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Identification of *Salmonella* Somatic and Flagellar Antigens by Modified Serological Methods

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This report describes two modified methods for the identification of *Salmonella* somatic (O) and flagellar (H) antigens. Over a period of 2 years, both modified methods were found to be approximately three times less labor intensive than the standard methods while requiring no more technical skill. The modified methods were as accurate as the standard methods in identifying the O and H antigens of 350 *Salmonella* isolates. Furthermore, 43 O antisera reacted exclusively with organisms possessing homologous O antigens when the modified and two standard methods were used. At the antiserum dilutions used for H antigen identification, H antisera did not react with O antigens or heterologous H antigens by either the modified or the standard method. Compared with the standard method for H antigen identification, the modified method was approximately 20 times more economical with respect to antisera and usually generated a 1.5- to 4-fold higher titer. Since the antisera stored for use in the modified method for H antigen identification were usually 100-fold more dilute than the antisera stored for the standard method, an antibody-stabilizing buffer was incorporated in the diluted antisera, allowing these reagents to be used for at least 9 to 16 months.

Standard serological methods for the identification of *Salmonella* O and H antigens have been described by a number of workers (3, 7). Alternate serological methods which are more economical or less labor intensive than the standard methods have also been developed (2, 6, 9, 10). For example, identification of *Salmonella* O antigens by coagglutination with antibody fixed to killed and stabilized protein A-bearing staphylococci consumes only 1/12th of the antiserum reagents used during standard slide agglutination tests; however, this method allows the expression of a substantial number of cross-reactions between O antibodies and heterologous O antigens (2). A new approach to the identification of O and H antigens involves the use of *Salmonella* O and H antibodies held within solid-phase diagnostic preparations, of which small quantities are transferred to glass slides and mixed with antigens. Protected by the solid-phase base, the antibodies maintain their specific activity and their ability to identify antigens accurately for 2.5 to 3 years (9). Guinée et al. (6) developed a mechanized microtiter method which was as accurate as standard methods in identifying the O and H antigens of approximately 2,000 *Salmonella* isolates. This method involves the staining and optical density standardization of each antigen. Another mechanized microtiter method developed by Shipp and Rowe (10) requires fewer preparatory steps, but its accuracy has not been compared with that of standard methods. Since the preparation and execution of most of these modified methods require expertise and skill, we saw the need to develop simple but reliable methods which would be especially suited for regions where highly trained technologists are in short supply. The purpose of this study was to develop modifications of the standard *Salmonella* O and H antigen identification methods which are technically simple while being accurate, economical, and less labor intensive than the standard methods.

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The technical simplicity and labor-saving features of our modified O and H antigen identification methods depend on the use of multiple sets of reactants, which are mixed on mechanically rotated slides. In contrast, the standard methods involve either labor-intensive manual mixing and rotation of reactant-bearing slides (O antigen identification methods) or tedious pipetting (H antigen identification method). The accuracy of both modified methods and the substantial economy of H antisera reagents used in the H antigen modified method are documented and present additional reasons for the use of the modified methods.

MATERIALS AND METHODS

Antisera. *Salmonella* O and H antisera were obtained from Difco Laboratories, Detroit, Mich., Roach Laboratories, Loganville, Ga., and the Centers for Disease Control (CDC), Atlanta, Ga. As specified by the manufacturers, working solutions of O antisera were prepared by reconstituting the lyophilized Difco antisera with 0.85% NaCl to a final dilution of 1:2; the CDC and Roach Laboratories liquid O antisera were diluted with 0.85% NaCl to a final dilution of 1:6. The strength of H antisera obtained from CDC, Difco, and Roach Laboratories was very similar, since they are usually used in the standard test for H antigen identification at a final dilution of 1:1,000. Given the similar strength of these H antisera, all the working solutions used in the standard test were prepared at a dilution of 1:10, a procedure recommended for CDC H antisera. Both diluted and undiluted O and H antisera were stored at 4°C.

Observation and estimation of serological reactions. Reactions were always observed by placing slides or tubes holding the reactants over an illuminated microscope substage mirror (diameter, 4.8 cm). Reactions were seen as reflections in the mirror. When the strength of reactions was estimated, the numbers 1, 2, 3, and 4 were used to represent reactions of progressively increasing strength (2); 0 signifies a lack of reaction.

Identification of O antigens. Slants composed of 10 ml of

TABLE 1. Outline of the methods used for the identification of *Salmonella* O antigens

Step	Method 1	Method 2	Method 3
1	Using a swab, the entire growth on an agar slant is suspended in 3.0 ml of 0.08% $HgCl_2$ in 0.85% NaCl; within 5 min the cells are dead.	Using a swab, the entire growth on an agar slant is suspended in 1.0 ml of 100 or 96% ethanol.	Drops from antiserum vials are dispensed into squares of a 2- by 3-in. or a 1- by 3-in. slide.
2	Drops from antiserum vials are dispensed into six squares of a 2- by 3-in. slide or into the rings of a ceramic ring slide.	The suspension is heated for 1 h in a 60°C water bath.	With a loop, growth from an agar slant is emulsified in a drop of antiserum.
3	Using a Pasteur pipette, a drop of *Salmonella* suspension is dispensed into each square or ring.	The dead cells are sedimented by centrifugation; the alcohol is decanted.	The slide is tilted back and forth for up to 1 min.
4	Six to eight slides are rotated horizontally for up to 6 min on a clinical rotator at 90 to 100 rotations per min.	The cells are suspended in 0.5 ml of physiological saline and incubated for 30 min at room temperature.	The reactions are recorded.
5	The reactions are recorded.	A loopful of the suspension is mixed with a loopful of antiserum on a 2- by 3-in. or a 1- by 3-in. slide. A 1- by 3-in. slide accommodates three reactions.	
6		The slide is tilted back and forth for up to 1 min.	
7		The reactions are recorded.	

veal infusion agar (Difco) were inoculated with *Salmonella* isolates and incubated for 18 to 24 h at 35°C. For all three O antigen identification methods, polyvalent *Salmonella* O antiserum A–I and Vi (Difco) was used first, followed by antisera directed against individual O groups A through I. If the polyvalent *Salmonella* antiserum failed to agglutinate the organisms, other polyvalent O antisera (e.g., Roach Laboratories antisera) were used, and then the individual components of the polyvalent antiserum with which the organisms had reacted were used.

Method 1 (our modified method), method 2 (described by Ewing [3]), and method 3 (described by Kauffman [7]) are outlined in Table 1. The slides described in Table 1 and used in all three methods were disposable Gold Seal Micro Slides (3 by 2 in. [7.62 by 5.08 cm]; Clay Adams, Parsippany, N.J.) which were divided into six squares by using a Markette, All Purpose Marker (EE Eberhard, Faber, Inc., Wilkes-Barre, Pa.). Occasionally, for method 1, nondisposable ceramic ring slides (Clay Adams) with 12 rings per slide were used. For methods 2 and 3, slides from Propper Manufacturing Co., Long Island City, N.Y. (1 by 3 in. [2.54 by 7.62 cm]) were also used.

Identification of H antigens and determination of H antiserum titers. Tubes containing 10 ml of tryptic soy broth (GIBCO Laboratories, Grand Island, N.Y.) were inoculated with *Salmonella* isolates and incubated for 18 to 24 h at 35°C. An equal volume of 0.6% Formalin in 0.85% NaCl was then added to each tube (3). The tubes were incubated for 16 to 18 h at room temperature to kill the organisms (4). For both H antigen identification methods, the antisera initially used contained antibodies homologous to the H phases most commonly found among *Salmonella* isolates of the identified O group. When an organism reacted with a multivalent H antiserum, monovalent H antisera were used to determine the exact antigenic composition of each phase. When only one H phase of a biphasic *Salmonella* isolate could be identified, the H phase-reversal procedure was used to identify the second phase (3).

When H antigens were identified by the standard method described by Ewing (3), 10 μl of a 1:10 dilution of most antisera (normal-strength antisera), 20 μl of a 1:10 dilution of Difco antisera x, z_{13}, z_{15}, and z_{28}, and 25 μl of 1:10-diluted Roach antiserum v followed by 1.0 ml of formalinized broth, were dispensed into tubes. The final approximate antiserum

TABLE 2. Identification of *Salmonella* serotypes by three O and two H antigen identification methods

Organism	O antisera[a] reacting with the organisms by:			H antigens identified by:		Serotype identified	Complete O and H antigenic makeup of serotype[b]
	Method 1	Method 2	Method 3	Standard method	Modified method		
1	6,7	6,7	6,7	r:1,5	r:1,5	*S. infantis*	6,7,14:r:1,5
2	6,8	6,8	6,8	z_{10}:e,n,x	z_{10}:e,n,x	*S. hadar*	6,8:z_{10}:e,n,x
3	1,4,5,12	1,4,5,12	1,4,5,12	i:1,2	i:1.2	*S. typhimurium*	1,4,[5],12:i:1,2
4	1,9,12	1,9,12	1,9,12	g,m	g,m	*S. enteritidis*	1,9,12:g,m:[1,7]
5	3,10	3,10	3,10	e,h:1,6	e,h:1,6	*S. anatum*	3,10:e,h:1,6
6	11	11	11	i:1,2	i:1,2	*S. aberdeen*	11:i:1,2
7	17	17	17	b:1,2	b:1,2	*S. kirkee*	17:b:1,2
8	18	18	18	z_4,z_{23}	z_4,z_{23}	*S. cerro*	6,14,18:z_4,z_{23}:[1,5]
9	35	35	35	z_4,z_{23}	z_4,z_{23}	*S. alachua*	35:z_4,z_{23}:−
10	55	55	55	k:z_{39}	k:z_{39}	*S. tranoroa*	55:k:z_{39}

[a] For some of the antisera, the numerical designations include antibodies which were present in the antisera before they were absorbed. To prevent cross-reactions between 0 antisera 6,7 and 6,8, the manufacturers had absorbed out most of the O6 antibodies from both antisera. For the same reason, most of the O1 and O12 antibodies had been eliminated from antisera 1,4,5,12 and 1,9,12.

[b] Identification of the O group and flagellar phases identifies the serotype, which, in turn, reveals the complete O and H antigenic makeup of the organism (3). An underscore beneath the designation for an O antigen indicates that the strain has been lysogenized. Brackets mean that the antigen may not be present. The negative sign indicates that the organism is monophasic.

TABLE 3. Strength of reactions of 10 O antisera with 10 O antigens in methods 1, 2, and 3

O antigen	Strength[a] of reaction with O antiserum[b] by each method														
	1,4,5,12 (Difco)			1,9,12 (Difco)			6 (Roach)			6,8 (Difco)			11 (Roach)		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1,4,[5],12[c]	3	3	3	0	0	0	0	0	0	0	0	0	0	0	0
1,9,12[c]	0	0	0	3.33 ± 0.33	3.67 ± 0.33	3.33 ± 0.33	0	0	0	0	0	0	0	0	0
6,7	0	0	0	0	0	0	2.67 ± 0.33	2.67 ± 0.33	2.33 ± 0.33	0	0	0	0	0	0
6,8	0	0	0	0	0	0	3.33 ± 0.33	3	2.67 ± 0.33	4	4	3.67 ± 0.33	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0	4	3.33 ± 0.33	4
22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
39	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
43	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Each value represents the relative mean strength of reaction of three independent tests ± standard error.
[b] Most of the O antibodies 1 and 12 had been removed from antisera 1,4,5,12 and 1,9,12, whereas most of the O antibodies 6 had been eliminated from antiserum 6,8.
[c] An underscore beneath the designation for an antigen indicates that the strain has been lysogenized. Brackets indicate that the antigen may not be present.

dilution in each tube was either 1:1,000 of a normal-strength antiserum; 1:500 of Difco antiserum x, z_{13}, z_{15}, or z_{28}; or 1:400 of Roach antiserum v. The tubes were incubated for up to 1 h in a 48 to 50°C water bath, and the reactions were recorded. For antiserum titer determinations, twofold dilutions were made in 0.85% NaCl, starting at a dilution of 1:10; reactants (consisting of 0.1 ml of antiserum dilution and 0.9 ml of formalinized broth) were incubated for 1 h.

When H antigens were identified by the modified method, normal-strength H antisera were prepared at a final dilution of 1:1,000. The weaker Difco antisera x, z_{13}, z_{15}, and z_{28} were diluted 1:500, whereas Roach antiserum v was diluted 1:400. The diluent (buffer) consisted of 0.85% NaCl, 0.05 M PO_4^{3-}, 0.5% phenol, and 0.2% bovine serum albumin (pH 7.2 ± 0.2). Aliquots (10 ml) of these diluted antisera were stored in screw-cap dropper bottles at 4°C. Reactions were tested on the 2- by 3-in. slides which had been divided into six squares or on the ceramic ring slides. One drop of antiserum reagent was dispensed into each ring or square, followed by a drop of formalinized broth, dispensed from a Pasteur pipette. The final antiserum dilution was 1:2,000 of normal-strength antiserum; 1:1,000 of Difco antiserum x, z_{13}, z_{15}, or z_{28}; or 1:800 of Roach antiserum v. Slides were rotated horizontally for up to 5 min at a speed of 90 to 100 rotations per min. The flocculation reactions were then recorded.

When the modified method was used for H antiserum titer determinations, the antisera were subject to twofold dilutions in buffer, starting at a dilution of 1:50. Antisera which had been stored at a dilution of 1:1,000 or 1:500 were diluted to 1:1,600 and then subjected to twofold dilutions. Reactants consisted of 50 μl of antiserum dilution and 50 μl of formalinized broth, dispensed from 0.2-ml pipettes. When antiserum titers were being determined, the slides were rotated for exactly 5 min.

RESULTS

Identification of O and H antigens. Three hundred *Salmonella* strains isolated from humans or food sources and 50 *Salmonella* stock cultures were each subcultured to agar slants and tryptic soy broth tubes and coded. For all 350

TABLE 4. H antiserum titers generated by the standard and modified methods during reactions with homologous H antigens

Principal specificity of H antiserum	Immunogen[a]	Source of antiserum	Antigen tested[a]	H antiserum titer[b] by:	
				Standard method	Modified method
b	1,4,[5],12:b:−	Difco	1,4,[5],12:b:1,2	8,533 ± 2,136	12,800
i	1,4,[5],12:i:−	CDC	1,4,[5],12:i:−	17,067 ± 4,272	34,133 ± 8,543
k	6,7,14:k:−	CDC	6,7,14:k:−	12,800	21,333 ± 4,272
lv	3,10:lv:−	Roach	1,4,12,27:lv:1,7	3,200	10,667 ± 2,136
lz_{13}	3:10:lz_{13}:−	Roach	3,10:lz_{13}:1,5	6,400	6,400
r	11:r:−	Difco	11:r:−	6,400	12,800
z_{35}	(1),3,10,(19):−:z_{35}	CDC	(1),3,10,(19):b:z_{35}	3,200	12,800
1,6	3,10:−:1,6	Roach	3,10:−:1,6	25,600	51,200
d	9,12,[vi]:d:[z_{66}]	CDC	6,8:d:1,5	6,400	6,400
g,m,s	6,7,14:g,m,[p],s:[1,2,7]	CDC	1,9,12:g,m:[1,7]	17,067 ± 4,272	42,667 ± 8,543
m	4,12:g,m:− or 6,7:m,t:−	Roach	1,9,12:g,m:[1,7]	2,133 ± 534	6,400
z_4,z_{23}	6,14,18:z_4,z_{23}:[1,5]	Roach	35:z_4,z_{23}:−	2,667 ± 534	3,200
z_{23}	6,14,18:z_4,z_{23}:[1,5]	Difco	35:z_4,z_{23}:−	6,400	10,667 ± 2,136
2	1,4,[5],12:−1,2	Difco	11:i:1,2	3,200	8,533 ± 2,136

[a] An underscore beneath the designation for an O antigen indicates that the strain has been lysogenized. Brackets indicate that the antigen may not be present. Parentheses indicate that the antigen is poorly developed and hence weakly agglutinable. The negative sign indicates that the serotype is monophasic or that the second phase has been substantially reduced or eliminated by the phase-reversal procedure.
[b] Reciprocal of the highest antiserum dilution (after addition of antigen) which still visibly reacts with the antigen; each value represents the mean serum antibody titer of three independent tests ± standard error.

TABLE 3—*Continued*

22 (Roach)			30 (CDC)			39 (Roach)			41 (CDC)			43 (CDC)		
1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	2.33 ± 0.33	2.33 ± 0.33	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	4	3.67 ± 0.33	3.67 ± 0.33	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	3.33 ± 0.33	3	2.67 ± 0.33	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	4	4	4	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	3	3	2.67 ± 0.33

organisms, the three methods for O antigen identification and the two methods for H antigen identification yielded the same results. The ability of the various methods to correctly identify O and H antigens is illustrated in Table 2, which shows the results obtained with 10 of the 350 *Salmonella* isolates.

Reactions between O antisera and homologous and heterologous O antigens. A total of 43 O antisera, each with a different O group or subgroup specificity which ranged from group 1,2,12 (group A) to group 66, were tested with 43 *Salmonella* strains, each of which was homologous to one of the antisera and, in some cases, partially homologous to other antisera. To detect all the weak reactions, all antigen-antiserum mixtures were rotated for the maximum time stipulated for each method (6 min for method 1 and 1 min for methods 2 and 3). Regardless of the method used, all the antisera reacted with organisms belonging to the O subgroup and/or the group against which the antisera were directed, whereas reactions between O antisera and heterologous O antigens never occurred. Reactions between 10 of the 43 antisera and 10 of the 43 *Salmonella* strains are listed in Table 3. This table shows that the use of method 1 often resulted in positive reactions which were stronger than those obtained with method 2, whereas method 3 often yielded the weakest reactions. Not withstanding the absence of cross-reactions in Table 3, cross-reactions did occasionally occur between O antisera 1,4,5,12 and 1,9,12 during the identification of the 350 *Salmonella* strains. The cross-reactions occurred regardless of the method used and were due to the incomplete absorption of O antibody 12 from the antisera as well as the strong representation of the O12 antigen at the surface of the cross-reacting organisms. By using the single-factor O antisera 4, 5, and 9, the O group of *Salmonella* strains was easily identified.

Reactions of H antisera with homologous antigens. The standard and modified methods were compared with respect to the sensitivities (titers) expressed when H antisera reacted with homologous antigens. Fourteen antisera were tested either with the serotype used to elicit each antiserum or with another organism sharing complete or partial H antigen homology with the immunogen. Titers which ranged from 3,200 through 51,200 were obtained when H antisera reacted with the corresponding immunogen or with an organism sharing partial or complete H antigen homology with the immunogen (Table 4). Titers obtained by the modified method were usually 1.5- to 4-fold higher than those ob-

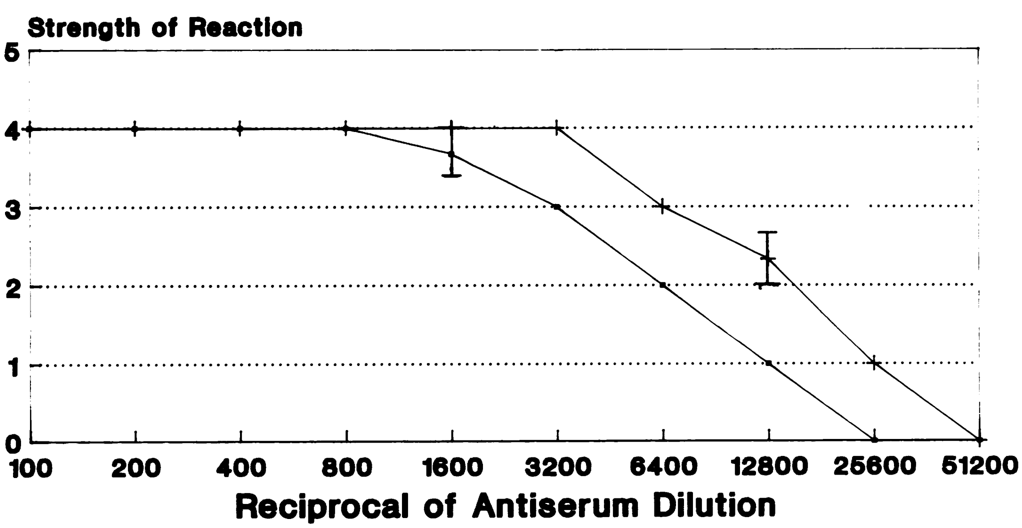

FIG. 1. Strength of reactions between various dilutions of H antiserum i (CDC) and antigen 1,4,[5],12:i:− when the standard (■) and modified (+) methods were used. Each value represents the mean of three independent tests ± standard error.

TABLE 5. Strength of flocculation reactions generated by the standard and modified methods with antiserum dilutions of 1:1,000 and 1:2,000

Principal specificity of H antiserum	Source of antiserum	Antigen tested[a]	Strength of reaction by:	
			Standard method[b]	Modified method[b]
b	Difco	1,4,[5],12:b:1,2	3	3
i	CDC	1,4,[5],12:i:−	4	4
k	CDC	6,7,14:k:−	3.67 ± 0.33	3
lv	Roach	1,4,12,27:lv:1,7	4	3
lz_{13}	Roach	3,10:lz_{13}:1,5	4	4
r	Difco	11:r:−	2.33 ± 0.33	3.67 ± 0.33
z_{35}	CDC	(1),3,10,(19):b:z_{35}	4	4
1,6	Roach	3,10:−:1,6	3	4
d	CDC	6,8:d:1,5	2.67 ± 0.33	3
g,m,s	CDC	1,9,12:g,m:[1,7]	3.67 ± 0.33	3
m	Roach	1,9,12:g,m:[1,7]	3.33 ± 0.33	3
z_4,z_{23}	Roach	35:z_4,z_{23}:−	3	3
z_{23}	Difco	35:z_4,z_{23}:−	3	3
2	Difco	11:i:1,2	3	3

[a] An underscore beneath the designation for an O antigen indicates that the strain has been lysogenized. Brackets indicate that the antigen may not be present. Parentheses indicate that the antigen is poorly developed and hence is weakly agglutinable. The negative sign indicates that the serotype is monophasic or that the second phase has been substantially reduced or eliminated by the phase-reversal procedure.

[b] Each value represents the relative mean strength of reaction of three independents tests ± standard error.

tained by the standard method. The greater efficiency of the modified method is also shown in Fig. 1, which illustrates that as an antiserum is subject to progressive dilution, the strength of the flocculation reaction decreases at a somewhat lower rate when the modified method is used in lieu of the standard method.

The results in Table 5 justify the routine use in the modified method of H antiserum which is always twofold more dilute than the antiserum used in the standard method; the table shows that the use of 1:2,000-diluted antisera in the modified method results in flocculation reactions which are relatively as strong as those generated by the 1:1,000-diluted antisera used in the standard method.

Reactions of H antisera with heterologous antigens. The manufacturers do not remove O antibodies from H antisera, and, consequently, such antisera also contain O antibodies directed against the immunogen. To determine the reaction capacity of such O antibodies, the first eight antisera listed in Table 4 were each tested with an organism possessing both homologous O antigens and heterologous H antigens which do not cross-react with the immunogen (3). Each of these eight antisera was also tested with an organism possessing both homologous O antigens and heterologous H antigens which are known to cross-react with the H antigen(s) of the immunogen at antiserum titers ranging from 400 to 6,400 (3). Organisms sharing only O antigens with the immunogens usually failed to react even with an antiserum dilution of 1:100, or they reacted only with low dilutions of 1:100 or 1:200 (Table 6). On the other hand, most of the eight H antisera reacted with organisms which shared O antigens and cross-reacting H antigens with the immunogen (Table 6); however, here, too, the titers of the antisera never exceeded 200, apparently because the manufacturers remove most of the cross-reacting H antibodies from the antisera.

Effect of storage on the performance of H antisera used for the modified method. A total of 20 monovalent and 20 multivalent H antisera were diluted 1:1,000 in buffer and stored for 9 months at 4°C. At the time of initial storage and 6 and 9 months thereafter, each antiserum was tested with two organisms, one possessing homologous and one pos-

TABLE 6. H antiserum titers generated by the standard and modified methods during reactions with homologous O antigens and heterologous H antigens

Principal specificity of H antiserum	Immunogen[a]	Source of antiserum	Titers obtained with organisms possessing homologous O antigens and heterologous, non-cross-reacting H antigens[b]			Titers obtained with organisms possessing homologous O antigens and heterologous, cross-reacting H antigens[b]		
			Antigen tested[a]	Standard method	Modified method	Antigen tested[a]	Standard method	Modified method
b	1,4,[5],12,:b:−	Difco	1,4,[5],12:f,g:[1,2]	<100	<100	1,4,[5],12:−:1,2	<100	100
i	1,4,[5],12:i:−	CDC	1,4,[5],12:f,g:[1,2]	<100	<100	1,4,[5],12:r:−	100	167 ± 33.4
k	6,7,14:k:−	CDC	6,7,14:e,h:e,n,z_{15}	<100	<100	6,7:−:lw	100	100
lv	3,10:lv:−	Roach	3,10:e,h:−	<100	<100	3,10:−:1,5	<100	100
lz_{13}	3,10:lz_{13}:−	Roach	3,10:e,h:−	<100	<100	3,10:−:1,6	<100	<100
r	11:r:−	Difco	11:−:1,2	133 ± 33.4	167 ± 33.4	11:i:−	167 ± 33.4	200
z_{35}	(1),3,10,(19):−:z_{35}	CDC	1,3,19:g,[s],t:−	<100	<100	1,3,19:z:−	<100	100
1,6	3,10:−:1,6	Roach	3,10:y:−	100	100	3,10:lv:−	<100	167 ± 33.4

[a] An underscore beneath the designation for an O antigen indicates that the strain has been lysogenized. Brackets indicate that the antigen may not be present. Parentheses indicate that the antigen is poorly developed and hence weakly agglutinable. The negative sign indicates that the serotype is monophasic or that the second phase has been substantially reduced or eliminated by the phase-reversal procedure.

[b] Reciprocal of the highest antiserum dilution (after addition of antigen) which still visibly reacts with the antigen; each value represents the mean serum antibody titer of three independent tests ± standard error.

TABLE 7. H antiserum titers generated by the modified method when freshly prepared and stored 1:1,000- or 1:500-diluted antisera, possessing the same specificity and manufacturer lot number, were used

Principal specificity of H antiserum	Source of antiserum	Antigen tested[a]	H antiserum titer[b] for:		Storage time (mo)
			Freshly prepared reagent	Stored reagent	
d	CDC	6,8:d:−	17,067 ± 4,272	25,600	9
e,h	Difco	3,10:e,h:−	6,400	5,333 ± 1,068	11
t	Difco	1,3,19:g,[s],t:−	42,667 ± 8,543	25,600	13
z_{13} (1:500)	Difco	3,10:l,z_{13}:−	12,800	12,800	14
g,m,s	Roach	1,9,12:g,m:[1,7]	17,067 ± 4,272	12,800	14
z_4,z_{32}	CDC	35:z_4,z_{23}:−	6,400	12,800	14
x	Roach	6,8:−:e,n,x	3,200	3,200	15
h	Roach	3,10:e,h:−	12,800	12,800	15
b	Roach	1,4,[5],12:b:−	12,800	17,067 ± 4,272	15
e,n,x	CDC	6,8:−:e,n,x	10,667 ± 2,136	6,400	16

[a] An underscore beneath the designation for an O antigen indicates that the strain has been lysogenized. Brackets indicate that the antigen may not be present. The negative sign indicates that the serotype is monophasic or that the second phase has been substantially reduced or eliminated by the phase-reversal procedure.

[b] Reciprocal of the highest antiserum dilution (after addition of antigen) which still visibly reacts with the antigen; each value represents the mean serum antibody titer of three independent tests ± standard error.

sessing heterologous H antigens. Whenever tested, all the antisera reacted strongly with organisms possessing the homologous antigen(s) but never reacted with any organism possessing heterologous antigens. Also, 10 1:1,000- or 1:500-diluted H antisera stored for 9 to 16 months at 4°C were each compared with an antiserum which was freshly diluted from a working solution, possessing the same specificity and manufacturer lot number as the stored antiserum. When tested with completely or partially homologous H antigens, the stored reagents generated titers which were equal or very similar to titers generated by the freshly prepared reagents (Table 7).

DISCUSSION

The three methods for O antigen identification were shown to possess equivalent accuracy with respect to both their ability to identify O antigens and the absence of cross-reactions of antisera with heterologous O antigens (Tables 2 and 3). None of the three methods require much technical skill. However, the methods are quite different in terms of safety, economy, and labor. Method 2 has the advantage of using killed organisms and in being economical, since only a loopful of antiserum is used per test. However, this method consists of many steps, and we found it to be the slowest method. Method 1 (our method of O antigen identification) has more steps than method 3 (Table 1). Nevertheless, over a period of 2 years, method 1 has been shown to be faster and considerably less tedious than method 3 because much labor is saved by mixing the reactants on a mechanical rotator. Furthermore, in method 3, slides have to be processed one at a time, whereas method 1 lends itself to assembly-line procedures. For example, while *Salmonella* strains are killed in the first $HgCl_2$-saline solution, additional *Salmonella* strains to be tested are suspended in tubes containing this solution. Furthermore, while six to eight slides are on the mechanical rotator, additional slides and reactants can be prepared. Method 1 also has the advantage over method 3 in that it employs killed organisms and enjoys the advantage over method 2 in that it uses $HgCl_2$, which, unlike ethanol, does not have to be removed prior to the serological testing.

The serological testing of organisms suspended in solutions containing Hg^{2+} was suggested by Bridges (1), who conducted slide agglutination tests with *Shigella* cells suspended in a solution composed of 0.1% HgI_2, 0.4% KI, and physiological saline. At CDC, *Shigella* cells are suspended and killed in physiological saline containing 0.1% HgI_2. The HgI_2 does not alter the antigens of the *Shigella* cells, and such suspensions are used for selective absorption of O antibodies from *Shigella* antisera (H. G. Wathen-Grady, personal communication).

In Table 2, the O and H antigens of 10 of the 350 *Salmonella* isolates identified are shown and exemplify the equivalent accuracy of the three O and two H antigen identification methods. However, correct identification of H antigens cannot be taken for granted; H antisera diluted up to 1/200 may still have enough O and H antibodies which react, respectively, with homologous O antigens and cross-reacting H antigens (Table 6). When low dilutions of H antisera are used, such reactions can potentially cause the misidentification of H antigens. In fact, Le Minor and Rohde (8) have used a method in which H antigens are tested with dilutions of H antisera on slides; to prevent O antibodies from interfering with H antigen identification, the H antisera are elicited by rough *Salmonella* mutants which lack the specific O group antigen(s). The high antiserum dilutions used in the standard and modified methods for H antigen identification obviate the need for such immunogens; after the addition of antigen, the H antiserum dilution in the standard method is usually 1:1,000, sometimes 1:500 (with Difco antisera x, z_{13}, z_{15}, and z_{28}), and rarely 1:400 (with Roach antiserum v), whereas the H antiserum dilutions in the modified method are always twofold higher (1:2,000, 1:1,000, and 1:800, respectively). The use of twofold-higher antiserum dilutions in the modified method compensates for the slightly higher sensitivity of this method as compared to the standard method (Tables 4 and 5; Fig. 1).

Both H antigen identification methods are technically simple. A distinct economic advantage of the modified method is that 0.1 ml of a 1:10 dilution of H antiserum can generate only 10 standard tests, but when it is mixed with 9.9 ml of buffer, about 200 modified tests can be conducted. Furthermore, the antisera used in the modified method, even though diluted 1:1,000, can be used for at least 9 to 16 months (Table 7).

The long shelf life of the reagents is due to the stabilizing effect of the bovine serum albumin-containing buffer, since

in phenolized physiological saline, such diluted antisera can be used for only a few weeks (R. Gruenewald, unpublished observations). For the stabilization of dilute antisera used for complement fixation or hapten inhibition tests, Wasserman and Levine (11) and Gruenewald and Stollar (5) used buffer containing 0.1% bovine serum albumin as their diluent. The addition of bovine serum albumin to dilute antisera helps to stabilize the antibodies by somewhat increasing the protein concentration, which is greatly reduced by the dilution procedure (B. D. Stollar, personal communication). Antisera diluted in the buffer often assume an amber color after prolonged storage; this does not affect their performance. Occasionally the diluted antisera lose their serological activity after variable periods of storage. Whether the loss of activity is due to bacterial contamination or other factors has not been determined. Such deterioration of serological activity can be monitored by an adequate quality control program.

The modified method is much less labor intensive than the standard method, since the usage of slides and Pasteur pipettes is less labor intensive than the use of calibrated pipettes and tubes. Furthermore, an advantage of the former method is that 6 and 12 reactions can be observed almost simultaneously on 2- by 3-in. slides and ceramic ring slides, respectively; in the standard method, reactions in tubes are observed one at a time. During the last 2 years, the modified methods for O and H antigen identification have reduced the labor time by approximately two-thirds. Our results show that this increased efficiency was accompanied by retention of accuracy and simplicity of methodology and by a substantial saving of H antiserum reagents.

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