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Monoclonal Antibody Enzyme-Linked Immunosorbent Assay for Identification of K99-Positive *Escherichia coli* Isolates from Calves†

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For *Escherichia coli* to produce diarrhea in animals it must possess the ability to attach to the epithelial cells of the intestine and to produce enterotoxins. Tests developed to differentiate pathogenic from nonpathogenic *E. coli* have relied on detection of adherence structures called pili or detection of the toxins. We utilized a monoclonal antibody to K99 pili in an enzyme-linked immunosorbent assay to detect the presence of K99 pili in *E. coli* isolated from calves. Twenty-three *E. coli* isolates that were known to be stable toxin positive were all shown to produce K99 pili. A 100% correlation also was shown between the presence of K99 antigen and production of stable toxin by *E. coli* isolates. Of the 251 isolates, 245 were negative by K99 enzyme-linked immunosorbent assay and stable toxin assay. The other six were positive on both tests. The enzyme-linked immunosorbent assay also was shown to be specific for K99 pili by antibody-blocking assays. The number of *E. coli* necessary for detecting K99 pili by enzyme-linked immunosorbent assay was determined to be 3.5×10^5 bacteria per ml.

Enterotoxigenic *Escherichia coli* (ETEC) strains are a common cause of diarrhea in newborn calves, as well as in other species. The colonization of the intestinal tract is aided by pili which allow the ETEC to adhere to the small intestines in calves, lambs, and pigs (10, 12, 14, 22, 28). Three pilus antigens have been characterized in animal ETEC: K88 and 987P, found principally in swine, and K99, found in bovine, ovine, and porcine species. The K88 pili have been reported to be associated with both stable toxin (ST) and labile toxin (LT) production (8, 9). The K99 and 987P pili have been associated only with ST production (7, 11, 15, 20, 21), regardless of the species from which the isolate was obtained.

There are several methods used to identify ETEC, usually by identification of one of the two pathogenic factors, the toxins or the pili. Toxin assays such as the infant mouse test (3) for ST or the Y-1 mouse adrenal gland cell toxicity assay (25) for LT have been useful but are restricted to laboratories with cell culture capabilities and access to a mouse colony. The identification of ETEC by pilus detection can be in the form of agglutination, fluorescent-antibody (1, 2), or enzyme-linked immunosorbent assay (ELISA) (4, 17, 19). The agglutination assay is rather insensitive and has been prone to nonspecific agglutination or to false negatives because of thick capsules on some isolates. The fluorescent antibody is a good technique, but many small laboratories do not have good fluorescent capabilities. The ELISA is the most sensitive and can be done with very little equipment.

The ELISA has been used to diagnose a variety of bacterial, viral, and fungal diseases (24). Its application to diagnosing *E. coli* infections in calves, pigs, and humans by detecting K99 protein (5), K88 protein (18), and LT (16, 30) have been described.

Any immunoassay relies on the specificity of the antibody used. The advent of monoclonal antibodies has increased the specificity of antibodies, but there have been questions about too much specificity.

We developed a rapid ELISA by using a monoclonal antibody for detection of K99 antigen. Good correlation was shown between presence of K99 antigen and ST production in clinical *E. coli* isolates from calves.

MATERIALS AND METHODS

Antibody and conjugate. A monoclonal K99 antibody (Molecular Genetics, Inc., Minnetonka, Minn.) was used in a standard sandwich ELISA. Five milligrams of ammonium sulfate-precipitated monoclonal mouse anti-K99 was conjugated to peroxidase by a modification of the method of Nakane and Kawaoi (23) as previously described (29).

Microtiter tray preparation. Polyvinyl microtiter plates (catalog no. 001-010-2101; Dynatech Laboratories, Inc., Alexandria, Va.) were rinsed with absolute ethyl alcohol and allowed to dry. Monoclonal anti-K99 globulin was diluted 1:1,000 in pH 9.6 buffer, and 0.1 ml was dispensed into each well. The plate was covered with sealing tape and incubated at 37°C for 90 min and then placed directly into a −20°C freezer. When an assay was to be done, the correct number of wells was cut from the plate, and that section was allowed to thaw at room temperature.

Washing and diluting solution. Washing steps and reagent dilutions were all done in 0.01 M phosphate-buffered saline, pH 7.2, containing 0.5% horse serum (Sterile Systems Inc., Logan, Utah) and 0.1% Tween 80.

Substrate. 2,2-Azino-di[(3-ethylbenzthiazoline sulfonic acid)] (ABTS; Sigma Chemical Co., St. Louis, Mo.) was prepared according to the procedure of Bartlett (26). To 10 ml of 0.05 M citric acid (pH adjusted to 4 with 1 NaOH), we added 100 μl of a 40 mM solution of ABTS and 100 μl of 3% H_2O_2.

E. coli isolates from intestinal tracts of calves with diarrhea submitted as clinical specimens to the Kansas State University Diagnostic Laboratory were used. Routinely, *E. coli* was isolated from intestinal contents on blood agar and Maconkey plates, and the isolates were transferred to Tergitol-7 plates for a separation by colony morphology (27). The isolated cultures were maintained on Trypticase soy agar slants (BBL Microbiology Systems, Cockeysville, Md.) at room temperature until tested.

Enterotoxin test. Isolates were examined for ST produc-

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tion by the method of Dean et al. (3). After culturing for 4 h in brain heart infusion broth, *E. coli* cells were transferred to 1 ml of brain heart infusion roller tubes and incubated at 37°C overnight. The cultures were injected intragastrically into 3-day-old mice, which were killed with chloroform 4 h postinjection and examined visually for characteristic fluid accumulation in the intestine.

Preparation of *E. coli* for K99 ELISA. Cultures on Trypticase soy agar were inoculated into E broth (6) and incubated at 37°C overnight. The broth was then swabbed onto an E agar plate and incubated at 37°C overnight. Suspensions for ELISA were made from these plates.

ELISA for K99. Frozen antibody-coated plates were thawed and washed three times with diluent. One hundred microliters of each *E. coli* suspension was dispensed into a microtiter plate well. The plates were incubated at 37°C on a shaker (Dynatech Laboratories) for 15 min. Plates were washed three times with diluent, and 100 μl of a 1:3,000 dilution of monoclonal K99-horseradish peroxidase was added. The plates were incubated as above for 10 min and then washed again three times with diluent. Substrate (75 μl per well) was added, allowed to react for 5 min, and then stopped with 75 μl of stopping reagent. The plates then were read visually.

Sensitivity of K99 ELISA on whole bacteria. A suspension of K99-positive *E. coli* was made in sterile phosphate-buffered saline. Part of this suspension then was diluted 10^1 through 10^6 in phosphate-buffered saline-Tween 80-horse serum and tested by ELISA. Viable *E. coli* counts also were determined by standard suspension plating techniques.

Quantitation of known K99-positive and -negative cultures. Stock K99-, K88-, and 987P-positive cultures were grown and checked for reaction in the ELISA. The average of 10 readings obtained with a through-the-plate reader (BioTek Instruments, Burlington, Vt.) were used to determine absorbance values on *E. coli* with each pili type. This was done to assure specificity of the antibody to the K99 pili.

Specificity of K99 ELISA-blocking test. Guinea pig antisera to K99, K88, and 987P and normal guinea pig sera were used to demonstrate the specificity of the ELISA. Serial twofold dilutions (1:2 to 1:4,096) of the K99 antisera were tested for blocking of the K99 monoclonal ELISA. Normal guinea pig sera and specific guinea pig antisera to K88 and 987P also were checked for presence of blocking antibody in the K99 ELISA at the 1:2 dilution. The antisera were allowed to react in the ELISA system after the bacteria suspension had been incubated and the plate washed. Incubation of the antisera was for 15 min on the shaker. All other steps in the ELISA procedure were unchanged for the procedure described above.

K99 ELISA of ST-positive cultures. Bovine *E. coli* isolates, previously shown to be ST positive by the infant mouse test, were tested for K99 production by ELISA. Cultures first were passed through E broth and then onto E agar. Suspensions were made from the E agar and checked by K99 ELISA.

K99 ELISA on clinical isolates. All *E. coli* isolated from calves less than 1-month-old that were submitted to the Veterinary Diagnostic Laboratory were ELISA tested for K99 and tested for ST production by the infant mouse test (3). A total of 251 *E. coli* isolates were tested.

RESULTS

The monoclonal anti-K99-coated polyvinyl plates were coated in batches of 10 and were found to be stable at −20°C for at least 6 months. These plates were first treated with absolute alcohol to remove any oil that might be present on the plates.

Addition of Tween 80 and horse serum to the washing and diluting solution was found to reduce nonspecific binding of whole bacteria to the polyvinyl plate but to allow specific antibody binding to take place.

Use of ABTS as a substrate allows for rapid determination of results (5 min), and the green color is easy to visualize.

Tergitol-7 medium prevented cocultivation and ELISA testing of piliated and nonpiliated *E. coli* strains.

For the *E. coli* to consistently express the K99 pili in culture it was necessary to use the E agar. Differences were observed in the amount of K99 pili produced by known K99-positive isolates when grown on sheep blood agar or Trypticase soy agar. Of the 23 known ST-positive cultures, 7 gave negative ELISA reactions when grown on blood agar or Trypticase soy agar.

The ELISA was able to detect the presence of K99 pili at 3.6×10^5 bacteria per ml. The maximum ELISA reaction was observed when a suspension contained 10^7 bacteria per ml.

Absorbance readings (at 414 nm) on 10 wells for each pilus type were as follows: K99, 1.620; K88, 0.008; 987P, 0.013. The differences were easily detected visually, so quantitation was discontinued.

Figure 1 shows the inhibition curve with guinea pig K99 antisera. Antisera to K88 and 987P and normal guinea pig sera are shown at the lowest dilution and had no inhibitory effect on the reaction seen in the K99 ELISA.

The 23 known ST-positive isolates all were found to be positive by the K99 ELISA. Of the 251 *E. coli* isolates

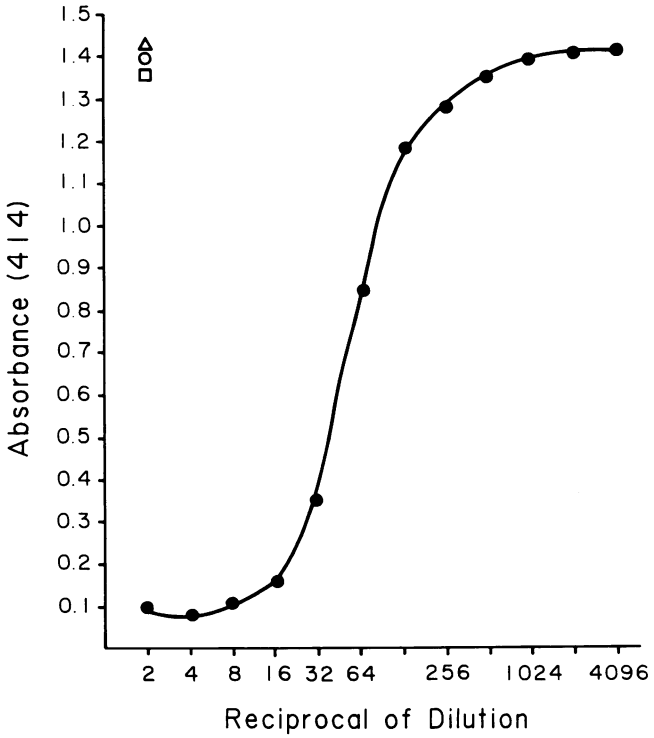

FIG. 1. Absorbance readings on K99 ELISA blocking test. Symbols: ●, guinea pig anti-K99 antisera added in twofold dilutions; △, normal guinea pig sera, 1:2; ○, guinea pig anti-K88 antisera, 1:2; □, guinea pig anti-987P antisera, 1:2.

tested, 243 were found to be negative by both the ST assay and the K99 ELISA. Six isolates were found to be positive by both the K99 ELISA and the ST assay.

DISCUSSION

A variety of results have been reported on the association of ST with the presence of the K99 antigen. Guinee and Jansen (7) showed 100% correlation between ST production and the presence of the K99 antigen. They also found that none of the non-enterotoxigenic strains possessed the K99 antigen. Other researchers have reported that 99 (13), 87 (11), and 77% (21) of the ST-positive strains possessed the K99 antigen. Kaechenbeeck et al. (13) reported that 21% of ST-negative strains were positive for the K99 antigen. These differences in percent association of ST to K99 antigen could be attributable to difficulties in K99 detection, but there exists the possibility of non-K99 adhesions being associated with ST production.

Results presented here would indicate that the K99 pili are closely associated with production of ST. A 100% correlation was shown between ST and presence of K99 pili in 23 stock known ST positives. In evaluation of the ELISA on 251 clinical isolates, 6 were shown to be K99 and ST positive and 243 were negative for ST and K99 pili.

We demonstrated that an ELISA with a monoclonal antibody to K99 protein is specific yet detects the K99 protein in a variety of pathogenic *E. coli* isolates. The specificity of the monoclonal anti-K99 was verified by specific antibody-blocking assays and by ELISA tests on other *E. coli* pili types.

We feel that a commercial kit could be assembled by using the methods described, which would offer a standardized method of identifying K99-positive *E. coli* to many laboratories. As monoclonal antibodies are developed for K88 and 987P, a kit could also be made to address neonatal pig diarrhea.

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