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Haemagglutinating and Adhesive Properties Associated with the K99 Antigen of Bovine Strains of *Escherichia coli*

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SUMMARY

The K99 antigen common to some bovine strains of *Escherichia coli* caused mannose-resistant haemagglutination of sheep erythrocytes and was shown to be responsible for the attachment of K99-positive bacteria to calf brush-border preparations because (i) strains grown at 18 °C did not produce K99 antigen, cause haemagglutination, or attach to brush borders; (ii) a K12 ($K99^+$) recombinant strain showed both haemagglutinating activity and attachment to brush borders whereas, before it received the K99 plasmid, the recipient strain was negative in both respects; and (iii) cell-free extracts of K99 antigen showed haemagglutinating activity and inhibited the attachment of K99-positive organisms to brush borders.

K99 antigen appears to be a virulence determinant in the pathogenesis of neonatal calf diarrhoea. It is readily demonstrated by haemagglutination and brush-border attachment tests.

INTRODUCTION

Most strains of *Escherichia coli* isolated from calves with diarrhoea (Smith & Halls, 1967) possess a common K antigen (Smith & Linggood, 1972) which has been designated K99 (Ørskov *et al.*, 1975). This antigen is transmissible and appears to be an important virulence determinant, probably because it enables K99-positive organisms to proliferate in the small intestine (Smith & Linggood, 1972).

The rapid proliferation of K88-positive strains of *E. coli* in the small intestine of pigs with neonatal diarrhoea (Smith & Jones, 1963) has been attributed to the ability of these organisms to attach to the intestinal epithelium (Arbuckle, 1970; Drees & Waxler, 1970*a*, *b*; Bertschinger, Moon & Whipp, 1972). Furthermore the K88 antigen has been shown to be responsible for the attachment both *in vivo* (Jones & Rutter, 1972) and *in vitro* (Jones, 1972; Jones & Rutter, 1972; Wilson & Hohmann, 1974; Sellwood *et al.*, 1975).

It seems reasonable to assume that K99 antigen may function in calves in a similar manner to K88 antigen in pigs. The haemagglutinating properties of K88 have been documented by Stirm *et al.* (1967*a*), Jones (1972) and Jones & Rutter (1974). Their results have indicated the usefulness of this test system in reflecting the adhesive properties of K88 antigen.

The object of this study was to investigate the haemagglutinating activity and morphology of K99 antigen and assess its significance in the attachment of K99-positive strains of *E. coli* to brush borders prepared from the epithelial cells of calf small intestine.

METHODS

Strains of E. coli. Seven K99-positive enteropathogenic calf strains, characterized by Ørskov *et al.* (1975), and a K12 strain, kindly supplied by Dr H. Williams Smith, Houghton Poultry Research Station, Huntingdon, were used. The seven K99-positive strains were B41 [O101:K99], B42 [O9:K35,K99], B44 [O9:K30,K99], B85 [O9:K99], B79 [O101:K32, K99], B111 [O101:K99], and B117 [O8:K85,K99].

The bacteria were grown on tryptose glucose yeast extract agar (TGXA; Schlechts & Westphal, 1966) modified to include the trace elements solution as used in Davis & Mingioli's minimal media (Cruickshank, 1965). Cultures were incubated at either 37 °C or 18 °C.

Preparation of antiserum. OK antisera against the seven K99-positive strains and against a K12 K99-positive recombinant strain were raised by giving rabbits a series of intravenous injections containing approximately 10^8 live organisms/ml (Sojka, 1965). O antisera were also prepared against O9, O8 and O101 antigens using boiled cultures of strains B44, B117 and B41.

Serological and haemagglutination tests. O and K agglutination tests and immunodiffusion tests were performed as described by Jones & Rutter (1972). For immunodiffusion tests, bacterial suspensions were frozen and thawed three times. Microhaemagglutination tests were done as described by Jones & Rutter (1974). Whole blood from guinea pigs, cows, fowl, goats and pigs was collected in sodium citrate (3·8 %, w/v, in distilled water). Washed packed human O cells (kindly supplied by the Blood Transfusion Centre, Oxford) and defibrinated sheep blood (Wellcome Reagents, Beckenham, Kent) were also used.

Electron microscopy. Bacteria were prepared for electon microscopic examination using the method of Stirm *et al.* (1967*a*). Specimen grids were prepared by a modification of the method of Gregory & Pirie (1973). The grids were metal shadowed in an AEI metrovac coating unit using gold–palladium alloy at an angle of 29°, and were examined in a Philips EM 300 electron microscope.

Cell-free K99 antigen. K99 antigen was extracted from K12 ($K99^+$) and B41 strains as described by Stirm *et al.* (1967*b*) for K88 antigen, except that the precipitate was collected at pH 4·5. The K99 antigen preparations were dissolved in 0·15 M-NaCl in phosphate buffer pH 7·0 (containing 0·03 M-Na_2HPO_4 and 0·02 M-KH_2PO_4) and stored at −20 °C.

Transfer of K99 plasmid. The prospective recipient strain of *E. coli* was a nalidixic-acid-resistant mutant of a K12 strain. The donor strain, B41 [O101:K99], was nalidixic-acid-sensitive.

The K99 plasmid was transferred by inoculating 0·02 ml of nutrient broth culture of K12 grown overnight and 0·02 ml of a similar culture of B41 into 100 ml of nutrient broth, and incubating at 37 °C for 18 h. This mated mixture was plated out on TGXA medium containing nalidixic acid (100 μg ml^{-1}); and colonies were picked and examined for the presence of the K99 antigen by slide agglutination tests. The sugar fermentation reactions of the K99-positive nalidixic-acid-resistant organisms were compared with those of both the donor and recipient strains to check that the recombinant was a K12 ($K99^+$) recombinant strain and not a nalidixic-acid-resistant mutant of strain B41.

Brush-border adhesion test. Brush borders were prepared from the small intestine of calves (1 to 2 days old), and the adhesion test was performed as described by Sellwood *et al.* (1975) except that the brush borders and the *E. coli* were finally washed and resuspended in phosphate buffer pH 7·0. Bacterial suspensions contained approximately 10^8 organisms/ml.

Inhibition of adhesion of K99-positive bacteria. A suspension (0·1 ml) of brush borders was centrifuged in a small conical tube and the supernatant fluid was discarded. K99 antigen

Table 1. *The inhibition of haemagglutinating activity of K99-positive strains of E. coli by selected antisera*

Dilutions of antisera (25 μl) were incubated with equal volumes of either 4 to 8 haemagglutinating units (1 haemagglutinating unit is the highest dilution of test material which caused complete haemagglutination) of bacterial suspension or cell-free K99 antigen at 37 °C for 2 h, then cooled on ice and 25 ml of a 3% (w/v) suspension of red-blood cells was added. Haemagglutination inhibition (HI) titres were the highest dilutions of serum that inhibited haemagglutination after 1 h at 0 to 4 °C. Bacterial agglutinin titres were recorded after bacterial suspensions had been incubated at 4 °C overnight with 0·15 M-NaCl, in place of red cells, and were expressed as the highest dilutions of serum causing bacterial agglutination. Titres are recorded as the reciprocal dilutions of antisera.

	B41 OK antiserum		K12 (K99) OK antiserum	
Strain	Agglutinin titre	HI titre	Agglutinin titre	HI titre
B41	1280	1280	5120	280
B42	1280	80	5120	26
B44	320	320	2560	24
B85	640	80	5120	140
B79	160	1280	1280	66
BIII	10	10	10	20
BII7	10	10	10	80
K12 ($K99^+$)	160	80	960	124
Cell-free K12 ($K99^+$)	NT	160	NT	133

NT, Not tested.

(0·1 ml) or K99 antiserum (0·1 ml) was added and the brush borders were resuspended and incubated at room temperature for 30 min. A suspension (0·1 ml) of *E. coli* strain B41 was then added, and the mixture was transferred to a small screw-capped vial (1 × 3 cm) and incubated at room temperature for 30 min on a rotary shaker operating at 30 rev. min^{-1}. The number of bacteria attached to 20 brush borders was counted using phase-contrast microscopy. In control tests, phosphate buffer was substituted for K99 antigen. Inhibition of bacterial adhesion is expressed as the difference between the number of bacteria attached to brush borders in the control and the number attached in the test, as a percentage of the control.

RESULTS

Properties of K99-positive strains grown at 37 °C

Freeze–thawed suspensions of the seven K99-positive strains showed a single identical precipitin line with antisera prepared in rabbits against each of the seven K99-positive strains. This line was still demonstrable after the antisera had been absorbed with homologous boiled cultures but was not present after absorption with live B41 organisms.

The OK antisera prepared against the seven K99-positive strains cross-reacted in K agglutination tests to various degrees. Strains BIII and BII7 gave low titres (< 1/40) with homologous and heterologous antisera.

Bacterial suspensions of the K99-positive strains caused haemagglutination of sheep red-blood cells; red cells from the other species tested either gave inconsistent results or did not show evidence of haemagglutination. The haemagglutination of sheep red-blood cells was not inhibited by the presence of 0·5% D-mannose, and did not completely elute at 37 °C although titres were reduced. Titres against strains BIII and BII7 were consistently lower than those against the other strains tested, and in some tests were less than 1/10, the lowest dilution tested. All samples of sheep cells tested were agglutinated by the K99-positive

strains; a minimum average of between 0·3 and 3 bacteria per red cell was necessary for complete agglutination. Mannose-resistant (m.r.) haemagglutinating activity could be inhibited with antisera prepared against the homologous or heterologous live organisms (Table 1). Haemagglutination was not inhibited by O antisera or by OK antisera previously absorbed with live K99-positive organisms.

All seven strains adhered to brush borders, although fewer organisms of strains B111 and B117 adhered compared with the other strains. At dilutions of K12 (K99+) antiserum of less than 1/10, bacterial agglutination occurred, which might account for the observed inhibition of adhesion of strain B41. At dilutions of antiserum of 1/10 or more, little or no agglutination occurred but adhesion of strain B41 to brush borders was inhibited.

Properties of the K99-positive strains grown at 18 °C

Strains grown at 18 °C do not produce the K99 antigen (Ørskov *et al.*, 1975). Suspensions of such strains showed no evidence of precipitin lines against homologous or heterologous OK antisera. Strains also showed no evidence of K99 antigen in K agglutination tests. Agglutination was observed against homologous OK antisera and OK antisera prepared against strains having the same O antigen: this was attributed to the presence of O agglutinins in the antisera. These strains were also negative in haemagglutination tests and did not adhere to brush borders. When cultures grown at 18 °C were used to absorb homologous antisera, there was no reduction in the haemagglutination inhibition titres of K agglutination titres compared with those of unabsorbed OK antisera.

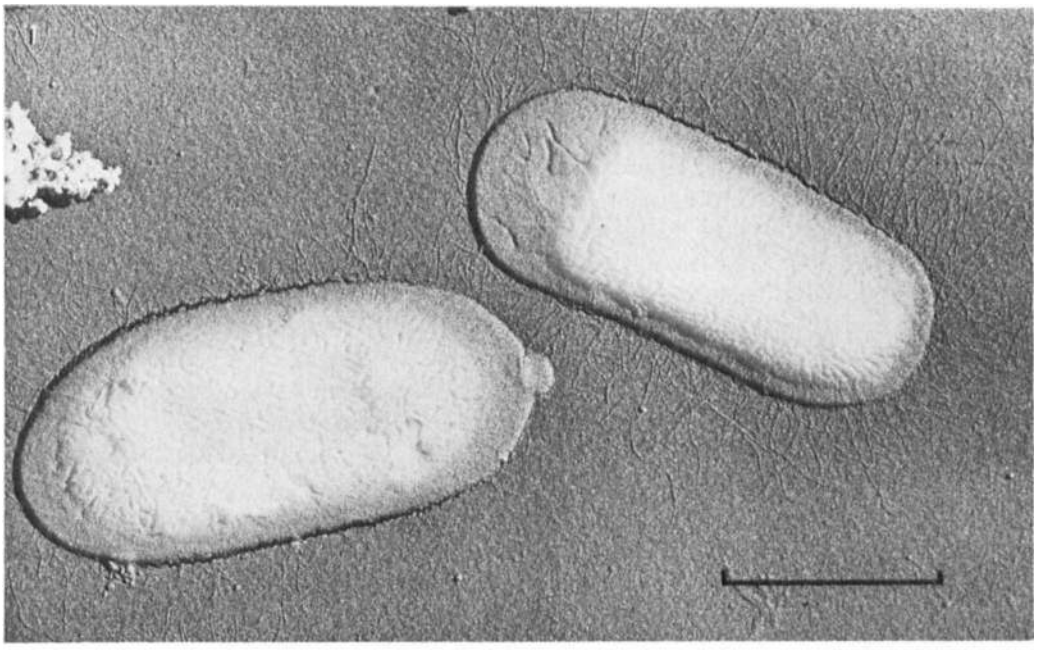
Properties of the K12 (K99+) recombinant strain

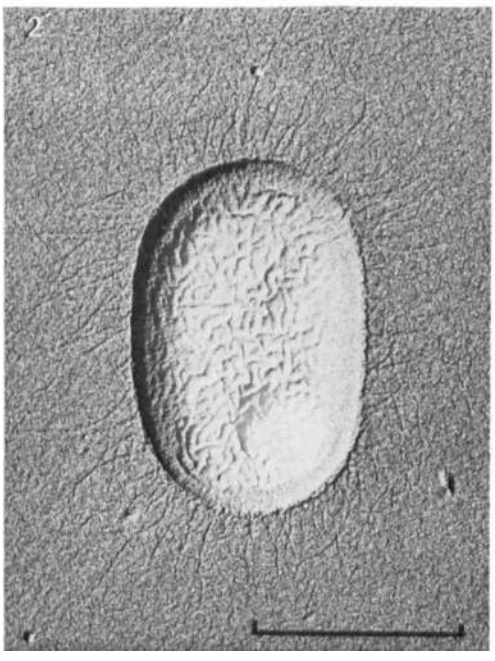
Before recombination, the K12 recipient strain showed no precipitin lines in gel diffusion tests against K99 antiserum and did not show agglutinating, haemagglutinating or adhesive activity. The K12 (K99+) recombinant strain gave a single line in gel diffusion tests that was identical to the line given by the K99+ strains, and agglutinated with OK antisera prepared against the other K99-positive strains. The K12 (K99+) strain also showed m.r. haemagglutinating activity with sheep red-blood cells. This activity could be inhibited with OK antisera prepared against the other K99-positive strains. OK antisera prepared against the recombinant strain also inhibited the haemagglutinating activity of the other K99-positive strains (Table 1). The recombinant strain adhered to calf brush borders, and adhesion could be inhibited with homologous antiserum and OK antiserum prepared against strain B41 at a dilution of 1/10.

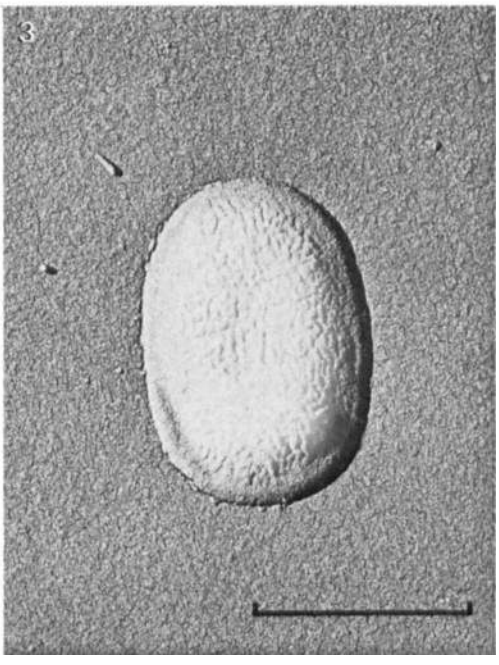
Electron micrographs of the K12 (K99+) strain showed fine branched filamentous projections covering the surface of the bacterial cell (Fig. 1) similar to those seen in electron micrographs of the donor strain B41 (Fig. 2). There was no evidence of such surface structures in the K12 recipient strain (Fig. 3).

Properties of cell-free K99 antigen

Cell-free K99 antigen gave a single identical line in gel diffusion against all the K99-positive antisera. These preparations caused m.r. haemagglutination of sheep red-blood cells, which could be inhibited by OK antisera prepared against the seven K99-positive strains. The m.r. haemagglutinating activity of cell-free K99 antigen eluted completely at room temperature and at 37 °C. Cell-free K99 antigen also caused an 85% inhibition in the adhesion of strain B41 to calf brush-border preparations.







All bar markers represent 1 μm.

Fig. 1. K12 (K99$^+$) recombinant strain, showing fine filamentous surface structures of K99 antigen.

Fig. 2. B41 [O101:K99] donor strain.

Fig. 3. K12 recipient strain, showing no evidence of surface structures.

DISCUSSION

When grown at 37 °C, the seven K99-positive strains of *E. coli* examined all caused m.r. haemagglutination of sheep red-blood cells and attached to brush borders prepared from the small intestine of calves. Two strains, B111 and B117, reacted poorly in K agglutination, haemagglutination and adhesion tests, possibly because they produce less K99 antigen than other strains. The haemagglutinating and adhesive properties were not exhibited by K99-positive strains grown at 18 °C, at which temperature the K99 antigen is not expressed (Ørskov *et al.*, 1975). Transfer of a plasmid specifying the K99 antigen to a K99-negative strain conferred both haemagglutinating and adhesive activity on the recombinant strain. This evidence suggests that the K99 antigen is responsible for both the haemagglutinating and adhesive properties of the K99-positive *E. coli* strains. The inhibition of haemagglutination and adhesion by homologous and heterologous antisera, and the absence of haemagglutination inhibition by O and OK antisera previously absorbed with K99-positive live organisms, support our view that a common factor is responsible for both haemagglutination and adhesion.

Gel-diffusion studies showed that all the K99-positive strains possessed a single common antigen when tested against seven OK antisera. The K12 K99-positive recombinant strain gave an identical precipitin line against the same antisera. These precipitating antigens were absent from K99-positive strains grown at 18 °C, and K99 antibody could not be absorbed from antisera by these bacteria. It was difficult to demonstrate precipitin formation with both cell-free K99 antigen and freeze–thawed bacterial suspensions – as it was with the K88 antigen of pig enteropathogenic *E. coli* (Ørskov *et al.*, 1971). Electron micrographs suggest that the K99 antigen may be present on bacteria as filamentous surface structures, similar to the K88 antigen (Stirm *et al.*, 1967*a*). If so, the large molecular size of the antigen might inhibit its diffusion in agar gels.

The haemagglutinating activity of cell-free K99 antigen and its ability to inhibit adhesion of K99-positive organisms to brush borders suggest that, with live bacteria, this antigen is responsible for m.r. haemagglutination and adhesion to brush borders. However, the m.r. haemagglutinating activity of cell-free K99 antigen always eluted completely at room temperature and at 37 °C whereas that of live K99-positive bacteria was inconsistently eluted at these temperatures. The reason for this difference is not known and is being investigated. K99-positive *E. coli* also adhere to brush borders prepared from the small intestine of pigs (Burrows & Sellwood, unpublished observations) of both K88 adhesive and K88 non-adhesive phenotypes (Sellwood *et al.*, 1975). However, these strains appear to be non-pathogenic for pigs (Smith & Linggood, 1971) since the enterotoxin they produce does not dilate ligated intestinal loops in pigs (Smith & Halls, 1967).

The K99 antigen of calf enteropathogenic strains of *E. coli* resembles the K88 antigen of pig enteropathogenic *E. coli* (Jones & Rutter, 1974) in its ability to adhere to intestinal epithelium, its haemagglutination properties and its filamentous structure. The K99 antigen may function in a similar manner to the K88 antigen by allowing adhesion of *E. coli* to the small intestine *in vivo* (Jones & Rutter, 1972) and thus enabling the organisms to proliferate and colonize the mucosa. The K99 antigen may therefore be regarded as a virulence determinant in the pathogenesis of neonatal calf diarrhoea.

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