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Detection and Characterization of the Coli Surface Antigen 6 of Enterotoxigenic *Escherichia coli* Strains by Using Monoclonal Antibodies

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We describe, for the first time, the production of monoclonal antibodies (MAbs) against coli surface antigen 6 (CS6) of enterotoxigenic *Escherichia coli* (ETEC) and their use for characterization and diagnosis of CS6. Two MAbs, MAbs CS6-20:11:9 and CS6-2A:14, were produced by immunizing mice with purified CS6 or CS6-containing bacterial extracts. The MAb specificity was demonstrated by enzyme-linked immunosorbent assay (ELISA), immunoblotting, and immunoelectron microscopy, which showed that the MAbs bound to CS6-expressing bacteria as well as to purified CS6 and CS6 structural subunits but not to CS6-negative bacteria or other purified ETEC colonization factors. By using bacterial recombinants, i.e., strains with a complete CS6 operon or parts thereof, it was found that both MAbs were specific for CssB, one of the two structural subunits of CS6. Although the MAbs bound specifically to the entire surface of CS6-expressing bacteria, no structure of CS6 could be identified. The usefulness of the MAbs for the detection of CS6 was evaluated in an inhibition ELISA and in a dot blot test. Ninety-two ETEC strains with known colonization factors were analyzed, and all CS6-positive strains were identified by either assay with MAb CS6-2A:14, whereas MAb CS6-20:11:9 failed to identify two CS6-positive strains; in no instance was any CS6-negative strain identified by either of the MAbs. Parallel analyses of 48 strains with a gene probe specific for the other structural subunit of CS6, i.e., CssA, and the MAb-based assays gave identical results, suggesting the simultaneous presence of both subunits.

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of diarrhea, especially in children in developing countries and in travelers to these areas (9). ETEC isolates colonize the small intestine by means of colonization factor (CF) and coli surface (CS) antigens. The subsequent production of heat-labile (LT) and/or heat-stabile (ST) enterotoxins results in diarrhea (9). The best-characterized CFs in human-derived ETEC strains are CFA/I (8) and CS1 to CS6 (29). In addition, a number of putative colonization factors (PCFs) have been described, e.g., CFA/III, CS7, CS17, PCFO9, PCFO20, PCFO159, and PCFO166 (14, 21, 35). CS6 is expressed either alone or in combination with either CS4, CS5, or CFA/III (25, 26, 34). It has been suggested that CS6 is a CF since ETEC strains expressing only CS6 have been shown to colonize rabbit intestine (32), and such strains have been identified as the only pathogen in patients with diarrhea (1b, 38); CS6 is also one of the most frequently identified CFs on clinical isolates (9). The CS6 operon has recently been shown to consist of four open reading frames which were designated *cssA*, *cssB*, *cssC*, and *cssD* (2). Both *cssA* and *cssB* were found to encode CS6 structural proteins. CssC belongs to the family of molecular chaperones, and CssD is a molecular usher.

As suggested by studies with both animals and human volunteers, the most prevalent CF and CS antigens are probably important components in future oral ETEC vaccines (33). Since the prevalence of the various CFs in ETEC isolates differs considerably among isolates from different geographic areas (10), epidemiological surveys of CF and CS factors on ETEC strains isolated from different locations is important in the design of candidate vaccines. Preliminary studies suggest that CS6 may be one of the most prevalent CFs worldwide (1b, 38). Therefore, it is important to develop suitable methods that could be used in different locations to identify this CF. Until recently, CS6 was identified either by immunodiffusion tests (1b) or by enzyme-linked immunosorbent assays (ELISAs) (12) with polyclonal anti-CS6 sera or with a CS6-specific polynucleotide probe (12). In this report, we describe the production and characterization of CS6-specific monoclonal antibodies (MAbs) and their use in the characterization of CS6 as well as in diagnostic assays.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The following reference strains, kindly provided by B. Rowe and M. M. McConnell (Colindale, Great Britain), were used: E11881/9 (O25:H42, ST positive [ST^+] and LT positive [LT^+], CS4 positive [$CS4^+$] and $CS6^+$), E11881/2 (O25:H42, ST^+ and LT^+, CS4 negative [$CS4^-$] and $CS6^-$), E17018/A (O167:H5, ST^+, $CS5^+$ and $CS6^+$), E17018/6 (O167:H5, ST negative [ST^-], $CS5^-$ and $CS6^-$), E11881/23 (O25:H42, ST^- and LT negative [LT^-], $CS4^+$ and $CS6^+$), and E11881/14 (O25:H42, ST^- and LT^-, $CS4^-$ and $CS6^+$). The *Escherichia coli* K-12 strain C600, provided by J. Kaper (Baltimore, Md.), was used as a negative control. Furthermore, six LT^+ *cssA* probe-positive strains (12, 13) and 92 ETEC strains (Table 1) that had previously been examined for expression of CFs were included in the study. The strains were maintained at −70°C in broth supplemented with 15% (vol/vol) glycerol and were cultured as described previously (7, 22) at 37°C overnight. Heat extracts were prepared as described previously (12).

Bacterial recombinants. Plasmids pDEP3, pDEP4, and pDEP5 encoding CS6 have been described previously (37). Site-directed mutagenesis of *cssB* was performed by partially digesting pDEP5 with 0.4 U of *Pst*I at 37°C for 30 min. The resulting linear fragments were extracted from an agarose gel (1%) with the GeneClean Kit (Bio 101, Inc., La Jolla, Calif.). T4 DNA polymerase (Gibco BRL, Gaithersburg, Md.) was used to transform the *Pst*I sites into blunt ends under the conditions specified by the manufacturer, and T4 DNA ligase (Gibco BRL) was added to recircularize the linear DNA fragments. Transformation of ligation mixtures to competent *E. coli*(PC2495) cells was performed by electroporation with a Gene-Pulser (Bio-Rad Laboratories, Richmond, Calif.) accord-

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ing to the protocol. Transformants were selected on Luria-Bertani agar plates supplemented with ampicillin. Since the *Pst*I site in the vector pBR322 is in the gene coding for β-lactamase, only pDEP5 derivatives with mutations in the *Pst*I site in the *cssB* gene can grow in the presence of this antibiotic. The correct mutants were identified by their *Pst*I restriction patterns. For two mutants, mutants pIVB3-736 and pIVB3-737, it was confirmed by determination of the nucleotide sequence that the sequence CCCTGCAGGT was altered to CCCG GT. The *cssA* and *cssB* genes were amplified in a PCR in which purified plasmid pDEP5 was used as the template and the following primers were used for *cssA*: 5′-GATTAAAGCTTTAGTAATGGTG-3′ and 5′-CCAGTATCTCGAGTACA TAGTAACC-3′. For *cssB* the following primers were used: 5′-ACTAGATCTA TGATTATGTTGAAAAAAATTATT-3′ and 5′-AATGCGGCCGCTTTTTTA TTGCTG-3′. Each 50-μl PCR mixture contained 20 pmol of the two primers, 100 ng of template DNA, 0.2 mM deoxynucleoside triphosphates (Pharmacia, Uppsala, Sweden), and 0.625 U of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.) in PCR buffer (Perkin-Elmer). PCR amplification was performed in an automated thermal cycler (Hybaid Omnigene) under the following conditions: 1 min at 95°C, 1 min at 40°C, and 1 min at 72°C for 35 cycles, followed by 10 min at 72°C. Each PCR mixture was electrophoresed in a 1% agarose gel in 1× TBE (Tris-borate-EDTA) buffer (pH 8.3). DNA fragments were removed from the gel by using the GeneClean Kit as described above. The fragments containing the *cssA* gene and the *cssB* gene were cut with the restriction endonucleases *Hin*dIII and *Xho*I and with *Bgl*II and *Not*I, respectively, under the conditions specified by the manufacturer (Pharmacia). Next, the fragments were ligated into plasmid pET-24 (Novagen, Inc., Madison, Wis.), resulting in pET-*cssA* and pET-*cssB*. Ligation mixtures were transformed to competent *E. coli* HMS174 cells as described above. Strains were grown in liquid Luria-Bertani medium containing kanamycin (30 μg/ml) and were induced with isopropyl-β-D-thiogalactopyranoside (0.3 mM). Expression of CssA and CssB was verified by silver staining (Bio-Rad Laboratories) of a sodium dodecyl sulfate (SDS)-polyacrylamide gel (see below).

Purification of CFs and CS factors. CFs (i.e., CFA/I, CS1, CS2, CS3, CS4, CS5, CFA/III, CS7, CS17, PCFO159, and PCFO166) were purified by ammonium sulfate fractionation followed by either negative DEAE-Sephadex column chromatography (6) or isopycnic cesium chloride gradient ultracentrifugation (110,000 × *g*, 18 h) (18). CS6 was purified by separating bacterial heat extracts from the CS6-only-expressing strain E11881/14 by SDS-polyacrylamide gel electrophoresis (PAGE) and then electroeluting the CS6 protein from the gel (12). The purity and concentration of the different preparations were determined as described previously (27).

Polyclonal antisera. Two rabbit serum samples against CS6 were used, i.e., serum sample R1296 specific for CS5 and CS6 and serum sample R1614 specific for CS4 and CS6 (12).

Production of MAbs. Inbred female BALB/c mice (age, 6 to 8 weeks) were immunized by two different regimens. One group of mice was given an initial intraperitoneal (i.p.) injection of 5 μg of purified CS6 in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.), followed by one i.p. injection of 2.5 μg of CS6 in Freund's incomplete adjuvant and two intravenous injections of 1 μg of antigen without adjuvant at 2-week intervals. Another group received an i.p. immunization with 5 μg of purified CS6 in RIBI adjuvant (TriChem ApS, Copenhagen, Denmark), followed by two i.p. booster immunizations with a heat extract of strain E11881/14 ($CS4^-$ $CS6^+$) after 3 and 5 weeks, and a fourth i.p. immunization consisted of 2.5 μg of CS6 without adjuvant (which was given 23 weeks after the last immunization). Four days after the last immunization the mice were sacrificed, the spleens were excised, and the spleen cells were fused with exponentially growing mouse myeloma cell lines (P3×63-Ag8 653 or F/O 367); MAbs were then produced as described previously (5). Ten days after the fusion, culture fluids were analyzed by ELISA to determine whether specific antibodies had been produced. Two hybrids, one from each immunization regimen, of 130 hybrids from four fusions with high CS6-specific titers were cloned and expanded. The culture fluids were frozen in aliquots at −30°C until further use, and the hybridoma cells were frozen in liquid nitrogen.

Determination of the isotypes and immunoglobulin concentrations of MAbs. The isotypes and immunoglobulin concentrations of the MAbs were determined as described previously (27).

ELISA. Hybridoma culture fluids were tested for CS6 antibodies in ELISAs by using purified CS6, CS6-expressing bacteria (E11881/14), or CS6-negative bacteria (E11881/2) as solid-phase antigens (27). Coating with purified CS6 was performed by incubating polystyrene microtiter plates (Dynatech Laboratories Ltd., Billingshurst, Great Britain) with CS6 (1 μg/ml) in phosphate-buffered saline (PBS; pH 7.2) at 37°C overnight. Whole bacteria (5×10^9 organisms/ml) were attached to poly-L-lysine-treated microtiter plates (17).

SDS-PAGE and immunoblotting. SDS-PAGE was performed in 16% polyacrylamide slab gels as described previously (16), except that 2-mercaptoethanol was omitted from the sample buffer. Bacterial heat extracts and purified antigens were separated at 200 V, immobilized onto nitrocellulose membranes (0.45-μm pore size; Sartorius AG, Göttingen, Germany) and either stained with amido black (0.1%; wt/vol) or reacted with MAb or polyclonal sera as described for the dot blot assay (see below).

Immunoelectron microscopy (IEM). Bacteria cultured on CFA agar or in rabbit ileal loops for 16 h (16) were applied to Formvar-carbon coated Ni grids (400 mesh) for 2 min. In some instances the bacteria were treated with 0.1% (vol/vol) liquefied phenol for 2 h at 37°C prior to being applied to the grids. The grids were then placed on drops of anti-CS6 MAb diluted 1/2 (original concentration or concentrated 10 times by ultrafiltration) in 0.1% (vol/vol) bovine serum albumin (BSA)–PBS–0.05% (vol/vol) Tween, for 15 min. Alternatively, rabbit polyclonal anti-CS6 sera were used at a dilution of 1/25. After rinsing, the grids were placed on drops of gold-labelled goat anti-mouse immunoglobulin G (IgG) (10 nm; British BioCell, Cardiff, Great Britain) or protein A (Amersham International plc, Amersham, Great Britain) diluted 1/30 in 0.1% BSA–PBS–0.05% Tween for 15 min. The grids were negatively stained with 1% (wt/vol) ammonium molybdate (pH 7.0) and were examined in a JEM-1200 EX electron microscope (Jeol Ltd., Tokyo, Japan). For control purposes, CS6-positive reference strains were tested for unspecific binding of gold-labelled goat anti-mouse IgG with a MAb against *E. coli* ST (ST-1:3).

Slide agglutination test. Slide agglutination was performed as described previously (20).

Inhibition ELISA. The capacities of different *E. coli* strains, whole bacteria (10^{11}/ml), or corresponding heat extracts to inhibit the binding of anti-CS6 MAbs (MAb CS6-20:11:9 diluted 1/1,000 and MAb 2A:14 diluted 1/800) to whole CS6-positive bacteria (E11881/14) or solid-phase-bound purified CS6 were evaluated by an inhibition ELISA (20). Strains that inhibited binding of the anti-CS6 MAb or sera by ≥50% were regarded as CS6 positive.

Dot blot test. The strains were analyzed for their reactivities with the anti-CS6 MAbs in a dot blot test. A nitrocellulose membrane (Sartorius AG) was soaked in PBS and was allowed to air dry; thereafter, 2 μl of whole bacteria diluted in PBS (10^{11}/ml) or corresponding heat extracts were applied to the membrane and blocking was performed with 1% (wt/vol) BSA (Sigma Chemical Company, St. Louis, Mo.)–PBS for 30 min with gentle agitation. All incubations were performed at room temperature. After washing twice in PBS, the nitrocellulose was incubated with anti-CS6 MAb and was diluted 1/30 in 0.1% BSA–PBS–0.05% Tween overnight with gentle agitation. The membrane was washed thrice in PBS–0.05% (vol/vol) Tween, and the anti-mouse IgG-horseradish peroxidase conjugate (Jackson Immuno Research Laboratories, West Grove, Pa.) diluted in 0.1% BSA–PBS–0.05% Tween was added. After incubation for 2 h the membrane was washed twice in PBS-Tween and once in PBS and was then developed for 5 to 10 min by using hydrogen peroxide (0.012%) as the substrate and 4-chloro-naphthol (Bio-Rad Laboratories) as the chromogen. Stained dots on a white background indicated positive results.

Colony hybridization assay. Colony hybridization for the detection of CS6-positive ETEC isolates was performed as described previously (12).

RESULTS

Among several hybridomas producing antibodies specific for CS6 obtained from the different fusions, two were selected for cloning and further studies, i.e., MAb CS6-20:11:9, which was produced by immunizing mice with purified CS6, and MAb CS6-2A:14, which was obtained by priming mice with purified CS6 and boosting them with the heat extract from a CS6-positive bacterium (E11881/14). Both MAbs were of the IgG1 isotype, and the concentrations of specific immunoglobulin in the culture fluids were 77 μg/ml for CS6-20:11:9 and 20 μg/ml for CS6-2A:14.

Specificities of the MAbs. The specificities of the MAbs were tested by ELISA, immunoblotting, and IEM. Analogous to the results obtained with polyclonal anti-CS6 sera, both MAbs failed to agglutinate CS6-expressing bacteria. The ELISA titers of MAbs CS6-20:11:9 and CS6-2A:14 against purified CS6 were 1/14,400 and 1/10,000, respectively; corresponding titers against $CS6^+$ bacteria were 1/3,000 and 1/2,200. No ELISA reactivity was seen with either MAb and a CS6-deficient strain. When the MAbs were tested by immunoblotting, both MAbs reacted strongly with the CS6 bands in bacterial heat extracts as well as with purified CS6 (Fig. 1), but MAb CS6-20:11:9 could be diluted to 10-fold lower immunoglobulin concentrations than CS6-2A:14. Furthermore, when testing the MAbs for their reactivities with other purified ETEC CFs, none of them gave a positive reaction (data not shown). Faint bands of approximately 40 kDa were, however, developed in heat extracts when using MAb CS6-20:11:9. When reacted with heat extracts of the *cssA* and *cssB* recombinants in immunoblots, i.e., pET-*cssA* and pET-*cssB*, both MAbs were shown to be specific for CssB (Fig. 1). Furthermore, the MAbs reacted only with recombinants retaining *cssB*, i.e., pDEP3, pDEP4, and pDEP5, but not with pIVB3-736 or pIVB3-737.

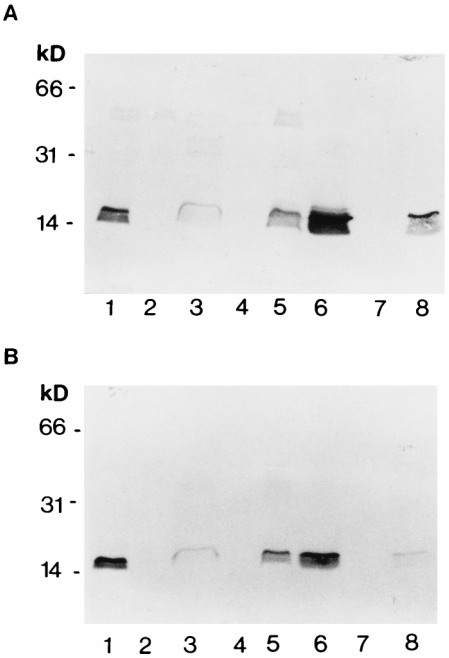

FIG. 1. Immunoblot analysis showing the CS6 reactivities and CssB specificities of MAb CS6-20:11:9 (A) and MAb CS6-2A:14 (B). The bacterial samples used were heat extracts (10^{10} bacteria/ml) of the following strains: E11881/23 (O25:H42, ST$^-$ LT$^-$, CS4$^+$ CS6$^+$) (lane 1), E11881/2 (O25:H42, ST$^+$ LT$^+$, CS4$^-$ CS6$^-$) (lane 2), E17018/A (O167:H5, ST$^+$ LT$^-$, CS5$^+$ CS6$^+$) (lane 3), E17018/6 (O167:H5, ST$^-$ LT$^-$, CS5$^-$ CS6$^-$) (lane 4), E11881/14 (O25:H42, ST$^-$ LT$^-$, CS4$^-$ CS6$^+$) (lane 5), purified CS6 (1 μg) (lane 6), pET-*cssA* (lane 7), and pET-*cssB* (lane 8).

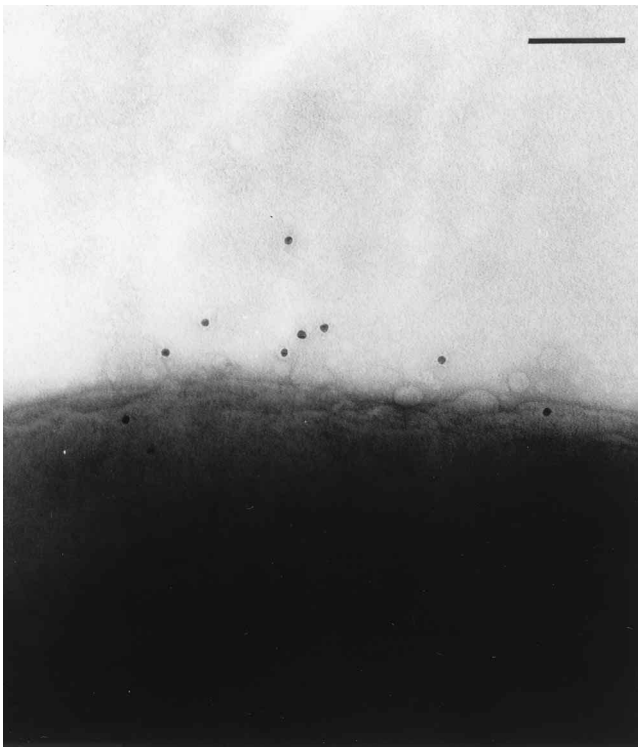

FIG. 2. Electron micrograph of immunogold-labelled bacteria showing the binding of the CS6-specific MAb CS6-20.11:9 to the CS6-positive ETEC strain E11881/14. Bar, 100 nm.

The reactivities of the MAbs were further examined by investigating by IEM their capability to bind to native CS6 on the surfaces of the bacteria. Bacteria cultured either in vitro or in vivo in rabbit ileal loops were tested. We also used phenol-treated bacteria in an attempt to stabilize the CS6 structure, since this has been shown to inactivate CS6$^+$ bacteria without affecting the antigenicity of CS6 (31). Both MAbs bound to the bacterial surface in a similar fashion and at a similar amount. The structure of CS6 could not be ascertained, although gold-labelled protrusions were visible on the surfaces of CS6-expressing bacteria (Fig. 2). The number of bound gold particles was low, despite the use of relatively high concentrations of the MAbs (i.e., 380 μg of immunoglobulin/ml). Nonspecific binding could be excluded since no labelling was seen when incubating CS6-expressing bacteria with a MAb against *E. coli* ST and then with the same anti-immunoglobulin–gold conjugate or when reacting the MAbs with CS6-negative bacteria. Polyclonal rabbit anti-CS6 sera revealed gold-labelled protrusions similar to those obtained with the MAbs.

We also attempted to produce MAbs against CssA by immunizing mice with heat extract or sonicate of pET-*cssA*. However, in spite of giving the mice several doses of such antigens, they did not respond or responded very poorly, suggesting that CssA is weakly immunogenic or that the doses given to the mice had been too low.

Development of diagnostic methods. A previously developed inhibition ELISA (12) has now been improved by replacing the polyclonal anti-CS6 serum with either of the two MAbs and by using whole CS6-expressing bacteria instead of purified CS6 as the solid-phase antigen (Fig. 3). The availability of the anti-CS6 MAbs also allowed for the development of a dot blot test (Fig. 4). Comparison of whole bacteria and heat extracts as antigens in both assays showed that whole bacteria gave somewhat lower background readings. MAb CS6-2A:14 had a more rapid and a stronger reaction in the dot blot assay than CS6-20:11:9 at similar immunoglobulin concentrations.

Testing of ETEC strains for expression of CS6. Ninety-two ETEC strains (Table 1) were analyzed for their expression of CS6 by using the dot blot test and the inhibition ELISAs developed. When whole bacteria or heat extracts of the strains were examined with MAb CS6-20:11:9 in the dot blot assay, 38 of the 40 known CS6$^+$ strains were positive for CS6; identical

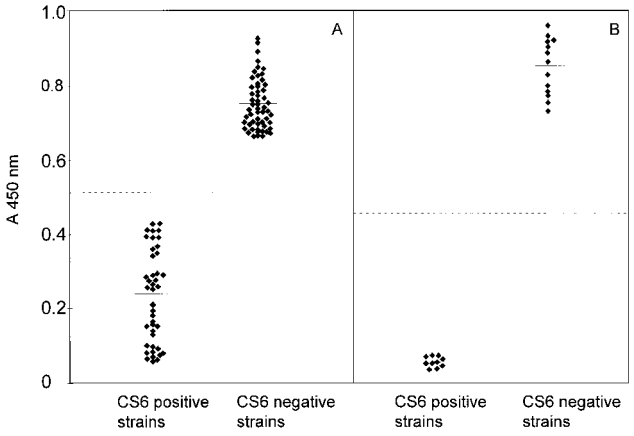

FIG. 3. Graphical presentation of the results obtained by the inhibition ELISA with MAb CS6-20:11:9 (A) and MAb CS6-2A:14 (B). The dashed line represents the 50% inhibition value. The absorbance at 450 nm was read after 15 to 20 min of enzyme reaction.

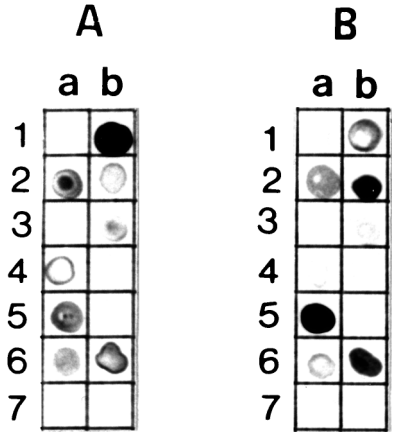

FIG. 4. Dot blot test with MAb CS6-20:11:9 (A) and MAb CS6-2A:14 (B) for the detection of whole CS6-expressing ETEC strains. CS6-expressing strains were applied in rows 2, 4, 5, and 6 of panel a and rows 1, 2, 3, and 6 of panel b. Final immunoglobulin concentrations were 2.6 μg/ml for MAb CS6-20.11:9 and 0.7 μg/ml for MAb CS6-2A:14.

results were obtained by the inhibition ELISA (Table 2). Two previously CS6-positive strains, i.e., Z 26-5-6 (CFA/III$^+$, CS6$^+$, and LT$^+$) (1a) and Z 214-2 (CS5$^+$, CS6$^+$, and ST$^+$) (19), were negative when they were tested as heat extracts and whole bacteria in either assay by using CS6-20:11:9, although the strains still expressed CFA/III and CS5, respectively. Reexamination of these two strains showed that they maintained their original toxin gene profiles and were positive by the CS6 colony hybridization assay and immunoblotting with MAb CS6-20:11:9. The remaining 52 strains were negative for CS6 by both assays. All except seven of the strains tested as whole bacteria gave results similar to those obtained with heat extracts. These seven strains that had previously been identified as being CS5$^+$, CS6$^+$, and ST$^+$ in our laboratory when heat extracts were tested (1a) were negative or only weakly positive for CS6 when whole bacteria were tested in either assay.

When using MAb CS6-2A:14, on the other hand, all 40 CS6$^+$ ETEC strains, including strains Z 26-5-6 and Z 214-2 and the seven CS5$^+$, CS6$^+$, and ST$^+$ strains, tested positive for CS6, irrespective of whether whole bacteria or heat extracts were used. The remaining 52 strains were negative for CS6. Comparable results were obtained when using the dot blot assay and the inhibition ELISA. Forty-eight of the 92 strains were analyzed by using the *cssA* probe and the MAb-based assays. The two tools for CS6 detection had excellent agreement. Thus, 31 strains tested positive with both the *cssA*-specific probe and the CssB-specific MAbs, and 17 strains were negative with both the probe and the MAbs.

We also tested six strains (all LT$^+$) (13), previously found to be positive with the *cssA* probe but phenotypically negative for CS6 when using polyclonal sera in inhibition ELISA (12), for their MAb reactivities. Neither MAb CS6-20:11:9 nor CS6-2A:14 was able to detect any production of CS6 by these strains. These strains were found to be positive for *cssB*, *cssC*, and *cssD* when they were tested by PCR (11).

DISCUSSION

In this report we describe the production of MAbs specific for CS6 of ETEC strains and their use in the identification and characterization of CS6. The specificities of the two MAbs CS6-20:11:9 and CS6-2A:14 were demonstrated by ELISA, immunoblotting, and IEM, which showed that the MAbs bound to CS6-expressing bacteria, purified CS6, and a CS6 structural subunit, i.e., CssB, but not to CS6-negative bacteria or purified non-CS6 CFs. Differences in the specificities of the two MAbs were observed. Thus, MAb CS6-20:11:9 gave a much stronger band than MAb CS6-2A:14 when reacting with CS6 subunits in immunoblots, even when MAb CS6-20:11:9 was tested at 10-fold lower immunoglobulin concentrations. MAb CS6-2A:14, on the other hand, yielded stronger signals than MAb CS6-20:11:9 in the dot blot test. On the basis of these results, we assume that MAb CS6-20:11:9 reacts with an epi-

TABLE 1. Bacterial strains used in the study (excluding the reference strains)

No. of strains	Enterotoxin profile	Antigenic designation[a]	Origin[b]
5	ST$^+$ LT$^+$	CFA/I$^+$	A, B
3	ST$^+$	CFA/I$^+$	B, C
1	—[c]	CS1$^+$	D
11	ST$^+$ LT$^+$	CS1$^+$ CS3$^+$	B, C, D, E
1	ST$^+$	CS1$^+$ CS3$^+$	B
1	—	CS2$^+$	D
7	ST$^+$ LT$^+$	CS2$^+$ CS3$^+$	B, C, E
1	ST$^+$	CS2$^+$ CS3$^+$	D
1	LT$^+$	CS2$^+$ CS3$^+$	E
2	ST$^+$ LT$^+$	CS3$^+$	D, E
1	LT$^+$	CS3$^+$	E
2	—	CS4$^+$	F
1	—	CS4$^+$ CS6$^+$	D
3	ST$^+$ LT$^+$	CS4$^+$ CS6$^+$	D, E
3	LT$^+$	CS4$^+$ CS6$^+$	E
1	—	CS4$^-$ CS6$^-$	D
5	ST$^+$ LT$^+$	CS5$^+$ CS6$^+$	C, E, G
13	ST$^+$	CS5$^+$ CS6$^+$	C, G, I
1	—	CS5$^-$ CS6$^-$	D
1	ST$^+$ LT$^+$	CS6$^+$	D
10	ST$^+$	CS6$^+$	C, G
1	ST$^+$	CS6$^+$	D
1	—	CS6$^-$	D
3	LT$^+$	CS6$^+$ CFA/III$^+$	C, F
1	—	CFA/III$^+$	F
2	LT$^+$	CS7$^+$	F
2	LT$^+$	CS17$^+$	C, F
1	LT$^+$	PCFO9$^+$	H
2	—	PCFO9$^-$	H
1	ST$^+$ LT$^+$	PCFO20$^+$	C
2	ST$^+$ LT$^+$	PCFO159$^+$	F
1	ST$^+$	PCFO166$^+$	C
1	—	PCFO166$^+$	F

[a] The antigenic designation of the strains was established by slide agglutination and immunodiffusion with polyclonal antisera or MAbs, hemagglutination, immunoblotting, ELISA, or colony hybridization.
[b] Origins: A, D. G. Evans (8); B, C. Åhrén (10); C, G. Viboud and M. Jouve (1b, 35); D, B. Rowe (4, 26, 28, 34); E, J. D. Clemens (3); F, M. M. McConnell (15, 22–24, 36); G, H. Sommerfelt (13, 30); H, P. Manning (14); I, C. Åhren (1).
[c] —, enterotoxin negative.

TABLE 2. Capability of anti-CS6 MAbs to detect CS6-expressing bacteria, as heat extracts, by dot blot assay and inhibition ELISA

Antigenic designation	No. of strains as determined by:					
	Dot blot assay				Inhibition ELISA with MAb 20:11:9	
	MAb 20:11:9		MAb 2A:14			
	+	−	+	−	+	−
CS6$^+$	38	2	40	0	38	2
CS6$^-$	0	52	0	52	0	52

tope that is better exposed on denatured than native CS6 since this MAb was obtained after immunization with SDS-PAGE-purified CS6, i.e., partly denatured protein. In contrast, MAb CS6-2A:14 seems to be specific for an epitope that is more strongly exposed on native CS6 than on denatured CS6. The development of bands other than the CS6-specific band with CS6-20:11:9 in immunoblots suggests the existence of proteins with epitopes similar to the MAb CS6-20:11:9-reactive epitope. The CssB specificity of the two MAbs was established in immunoblots with recombinant strains expressing either of the two subunits, i.e., pET-*cssA* and pET-*cssB*, or the strains with a complete or partial CS6 operon, i.e., the pDEP and pIVB recombinants. Although IEM showed that the MAbs bound specifically to protrusions both at the outer membrane as well as at some distance from the bacterium, we could not identify a structure for the CS6 protein. However, we cannot exclude the possibility that CS6 has a fine fibrillar structure, as suggested previously (19).

The availability of CS6-specific MAbs has facilitated the development of a dot blot test for the detection of CS6 which was impossible to develop with polyclonal sera due to nonspecificity. In this assay, both whole bacteria and heat extracts could be used as the antigen, although heat extracts gave rise to some background staining when MAb CS6-20:11:9 was used. This additional reactivity may correspond to the non-CS6-specific bands developed with MAb CS6-20:11:9 in immunoblots. The intensities of the developed dots, corresponding to different CS6-positive strains, varied considerably. This suggests that the expression of CS6 on the bacterial surface is quite variable. This notion was reinforced by the results obtained in the inhibition ELISA; i.e., the CS6-positive strains gave absorbance values over a broad range.

The usefulness of the MAb-based inhibition ELISA and the dot blot tests in epidemiological studies was evaluated with a number of ETEC strains with known CF profiles. The analysis revealed that all strains designated CS6 positive were found to be positive when using MAb CS6-2A:14 and either whole bacteria or heat extracts. When using MAb CS6-20:11:9, however, seven of the strains gave positive results only when using heat extracts, whereas whole bacteria gave results ranging from negative to weakly positive. This difference might be due to scarce or faulty surface expression of the CS6 subunits. Two strains, Z 26-5-6 and Z 214-2, gave discrepant results with the two MAbs; these discrepant results were probably due to the difference in epitope specificity. The remaining 52 strains, designated CS6 negative, were found to be CS6 negative when using both MAb CS6-20:11:9 and MAb CS6-2A:14. Six LT- and probe-positive strains, which also were positive for *cssB*, *cssC*, and *cssD*, tested negative for CS6 by PCR when using either MAb. A previous study showed that these probe-positive strains were negative by ELISA with polyclonal anti-CS6 sera (12). Thus, it seems that for some yet unexplained reason, these strains are incapable of expressing CS6, in spite of being positive by all available genotypic CS6 tests. The results emphasize the difference between PCR and probes and the MAbs in detection capability, i.e., the difference between genotypic and phenotypic characterization of bacterial strains. Thus, these strains are genotypically CS6 positive but phenotypically CS6 negative.

Forty-eight strains were also tested for CS6 by using the *cssA* probe, and the results correlated with those obtained by the MAb-based assays. These results indicate the simultaneous presence of *cssA* and *cssB* in the genomes of the bacteria and emphasize the potential of these MAbs for use as diagnostic tools. The MAb-based inhibition ELISA and dot blot tests described in this study may thus be useful for future epidemiological studies with human-derived ETEC isolates. Since all strains described to date express CS4 and CS5 together with CS6, the characterization of ETEC strains with regard to CS4 and CS5 needs to be performed only if the strains test positive for CS6.

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