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Detection of the K99 Antigen by Means of Agglutination and Immunoelectrophoresis in *Escherichia coli* Isolates from Calves and Its Correlation with Enterotoxigenicity

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The common antigen of calf enterotoxigenic strains of *Escherichia coli*, recently established as the K99 antigen, was studied by means of the slide agglutination test and immunoelectrophoresis. Specific antisera were obtained by absorption of crude antisera with ultrasonicates of autologous cells grown at 18 C or by injection into rabbits of the purified K99 antigen obtained by preparative electrophoresis. The K99 antigen was usually undetectable in calf enterotoxigenic *E. coli* cultures with capsular K antigens of the A variety grown at 37 C on commercially available nutrient agar plates designed for the isolation of *Enterobacteriaceae*, but was rapidly detectable when grown on a buffered semisynthetic medium at pH 7.5 (Minca medium). An alternative procedure for the isolation and identification of calf enterotoxigenic *E. coli* strains from feces, using the Minca medium, is proposed. K99 was found in 70 of 74 strains of *E. coli*, the enterotoxigenicity of which was established in the ligated gut test in calves. None of the 20 cultures negative in the ligated gut test possessed K99 antigen. The K99 antigen is therefore probably a useful diagnositic tool for the identification of calf enterotoxigenic *E. coli* strains, taking into account that K99 and enterotoxigenicity are controlled by different plasmids.

It has been established by means of the ligated gut test (LGT) that pig enteropathogenic strains of *Escherichia coli* produce one or more toxins (15). Many of these enterotoxigenic strains, although belonging to different OK groups, have the K88 antigen in common (10). This antigen was shown to be a protein (19), to have a filamentous, fimbria-like morphology (18), not be be formed at 18 C (10), and to be plasmid controlled (9). The K88 antigen plays an essential role in the pathogenesis of neonatal diarrhea in pigs. It enables the bacteria to attach to the wall of the small intestine, to overcome gut mobility, and to reach large numbers in the small intestine (3). The K88 antigen is generally used as a diagnostic tool for the detection of pig enteropathogenic strains of *E. coli*, although K88 is not carried by all pig enteropathogenic strains (17).

Enteropathogenic *E. coli* strains apparently also cause diarrhea in calves (17). The enterotoxigenicity of a number of strains has been established (6, 15). Serotyping of such strains showed that many of them belonged to O groups O8, O9, and O101 and possessed the thermostable A type of capsular K antigen (17). However, the identification of calf enterotoxigenic strains by means of serotyping is hampered by the fact that strains with the same serotype are often also found in healthy calves (Guinée, unpublished observations). Sojka (personal communication to Smith and Linggood [16]) observed that many calf and lamb enteropathogenic *E. coli* strains possess a closely related or common capsular antigen although having different O and K antigens. Smith and Linggood termed this antigen, for convenience, the "common K antigen" and found that it was plasmid controlled (16). The common K antigen has recently been accepted as a K antigen by the World Health Organization Collaborative Centre for Reference and Research on Escherichia and designated K99 (11). The detection of this common K antigen was found to be difficult, probably because many of the strains possessing the common K antigen grow as mucoid, heavily encapsulated colonies.

Because of its possible significance for the identification of calf enterotoxigenic *E. coli* strains, we undertook to study this antigen. From the onset of our studies, it was taken into account that K99 may have properties similar to those of K88, i.e., that it may be a thermolabile antigen that is not formed at 18 C. The aim of this study was to prepare specific K99 antisera and to establish the correlation between enterotoxigenicity and the presence of K99 antigen. It was expected that a special nutrient

medium might be required for optimal development of K99 antigen.

MATERIALS AND METHODS

Strains. The standard strains of *E. coli* were obtained from F. Ørskov (Collaborative Centre for Reference and Research on Escherichia, World Health Organization, Statens Seruminstitut, Copenhagen, Denmark). W. J. Sojka (Central Veterinary Laboratory, Weybridge, Surrey, U.K.) kindly supplied the following strains: B41, B42, B44, B79, B111, B117, WS9, WS10, and WS11. Strains designated with the prefix H were sent to us for serotyping by several veterinary laboratories. The majority of these strains came from L. Myers (Veterinary Research Laboratory, Montana State University, Bozeman) and I. Bijlsma (Institute of Veterinary Bacteriology, University of Utrecht, Utrecht, The Netherlands). All strains were of bovine origin.

Media. Nutrient broth, nutrient agar, and MacConkey agar were purchased from Difco. D5 medium (14) was used with 0.1% glucose (8). Preliminary studies revealed that the K99 antigen is difficult to detect by means of slide agglutination when the strains supposed to possess K99 are grown on these nutrient media. A medium allowing better detectability of K99 was therefore developed. Two factors were thought to influence the detectability of K99 antigen: (i) abundant K polysaccharide formation might mask the K99 antigen; and (ii) the formation of K99 might be pH dependent. Preliminary experiments indicated that strains with K antigens of the A variety [K(A) antigens] grew less mucoid on a synthetic medium with 0.1% amino acids and 0.1% carbohydrate than on nutrient agar, without losing their agglutinability in OK antisera. Upon further reduction of amino acids, many of the strains failed to grow sufficiently for diagnostic work. By further reduction of the concentration of carbohydrate, the agglutinability of the polysaccharide K antigen was impaired. The pH of the medium was adjusted to several values in the range of 5 to 8 by means of Sörensen 0.067 M phosphate buffer and the detectability of K99 tested in slide agglutination. The detectability was found to be optimal at pH 7.5. The pH of the medium was found to remain above 7.2 during incubation. When the medium with pH 7.5 was enriched by increasing the concentration of amino acids to 0.25% or by addition of 0.1% yeast extract (Difco), strains with K(A) antigens tended to grow more mucoid and their K99 antigen became more difficult to detect in the slide agglutination. The detectability of K99 also considerably decreased when the concentration of carbohydrate was increased from 0.1 to 0.5%. Strains with K(A) antigens did grow more mucoid in some instances, but the pH of the medium always fell below 7.0 during incubation. It was concluded that a constant pH of 7.5 is optimal for the development of K99 and that keeping the amount of nutrients (amino acids and carbohydrate) relatively low prevents the masking of K99 by abundant K polysaccharide formation. The finally adopted composition of the medium that we called Minca medium (minimal casein medium) is as follows: KH_2PO_4 (Merck), 1.36 g; $Na_2HPO_4 \cdot 2H_2O$ (Merck), 10.1 g; glucose (B.D.H.), 1 g; trace salts solution, 1 ml; Casamino Acids (Difco), 1 g; agar (Difco), 12 g; and distilled water, 1,000 ml. The pH is 7.5. The trace salts solution contained, per liter: $MgSO_4 \cdot 7H_2O$ (Merck), 10 g; $MnCl_2 \cdot 4H_2O$ (Merck), 1 g; $FeCl_3 \cdot 6H_2O$ (Merck), 0.135 g; and $CaCl_2 \cdot 2H_2O$ (Merck), 0.4 g.

Serotyping of *E. coli*. The O antigen was determined with a mechanized microtechnique described earlier (2). The technique was further developed for the typing of the K antigen. The growth of a nutrient agar slope, incubated at 37 C for 18 h, was suspended in 9 ml of formalinized (0.5%) NaCl (0.5%) solution containing 0.009% gentian violet (Merck) and tested with all standard OK antisera. By means of a multiple dropper (2), 0.037 ml of each OK antiserum is delivered into the cups in a plastic tray. The antisera used in the multiple dropper are diluted 50% less than those used in the slide agglutination. A drop of antigen is added manually by means of a 5-ml pipette. The tray is shaken on a rotary shaker (150 strokes/min) for 10 min at room temperature and then read on an illuminated screen. Positive or suspected positive reactions must be confirmed with the slide agglutination test using OK antisera since the mechanical procedure gives no information about the type of agglutination (O or K agglutination). Strains not reacting in any of the OK antisera and not reacting in their homologous O antisera by slide agglutination were designated K? or K(A)? if the O antigen could only be detected after heating at 120 C for 2.5 h. Strains reacting in the living state in pure O antiserum were designated K−.

Production of ultrasonicates (US). Cells were grown in petri dishes (15 cm in diameter) containing approximately 60 ml of medium for 18 h at 37 C or for 24 h at 18 C. The cells were suspended in Sörensen 0.067 M phosphate buffer, pH 7.5, to give a concentration of 5×10^{10} cells/ml. Six milliliters of this suspension in a 50-ml coniform borosilicate centrifuge tube (Schott, Mainz, Germany) was ultrasonicated (Braun Sonic, 300 S) with 60% output for 3 min at 0 C. The disrupted bacteria were centrifuged at 20,000 × *g* for 30 min at 4 C. The supernatants of the ultrasonicates (US 37 and US 18) were found to contain 8 to 15 mg of protein per ml as estimated by the method of Lowry et al. (5). When used for absorption, some US were concentrated to approximately 1/10 of their original volume by means of dialysis in a bag against Sephadex G-200 powder.

Production and absorption of K99 antisera. Unless otherwise stated, the sera were prepared by the methods described for the production of OK antisera by Kauffmann (4). Alum-precipitated antigens were prepared according to Proom (12). The removal of nonspecific antibodies was done either by adding 5×10^{10} viable cells to 1 ml of crude antiserum and keeping the absorption mixture at 18 C for 24 h or by mixing equal parts of US and antiserum or concentrated US and antiserum in a 1:10 ratio and keeping the mixture at 4 C for 72 h. The agglutinate or precipitate was removed by centrifugation at 20,000 × *g* for 30 min at 4 C.

Immunoelectrophoresis (IE). The method of Scheidegger (13) as described by Ørskov et al. (8) was used, except that 8 to 10 tests were performed on

one 7 to 20-cm glass plate overlaid with 40 ml of Noble agar (Difco) instead of on 8 to 10 separate glass slides.

Preparative electrophoresis. For preparative purposes, the same agar plates used for IE were used. In the middle of the plate, a strip of gel of about 2-mm width was removed and replaced by a liquid antigen-agar mixture (1:1). In this way, approximately 0.75 ml of US could be separated by means of electrophoresis. To locate the desired antigen after electrophoresis, two IE patterns were punched at the edges of the agar layer, the holes of which were filled with the antigen solution. After electrophoresis, the gel at the cathodic side was divided into six strips, and each strip was taken off separately and frozen at −20 C. The unabsorbed antiserum was added to the two wells, and the plate was incubated at room temperature for 18 h. After reading the IE plate, the strip containing the desired antigen was mixed with 5 ml of complete Freund adjuvant (Difco) and injected into a rabbit via the intramuscular route at weekly intervals two or three times.

Double diffusion (Ouchterlony). Double diffusion was performed on the same agar plates used for IE. The plates were washed for 3 days in running tap water and dried before straining with Coomassie brilliant blue (5 g of dye [Serva] in 900 ml of 50% ethyl alcohol and 100 ml of glacial acetic acid). Destaining was done with the same solution without dye.

Blending and heating of cells. Blending was found to be a useful method for the removal of flagellae from *Salmonella* and *E. coli* (1) and the removal of F pili from *E. coli* (7), and was also successfully used to isolate fimbriae from *Salmonella* (V. Aleksic, personal communication). Suspensions of 5 $\times 10^{10}$ viable cells per ml were blended in a 100-ml beaker in a Sorvall Omnimixer at 8,000 rpm for 10 min at 4 C and centrifuged at 20,000 $\times g$ for 30 min at 4 C. Heating was used for release of K99 antigen as described by Stirm et al. with respect to K88 antigen (19). A suspension of 5×10^{10} viable cells per ml was heated in a water bath at 60 C for 20 min and centrifuged at 20,000 $\times g$ for 30 min at 4 C.

Enterotoxigenicity test. The strains received from Sojka were reported to be enterotoxigenic when tested in the LGT (15). The enterotoxigenicity of the other strains used in this study was established by means of the LGT by L. L. Myers (manuscript in preparation) or by I. Bijlsma (manuscript in preparation).

RESULTS

Evaluation of K99 antisera by means of slide agglutination. Three K99 antisera were prepared with three different strains. The first strain used was WS10 (O8:K85; see also reference 11) because C. Wray (personal communication) reported that it possessed K99 antigen. WS10 antiserum strongly agglutinated strain WS10, but did not agglutinate or only weakly agglutinated the other strains of Sojka reported to possess K99 antigen. The majority of these strains have polysaccharide K antigens of the A type and grow as mucoid, heavily encapsulated colonies on nutrient agar. The more transparent growth, which arises after a few days at room temperature, was weakly agglutinated by WS10 antiserum in a few cases. This observation actuated the development of Minca medium. When grown on Minca medium, all strains received from Sojka were agglutinated in WS10 antiserum. WS10 antiserum absorbed with cells of strain O8 and strain O141:K85 still agglutinated all the strains grown on Minca medium at 37 C. When grown at 18 C, only strains WS9, WS10, WS11, and B117, all having seroformula O8:K85, were agglutinated (Table 1). This may indicate that the O and K antigens of strain WS10 are not entirely identical to those of the standard strains O8 and O141 and that K99 antigen is not formed at 18 C. WS10 antiserum was therefore absorbed with a concentrated US 18 of the autologous strain WS10. This antiserum reacted with none of the cultures grown on Minca medium at 18 C, whereas after growth on Minca medium at 37 C all cultures except WS11 and B117 were agglutinated. This might indicate that strains WS11 and B117 did not possess K99 antigen (Table 1).

Antisera were also prepared with two other strains. Strain H416 (O101:K(A)?:NM) was used because it behaved exceptionally in the IE test (see Results). Strain B41 was used for antiserum production because this strain was recently established as the type strain for K99 (11). Slide agglutination tests with these antisera, absorbed with concentrated US of the autologous strains, yielded results that were similar to those obtained with absorbed WS10 antiserum.

Evaluation of K99 antisera by means of IE. WS10 (O8:K85) antiserum showed many precipitation lines with US of WS10 and also, as expected, with US of the standard strains, O8 and O141:K85. Absorption of WS10 antiserum with cells of the latter two strains removed all lines except three. One of these was found to represent a thermostable O or K antigen that was also found in WS10 US 18. This confirms the earlier assumption that the OK antigens of WS10 are not entirely identical to O8 or O141:K85. Absorption of crude WS10 antiserum with concentrated WS10 US 18 removed all lines except two, representing thermolabile antigens in WS10 US 37. The two lines (lines 1 and 2) were both at the cathodic side. Line 1, nearest to the antigen hole, was strong, whereas line 2 was weak. US of a number of enterotoxigenic strains were tested in IE using absorbed WS10 antiserum (see Results). Strain H416 was the only enterotoxigenic strain tested in IE that did not form line 1, but it formed a

TABLE 1. *Slide agglutination tests with strains grown at 18 and 37 C using crude or absorbed WS10 antiserum*

Strain		Agglutination in WS10 antiserum						
		Crude			Absorbed with cells of O8 and O141:K85		Absorbed with US 18 of WS10	
No.	Serotype	Nutrient agar[a]	Transparent growth on nutrient agar after a few days at room temperature	Minca	Minca	Minca, 18 C	Minca	Minca, 18 C
WS10	O8:K85	+	ND[b]	+	+	+	+	−
WS9	O8:K85	+	ND	+	+	+	+	−
WS11	O8:K85	+	ND	+	+	+	−	−
B117	O8:K85	+	ND	+	+	+	−	−
B42	O9:K(A)35	−	±	+	+	−	+	−
B44	O9:K(A)30	−	±	+	+	−	+	−
B111	O101:K(A)30	−	−	+	+	−	+	−
B79	O101:K(A)32	−	−	+	+	−	+	−
B85	O9:K−	+	ND	+	+	−	+	−
B41	O101:K−	+	ND	+	+	−	+	−
	O141:K85	+	ND	+	−	ND	ND	ND

[a] Medium on which cells were grown. All agglutinations were done with cultures grown at 37 C unless otherwise stated.

[b] ND, Not done.

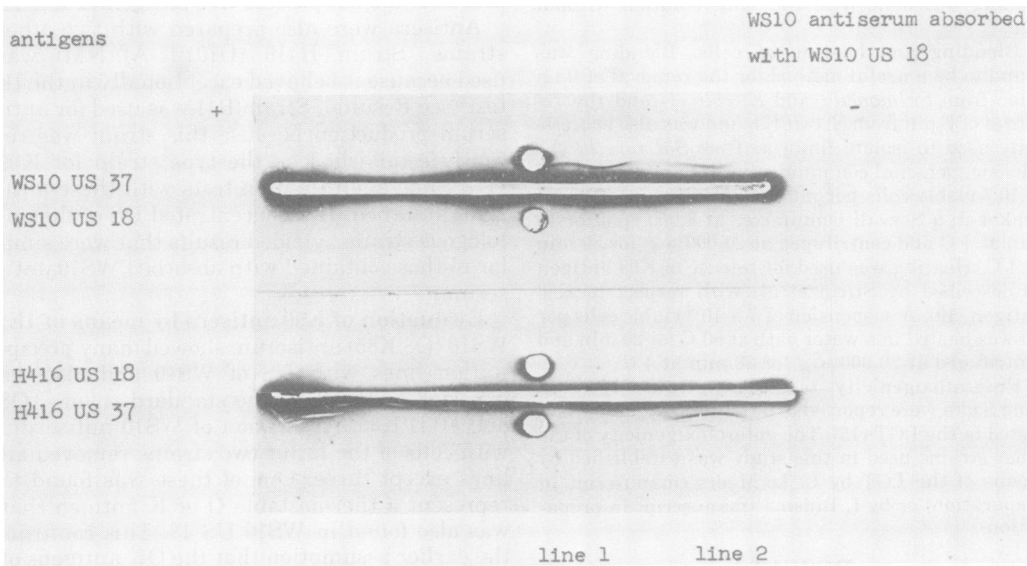

FIG. 1. *Immunoelectrophoretic patterns obtained with ultrasonicates of cells grown on Minca medium at 37 C (US 37) or 18 C (US 18) and WS10 antiserum absorbed with WS10 US 18.*

exceptionally clear line 2 (Fig. 1, bottom). Assuming that line 2 represented the K99 antigen, an antiserum was prepared with strain H416 and absorbed with concentrated H416 US 18. The antiserum prepared with strain B41 as the K99 standard strain (11) was also absorbed with concentrated B41 US 18. The results of IE tests using these three absorbed antisera and US 37 and US 18 of the three strains are summarized in Fig. 2 and Table 2. US 37 of strain WS10 and B41 formed lines 1 and 2 in all three antisera. None of these precipitation lines was formed by any of the US 18 or by US 37 after heating at 100 C.

Isolation of K99 antigen by means of blending and heating. Supernatants of heated or blended suspensions of strains H416 cells gave no precipitation lines when tested in IE against

K99 antisera. Nevertheless, the native supernatants were injected into rabbits intravenously and as alum-precipitated antigens intramuscularly. The immune response was measured in IE using as antigens WS10 US 37 and US 18, H416 US 37 and US 18, and the supernatants used for immunization.

Line 2 against H416 US 37 and lines 1 and 2 against WS10 US 37 were observed with both antisera, in addition to other lines that were also formed using US 18 and that could be removed by means of absorption with US 18. No precipitation was seen with the supernatants used for immunization. This may indicate that the supernatants contained enough K99 antigen to provoke antibodies, but not enough to produce visible precipitation lines in IE.

Preparative electrophoresis of K99 antigen from strain H416. Line 2 was found to be strong and well separated from other precipitation lines when H416 US 37 was tested in IE using unabsorbed H416 antiserum. Therefore, it seemed worthwhile to isolate the K99 antigen from H416 US 37 by means of preparative electrophoresis and to use it for preparation of specific K99 antiserum. After two or three injections at weekly intervals, a good immune response was obtained. One precipitation line (line 2) was observed with US 37 of the autologous strain, and two lines (lines 1 and 2) were

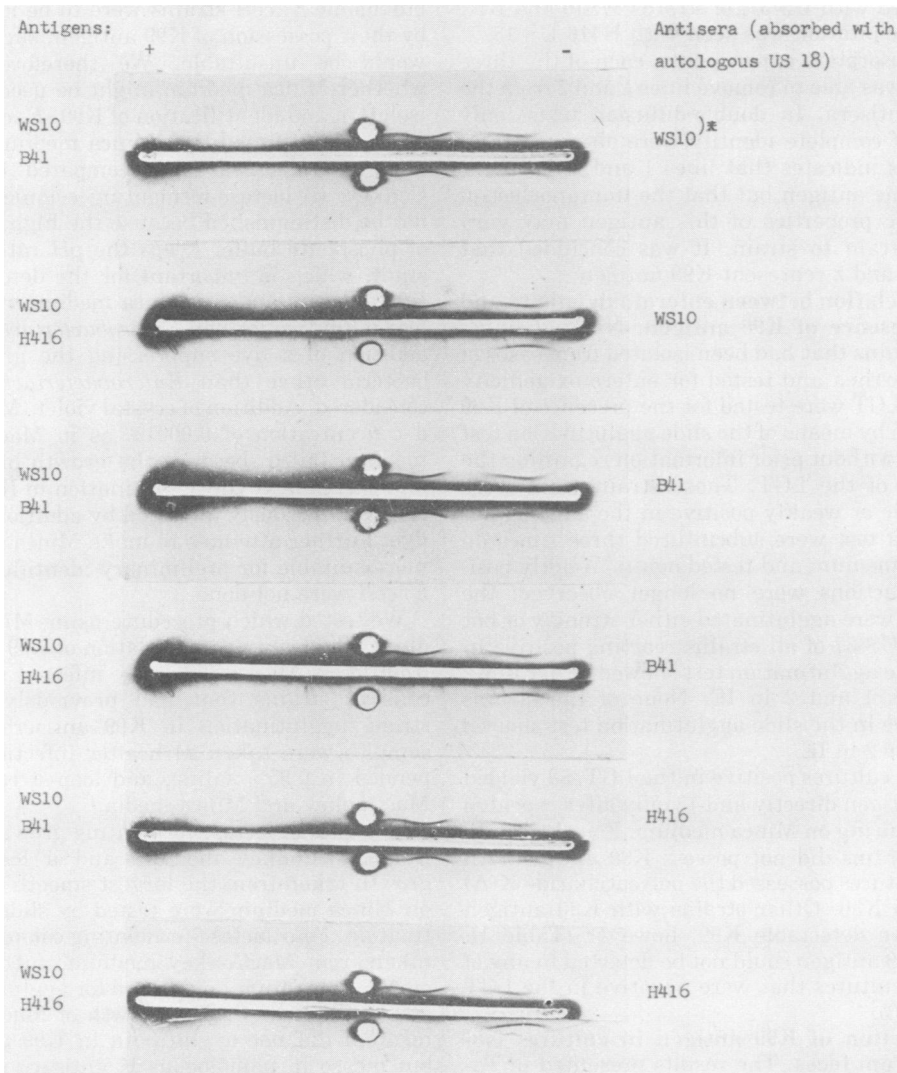

FIG. 2. *Immunoelectrophoretic patterns obtained with US 37 and three antisera absorbed with autologous US 18.*

TABLE 2. *Immunoelectrophoresis with ultrasonicates from strains grown on Minca medium at 37 C (US 37) using absorbed antisera presumed to contain K99 antibodies only*

Antigen[a]	Antisera absorbed with autologous US 18					
	WS10		H416		B41	
	Line 1	Line 2	Line 1	Line 2	Line 1	Line 2
WS10 US 37	+	±	+	±	+	±
H416 US 37	−	++[b]	−	++	−	++
B41 US 37	±	±	±	±	+	+

[a] No precipitation was observed with US 18 or with US 37 heated at 100 C for 30 min.

[b] ++, Precipitation line was very clear.

observed with US 37, of strains WS10 and B41. No precipitation was seen with H416 US 18.

In absorption experiments, each of the three US 37 was able to remove lines 1 and 2 from the four antisera. In double-diffusion tests, only lines of complete identity were observed (Fig. 3). This indicates that lines 1 and 2 represent the same antigen but that the immunoelectrophoretic properties of this antigen may vary from strain to strain. It was concluded that lines 1 and 2 represent K99 antigen.

Correlation between enterotoxigenicity and the presence of K99 antigen. Ninety-four *E. coli* strains that had been isolated from cases of calf diarrhea and tested for enterotoxigenicity in the LGT were tested for the presence of K99 antigen by means of the slide agglutination test and IE without prior information regarding the results of the LGT. Those strains that were negative or weakly positive in the slide agglutination test were subcultured three times on Minca medium and tested again. Weakly positive reactions were no longer observed: the strains were agglutinated either strongly or not at all. US 37 of all strains reacting positive in the slide agglutination test showed either line 2 or lines 1 and 2 in IE. None of the strains negative in the slide agglutination test showed line 1 or 2 in IE.

Of 74 cultures positive in the LGT, 58 yielded K99 antigen directly and 12 only after repeated subculturing on Minca medium. Four LGT-positive strains did not possess K99 antigen. All four cultures possessed the polysaccharide K(A) antigen K30. Other strains with K30 antigen did have detectable K99, however (Table 1). The K99 antigen could not be detected in any of the 20 cultures that were negative in the LGT (Table 3).

Detection of K99 antigen in cultures isolated from feces. The results presented in Table 1 indicate that K99$^+$ cultures of *E. coli* possessing K antigens of the A type are not agglutinated in K99 antisera when grown on nutrient agar. It was therefore expected that the K99 antigen of such strains would also be difficult or impossible to detect by means of slide agglutination when the cultures were grown on nutrient media commonly used for isolation and preliminary identification of *Enterobacteriaceae*. This hypothesis was confirmed by testing the 70 K99$^+$ strains mentioned above for the presence of K99 antigen after they had been cultured on MacConkey medium: none of the cultures carrying K(A) antigens was agglutinated by the K99 antisera.

It is common practice to use MacConkey and similar media for the isolation of enteropathogenic *E. coli* from feces. If, however, calf enterotoxigenic *E. coli* strains were to be identified by their possession of K99 antigen, such media would be unsuitable. We therefore tested whether Minca medium might be used for the isolation and identification of K99$^+$ *E. coli* from feces. We expected that Minca medium would have two disadvantages compared to MacConkey: (i) lactose-fermenting colonies would not be distinguished because the high amount of phosphate buffer keeps the pH rather constant, which is important for the detection of K99 antigen; and (ii) Minca medium would be less inhibitive for non-*Enterobacteriaceae*. The addition of a dye suppressing the growth of bacteria other than *Enterobacteriaceae* was considered. Addition of crystal violet (Merck) at a concentration of 0.0001% as in MacConkey medium failed, because the growth of *E. coli* and particularly the agglutination in K99 antisera was seriously impaired by addition of this dye. Further attempts to make Minca medium more suitable for preliminary identification of *E. coli* were not done.

We tested which procedure using Minca medium would enable the isolation of K99$^+$ *E. coli* from feces. Mice were orally infected with 10^9 cells of strains that had previously shown strong agglutination in K99 antisera. Fecal samples were taken 24 h after infection, suspended in 0.85% saline, and loop-streaked on MacConkey and Minca media.

A loopful of lactose-fermenting growth taken from MacConkey medium and a loopful of growth taken from the largest smooth colonies on Minca medium were tested by slide agglutination. Four lactose-fermenting colonies were taken from MacConkey medium, subcultured on Minca medium, and tested for agglutination after 18 h at 37 C. The growth of MacConkey medium did not agglutinate in K99 antisera but did so in homologous K antiserum. After subculturing on Minca medium, most colonies agglutinated in K99 antiserum. However, only

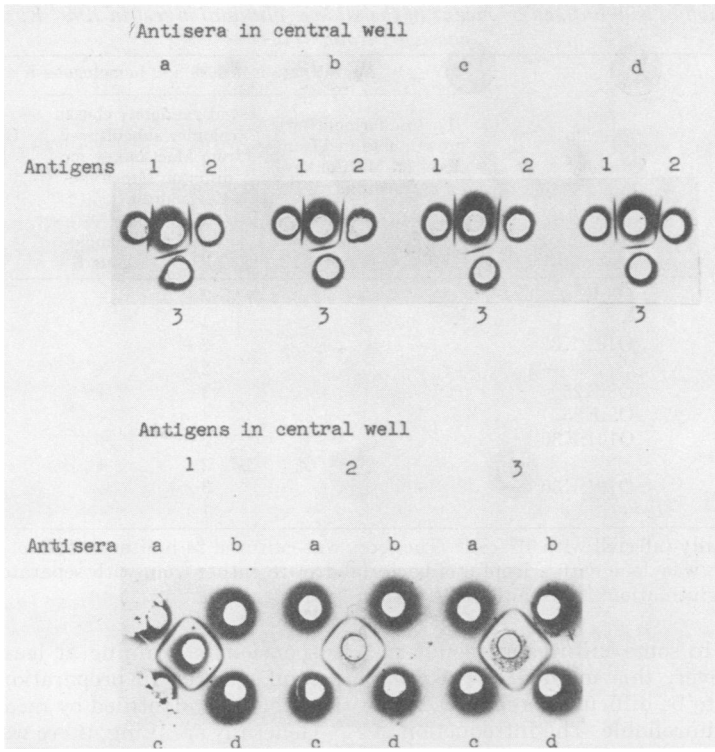

FIG. 3. *Double-diffusion tests with US 37 and absorbed antisera. (a) WS10 antiserum absorbed with WS10 US 18; (b) B41 antiserum absorbed with B41 US 18; (c) H416 antiserum absorbed with H416 US 18; (d) H416 antiserum prepared by means of preparative electrophoresis. 1, WS10 US 37; 2, B41 US 37; 3, H416 US 37.*

TABLE 3. *Correlation between enterotoxigenicity and the presence of K99 antigen after (repeated) culturing on Minca medium*[a]

Entero-toxicity[b]	K99 antigen is detectable on Minca medium:		No. of isolates
	Directly	Only after repeated cul-turing	
+	+		58
	− or ±	+	12
	−	−	4[c]
−	−	−	20

[a] K99 antigen was tested by means of the slide agglutination test and IE.

[b] Determined by means of the ligated gut test using calf intestine.

[c] All four cultures had K(A) antigen K30.

two of four colonies of strain B79 reacted in K99. Apparently, K99 was lost. Many O101:K30 strains agglutinated only weakly after subculturing. Direct plating of the fecal suspension on Minca medium always yielded bacterial growth that reacted strongly in K99 was well as in its homologous K antiserum. A summary of these results is given in Table 4.

DISCUSSION

Most calf enterotoxigenic strains of *E. coli* possess K antigens of the A variety and grow as mucoid, heavily encapsulated colonies on common nutrient media. The detection of K99 antigen was hampered in the initial phase of this study. It was thought that K99 antigen was masked by abundant K polysaccharide formation.

Ørskov et al. reported that the reaction in K99 antiserum was dependent on the K (polysaccharide) antigen and the medium. The K99 antigen was most easily demonstrated in the more transparent colonies, which had the least amount of polysaccharide K antigen (11). Similar observations were made in this study. The K99 antigen could usually not be detected in strains with K(A) antigens grown on nutrient media at 37 C. In some instances, the more transparent growth that arises after further incubation at room temperature was weakly agglutinated in K99 antisera. This does not seem to agree with the observation that K99 is not formed at 18 C. The fact that the room temperature may range from 20 to 25 C offers some explanation for the fact that K99 antigen

TABLE 4. *Detection of K99 antigen by means of the slide agglutination test in K99⁺ E. coli cultures after passage through mice*[a]

Strain	Serotype	Agglutination in K99 and homologous K antisera of:					
		Lactose-fermenting growth obtained from feces on MacConkey medium[b]		4 at randomly chosen colonies subcultured from MacConkey medium on Minca medium		Growth obtained from feces on Minca medium	
		K99	Homologous K	K99	Homologous K	K99	Homologous K
WS10	O8:K85	−[c]	+	3+	+	+	+
				1−	−		
B79	O101:K32	−	+	2+	+	+	+
				2−	+		
H1915	O8:K25	−	+	4+	+	+	+
H1301	O9:K35	−	+	4+	+	+	+
B44	O101:K30	−	+	2+	+	+	+
				2±	+		
H1939	O101:K30	−	+	3±	+	+	+
				1−	+		

[a] Mice were orally infected with 10^{9} cells. The feces was cultured 24 h after infection.
[b] Agglutination was done with a loopful of bacterial growth rather than with separate colonies.
[c] +, Strong agglutination; ±, Weak agglutination; −, no agglutination.

may be formed in some cultures at room temperature. However, this method of detecting K99 was found to be difficult to reproduce and was considered unreliable. The introduction of Minca medium considerably contributed to the detectability of K99 antigen and to the reproducibility of the results. Minca medium contains fewer amino acids and less carbohydrate than common nutrient media and, in contrast to media commonly used for the isolation of *Enterobacteriaceae*, its pH remains nearly constant during incubation. We concluded that the improved detectability of K99 on Minca medium was due to optimal development of K99 at pH 7.5 on one hand and decreased development of K polysaccharide on the other. A positive correlation was found between the agglutination in absorbed antisera and the appearance of one or two precipitation lines in IE. The agglutination test and the IE were negative with US prepared from cultures grown at 18 C, which confirms the observations of Ørskov et al. (11). Ørskov et al. (11) reported that attempts to demonstrate K99 by means of IE failed, whereas our attempts were successful. This is easily explained by the fact that different techniques were used; Ørskov et al. (11) used the techniques of heating and blending for the release of K99. These techniques had previously been used successfully for the release of K88 by Stirm et al. (19). In our experiments this technique did not release enough K99 antigen to give a precipitation line in IE, but released enough to provoke antibodies when injected into rabbits. Only ultrasonication of cell suspensions containing at least 5×10^{10} cells per ml resulted in preparations in which the K99 could be identified by means of IE.

Generally speaking, there was a positive correlation between enterotoxigenicity, as measured in the LGT, and the presence of K99 antigen. The detection of K99 antigen may therefore be a significant diagnostic tool for the identification of enterotoxigenic strains. However, K99 antigen could not be detected by agglutination in strains with K antigens of the A variety when grown on nutrient media such as MacConkey. When suspected colonies primarily isolated from feces on MacConkey medium were subcultured on Minca medium, the K99 antigen could be detected in most cases. But in some instances the K99 antigen could not be detected, as was the case with strain B79 in our experiments, K99 was easily detected when the feces was directly cultured on Minca medium in these experiments. We would therefore recommend using Minca medium for the primary isolation of enterotoxigenic *E. coli* from field cases of enterotoxicosis. Colonies reacting with K99 antigen should then be confirmed biochemically as *E. coli*.

Not only can K99 antigen be masked by K polysaccharide formation, but it is probably also easily lost. This agrees with the observation of Smith and Linggood (16) that K99 antigen is plasmic controlled. The fact that, for instance, strain B117 possesses K99 antigen in the hands of Ørskov et al. (11) and not in our hands may thus be explained in terms of plasmid segregation. Assuming that production of

enterotoxin and K99 are controlled by different plasmids, the possibility should be considered that *E. coli* strains possessing K99 but lacking enterotoxin may occur. It is not known whether non-enterotoxigenic *E. coli* strains with "enteropathogenic serotypes" and possessing the K99 antigen do occur in healthy calves.

K99 antigen resembles K88 antigen in several respects: (i) it is shared by *E. coli* strains belonging to different O groups; (ii) it is not formed at 18 C; and (iii) it is thermolabile. K88 is essential in the pathogenesis of *E. coli* enterotoxicosis in pigs because it mediates adherence of the bacteria to the intestinal wall (3). It is conceivable that K99 antigen plays a similar role in the pathogenesis of *E. coli* enterotoxicosis in calves and lambs.

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