A REVIEW

The application of magnetic separations in applied microbiology

I. Safarik, M. Safariková and S.J. Forsythe

Department of Biochemistry and Biotechnology, Institute of Landscape Ecology, České Budejovice, Czech Republic and
Department of Life Sciences, The Nottingham Trent University, Nottingham, UK


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1. INTRODUCTION

The separation and detection of a specific microbial species from a mixed culture is a fundamental microbiological technique. Sometimes it can be achieved by the use of direct plating on to selective media. However, this is often inefficient without previous enrichment steps especially when stressed (e.g. heat-damaged) cells must be isolated. In food microbiology specific organisms must be detectable from a mixed and often dominate background flora. Subsequently the isolation of food-borne salmonellae requires four stages and at least four different growth media (Blackburn 1993). Hence the total time from sampling the food to obtaining a result can be measured in days. These time consuming stages are due to waiting for the target organism to multiply faster than potential contaminating background flora. One of the possibilities for shortening the isolation and detection period is to replace the selective enrichment stage with a non-growth related procedure. This can be achieved by specific magnetic separation of the target organism directly from the sample or the pre-enrichment medium. Isolated cells can then be identified by standard microbiological procedures.

In this review magnetic separations for the detection of target bacteria or their metabolites will be made with special reference to applications in the food industry. Additionally other areas of applied microbiology, such as waste-water treatment, where magnetic separations are applicable.
will be covered (see Table 1 for a review of applications). Emphasis will be placed on the range of capturing methods and end detection systems as opposed to a review of all specific organisms studied to date. A review of specific applications of immuno-magnetic separation in clinical microbiology has already been published by Olsvik et al. (1994).

2. EQUIPMENT AND PROCEDURES NECESSARY TO PERFORM MAGNETIC SEPARATIONS

2.1 Magnetic and super-paramagnetic particles and magnetization of microbial cells

Many magnetic or magnetizable carriers can be used for magnetic separations. Most of the particles used are super-paramagnetic, that is they only exhibit magnetic properties in the presence of an external magnetic field. They can be easily removed from a suspension with a magnetic separator. Since there is no magnetic remanence the particles are not attracted to each other and therefore they can be easily suspended into a homogenous mixture in the absence of any external magnetic field (Lea et al. 1988).

Probably the most often used magnetic carriers are produced by Dynal A/S (Oslo, Norway). These super-paramagnetic, polystyrene-based particles have diameters of 2.8 μm (Dynabeads® M-280) and 4.5 μm (Dynabeads® M-450). Very small magnetizable particles of gamma-Fe₂O₃ (approx. 5–10 nm in diam.) are formed during the magnetization process in the pores of the matrix and are homogeneously distributed in the whole volume of the particle. The pores are partially filled with a polymeric material and thus the surface area is lowered. The polymer material is a source of the functional groups, which are used for the activation of the particles. The Dynabeads® M-450 particles have a surface area of 3–5 m² g⁻¹, a density of 1.5 g cm⁻³ with an iron content of 20% (w/v) and the number of particles in 1.0 mg is 1.7 × 10¹⁰. Both M-280 and M-450 carriers can be obtained in non-activated and tosyl-activated form. Coated Dynabeads® with covalently immobilized streptavidin or secondary antibodies against selective primary antibodies are commercially available from Dynal (UK) Ltd, Wirral. An alternative magnetic sorbent is BioMag (Metachem Diagnostics Ltd, Northampton). Particles of this carrier (0.5–1.5 μm in diam.) are formed by silanized magnetic iron oxide, carrying on the surface amino-, carboxy- or thiol-groups. The surface area is greater than 100 m² g⁻¹, the density is 2.5 g cm⁻³ and the number of particles in 1.0 mg is 5 × 10¹⁰. Due to the small size of the particles the spontaneous sedimentation is low, not exceeding 4% during 30 min (Groman et al. 1985).

Other magnetic carriers used include chromium dioxide particles, which exhibit high protein uptake capacity (40 mg g⁻¹) allowing rapid capture kinetics and high binding capacity (Birkmeier et al. 1987; Obzansky et al. 1990; Widjojoatmodjo et al. 1991, 1992; Torensma et al. 1992; Fluit et al. 1993a). Blackburn et al. (1991) used polystyrene para-magnetic microparticles (1–2 μm diam., Polysciences Ltd, Northampton), magnetic polycarboxylate particles (1–10 μm diam., Scipak, Sittingborne, Kent, UK) and polystyrene-divinylbenzene magnetic particles (0.7 μm diam., Sepadyn Indianapolis, USA).

Alternatively, microbial cells can be made magnetizable by the direct adsorption of submicron particles of magnetic iron oxides on to their surface (Dauer and Dunlop 1991). In the case of Saccharomyces cerevisiae, attachment of the gamma-Fe₂O₃ particles to the yeast surface occurs irrespective of the solution pH and surface charge and is essentially irreversible. Commercially available fine particles of magnetite (Fe₃O₄) or gamma-Fe₂O₃ can be co-

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immobilized, together with a suspension of microbial cells, into the structure of an appropriate polymer, such as alginate gel (Larsson and Mosbach 1979; Birnbaum and Larsson 1982).

Another means of making microbial cells magnetizable is to coat them with erbium ions. The para-magnetic trivalent lanthanide erbium ion, Er\textsuperscript{3+}, has an exceptionally high atomic magnetic moment of 9.3 Bohr magnetons. After addition as ErCl\textsubscript{3} (final concentration 5 mmol dm\textsuperscript{-3}) to bacterial suspensions, it imparts the magnetic moment of the bacterial cells by ionic binding to the cell surface (Zborowski et al. 1991, 1992, 1993). Modified cells of Gram-positive bacteria exhibit a stronger interaction with a magnetic field due to the fact that they can adsorb substantially higher amounts of Er\textsuperscript{3+} ions than the cells of Gram-negative bacteria. Magnetically-modified microbial cells can be separated in various types of ferrographs or by thin layer magnetophoresis. Magnetic separation of microbial cells can be useful for the determination of the total number of micro-organisms, e.g. during analysis of urine or water samples. Magnetic labelling of cells with Er\textsuperscript{3+} ions does not influence the possibility of their further characterization, e.g. by Gram staining or immunofluorescence procedures. The magnetic deposition protocol allows quantitative detection of \textit{E. coli} down to the concentrations of 10\textsuperscript{5} cfu ml\textsuperscript{-1}.

A procedure for the preparation of magnetized bacterial cells has been described, based on the precipitation of ferro ions as hydrogen phosphates or sulphides on their surface (Ellwood et al. 1992). Such bacteria, due to the specific properties of the altered cell wall, could be used for the clean up of environmental pollution. These applications include the removal of heavy metals, radionuclides and toxic agricultural chemicals from effluents.

Magneto tactic bacteria contain magnetosomes which are composed of magnetite (Fe\textsubscript{3}O\textsubscript{4}) covered by a stable lipid membrane. Due to their small size (50–100 nm diam.) the particles can be easily dispersed in solution. Isolated magnetosomes can be activated with appropriate bifunctional reagents (e.g. glutaraldehyde and N-succinimidyl 3-(2-pyridyldithio)propionate) for binding appropriate affinity ligands (Nakamura et al. 1993). Subsequently they can be used as a solid support for the immobilization of biologically active compounds (Matsunaga and Kamiya 1987; Matsunaga 1991; Nakamura and Matsunaga 1993; Nakamura et al. 1991, 1993).

### 2.2 Affinity ligands: antibodies, lectins and agglutinins

Specific monoclonal or polyclonal antibodies are frequently used as affinity ligands to bind target cells. The use of antibodies immobilized on magnetic carriers for capturing target cells is termed immuno-magnetic separation. Alternatively, lectins and agglutinins have been used as the affinity ligands (Payne \textit{et al.} 1992, 1993a,b). It should be recognized that the separation procedure can only be as good as the specificity of the ligand and that the ligand’s specificity could be altered after covalent immobilization to the magnetic particle.

Polyclonal antibodies often exhibit unwanted cross-reactions and it is difficult to prepare them in a reproducible form. Therefore, monoclonal antibodies often exhibit better properties. In some cases, a mixture of various monoclonal and polyclonal antibodies is immobilized on a magnetic carrier to enable the binding of a wide range of serovars. The Dynabeads\textsuperscript{®} anti-Salmonella uses a mixture of polyclonal and monoclonal antibodies and reacts with more than 1400 salmonella serovars which cover 99-4% of salmonella isolates from humans and animals in Europe and USA over the last 10 years. In contrast, the EiaFoss product uses monoclonal antibodies against salmonella flagellar antigens (Krusell and Skovgaard 1993). In the case of \textit{E. coli} strains, specific surface epitopes, such as fimbiae associated with the ability to induce disease, can be used as targets for immuno-magnetic separation (Fratamico \textit{et al.} 1992).

Various lectins and agglutinins isolated from \textit{Agaricus bisporus}, \textit{Triticum vulgare}, \textit{Lumbricus terrestrial} and \textit{Limulus polyphemus} have been studied as capturing ligands conjugated to magnetic particles (Patchett \textit{et al.} 1991; Payne \textit{et al.} 1992, 1993a,b; Grant \textit{et al.} 1993). Payne \textit{et al.} (1992) demonstrated the capture of \textit{Staphylococcus aureus}, \textit{E. coli}, listeria and salmonella cells by lectin-conjugated magnetic particles. Efficiency of capture by \textit{Helix pomatia} lectin was greater for Gram-positive bacteria compared to Gram-negative organisms. \textit{Agaricus bisporus} lectin removed \textit{L. monocytogenes} and \textit{Staph. aureus} from undiluted milk and ground beef. Further work by Payne \textit{et al.} (1993a,b) showed that lectin-based magnetic separation gave ‘cleaner’ microbiological samples, i.e. less carryover of background flora. In some cases there was increased sensitivity, for example the detection of \textit{Salm. enteritidis} in beef samples was enhanced by two orders of magnitude compared to conventional plating techniques. Therefore lectin-based microbial separation procedures could be used for the separation of pathogenic bacteria from food.

Magnetic particles available from Dynal A/S (Dynabeads\textsuperscript{®} M-280) and Advanced Magnetics (BioMag) are already coated with a secondary antibody. Therefore primary antibodies, specific against selected surface structures of the separated cells, can be immobilized on these sorbents enabling the \textit{F\textsubscript{ab}} regions of the primary antibody to reach the optimum position for the capture of the isolated cells. Magnetic particles with immobilized streptavidin (e.g. Dynabeads\textsuperscript{®} M-280 Streptavidin and BioMag...
Streptavidin) can be used for immobilization of biotinylated antibodies and lectins.

2.3 Magnetic particle separators

Strong permanent magnets or electromagnets can be used for the separation of magnetic particles and their complexes with adsorbed (immobilized) cells. Magnetic particle separators, employing strong permanent magnets (based on the rare earth metals) are most suitable due to their ease of handling. Several types of magnetic separators have been described and are commercially available (Groman et al. 1985; Hardwick et al. 1990; Miltenyi et al. 1990; Ellwood et al. 1992).

3. IMMUNO-MAGNETIC SEPARATION APPLICATIONS

3.1 General procedure

In the direct immuno-magnetic separation technique, immuno-magnetic particles specific for the target organism are suspended in an aliquot of the mixed cell suspension. After an appropriate incubation time the immuno-magnetic particles with bound target cells are separated from the suspension with a magnetic particle separator, the remaining suspension is removed and magnetic particles are washed several times with a suitable solution.

Alternatively, the indirect technique can be used in which primary antibodies are added to the microbial suspension and, after their binding on the target cell-surface structures, magnetic particles with immobilized secondary antibodies (antibodies against the primary antibodies) are added. After the interaction of primary and secondary antibodies the whole complex is removed from the suspension with the aid of a magnetic separator.

The amount of immuno-magnetic particles required for capturing the target cells differs according to the application. It has been reported for salmonellae and E. coli O157 : H7 that the highest recovery of the isolated target cells can be achieved with high concentrations of immuno-magnetic particles at 2 × 10⁶–10⁷ particles ml⁻¹ (Skjerve et al. 1990; Vermunt et al. 1992; Fratamico et al. 1992). A general guideline is that the ratio of immuno-magnetic particles to target cells should be between 3 : 1 to 20 : 1. The recovery of target cells is affected by the incubation period (usually 10–60 min) with the immuno-magnetic particles. An increased incubation period substantially increases the recovery of the target cells (Skjerve et al. 1990; Vermunt et al. 1992; Fratamico et al. 1992). A prolonged incubation time, however, can lead to the non-specific binding of the non-target micro-organisms. Non-target organism carry-over can be reduced by washing the beads several times in saline solution and changing the test tubes with each wash (Okrend et al. 1992). Addition of detergents such as Tween-20 (0.05–0.1% w/v) or protamine to the incubation mixture usually decreases the non-specific adsorption (Morgan et al. 1991; Fratamico et al. 1992; Okrend et al. 1992; Vermunt et al. 1992). Tween-20 is also used in the washing procedure to remove non-specifically bound cells.

An important advantage of immuno-magnetic separations is the ability to capture and detect dead or severely-damaged micro-organisms which cannot be detected by the standard cultivation conditions (Hornes et al. 1991; Mansfield and Forsythe 1993). The detection of dead organisms is achieved using polymerase chain reaction technique (see Section 4.6).

3.2 Anti-Salmonella magnetic beads

In 1984, Mattingly published the first paper on the immuno-magnetic separation of salmonella from food and faecal samples. His procedure used a myeloma protein (M467) and a hybrid antibody (61H4) which were conjugated to a polycarbonate-coated metal bead. The magnetic beads were able to capture salmonella cells at a density of 10⁹ even in the presence of a 3 log order greater number of other bacteria. Bird et al. (1989) tested magnetic particles coated with anti-Salmonella antibodies to capture salmonella from pre-enrichment broths. After washing the beads immobilized cells were transferred to an impedance system for final detection. Later, Lim and Ko (1990) used a two-stage assay involving Dynabeads® coated with anti-murine antibodies and coloured latex particles coated with salmonella specific antibodies. On testing this assay on sensitized (and control) murine sera, there was a 17 out of 18 positive agreement with the immune sera and 8 out of 9 negative results for normal sera. Luk and Lindberg (1991) coated magnetic beads with monoclonal antibodies specific for the O antigen 6,7 of salmonella lipopolysaccharide. These beads were specific for salmonella serogroup C1 and were used to detect salmonella in blood and faeces. The detection limit was 1–0 × 10³ to 10⁴ bacteria ml⁻¹. Other workers have concentrated on detecting salmonella in foods. The first application to food microbiology was by Skjerve and Olsvik (1991). They reported the separation of salmonella cells in Brie, dried milk, yogurt, meat and vegetables, with a detection limit of 1–0 × 10² cells g⁻¹ for certain foods. A large-scale comparison of immuno-magnetic separation of salmonellae from foods with conventional techniques was reported by Mansfield and Forsythe (1993). They compared immuno-magnetic separation using anti-Salmonella Dynabeads® with the three EC procedures for 120 food samples, half of which were spiked with low levels of salmonella (five serovars used). The results confirmed that anti-Salmonella magnetic beads were as efficient as a
selection stage as selenite broth, the most efficient of the EC procedures. Additionally, the total time required for sampling and detection could be reduced by 1 d due to the replacement of the selective enrichment step (overnight incubation) with the immuno-magnetic separation (10 min). Of particular interest was the greater recovery of stressed salmonellae from herbs and spices confirming that immuno-magnetic separation could capture injured target organisms which were unable to recover in standard selective enrichment broths. This observation was followed by an 11 laboratory ring-trial involving spiked herbs and spices which compared a wide range of ‘in-house’ salmonella detection procedures with a standardized immuno-magnetic separation technique. Although inter-laboratory recoveries varied, the overall results showed that salmonella were as efficiently recovered by immuno-magnetic separation as by ‘in-house’ techniques and saved 24 h in the total detection time (L.M. Mansfield, personal communication).

3.3 Detection of pathogenic strains of Escherichia coli

Escherichia coli is a commensal organism in the human large intestine; however, a number of strains are pathogenic to man. Therefore there is a problem with the isolation and differentiation of the pathogenic strains from the more numerous non-pathogenic variety. This can be partially achieved with specific immunodeterminants, especially adhesion factors which are pathogenicity factors. For example, fimbiae enhance attachment to the small intestine wall. Lund et al. (1988) used magnetic beads coated with monoclonal antibodies to the K88 antigen to detect K88-positive E. coli from mixed cultures. The level of detection was $4 \times 10^4$ cfu ml$^{-1}$. This work was followed by the use of immuno-magnetic enrichment for F4-positive enterotoxigenic E. coli and oligonucleotide probes to heat-labile and heat-stable enterotoxins (LT1 and ST1 respectively; Lund et al. 1991).

Escherichia coli O157 : H7 is a pathogenic strain which causes haemorrhagic uraemic syndrome (HUS) and haemorrhagic colitis (HC). HUS can be fatal, especially in young children, as demonstrated by recent outbreaks in the UK. Fortunately immunological differences between pathogenic and non-pathogenic E. coli strains can be exploited by immuno-magnetic separation for E. coli O157 : H7 detection. Fratamico et al. (1992) produced sheep anti-rabbit IgG magnetic beads coated with rabbit antiserum against E. coli O157 for an immuno-magnetic separation procedure. The method successfully recovered E. coli O157 : H7 from overnight enrichment cultures of meat samples at a density of 1 cell ml$^{-1}$. Okrend et al. (1992) similarly coated magnetic beads with E. coli O157 : H7 specific antibody for a separation procedure for meat samples. The authors noted there was competition between E. coli O157 : H7 and non-H7 strains for sites on the magnetic beads and this reduced isolation efficiency. Application of immuno-magnetic separation for verotoxigenic strains of E. coli for epidemiology studies has been demonstrated (Chapman and Wright 1993; Wright et al. 1994; Chapman et al. 1994). In 1993 Chapman and Wright published the first incident of E. coli O157 infection in the UK in which a suspect food source was microbiologically confirmed and this was achieved using immuno-magnetic separation. The route of transmission was cattle to man via milk that was either contaminated by the udder or excreted in the milk. These workers found the detection limit of E. coli O157 : H7 was 200 cells g$^{-1}$ bovine faeces by conventional techniques, whereas immuno-magnetic separation had a detection limit of 2 organisms g$^{-1}$. Over a 4 month period of sampling rectal swabs, immuno-magnetic separation produced isolates compared to 23 by conventional methods.

3.4 Detection of Listeria monocytogenes

Despite the reduced number of clinical listeriosis cases since the food scares of 1988 and 1989, listeria still remains an organism of commercial importance. At the moment guidelines for the number of listeria cells permitted in food varies from country to country. However, it is only certain serovars of the species L. monocytogenes which are of medical importance.

The first published work on the use of immuno-magnetic separation for the isolation of listeria was by Skjerve et al. (1990). They coated magnetic beads with monoclonal antibodies raised against L. monocytogenes flagella and studied immuno-capture of L. monocytogenes using agglutination, dot blots and viable enumeration on Oxford agar. The sensitivity of the immuno-magnetic assay was $2 \times 10^2$ cells ml$^{-1}$ in enriched foods. This work was followed up by Fluit et al. (1993a) with a magnetic immuno-polymerase chain reaction (PCR) assay (MIPA) for L. monocytogenes which is described in more detail later (Section 4.5).

A commercial immuno-magnetic separation system was capable of separating Listeria from food and environmental samples (VICAM Listertest, VICAM, Sommerville, USA). After a post-enrichment, it is possible to perform a detection within 24 h (Betts 1994).

3.5 Detection of Vibrio haemolyticus and Yersinia enterocolitica food-poisoning organisms

Numerous other food-poisoning bacteria can be magnetically separated from foods or faecal matter. Tomoyasu (1992) developed an immuno-magnetic method to specifically isolate K serovars of Vibrio parahaemolyticus. This method successfully isolated the same V. haemolyticus K
serovar from food incriminated in causing food-poisoning and from a patient. *Yersinia enterocolitica* detection in spiked food and water samples using immuno-magnetic separation has been reported by Kapperud *et al.* (1993). The initial isolation of the *Y. enterocolitica* was achieved with magnetic particles coated with antibodies against serogroup O : 3. With this technique all common pathogenic groups (O : 3, O : 5, 27, O : 8, O : 9, O : 13 and O : 21) were detectable and could be differentiated from *Y. pseudotuberculosis* and non-pathogenic yersinia. The detection level was 10–30 cfu g⁻¹ meat without overnight enrichment. This could be improved to 2 cfu g⁻¹ meat using overnight pre-enrichment provided the background flora was less than 10⁷ cfu g⁻¹. The procedure used a combined immuno-magnetic separation and PCR technique (DIANA) described in Section 4.7.

3.6 Detection of *Staphylococcus aureus* in mastitis

Immuno-magnetic particles coated with polyclonal antibodies against encapsulated *Staph. aureus* can be used to capture the organism directly from milk without subcultivation. Johne *et al.* (1989) reported that nearly all clinical isolates were encapsulated and this masked the detection of protein A.

4. END DETECTION PROCEDURES

A number of end detection systems have been used in conjunction with immuno-magnetic separation. Some research groups have made the whole procedure more rapid than the traditional technique by combining immuno-magnetic separation with standard ELISA protocols, whereas others are taking advantage of current developments in PCR techniques (i.e. MIFA, DIANA). Unfortunately for financial reasons many routine food laboratories are only able to carry out standard plating techniques after immuno-magnetic separation. However, although this may lose one day in obtaining the initial positive result, it should be borne in mind that all salmonellae isolates from food have to be sent to Public Health Laboratories for further analysis (i.e. serotyping and phage-typing). Hence an aliquot of the samples for ELISA and PCR analysis must be retained for colony isolation prior to treatments that kill any viable target cells.

4.1 Microscopy and fluorimetry

Micro-organisms attached to the magnetic beads can be visualized by acridine orange staining and examined under fluorescence microscopy (Lund *et al.* 1988). A second fluorescence-labelled antibody against other epitopes on the microbial cell surface can be used also to confirm the identity of the separated cells (Drancourt *et al.* 1992). Nakamura *et al.* (1993) immobilized fluorescein isothiocyanate (FITC) conjugated monoclonal anti-*E. coli* antibody on bacterial magnetic particles using a heterobifunctional reagent, N-succinimidyld 3-(2-pyridylithio)propionate.

Scanning electron microscopy can be used to confirm binding of the microbial cells to the magnetic particles (Johne and Jarp 1988). The electron microscopy of viruses can be simplified using a specific antibody or protein A labelled magnetic particles. These sorbents selectively bind viruses or antibody-treated virus particles on the electron microscope grid by the action of an electromagnet (Mizutani *et al.* 1989).

4.2 Plate and liquid culture methods

Magnetic particles with the bound cells can be inoculated on to the surface of appropriate selective or differential agar or into an appropriate liquid medium. Micro-organisms do not need to be detached from the beads since attachment to the immuno-magnetic beads has no effect on their growth and the cells can continue to multiply if nutritional requirements are provided (Torensma *et al.* 1993). However, the number of colonies grown on the agar need not be the same as the number of cells in the sample. This is because some of the magnetic particles will carry more than one cell on their surface or several cells can be attached to a cluster of beads, both cases result in the formation of a single colony (Skjerve *et al.* 1990). A general rule is that a colony represents up to six target organisms clumped together.

In some cases, sub-lethally injured target cells are non-culturable since they are unable to multiply in the subsequent growth medium due to the carryover in pipetting of growth inhibitors. However, immuno-magnetic separation captures the cells and minimizes the carryover of growth inhibitors. *Salmonella virchow* was only recovered from herb and spice samples by immuno-magnetic separation since the injured cells (due to antimicrobial agents in herbs and spices) did not recover in the standard selective broths (Mansfield and Forsythe 1993).

4.3 Impedance technology

Impedance microbiology relies upon the metabolism of the target organism to change the impedance (or conductance, depending on the equipment in use) of the growth medium. The larger the initial number of organisms the shorter the time taken for an impedance change to be detectable. Therefore since immuno-magnetic separation concentrates the target organism it follows that combining the two procedures would result in a detection time sooner than if the
sample had been directly plated on to a selective agar or the initial (pre-enriched) sample was directly analysed by impedance.

The rapid isolation of salmonella from skimmed milk powder was combined with impedance technology by Parmar et al (1992). Spiked milk powders were pre-enriched in buffered peptone water for timed periods before enrichment and detection in selenite-cysteine trimethylamine-N-oxide manitol broth by direct impedance (RABIT, Don Whitley Scientific Limited, Shipley, UK). Spiked milk powders (20 salmonella cells 25 g⁻¹) incubated in BPW for 6 h gave detection times of 7.5 h. The conductance curve differentiated between salmonellae and Cit. freundii which also bind to the anti-Salmonella Dynabeads®.

4.4 Latex bead and agglutination tests

Lim (1990) developed a rapid test for the detection of Salm. typhi endotoxin with two types of latex particles. The indicator latex particles were coated with antibodies specific for one antigenic determinant of the endotoxin, while the magnetic particles were carrying immobilized antibody against a different determinant. In comparison with a conventional slide latex agglutination test (sensitivity 25 ng ml⁻¹), the level of detection was 1, 4-9, 12-5 and 28-7-fold better after 5, 15, 30 and 60 min incubation.

Since protein A binds to the Fc region of antibodies, Dynabeads® M-450 coated with sheep IgG have been used in an agglutination test for the detection of Staph. aureus surface protein A. Scanning electron micrographs demonstrated the binding of protein A-positive bacteria to the particle surface, thus bridging the beads and forming the agglutination lattice (Johne and Jarp 1988).

4.5 ELISA detection of target antigens and immuno-magnetic separation

ELISA kits for detecting pathogenic micro-organisms generally require about 1 x 10⁶ cells ml⁻¹. Therefore pre-enrichment and enrichment incubation periods for the growth of the target organism may be required. However, prior immuno-magnetic separation for the target organism shortens the time taken to obtain 1 x 10⁶ cells.

The EiaFoss automated system for salmonella detection uses immuno-magnetic separation of cultures enriched in specially formulated pre-enrichment media (Krusell and Skovgaard 1993). After pre-enrichment the salmonellae are immunocaptured with magnetic particles (Dynal A/S, Oslo) and boiled for 15 min. A second antibody conjugated with β-galactosidase is then added which cleaves the substrate 4-methyl-umbelliferyl-β-galactoside (4-MUG) to yield the fluorescent product 4-methyl-umbelliferone (4-MU) which is quantified by a fluorescence detector.

The combined use of IMS-ELISA has been used for the detection of Clostridium perfringens type A enterotoxin (Cudjoe et al. 1991). The assay had a detection limit of 2.5 ng ml⁻¹ enterotoxin in homogenates of faeces and inoculated meat extracts, the effective assay time being 4 h. This level of sensitivity was comparable to conventional enterotoxin assays requiring 24 h or longer.

Erwinia chrysanthemi and Erw. carotovora subsp. atroseptica can be detected in potato peel extracts with BioMag immuno-magnetic beads to capture the target cells and antigenic cell products prior to SDS-PAGE and Western blotting analysis (van der Wolf et al. 1994). The immuno-capture was necessary to reduce background reactions and lowered the detection level by 10- to 100-fold. This procedure verified positive ELISA results and detection thresholds were generally comparable.

4.6 Magnetic separation with DNA hybridization and amplification techniques

The Magnetic Immuno PCR Assay (MIPA) combines the polymerase chain reaction after immuno-magnetic separation of the target cells (Widjojoatmodjo et al. 1991). The purpose of the immuno-magnetic separation was to remove the PCR inhibitory compounds from a sample without loss of sensitivity through dilution (Widjojoatmodjo et al. 1992; Powell et al. 1994).

The oligonucleotide primers selected should be specific for the target genus. Widjojoatmodjo et al. (1991) used primers from the origin of replication for Salm. typhimurium to amplify a 163 base pair region. The primer sequence was sufficiently specific to salmonella for amplification to occur with 25 salmonella strains tested but not with 19 other species of Enterobacteriaceae. The MIPA sensitivity was 100 salmonella cells even in the presence of 1 x 10⁷ E. coli cells and the total sampling time was 5 h. Detection of salmonella in faecal material was demonstrated by Widjojoatmodjo et al. (1992) with monoclonal antibodies specific for salmonella serogroups A to E. The faecal samples had to be diluted 10-fold (compared to the standard 500-fold) and 1μg of T4 gene 32 protein was added to the PCR reaction mixture to increase the accessibility of the template DNA to the DNA polymerase.

Two MIPA procedures for L. monocytogenes have been described (Fluit et al. 1993a). They compared two primers, one for the gene encoding the delayed-hypersensitivity factor and the other to the listeriolysin O gene. A combination of immuno-magnetic separation and the listeriolysin O gene primers gave better results. The latter detected L. monocytogenes in spiked samples of Port Salut cheese at 1 organism g⁻¹ after preliminary enrichment in
Listeria Enrichment broth and secondary enrichment in Fraser broth. The total time required from sampling to positive result was 55 h.

Various modifications of PCR are described for MIPA technique, e.g. nested PCR, with two nested pairs of primers in a two-step polymerase chain reaction (Kapperud et al. 1993). The nested PCR system reduces the possibility of false results since the inner set of primers functions only when the outer pair has amplified the correct sequence. Therefore the inner primers serve as controls for the first outer primer set. The number of cycles required in the second PCR is decreased because of the increased number of templates from the first PCR cycling (Olsvik et al. 1991b).

4.7 Magnetic separation of PCR products, DIANA

Amplified fragments of nucleic acids, prepared by MIPA and ordinary PCR, can be detected and identified by a procedure called DIANA (Detection of Immobilized Amplified Nucleic Acids). This procedure does not require electrophoresis, hybridization or restriction enzyme analysis for the identification of the PCR products. The basic principle of this procedure is the use of two sets of primers for PCR. The outer set is not labelled, while the inner set of primers is labelled; one of the primers is biotinylated on the 5'-end, the second one is labelled with a suitable radio-nuclide or with a tail of a partial sequence of the lac operator (lacOp) gene. At first, the PCR is performed with the outer set of primers (30–40 cycles); a great amount of the amplified sequence of DNA is formed. The second PCR is performed with the inner set of labelled primers (10–20 cycles). The amplified biotinylated DNA is selectively bound by streptavidin-coated magnetic beads. After washing the magnetic particles with radiolabelled DNA are suspended in a liquid scintillator and radioactivity is measured. Alternatively, if the inner primer was labelled with lacOp sequence, a fused protein composed from the lac repressor protein (lacI) and the enzyme β-galactosidase is added. After binding of the fused protein to the lacOp sequence and subsequent washing, the enzyme activity of this complex is measured after addition of a chromogenic substrate (Hornes et al. 1991; Olsvik et al. 1991a; Kapperud et al. 1993).

The comparison of gel electrophoretic detection of PCR products with a DIANA procedure, which enables easy visualization of amplified fragments in a microtitre plate format with an optical density reader, showed complete agreement in their discrimination between positive and negative samples (Kapperud et al. 1993).

DIANA has been applied to a number of immuno-magnetic separation assays including enterotoxigenic E. coli, Y. enterocolitica and Chlamydia trachomatis (Olsvik et al. 1991a,b; Hedrum et al. 1992; Kapperud et al. 1993). Olsvik et al. (1991a,b) used a two-step PCR procedure using primers in a nested configuration for the heat-stable enterotoxins I and II (SL1 and SL11) of enterotoxigenic E. coli (ETEC). The product from the first PCR cycle was used as the template DNA for the second PCR cycle where labelled primers were used. By biotin-labeling the double-stranded DNA fragments they could be separated from solution with streptavidin-coated magnetic beads. The assay could detect five ETEC. E.coli cells in 5ml and there was no interference from 100-fold excess of SL1 negative strains. A two-step DIANA procedure was developed by Kapperud et al. (1993) with two nested pairs of primers specific for the yadA gene. The initial isolation of the Y. enterocolitica was achieved with magnetic particles coated with antibodies against serogroup O : 3. By this technique all common pathogenic groups were detectable and could be differentiated from non-pathogenic verminiae. The detection level was 10–30 cfu g⁻¹ meat without overnight enrichment. This was improved to 2 cfu g⁻¹ meat by overnight pre-enrichment provided the background flora was less than 10⁷ cfu g⁻¹. A fully automated process could be developed for detecting the PCR products colorimetrically (DIANA) in a microtitre plate format with an optical reader.

It should be noted that detection by PCR does not mean that a viable target cell was present in the sample since the target DNA could originate from dead cells. Therefore application of this technique to food microbiology has to be in parallel with the colony isolation and confirmation.

5. APPLICATION OF MAGNETIC CARRIERS AND IMMOBILIZATION OF BACTERIA

5.1 Use of magnetite in waste-water treatment

The Siroflocc process clarifies and decolorizes water with magnetite particles which are subsequently regenerated (MacRae and Evans 1983, 1984; Bolto and Spurling 1991). Sewage is mixed with a magnetite slurry under acidic (pH 4.5–5.5) conditions. At these pH values the majority of impurities in water (together with the bacterial cells) are negatively-charged and hence adhere to the surface of the magnetite which has a positive charge. After mixing the suspension passes through a flocculating magnet which causes the magnetite and cells to clump together. The magnetite and cells settle in a clarifier gravimetrically and the magnetite can be liberated by addition of sodium hydroxide to give a pH of 10–11. The microbial mass can be further separated using a dissolved air flotation unit. The cells of Rhodopseudomonas palustris and Alcaligenes eutrophus, immobilized on magnetite, can be used for the removal of
poly-chlorinated hydrocarbons and pesticides during wastewater treatment (MacRae 1985, 1986). Unidentified microorganisms, immobilized with magnetic particles, have been used for batch and continuous mode removal of phenol contaminated water (Ozaki et al. 1991). In the continuous mode almost all the phenol and other readily degradable compounds were removed simultaneously over a 40 d period.

5.2 Industrial applications of microbial cells immobilized on magnetic carriers

The cells of Mycobacterium sp. have been immobilized by adhesion of magnetic iron oxides to the cell surface. The magnetically immobilized bacteria were used for side-chain degradation of cholesterol into androsta-1,4-diene-3,17-dione. The magnetic bacteria behaved as free cells in the transformation media and no mass transfer limitations were observed (Flygare and Larsson 1987). Saccharomyces cerevisiae cells, immobilized in magnetic alginate gel (Larsson and Moshbach 1979; Birnbaum and Larsson 1982), or adsorbed on surface-modified magnetite (Al-Hassan et al. 1991) have been used for the production of ethanol.

6. CONCLUSIONS AND FUTURE DEVELOPMENTS

The application of immuno-magnetic separation as a concentration method has been applied to many isolation procedures in microbiology. Reduced detection times for food-poisoning bacteria have been reported as well as clinical isolation of pathogenic bacteria in blood and faecal samples. Current developments with a combination of immuno-magnetic separation, nested PCR and colorimetric DNA visualization procedures (DIANA) make a fully automated analytical process possible.

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8. REFERENCES


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