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Short Communication

ELISA to determine anti-K99 pilus antibody in the sera of normal and diarrhoeic calves

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Abstract

An ELISA to detect circulating antibodies against K99 pili, a major attachment factor to intestinal epithelial cells of *Escherichia coli* in calves, was performed. Two methods of K99 pili purification were attempted. Best results in terms of purity of the K99 antigen were achieved following the method described by Karkhanis and Bhogal (1986). This procedure included a heat shock at 65°C during 25 min to release the pili and ultracentrifugation steps to purify the antigen. SDS-PAGE showed an 18 KDa major band, identified as the K99 pilus antigen after immunoblotting against reference antisera. The purified K99 antigen was then adsorbed to the ELISA microplates. High optical density was obtained in the ELISA using a pool of sera from immunized cows. No differences in antibody levels ($P \geq 0.05$) could be detected between clinically healthy calves and those showing diarrhoea.

Keywords: Escherichia coli; ELISA; K99 antigen; Cow; Pilus

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) strains are associated with significant economic loss due to diarrhoea in newborn calves (Acres, 1985). Adherence to intestinal mucosal cells and production of enterotoxins are the two main virulence factors for enteropathogenicity of ETEC (Tzipori, 1981). The K99 pilus is the major attachment factor of most strains of ETEC in calves (Gaastra and De Graaf, 1982). Since K99 antigen is a major virulence factor of ETEC strains in calves, the detection of antibodies directed against this adhesin is of great value. Passive antibody against the K99 antigen prevents severe fatal enteric collibacillosis when ingested by newborn calves soon after birth. The K99 antibody

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prevents ETEC diarrhoea by acting locally in the small intestine to prevent colonization (Acres, 1985). Cow colostrum contains large amounts of immunoglobulin (Ig) G1, the major Ig transferred passively to calves. Intestinal absorption of Igs by the newborn calves is nonselective. Molecules larger than 70 kDa, which includes all Ig molecules, are taken up by lymphatics and enter into circulation via the lymphatic duct. Also, most of the Ig G1 that is cleared from the blood of young calves is transferred back into the gut, providing protection against enteric pathogens (Banks and McGuire, 1989). Therefore, serum IgG levels against K99 antigen in newborn calves should correlate with passive immunity in the intestine against ETEC.

The determination of acquired antibody levels after vaccination against the K99 antigen in cows has been carried out previously using agglutination tests, indirect haemagglutination tests, haemoagglutination-inhibition tests, and agar gel diffusion tests (Morris et al., 1980; Altmann and Mukkur, 1983). This paper deals with the standardization of an ELISA for detecting specific antibodies against K99 antigen of *Escherichia coli* (*E. coli*) in calves. Two methods for pilus purification were compared.

2. Material and methods

Serum samples. Sera to be analyzed through ELISA were obtained from the following sources: (a) Twenty calves with diarrhoea caused by *E. coli* K99 positive and thirty clinically normal animals. These calves were ten days old or less and they were bled during the diarrheic episode. All samples were obtained from nonvaccinated herds. (b) Negative controls: Sera from eighteen newborn colostrum-deprived calves. (c) Positive controls: A pool of sera from five cows immunized twice subcutaneously was used. Immunizations with 1 ml of formaldehyde inactivated *E. coli* B44 culture, were conducted at seven day intervals using complete Freund's adjuvant (50% v/v).

Reference strain and K99 pili. The *E. coli* (K99+) (09:K30:H−) B44 strain and a purified K99 pili were obtained from the *E. coli* Reference Center (Pennsylvania State University, USA).

Reference antibodies. A monoclonal antibody (*E. coli* Reference Center, Pennsylvania State University, USA) and a polyclonal antiserum (IFFA-Merieux, Lyon, France) both against K99 pili were used in the assays.

Confirmation of the presence of the K99 antigen in the reference strain. The *E. coli* B44 strain was grown in Minca broth medium, as described by Guinee et al., (1976). Negative staining and subsequent electron microscopy observations were then conducted to observe the fimbrial morphology. The presence of K99 pili on the bacteria was confirmed by slide agglutination tests using the reference antibodies.

Purification of K99 pili. Two methods were performed according to the following authors: a) De Graaf et al., (1981); and b) Karkhanis and Bhogal (1986). In both cases bacteria were grown in Minca-polivitex broth at 37°C with shaking (200 rpm) for 18 h. Pilus

detachment from bacterial cells was achieved by sonication using the method of De Graaf et al., (1981) and by heat shock (65°C for 20 min) in the method of Karkhanis and Bhogal (1986). Protein concentration was measured by the method of Bradford (1976). The presence of K99 pili in the purified material was determined by double immunodiffusion tests (Isaacson, 1977), using the reference antibodies. The purity and the molecular weight (MW) of the protein was assayed by SDS-PAGE (Laemmli, 1970) using the reference pili as control.

Immunoblotting. (Towbin et al., 1979). The putative K99 pili, included in the polyacrylamide gels, were transfered to nitrocellulose sheets and then identified employing specific rabbit anti K99 serum as first antibody, and I^{125} labelled goat anti-rabbit IgG immunoglobulins (Sigma, St. Louis, USA) as a second antibody. The immunoglobulin iodination was carried out according to Fraker and Speck (1978). Autoradiographs were obtained after 24 h exposure at −72°C.

ELISA. Optimum antigen concentration and sera dilution was determined using dilutions of positive and negative sera and utilizing different antigen concentrations coating the plates. ELISAs were performed as previously described (Voller and Bidwell, 1986). Briefly, microplates were coated with 100 μl per well of a suspension containing 2 μg of purified pili (obtained according to Karkhanis and Bhogal, 1986) in PBS pH 9.6. After an overnight incubation at 4°C, plates were blocked with bovine serum albumin (BSA). All serum samples were diluted 100-fold in PBS-BSA 1% before being added in triplicate to the plates. Three washes were performed with PBS/Tween 20 (0.05% v/v) after an incubation period of 1 h in a plate shaker at room temperature. A rabbit anti-bovine IgG conjugated with horseradish peroxidase (Sigma, St. Louis, USA) diluted 1/1000 in PBS was added in a volume of 100 μl per well. After washing, the antigen antibody reaction was detected by adding the enzyme substrate 3,3,5,5 tetramethylbenzidine (Idexx Corp., Portland, USA). Optical densities (ODs) were determined in a Nunc ELISA reader at 620 nm.

Statistical Procedures. Statistical analysis was carried out using the Mann-Whitney U test and the Kruskall-Wallis one-way analysis of variance by ranks (Siegel, 1956).

3. Results and discussion

The expression of pili by the reference strain after cultivation in Minca broth was confirmed since fimbriae showing typical morphology (Korhonen et al., 1980; Altmann et al., 1982) were observed by electron microscopy. Furthermore, the monoclonal and the polyclonal reference antibodies recognized these pili as K99 by agglutination tests.

The presence of K99 pili in the purified material of the both procedures used (De Graaf et al., 1981, and Karkhanis and Bhogal, 1986) was proved by both immunodiffusion and immunoblotting assays.

After employing the purification method described by De Graaf et al., (1981) a final protein concentration of 1.92 μg/μl was obtained. A major 18 kDa band was observed by SDS-PAGE but high contamination with other proteins was also apparent. This could be

explained because many proteins are released after the lysis of some bacteria caused by the sonication used in this method. Furthermore, some outer membrane proteins can remain attached due to their hydrophobic bonds to pili. This can be avoided by heat treatment (Karkhanis and Bhogal, 1986).

Employing the method described by Karkhanis and Bhogal (1986), a final protein concentration of 0.5 $\mu g/\mu l$ was obtained. Best results in terms of purity of the K99 antigen was achieved following this procedure. SDS-PAGE showed an 18 kDa major band identical to the migration pattern shown by the reference pili and no other protein contamination was detected. The K99 antigen utilized for the ELISA proved to be sufficiently pure and specific because of its electrohoretic profile and recognition by both immunodiffusion and immunoblotting assays (Fig. 1.)

ODs of healthy calves sera through ELISA showed a mean value ($\bar{x}$) of 0.008 and a standard deviation (SD) of 0.012. Sera from diarrhoeic calves showed a $\bar{x} = 0.007$ and a SD = 0.010. Sera from colostrum deprived calves showed a $\bar{x} = 0.005$ and a SD = 0.007. The pool of sera from the five immunized cows demonstrated an OD of 0.850. When these cow sera were tested individually they showed a $\bar{x} = 0.745$ and a SD = 0.596.

Differences were not found ($P \geq 0.05$) among the means of the ODs of the sera of diarrhoeic animals, the non-diarrhoeic ones and the negative controls. This result was not unexpected since the samples were obtained from unvaccinated herds. The low antibody levels detected on healthy calves suggest that they may be susceptible to ETEC.

Fig. 1. Immunoblotting of purified K99 pili (according to Karkhanis and Bhogal, 1986) using specific rabbit K99 antiserum.

A significant difference ($P \leq 0.05$) was detected between the antibody levels obtained in the pool sera from the immunized cows and the ODs of the colostrum deprived calves. Nevertheless these immunized cows had great individual differences in the antibody response to the antigen (SD = 0.596) showing ODs from 0.06 to 1.30, thus these results validate the use of pooled sera as positive control for this test.

ELISA, employing purified pili K99, showed to be useful for monitoring the humoral immunitary status against ETEC in bovine herds and could be used to evaluate immunization programs.

Our purified K99 antigen has been used by Bustos et al., in further studies on antibody determination in calves through ELISA and radioimmunoassay, obtaining a high correlation between both procedures (Unpublished observation).

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