Use of Monoclonal Antibodies Specific for the α Determinant of K88 Pili for Detection of Enterotoxigenic Escherichia coli in Pigs†

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Monoclonal antibodies directed against the α determinant of K88 pili from porcine enterotoxigenic Escherichia coli which react with all three K88 variants have been produced. These antibodies have been used for diagnosis of porcine enterotoxigenic E. coli in a direct enzyme-linked immunosorbent assay with sensitivity to 50 ng of pilus protein per ml.

Both pilus adhesins and enterotoxins of enteropathogenic Escherichia coli are involved in pathogenesis of noninvasive diarrheal disease (6). The K88 pilus protein antigen (19, 20) is associated with swine infections (1, 14) and has at least three antigenic variants, ab (14, 15), ac (15, 16), and ad (7), of which ac and ad currently predominate (7, 25).

The detection of the K88 antigen in porcine E. coli is indicative of the enteropathogenicity of the strain, because K88-positive strains are nearly always also enterotoxigenic (18). The usual methods of detecting E. coli with the K88-type pili have been seroagglutinations (3, 4, 26), immunofluorescence (2, 4), or enzyme-linked immunosorbent assay (ELISA) (10, 12) using polyclonal antibodies. Monoclonal antibodies (MAb) have the advantages of increased sensitivity and specificity, while being available in an unlimited supply. The objectives of these experiments were to produce and characterize MAb specific for the α determinant of K88 pili which react with all three K88 variants (ab, ac, and ad) and to use these antibodies in an ELISA for the detection of K88-positive E. coli isolated from swine.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The following E. coli strains were either purchased from the E. coli Reference Center (Pennsylvania State University) or were provided by the National Animal Disease Center (Ames, Iowa): NADC 1260-G1108E O141:K85:K88aø:H14; 80-2575 46.1 O157: K88ac:H43; Morris O8:K87:K88ad (gift of Richard Wilson); O263:K87:K88ab; O101:K88−:K99+; O8:K87:K88ab:H19; O149:K91:K88ac; O8:K:K88ad:NM; O101:K:K99:NM; and O9:K103:987P.

A total of 142 K88-positive cultures from eight swine-producing states which were identified as K88 positive by either the E. coli Reference Center or the Animal Disease Research and Diagnostic Laboratory (Brookings, S.Dak.) were obtained so that isolates from a wide geographic area could be compared with those sent to the Kansas State University Department of Veterinary Diagnostic Investigation. The number of isolates from each of the eight states was as follows: 46, South Dakota; 39, Iowa; 20, Minnesota; 13, Nebraska; 12, Illinois; 3, Pennsylvania; 1, Indiana; and 1, Missouri, along with 7 isolates for which the state of origin was unknown.

Blood agar plates were used for expression of K88 and 987P pili (5). E. agg (24) was used for the expression of K99 pili (5). Tetrad (T-T) agar (Difco Laboratories) was used for separation of mixed cultures of E. coli and for separation from Proteus species on the basis of color and colony morphology (17). Cultures were grown at 37°C.

Purification of K88 pili. The K88 antigen was purified by using a modification (5) of the method of Stirm et al. (19). As described by Isaacson (8), an additional extraction and isoelectric precipitation were performed on the cell pellet. The material was subjected to two more isoelectric precipitations to obtain a pure pilus preparation. The final pilus preparation was analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9). Sodium dodecyl sulfate-polyacrylamide gels were electroblotted to nitrocellulose paper as described by Towbin et al. (23). An immunoperoxidase assay was used to detect antigen bands on the nitrocellulose sheets.

Evaluation of MAb specificity and sensitivity. The ELISA used for hybridoma screening and titer determination was a modification of the procedure described by Stocker et al. (21) with K88 pilus-coated microtiter wells incubated with hybridoma supernatants. Anti-K88 antibodies were identified by incubation with rabbit anti-mouse immunoglobulins conjugated with horseradish peroxidase (Cappel Laboratories) and detected with o-phenylenediamine (Sigma Chemical Co.). Results were determined visually.

The ELISA procedure for MAb specificity was performed as described except that aliquots of MAb were plated and then serial dilutions of various pilus preparations were incubated. The presence of bound pili was detected by using polyclonal rabbit anti-K88 antisera (10, 12) followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated antibodies and substrate.

To determine the sensitivity of the 2C1.16 MAb for use as a clinical tool, an ELISA utilizing MAb-coated plates as well as a MAb-horseradish peroxidase conjugate (MAb-HRPO) to detect pili was performed. Serial dilutions of the K88ab
pili were incubated in the MAb-coated plates, after which they were incubated with the MAb-HRPO. The ELISA was then developed as previously described.

**Diagnostic ELISA procedures.** The procedure used for the diagnostic ELISA has been previously described (10–12). Unconjugated anti-K88a MAb were diluted 1:2,000 and applied as a coating to polyvinyl chloride microtiter plates (Dynatech Laboratories, Inc., Chantilly, Va.) as previously described (11). The anti-K88a peroxidase-conjugated MAb (13, 22) were diluted 1:2,500 in ELISA wash diluent consisting of 0.01 M phosphate-buffered saline, pH 7.2, with 0.1% polyoxyethylene sorbitan mono-oleate (Tween 80)–0.5% equine serum. Results were determined visually.

**RESULTS AND DISCUSSION**

MAb 2C1.16 (Fig. 1) showed a capability of detecting any K88 variant, therefore demonstrating an affinity for the K88a antigenic site common to all K88-piliated *E. coli* organisms. Neither depiliated *E. coli* nor *E. coli* bearing 987P or K99 pili could be detected in these screenings when the appropriate homologous polyvalent antisera were utilized (data not shown).

An immunoblot assay was also performed to verify the antigen-antibody binding specificity. MAb 2C1.16 gave a strong reaction to the free subunits of the K88ab pili which appeared as a strong band with a molecular weight of approximately 16,000 (Fig. 2).

Figure 3 demonstrates the titration curve for the ELISA utilizing MAb-coated plates as well as MAb-HRPO to detect serial dilutions of K88ab pili. The sensitivity for this assay ranged from 50 to 80 ng/ml for purified pili.

The procedure described for the diagnostic ELISA was used to determine the specificity of the ELISA utilizing the 2C1.16 MAb specific for the a determinant against whole piliated *E. coli*. Assays were performed on the various *E. coli* suspensions standardized with a McFarland nephelometer standard corresponding to $1.5 \times 10^8$ bacteria per milliliter. The mean light absorbance from anti-K88 a specific

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**FIG. 1.** Determination of MAb specificity by ELISA. Plates were coated with a 1:1,000 dilution of MAb 2C1.16. Incubation was with serial dilutions of purified pili preparations, and quantitation was by detection with rabbit polyclonal anti-K88 antigen and HRPO-goat anti-rabbit antisera at A490. Symbols: O, O263:K88ab; Δ, O157:K88ac; ●, O8:K88ad.

**FIG. 2.** Immunoblot analysis of MAb 2C1.16. Shown is polyacrylamide gel electrophoresis of 20 µg of K88ab purified pili preparation transferred to nitrocellulose.

**FIG. 3.** Titration of K88 pili by ELISA. MAb 2C1.16 was plated at dilutions of 1:1,000 and then incubated with serial dilutions of K88ab pili. Bound pili were detected by incubation with a 1:2,000 dilution of 2C1.16-HRPO.
TABLE 1. MAb ELISA results for K88 pilus antigens

<table>
<thead>
<tr>
<th>Yr</th>
<th>No. of specimens tested</th>
<th>No. of specimens K88 positive</th>
<th>% Specimens K88 positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1986</td>
<td>52</td>
<td>11</td>
<td>21.15</td>
</tr>
<tr>
<td>1986</td>
<td>74</td>
<td>12</td>
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<td>1987</td>
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<td>490</td>
<td>79</td>
<td>16.12</td>
</tr>
<tr>
<td>1990</td>
<td>737</td>
<td>69</td>
<td>15.78</td>
</tr>
<tr>
<td>Total</td>
<td>2,173</td>
<td>346</td>
<td>15.92*</td>
</tr>
</tbody>
</table>

* Specimens came from pigs 6 weeks of age or less and were submitted to the Kansas State Veterinary Diagnostic Investigation between 1 January 1985 and 31 December 1990.

Only specimens requested by field veterinarians were tested.

Percentage of total number of specimens.

K88 E. coli with no significant background readings for K99 or 987P.

Using an antibody specific for the α determinant of K88 pili enables us to detect all three K88 variants while maintaining the sensitivity and specificity of the MAb ELISA. The ab and ac variants consistently produce stronger ELISA reactions than does the ad variant. The reason for the low ELISA reactions for the K88ad is unknown. It could be that either the α determinant is repeated fewer times per pilus unit in the ad variant or perhaps that fewer total pili are expressed per bacterium. The positive ELISA reaction for the ad is still easily determined visually because of the low background of the negative wells.

Before the MAb ELISA was incorporated for daily diagnosis of clinical isolates, all K88-positive isolates previously diagnosed were retested by both of the MAb and the polyclonal antibody (10, 12) ELISAs. A total of 443 E. coli cultures isolated from 1976 to 1985, which were previously shown to produce a heat-labile enterotoxin or K88 pili, were assayed. Two hundred and seventy-three isolates retained the ability to express the K88 antigen. Including the 142 K88-positive cultures collected from eight other swine-producing states, 415 isolates were used to compare the polyclonal antibody ELISA with the MAb ELISA. The bacterial suspensions used in the polyclonal antibody ELISA were subsequently checked by using the MAb ELISA specific for the α determinant. All 415 suspensions produced a stronger reaction (data not shown) with the MAb ELISA than with the polyclonal antibody ELISA. However, the reactions from both ELISAs on pure cultures were easily determined visually.

Intestines or feces from all pigs less than 6 weeks of age, which were sent to the Kansas State University Department of Veterinary Diagnostic Investigation, were cultured within 24 h and tested for K88 E. coli. Specimens were cultured for isolated colonies on blood agar and on T-7 agar at 37°C. Clinical isolates and positive controls from blood agar were suspended to a concentration which visually approximates a McFarland nephelometer standard corresponding to approximately 1.5 × 10⁹ bacteria per milliliter. Suspensions were made in ELISA wash diluent.

A total of 2,173 porcine specimens submitted from 1 January 1985 through 31 December 1990 were tested within 48 h of arrival for E. coli possessing the K88 antigen. A total of 346 (15.92%) were positive for K88. Table 1 shows the yearly results of the K88 ELISA.

The low numbers of cases diagnosed in 1985 and 1986 are due to the fact that the only specimens that were tested were those requested by field veterinarians. Beginning on 1 January 1987, specimens from all pigs less than 6 weeks of age were tested regardless of clinical signs. This was necessary because often case histories are incomplete or illegible and the clinical signs are sometimes misleading.

When we were originally developing the MAb ELISA for clinical isolates, specimens were inoculated only onto T-7 agar, from which each colony type was inoculated onto blood agar for expression of K88 pili. This allowed for pure cultures to be tested but resulted in as many as eight isolates per case. With this method, there was little advantage of the MAb ELISA over the polyclonal antibody ELISA except for the availability of the antibodies. Later, we began testing mixed cultures of E. coli, including those overgrown with Proteus sp., which had been inoculated from the specimen directly onto blood agar. The MAb ELISA was sensitive and specific enough to detect K88 E. coli overgrown with Proteus sp. whereas the polyclonal antibody ELISA was not. Pure cultures of K88 E. coli can be easily isolated from mixed E. coli cultures from blood agar because K88 E. coli organisms are nearly always beta-hemolytic. Pure cultures of K88 E. coli can be easily isolated from those overgrown with Proteus sp. by streaking for isolation on T-7 agar and subsequent transfer to blood agar. Proteus sp. appears purple on T-7 agar whereas K88 E. coli appears brown to amber.

Performing the ELISA on mixed cultures saves material, money, and time and allows for results to be reported to field veterinarians the day following arrival at the laboratory. This ELISA enables us to test large numbers of specimens rapidly and economically and at the same time allows for the isolation of K88 E. coli for antibiotic sensitivities so that effective antimicrobial agents can be administered to the affected animals as soon as possible.

ACKNOWLEDGMENTS

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REFERENCES