated in the course of a pathological event in young animals, but further studies are needed to illustrate the hypothesis of the carriage of O103 *E. coli* by healthy adults. Ribotyping and RAPD fingerprinting led to identical results in the definition of clones for about 3/4 of the strains studied. However, a more precise grouping of strains was obtained when both methods were used in conjunction. Therefore, the concomitant use of ribotyping and RAPD appears as a convenient procedure for epidemiological studies, and can be applied in future studies to trace the potential dissemination of particular strains in the rabbit production line, from selection flocks to the final step of rabbit-fattening farms.

ACKNOWLEDGMENTS

We thank A. Milon for providing several rabbit *E. coli* strains including RDEC-1 strain and J. F. Humbert for his help in RAPD assay. This work was supported in part by a grant from the Région Centre, France. S.L.S. is the recipient of a 'Région Centre/INRA' fellowship.

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