IDENTIFICATION OF SALMONELLAE OF SEROGROUP C1 BY IMMUNOFLUORESCENCE AND CO-AGGLUTINATION WITH ANTISERUM AGAINST AN OLIGOSACCHARIDE-PROTEIN CONJUGATE

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SUMMARY. Antiserum specific for salmonella O7 antigen was raised by immunisation of rabbits with an artificial conjugate consisting of oligosaccharide and bovine serum albumin (Os-BSA). The oligosaccharide was a pentasaccharide isolated after cleavage of the O antigen polysaccharide chain of *Salmonella thompson* (O antigen 6, 7) with *endo*-glycanase from bacteriophage 14. The usefulness of the *S. thompson* Os-BSA antiserum for rapid and accurate identification of isolates of *Salmonella* of serogroup C1 (O6, 7) was shown by indirect immunofluorescence tests in which 77 strains of *Salmonella* of serogroup C1 were correctly identified from among 848 intestinal strains investigated. The finding that three strains of *Escherichia coli* and most strains of *Candida* were also positive in immunofluorescence tests with this antiserum is readily explained by the known structural similarities among the antigenic determinants of *E. coli*, *Candida* and *Salmonella* of serogroup C1. The specificity of the antiserum for the O7 antigen determinant was further demonstrated in enzyme-linked immunosorbent assay tests and in co-agglutination tests with staphylococci sensitised with *S. thompson* Os-BSA antiserum.

INTRODUCTION

Because identification of salmonellae is based on conventional cultural procedures and biochemical and serological tests, which are time consuming and expensive, there is a need for diagnostic tools that are rapid, accurate and cheap. In this respect, other systems such as immunofluorescence (IFL) and co-agglutination (COA) have been investigated (Cherry and Moody, 1965; Kronvall, 1973; Thomason and Hebert, 1974; Cherry *et al.*, 1975). However, their diagnostic potential is highly dependent on the specificity of the antibody preparations used; the lack of specificity of many conventional antisera, i.e., those raised against whole killed bacteria, has limited their use in these alternative methods (Thomason and Hebert, 1974).

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Classification of salmonellae into serogroups is based on the specificity of their O antigens (Kauffmann, 1966), the determinants of which reside in the polysaccharide chain of the cell-wall lipopolysaccharide (LPS). The O polysaccharide chains of salmonellae from serogroups A (O antigens 1, 2, 12), B (O₁, 4, (5), 12), C₂ (O₆, 8), C₃ (O₈, 20), D (O₁, 9, 12) and El-4 (O₃, 10; O₃, 15; O₃, 34; O₁, 3, 19) are polymers of oligosaccharide-repeating units comprising from three to five monosaccharide residues (Kenne and Lindberg, 1982). We have reported elsewhere the preparation of a series of synthetic disaccharide-bovine serum albumin (BSA) conjugates, representative of O antigens 2, 4, 8 and 9, that elicited antisera specific for their respective bacterial O antigens and were of use, therefore, in identification by IFL and COA techniques (Svenungsson and Lindberg, 1977, 1978*a* and *b*, 1979; Svenungsson, Jörbeck and Lindberg, 1979). Although 75% of the salmonellae isolated in Sweden are of serogroup B, C₂, C₃ or D, about 15% belong to serogroup C₁ (O₆, 7). However, because the structure of the polysaccharide chain that determines the O antigenic specificity of salmonellae of serogroup C₁ has not been definitively established (Fuller and Staub, 1968), the use of a defined antigenic determinant as hapten in an artificial immunogen was not possible. A series of oligosaccharides has been isolated from the O polysaccharide of *Salmonella thompson* strain IS40 (O₆₁, 6₂, 7) (Bruse, Wrangsell, Lönngren and Svenson, unpublished results). In this paper, we describe an antiserum with specificity for antigen O₇, prepared against one of these oligosaccharides covalently coupled with BSA, and discuss its usefulness for the identification of isolates of salmonellae of serogroup C₁ by IFL and COA tests.

MATERIALS AND METHODS

Bacterial and yeast strains. *S. cholerae-suis* strain 5210 was kindly provided by Dr L. Le Minor, Institut Pasteur, Paris. Other strains of bacteria were from the culture collection of the Department of Bacteriology, National Bacteriological Laboratory, Stockholm, or were fresh cultures isolated from faeces in the course of examination for pathogenic intestinal bacteria. The isolation and identification of the different bacteria were performed as described by Svenungsson *et al.* (1979). *Staphylococcus aureus* strain Cowan 1 was used for the preparation of the COA reagent. *Candida albicans* (ATCC2091), *C. curvata* (ATCC10567), *C. pseudotropicalis* (PHLS3106), *C. zeylanoides* (ATCC7351), *C. tropicalis* (ATCC750), *C. guilliermondi* (ATCC6260), *C. stellatoidea* (ATCC11006), *C. parapsilosis* (ATCC7330) and *C. krusei* (ATCC6258) were from the mycology culture collection of the National Bacteriological Laboratory, Stockholm.

Bacteriophage 14 was kindly provided by Dr L. Le Minor, Institut Pasteur, Paris.

Lipopolysaccharide preparations from *S. typhimurium* strain SH4809 (O₄, 5, 12) and the rough strain TV160 were available from other studies (Svenson and Lindberg, 1979); those from *S. thompson* strain IS40 (O₆₁, 6₂, 7), *S. cholerae-suis* strain 5210 (O₆₂, 7) and *S. newport* strain IS50 (O₆₁, 8) were prepared as described by Hellerqvist *et al.* (1968).

Preparation of oligosaccharides. Oligosaccharides from *S. thompson* strain IS40 were prepared as follows (Bruse, Wrangsell, Lönngren and Svenson, unpublished results): LPS from *S. thompson* was treated with 0.15M NaOH at 100°C for 60 min. After extraction of released fatty acids, the residual solution was centrifuged at 5000 *g* for 20 min and the clear supernate was extensively dialysed against 5mM ammonium carbonate buffer (pH 7.0). The mixture of oligosaccharides obtained after treatment of the O polysaccharide with *endo*-mannosidase from bacteriophage 14 (specific for bacteria of serogroup C₁) was separated on Bio-Gel P4 (Bio Rad Laboratories, Richmond, Calif).

Oligosaccharide-bovine serum-albumin conjugate. An oligosaccharide of mol. wt *c.* 850 was covalently coupled to BSA as described by Svenson and Lindberg (1979) and the resulting conjugate, containing oligosaccharide and BSA in a molar ratio of *c.* 10:1, is referred to as *S. thompson* Os-BSA.

Immunisation procedure. The *S. thompson* Os-BSA conjugate suspended in Freund's complete adjuvant (1:1) was injected in 50- μ g amounts directly into the popliteal lymph nodes of rabbits on days 1, 15, 56 and 71. Rabbits were bled before injection and at day 85.

Enzyme-linked immunosorbent assay (ELISA). Performance of the ELISA test and preparation of the sheep anti-rabbit immunoglobulin alkaline-phosphatase conjugate specific for rabbit immunoglobulins IgG, IgM and IgA followed the techniques of Carlsson, Hurvell and Lindberg (1976).

Antiserum against the *Salmonella* O9 determinant, i.e., 3-O- α -(tyvelopyranosyl)- α -D-mannopyranoside covalently linked to BSA, was available from another investigation (Svenungsson and Lindberg, 1977). Antiserum against salmonella factor O7, prepared according to the method of Kauffmann (1966), was used in slide agglutination tests.

Co-agglutination studies. The preparation of staphylococci sensitised with *S. thompson* Os-BSA antiserum has been described (Svenungsson and Lindberg, 1978b). The COA reagent was stable for at least 2 weeks when stored at 4°C. A few bacterial colonies from the culture under investigation were suspended in a drop of the COA reagent on a glass slide, which was rocked back and forth for 2 min. Agglutination clearly visible by the naked eye was registered as 2+ and, when a magnifying glass was needed for its observation, as 1+.

Immunofluorescence studies. The indirect IFL method was that of Svenungsson and Lindberg (1977). The sheep anti-rabbit immunoglobulin-fluorescein isothiocyanate conjugate (fluorescein:protein molar ratio of 2.2:1) was from the Department of Immunology, National Bacteriological Laboratory, Stockholm. The working dilution of the conjugate was the highest dilution (1 in 40) that gave a 4+ reaction. A Leitz Orthoplan fluorescence microscope with incident light and a mercury HBO-200 lamp as light source was used. Fluorescence reactions were graded as described by Karlsson, Thore and Kudynowski (1975); 4+ and 3+ reactions were judged as positive, and 2+ and 1+ reactions as negative. In the performance of the IFL tests, the identity of a strain was not known to the examiner.

RESULTS

The specificity of the antiserum raised in rabbits against the *S. thompson* Os-BSA conjugate was first evaluated by ELISA (table I), which indicated a 400-fold increase in titre of a hyperimmune serum against LPS of *S. cholerae-suis* strain 5210 (O₆, 7) and no significant increase in titre against LPS from *S. newport* (O₆, 8). This finding suggested that the antibodies elicited were directed against the O7 antigen determinant. There was no increase in titre against LPS from two heterologous strains of *S. typhimurium*.

TABLE I

ELISA titration of rabbit antiserum elicited against S. thompson Os-BSA conjugate

Lipopolysaccharide coating antigen from strain (O antigens)	ELISA titre* (10 ³) of	
	preimmune serum	immune serum
<i>S. cholerae-suis</i> 5210 (O ₆ , 7)	4	1590
<i>S. newport</i> IS50 (O ₆ , 8)	< 1	7
<i>S. typhimurium</i> SH4809 (O ₄ , 12)	< 1	< 1
<i>S. typhimurium</i> TV161	< 1	< 1

* The serum dilution giving a change in optical density (400 nm) of 0.1 "per" 100 min.

Indirect immunofluorescence studies

In tests with *S. cholerae-suis* strain IS36 (O₆, 7), the endpoint titre of two hyperimmune sera from different rabbits that gave a 3+ reaction was 160. Fluorescence was not obtained in tests with salmonellae of serogroups other than C1, even when the serum was used at a dilution of 1 in 5. Neither preimmunisation sera nor serum against BSA gave fluorescence with any strain of *S. cholerae-suis*.

Known salmonellae. The specificity of the *S. thompson* Os-BSA antiserum indicated in ELISA tests was confirmed in IFL tests with 243 strains of *Salmonella* of known serogroups (table II), most of which were of commonly isolated serogroups. Of

TABLE II
Indirect immunofluorescence with S. thompson Os-BSA antiserum by salmonellae of different O serogroups

Salmonella serogroup (O antigens)	Number of strains positive by immunofluorescence/number of strains tested
A (O1, 2, 12)	0/2
B (O1, 4, 5, 12)	0/117
C1 (O6, 7)	49/50
C2 (O6, 8)	0/34
C3 (O8, 20)	0/5
D (O1, 9, 12)	1/19
E1-4 (O3, 10; O3, 15; O3, 34; O1, 3, 19)	0/10
G2 (O13, 23)	0/3
K (O18)	0/1
O38	0/1
O51	0/1
All	50/243

the 50 isolates of serogroup C1 tested, 49 fluoresced strongly with the antiserum against *S. thompson* Os-BSA (table II); the exceptional strain, *S. bareilly* (O₆, 7), which was scored as negative or weakly positive at the working dilution of serum, was strongly positive in tests with serum at a dilution of 1 in 20. One isolate, *S. panama* (O1, 9, 12) from serogroup D, was positive in similar tests (see below). The other 192 strains of *Salmonella* tested gave negative reactions in IFL tests with *S. thompson* Os-BSA serum.

Unidentified intestinal bacteria from faeces. The investigation was extended to include tests with cultures of 324 unidentified enterobacterial strains freshly isolated from faeces (table III). Part of a colony, considered presumptively on morphological grounds to be *Salmonella*, from a culture on either a deoxycholate-citrate agar or a brilliant green-phenol red agar, was suspended in phosphate-buffered saline on a glass slide for investigation by immunofluorescence. Part of the same colony was examined by routine biochemical and serological tests. Of the 324 strains tested, 27 that fluoresced strongly were salmonellae of serogroup C1 (O₆, 7); another 214 strains of *Salmonella* from serogroups other than C1 and 83 strains of other enterobacteria, primarily isolates of *Citrobacter* and *Enterobacter*, were negative (table III).

TABLE III

Indirect immunofluorescence with S. thompson Os-BSA antiserum by enterobacteria isolated from faeces

Salmonella serogrup (0 antigens)/ number of serotypes	Number of strains positive by immunofluorescence/ number of strains tested
B (O1, 4, 5, 12)/10	0/113
C1 (O6, 7)/7	27/27
C2 (O6, 8)/4	0/46
D (O1, 9, 12)/2	0/48
E1 (O3, 10)/1	0/3
E4 (O1, 3, 19)/1	0/2
G1 (O13, 22)/1	0/1
P (O38)/1	0/1
Not salmonella	0/83
All	27/324

Intestinal bacilli. The specificity of the antiserum against *S. thompson* Os-BSA was further assessed in tests with 364 isolates of intestinal bacilli (table IV). Apart from three strains of *E. coli*, all other enterobacteria and all strains of *Pseudomonas*, *Aeromonas*, *Acinetobacter*, *Vibrio*, *Pasteurella* and *Bacteroides* tested were negative in the IFL test. Of the three strains of *E. coli* that were positive in the IFL test, one belonged to serogroup O112ab; the other two were not typable.

Candida strains. Because cross reactions between salmonellae of serogroup C1 and strains of *Candida* are well known, isolates of nine different species of *Candida* were assayed in IFL tests with *S. thompson* Os-BSA antiserum. Apart from the isolate

TABLE IV

Indirect immunofluorescence with S. thompson Os-BSA antiserum of intestinal bacilli other than salmonellae

Species or group	Number of strains* positive by immunofluorescence/ number of strains tested
<i>Escherichia coli</i>	3/104
<i>Enterobacter</i> spp.	0/78
<i>Citrobacter</i> spp.	0/35
<i>Proteus</i> spp.	0/28
<i>Klebsiella</i> spp.	0/19
<i>Shigella</i> spp.	0/15
<i>Yersinia enterocolitica</i>	0/5
<i>Serratia</i> spp.	0/2
<i>Aeromonas</i> spp.	0/6
<i>Pseudomonas</i> spp.	0/7
<i>Acinetobacter</i> spp.	0/2
<i>Vibrio</i> spp.	0/4
<i>Pasteurella</i> spp.	0/1
<i>Bacteroides</i> spp.	0/58
All	3/364

* Includes 83 strains of enterobacteria from table III

of *C. curvata*, strains of *Candida* were strongly positive. However, the possibility of misidentification of *Candida* as *Salmonella* is unlikely in view of major differences in their shape and size.

Co-agglutination

The applicability of COA was investigated in tests with 129 strains of *Salmonella* from serogroups A to E and O35. Each of the 22 isolates of *Salmonella* from serogroup C1 agglutinated strongly with the *S. thompson* Os-BSA antiserum-sensitised reagent (table V). Another 107 strains from serogroups other than C1 were negative.

TABLE V

Co-agglutination of salmonellae with S. thompson Os-BSA antiserum-sensitised staphylococci

Salmonella serogroup (O antigens)	Number of strains positive by co-agglutination/ number of strains tested
A (O1, 2, 12)	0/5
B (O1, 4, 5, 12)	0/58
C1 (O6, 7)	22/22
C2 (O6, 8)	0/12
C3 (O8, 20)	0/6
D (O1, 9, 12)	0/18
E1 (O3, 10)	0/1
E4 (O13, 19)	0/6
O35	0/1
All	22/129

DISCUSSION

The pentasaccharide from LPS of *S. thompson* (O6₁, 6₂, 7), used as hapten in covalent linkage to an immunogenic carrier protein, apparently elicited antibodies specific for antigen O7 as demonstrated in ELISA, IFL and COA tests. Most of the information was obtained from IFL studies, which showed that among 848 isolates of intestinal bacteria, 76 of 77 strains of *Salmonella* of serogroup C1 were correctly identified at the routine working dilution of serum. In view of our previous observations (Svenungsson and Lindberg, 1977, 1978*a* and *b*) that strains after storage often gave weaker reactions in IFL tests than those of fresh clinical isolates, it was not unexpected that the exceptional strain of serogroup C1 that gave anomalous results in IFL tests was an isolate of *S. bareilly* from a stock-culture collection.

An unexpected positive reaction in IFL tests with the *S. thompson* Os-BSA antiserum was given by an isolate of *S. panama* (O9, 12). However, the finding that the bacterial cells from that group-D culture that stained with antiserum specific for antigen O9 outnumbered those stained with the *S. thompson* antiserum by 100 to 1 suggested that the culture of *S. panama* was possibly contaminated with small numbers of salmonellae of serogroup C1 (which we have not isolated). It should not be considered as indicating poor specificity of the *S. thompson* Os-BSA antiserum.

Confirmatory evidence of the specificity of the *S. thompson* Os-BSA antiserum was obtained from ELISA tests (table I) and COA studies (table V).

Although the detailed structure of the oligosaccharide of *S. thompson* has not yet been determined, it is probably composed of *N*-acetyl-D-glucosamine and D-mannose residues (in a molar ratio of 1:4), with the D-mannose residues joined in 1, 2 linkages and the *N*-acetyl-D-glucosamine residue substituted at C3 (Lönngren, Svenson and Lindberg, unpublished results). Work is in progress to establish the anomeric configurations. Because mannan structures are of common occurrence in micro-organisms, we did not expect to elicit against the mannose-rich oligosaccharide of the *S. thompson* Os-BSA conjugate antibodies of such high specificity as those elicited against the previously described synthetic dideoxyhexopyranosyl 1- α -3 mannopyranoside haptens representative of the antigens O2, O4, O8 and O9. Thus, the observation that three of 104 strains of *E. coli* examined were positive in IFL tests was not unexpected, because cross reactions between *Salmonella* of group C1 and *E. coli* have been described (Ørskov *et al.*, 1977). In yeasts, a mannan covalently linked to protein represents a principal cell-wall constituent (Ballou, 1976). The mannan has an α 1, 6-linked polymannose backbone with branches of α 1,3- and α 1,2-linked D-mannose residues. Because the structures of the oligosaccharide of *S. thompson* and the mannan of *Candida* are similar, the observation that most species of *Candida* stained with the *S. thompson* Os-BSA antiserum was expected.

The *S. thompson* Os-BSA antiserum was as specific for antigen O7 as a conventional antiserum, i.e., one prepared by immunisation with heat-killed bacteria and absorbed with five different strains of *Salmonella* (data not shown). However, because the pentasaccharide contains several different regions, it is likely that antigen O7 contains several antigenic determinants, one of which, if identifiable from further structural studies, may be used as hapten for production of an antiserum wholly specific for salmonellae of serogroup C1.

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