Rapid Detection of Salmonellae in Poultry with the Magnetic Immuno-Polymerase Chain Reaction Assay

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Rapid detection of salmonellae in chicken meat was accomplished by using the magnetic immuno-polymerase chain reaction assay (MIPA). A direct polymerase chain reaction assay performed with chicken meat spiked with Salmonella typhimurium resulted in poor sensitivity (approximately 10^7 CFU/g of meat). The use of immunoseparation with a Salmonella serogroup B-specific monoclonal antibody improved the sensitivity, but enrichment was required for the detection of low levels of contamination. Enrichment for 6 h in either buffered peptone water, lactose broth containing tertitol-7, or selenite-cystine broth resulted in the detection of an initial inoculum of 100 CFU per g of meat. Enrichment of the salmonellae present on 25 g of spiked chicken meat for 24 h in either buffered peptone water or selenite-cystine broth before detection by the MIPA yielded a detection limit of approximately 0.1 CFU/g of meat. A detection limit of approximately 1 CFU/g of meat was obtained when the spiked meat was stored at −20°C before enrichment for 24 h and analysis with the MIPA. Although the MIPA was developed for S. typhimurium, a MIPA in which a panel of six monoclonal antibodies specific for Salmonella serogroups A through E was used detected the presence of 0.1 CFU of Salmonella enteritidis per g of chicken meat. These data indicate that the method is applicable to other commonly isolated serotypes.

Salmonellae are some of the major causes of food poisoning in the developed world. Food products like meat, eggs, poultry, and chocolate are common sources of salmonellosis (2, 7, 12, 18). Therefore, a rapid diagnostic test is needed. Conventional methods for the isolation of Salmonella spp. require 5 days for the detection of small numbers of often stressed organisms (6). In recent years methods based on either immunoassays (3, 9, 12, 14, 17, 18) or DNA probes (4, 5, 15, 21) have been developed. Most prominent among the immunoassays is the Salmonella-Tek assay (Organon-Teknika Corp., Durham, N.C.) (18). This immunoassay utilizes two monoclonal antibodies which recognize antigens from heat-killed salmonellae. This test, however, still requires 48 h to complete (4), but with enrichment in Salmo-syst broth the total time required can be reduced to 31 h (18). Several DNA probes for Salmonella spp. have been developed, but only a probe that recognizes rRNA is commercially available (Gene-Trak Systems, Framingham, Mass.). Detection is based on the hybridization of a polyoxyethyleneylc acid-tailed capture probe and a fluorescein-labeled detector probe for Salmonella rRNA. The resulting hybrid is captured on a dipstick coated with polyoxyethylymydic acid and is detected by colorimetry by using an antifluorescein-antibody conjugate with horseradish peroxidase. Nevertheless, the earliest results are obtained on day 3 (4, 15, 21). Another major drawback of these tests is the relatively large number of false-positive results obtained (15).

Recently, magnetic separation technology was introduced to make the assays faster and more specific (10, 11, 19). Previously, we described (19) the development of the magnetic immuno-polymerase chain reaction assay (MIPA). This assay utilizes magnetic particles coated with monoclonal antibodies to extract bacteria from a sample. Trapped bacteria are lysed, and the supernatant, which contains bacterial DNA, is then subjected to the polymerase chain reaction (PCR). A MIPA performed with a Salmonella group B-specific monoclonal antibody and Salmonella-specific PCR primers based on the origin of DNA replication was capable of detecting 10 CFU of Salmonella typhimurium in the presence of 10^7 CFU of Escherichia coli (19). In this paper we show that when the MIPA is used in combination with enrichment, as few as 0.1 CFU of Salmonella spp. per g of poultry meat can be detected reliably within 30 h.

MATERIALS AND METHODS

Media. Most of the media used were obtained from Oxoid, Basingstoke, United Kingdom; the exceptions were sodium hydrogen selenite, which was purchased from Merck, Darmstadt, Germany, and tertitol-7, which was bought from Sigma Chemical Co., St. Louis, Mo.

Bacteria. The Salmonella serotypes used belong to Salmonella choleraesuis subsp. choleraesuis. The two strains used are referred to below as S. typhimurium and Salmonella enteritidis. Unless otherwise indicated, S. typhimurium was used.

Immunomagnetic separation. Magnisort M magnetic chromium dioxide particles coated with goat immunoglobulins specific for murine immunoglobulin G (IgG) and IgM (DuPont, Wilmington, Del.) were incubated with 75 μl of hybridoma culture supernatant for 5 min at room temperature with continuous shaking. The magnetic particles were recovered by magnetic force, and the supernatant was discarded. Bacteria from a fresh culture were suspended in saline and then added to the magnetic particles. After 15 min at room temperature with continuous shaking, the magnetic particles were recovered by magnetic force, washed three times with saline, and finally resuspended in 20 μl of water for use in the PCR or in saline for plating onto Giston agar or salmonella-shigella agar plates. The plates were incubated overnight at 37°C, and the colonies were counted. Unless otherwise

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indicated, 5 μl of Magnisort M and monoclonal antibody 71.40 were used.

Monoclonal antibodies. The generation of serogroup-specific monoclonal antibodies has been described by Torensma et al. (16). The following six monoclonal antibodies were used in this study: monoclonal antibody 47.15 (IgM), specific for serogroup A; monoclonal antibody 71.40 (IgG1), specific for serogroup B; monoclonal antibody 72.4 (IgG3), specific for serogroup C1; monoclonal antibody 35.71 (IgG1), specific for serogroup C2; monoclonal antibody 41.11 (IgG1), specific for serogroup D; and monoclonal antibody 42.77 (IgG3), specific for serogroup E.

PCR. The resuspended beads were heated to 95°C for 5 min to lyse the bound bacteria. The beads were recovered by centrifugation, and the resulting supernatant was utilized in the PCR. The PCR was carried out as described by Widjojoatmodjo et al. (19), except that a total volume of 50 μl was used instead of 100 μl. Briefly, the PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, deoxyribonucleoside triphosphates (dNTP) (each at a concentration of 100 μM), primers (each at a concentration of 0.5 μM), and 1.25 U of Taq polymerase (Cetus, Emeryville, Calif.). The primers were selected in the bacterial origin of chromosome DNA replication (oriC). Primer 1 (5'-TTATTAGGATCGGCAGGC) was located between bp 60 and 79, and primer 2 (5'-AAAGAATAACCGTTGTT) (CAC) was located between bp 204 and 223 of the previously published sequence of Salmonella oriC (8). Amplification was carried out with a Thermocycler apparatus (Perkin-Elmer Instruments, Norwalk, Conn.) for 35 cycles and was followed by a final 10-min extension at 72°C. Each cycle consisted of 1 min at 94°C, 1 min at 58°C, and 1 s at 72°C. The PCR incubation mixtures were analyzed for product formation on an ethidium bromide-stained agarose gel. Confirmation was accomplished by Southern hybridization with a digoxigenin-labeled probe as described by Widjojoatmodjo et al. (19). Briefly, the probe for Southern analysis was labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany) by the PCR. The PCR reaction mixture was analogous to the PCR mixture described above, except that the PCR mixture contained different dNTP concentrations (100 μM dATP, 100 μM dCTP, 100 μM dGTP, 90 μM dTTP, and 10 μM digoxigenin-11-dUTP). The number of cycles was 24. S. typhimurium DNA was used as the target DNA. The identity of the probe was confirmed by restriction enzyme analysis. Hybridization and detection were performed as specified by Boehringer Mannheim.

Processing of chicken meat samples. Samples (10 and 25 g) of chicken meat were treated for 2.5 min in a stomacher, and the volumes were brought up to 100 and 250 ml, respectively, with medium. The media used were buffered peptone water (BPW), lactose broth containing tertitol-7 (LB+), and selenite-cystine broth (SC). The resulting suspensions were spiked with S. typhimurium. The cultures were incubated at 37°C for the times indicated below. After thorough mixing, large particles were allowed to settle for 15 min at room temperature. A 1-ml portion of supernatant from each suspension was subjected to extraction with magnetic beads.

RESULTS

Recovery of Salmonella spp. with monoclonal antibody-coated magnetic beads. The sensitivity of the MIPA is dependent, among other things, on the number of bacteria that are recovered from a sample by using magnetic beads coated with a specific monoclonal antibody. The optimal extraction procedure was determined by using several sample sizes and different amounts of beads. Sample sizes of 0.1, 0.5, and 2 ml in 96-, 24-, and 6-well plates, respectively, containing 3 × 10⁴ CFU of S. typhimurium per ml were incubated with 2, 5, or 10 μl of monoclonal antibody-coated magnetic beads and after collection were plated onto Giston agar plates. The best recovery was obtained with 2-ml samples and 10 μl of magnetic beads coated with monoclonal antibodies (Fig. 1). However, a sample size of 1 ml and 5 μl of monoclonal antibody-coated magnetic beads were used since the preparations could be handled in either 24-well plates or 1.5-ml Eppendorf tubes.

Sensitivity of direct PCR and direct MIPA with chicken meat. The direct PCR with spiked meat suspensions was inhibited 100-fold compared with the PCR performed with pure cultures, resulting in a detection limit of approximately 1 × 10⁷ CFU of Salmonella sp. per g of meat. The performance of the MIPA was assessed with spiked chicken samples. Directly after treatment in a stomacher, the samples were analyzed with the MIPA. The presence of 1 × 10⁶ CFU/g of meat gave a clear band, while the presence of 1 × 10⁸ CFU/g could not be detected.

Enrichment of Salmonella spp. Enrichment was required to increase the sensitivity of the assay. Three media, BPW, LB+; and S/C, were tested for their ability to support the growth of Salmonella spp. Meat suspensions in these media were spiked with either 1 or 100 CFU of S. typhimurium per ml. At various times 100-μl portions were plated onto salmonella-shigella agar. Pale H₂S-producing colonies were not observed on plates containing samples which were not inoculated with Salmonella sp. Therefore, only pale and H₂S-producing colonies were counted on salmonella-shigella agar plates, and no further determination of these colonies was made. Optimal growth of Salmonella sp. was obtained in BPW, especially when the initial numbers of Salmonella sp. were low (Fig. 2). However, most of the results with the MIPA were negative; the only exception was the high enrichment in S/C of an initial inoculum of 100 CFU/ml.

Sensitivity of MIPA after enrichment. Direct addition of 1-, 2-, or 5-μl samples of both BPW and S/C enrichments of chicken meat spiked with 25 CFU of Salmonella sp. per g to
the PCR incubation mixtures did not produce any amplification product when the preparations were analyzed by agarose gel electrophoresis.

The sensitivity of the MIPA in combination with a 24-h enrichment step was examined. To ensure that the meat samples were free of *Salmonella* spp., 10 25-g portions of two chicken brands were tested with both the MIPA and the conventional Association of Official Analytical Chemists Bacteriological Analytical Manual method (1). Three portions of one brand were contaminated with *Salmonella* spp. Chicken samples from a different brand were negative and were used for subsequent studies. Chicken meat samples were spiked with either 1, 0.1, 0.01, or 0 CFU of *Salmonella typhimurium* per g of meat, and this was followed by enrichment for 24 h in either S/C or BPW. The presence of 0.1 CFU/g of meat after enrichment in BPW was detected by using agarose gel analysis. No amplification products were detected after enrichment in S/C by using agarose gel analysis, but after Southern hybridization the presence of 0.1 CFU/g of meat was also detected.

**General applicability of the MIPA for the detection of *Salmonella* spp.** A panel of monoclonal antibodies specific for *Salmonella* serogroups A through E was used to detect *S. enteritidis* in 25-g samples spiked with 1, 0.1, 0.01, or 0 CFU/g of meat after enrichment in either S/C or BPW. Figure 3A shows that *Salmonella* sp. was detected by agarose gel analysis in the sample spiked with 0.01 CFU/g of meat after enrichment in S/C. In BPW the presence of 0.1 CFU of *Salmonella* sp. per g of meat was detected. Southern hybridization of the amplification products did not alter the detection limit for either enrichment medium (Fig. 3B).

**MIPA of samples containing damaged *Salmonella* spp.** Recovery of damaged *Salmonella* spp. was studied by inoculating chicken meat with either 0 or 1 x 10^7 CFU of *Salmonella* sp. per g of meat. Inoculated chicken was frozen, thawed the next day and subsequently prepared for enrichment. Freezing and thawing reduced the number of viable cells approximately 10-fold. Suspensions in BPW, LB^+, and S/C were incubated for 6 h at 37°C. Portions (100 μl) were plated onto Giston agar and salmonella-shigella agar. The results showed that higher numbers of *Salmonella* sp. were found in BPW than in either LB^+ or S/C (Table 1). However, the number of non-*Salmonella* bacteria was also higher in BPW. No salmonellae were found in nonspiked meat samples. The MIPA was also performed to determine whether the high numbers of contaminating bacteria had any influence on the detection of *Salmonella* sp. in the assay. All of the samples that were spiked with *Salmonella* sp. gave positive results in the MIPA (data not shown).

The sensitivity of the MIPA with poultry spiked with damaged *Salmonella* sp. was determined. A total of 25 g of chicken meat was inoculated with 1 or 0.1 CFU of *Salmonella* sp. per g, frozen, thawed the next day, and treated in a stomacher either in 125 ml of BPW, 125 ml of LB^+, or 125 ml of S/C. After treatment another 100 ml of medium was added. The resulting suspensions were cultured overnight at 37°C. A 1-ml sample from each culture was subjected to the MIPA; 1 CFU of *Salmonella* sp. per g of meat could be detected after enrichment in either BPW or S/C. The MIPA was negative with LB^+ and when the inoculum was 0.1 CFU/g when the preparations were analyzed on agarose gels.

**DISCUSSION**

Previously, we described a MIPA for the detection of *Salmonella* spp. in which monoclonal antibodies that are specific for live *Salmonella* spp. and a PCR based on *Salmonella*-specific primers chosen in the origin of bacterial chromosome DNA replication (oriC) were combined (16, 19). The use of the MIPA allows the circumvention of *Taq* polymerase inhibition by components of food, enrichment media, or large amounts of non-*Salmonella* DNA. Indeed, the direct PCR with spiked meat suspensions was inhibited 100-fold compared with the PCR with pure cultures, resulting in a detection limit of approximately 1 x 10^7 CFU of *Salmonella* spp. per g of meat, suggesting that immunomagnetic separation can reduce inhibition of the PCR. Inhibition of the PCR is probably due to blood products present in meat, which have also been found to inhibit the direct PCR in blood specimens (13). A comparable improvement in sensitivity was described by Widjojoatmodjo et al. (20) when the MIPA was used to detect *Salmonella* spp. in fecal samples. In these samples inhibition was probably due to bile salts and degradation products of heme, such as bilirubin. The inhibitors bound to proteins present in the sample and are probably released when the sample is heated to 94°C in the PCR. Thus, the MIPA reduces the amount of inhibitors present, but inhibitors which adhere to the bacteria are not removed, resulting in residual inhibition of the PCR.
FIG. 3. (A) Agarose gel analysis of MIPA products of S. enteritidis-spiked chicken meat after overnight culture in S/C (lanes 1 through 5) and BPW (lanes 6 through 10). The inocula used were 1 CFU/g of meat (lanes 1 and 6), 0.1 CFU/g (lanes 2 and 7), 0.01 CFU/g (lanes 3 and 8), 0.001 CFU/g (lanes 4 and 9), and 0 CFU/g (lanes 5 and 10). Lane 11 contained a negative MIPA control. Lane 12 contained a phage lambda PstI digest as a marker. Lane 13 contained a positive PCR control, and lane 14 contained a negative PCR control. (B) Southern blot of the agarose gel shown in panel A, obtained with a digoxigenin-labeled probe specific for the amplified product.

Adherence of bacteria to the large particulate matter which was allowed to settle also lowers the level of recovery. Enrichment is required to obtain improved sensitivity. A culture of Salmonella spp. in BPW showed the highest level of recovery, but contaminating flora grew equally well in this medium. The selective media S/C and LB+ produced lower numbers of Salmonella spp., but also lower numbers of contaminating flora. Since LB+ performed more poorly in the MIPA, this enrichment medium was not fully investigated. Direct PCR with samples of BPW and S/C enrichments of Salmonella-spiked chicken meat did not result in the detection of 1 CFU/g, which was probably due to the
TABLE 1. Growth of S. typhimurium damaged by freezing and thawing in BPW, LB+, and S/C in the presence of 10% (wt/vol) chicken meata

<table>
<thead>
<tr>
<th>Medium</th>
<th>No. of CFU/ml 4 h after thawing</th>
<th>No. of CFU/ml 6 h after thawing</th>
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<tbody>
<tr>
<td>BPW</td>
<td>$5 \times 10^5$</td>
<td>$1 \times 10^8$</td>
</tr>
<tr>
<td>LB+</td>
<td>$3 \times 10^5$</td>
<td>$3 \times 10^7$</td>
</tr>
<tr>
<td>S/C</td>
<td>$2 \times 10^5$</td>
<td>$5 \times 10^5$</td>
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a The input was $10^5$ CFU/g of meat.

presence of PCR-inhibitory substances. This indicates the need for immunomagnetic separation. Indeed, with the MIPA, the presence of 0.1 CFU of Salmonella spp. per g could be detected by using 25-g meat samples. A similar sensitivity was obtained when a panel of six monoclonal antibodies specific for serogroups A through E was used to detect S. enteritidis. This demonstrates the applicability of this method for other commonly isolated Salmonella serotypes. Even damaged salmonellae were detected after storage of contaminated meat at $-20^\circ$C. Our results show that LB+ is ineffective, whereas both BPW and S/C had a detection limit of 1 CFU/g. This detection limit is comparable to those reported for the Salmonella-Tek and Gene-Trak assays in which enrichment cultures are used. These assays require 48 h, but Van Poucke (18) demonstrated that with an improved medium (Salmosyst) the detection time for the Salmonella-Tek assay could be reduced to 31 h. The MIPA described here requires a total time of 30 h. Given the necessary short enrichment incubation, determination in 1 work day is not yet feasible, but the samples can be collected and prepared for overnight culture in enrichment broth. The next day MIPA and the detection of amplification products can be carried out on several samples simultaneously. The MIPA in combination with a 24-h enrichment step overcomes the disadvantages of both enzyme assays and the PCR alone. Non-Salmonella bacteria, which are either bound nonspecifically to the beads or cross-react specifically with the monoclonal antibodies (16), are not detected in the PCR when Salmonella-specific primers are used. Furthermore, material which inhibits the Taq polymerase is removed during immunomagnetic separation. This approach results in the detection of 2.5 CFU of Salmonella spp. per 25 g of meat and 25 CFU/25 g of meat after the salmonellae are damaged by freezing and thawing.

REFERENCES


