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QUALITY EVALUATION OF COMMERCIAL ANTI-SALMONELLA ANTIBODIES FOR IMMUNOMAGNETIC SEPARATION USING WHOLE-CELL DOT BLOT

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The selection of antibodies with the desired specificity, immunoreactivity, purity and integrity is a key step in developing an effective immunosorbent for immunomagnetic separation (IMS). The choice of methods for characterizing the antibodies is limited, especially with antibodies specific to surface cell structures. In this study, four commercial anti-Salmonella antibodies were evaluated from the point of view of their immunoreactivity with the cells of Salmonella Typhimurium and of their purity. For these purposes, traditional SDS-PAGE analysis with subsequent silver staining and a newly adapted whole-cell dot blot technique were applied. Based on this testing, monoclonal anti-core LPS Salmonella antibodies from MyBiosource unambiguously demonstrated the highest immunoreactivity to Salmonella Typhimurium cells, whereas cross-reactivity with the closely related

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G- bacteria, Escherichia coli and Citrobacter braakii was not observed. Such antibodies will be subsequently used for the preparation of an anti-Salmonella immunosorbent and applied in IMS. The immunoreactivity and selectivity of anti-Salmonella poly- and monoclonal antibodies are also discussed.

Introduction

The immunomagnetic separation (IMS) of bacterial cells is a well-established and described technique based on the principle of bioaffinity chromatography. It is widely exploited in the food industry [1] and clinical microbiology [2]. IMS serves as a selective enrichment method without time-consuming pre-incubation steps. Reduced times for the detection of pathogens together with no need for complicated and expensive instruments makes this technique the method of choice for many laboratories. Isolated cells can be identified and counted by standard microbiological plating procedure [3] or PCR [4]. In recent years, there has been increasing interest in combining IMS with advanced end-detection analytical systems such as microfluidic lab-on-a-chip devices [5] or biosensors [6,7].

High-quality magnetic microparticles covered with specific antibodies, known as an immunosorbent, are a key prerequisite to performing successful IMS. Thus, the selection of the proper antibodies with the desired specificity as well as sensitivity towards the target antigen is an essential initial step in the preparation of such an immunosorbent. There are usually several antibodies of the same specificity available from different suppliers on the market, so the quality of antibodies often needs to be compared and then the right one for a particular application selected. The antibodies can be evaluated from various points of view — purity, source, presence of glycosylation and affinity or immunoreactivity. These parameters can be significantly affected by the method of manufacture and storage conditions. For testing the immunoreactivity between proper antibodies and cells, methods like western blot or dot blot can be applied. Whereas the preparation of bacterial samples for western blotting typically involves cell lysis followed by SDS-PAGE, during dot blotting the lysis is not essential when examining the immunoreactivity of the cell surface antigen. Moreover, since the cells are native when spotted on the membrane, the interaction should not significantly differ from the reaction conditions of IMS. Therefore, a dot blot method adapted for the whole-bacterial cell was chosen for comparing the immunoreactivity of antibodies for subsequent IMS in this study.

The dot blot or dot-immunobinding assay was first introduced by Hawkes *et al*. in 1982 [8]. The precise principle is described elsewhere [9,10]. Briefly, the antigen is applied in the shape of dots on the membrane and free reactive groups which could nonspecifically react with antibodies are then blocked with a blocking agent. The blotting membrane is then incubated with the specific primary

antibodies. The resultant immunocomplexes are detected by a secondary antibody conjugated with the enzyme and visualized after the addition of a specific substrate. The reaction scheme is shown in Fig. 1. Dot blotting with bacteria is usually accompanied by the addition of an extra step for bacteria lysis or DNA extraction. This is typically followed by DNA hybridization assays to identify the organisms [11-13] or searching for a specific gene [14,15]. When the detection or characterization of a pathogen of interest in food [16-18] or clinical specimens [19,20] or the determination of surface protein expression [21,22] is required, whole-cell dot blotting without cell lysis can be successfully utilized.

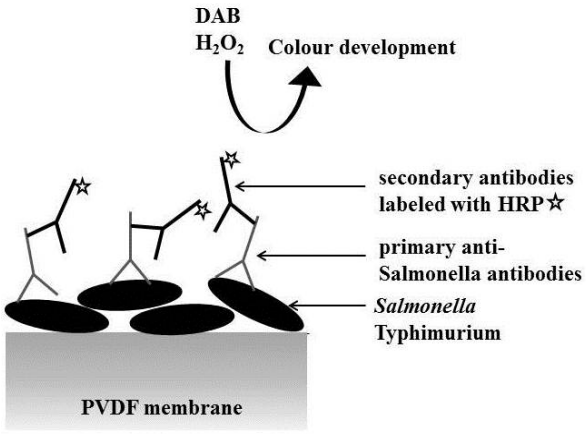

Fig. 1 Scheme of whole-cell dot blot method

In this paper, four commercial antibodies with specificity against *Salmonella* sp. were selected as promising candidates for the preparation of an immunosorbent for IMS. The quality of the antibodies was confirmed by SDS-PAGE. Their immunoreactivity against *Salmonella* Typhimurium bacteria was evaluated by a dot blot technique using a whole-cell suspension. In contrast to the above-mentioned works, which have all been done on a nitrocellulose or nylon membrane, a PVDF membrane was selected for our study because it retains the target protein very strongly and reduces nonspecific protein binding that can obscure high-sensitivity detection. The specificity and selectivity of the antibodies towards the control organisms from the family of *Enterobacteriaceae* — *Citrobacter braakii* and *Escherichia coli* were also tested.

Materials and Methods

Reagents and Chemicals

Lipopolysaccharides from *Salmonella enterica* serotype Enteritidis, bovine serum albumin (BSA), Tween 20, $NiCl_2$, acrylamide, *N,N*'-methylen-*bis*-acrylamide,

N,N,N',N'-tetramethylendiamine (TEMED), ammonium persulfate, glutaraldehyde, silver nitrate, goat anti-mouse IgG with HRP (horseradish peroxidase) and goat anti-rabbit IgG with HRP were purchased from Sigma Aldrich (St. Louis, MO, USA). 3,3'-Diaminobenzidine tetrahydrochloride (DAB), Precision Plus Protein Standards Unstained and PVDF membrane (Immuno-Blot PVDF Membrane, 0.2 μm) were acquired from Bio-Rad (Hercules, CA, USA). Mouse monoclonal antibodies (monoAb): anti-LPS core IgG2a Clone No M9011222 (concentration: 3.35 mg ml^{-1}) were acquired from MyBiosource (San Diego, CA, USA) and anti-LPS core IgG2a with ID GWB-115B3D (concentration: 0.1 mg ml^{-1}) were produced by GenWay Biotech (San Diego, CA, USA). Polyclonal antibodies (polyAb): rabbit anti-Salmonella sp. Antibodies (concentration: 4-5 mg ml^{-1}) were provided by Virostat (Portland, ME, USA) and goat antibodies of the same specificity (lyophilized form, 1 mg) were from KPL (Gaithersburg, MD, USA). Rabbit anti-goat IgG-HRP from Dako (Glostrup, Denmark) was provided by Institut Pasteur, Paris, France. Other chemicals were of analytical grade and obtained from Penta (Chrudim, the Czech Republic). All buffers were prepared from ultrapure water filtered through a TKA Smart2Pure system (Thermo Scientific TKA, Niederelbert, Germany).

Bacterial Strains and Their Cultivation

A culture of *Salmonella enterica* serovar Typhimurium (ATCC 43971) was provided by Institut Pasteur, Paris, France. *Escherichia coli* CCM 3954 and *Citrobacter braakii* CCM 158 were obtained from the Czech Collection of Microorganisms (Masaryk University, Brno, the Czech Republic). Bacterial strains were cultured on nutrient agar No. 2 (HiMedia, Mumbai, India) at 37 °C. Overnight cultures of all bacterial strains were suspended in phosphate-buffered saline (PBS) to an optical density of 1.5 at 600 nm (measured on BioPhotometer Eppendorf AG, Hamburg, Germany). For their application to the blotting membrane, the bacterial suspension was diluted with PBS in a 1:1 ratio. Various dilutions of the above suspension were tested to optimize the appropriate concentration of bacteria to be applied to the membrane (from 1:200 up to 2:1 (v/v)).

SDS-PAGE and Silver Staining

All antibodies were analyzed by SDS-PAGE followed by silver staining. Samples (1 μg antibody per well) were mixed with Laemmli sample buffer (1:1) and boiled at 100 °C for 2 min. The samples were loaded onto a 0.75 mm Tris-glycine gel (10 % [w/v] separating gel and 5 % [w/v] focusing gel) according to Laemmli [23].

Electrophoresis was performed using a Mini-Protean system (Bio-Rad, Hercules, CA, USA) at 180 V in Tris/glycine/SDS running buffer. The visualization of antibodies in the gel was done using a staining method with silver nitrate [24] and captured with a Nikon Coolpix 5000 digital camera (Nikon, Tokyo, Japan).

Dot Blot

The dot blot experiment was performed according to the previously published method [25] but omitting the step with the chaotropic agent. For this experiment, a Dot-blot DHM-96 unit manifold from Scie-Plast (Cambridge, UK) and vacuum pump rated at 600 mm Hg (0.8 bar) were used. The suspensions of bacterial cells (see above) were spotted on the PVDF membrane in a volume of 100 µl. Lipopolysaccharides and BSA were spotted at a concentration of 5 µg per 100 µl of PBS. After the application of the bacterial suspensions, controls and blanks, the membrane was air dried and cut into four rectangles; each containing 6 spots (see Fig. 2). Incubation with primary and secondary antibodies was carried out in Petri dishes (diameter of 5 cm) in a total volume of 3 ml. A dilution factor of 1:1 000 was used to dilute the primary and secondary antibodies. The HRP was quantified using DAB chromogen and H_2O_2 as a substrate. A positive reaction appeared as a brown spot on the membrane, which could be clearly distinguished from a colorless spot (negative reaction). For details, see Ref. [25]. The intensity of the spots was densitometrically quantified using the free software ImageJ (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA). Each experiment was repeated three times.

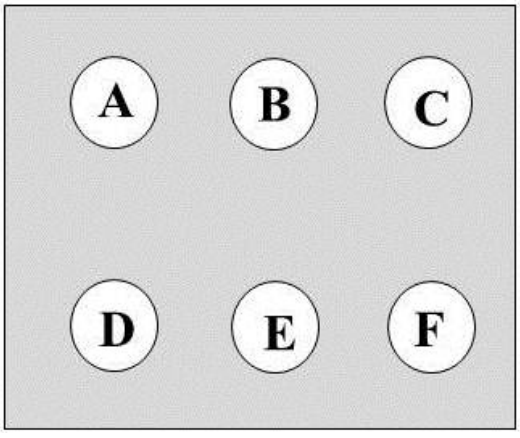

Fig. 2 Scheme of application of samples and controls to be tested by whole-cell dot blot method. A – lipopolysaccharide; B – bovine serum albumin; C – PBS buffer; D – *Salmonella* Typhimurium; E – *Escherichia coli*; F – *Citrobacter braakii*

Results and Discussion

The aim of our study was to evaluate the immunoreactivity between whole intact cells of *Salmonella* Typhimurium and four types of commercially available antibodies. Currently, even though suppliers offer a wide range of specific monoclonal as well as polyclonal antibodies, detailed information about their precise specificity (antigenic determinants) or purity are often unavailable. Therefore, a preliminary confirmation of the purity, integrity and immunoreactivity of the purchased antibodies is an initial necessary step in the preparation of the immunosorbent. Additionally, the antibodies are usually the most expensive component of the prepared immunosorbent. A fast, reliable and reproducible confirmation method before commencing the immobilization of antibodies is, therefore, highly valuable. SDS-PAGE for verifying purity and wholeness together with a dot blot for examining immunoreactivity are a few of the methods usable for such purposes. The results of these mutually complementary methods provide the information needed to prepare a high-quality immunosorbent.

Table I List of commercial anti-*Salmonella* antibodies selected for this study

Clonality	Host animal	Isotype	Immunogen	Supplier	Form
polyclonal	goat	ND	Various strains of *Salmonella*	KPL	lyophilized
	rabbit	ND	Mixture of *S.* Enteritidis, *S.* Typhimurium, *S.* Heidelburg	Virostat	0.01 M PBS pH 7.2 with 0.1% NaN_3
monoclonal	mouse	IgG2a	LPS core of Salmonella	GenWay Biotech	
	mouse	IgG2a	LPS core of Salmonella	MyBiosource	

LPS…lipopolysaccharide
ND…not defined

The first criterion for the selection of antibodies from a large number of commercially available antibodies was the absence of stabilizing agents (e.g., BSA, trehalose, gelatin) in the shipping solution. Such stabilizing agents can reduce the binding efficiency of antibodies during the preparation of the immunosorbent. With monoclonal antibodies, specificity against the core oligosaccharide region of the lipopolysaccharide (LPS) was sought, since it is a relatively conserved structure. More than 2 000 serotypes of Salmonella share only two closely related core types that are identical in all serogroups of the genus *Salmonella* [26-28]. Two types of monoAb and polyAb without any stabilizers

were thus selected for the subsequent testing (Table I). All antibodies were shipped in phosphate buffer containing sodium azide, which serves as a preservative and is easily removable by gel filtration or dialysis. The second criterion was the integrity and purity of the antibodies. Therefore, an SDS-PAGE analysis was performed. According to the results from electrophoresis (see Fig. 3), both monoclonal and polyclonal antibodies seemed to be pure, intact and non-aggregated.

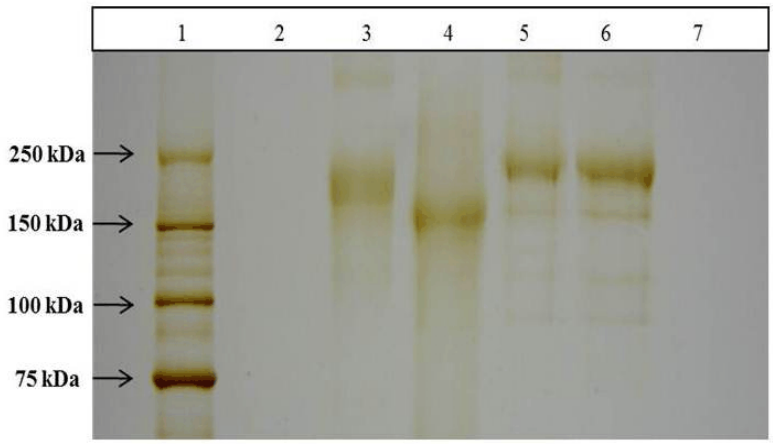

Fig. 3 SDS-PAGE analysis followed by silver staining Lanes. 1 – molecular weight marker (10-250 kDa); 2 – empty; 3 – goat polyAb from KPL; 4 – rabbit polyAb frm Virostat; 5 – mouse monoAb from GenWay Biotech; 6 – mouse monoAb from MyBiosource; 7 – empty

The final aspect of the selection of antibodies for IMS was their immunoreactivity towards Salmonella cells, which was tested by whole-cell dot blot. To be sure that the intensity of the dots corresponded purely to the specific interaction between the antigen and corresponding primary antibody, several control samples needed to be included in the assay. Commercial lipopolysaccharide (LPS), a highly immunogenic determinant and a major constituent of the cell wall of most G-bacteria, served as a positive control. The non-specific interactions of the reaction compounds are frequent secondary undesirable effects that interfere with immunoassays. Whereas suppression of these effects was almost unfeasible, various types of negative controls and blanks were included in our experiments. The reaction environment (PBS buffer) and bovine serum albumin (BSA) were used as the control of the non-specific sorption of not only the primary but also the secondary antibodies (blank). The selectivity of the chosen antibodies was verified by reaction with closely related G-bacteria from the family of *Enterobacteriaceae*, *Escherichia coli* and *Citrobacter braakii,* which served as the negative controls of our immunoassay. To optimize the concentration of bacteria applied to the PVDF membrane, serial dilutions of

Salmonella (from 1:200 to 2:1 (v/v)) were performed (results not shown). An intensive and uniform coloring of the spot was achieved when the suspension was spotted on the membrane in a volume ratio of 1:1. This dilution was used for all subsequent experiments.

Fig. 4 Image of whole-cell dot blot immunoassay. Part 1: immunoreactivity of polyAb from KPL; Part 2: immunoreactivity of polyAb from Virostat; Part 3: immunoreactivity of monoAb from GenWay; Part 4: immunoreactivity of monoAb from MyBiosource — for application scheme, see Fig. 2

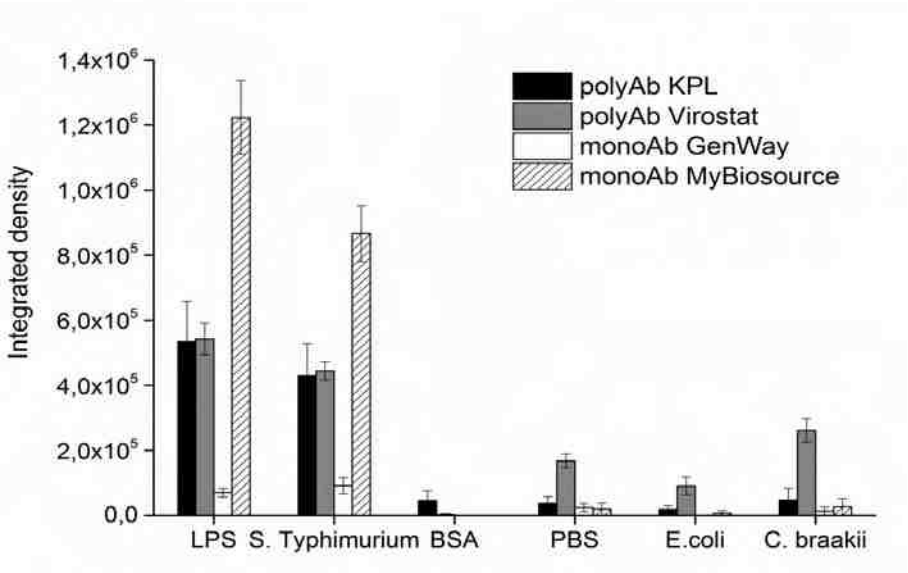

Fig. 5 Colour density of spots as function of immunoreactivity of particular antibodies with corresponding antigens and controls. LPS – positive control of immunoassay; BSA, PBS – blank of immunoassay; *Escherichia coli*, *Citrobacter braakii* – negative control of immunoassay

The results of whole-cell dot-blot immunoassay are shown in Figs 4 and 5. The colour intensity of the spots, as an expression of specific immunoreactivity level, was evaluated quantitatively by densitometric analysis using ImageJ software. Generally, polyclonal antibodies are usually connected with a lower intensity of the spots and higher background signal during dot blotting. Also, cross-reactivity with other bacteria can be expected due to the structural similarities among related species. In comparison, monoclonal antibodies specifically prepared against the particular antigenic determinant should possess an exceedingly high immunoreactivity to the corresponding antigen. The results

of both tested polyclonal antibodies were in agreement with our expectations (part 1 and 2 in Fig. 4). Their immunoreactivity to LPS and *Salmonella* were comparable, and a level of cross-reactivity to *Escherichia coli* and *Citrobacter braakii* was observed. Rabbit polyclonal antibodies from Virostat were found to be more cross-reactive than the goat antibodies from KPL. With the tested monoclonal antibodies, our results were surprising. While the antibodies from MyBiosource (part 4 in Fig. 4) proved to be excellent in terms of their reactivity to both LPS and *Salmonella*, the immunoreactivity of GenWay antibodies (part 3 in Fig. 4) was significantly reduced. This phenomenon was further studied, and their immunoreactivity with secondary antibodies with HRP was confirmed (data not shown). Based on this fact, we assume that the GenWay antibodies do not exhibit the claimed specific immunoreactivity towards *Salmonella* Typhimurium.

Conclusion

In this study, SDS-PAGE and dot-blot evaluation of commercial anti-Salmonella antibodies was performed. The SDS-PAGE analysis indicated the high quality (purity and wholeness) of all the tested antibodies and their applicability to immunosorbent preparation. Nevertheless, the results obtained from the whole-cell dot blot demonstrated that the immunoreactivities of the various antibodies were significantly different. The monoAb from MyBiosource seemed to be the best of the tested antibodies for the IMS of *Salmonella*. These results confirmed our long-term practical experience that not all of the commercially available antibodies against the desired antigen fulfill the criteria needed for IMS.

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