

COLICIN PRODUCTION AND SERUM RESISTANCE IN PATHOGENIC *Escherichia coli* STRAINS ISOLATED FROM HUMANS IN NORTH EAST BRAZIL

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ABSTRACT

Six pathogenic *Escherichia coli* strains isolated in Recife (Brazil) were analyzed for the presence of virulence-associated factors. All strains were resistant to the human serum complement lytic effect, carried antibiotic-resistant markers and harbored plasmids of diverse molecular weights. One isolate, *E. coli* 3116 strain, was proficient in the synthesis of colicin V and hemolysin. The *E. coli* 3116 strain contained a 128 MD plasmid which coded for colicin V, haemolysin production, and resistance to antibiotics, as could be demonstrated by conjugation and plasmid curing experiments. Eletrophoretic analysis of transconjugants and plasmid-cured derivatives of the 3116 strain showed that serum resistance was not a plasmid coded trait. The distinct genetic basis of colicin V production and serum resistance is discussed.

INTRODUCTION

Escherichia coli is one of the most important causes of intestinal and extra intestinal infections in man and livestock. Pathogenic *E. coli* usually harbor plasmids conferring specific virulence properties which render the bacterial cell able to face mammalian defense mechanisms. Production of enterotoxins, hemolysins, colicins, adherence and colonization factors, expression of iron uptake systems and resistance to the bactericidal action of serum, are some virulence-associated factors determined by

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plasmids in pathogenic *E. coli* (Smith, 1974; Cooke and Ewins, 1975; Evans *et al.*, 1975; Minshew *et al.*, 1978; Scaletsky *et al.*, 1983; Silveira *et al.*, 1987).

The bacteriolytic activity mediated by activated serum complement is an important mammalian defense mechanism. On the other hand, resistance to the lytic action of serum is frequently found in pathogenic *E. coli* (Taylor, 1983). Some authors reported the association between serum resistance and colicin V coding (Col V) plasmids (Binns *et al.*, 1979). Indeed, several reports have indicated the involvement of Col V plasmids with increased invasiveness and pathogenicity (Smith, 1974; Smith and Higgins, 1976, 1980; Davies *et al.*, 1981). Col V plasmids have also been found associated with improved adhesion to intestinal epithelium and expression of iron uptake systems (Williams and Warner, 1980; Clang and Savage, 1981).

In this work, pathogenic *E. coli* strains isolated from hospitalized patients in Recife, in North Eastern Brazil, were analysed for expression of serum resistance, colicin production and presence of plasmids. All six strains tested were resistant to normal human serum and all contained one or more plasmids. In one strain, a 128 MD plasmid encoded Col V.

MATERIALS AND METHODS

Bacterial strains

The source and characteristics of bacterial strains used in this work are listed in Table I. Six pathogenic *E. coli* (EPEC), serogroups O111 and O125 were isolated and kindly donated by the Bacteriology Division of the Fundação de Saúde Amauri de Medeiros (FUSAM).

Growth conditions

All strains were grown on YT medium at 37°C shaking unless otherwise stated. Hemolysin production was tested in strains grown on YT plates containing 5% of defibrinated sheep blood at 37°C.

Determination of drug resistance and colicin production

Antibiotic resistance was determined by the agar diffusion method with commercial antibiotic paper discs (Bauer *et al.*, 1966). Minimum inhibitory concentrations (MIC) were estimated by plating diluted stationary phase cells in YT plates containing serial dilutions of each antibiotic with concentrations ranging from 1000 µg/ml to 25 µg/ml. The drugs tested were: ampicillin (Ap); chloramphenicol (Cm);

Table I - List and source of *E. coli* strains used in this study.

Strain no.	Characteristics	Year of isolation	Source/Origin
1980	Wild isolate (faeces)	1988	FUSAM
2571	Wild isolate (faeces)	1988	FUSAM
3116	Wild isolate (faeces)	1988	FUSAM
5919	Wild isolate (faeces)	1988	FUSAM
1075	Wild isolate (faeces)	1988	FUSAM
1947	Wild isolate (urine)	1988	FUSAM
J53	F-Nal ^R	—	Our laboratory
BZB2101	pCo1A - CA31	—	Pugsley, 1985
BZB2102	pCo1B - K260	—	Pugsley, 1985
BZB2103	pCo1D - CA23	—	Pugsley, 1985
BZB2104	pCo1E1 - K53	—	Pugsley, 1985
BZB2125	pCo1E2 - P9	—	Pugsley, 1985
BZB2107	pCo1E4 - CT9	—	Pugsley, 1985
BZB210	pCo1E7 - K317	—	Pugsley, 1985
BZB2115	PCo1 Ib- P9drd	—	Pugsley, 1985
BZB2116	pCo1K - K235	—	Pugsley, 1985
PAP-1	pCHAP ₂ - p(Co1 M)	—	Pugsley, 1985
BZB2123	pCo1N - 284	—	Pugsley, 1985
PAP2	pCHAP 2 (Co1S4)	—	Pugsley, 1985
PAP222	pCo1V - K270	—	Pugsley, 1985
BZB1011	Col ^S	—	NCTC-PHL
22R80	Col ^S	—	NCTC-PHL
22R915	Col V ^R Col B ^R	—	NCTC-PHL
39R861	Plasmid reference strain	—	NCTC-PHL

Col^S, colicin sensitive; Col^R, colicin resistant.

National Collection of Type Cultures, Central Public Health Laboratory, London, England.

streptomycin (Sm); kanamycin (Km); tetracycline (Tc); gentamycin (Gm); nalidixic acid (Nal); trimethoprim (Tm) and rifampicin (Rif).

Colicin production was assayed as described by Ozeki *et al.* (1962). Briefly, colonies were transferred to YT plates with toothpicks and incubated at 37°C for 18 h. Cells were killed by chloroform vapors and the plates were overlaid with 3 ml of YT soft agar (0.7% agar) containing 0.1 ml of an overnight culture of strain BZB 1011 or 22R80. Colicin-producing colonies were further typed with specific colicin-indicator strains listed in Table I.

Conjugation and plasmid-curing experiments

Plasmid transfer was carried out in conjugation experiments with mating mixtures of exponential phase donor and recipient cells (1:10). After incubation at 37°C for 3 or 24 hours without shaking, conjugation mixtures were plated on antibiotic-containing selective plates. Cotransference of unselected markers were checked with 10, randomly chosen, transformant colonies from each selective plate after purification in the same medium.

Plasmid-curing by the sodium dodecyl sulphate and high temperature incubation method was carried out as previously described (Jungmann and Ferreira, 1987).

Serum resistance assays

Serum resistance in *E. coli* strains was determined by a rapid turbidimetric assay in microtitration plates (Pelkonen and Finne, 1987). Overnight bacterial cultures were diluted 1:10 in fresh YT medium and incubated at 37°C for 90 min with shaking. Cultures were cooled down (4°C), centrifuged (2500 x g, 15 min) and resuspended in cold phosphate-buffered saline pH 7.4 and kept on ice until use. The cell suspension (175 µl) was pipetted into wells of a microtiter plate followed by addition of 100 µl freshly collected normal human serum (NHS) (36% final concentration). Plates were briefly shaken and incubated at 37°C. Absorbance of the samples was measured in a BIO-RAD EIA-spectrophotometer model 2550 after 0,30, 60 and 90 minutes at 630 nm. Screening of serum resistant strains was usually performed after 150 minutes incubation with NHS.

Purification and electrophoresis of plasmid DNA

Plasmid DNA were isolated according to the method of Casse *et al.* (1979). DNA electrophoresis was carried out in horizontal 5 mm thick, 0.8% agarose gels with Tris-acetate running buffer at 60 V during 4-6 hours.

Other methods

Serogroup determinations were kindly performed by Dr. E. Hoffer (Departamento de Bacteriologia, Fundação Oswaldo Cruz, Rio de Janeiro).

RESULTS

Genetic characterization of pathogenic E. coli strains

Six pathogenic *E. coli* strains were isolated from diarrheic patients and from a urinary tract infection at FUSAM, Recife, during the month of May, 1988. A preliminary screening with NHS showed that all strains were resistant to the lytic action of serum complement (Table II).

Table II - Genetic characteristics of the pathogenic *E. coli* strains studied.

Strain	Serum resistance	Colicin production	Hemolysin production	Antibiogram
1980	+	-	-	Ap Cm Sm Tm Km Tc
1947	+	-	-	Ap Cm Sm Tm Tc
2571	+	-	-	Ap Cm Sm Tm Km Tc
3116	+	+ (Col V)	+	Ap Cm Sm Tm Tc
5919	+	-	-	Ap Cm Sm Tm Km
1075	+	-	-	Ap Cm Sm Tm Km Tc

Colicin production was evaluated with the indicator strains 22R80 and BZB 1011 and only one isolate was positive. Further analysis with specific colicin indicator strains indicated that the *E. coli* strain 3116 was a colicin V producer since it was resistant to colicin produced by the PAP222 (Col V) strain. Additional confirmation of the colicin produced by the 3116 strain was carried out with the colicin resistant indicator strain 20R915 (Table II).

Hemolysin production was also found only in strain 3116. All six strains were resistant to several antibiotics (Ap, Cm, Sm, Tm, Km, Tc) at high levels (Table II).

Plasmid content of the E. coli strains analysed

The six *E. coli* strains harbored plasmids of diverse molecular weights. Table III shows that the number of plasmid types in each strain varied from one to five with molecular weight ranging from 0,84 to 128 MD. A unique 128 MD plasmid was found in the colicin V producing strain 3116. This strain was selected for further analysis to investigate the potential correlation of serum resistance and colicin V production with the presence of its plasmid.

Table III - Plasmid content of the *E. coli* strains analysed.

Strains	Plasmid contents (M.D.)
1980	30,00
	0,84
5919	85,0
	52,0
	28,0
	5,0
	1,0
2571	120,0
	70,0
3116	128,0
1947	120,0
	110,0
	80,0
	4,6
	2,8
1075	52,0

Plasmidial traits of the E. coli 3116 strain

Identification of genetic characteristics determined by the 128 MD plasmid in strain 3116 was carried out by conjugation experiments with the *E. coli* strain J53 as recipient. Table IV shows conjugation data after 24 hours of mating using tetracycline resistance as the selective marker. Transconjugants were analysed for the presence of unselected genetic characteristics. All clones investigated received the same antibiotic resistance pattern from the donor strain as well as hemolysin and colicin V production ability. Plasmid-curing experiments based on the SDS-high temperature incubation method (Jungmann and Ferreira, 1987) further demonstrated the linkage between antibiotic resistance markers, hemolysin and colicin production (data not shown). Gel electrophoresis of transconjugants and plasmid-cured derivatives of the *E. coli* 3116 strain confirmed the involvement of the 128 MD plasmid with the expression of antibiotic resistance, hemolysin and colicin V production (Figure 1).

Table IV - Transfer of genetic markers after conjugation of *E. coli* 3116 and *E. coli* K12 J53.

Parental strains	Selective plate	Transfer frequency ^a	Cotransferred unselected markers ^b
<i>E. coli</i> 3116 (donor)			
x	Nal Tc	5×10^{-4}	Ap Cm Sm Tm Hyl ⁺ Col V ⁺
<i>E. coli</i> K12 J53 (recipient)			

Hyl⁺ - Hemolysin production.

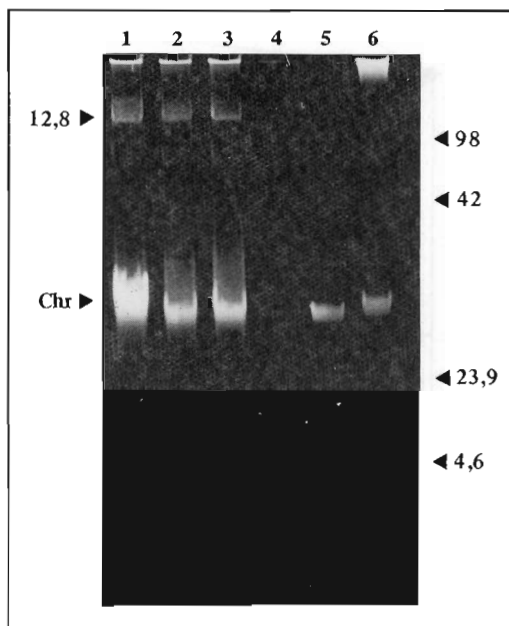
a - Twenty four hour mating.

Conjugation efficiency was calculated by the ratio:

$$\frac{\text{Titer of transconjugants}}{\text{Titer of donors}}$$

b - Ten randomly chosen colonies were checked for each marker.

Figure 1 - Gel electrophoresis of plasmid DNA of *E. coli* 3116 and derivatives. 1 - *E. coli* 3116; 2 - Transconjugant, clone 1; 3 - Transconjugant, clone 2; 4 - *E. coli* 3116 plasmid-cured, clone 1; 5 - *E. coli* 3116 plasmid-cured, clone 2; 6 - *E. coli* strain 39R861. Molecular weight markers are indicated on the right side. The 98 MD marker, used for estimation of the plasmid molecular weight is indicated, though it was too faint to photograph.



Serum resistance in the E. coli 3116 strain

Transconjugants and plasmid-cured derivatives of the *E. coli* 3116 strain were analysed for expression of resistance to NHS. Figure 2 shows turbidimetric data of two

plasmid-cured derivatives and two transconjugants of the 3116 strain in serum resistance assays. The mass increase patterns of the two plasmid-cured clones were a little less developed than the behavior detected with the original wild strain after a 90 min incubation with NHS. On the other hand, two clones of the *E. coli* K12 J53 carrying the 128 MD showed a lytic pattern quite similar to plasmid-free cells (Figure 2). These results indicate that serum resistance in strain 3116 is not coded by the Col V coding plasmid.

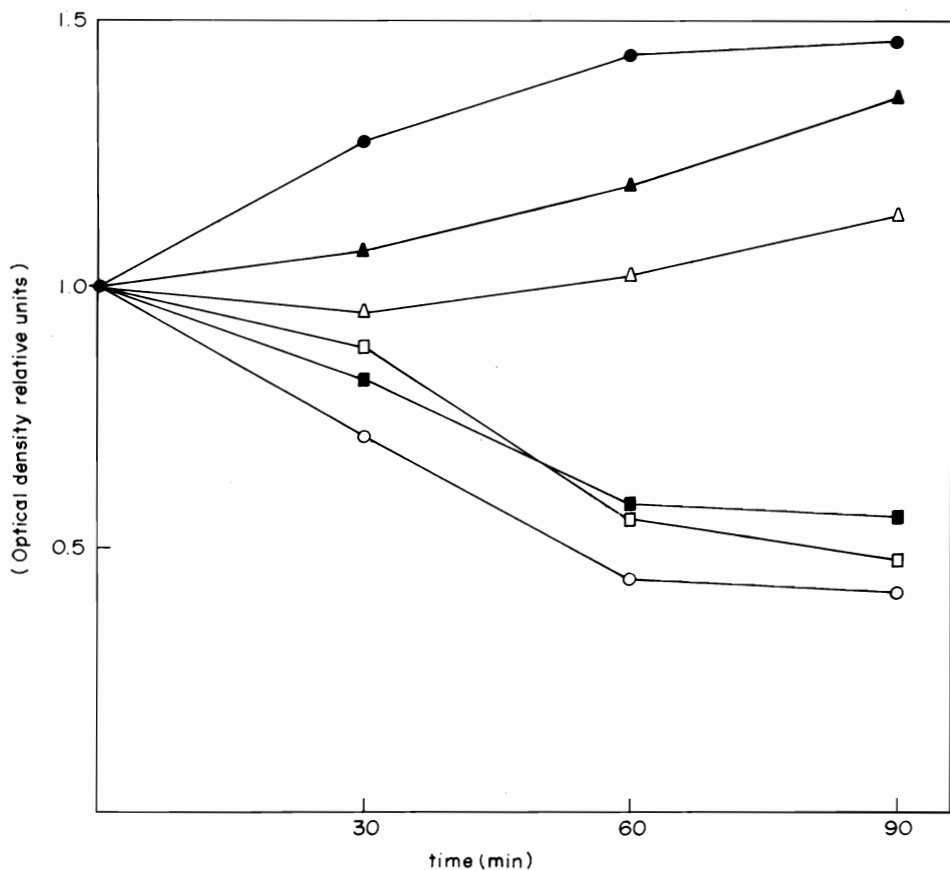


Figure 2 - Serum resistance of *E. coli* 3116 and derivatives. Turbidimetric time course experiments were carried out as described in Materials and Methods. The symbols represent: (●) *E. coli* 3116; (○) *E. coli* K12 J53; (■) Transconjugant clone 1; (□) Transconjugant clone 2; (▲) Plasmid-cured clone 1; (△) Plasmid-cured clone 2.

DISCUSSION

The complement system plays an important role as a mammalian defense mechanism by promoting the inflammatory response through the generation of chemotactic factors and anaphylatoxins, mediating opsonization and phagocytosis, and direct killing of susceptible Gram-negative bacteria (Taylor, 1983). The high incidence of serum-resistance in Gram-negative organisms causing diverse infections in human and other animals, as upper urinary tract infections and bacteremias, further emphasizes the relevance of the bactericidal action of the serum complement (Vosti and Randall, 1970; Gower *et al.*, 1972; Hughes *et al.*, 1982). Serum resistance in *E. coli* isolates was frequently ascribed to nonchromosomal genes as in the cases of the R6-5, R100, and Col V I-K94 plasmids (Binns *et al.*, 1979; Ogata and Levine, 1980).

In this work serum resistance was observed in all *E. coli* strains isolated from patients with intestinal infections or with urinary tract infection. Even though, the number of strains analysed was reduced and all of them belonged to only two serogroups of EPEC, the expression of serum resistance in bacteria isolated from an intestinal environment is unusual, since these cells are usually not exposed to the complement activity of serum. The possibility that the EPEC isolates express serum resistance due to a clonal dispersal of an invasive pathogen was rejected by the appearance of diverse plasmid contents. A possible selective advantage of serum resistant bacteria in alimentary tract infection should await future analysis of a larger number of isolates.

Serum resistance in Gram-negative bacteria has been correlated to alterations in the lipopolysaccharide moiety or with the presence of specific outer membrane proteins, both features coded by plasmid (Moll *et al.*, 1980; Joiner, 1985; Hackett *et al.*, 1987). However, the analysis of transconjugants and plasmid-cured derivatives of the pathogenic *E. coli* strain 3116 clearly disclosed the non-plasmidial nature of serum resistance in this strain. These observations and the distinct plasmid content of each serum-resistant strain demonstrated that resistance to the lytic action of human serum complement can also be coded by chromosomal genes. At present, no evidence is available for the involvement of outer membrane proteins or lipopolysaccharides on the serum-resistance of the strains analysed in this work. Studies on the molecular mechanisms of serum resistance in these strain could contribute to a better knowledge of chromosomal and plasmid-coded serum resistance processes and are under investigation.

Production of colicin V was considered an important virulence factor in *E. coli* by some authors (Smith, 1974). Additional studies clearly demonstrated that other characteristics determined by Col V plasmids, such as serum resistance and adhesion to mouse intestinal epithelium, were associated with enhanced virulence in *E. coli* but not with colicin V production itself (Binns *et al.*, 1979; Quackenbush and

Falkow, 1979; Clancy and Savage, 1981). The lack of involvement between the Col V plasmid and serum-resistance in the strain 3116 offers additional evidence that both characteristics are not strictly linked in nature. Based on these results, the use of colicin V production as an indication for the presence of virulence determinants in *E. coli* should be carefully considered. The isolation of the strain 3116 from an intestinal infection further emphasizes such concern.

The ubiquitous location of serum resistance genes on plasmids and chromosomes suggests a possible transposable genetic nature. The location of serum resistance genes in transposons would have a considerable relevance on the understanding of the dispersal pattern of this characteristic in pathogenic *E. coli* and other bacterial pathogens and deserve special interest.

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RESUMO

Seis linhagens de *Escherichia coli* patogênicas, isoladas no Recife (Brazil), foram analisadas quanto à presença de fatores associados à virulência. Todas as linhagens foram resistentes à ação lítica do complemento no soro humano, carregavam marcas de resistência a antibióticos e albergavam plasmídeos de pesos moleculares diversos. Um isolado, a linhagem *E. coli* 3116, mostrou-se capaz de sintetizar colicina V e hemolisina. A linhagem *E. coli* 3116 continha um plasmídeo de 128 MD que codificava a produção de colicina V e hemolisina além de resistência a antibióticos, como pode ser demonstrado por experimentos de conjugação e cura do plasmídeo. Análises eletroforéticas de transconjugantes e derivados curados da linhagem 3116 mostraram que a resistência ao soro não era uma característica codificada pelo plasmídeo. A origem genética distinta da produção de colicina V e a resistência ao soro são discutidas.

REFERENCES

- Bauer, A.W., Kirby, W.M., Sherris, J.C. and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disc method. *Amer. J. Clin. Path.* 45: 493-496.
- Binns, M.M., Davies, D.L. and Hardy, K.G. (1979). Cloned fragments of the plasmid Col V, I-K94 specifying virulence and serum resistance. *Nature* 279: 778-781.
- Casse, F., Boucher, C., Julliot, J.S., Michel, M. and Denairé, J. (1979). Identification and characterization of large plasmids in *Rhizobium melilot* using agarose gel electrophoresis. *J. Gen. Microbiol.* 113: 229-242.
- Clancy, J. and Savage, D.C. (1981). Another colicin V phenotype adhesion *in vitro* of *Escherichia coli* to mouse intestinal epithelium. *Infect. Immun.* 32: 343-352.

- Cooke, E.M. and Ewins, S.P. (1975). Properties of strains of *Escherichia coli* isolated from a variety of sources. *J. Med. Microbiol.* 8: 107-111.
- Davies, D.L., Falkiner, F.R. and Hardy, K.G. (1981). Colicin V production by clinical isolates of *Escherichia coli*. *Infect. Immun.* 31: 574-579.
- Evans, D.G., Silver, R.P., Evans Jr., D.J., Chase, D.G. and Gorbach, S.L. (1975). Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. *Infect. Immun.* 12: 656-667.
- Hackett, J., Wyk, P., Reeves, P. and Mathan, V. (1987). Mediation of serum resistance in *Salmonella typhimurium* by an 11-kilodalton polypeptide encoded by the cryptic plasmid. *J. Infect. Dis.* 155: 540-549.
- Hughes, C., Phillips, R. and Roberts, A.P. (1982). Serum resistance among *Escherichia coli* strains causing urinary tract infection in relation to O type and the carriage of hemolysin, colicin and antibiotic determinants. *Infect. Immun.* 35: 270-275.
- Joiner, K.A. (1985). Studies on the mechanisms of bacterial resistance to complement-mediated killing and on the mechanism of action of bactericidal antibody. *Curr. Top. Microbiol. Immunol.* 121: 99-133.
- Gower, P.E., Taylor, P.W., Koutsaimanis, K.J. and Roberts, A.P. (1972). Serum bactericidal activity in patients with upper and lower urinary tract infections. *Clinical Sci.* 43: 13-22.
- Jungmann, D.M. and Ferreira, L.C.S. (1987). Plasmid curing in *Escherichia coli* and *Salmonella typhimurium* by treatment with sodium dodecyl sulphate and high temperature incubation. *Rev. Microbiol.* 18: 178-183.
- Minshew, B.H., Jorgensen, J., Swanstrum, H., Grootes, G.A., Reevecamp, and Falkow, S. (1978). Some characteristics of *Escherichia coli* strains isolated from extraintestinal infections of humans. *J. Infect. Diseases* 137: 648-654.
- Moll, A., Manning, P.A. and Timmis, K.N. (1980). Plasmid-determined resistance to serum bacterial activity: a major outer membrane protein, the *trt* gene product is responsible for plasmid-specified serum resistance in *Escherichia coli*. *Infect. Immun.* 28: 359-367.
- Ogata, R.T. and Levine, R.P. (1980). Characterization of complement resistance in *Escherichia coli* conferred by the antibiotic resistance plasmid R 100. *J. Immunol.* 125: 1494-1498.
- Ozeki, H., Stocker, B.A.D. and Smith, S. (1962). Transmission of colicinogeny between strains of *Salmonella typhimurium* grown together. *J. Gen. Microbiol.* 28: 671-687.
- Pelkonen, S. and Finne, J. (1987). A rapid turbidimetric assay for the study of serum sensitivity of *Escherichia coli*. *TEMS Microbiol. Lett.* 42: 53-57.
- Pugsley, A.P. (1985). *Escherichia coli* K12 strains for use in the identification and characterization of colicins. *J. Gen. Microbiol.* 131: 369-376.
- Quackenbush, R.L. and Falkow, S. (1979). Relationship between colicin V activity and virulence in *E. coli*. *Infection Immunity* 24: 562-564.
- Scaletsky, I.C.A., Silva, M.L.M., Reis, M.H.L., Affonso, M.H.T. and Trabulsi, L.R. (1983). Ent plasmid, col plasmids and resistance in *Escherichia coli* strains of human origin. *Braz. J. Genetics* 6: 1-14.

- Silveira, W.D., Yano, T., Azevedo, J.L. and Castro, A.F.P. (1987). Plasmid-mediated production of a new colonization factor (F42) in enterotoxigenic *Escherichia coli*. *Braz. J. Genet.* 10: 635-646.
- Smith, H.W. (1974). A search for transmissible pathogenic characteristics in invasive strains of *Escherichia coli*: the discovery of a plasmid-controlled toxin and a plasmid-controlled lethal character closely associated or identical, with colicine V. *J. Gen. Microbiol.* 83: 95-111.
- Smith, H.W. and Huggins, M.B. (1976). Further observations on the association of the colicine V plasmid of *Escherichia coli* with pathogenicity and with survival in the alimentary tract. *J. Gen. Microbiol.* 92: 335-350.
- Smith, H.W. and Huggins, M.B. (1980). The association of the O18, H1 and H7 antigens and the Col V plasmid of a strain of *Escherichia coli* with its virulence and immunogenicity. *J. Gen. Microbiol.* 121: 387-400.
- Taylor, P.W. (1983). Bactericidal and bacteriolytic activity of serum against gram-negative bacteria. *Microbiol. Reviews* 47: 53-83.
- Vosti, K.L. and Randall, E. (1970). Sensitivity of serologically classified strains of *Escherichia coli* of human origin to serum bactericidal system. *Am. J. Med. Sci.* 259: 114-119.
- Williams, P. and Warner, P.J. (1980). Col V plasmid-mediated colicin V-independent iron uptake system of invasive *E. coli*. *Infect. Immun.* 26: 925-932.

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