Sensitive and Rapid Immunoassays for *Salmonella enteritidis*

J.-L. Liu, C.E. Benson, D.S. Munro, and B. Wolf*

University of Pennsylvania, School of Veterinary Medicine, New Bolton Center, Kennett Square, Pennsylvania

In order to develop more sensitive, specific, and rapid immunoassays to detect *Salmonella enteritidis* in food supplies, we have applied various approaches by using several different antibody preparations. Utilizing ELISA in both a plate and immunodot assay, we employed (i) a polyclonal rabbit antiserum to a boiled suspension of the organism; (ii) a monoclonal antibody to the cell surface of the bacterium; and (iii) mouse antisera to two oligosaccharides each containing the rare sugar tyvelose, and exhibited by *S. enteritidis*, a member of the group D salmonellae. We showed that the polyclonal antiserum and monoclonal antibody IG-10 to the cell surface could specifically detect from 10^2 to 10^3 organisms in a 10- μ l sample in the plate and immunodot assay. Both assays are read in 4–5 hr. Further, in the mice immunized to the trisaccharide, (α -

D-galactose- α -tyvelose- α -D-mannose), as well as those mice immunized to the tetrasaccharide, (α -D-galactose- α -tyvelose- α -D-mannose- α -L-rhamnose), specificity to tyvelose was determined by inhibition studies. The inhibitors of the antisera to the trisaccharide included the single sugar tyvelose conjugated to bovine serum albumin (BSA), a tetrasaccharide in which tyvelose is excluded, but contains α -D-galactose, α -ascarose, α -D-mannose, and α -L-rhamnose (conjugated to BSA), and others. The inhibition studies suggest that the mouse antisera are specific for tyvelose and also contain antibodies for mannose and rhamnose. The antibodies that have been made to the unique sugar tyvelose should improve the specificity in assays for *S. enteritidis*. J. Clin. Lab. Anal. 15:165–170, 2001. © 2001 Wiley-Liss, Inc.

Key words: rapid immunoassays; *S. enteritidis*

There is an essential need to develop sensitive and specific procedures to detect a variety of infectious microorganisms in foods as well as in unprocessed plants and animals. Although other tests are available, immunoassays provide direct and often ultra-high sensitivity without the need to purify the target organism. In the past several years, we have been engaged in developing immunoassays for infectious microorganisms and for a number of drugs including sulfonamides and gentamicin, which are found as extra-label residues in milk. This has resulted in development of a rapid immunodot assay using a high-affinity monoclonal antibody to gentamicin suitable for field testing (1).

In the present work, we compared several immunoassays to detect *Salmonella enteritidis* in order to devise a rapid field test. We utilized in an ELISA (i) a polyclonal rabbit antiserum, (ii) a mouse monoclonal antibody to the bacterial cell surface, and (iii) mouse antisera to two oligosaccharides, each containing reactivity to the rare sugar tyvelose, present only in group D salmonellae (of which *S. enteritidis* is a member). For testing, both a plate and an immunodot ELISA were employed.

MATERIALS AND METHODS

Immunizations

The strains of *Salmonella* used in immunization and adsorptions were all acquired from the United Kingdom's Na-

tional Collection of Type Cultures. Rabbits were immunized with a boiled whole-cell suspension of *S. enteritidis* (NCTC:12694/ATCC 13076) according to standard methods to provide high-titered antisera (approximately 1:16,000–1:32,000). An antiserum was cross-adsorbed with a range of serotypes as recommended by the WHO (2) [*S. paratyphi* A (NCTC:5702), *S. typhimurium* (NCTC: 5711, *S. senftenberg* (NCTC: 3158), *S. onderstepoort* (NCTC: 5795), *S. illinois* (NCTC 8498), *S. nairembé* (SSRL/Institute Pasteur), *S. abortus equi* (NCTC: 5727)] to remove the three minor subfactors of antigen 12. In addition, one culture each of *Escherichia coli*, *Citrobacter freundii*, and *Proteus vulgaris* from clinical submissions to the Scottish Salmonella Reference Laboratory (SSRL) that had previously been determined by SSRL to carry extraneous subfactors of factor 12 were included in the panel of absorbing strains. These strains had been submitted to SSRL as putative salmonellae on the basis of their

*Correspondence to: B. Wolf, School of Veterinary Medicine, 382 West Broad Street, Kennett Square, PA. E-mail: bwolf@vet.upenn.edu

Received 29 March 2000; Accepted 18 December 2000

reaction with commercially available antisera. Prior to use, the immunizing and absorbing cultures were re-serotyped by the SSRL using standard methods (2) to confirm serotype and the absorbed serum was tested by tube titration (1/4) to ensure that all activity to the subfactors of the 12 antigen complex had been removed. Other group D serotypes, i.e., *S. berta*, *S. dublin*, *S. panama*, or *S. wangata* were not tested for their reactivity with the polyclonal antiserum.

Mice were immunized with a cell lysate of *S. enteritidis* and monoclonal antibodies from hybridomas were obtained after hybridization with sp 2/0-Ag 14 myeloma cells (2). Other mice were immunized with the tyvelose-containing tri- and tetrasaccharides 149-1 and 195-1 conjugated to bovine serum albumin (BSA) as shown in Figure 4. Glycoconjugate structure 149-1 in the figure shows the trisaccharide α -D-galactose $p(1\rightarrow2)$ -[α -tyvelose (1 $\rightarrow$ 3)] α -D-mannose conjugated to bovine serum albumin (BSA) at the mannose sugar. Structure 195-1 is the tetrasaccharide α -D-galactose (1 $\rightarrow$ 2)-[α -tyvelose (1 $\rightarrow$ 3)] α -D-mannose $p(1\rightarrow4)$ - α -L-rhamnose p conjugated to BSA at rhamnose. Other structures in the figure are 159-1, α -tyvelose (1 $\rightarrow$) conjugated to BSA, 189-1 which does not contain tyvelose but is α -D-galactose $p(1\rightarrow2)$ -[α -ascarose (1 $\rightarrow$ 3)]- α -D-mannose $p(1\rightarrow4)$ α -L-rhamnose p -conjugated to BSA and structure 161-1, β -D-glucosaminacetate- α -L-rhamnose p also conjugated to BSA (3).

Fig. 1. Plate test using an adsorbed rabbit antiserum to *S. enteritidis* in which the bacteria could be detected routinely at levels of 10^3 organisms in a 10 μ l sample.

ELISAs

Two immunoassays were performed. The first, a plate test, utilizes boiled *S. enteritidis*, Y8P2 (Benson strain), which was initially diluted to amounts varying from 10^7 to 10^2 organisms in carbonate buffer (pH 9.6) was applied to 96-well immunoassay plates. This approach was chosen for the following reasons. As has been verified in a number of cases, this does not alter the antigenic configuration of the bacterial organism, and the results obtained as described in this paper would apply to testing of the bacterium in foods. A simple direct assay was deemed to be necessary because we thought that it would be more amendable for testing in the field and would yield results more quickly. In addition, as has been observed by others, there was a danger that trying to extract the relevant antigen from bacteria often denatures epitopes. If fewer reagents were used, backgrounds would be lower, interlaboratory reproducibility would be greater, as would assessment of assay precision. False positives and negatives might be minimized as well. Numerous positive and negative controls were run. They included the antigen alone without antibody, buffer control, polyclonal and monoclonal antibody alone, heterologous antisera and heterologous monoclonals, and in the case of the hybridoma antibody, culture medium controls. After incubation (1 hr, 4°C), 0.5% glutaraldehyde was added to crosslink the bacteria and create a bacterial mat. Using a

Fig. 2. Representative plot in a plate assay in which 10^3 organisms/10 μ l were detected using the monoclonal antibody IG-10 specific for the bacterial surface with no cross-reactivity, as noted in the text.

complexed matrix has been shown by others to lead to a positive reaction, whereas uncomplexed antigen gave negative results (4). This step also considerably enhanced the sensitivity of the reaction. After the plates were washed in phosphate-buffered saline (PBS)/1% Tween and blocking buffer [(PBS) + 1% Tween + 10% fetal calf serum)] was added, the antiserum or monoclonal antibodies were added in predetermined dilution, the plates were washed, and either biotinylated anti-rabbit IgG or biotinylated anti-mouse IgG was added. Alkaline phosphatase-conjugated streptavidin and the coloring solution, p-nitriphenyl phosphate (pNPP), were then added. The yellow color is read quantitatively at 405 nm after 30–60 min, with the procedure taking 5–6 hr.

The second assay, an immunodot ELISA, was performed on nitrocellulose paper strips. First, bacteria in counts ranging from 10^5 to $10^1/\mu\text{l}$ were dotted on the paper strips in carbonate buffer, pH 9.6 for 30 min, at room temp. The strips were soaked in PBS buffer containing 0.2% glutaraldehyde solution for 15 min to enhance the reaction. After being blocked in PBS + 1% Tween + 10% fetal calf serum, the strips were incubated with biotinylated antiserum or biotinylated monoclonal antibodies. After alkaline phosphatase-streptavidin was applied, the blue-colored dots were visualized with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) plus nitro blue tetrazolium (NBT). The reaction was complete in 4–5 hr.

RESULTS

Initially, we tested the antiserum to the entire organism, which had been cross-adsorbed with a variety of salmonellae as well as *E. coli*, *C. freundii*, and *P. vulgaris* in the plate test and dot assay. We found in the plate test that with a 1:500 dilution of the antiserum we could routinely detect approximately 10^3 organisms in a 10- μl sample (Fig. 1). The background optical density (OD) level in the negative controls ranged from 0.041 for the well containing 10^6 organisms/10 μl to 0.039 for the wells containing 10^3 organisms/10 μl . The positive readings were thus about 4.5 to 5-fold above the negative controls. Although the curve was basically flat at 10^4 and 10^3 , because both were about 5-fold above background these were deemed to be positive. In the tests for specificity against the aforementioned cross-reactive bacteria, we were unable to detect them at levels of $10^5/10 \mu\text{l}$ (not shown). In the immunodot assay, 10^2 *S. enteritidis* in 10 μl could be repeatedly detected with no discernible cross-reactivity against the same organisms as in the plate test.

In addition, we also employed the monoclonal antibody IG-10, previously reported by our laboratory (4) in both the plate and immunodot assays. This antibody had been raised using a cell lysate from *S. enteritidis* and previously evaluated against a number of salmonellae represented by groups B, C1, C2, C3, C4, D1, E1, E2, E3, E3, G2, K, and R. As

Dot-ELISA for *Salmonella enteritidis* using monoclonal antibodies to lipopolysaccharide (1G 10)

Fig. 3. Data of the immunodot assay using the monoclonal antibody IG-10 specific for the bacterial surface. The assay could detect 10^4 organisms/ml or 10^2 organisms/10 μl on the nitrocellulose filter.

Fig. 4. Structures of the oligosaccharides used to immunize mice against tyvelose and surrounding monosaccharides (149-1 and 195-1). Inhibitors employed were 159-1, 189-1, and 161-1 (see text).

described in their paper (4) only *S. enteritidis* (D1), *S. dublin* (D1), and *S. berta* (D1) reacted with the IG-10 antibody. Other enteric bacteria were tested with no cross-reactivity (*E. coli*, *Serratia marcescens*, *C. freundii*, *Klebsiella pneumoniae*, *Proteus vulgaris*: $10^5/10\ \mu\text{l}$). To achieve a more sensitive ELISA, we used the method of cross-linking the bacteria into an aggregate in the well plus utilizing the biotinylated antibodies, detected with alkaline phosphatase-conjugated streptavidin, plus *p*-nitrophenyl phosphate (pNPP). We wished also to evaluate a immunodot method. We were indeed successful in raising the sensitivity of the ELISA so that fewer organisms could be detected in each of the assays (Fig. 2 and 3), than in our previous report, and these figures show in the lower limit of detection a considerable increase in sensitivity. Figure 2 shows a representative plot in a plate assay in which 10^3 organisms/ $10\ \mu\text{l}$ are routinely detectable for the monoclonal antibody (IG-10) to the bacterial cell surface. This is a 10-fold increase in sensitivity of previous findings (4). In ad-

dition, *E. coli*, *C. freundii*, and *P. vulgaris* did not react in the monoclonal antibody-based assay. Figure 3 shows the data of the immunodot assay, also using the monoclonal antibody IG-10. The assay could detect 10^4 organisms/ml or 10^2 organisms/ $10\ \mu\text{l}$ on the nitrocellulose filters with no cross-reactivity to the three organisms used in the plate test described above.

Although the presence of tyvelose has been confirmed for *S. enteritidis*, to date there have been no reports on the induction of antibodies to this sugar. Dr. David Bundle succeeded in conjugating a number of oligosaccharides to BSA, shown in Fig. 4, three of which contained tyvelose and two of which contained other sugars that could be used as negative controls. After we injected the trisaccharide-BSA (149-1) and the tetrasaccharide-BSA (195-1) (Fig. 4) each of which contained tyvelose, into individual mice, the antisera were run in a plate test to determine their potency. Titers of 1:640 in pooled antisera were achieved in both 149-1 and 195-1. We selected

Fig. 5. Inhibition data in which a mouse antiserum to the tetrasaccharide 195-1 was inhibited with either the immunizing antigen (195-1) or alternatively with tyvelose-containing sugars or those not containing tyvelose (see text).

the antisera to 195-1 to test for the specificity to tyvelose. Inhibition assays were performed using the antigens 149-1, 195-1, 159-1, and 161-1, each starting at the concentration of 1 µg/ml which was diluted from 1:50–1:6,400, as shown (Fig. 5). The tyvelose–BSA conjugated (structure 159-1) was especially important to evaluate, and it showed good inhibition. It is interesting that structures 189-1 and 161-1 also gave good inhibition and this is interpreted to indicate that the sera also contained antibodies to rhamnose and mannose. Tests will be performed to validate this assumption. These assays employed a horseradish peroxidase-base plate ELISA and an agglutination test. The ELISA assay was able to detect a lower limit of 10^5 – 10^6 organisms per 0.1 ml (10^4 organisms).

DISCUSSION

Our objective was to compare several immunoassays in order to determine the most suitable tests so that ultrasensitive and rapid methods could be devised for *S. enteritidis* in foods. We employed plate and immunodot ELISAs, both of which could be performed in 4–6 hr. The sensitivity for the polyclonal antiserum and the monoclonal antibody to the bacterial cell surface was 10^3 organisms/10 µl in the plate test. The sensitivity in the immunodot assay was somewhat higher at 10^2 organisms/10 µl using the polyclonal and the monoclonal antibody.

The objective in immunizing mice to the tyvelose-contain-

ing oligosaccharides was to develop monoclonal antibodies for sensitive assays to the sugar. Dr. Bundle has published research on carbohydrate-containing epitopes of serogroup B salmonellae (7,8), and recently he and his group have reported on tyvelose-containing epitopes in *Trichinella spiralis* (8,9). In assaying mouse antisera, we found that specific antibodies were formed to tyvelose as judged by inhibition analyses. Antibodies to the adjacent sugars rhamnose and mannose residues were apparently also formed. However, although antibodies to these adjacent sugars were seen, specific reactivity to tyvelose alone was evident as judged by the inhibition with the single tyvelose sugar. Because this is the case, it is important to report this finding. To date we have not succeeded in constructing hybridomas that secrete anti-tyvelose antibodies. This may be due to an underrepresentation of cells undergoing hybridization as a result of a less intense immunization protocol. A more intensive immunization may correct this problem.

Several ELISA assays to detect *S. enteritidis* have been reported. It was found that monoclonal antibodies to fimbriae showed cross-reactivity to other fimbriae, indicating heterogeneity among salmonellae (10). An indirect ELISA to a combination of flagellar and fimbrial antigens showed high sensitivity with decreased cross-reactivity upon absorption (11). An indirect ELISA for flagellin was also used to screen for flagellin antibody in commercial flocks of chickens with high sensitivity, but no data were given as to the numbers of bacterial cells which could be detected (13). There also have been publications on immunodot assays for *S. enteritidis* (14,15). Both of these papers show the immunodot assay to be sensitive and specific. However, we wished to enlarge the repertoire of tests to include more sensitive and specific assays. The advantage of our reported tests are evident in that they are highly sensitive and specific and can be performed routinely by nonspecialized personnel in the field, especially if test kits were made available. Indeed, an added advantage is that both procedures can be completed in 4–5 hr. The sensitivity has been increased in our plate and immunodot ELISAs utilizing an absorbed antiserum as well as a monoclonal antibody to the bacterial cell surface. Likewise, for the first time we have detected antibodies to a single epitope, tyvelose, which should afford a highly specific immunoassay for the organism. The ultimate aim of course is to utilize these procedures in the detection of the organism in food supplies; this may be readily accomplished, as we have earlier reported testing for the organism in eggs and other field samples and have the procedures at hand (4).

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