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Serotyping of *Listeria monocytogenes* by Enzyme-Linked Immunosorbent Assay and Identification of Mixed-Serotype Cultures by Colony Immunoblotting

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Routine analysis of *Listeria monocytogenes* by serotyping using traditional agglutination methods is limited in use because of the expense and limited availability of commercially prepared antisera and intra- and interlaboratory discrepancies arising from differences in antiserum preparation and visual determination of agglutination. We have adapted a commercially available set of *L. monocytogenes* antisera to an enzyme-linked immunosorbent assay (ELISA) format for high-throughput, low-cost serotype determination. Rather than subjective visualization of agglutination, positive antigen and antiserum reactions were scored by a quantitative, colorimetric reaction. ELISA serotyping of 89 of 101 *L. monocytogenes* isolates agreed with slide agglutination serotyping data, and 100 previously uncharacterized isolates were serotyped unambiguously by the ELISA method. In addition, mixed-serotype cultures of *L. monocytogenes* were identified by a colony immunoblot procedure, in which serogroup 1/2 and serogroup 4 colonies were discriminated by differential staining.

Listeria monocytogenes is a gram-positive, food-borne bacterial pathogen that causes listeriosis in susceptible individuals (4). In addition, *L. monocytogenes* is a widespread, saprophytic bacterium that can be found not only in association with soil, plants, and animal waste (5, 8, 23, 24) but also as a persistent organism in food and dairy processing environments (3, 14). All of these environments are potential sources for contamination of fresh and prepared foods with *L. monocytogenes*, which, in turn, poses a significant public health risk in terms of the potential for listeriosis outbreaks. In order for these risks to be minimized, subtyping of *L. monocytogenes* isolates has been undertaken in several laboratories in recent years to begin to identify type-specific factors contributing to virulence, persistence, and/or transmissibility of the bacterium relative to its outbreak potential (7, 10–13, 20, 22).

Current methods to subtype *L. monocytogenes* include DNA fingerprinting using specific (7) or random (10, 12) PCR primers, ribotyping (10, 12, 13), and pulsed-field gel electrophoresis (1). However, groupings based on these methods are still often compared to groupings based on serotype, using an agglutination method and subgrouping scheme developed by Seeliger and Höhne (18). This method differentiates *L. monocytogenes* into 12 different serotypes based on the reactions of somatic (O) and flagellar (H) antigens with a series of polyvalent and monovalent antisera. Serotyping of *L. monocytogenes* isolates is not routinely performed outside of public health reference laboratories due to the limited availability and high cost of commercially produced antisera and to the inconvenience and reliability issues associated with producing one's own antisera using specific reference strains of *L. monocytogenes*. Conversely, using a reference laboratory for serotyping large numbers of *L. monocytogenes* isolates may also be prohibitively costly or time-consuming for individual laboratories. We sought to adapt a commercial serotyping kit to a format that would be cost-effective, reliable, and of sufficiently high throughput to facilitate serotype determination of *L. monocytogenes*. Using a 96-well enzyme-linked immunosorbent assay (ELISA) format instead of agglutination as a means to score reactions with each antiserum, this method provides a semiquantitative measurement of positive and negative reactions and requires only a fraction of the antisera used in the agglutination assay. In addition, we used antisera from the same serotyping kit in colony immunoblot experiments to identify mixed-serotype *L. monocytogenes* cultures, as an initial method to resolve ambiguous intra- and interlaboratory serotyping results.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *L. monocytogenes* strains used in this study are listed in Table 1. Isolates were confirmed as *L. monocytogenes* by production of turquoise colonies on BCM *L. monocytogenes* plating medium (Biosynth International, Naperville, Ill.) (15) and by PCR amplification of an *iap* gene fragment using *L. monocytogenes*-specific primers (2). Stock cultures of all strains were stored at −80°C in Bacto tryptic soy broth without dextrose (Difco) containing 0.6% (wt/vol) yeast extract (Difco) (TSYE) and 1 M glycerol. Working cultures were maintained on TSYE agar and grown at 30°C. Prior to serotype determination, single colonies of each strain were inoculated onto brain heart infusion (BHI) (Difco) motility plates containing 0.3% (wt/vol) agar and grown for 24 h at 30°C. Bacteria from the edges of the motility plate-grown colonies were then inoculated into 5 ml of BHI broth and incubated overnight at 30°C.

Serotyping. One hundred one *L. monocytogenes* isolates were serotyped by both the agglutination method and the ELISA method. The two methods were performed independently by two different laboratories. Twenty-six strains were tested three times using three different lots of serotyping reagents (Table 1).

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TABLE 1. *L. monocytogenes* strains used in this study

Isolate	Strain no.	Origin	Serotype by:		Source
			Agglutination	ELISA	
10403	RM2194	Human, clinical	1/2a	1/2a	D. Portnoy
32490G	RM2985	Bulk milk	1/2a	1/2a	USDA[a]
35568A	RM2989	Bulk milk	1/2a	1/2a	USDA
10867C	RM2990	Bulk milk	1/2a	1/2a	USDA
51772	RM3015	Cheese	1/2a	1/2a	ATCC[b]
19111	RM3023	Poultry	1/2a	1/2a	ATCC
J0098	RM3029	Food	1/2a	1/2a	CDC[c]
12443	RM3102	Monkey, clinical	1/2a	1/2a	R. Rabourne
FSL-J2-020	RM3152	Cow	1/2a	1/2a	M. Wiedmann
FSL-C1-056	RM3160	Human, sporadic	1/2a	1/2a	M. Wiedmann
FSL-J2-054	RM3161	Sheep	1/2a	1/2a	M. Wiedmann
FSL-J2-031	RM3163	Cow	1/2a	1/2a	M. Wiedmann
FSL-J2-066	RM3164	Sheep	1/2a	1/2a	M. Wiedmann
FSL-J2-063	RM3165	Sheep	1/2a	1/2a	M. Wiedmann
FSL-J1-101	RM3175	Human, sporadic	1/2a	1/2a	M. Wiedmann
FSL-N3-031	RM3184	Food, sporadic	1/2a	1/2a	M. Wiedmann
FSL-R2-499	RM3185	Human, epidemic	1/2a	1/2a	M. Wiedmann
750[f]	RM3370	Environmental	1/2a	1/2a	WADOH[d]
841[f]	RM3372	Environmental	1/2a	1/2a	WADOH
1155[f]	RM3373	Human	1/2a	1/2a	WADOH
1157[f]	RM3374	Human	1/2a	1/2a	WADOH
1160[f]	RM3376	Human	1/2a	1/2a	WADOH
1162[f]	RM3378	Human	1/2a	1/2a	WADOH
1163[f]	RM3379	Environmental	1/2a	1/2a	WADOH
1165[f]	RM3381	Environmental	1/2a	1/2a	WADOH
1166[f]	RM3382	Human	1/2a	1/2a	WADOH
1445[f]	RM3385	Blood vessel	1/2a	1/2a	WADOH
17209	RM2991	Sheep brain	1/2b	1/2b	I. Wesley
16888	RM2995	Cow brain	1/2b	4b	I. Wesley
G848	RM3024	Unknown	1/2b	1/2b	CDC
FSL-J2-064	RM3155	Cow	1/2b	1/2b	M. Wiedmann
FSL-J1-177	RM3156	Human, sporadic	1/2b	1/2b	M. Wiedmann
FSL-J2-035	RM3157	Goat	1/2b	1/2b	M. Wiedmann
9900101[f]	RM3368	Environmental	1/2b	1/2b	WADOH
9900104[f]	RM3369	Human	1/2b	1/2b	WADOH
842[f]	RM3371	Blood vessel	1/2b	1/2b	WADOH
1159[f]	RM3375	Human	1/2b	1/2b	WADOH
1164[f]	RM3380	Human	1/2b	1/2b	WADOH
G-3321	RM3014	Human	1/2c	1/2c	CDC
H9666	RM3017	Blood	1/2c	1/2c	CDC
H9333	RM3018	Blood	1/2c	1/2c	CDC
H9066	RM3019	Mushrooms	1/2c	1/2c	CDC
H9067	RM3020	Cheese	1/2c	1/2c	CDC
H7973	RM3021	Blood	1/2c	1/2c	CDC
FSL-J1-094	RM3166	Human, sporadic	1/2c	1/2, nonmotile	M. Wiedmann
9900096[f]	RM3367	Environmental	1/2c	1/2c	WADOH
J0095	RM3026	Pie	3a	3a	CDC
FSL-C1-115	RM3167	Human, sporadic	3a	1/2a	M. Wiedmann
FSL-J1-169	RM3158	Human, sporadic	3b	1/2b	M. Wiedmann
J0096	RM3027	Chicken	3c	3c	CDC
FSL-J1-049	RM3159	Human, sporadic	3c	3c	M. Wiedmann
FSL-J1-031	RM3168	Human, sporadic	4a	4c	M. Wiedmann
FSL-J1-168	RM3169	Human, sporadic	4a	4c	M. Wiedmann
FSL-X1-010	RM3171	Unknown	4a	4a	M. Wiedmann
F2379	RM2199	Cheese, outbreak	4b	4b	D. Portnoy
8807-X2	RM2983	Sheep brain	4b	4b	WADDL[e]
013668A	RM2984	Cow brain	4b	4b	WADDL
35584A	RM2986	Bulk milk	4b	4b	USDA
2149	RM2987	Human, clinical	4b	4b	I. Wesley
2219	RM2988	Coleslaw	4b	4b	I. Wesley
2223	RM2992	Cucumber	4b	4d/4e	I. Wesley
13565A	RM2996	Bulk milk	4b	4b	USDA
11056A	RM2997	Bulk milk	4b	4b	USDA
2207	RM2998	Human, stillbirth	4b	4b	I. Wesley
19115	RM3013	Human	4b	4b	ATCC
J0097	RM3028	Human	4b	4b	CDC

Continued on following page

TABLE 1—*Continued*

Isolate	Strain no.	Origin	Serotype by:		Source
			Agglutination	ELISA	
G3990	RM3098	Cheese	4b	4b	R. Rabourne
G3982	RM3099	Cheese	4b	4b	R. Rabourne
H7550	RM3100	Hot dog	4b	4b	R. Rabourne
ScottA	RM3101	Human, clinical	4b	4b	R. Rabourne
12375	RM3103	Monkey, clinical	4b	4b	R. Rabourne
FSL-J1-225	RM3150	Human, clinical	4b	4b	M. Wiedmann
FSL-N1-225	RM3151	Human, epidemic	4b	4b	M. Wiedmann
FSL-J1-110	RM3153	Food, epidemic	4b	4b	M. Wiedmann
FSL-C1-122	RM3154	Human, sporadic	4b	4b	M. Wiedmann
FSL-J1-158	RM3173	Goat	4b	4b	M. Wiedmann
FSL-J1-108	RM3176	Human, epidemic	4b	4b	M. Wiedmann
FSL-J1-116	RM3177	Human, epidemic	4b	4b	M. Wiedmann
FSL-J1-119	RM3178	Human, epidemic	4b	4b	M. Wiedmann
FSL-J1-126	RM3179	Human, epidemic	4b	4b	M. Wiedmann
FSL-N1-227	RM3180	Food, epidemic	4b	4b	M. Wiedmann
FSL-N3-008	RM3181	Food, epidemic	4b	4b	M. Wiedmann
FSL-N3-013	RM3182	Food, epidemic	4b	4b	M. Wiedmann
FSL-N3-022	RM3183	Food, epidemic	4b	4b	M. Wiedmann
9900094[f]	RM3366	Human, spinal fluid	4b	4b	WADOH
1161[f]	RM3377	Human	4b	4b	WADOH
1167[f]	RM3383	Human	4b	4b	WADOH
1329[f]	RM3384	Human	4b	4b	WADOH
2140[f]	RM3386	Human, spinal fluid	4b	4b	WADOH
2150[f]	RM3387	Tissue	4b	4b	WADOH
2172[f]	RM3388	Stool	4b	4b	WADOH
F-4565[f]	RM3390	Human	4b	4b	CDC
G-1092[f]	RM3391	Human	4b	4b	CDC
19116	RM3022	Chicken	4c	4c	ATCC
J0099	RM3030	Bull	4c	4c	CDC
FSL-X1-009	RM3170	Unknown	4c	4b	M. Wiedmann
FSL-X1-008	RM3172	Unknown	4c	4b	M. Wiedmann
36467C[f]	RM3389	Bulk milk	4c	4c	USDA
J0094	RM3025	Human	4d	4d	CDC
19118	RM3016	Chicken	4e	4b	ATCC
FSL-M1-004	RM3162	Human, sporadic	N/A	3a	M. Wiedmann

[a] USDA, U.S. Department of Agriculture.
[b] ATCC, American Type Culture Collection, Manassas, Va.
[c] CDC, Centers for Disease Control and Prevention, Atlanta, Ga.
[d] WADOH, Washington State Department of Health, Olympia.
[e] WADDL, Washington Animal Disease Diagnostic Laboratory, Pullman.
[f] Tested by three different lots of serotyping reagents.

(i) Agglutination method. Serotyping was performed by a slide agglutination assay using commercially prepared antisera (*Listeria* antiserum Seiken kit; Denka Seiken Co., Tokyo, Japan) according to the manufacturer's instructions.

(ii). ELISA method. Cultures of *L. monocytogenes* grown as described were pelleted by centrifugation (14,000 × *g* for 5 min) and resuspended in an equal volume of 0.2% (wt/vol) NaCl. Cells to be used for O-antigen determination were autoclaved for 30 min at 121°C, allowed to cool to room temperature, centrifuged and resuspended in 0.2% NaCl to an optical density at 600 nm of 0.3 to 0.4. Cells to be used for H-antigen determination were centrifuged, resuspended in an equal volume of 4% (wt/vol) formaldehyde–0.2% NaCl, and incubated at room temperature for 1 h. Cells were then washed, pelleted by centrifugation, and resuspended in 0.2% NaCl to an optical density at 600 nm of 0.3 to 0.4. Prepared cells were added (70 μl/well) to ELISA well strips (MaxiSorp flat-bottom well strips; Nalge Nunc International, Rochester, N.Y.) and allowed to dry at 40°C overnight. All subsequent steps were performed at room temperature. After rinsing the wells with distilled, deionized water (ddH_2O), nonspecific surfaces were blocked with 200 μl/well of casein blocking solution (0.5% casein, 30 mM NaN_3, 150 mM NaCl, 10 mM Tris-HCl [pH 7.5]) for 1 h. After removing blocking solution and rinsing with ddH_2O, individual antisera (*Listeria* antiserum Seiken kit) were diluted in dilution buffer (1% bovine serum albumin, 0.1% Tween 20, 2.7 mM KCl, 15 mM NaN_3, 150 mM NaCl, 10 mM Tris-HCl [pH 7.5]), and 100 μl was added to each well and incubated for 1 h. Wells were washed twice with wash buffer (0.1% Tween 20, 150 mM NaCl, 10 mM Tris-HCl [pH 7.5]) and twice with ddH_2O. Alkaline phosphatase-conjugated goat anti-rabbit antibody (Zymed Laboratories, South San Francisco, Calif.) was diluted 1:1,000 in dilution buffer, and 100 μl was added to each well and incubated for 1 h. After washing the wells as before, 100 μl of 1-mg/ml *p*-nitrophenyl phosphate in 1 M diethanolamine–0.5 mM $MgCl_2 \cdot 6H_2O$, pH 9.8, was added to each well and incubated for 30 min. *p*-Nitrophenyl phosphate hydrolysis was measured as A_{405} using a SpectraMax 340 microplate reader (Molecular Devices Corp., Sunnyvale, Calif.). Average values of duplicate reactions of each strain for each O-factor antiserum and H-factor antiserum were calculated relative to the maximum O-factor antiserum and H-factor antiserum reactions, respectively, for that strain. For each strain-antiserum combination, duplicate wells containing cell suspensions incubated without primary antisera and antisera incubated without cell suspensions were included as negative controls. Serotypes were assigned according to the scheme described by Seeliger and Höhne (18).

Colony immunoblotting. Colonies of *L. monocytogenes* grown on BHI plates at 30°C were transferred by colony lift to nitrocellulose filters (pore size, 0.45 μm; Schleicher and Schuell, Keene, N.H.) for 10 min. Filters were washed (twice for 10 min each) in wash buffer to remove cell debris and incubated in 20 ml of casein blocking solution for 1 h. As the primary antibody, *L. monocytogenes* O-factor antiserum I/II (from the *Listeria* antiserum Seiken kit), which is specific for all serotype 1/2 and 3 strains, was diluted 1:2,000 in 10 ml of dilution buffer and incubated with blocked filters for 30 min. Filters were then washed (twice for 10 min each) with 20 ml of wash buffer and incubated for 30 min with 10 ml of a 1:2,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit antibody. After washing (twice for 10 min each) with 20 ml of wash buffer, colonies positively reacting with antiserum I/II were detected using 10 ml of naphthol-AS-phosphate (0.2 mg/ml)–Fast Red TR (0.1 mg/ml; multicolor detection set

Red; Roche Molecular Biochemicals, Indianapolis, Ind.). After color development, alkaline phosphatase was inactivated by incubating the filters for 10 min in 50 mM EDTA, pH 8.0, at 80°C. The filters were then blocked as before, and the same procedure was performed, except that *L. monocytogenes* O-factor antiserum V/VI (from the *Listeria* antiserum Seiken kit), which is specific for all serotype 4 strains, was used as the primary antibody. Alkaline phosphatase activity specific to positive reactions with antiserum V/VI were detected using 10 ml of naphthol-AS-GR-phosphate (0.2 mg/ml)–Fast Blue B (0.35 mg/ml; multicolor detection set Green; Roche).

RESULTS

Development of ELISA-format serotyping method. The ELISA protocol to serotype *L. monocytogenes* isolates was adapted from standard immunoassay methods (9), using the slide agglutination protocol of the *Listeria* antiserum Seiken serotyping kit as a guide for sample preparation. Strains J0094, J0095, J0096, J0097, J0098, and J0099 (serotypes 4d, 3a, 3c, 4b, 1/2a, and 4c, respectively) were used as serotype reference strains (Table 1) in initial experiments to determine the proper dilution level for discrimination between positive and negative reactions for all antisera. We determined that the O-factor and H-factor antisera gave suitable results at dilutions of 1:1,000 and 1:500, respectively (data not shown). All subsequent ELISA serotype determinations were performed using antisera diluted to these levels.

Using the serotype reference strains, cell preparation methods were compared. Cell suspensions that were autoclaved for 30 min, boiled for 1 h, incubated at 65°C for 1 h, or not treated reacted similarly to the appropriate positive O-factor antisera. However, autoclaved cells tended to react less to negative O-factor antisera relative to cells treated by the other methods; that is, lower background reactions were observed. In addition, formaldehyde-treated cells were prone to give false-positive reactions to O antiserum IX (data not shown). Since the ELISA method was to be compared directly to the antiserum kit slide agglutination method, which recommended using autoclaved cells, all subsequent O-antigen determinations were performed with autoclaved cells. Similarly, to directly compare H-antigen determination by the ELISA method to that by the slide agglutination method, isolates initially were subcultured three times on BHI motility agar, as recommended in the slide agglutination kit protocol, to increase the proportion of fully flagellated cells and thereby render a robust reaction with the appropriate H-factor antisera. Subsequent experiments showed that a single 24-h passage on BHI motility agar was sufficient in the ELISA protocol to yield accurate reactions with H-factor antisera. Treatment of the cell suspensions by autoclaving was less effective than treatment with formaldehyde in preserving H-antigens for serotyping. Therefore, formaldehyde-treated cell suspensions and autoclaved cell suspensions were considered optimal for determining H-antigens and O-antigens, respectively.

Interpretation of ELISA reactions. As with the slide agglutination method, serotype assignment using the ELISA method is a three-step process. First, the reactions of O-factor antisera I/II and V/VI are compared; strains that react positively to antiserum I/II will react negatively to antiserum V/VI, and vice versa. Strains reacting positively with antiserum I/II are differentiated into serogroup 1/2 by a positive reaction to O-factor antiserum I and serogroup 3 by a positive reaction to O-factor antiserum IV. These serogroups are further differentiated into serotypes by their specific reaction to H-factor antisera (Table 2). Strains reacting positively with antiserum V/VI (i.e., serogroup 4) are differentiated into serotypes 4a, 4ab, 4b, 4c, 4d, and 4e by their reactions to O-factor antisera VI, VII, VIII, and IX; all serogroup 4 strains react identically to H-factor antisera (Table 2). Positive and negative reactions were scored relative to the maximal positive reaction of each strain to all O-factor and H-factor antisera, with negative reactions typically at levels less than 25% of the maximal positive reaction for O-antigens (Fig. 1 and 2). Reactions to H-factor antisera were generally weaker than those to O-factor antisera, and so interpretation of results was more subjective, especially for 1/2c and 3c strains (Fig. 1C and E). Nevertheless, graphic interpretations of data obtained using the ELISA method were less ambiguous than visual positive-negative interpretations obtained using the slide agglutination method.

TABLE 2. Antigen components of each *L. monocytogenes* serotype

Serotype	O-antigens[a]	H-antigens
1/2a	I, II	A, B
1/2b	I, II	A, B, C
1/2c	I, II	B, D
3a	II, IV	A, B
3b	II, IV	A, B, C
3c	II, IV	B, D
4a	(V), VII, IX	A, B, C
4ab	V, VI, VII, IX	A, B, C
4b	V, VI	A, B, C
4c	V, VII	A, B, C
4d	(V), VI, VIII	A, B, C
4e	V, VI, (VIII), (IX)	A, B, C

[a] Antigens in parentheses may not be present in all isolates.

Comparison of ELISA and slide agglutination to assign serotypes. The ELISA serotyping method was compared to the slide agglutination method using 101 different *L. monocytogenes* isolates (Table 1). Of these strains, results obtained by the ELISA method matched those obtained by slide agglutination for 89 of the 101 isolates (88%). For isolates of the clinically important serotypes 1/2a, 1/2b, and 4b, the ELISA method agreed with the slide agglutination method for 27 of 27 strains (100%), 10 of 11 strains (91%), and 38 of 39 strains (97%), respectively. Conversely, one isolate (19118) that was designated serotype 4e by slide agglutination was identified as serotype 4b by ELISA due to a negative reaction with O-factor antiserum IX, and one isolate that was designated serotype 4b by slide agglutination was identified by ELISA as serotype 4d/4e due to a positive reaction with O-factor antiserum VIII. It is noteworthy that in the serotyping scheme presented by Seeliger and Höhne (18) (Table 2), not all serotype 4e strains necessarily contain O-antigens VIII and IX. Such strains would be indistinguishable from serotype 4b. Also, the scheme cannot distinguish between serotypes 4e and 4d solely by the presence of O-antigen VIII (Table 2). During testing, two strains (FSL-J1-094 and G3990) were nonmotile on motility agar, and a third strain (FSL-J1-116) was delayed in motility, requiring a second passage on motility agar. Because of this defect, strain FSL-J1-094 (serogroup 1/2) could not be typed fully due to the lack of H-factor antiserum-reactive flagella; strains G3990 and FSL-J1-116 were both typed as 4b, so H-antigen determinations were not necessary.

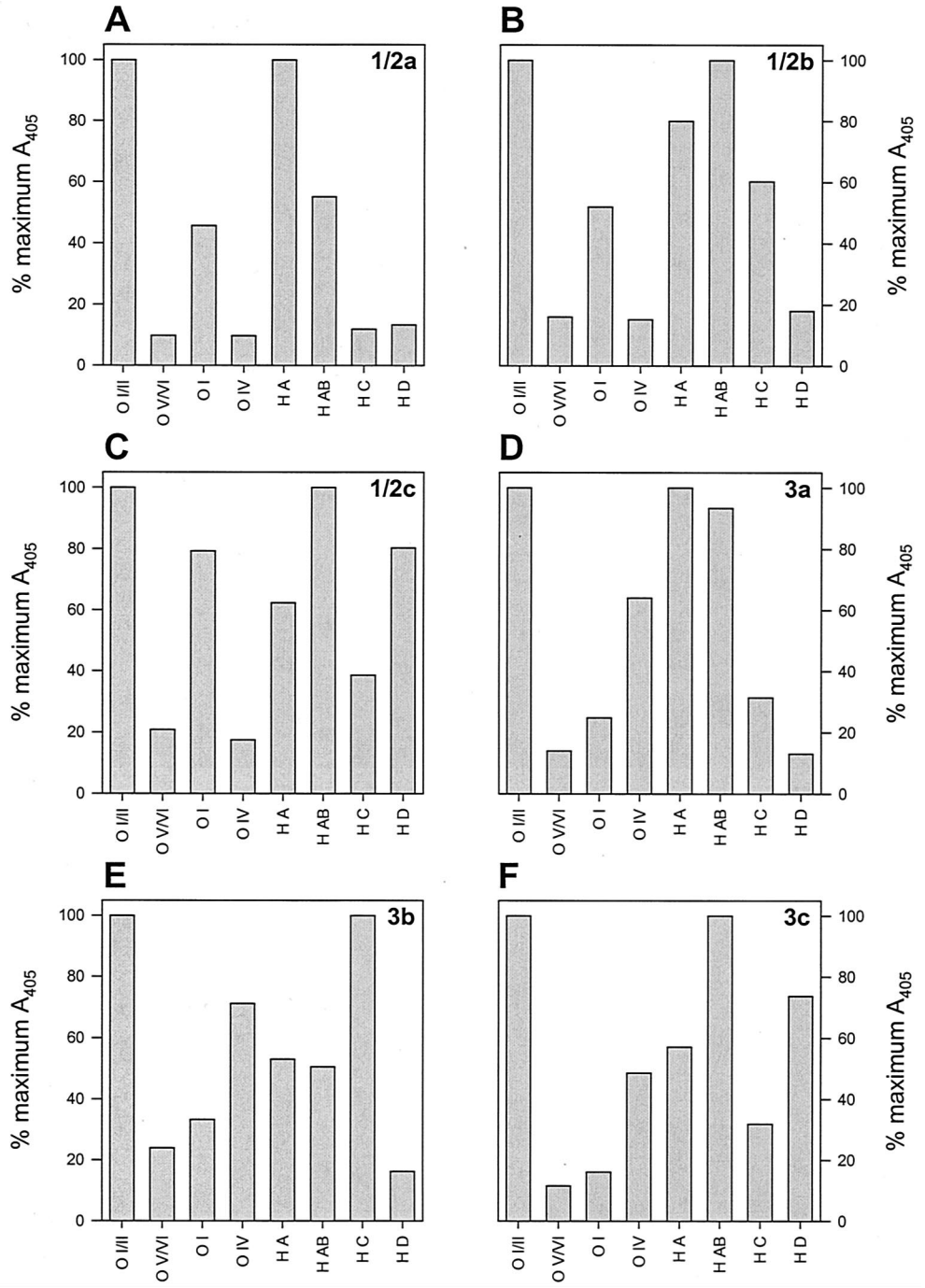

FIG. 1. ELISA O-antigen and H-antigen reactions of serogroup 1/2 and 3 *L. monocytogenes* strains. (A) Strain J0098, serotype 1/2a; (B) strain G848, serotype 1/2b; (C) strain G-3321, serotype 1/2c; (D) strain J0095, serotype 3a; (E) strain cpp81, serotype 3b; (F) strain J0096, serotype 3c.

In addition to those strains serotyped by both methods, 97 *L. monocytogenes* strains that previously were not serotyped were characterized by the ELISA protocol. These strains were isolated from various sources, including produce, meats and dairy products, food processing environments, animals and humans, soils, and environmental samples. The majority of these isolates (86%) were serotyped unambiguously as 1/2a (17 strains), 1/2b (39 strains), or 4b (27 strains) by the ELISA protocol. The remaining isolates were serotyped as 1/2c (5 strains), 3b (3 strains), 4a (2 strains), 3a (1 strain), 4ab, (1 strain), 4c (1 strain), and 4d (1 strain).

Identification of mixed-serotype cultures. Initial comparisons of the serotyping methods using *L. monocytogenes* strain 19118 (Table 1) were problematic in that slide agglutination resulted in serotype 4e, while independent ELISA tests on cultures from two separate colony picks identified the strain as serotype 4b and 1/2c. Since the initial discrimination between positive and negative reactions to O-factor antisera I/II and

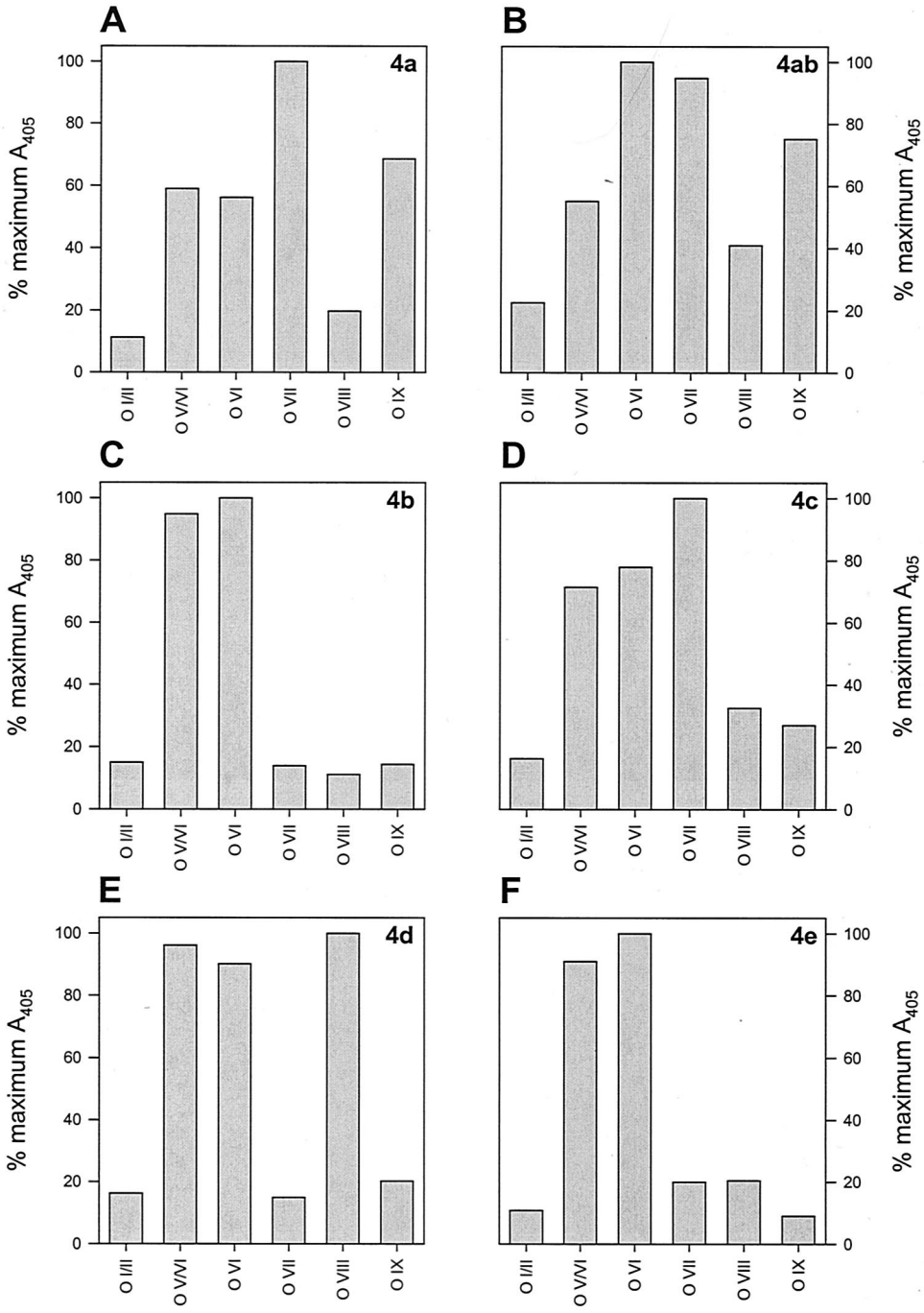

FIG. 2. ELISA O-antigen reactions of serogroup 4 *L. monocytogenes* strains. (A) Strain FSL-X1-010, serotype 4a; (B) strain JL2-10, serotype 4ab; (C) strain J0097, serotype 4b; (D) strain J0099, serotype 4c; (E) strain J0094, serotype 4d; (F) strain 19118, serotype 4e. Note that serotype 4e is indistinguishable from 4b in this case.

V/VI generally is very robust, we hypothesized that the stock culture of strain 19118 contained a mixture of these two serotypes rather than hypothesizing that the discrepancy resulted from inconsistent performance of the ELISA. To test this hypothesis, a colony immunoblot assay was developed using the O-factor antisera I/II and V/VI to discriminate between serogroups 1/2 and 4. Colony lifts on nitrocellulose were probed sequentially using each O-factor antiserum as the primary antibody and a unique color alkaline phosphatase substrate to correspond to each primary antibody. Using this method, colonies of serotype 1/2a reference strain J0098 and serotype 4b reference strain J0097 could be distinguished on a plate containing a mixture of both strains (Fig. 3C). No false-positive colonies resulting from cross-reaction of O-factor antiserum I/II with strain J0097 or O-factor antiserum V/VI with strain J0098 were observed (Fig. 3A and B). Performing this

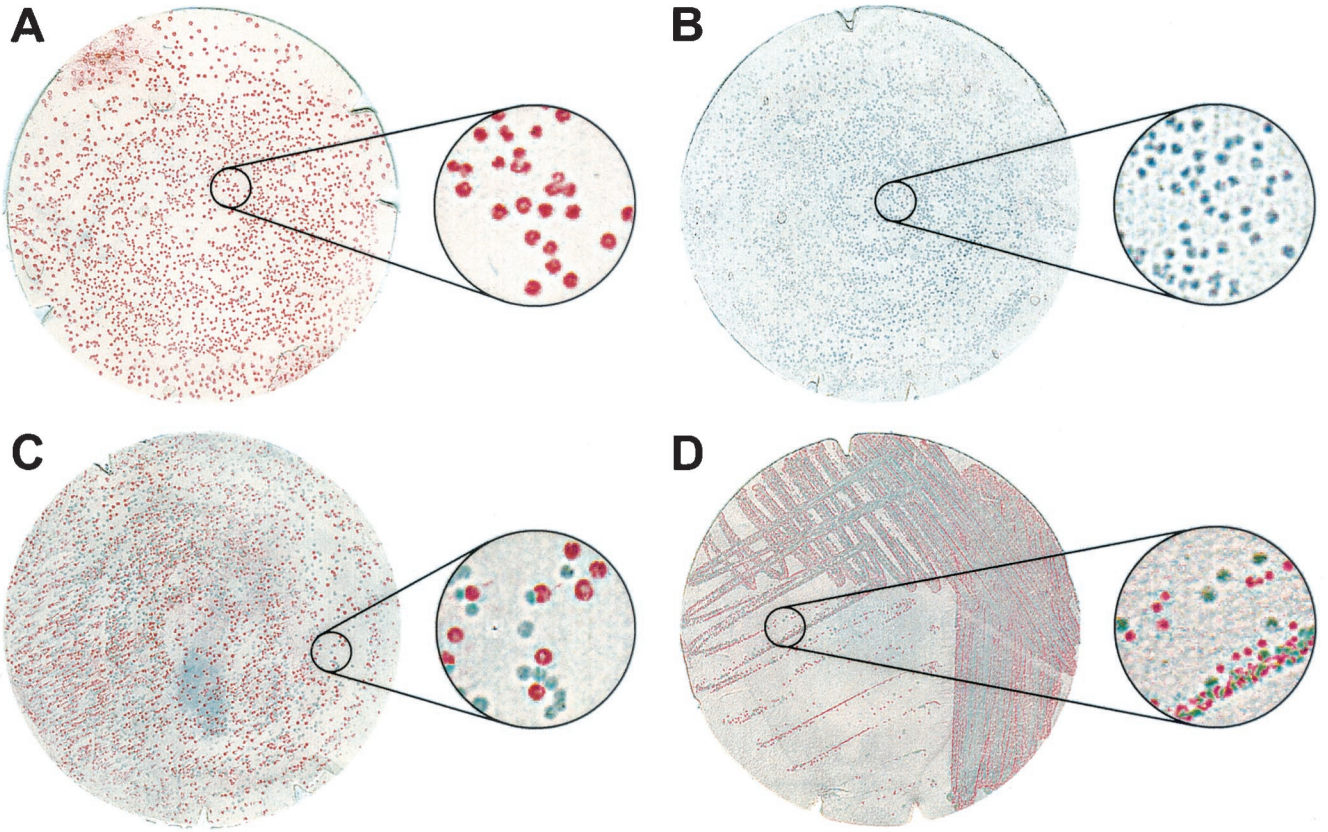

FIG. 3. Colony immunoblotting of single- and mixed-serotype cultures of *L. monocytogenes*. Blots of serotype 1/2a strain J0098 (A), serotype 4b strain J0097 (B), or a mixture of strains J0098 and J0097 (C) were probed sequentially with O-factor antiserum I/II (stained red) and O-factor antiserum V/VI (stained green). (D) A streak plate of strain 19118 was blotted and probed sequentially with O-factor antiserum V/VI (stained red) and O-factor antiserum I/II (stained green). Insets are magnifications (×5) of the indicated region of each filter to show detail.

colony immunoblot procedure on a plate streaked for individual colonies of strain 19118 confirmed the presence of serogroups 1/2 and 4 (Fig. 3D), consistent with the initial, seemingly conflicting ELISA serotyping results, and demonstrated that this was, in fact, a mixed culture of serotype 4b strain 19118 contaminated with a serotype 1/2c strain.

DISCUSSION

Serotyping of *L. monocytogenes* as a primary subtyping method has been in use for decades, even though it has been noted repeatedly that serotyping is ambiguous, sometimes variable within and between laboratories, and limited in its usefulness to demonstrate a correlation of strains between outbreaks of listerial infections (16, 17, 19). The ELISA serotyping protocol described in this work is a means to attempt to rectify the ambiguities and intra- and interlaboratory variability inherent to the traditional serotyping method, while at the same time allowing laboratories to perform more analyses with significant cost and time savings. Because serotype reactions depend on the quality of the antisera used, which in turn depends on which standardized strains and antigen preparation methods are chosen, the likelihood of inaccurate or inconsistent assignment of serotype rises when laboratories prepare their own antisera, especially for nonclinical isolates (i.e., those not of serotypes 1/2a, 1/2b, or 4b) (6, 16). The standard slide agglutination method relies on visual acuity and judgement to produce accurate data; this may be a substantial source of variability when comparing data between individuals in a laboratory and between different laboratories. In addition, the agglutination assays typically use 10 to 100 μl of antiserum per reaction, depending on methodology. Using the commercially prepared antiserum kit and dilution factors described, the ELISA protocol requires 0.2 μl of each O-factor antiserum and 0.4 μl of each H-factor antiserum to determine the serotype of one isolate in duplicate, so the contents of the kit are sufficient to perform ELISA serotyping in duplicate on 10,000 strains. Since the titers of the antisera vary on a lot-to-lot basis, the dilution levels used with each kit must be optimized. The use of a commercially prepared set of antisera and a semiquantitative ELISA format greatly reduce the variability of antiserum quality as well as the inconsistencies in judgement associated with weakly agglutinating antigen-antiserum combinations. Using *L. monocytogenes* strains of known serotypes as references (Fig. 1 and 2), serotypes of uncharacterized isolates can be assigned easily and with little ambiguity. With the exception of a strain previously serotyped as 4e by agglutination, which was consistently serotyped as 4b by ELISA, strains representative of all serotypes were typeable by this method. The distinctions between serotypes 4b and 4e and between serotypes 4d and 4e

are poorly defined in the original scheme (Table 2). Modifications to this scheme proposed by Garcia et al. (6) make the distinctions marginally clearer, in that all serotype 4e strains, and some serotype 4b strains, were proposed to contain O-antigen IX. In contrast, neither of these serotypes reacted positively to O-factor antiserum IX in the ELISA, using cells prepared as described. Also, two strains typed as 4a by agglutination were typed as 4c by ELISA, the difference resulting from negative reactions to O-factor antiserum IX in the ELISA. These data suggest that O-antigen IX may be unstable during cell preparation or intermittently expressed at sufficient levels to detect by ELISA in certain *L. monocytogenes* strains, and that further optimization of O-factor antiserum IX reaction conditions is necessary for consistent use in the ELISA method.

Another source of variability in serotype identification may arise when *L. monocytogenes* cultures contain mixtures of strains of different serotypes. This scenario arose when our laboratories assigned different serotypes, most obviously in serogroups 1/2 and 4, to apparently identical samples of particular strains (Table 1). The discrepancy was investigated by developing a serogroup-specific colony immunoblot method, which could distinguish these serogroups by differential staining (Fig. 3). *L. monocytogenes* isolates are routinely obtained through enrichment procedures, the result of which may contain more than one strain of a particular serotype. For example, single-colony picks from an enrichment culture from one soil sample showed that the soil contained two different serotypes of *L. monocytogenes* (strains TP1-1 and TP1-3; data not shown). Picking one or a small number of colonies following an enrichment protocol for serotyping may therefore not include all serotypes present in the original sample. Alternatively, the colony immunoblot method could be used on entire streak or spread plates to quickly ascertain whether they contained mixtures of, in this case, serogroup 1/2 or 3 and serogroup 4 strains. This assay could be developed further in order to distinguish between serogroups 1/2 and 3, and different serogroup 4 types.

As an epidemiological tool, serotyping is not sufficiently discriminatory to determine positive correlations between food-borne isolates and clinical isolates in cases of listeriosis outbreaks, though a negative relationship between isolates could be demonstrated by differing serotype results in these instances (19). More-discriminatory molecular methods are currently in use (7, 10, 12, 13, 16, 20, 22) and offer a greater degree of confidence in determining epidemiological relatedness of food-borne and clinical *L. monocytogenes* isolates. However, because serotype designation has been correlated with virulence potential (only 3 of the 14 serotypes cause a vast majority of human listeriosis cases (21), serotyping does provide relevant subtyping information. The ELISA and colony immunoblotting techniques described in this paper are useful means to quickly and economically serotype *L. monocytogenes* isolates and confirm the serotype uniformity of a culture before more-detailed epidemiological analyses are performed.

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