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Murine Monoclonal Antibodies against *Escherichia coli* O4 Lipopolysaccharide and H5 Flagellin

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Two murine monoclonal antibodies (MAb), 2C5-F10 and 8D1-H10, reactive with *Escherichia coli* O4 and H5 antigens, respectively, were generated and characterized. Enzyme immunoassays and immunoblots demonstrated that MAb 2C5-F10 reacted specifically with lipopolysaccharide O antigen of *E. coli* O4 isolates, while MAb 8D1-H10 reacted with *E. coli* strains expressing H5 flagella.

Escherichia coli expressing O4 lipopolysaccharide (LPS) and/or H5 flagellin frequently causes extraintestinal infections in humans and domestic animals (1, 10, 19). *E. coli* O4 organisms are common etiologic agents of human, canine, and feline genitourinary tract infections, manifested as pyelonephritis, cystitis, prostatitis, urosepsis, and asymptomatic bacteriuria (4, 8, 13, 25, 31). In particular, the *E. coli* O4:H5 serotype is a potent human uropathogen, especially the disseminated virulent J96 clone (14, 15). *E. coli* O4 and/or H5 antigens are also associated with enteric disease. Shiga toxin-producing *E. coli* (STEC) O4:H-negative (26), O4:H5 (11), O4:H10 (29), O2:H5 (5, 27), and O75:H5 (5) strains have caused hemorrhagic colitis and hemolytic uremic syndrome cases, while non-STEC O4 and enteroaggregative O4 have caused pediatric diarrhea outbreaks (6, 7). STEC O4:H-negative (28), O4:H4 (3), O4:H21 (24), O4:H25 (30), and O2:H5 (23) have been isolated from healthy cattle, while STEC O4 and non-STEC O4 have been associated with calves (18), pigs (9), and lambs (2) with diarrhea. While the *E. coli* O4 and H5 antigens are both markers of strain pathogenic potential, the O4 antigen moiety may itself function as a urovirulence factor (22).

Bacteria reference laboratories usually perform *E. coli* O and H serotyping using agglutination assays and rabbit hyperimmune antisera against reference O ($n = 181$) and H ($n = 52$) antigens (20; R. A. Wilson, personal communication). However, absorbed anti-*E. coli* O4 polyclonal antibodies (PAb) may cross-react with *E. coli* possessing O antigens 12, 13, 16, 18, 19, and 102 (20). Furthermore, *E. coli* expressing H antigens 1, 4, 8, 12, 38, 44, and 56 may cross-react with anti-H5 PAb (R. A. Wilson, personal communication). Anti-*E. coli* O4 and H5 monoclonal antibodies (MAb) have not been reported but offer potential diagnostic specificity, reagent quality, and typing assay format flexibility advantages over conventional agglutinating PAb. We generated anti-O4 and anti-H5 MAb in order to possess accurate serotyping reagents for use in ongoing and planned epidemiologic surveys for pathogenic and zoonotic *E. coli* in livestock.

MAb were produced from splenocytes of BALB/c mice immunized with *E. coli* O4:K3:H5 (*E. coli* Reference Center [ECRC] U4-41, the O4 and H5 reference strain) (20) whole-bacterium lysate and boosted with semipurified H5 flagellin (12). Immunization, hybridoma and ascites production, and MAb screening, isotyping, and characterization protocols were as previously described (12, 21). One anti-*E. coli* O4 MAb (2C5-F10; immunoglobulin M [IgM] isotype) and one anti-H5 MAb (8D1-H10; IgG1 isotype) were generated and characterized. MAb diagnostic sensitivity and specificity were estimated by enzyme-linked immunosorbent assay (ELISA) reactivity with whole-bacterium lysate preparations from 350 antigenically diverse gram-negative bacterial (272 *E. coli* and 78 non-*E. coli*) strains. The *E. coli* subset consisted of 8 O4:H5 strains, 4 O4:non-H5 strains, 8 non-O4:H5 strains, and 252 non-O4:non-H5 (including 1 non-O4:H autoagglutinable [HA]) strains of various O:H serotypes including 12 non-O4 isolates reported to react with anti-O4 PAb (O12 [$n = 2$], O16, O18 [$n = 5$], O19 [$n = 3$], O102) and 33 non-H5 isolates reported to react with anti-H5 PAb (H1 [$n = 5$], H4 [$n = 14$], H8 [$n = 5$], H12 [$n = 4$], H38 [$n = 3$], H44, and H56). Overall, *E. coli* isolates possessed 102 different O antigens and 51 different H antigens. Non-*E. coli* strains consisted of 50 *Salmonella* spp. and 28 other gram-negative bacteria from 22 genera. For ELISA, bacterial-antigen-coated plates were sequentially incubated with MAb (diluted ascites fluid), horseradish peroxidase (HRP)-conjugated antibody against mouse IgG plus mouse IgM (anti-mouse IgG+IgM), and 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate(6)] solution (ABTS peroxidase substrate) (12). ELISA optical density at dual wavelengths of 405 and 490 nm ($OD_{405/490}$) was measured, and $OD_{405/490}$ of >0.200 was considered positive (12). Dot box plots (16) of MAb ELISA $OD_{405/490}$ values for bacterial antigen subsets were generated (Prism 3.0; Graph Pad Software Inc., San Diego, Calif.), and MAb diagnostic-sensitivity and -specificity point estimates with exact binomial 95% confidence intervals (CI) were calculated (Epi Info 6.0; Centers for Disease Control and Prevention, Atlanta, Ga.). Sensitivity was defined as the number of MAb ELISA-positive isolates per the total number of isolates tested possessing the target (O4 or H5) antigen. Specificity was defined as the number of MAb ELISA-nonreactive isolates per the total number of isolates tested that did not possess the target antigen.

MAb 2C5-F10 was ELISA reactive with 12 *E. coli* O4 iso-

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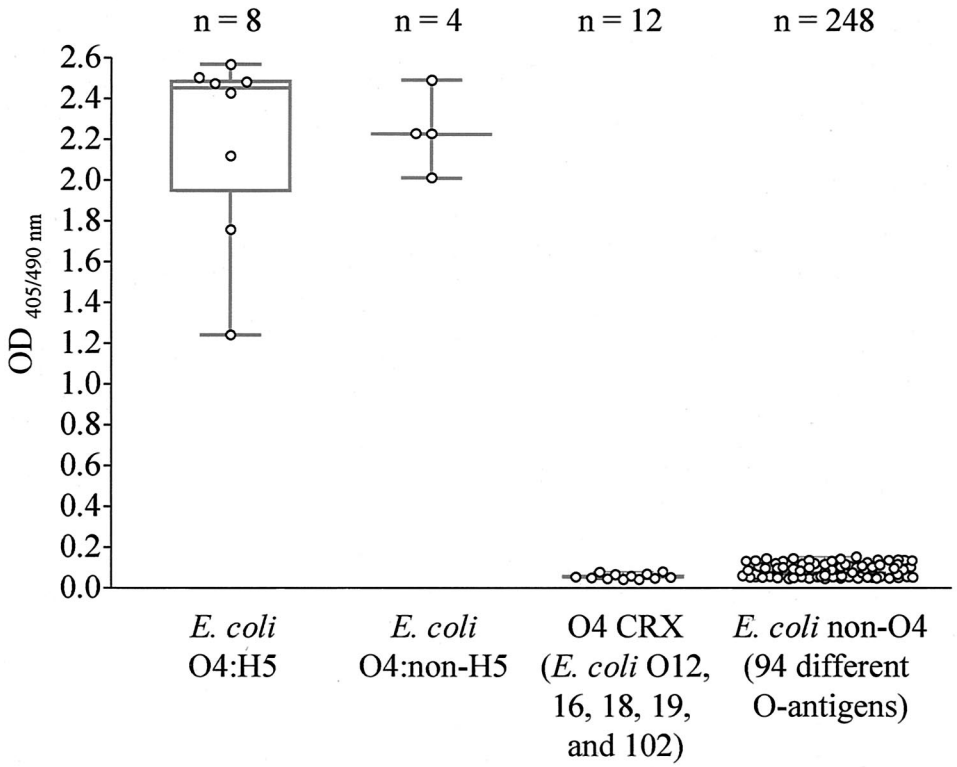

FIG. 1. Dot box plot of ELISA reactivities of 272 whole-bacterium lysates with anti-*E. coli* O4 MAb 2C5-F10. ELISA plates coated with whole-bacterium antigens were incubated sequentially with MAb 2C5-F10 (ascites fluid; 1:32,000), anti-mouse IgG+IgM-HRP conjugate, and ABTS peroxidase substrate. A MAb ELISA $OD_{405/490}$ of >0.200 was considered positive. Dots represent means of duplicate OD values. The bottom and top edges of the superimposed box plots are the 25th and 75th distribution percentiles, respectively; the central horizontal line represents the median (50th percentile), and the central vertical lines extend from the box as far as the data extend (range). CRX, non-*E. coli* O4 bacteria known to cross-react with anti-*E. coli* O4 PAb. Data for MAb ELISA reactivity with 78 non-*E. coli* isolates are not shown.

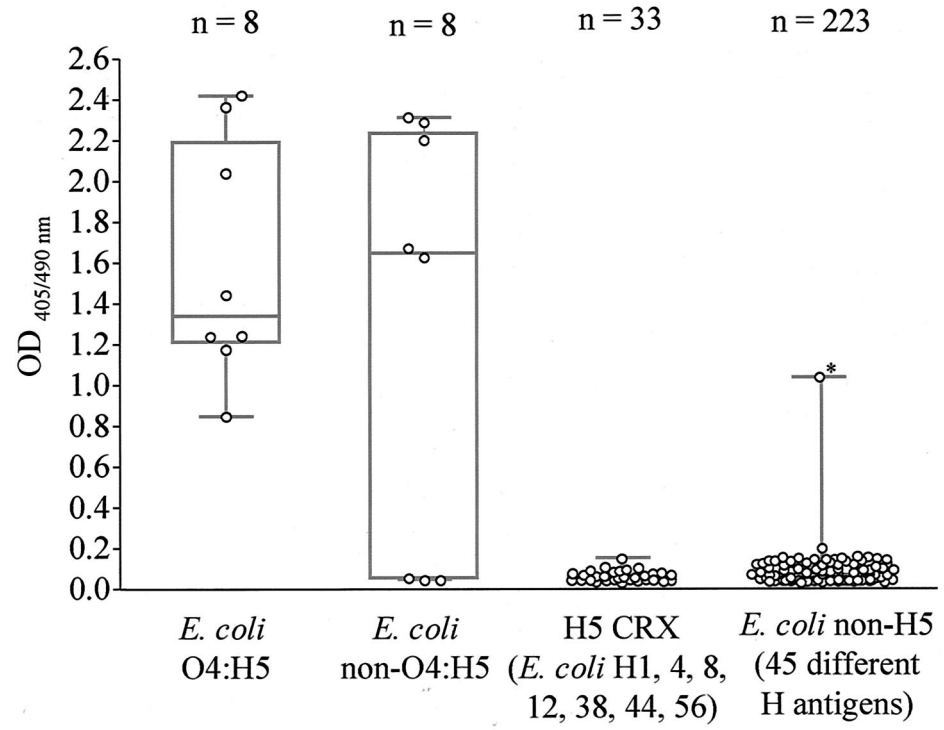

FIG. 2. Dot box plot of ELISA reactivities of 272 whole-cell bacterium lysates with anti-*E. coli* H5 MAb 8D1-H10. ELISA plates coated with whole-bacterium antigens were incubated sequentially with MAb 8D1-H10 (ascites fluid; 1:50,000), IgG+IgM-HRP, and ABTS solution. OD values represent means of duplicate wells. For dot box plot interpretation, see the legend for Fig. 1. CRX, non-*E. coli* H5 bacteria known to cross-react with anti-*E. coli* H5 PAb; *, datum point for *E. coli* O2:HA (autoagglutinable) 92–0609, which was presumptively H5 based on Western immunoblotting (Fig. 4). Data for MAb ELISA reactivity with 78 non-*E. coli* isolates are not shown.

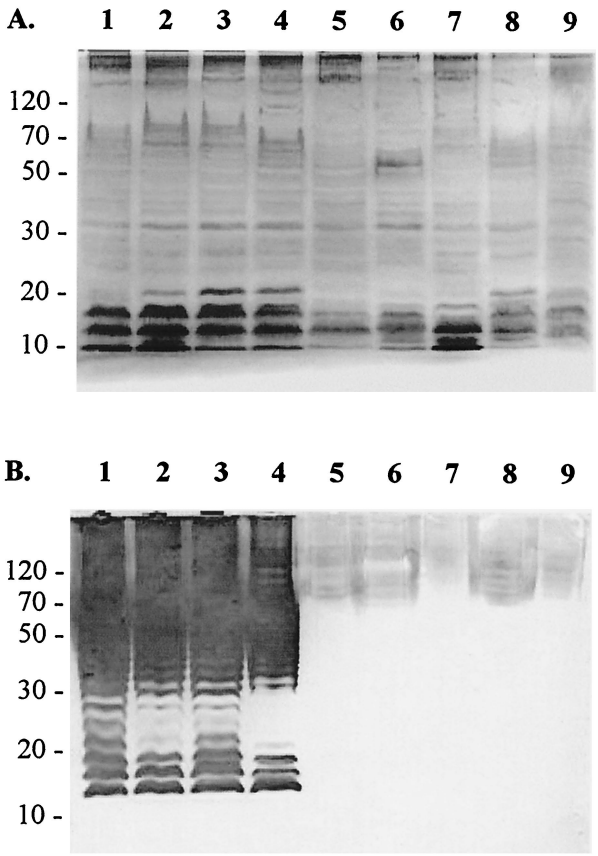

FIG. 3. SDS-PAGE and Western blots of MAb 2C5-F10 (anti-*E. coli* O4) on whole-bacterium antigens (25 μg per well). Lane 1, *E. coli* O4:H5 U4-41; lane 2, *E. coli* O4:H5 J96; lane 3, *E. coli* O4:H5 CA002; lane 4, O4:H32 90-1870; lane 5, *E. coli* O-negative:NM CDC 3377-85; lane 6, O-negative:H-negative 90-1870; lane 7, *E. coli* O18:H5 94-0061; lane 8, *E. coli* O12:H6 H9; and lane 9, *E. coli* O13:H11 SU 4321-41. After electrophoresis, one gel was silver stained (A). The other gel was transferred onto a polyvinylidene difluoride membrane and incubated with MAb 2C5-F10 (cell culture medium; 1:2) (B). MAb-reactive bands were revealed with rabbit anti-mouse IgG+IgM-HRP (1:1,000) and diaminobenzidine substrate. Molecular masses (in kilodaltons) are indicated on the left.

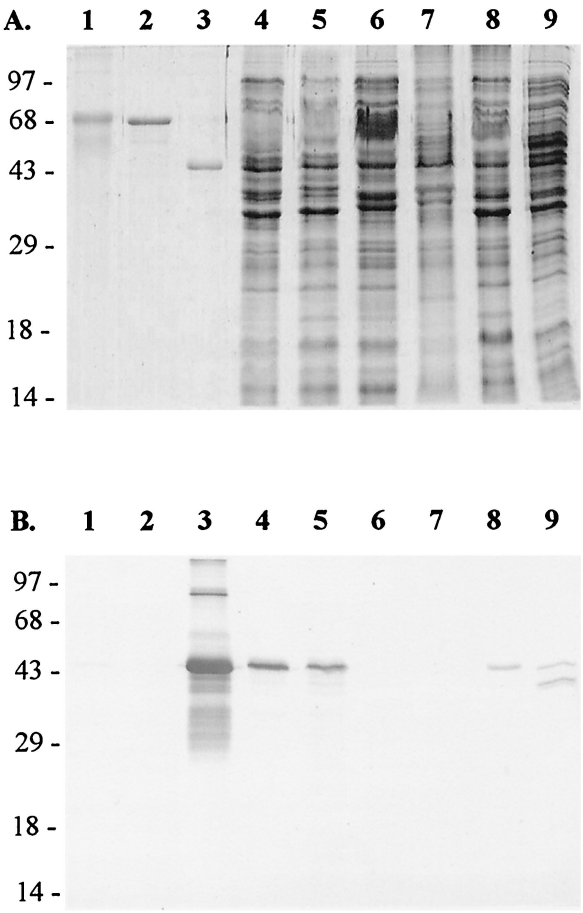

FIG. 4. SDS-PAGE and Western blots of MAb 8D1-H10 (anti-*E. coli* H5) on partially purified flagellins (lanes 1 to 3; 2 μg per well) and whole-bacterium antigens (lanes 4 to 9; 24 μg per well). Lane 1, *E. coli* O157:H7 43895; lane 2, *E. coli* O9:H12 Bi316-42; lane 3, *E. coli* O4:H5 U4-41; lane 4, *E. coli* O4:H5 J96; lane 5, *E. coli* O75:H5 CL35; lane 6, *E. coli* O166:H-negative 94-0287; lane 7, *E. coli* O139:H56 SN3N/1; lane 8, *E. coli* O4:H5 91-1750; and lane 9, *E. coli* O2:HA 94-0609. After electrophoresis, one gel was Coomassie brilliant blue stained (A). The other gel was transferred onto a polyvinylidene difluoride membrane and incubated with MAb 8D1-H10 (cell culture medium; 1:1,000) (B). MAb-reactive bands were revealed with rabbit anti-mouse IgG+IgM-HRP (1:1,000) and diaminobenzidine substrate. Molecular masses (in kilodaltons) are indicated on the left.

lates (sensitivity, 100%; 95% CI, 73.5 to 100) and nonreactive with 260 non-O4 *E. coli* and 78 non-*E. coli* isolates (specificity, 100%; 95% CI, 98.9 to 100) (Fig. 1). Significantly, there was no MAb ELISA reactivity with 12 bacteria that cross-react with anti-O4 PAb.

MAb 8D1-H10 reacted with 13 of 16 *E. coli* H5 isolates (sensitivity, 81.3%; 95% CI, 54.4 to 96.0) (Fig. 2); the three nonreactive strains were *E. coli* O75:H5 isolates. In spite of repeated attempts to induce and select for motility using flagellum broth and semisolid motility agar (12), these three strains remained nonmotile in our laboratory both macroscopically in media and microscopically under phase-contrast microscopy. This suggests that these *E. coli* O75:H5 strains may have been nonreactive with the anti-H5 MAb due to lack of expression of flagellar antigens (12). This MAb did not react with 78 non-*E. coli* strains but reacted with 1 of 255 non-H5 *E. coli* (specificity, 99.6%; 95% CI, 97.9 to 99.9) strains (Fig. 2). The cross-reactive strain was *E. coli* O2:HA (ECRC 94-0609), which possesses an unknown H-antigen.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western immunoblotting using whole-bacterium lysates, semipurified flagellin, and LPS preparations from *E. coli* O4 and H5 and non-O4 and non-H5 antigens confirmed MAb O4 and H5 specificity (12, 21) (Fig. 3 and 4). Reactive bands from gels transferred onto polyvinylidene difluoride membranes and incubated with MAb were revealed with HRP-conjugated rabbit anti-mouse IgG+IgM and diaminobenzidine substrate. The ladder pattern characteristic of LPS was observed on silver-stained gels and membranes incubated with anti-O4 MAb 2C5-F10 (Fig. 3), which reacted only with *E. coli* O4:H5 (lanes 1 to 3) and O4:H32 (lane 4), but not with four other whole-bacterium preparations (Fig. 3B).

TABLE 1. ELISA reactivities of MAb 2C5-F12 (anti-O4) and 8D1-H10 (anti-H5) to 37 *E. coli* whole-bacterium antigens

Isolate group	O-H serotype	Strain	ELISA reactivity of:	
			MAb 2C5-F12 (anti-O4)	MAb 8D1-H10 (anti-H5)
O4:non-H5	O4:NM	Bos 021	+	−
	O4:NM	Bos 038	+	−
	O4:NM	Bos 046	+	−
	O4:NM	Bos 105	+	−
	O4:NM	PM8	+	−
	O4:H1	Afr 015	+	−
	O4:H1	Bos 040	+	−
	O4:H1	V31	+	−
	O4:H-multiple[a]	Z	−	−
O4:H5	O4:H5	CP9	+	+
	O4:H5	R28	+	+
	O4:H5	518	+	+
non-O4:H5	O85/157:H5	Bos 023	−	+
non-O4:non-H5	Ox7w:H16	Y	−	−
	O15:H1	2H17	−	−
	O15/40:H1	V32	−	−
	O17:H8	V11	−	−
	O18ac:H7	2H25	−	−
	O18:Hagge[b]	V	−	−
	O18:H23	C	−	−
	O19:H27	G	−	−
	O36:nt[c]	U	−	−
	O59:nt	B	−	−
	O63w:H6	R	−	−
	O64:H21	PM1	−	−
	O73w:nt	F	−	−
	O75:NM	N	−	−
	O82:H12	X	−	−
	O84:H-	V12	−	−
	O86:H12	M	−	−
	O113:H-	E	−	−
	O143:H-	2V5	−	−
	O149w:H8	I	−	−
	O152:Hnt	IA	−	−
	O169w:H8	L	−	−
	Ont:H7/24	D	−	−
	Ont:H16	A	−	−

[a] Serotyped as O82:H-multiple on resubmission to ECRC after MAb testing.
[b] agge, autoaggregative.
[c] nt, nontypeable.

Similar results were obtained using semipurified LPS as antigen (data not shown).

Specificity of MAb 8D1-H10 for H5 antigen was confirmed by SDS-PAGE of semipurified flagellins and whole-bacterium preparations followed by immunoblotting (Fig. 4). MAb 8D1-H10 reacted predominantly with purified flagellins (lane 3) and whole-bacterium preparations (lanes 4, 5, and 8) of *E. coli* H5 at the expected molecular mass of 46 kDa (17). MAb 8D1-H10 immunoblotting of ELISA-reactive *E. coli* O2:HA 94-0609 produced a doublet of reactive bands at M_r of ≈46 and ≈37 kDa. The 46-kDa reactive band of this autoagglutinable strain was of a size consistent with H5 flagellin; the 37-kDa band could be an H5 degradation product or a non-H5 flagellin.

To validate MAb performance, a second panel of 37 *E. coli* O4, H5, non-O4, and non-H5 human clinical isolates (kindly provided by J. R. Johnson, University of Minnesota, St. Paul) was tested blindly for ELISA reactivity separately with each MAb (Table 1). The anti-O4 MAb reacted with 11 of 12 *E. coli* O4 strains and with 0 of 25 non-O4 strains. The nonreactive *E. coli* O4:H-multiple strain Z was serotyped as O82:H-multiple when submitted to the ECRC after MAb ELISA testing. The anti-H5 MAb reacted only with the four *E. coli* H5 strains.

In conclusion, we produced MAb against the *E. coli* O4 and H5 antigens that appeared to be sensitive and specific as assessed by ELISA and immunoblotting. These MAb have potential clinical use as immunodiagnostic or serotyping reagents, especially if used in combination with other phenotypic or genotypic pathogenicity markers. They may be of particular utility for experimental or epidemiologic studies of extraintestinal *E. coli* O4:H5 infections.

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