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Evaluation of the Magnetic Immuno PCR Assay for Rapid Detection of *Salmonella*

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A new technique, the Magnetic Immuno PCR Assay (MIPA), has been developed for the detection of *Salmonella*. The assay utilizes magnetic particles coated with monoclonal antibodies against *Salmonella* to extract these bacteria from the sample. Trapped bacteria are lysed, and the supernatant, which contains bacterial DNA, is then subjected to the polymerase chain reaction (PCR) using primers from the *Salmonella typhimurium* origin of DNA replication to amplify a 163 bp region. The specificity of the primer set was tested in the PCR; amplification occurred with all 25 *Salmonella* strains tested but not with 19 other species of *Enterobacteriaceae* tested. A sensitivity of 100 cfu *Salmonella typhimurium* was achieved for the MIPA by visualization of the amplified products by ethidiumbromide stained agarose gel electrophoresis. A ten-fold higher sensitivity was obtained by Southern blotting of the amplified products. The presence of 10^7 cfu *Escherichia coli* did not interfere with these detection levels. The MIPA thus specifically detected 100 cfu of *Salmonella* within 5 h and may be potentially useful for rapid detection of *Salmonella* in clinical specimens and food.

In clinical specimens or food products, potentially pathogenic bacteria may be present in low numbers among large numbers of contaminating microorganisms. For the isolation of these potential pathogens, selective and differential media are used. For example, the classical procedures for *Salmonella* detection involve culture on Salmonella/Shigella agar and enrichment in selenite cystine broth, followed by biochemical and serological identification (1, 2). These culture methods are time consuming and labor intensive.

Since the first enzyme immunoassay (EIA) for *Salmonella* was reported by Krysinski and Heimsch (3), several EIAs have been developed using polyclonal and/or monoclonal antibodies to detect *Salmonella* in food (4, 5) and clinical samples (6). Although the assay time is reduced by one or two days, samples to be screened must be enriched in growth medium before assaying in an EIA test system. Disadvantages of the immunochemical detection are the difficulty of discriminating between related microorganisms by antisera and the achievable sensitivity of 10^6 cells per ml.

The use of DNA technology offers a new alternative approach to the detection of microorganisms. Detection of *Salmonella* in food and clinical samples by hybridization with a DNA probe specific for *Salmonella* has been shown to be feasible (7–9), but enrichment is still required. Amplification of DNA sequences by the polymerase chain reaction (PCR) (10) using primers specific for a given microorganism is a promising improvement with regard to speed and sensitivity. However, amplification of the target DNA sequence can be inhibited by large amounts of contaminating DNA and/or interfering factors in the sample, such as with feces (11).

This report describes a new detection system, the Magnetic Immuno PCR Assay (MIPA), which overcomes the disadvantages of both the immunochemical and PCR methods. The MIPA combines selective separation of certain bacteria using magnetic chromium dioxide particles coated with monoclonal antibodies (12) from a test sample, followed by amplification of the DNA in the PCR reaction. The origin of DNA replication, which is strongly conserved in *Es-*

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cherichia coli, Salmonella typhimurium and a number of other gram-negative species (13, 14), was chosen as the target DNA region for PCR amplification.

Materials and Methods

Materials. Magnisort M magnetic chromium dioxide particles were purchased from DuPont, USA. Ampli-Taq DNA polymerase was purchased from Cetus, USA. Deoxynucleotides, digoxigenin-11-dUTP and digoxigenin labelling reagents were from Boehringer, Germany. *Hae*III digested ΦX174 DNA was from Pharmacia LKB, Sweden. Bacterial strains were obtained from the University Hospital Laboratory in Utrecht and the National Institute of Public Health and Environmental Protection (Bilthoven, The Netherlands). The number of cfu was determined by plating on blood agar plates for 16 h at 37 °C. Monoclonal antibodies were raised against *Salmonella* bacteria and belonged to the immunoglobulin G subclass.

Primers. Oligonucleotide primers were synthesized on a Pharmacia LKB Gene Assembler Plus synthesizer (Pharmacia AB, Sweden) using phosphoramidite chemistry. Primer 1 was 5'-TTATTAGGATCGCGCCAGGC-3' and primer 2 was 5'-AAAGAATAACCGTTGTTCAC-3'. After deprotection and cleavage, the oligonucleotides were purified by chromatography on NAP10 columns.

Bacterial Capture. The assays were conducted in 96-well microtitration plates. Dilutions were made in phosphate-buffered salt solution with 1 % gelatin (gPBS, pH 7.4). Seventy-five microlitres of the hybridoma culture supernatant (production of hybridomas will be described elsewhere) was incubated with 50 µl (1:25 diluted) of Magnisort M magnetic chromium dioxide particles coated with goat immunoglobulins specific for murine IgG and IgM (DuPont, USA) for 5 min at room temperature with continuous shaking. The magnetic particles were recovered by magnetic force and the supernatant was discarded. Bacteria of a fresh culture were suspended in gPBS (100 µl) and added to the magnetic particles. After incubating for 15 min at room temperature with continuous shaking, the magnetic particles were recovered by magnetic force, washed three times with gPBS, and resuspended in distilled water after the final wash step. The sample then was incubated for 5 min at 100 °C, followed by a brief centrifugation step. A 20 µl portion from the supernatant was subjected to the PCR.

Amplification by PCR. A PCR reaction mixture (50 µl) consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % (w/v) gelatin, 100 µM each dNTP, 0.5 µM each primer and 0.625 U Ampli-Taq polymerase and was covered with 50 µl of mineral oil. Amplification was conducted on a Perkin-Elmer Thermocycler (Perkin-Elmer Instruments, USA) for 35 cycles, followed by a final 10 min extension at 72 °C. One cycle consisted of a denaturation step at 94 °C for 1 min, a primer annealing step at 50 °C for 1 min, and an extension step at 72 °C for 1 sec. Amplification products (20 µl) were electrophoresed on a 1.5 % agarose gel, visualized by ethidium-bromide staining and photographed under UV light. *Hae*III digested ΦX174 DNA was used as size marker. The gel was Southern blotted to Zeta-probe membrane (Biorad, USA) by standard methods (15) and subjected to hybridization.

Probe. The probe for Southern analysis was labelled with digoxigenin-11-dUTP by the PCR. The PCR reaction procedure was analogous to the PCR mix for the MIPA amplification, but the PCR mix differed in the dNTPs concentration: 100 µM dATP, 100 µM dCTP, 100 µM dGTP, 90 µM dTTP and 10 µM digoxigenin-11-dUTP. *Salmonella typhimurium* DNA was used as target DNA.

Hybridization and Detection of the Digoxigenin Probe. Hybridization and detection was according to the protocol supplied by the manufacturer.

Results

PCR Amplification. The PCR technique was used to amplify a 163 bp sequence of the origin of DNA replication sequence. Primers were designed which contained sequences taken from the origin of replication of *Salmonella typhimurium*.

The specificity of the primer set was tested in the PCR with 25 *Salmonella* strains and 19 non-*Salmonella Enterobacteriaceae* (Table 1). Bacteria (10^7 cfu) were lysed for 5 min at 100 °C, and after a brief centrifugation step the supernatant was used directly for the PCR. After amplification a DNA fragment of expected size was observed not only with the *Salmonella typhimurium* strains but with all *Salmonella* tested. No amplification was observed with the *Enterobacteriaceae* tested, such as *Shigella, Escherichia coli, Citrobacter* and *Pseudomonas.* This indicates that the primer set is *Salmonella* specific.

Sensitivity and Specificity of the MIPA. At first, the PCR was conducted with the bacteria bound to the magnetic particles after extraction of *Salmonella typhimurium* by the monoclonal antibodies bound to the magnetic particles. However, amplification was inhibited by the presence of these magnetic particles. Therefore, samples were incubated for 5 min at 100 °C to lyse the bacterial cells. After a brief centrifugation step, the supernatant containing bacterial DNA was subjected to the PCR. Figure 1, lane 1, shows the results of 10^7 cfu *Salmonella typhimurium* assayed with the MIPA after amplification by the PCR.

The presence of a large number of non-*Salmonella* bacteria could interfere with the assay system; therefore, a serial dilution of *Salmonella typhimurium* ranging from 10^5 to 10 cfu was mixed with 10^7 cfu *Escherichia coli* and tested in the MIPA.

Table 1: PCR amplification results for *Salmonella* and other enterobacterial strains.

Strain	PCR amplification
Salmonella durazzo	+
Salmonella paratyphi A	+
Salmonella typhimurium S2	+
Salmonella typhimurium 17.24	+
Salmonella typhimurium 14H	+
Salmonella bredeny	+
Salmonella derby	+
Salmonella heidelberg	+
Salmonella brandenburg	+
Salmonella saintpaul	+
Salmonella paratyphi B	+
Salmonella abortus equi	+
Salmonella infantis	+
Salmonella newport	+
Salmonella betem	+
Salmonella paratyphi C	+
Salmonella panama	+
Salmonella typhosa	+
Salmonella enteriditis	+
Salmonella shangani	+
Salmonella give	+
Salmonella pretoria	+
Salmonella atlanta	+
Salmonella worthington	+
Salmonella arizona	+
Escherichia coli	–
Escherichia coli 07K1	–
Klebsiella pneumoniae 1.100	–
Klebsiella pneumonia 1.88	–
Klebsiella oxytoca	–
Citrobacter freundii	–
Citrobacter diversus	–
Serratia liquefaciens	–
Serratia marcescens	–
Enterobacter agglomerans	–
Enterobacter aerogenes	–
Enterobacter cloacae	–
Proteus mirabilis	–
Morganella morganii	–
Proteus vulgaris	–
Pseudomonas aeruginosa	–
Shigella flexneri	–
Shigella dysenteriae	–
Yersinia enterolitica	–

As shown in Figure 1, 10^2 cfu *Salmonella* were detectable on agarose gel electrophoresis; probe hybridization of the Southern blot obtained a detection level of 10 cfu. These detection levels observed in the MIPA with a 10^5 to 10^6-fold excess of *Escherichia coli* present in the sample are equal to the detection levels observed for *Salmonella typhimurium* tested directly in the PCR (data not shown). No amplification was observed in the control experiments with *Escherichia coli* (10^7 cfu) and gPBS.

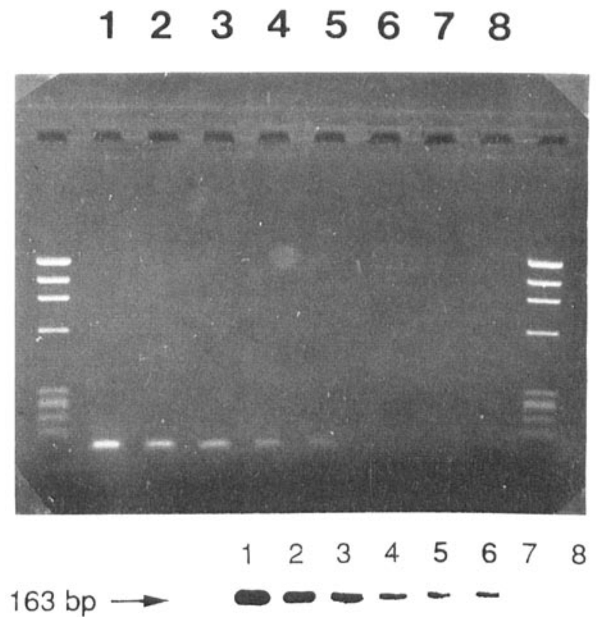

Figure 1: Top: gel electrophoresis of MIPA-amplified products. Bottom: Southern hybridization of MIPA-amplified products with the digoxigenin-labelled probe. Lane 1: MIPA product obtained with 10^7 cfu *Salmonella typhimurium*. Lanes 2-7: MIPA products obtained with 10^5, 10^4, 10^3, 10^2, 10 and 0 cfu of *Salmonella typhimurium* in the presence of 10^7 cfu *Escherichia coli*. Lane 8: negative control, gPBS. Marker lanes contain *Hae*III digested fragments of ΦX174 DNA (1358, 1078, 872, 603, 310, 281, 234, 194, 118 and 71 bp).

Discussion

The use of monoclonal antibodies coupled with magnetic particles to bind the *Salmonella* before applying the PCR has several advantages. Firstly, *Salmonella* bacteria are specifically separated from the sample, resulting in a sample for the PCR technique with few or no interfering DNA or other factors that may inhibit the PCR amplification. Secondly, primers for PCR are *Salmonella* specific; neither non-specific binding to the magnetic particles nor cross-reactivity by the monoclonal antibody is measured with the PCR. Thirdly, the separation of *Salmonella* by monoclonal antibodies leads to a concentration of bacteria.

The total time needed for the detection of *Salmonella* with the MIPA technique is only 5 h (1 h magnetic immuno step plus 3 h PCR plus 1 h gel electrophoresis). Analysis of the PCR product after 35 cycles on an agarose gel resulted in a sen-

sitivity of 10^2 cfu. The detection limit for probe hybridization is 10 cfu, 10-fold more sensitive. Further studies must be conducted to optimize the sensitivity of the system, e.g. optimizing the magnetic immuno step, and to study the applications of the MIPA for the detection of *Salmonella* in clinical and/or food samples.

In this paper we have demonstrated our newly developed Magnetic Immuno PCR Assay for the detection of *Salmonella* bacteria. The Magnetic Immuno PCR Assay is not restricted to *Salmonella* bacteria but can be expanded to detect other microorganisms having specific monoclonal/ polyclonal antibodies with a specific DNA primer set for a given microorganism.

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References

1. **Helrich K (ed):** Official methods of analysis. Association of Official Analytical Chemists, Arlington, VA, 1990, p. 467–492.
2. **Van Leusden FM, van Schothorst M, Beckers HJ:** The standard *Salmonella* isolation method. In: Corry JEL, Roberts D, Skinner FA (ed): Isolation and identification methods for food poisoning organisms. Academic Press, London, 1982, p. 35–49.
3. **Krysinski EP, Heimsch RC:** Use of enzyme-labeled antibodies to detect *Salmonella* in foods. Applied and Environmental Microbiology 1977, 33: 947–954.
4. **Eckner KF, Flowers RS, Robison BJ, Mattingly JA, Gabis DA, Silliker JH:** Comparison of *Salmonella* Bio-Enza Bead immunoassay method and conventional culture procedure for detection of *Salmonella* in foods. Journal of Food Protection 1987, 50: 379–385.
5. **Van Poucke LSG:** Salmonella-TEK, a rapid screening method for *Salmonella* species in food. Applied and Environmental Microbiology 1990, 56: 924–927.
6. **Araj GF, Das Chugh T:** Detection of *Salmonella* spp. in clinical specimens by capture enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 1987, 25: 2150–2153.
7. **Fitts R, Diamond M, Hamilton C, Neri M:** DNA-DNA hybridization assay for detection of *Salmonella* spp. in foods. Applied and Environmental Microbiology 1983, 46: 1146–1151.
8. **Gopo JM, Melis R, Filipska E, Meneveri R, Filipski J:** Development of a *Salmonella*-specific biotinylated DNA probe for rapid routine identification of *Salmonella*. Molecular and Cellular Probes 1988, 2: 271–279.
9. **Scholl DR, Kaufmann C, Jollick JD, York CK, Goodrum GR, Charache P:** Clinical application of novel sample processing technology for the identification of salmonellae by using DNA probes. Journal of Clinical Microbiology 1990, 28: 237–241.
10. **Saiki RK, Gelfand DH, Stoffel S, Sharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA:** Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988, 239: 487–491.
11. **Allard A, Girones R, Juto P, Wadell G:** Polymerase chain reaction for detection of adenoviruses in stool samples. Journal of Clinical Microbiology 1990, 28: 2659–2667.
12. **Birkmeyer RC, Diaco R, Hutson DK, Lau HP, Miller WK, Neelkantan NV, Pankratz TJ, Tjeng SY, Vickery DR, Yang EK:** Application of novel chromium dioxide magnetic particles to immunoassay development. Clinical Chemistry 1987, 33: 1543–1547.
13. **Kornberg A:** Supplement to DNA replication. W.H. Freeman, San Fransisco, 1982, p. 145–152.
14. **Zyskind JW, Cleary JM, Brusilow WSA, Harding NE, Smith DW:** Chromosomal replication origin from the marine bacterium *Vibrio harveyi* functions in *Escherichia coli: oriC* consensus sequence. Proceedings of the National Academy of Sciences of the USA 1983, 80: 1164–1168.
15. **Maniatis T, Fritch EF, Sambrook J:** Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.