GENETIC STUDIES OF VIRULENCE FACTORS OF AVIAN *Escherichia coli* STRAINS

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ABSTRACT

Ten pathogenic ***E. coli*** *strains were examined for plasmid types and pathogenicity. Some strains were found to produce colicin and to have drug resistance determinants on a single conjugative plasmid while others harbored apart Col V and R plasmids. These plasmids were derepressed with a transfer frequency of around* 10^{-6} *and* 10^{-1} *and did not induce serum resistance or virulence in the recipient cells of* ***E. coli*** K_{12} *and of normal flora* ***E. coli*** *strains of chickens. It was the verified that, in the strains studied, the serum resistance and the determinants for virulence are coled by cromossomal genes or have their expression dependant on chromossomal genes.*

KEY-WORDS: *Virulence factors; Avian* ***Escherichia coli****; Plasmids; Conjugation.*

INTRODUCTION

Escherichia coli is an important cause of diarrhoea and generalized infections in man and livestock (Binns et al., 1979). The two major determinants of virulence in enterotoxigenic **E. coli**, production of enteroxin and formation of colonization pili, are usually carried on plasmids (Smith & Halls, 1968; Orskov & Orskov, 1966).

Smith (1974, 1976) found that plasmids could also enhance the virulence of invasive strains responsible for generalized infections. Most of the invasive **E. coli** isolated from calves, lambs and chicks have a plasmid coding for the synthesis of colicin V, an antibacterial protein (Col plasmid).

Investigations on correlations between colicinogenicity and virulence have been made by identification of several Col V-plasmid-associated characteristics which may be implied in pathogenicity. For example, p Col V, I-K_{94} has been known to encode a genetic determinant for serum resistance (Binns et al., 1979). Several other Col V plasmids, but not p Col V, I-K_{94}, can determine systems involved in iron sequestration (Stuart et al., 1980; Williams, 1979). The p Col V-B_{188} confers on the host cells enhanced adhesion to mouse intestinal epithelium **in vitro** (Clancy & Savage, 1981). Although colicin V has been reported to serve as a virulence factor (Ozane et al., 1977), neither colicin V nor immunity to colicin V was found to be responsible for plasmid mediated - virulence enhancement (Quackenbush and Falkow, 1979; Milch et al., 1984; Vidotto et al., 1991).

The Col V plasmids increase bacterial resistance to the bactericidal action of serum complement. However, some R plasmids are also responsible for serum resistance (Moll et al., 1980; Reynard & Beck, 1976; Reynard et al., 1978).

How bacteria harboring R plasmids become resistant to complement is unclear (Taylor, 1983) but one mechanism of resistance has been determined by Moll et al. (1980). They report that the plasmid R_{6-5} resistance to complement is mediated by the **tra** T protein (tra Tp). This protein has previously been identified as an outer – membrane protein responsible for preventing unproductive conjugation between bacteria carrying these plasmids. Studies with Col V plasmids also showed that the serum resistance could be associated with a structure located on the bacterial cell that may either prevent binding of the membrane attack complex of the complement cascade or inhibit its action (Binns et al., 1979; Nilius & Savage, 1984). Although the serum resistance encoded by Col V plasmids does not appear to be related to a gene of the transfer system itself, there seems to be a complex relationship between the serum resistance and conjugation functions (Nilius & Savage, 1984).

In our laboratory, we verified that **E. coli** strains isolated from chickens with colisepticemia exhibited serum resistance, colicin production, drug-resistance and plasmids of high molecular weight (Vidotto et al., 1990).

The purpose of our work was to study different types of avian **E. coli** strains plasmids, such as plasmids that encode serum resistance, colicin production and resistance to antimicrobial drugs, and to determine their correlation with pathogenicity.

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TABLE I – CHARACTERISTICS OF BACTERIAL STRAINS USED IN THIS STUDY

E. coli Strains	Serotype	Genetic characteristics: colicin production	serum resistance	drug resistance
UEL 1	050: H5	+ (Col V,I)	+	Su
3	0X12: H14	+ (Col V,E_1)	I[c]	Su Tc Sm Ap
9	050: H9	+ (Col V)	+	Su Sm
10	050: H5	+ (Col V)	+	Su Tc Sm Cm
13	050: H⁻	+ (Col V,I)	+	Tc Km
14	045: H⁻	+ (Col V,I)	+	Su Tc
17	078: ND[a]	+ (Col V,I)	+	Su Tc Sm Cm Ap
24	050: H9	+ (Col V,I)	+	Su
27	036: Hund[b]	+ (Col V,I)	+	Su Tc
28	0und: H28	+ (Col und)	+	Su Tc Sm Cm Ku
K12.711F⁻	Rough	–	–	Nal
Normal Flora (FN)	0und	–	+	–

Su (sulfadiazine); Tc (tetracycline); Sm (streptomycin); Ap (ampicillin); Cm (chloramphenicol); Km (kanamycin); Nal (nalidixic acid)
a. ND, not done
b. und, undetermined
c. I, intermediate

MATERIALS AND METHODS

Bacterial strains

The characteristics of bacterial strains used in this work are listed in Table I. The 10 pathogenic **E. coli** strains used in this study were isolated in Londrina, Brazil, during 1980-1983. They came from chickens affected with colibacillosis, possess virulence factors and most are resistant to several antimicrobiol drugs (Vidotto et al., 1990).

Colicin production

To determine the capacity of whole cells to produce colicin, single colonies were transfered to tryptone plates with tooth-picks. After incubation at 37ºC for 12 to 18h, cells were killed with cloroform vapors, and the plates were overlaid with 3ml of soft tryptone agar (0.7% agar), containing 1ml of an overnight culture of strain MA335 diluted 10^{-1}. Colicin producing colonies showed a clear killing zone surrounding the cell colony. The **E. coli** K_{12} MA335 sensitive to all colicin was used as an indicator and the **E. coli** strains 20R675 (Col E1), 22R81 (Col I), 22R82 (Col Ia), 22R83 (Col Ib) and 22R 915 (Col V) were used to type the colicin.

Serum resistance assays

Blood was obtained from five healthy rabbits (N-RS), allowed to clot at room temperature for 30 min., kept for 2h at 4ºC, centrifuged at 4ºC, pooled and stored in aliquots at –20ºC before use.

The determination of bacterial resistance to the lethal activity of serum was determined by a rapid turbidimetric assay in microtitration plates (Pelkonen and Fine, 1987, with some modifications). Overnight bacterial cultures were diluted 1:100 in fresh Luria broth (LB) and incubated with shaking at 37ºC for 90 min. Cultures were cooled to 4ºC, centrifuged (2,500g, 15 min.) and resuspended in cold phosphate-buffered saline pH 7.4 (2 X 10^7 bacteria/ml) and kept on ice until use. The cell suspension (175ul) was pipetted into wells of a microtiter plate followed by addition of 100ul normal serum (N-RS) (36% final concentration). Plates were briefly shaken and incubated at 37ºC.

Absorbance at 620nm (A 620) of the samples was measured at 0, 30, 60 and 90 min. (Titertek Multiskan Spectophotometer, model 340). The instrument was set to zero absorbance with the blanks in the first row in order to eliminate the background absorbance of the serum. The sensitivity to N-RS was measured in triplicate.

Conjugation experiments

Matings in liquid medium were carried out by adding 1.2 ml of exponential phase donor and 0.4ml of recipient cells and incubating the mixtures for 2h at 37ºC without shaking. Transconjugants resistant to drugs were selected on LB agar containing the drugs to which the donor strains were resistant or on minimal medium A of Davis and Mingioli containing sulfa. Both kinds of plates had nalidixic acid (Nal). The transfer of colicin production was tested with 50-100 colonies grown on the selective plates containing Mac Conkey agar plus Nal. The retransfer of plasmids was performed with normal flora **E. coli** as the recipient strain.

The transfer frequency for a given antibiotic resistance determinant was expressed as the number of resistant transconjugants divided by the number of recipient cells.

Plasmid-curing experiments

Plasmid-curing was performed by the orange-acridine method (Watanabe & Fukasawa, 1961), and by the sodium dodecyl sulphate and high

temperature incubation method (Jungmann & Ferreira, 1987).

Isolation and eletrophoresis of plasmid DNA

Preparation of large plasmids was carried out according to the method of Kado and Liu, 1981. Plasmid DNA was subjected to electrophoresis in vertical 0.8% agarose gel with Tris-borate (Meyers et al., 1976).

Pathogenic tests

The pathogenicity of strains and transconjugants was evaluated in one-day-old susceptible broiler chickens. Five birds per isolate were inoculated subcutaneously with 0.5ml of nutrient broth culture containing 1.0 X 10^8 colony-forming units (CFU) of **E. coli**. Birds were maintaned for seven days after inoculation and monitored daily for mortality.

RESULTS

Plasmid content of the E. coli studied

The ten **E. coli** harbored plasmids of diverse molecular weights. Table II shows that the number of plasmid in each strain varied from one to three with molecular weights ranging from 110 to 12Md.

TABLE II – PLASMID CONTENT OF *E. coli* STRAINS STUDIED

Strains	Plasmid contents (Md)
1	pMV1 (78)
3	pMV3a (90) pMV3b (41) pMV3c (12)
9	pMV9a (110)
10	pMV10a (90) pMV10b (66)
13	pMV13a (110) pMV13b (78)
14	pMV14 (110)
17	pMV17a (80) pMV17b (58)
24	pMV24 (90)
27	pMV27a (90) pMV27b (58)
28	pMV28a (100) pMV28b (90) pMV28c (58)

Transfer of genetic markers by conjugation

From the 10 strains used in direct-mating experiments with **E. coli** K_{12} 711, after selection for antibiotic resistance markers, four transferred drug resistance markers. These plasmids showed high transfer frequency (10^{-6} to 10^{-1}) (Table III). In three strains, the colicin production was found to be transferred along with drug resistance. The UEL 17 transferred Ap Sm Col V,I; UEL 27, Tc Col V,I and UEL 28, Tc Sm Col V,I (Table III and Fig. 1B).

Table IV shows conjugation data after 24 hours of mating using the colicin test on 50-100 colonies grown on selective plates containing only Nal. The strains UEL 1, 3, 10 and 13 transferred colicin production. The transconjugants R^+ and Col V^+ were retransferred to normal flora **E. coli** strain. All transconjugants were used for agarose gel electrophoresis (Fig. 1A, 1B).

TABLE III – TRANSFER OF DRUG RESISTANCE MARKERS AFTER CONJUGATION OF PATHOGENIC *E. coli* WITH *E. coli* 711 AS THE RECIPIENT STRAIN

Donor UEL	Selective plate	Transfer frequency[a]	Contransferred markers[b]
13	Nal Tc	3.0×10^{-6}	Tc Km
17	Nal Tc Nal Ap	1.1×10^{-3} 3.8×10^{-2}	Tc Su Sm Cm Ap Sm Col V, I
27	Nal Tc	1.2×10^{-3}	Tc Col V,I
28	Na Tc	1.0×10^{-1}	Tc Sm Col V

a. Two hour mating
b. Ten randomly chosen colonies were checked for each marker.

TABLE IV – TRANSFER OF COLICIN MARKER FOR CONJUGATION OF PATHOGENIC *E. coli* WITH *E. coli* 711

Donor UEL	Selective plate	Nº of colonies tested[a]	Col
1	Nal	50	5 / Col V, I
3	Nal	50	8 / Col V,E1
9	Nal	100	0
10	Nal	50	2
13	Nal	50	2 / Col V, I
14	Nal	100	0
24	Nal	100	0

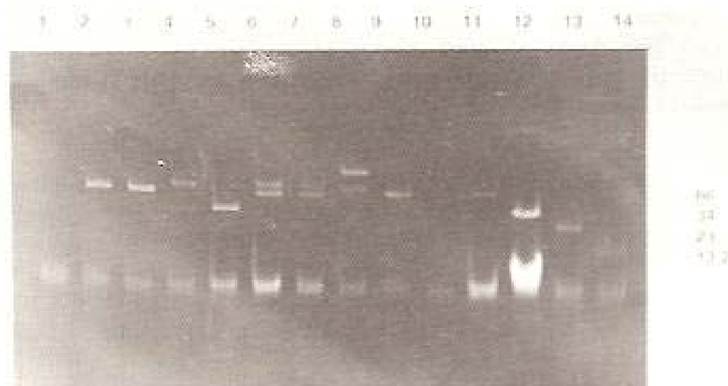

FIGURE 1 – Electrophoresis of plasmid DNA of pathogenic *E. coli* strains.
A) 1- *E. coli* 711; 2- UEL 1; 3- transconjugant 711 (pMV1); 4- UEL 3; 5- transconjugant 711 (pMV3b); 6- UEL 10; 7- transconjugant 711 (pMV10a,b); 8- UEL 13; 9- transconjugant 711 (pMV13b); 10- UEL 13 plasmid-cured; 11, 12, 13 and 14-molecular weight markers (Md)

B) 1-*E. coli* 711; 2- UEL 17; 3- transconjugant 711 (pMV17a,b); 4- transconjugant 711 (pMV17b); 5- UEL 27; 6- transconjugant 711 (pMV27b); 7- UEL 28; 8- transconjugant 711 (pMV28c); 9; 10; 11 and 12- molecular weight markers (Md)

Plasmid-curing experiments

Among five strains (UEL 13, 14, 17, 27 and 28) utilized in plasmid-curing experiments, only UEL 13 was cured. The plasmid-cured-derivatives lost antibiotic resistance markers and colicin production, but did not lose the serum resistance and virulence for one-day-chickens. Gel electrophoresis (Fig. 1) of these derivatives confirmed the involvement of the plasmids only with the expression of antibiotic resistance and colicin production.

Serum resistance

The transfer of serum resistance was studied by testing the R^+ and Col V^+ transconjugants. After screening with normal rabbit serum (N-RS), all the transconjugants were sensitive to the lytic action of serum. The transconjugants of **E. coli** k_{12} 711 carrying the 110 and 78Md plasmids of **E. coli** UEL 13 showed a lytic pattern similar to plasmid-free cells and were sensitive to serum (Fig. 2). The plasmid-cured derivatives of the **E. coli** strain UEL 13 also were analysed for expression of resistance to N-RS and showed a mass increase a little less developed than the behavior detected with the original wild strain (Fig. 2). Fig. 2 includes turbidimetric data of the normal flora **E. coli** strain in assays demonstrating resistance to serum.

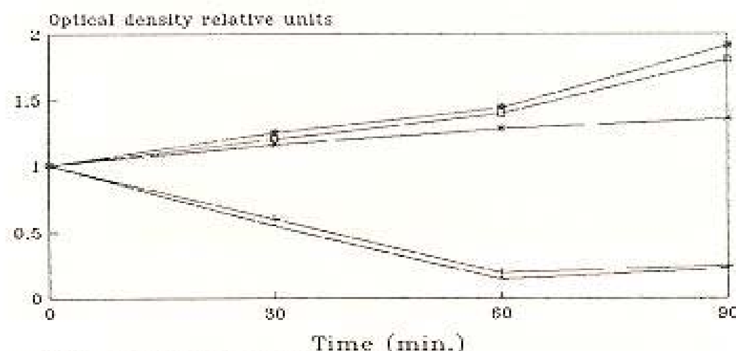

Fig.2 – Serum resistance of *E. coli* strains
— E. coli K12 711 —+— 711 (pMV13 a,b) —*— UEL13
□ UEL13 Plasmid-cured * E. coli FN

Pathogenicity tests

The transconjugants R^+, Col V^+ and R^+ / Col V^+ obtained in the mating experiments with **E. coli** K_{12} 711 and **E. coli** F.N. strains were inoculated in groups of five one-day-chiks. None of them expressed virulence.

The plasmid-cured derivate of the pathogenic **E. coli** strains UEL 13 was also examined for pathogenicity and proved pathogenic to one-day-old chiks.

DISCUSSION

A high proportion of **E. coli** strains responsible for generalized infections in livestock showed multiple resistance to drugs, colicin V production and the virulence factors such as serum resistance and aerobactin production (Vidotto et al., 1990).

In this study we verified the kind of plasmids contained in avian **E. coli** strains and their correlation to pathogenicity. Of the 10 strains studied, the determinant for colicin (V, I and E1) was found on transmissible plasmids in seven strains. Three of these strains (UEL 17, 27 and 28) showed the colicin production and drug resistance determinants on a single plasmid as indicated by the results of the mating experiments and by the appearance of a common single band in agarose gels (Fig. 1). These results showed that Col plasmid often carries genes for drug resistance or coexists in strains that harbor the R plasmid. Besides, the Col and R plasmids are transmited to other strains by high frequency, around 10^{-6} a 10^{-1}. Hence, the high frequency of colicinogenic strains among the pathogenic **E. coli** may be due to the selective pressure of antimicrobial drugs.

A high percentage of colicinogenic strains are also resistant to serum, a characteristic that is associated with certain Col V plasmids (Timmis et al., 1981) and with some R plasmids (Fietta et al., 1977; Reynard & Beck, 1976; Reynard et al., 1978; Abul-Mihl et al., 1987; Moll et al., 1980). These plasmids increase the ability of **E. coli** to escape from the bactericial action of complement (Moll et al., 1980; Ogata & Levine, 1980). The plasmid-mediated ability to resist bactericidal activity may reside in the **iss** determinant present in some Col V plasmids (Binns et al., 1979, 1982), in the Col V-specified outer membrane proteins (Moores & Rowbury, 1981) or in proteins analogous to **tra** T gene product of conjugative R plasmids (Mool et al., 1980; Ogata & Levine, 1980).

In this study, however, the transconjugants Col V^+ and R^+ did not express serum resistance. This could be due to a lack of expression in the host strain since the level of serum resistance is associated with the O antigens of **E. coli** lipopolysaccharides. Rough **E. coli** strains of the K-12 type, that lack O-specific polysaccharides due to a mutation at the **rfb** locus (Orskov et al., 1977), are highly sensitive to the bactericidal activity of serum (Mool et al., 1979; Timmis et al., 1981). Nevertheless, the **E. coli** K-12 strains were used as hosts in order to determine serum resistance (Nilius & Savage, 1984). The plasmid-cured derivatives of the pathogenic **E. coli** strains UEL 13 also remained

resistant to serum (Fig. 2). These observations demonstrated that the resistance to the lytic action of serum can also be coded by chromosomal genes. Similar results were found in pathogenic **E. coli** strains (EPEC) isolated in northeast Brazil (Brennand et al., 1989).

The transconjugants R^+, Col^+ obtained from the recipient strains K12 711 and normal flora **E. coli** were inoculated into one-day-old chicks for pathogenicity tests. None expressed virulence. This suggests that the determinants for virulence may be on chromosomal genes or on plasmids, although not expressed in the host strains. Binns et al., 1979, cloned the determinant for serum resistance of Col V, I-K94 plasmid and verified that this clone increases the virulence of **E. coli**, causing an approximately 100-fold reduction in LD_{50} for chicks. But, these authors utilized a recently isolated **E. coli** strain, serotype 078:K80, which was more suitable for testing the effects of plasmids on the pathogenicity of **E. coli** for experimental animals. Also the fragment cloned was in pBR_{322}, which is a multicopy plasmid (Bolivar et al., 1977) and produces a high level of serum resistance.

The **E. coli** isolated from normal feces of chicken, used as recipient strain, plasmidless and apathogenic, showed serum resistance. This fact suggests that serum resistance is not the only virulence factor.

A high percentage (65%) of enteric flora from healthy people showed serum resistance (Vidotto, unpublished data), and this seems to bem significant in urinary tract infections and generalized infections in man.

Similary, a high percentage of normal flora **E. coli** strains of chickens are serum resistance (unpublished data) and the serum-resistance was correlated to virulence of avian **E. coli** (Vidotto et al., 1990). Therefore, other virulence factors, such as the aerobactin production and cellular invasion, must be included and are under investigation.

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RESUMO

Dez amostras de **E. coli** *patogênicas para aves foram analisadas quanto a associação entre os plasmídios R e Col e a patogenicidade. Foram encontradas amostras com determinantes de resistência a drogas e produção de colicina em um único plasmídio conjugativo e amostras que transportavam plasmídios Col V e R. Estes plasmídios (R e/ou Col) eram desreprimidos, apresentando altas freqüências de transferência (10^{-1} a 10^{-6}) e não determinavam resistência ao soro, nem virulência em amostras receptoras de* **E. coli** *K_{12} e de flora normal de galinha. Assim, foi verificado que nas amostras estudadas, a resistência ao soro não é codificada por plasmídios e que os determinantes para virulência podem estar em genes cromossomais ou dependem de genes cromossomais para sua expressão.*

PALAVRAS-CHAVE:* Escherichia coli; Fatores de virulência; Plasmídios; Conjugação.*

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PREVALÊNCIA DE ANTICORPOS ANTI-*Toxoplasma gondii* EM CÃES ATENDIDOS NO HOSPITAL VETERINÁRIO DA UEL-PR.

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FREIRE, R.L. et al. Prevalência de anticorpos anti-*Toxoplasma gondii* em cães atendidos no Hospital Veterinário da UEL - Pr. *Semina:* **Ci. Agr., Londrina, v. 13, n. 1, p. 66-69, mar. 1992.**

RESUMO

No período de 1985 a 1990 foram realizados 254 exames de Imunofluorescência Indireta (RIFI-IgG) em cães com sinais clínicos compatíveis com a toxoplasmose. Verificou-se que 75,98% dos animais reagiram ao ***Toxoplasma gondii****, com título variando entre 16 e 65.536. Quanto à faixa etária, a prevalência foi de: até 1 ano 67,02%; 1 a 7 anos 80,45%; acima de 7 anos 85,18%. Estes resultados evidenciaram que a infecção está amplamente disseminada em nosso meio, sendo no entanto pouco diagnosticada quando avaliada apenas por exames clínicos.*

PALAVRAS-CHAVE: *Toxoplasmose,* ***Toxoplasma gondii****, Cães.*

INTRODUÇÃO

A toxoplasmose é uma zoonose de distribuição mundial, causada pelo **Toxoplasma gondii**. Trata-se da parasitose mais frequente no homem e talvez nos outros animais homeotérmicos, porém, com baixa morbidade e mortalidade (APT et al, 1973).

Devido a sua importância em Saúde Pública como

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