

CHARACTERIZATION AND UTILIZATION OF MONOCLONAL ANTIBODIES REACTIVE TO *YERSINIA PSEUDOTUBERCULOSIS*

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Abstract. The 3 murine monoclonal antibodies, Yps1, Yps2 and Yps3 reactive to *Y. pseudotuberculosis* can be stabilized and all were found to be of IgG type. Monoclonal antibody, Yps1, recognized a glycoprotein antigen of the organism with reactivity at the 55-75 kDa region, while Yps2 and Yps3 recognized protein antigens of *Y. pseudotuberculosis* 65 kDa and 26-28 kDa molecular weight regions, respectively. The specificity of monoclonal antibodies was tested using dot ELISA and Western blotting with whole cell organisms or whole cell sonicated soluble antigens of different *Yersinia* species, *Salmonella typhi*, *Klebsiella pneumoniae*, *Streptococcus abortus-equi* and *Escherichia coli*. Monoclonal antibody, Yps1 exhibited cross-reactivity with soluble antigens and whole cell preparations of *Y. pestis*. Yps2 cross-reacted to soluble antigens of all the tested bacteria. Reactivity of monoclonal antibody, Yps3 was restricted to *Y. pseudotuberculosis* and *Y. pestis* with soluble antigen preparations. No reaction was observed with Yps2 and Yps3 to whole cell organism preparations from tested bacteria including *Y. pseudotuberculosis*. The co-agglutination reagent prepared by sensitizing staphylococcal cells with Yps1 monoclonal antibody produced a positive agglutination with all the 4 *Y. pseudotuberculosis* isolates and the 3 *Y. pestis* strains tested. Sandwich dot ELISA using monospecific antisera as a capture antibody and a monoclonal antibody, and Yps3 as a revealing antibody had a high level of specificity in detecting *Y. pseudotuberculosis* antigens.

INTRODUCTION

Y. pseudotuberculosis, primarily a food pathogen, is mainly associated with gastrointestinal infections in humans. Infections may result in enterocolitis, lymphadenitis, acute appendicitis and uveitis (Hannuksela, 1977). Frequently systemic dissemination may lead to septicemia, endocarditis, septic arthritis and cellulitis. In Humans, the persistence of *Y. pseudotuberculosis* has been found to be associated with the immune status and the HLA-B27 antigen. These manifestations are more common in immuno-compromised hosts or in those with underlying disorders such as iron overload, thalassemia, aplastic anemia, sickle cell disease and defects in iron metabolism (Hoogkamp-Korstanje, 1996). The secondary immunologically mediated sequelae of acute *Y. pseudotuberculosis* infection, such as arthritis, erythema nodosum, Reiter's syndrome and glomerulonephritis have been predominantly

reported in patients who are HLA-B27 positive.

Although worldwide incidences of *Y. pseudotuberculosis* infections are on the increase, there are only a few reports of *Y. pseudotuberculosis* infections from India, from some animal species (Behra *et al*, 1984; Srivastava *et al*, 1978; Jayaramman and Sethumadavan, 1973). The main probable reason for the absence of reported human infections from India and in other developing countries is because of a low awareness of this organism in clinical practice. Specific attempts are often not made to isolate and identify these bacteria from clinical samples.

The present work was initiated with the objectives of developing an immuno-based rapid identification system for *Y. pseudotuberculosis*. To prepare reagents for immuno-identification, polyclonal hyper-immune sera was raised in rabbits and murine monoclonal antibodies were generated following fusion of spleenocytes from immunized mice with the Sp2/0 myeloma cell line.

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MATERIALS AND METHODS

Animals

New Zealand male white rabbits 1 to 1.5 kg,

inbred balb/C mice weighing 15 to 20 g were obtained from animal house of DRDE, Gwalior, India.

Bacterial cultures

Standard cultures of *Y. pseudotuberculosis* O:1A, *Y. pestis* A1122, *Y. enterocolitica* O:9 were obtained from WHO sources. Standard cultures of *Y. kristensenii*, *Y. intermedia*, *Y. fredericksonii*, *Y. enterocolitica* O:3, *Y. enterocolitica* O:5, *Y. enterocolitica* O:13, *Y. enterocolitica* O:21 were obtained from a laboratory in Norway. Clinical isolates of *Y. pestis* (101, 111, 112 and 113) were obtained from Microbiology Division of DRDE, Gwalior. *Escherichia coli*, *Salmonella typhi*, *S. abortus-equi*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Citrobacter koseri*, *Enterobacter cloacae* and *Escherichia aerogenes* were obtained from Institute of Microbial Technology, Chandigarh, India.

Preparation of polyclonal hyper-immune sera

Two New Zealand male rabbits weighing 1-1.5 kg were used for raising sera against *Y. pseudotuberculosis*. These rabbits were immunized at weekly intervals with 100 µg of *Y. pseudotuberculosis* sonicated soluble antigen. The first dose was prepared with complete Freund's adjuvant and subsequent doses were prepared with Freund's incomplete adjuvant. All the doses were given intradermally. The titer of hyper-immune sera was checked by dot ELISA and plate ELISA.

Preparation of mono-specific antisera

Y. pestis A1122, *Y. enterocolitica* O:9, *Y. enterocolitica* O:5, *Y. kristensenii*, *Y. intermedia*, *Y. fredericksonii* and *S. typhi* were inoculated on BHI agar plates and incubated at 37°C for 48 hours. The growth obtained was harvested and washed thrice with sterile PBS and centrifuged at 10,000 rpm for 20 minutes at 4°C. Hyper-immune sera with a high titer to *Y. pseudotuberculosis* was allowed to adsorb sequentially with bacterial pellets obtained from the mentioned bacteria for 3 hours at room temperature with constant shaking. Bacteria were removed by centrifugation at 10,000 rpm for 20 minutes. The supernatant was collected and stored at -20°C in aliquots. Thiomersal solution 1% was added to aliquots as a preservative. The specificity of mono-specific sera was tested by dot ELISA. Sonicated antigens

were prepared from different *Yersinia* species, *E. coli*, *K. pneumoniae* and *S. typhi* and nitrocellulose tips of combs were coated with 2 µl of 100 µg/ml concentration of antigen in sodium bicarbonate buffer, pH 9.6. Antigen coated strips were blocked with 5% skimmed milk solution in PBS kept at 4°C overnight. Blocked antigen coated strips were reacted with mono-specific sera at different dilutions (1:1,000, 1:2,000 and 1:5,000).

Generation of monoclonal antibodies

BALB/c mice were immunized with 25 µg of *Y. pseudotuberculosis* sonicated soluble antigen at weekly intervals for 4 weeks. Three days prior to fusion, immunized mice were given 3 injections intra-peritoneally. Lymphocytes from the spleen of these immunized mice were fused with the mouse myeloma cell line, Sp2/0, in the presence of polyethylene glycol. The procedure for the fusion was followed as suggested by Kohler and Milstein (1975) with minor modifications. The supernatant of hybrid containing wells were tested for the presence of antibodies against *Y. pseudotuberculosis* by dot ELISA. Consistently positive wells were cloned and sub-cloned thrice to obtain stable hybrids.

Characterization of monoclonal antibodies

The immunoglobulin class of the monoclonal antibody was determined by dot ELISA using HRP labeled goat antimouse IgG and IgM conjugates (Sigma chemicals, USA). The nature of the epitope recognized by the generated monoclonal antibody was determined by treating the coated antigens with trypsin (concentration varying from 0.5 µg -5 µg per well) followed by a standard ELISA for the protein type of epitopes and a periodate treatment of antigens was done for the glycoprotein type of epitopes. The procedure followed for sodium-meta-periodate (Sigma chemicals, USA) mediated oxidation of sugar residues in the *Y. pseudotuberculosis* antigen coated ELISA plates was as per the protocol given by Woodward and associates (1985) with minor modifications.

Specificity testing of monoclonal antibodies

Using soluble extracts of sonicated antigens and/or whole cell organisms of different species of genus *Yersinia* (*Y. pseudotuberculosis* O:1A, *Y. enterocolitica* O:5, *Y. enterocolitica* O:8, *Y.*

enterocolitica O:3, *Y. enterocolitica* O:9, *Y. enterocolitica* O:13, *Y. enterocolitica* O:21, *Y. intermedia*, *Y. fredericksonii*, *Y. kristensenii*, *Y. pestis* A1122 and 4 clinical isolates of *Y. pestis*) and certain species of the family enterobacteriaceae (*E. coli*, *S. abortus-equi*, *S. typhi* and *K. pneumoniae*) dot ELISA and/or Western blotting was performed for specificity testing. For dot ELISA soluble antigen was coated at 1-2 µl volume at 100 µg/ml concentration and whole cell organisms were coated at approximately 10⁵ organisms/ml concentration.

SDS-PAGE and Western blotting

SDS-PAGE was performed by the method of Laemmli (1970). The samples prepared from pure growth of different *Yersinia* species, *S. typhi*, *K. pneumoniae* and *E. coli* in 2X lysis buffer (0.0625 M tris, 2% SDS, 10% glycine, 5% β-mercaptoethanol, 0.001% bromo-phenol blue, pH 6.8) were electrophoretically separated in a 10% separating gel and 3.6% stacking gel in the presence of 0.5% SDS at a constant current of 15 mA per gel for 3 hours in an electrophoretic cell [Hoeffer's mini gel apparatus containing electrode buffer (pH 8.6)]. Standard low and high molecular weight markers were included in each run. The separated polypeptides were transferred onto 0.45-mm-pore size nitrocellulose membrane by the method described by Towin *et al* (1979) using 25 mM tris hydrochloride-192 mM glycine buffer (pH 8.3), containing 20% methanol at a constant voltage of 45 volts for 1 hour in a transblot cell (Hoefer transfer apparatus, USA). The free sites of the nitrocellulose membrane were saturated by incubating in 5% skimmed milk powder in PBS, overnight at 4°C. Membranes were washed 3 times with PBS-Tween, each with shaking for 5 minutes and incubated with different monoclonal antibodies. Following a further 3 washings in PBS-Tween, incubation was carried out with anti-rabbit IgG-HRP conjugate (Sigma chemicals, USA) diluted at 1:1,000 in PBS at 37°C for 1 hour. After 3 washings of 5 minutes each in PBS-Tween, the nitrocellulose membranes were developed with DAB-H₂O₂ substrate.

Co-agglutination test for antigen detection

S. aureus Cowan 1 strain was inoculated in 2 liters of nutrient media and was incubated at 37°C for 10 hours. Culture was centrifuged at 7,000 rpm for 20 minutes and the pellet obtained

was washed thrice with PBS by centrifugation at 7,000 rpm for 20 minutes. The pellet was then suspended in PBS containing 0.5% formalin for 1 hour. The cell suspension was centrifuged and the pellet was washed thrice with PBS and was heat inactivated at 80°C for 30 minutes. The test reagent was prepared by mixing 5 ml of supernatant from clones Yps1 and Yps3 respectively with 5 ml of stock solution of *S. aureus* Cowan1 strain at room temperature for 3 hours by continuous shaking. This was centrifuged at 5,000 rpm for 20 minutes and the pellet was given 3 washings with PBS. The pellet obtained was suspended in 5 ml of sterile PBS. The control reagent was prepared similarly, where instead of monoclonal antibodies normal mice antisera was used.

Identification of *Y. pseudotuberculosis* by co-agglutination test

A homogeneous bacterial cell suspension of different species of *Yersinia*, *E. coli*, *S. typhi*, *C. koseri*, *E. cloacae* and *K. pneumoniae* was prepared in glycine saline buffer, pH 8.8. One drop of approximately 25 µl of bacterial suspension and 1 drop of test reagent were mixed and observed for an agglutination reaction within 2 minutes. Simultaneously, the culture of *Y. pseudotuberculosis* mixed with the control reagent was also tested. The reagent prepared from monoclonal antibodies was then utilized to identify the surveillance isolates of *Y. pseudotuberculosis* and the strains of *Y. pestis* in a slide co-agglutination test following the same procedure used to test the specificity.

Sandwich ELISA for antigen detection

The mono-specific polyclonal antibody to *Y. pseudotuberculosis* was utilized as a capture antibody and the Yps3 monoclonal antibody as a revealing antibody in a sandwich dot ELISA. The specificity of the sandwich ELISA was tested with the sonicated soluble antigen of different *Yersinia* species, *E. coli*, *K. pneumoniae* and *S. typhi* at a concentration of 2.5 µg/ml. For coating, capture antibodies were used at a 1:200 dilution.

RESULTS

Specificity of mono-specific polyclonal sera

At a 1:3,000 dilution, the mono-specific antibody reacted only with *Y. pseudotuberculosis*

in the dot ELISA. The reaction with different *Yersinia* species, *E. coli*, *S. typhi* and *K. pneumoniae* was not observed at this dilution. The titer of mono-specific polyclonal sera was 1:12,800 in the dot ELISA.

Generation of monoclonal antibodies

After 12 days of fusion 90% of wells of tissue culture plates were found to contain hybrids. Of these, 26 wells had hybrids producing antibodies reactive to *Y. pseudotuberculosis* when tested with the dot ELISA. Cells from all the 26 reactive wells were put onto a limiting dilution, 2 to 3 times to get clones originating from a single cell in a well regularly checked by the dot ELISA for antibody production. Finally 3 stable antibody producing clones were obtained and named Yps1, Yps2 and Yps3.

Characterization of monoclonal antibodies

All the 3 monoclonal antibodies were of the IgG type. Trypsin treatment affected the reactivity of all 3 monoclonal antibodies even at the lowest concentration of 0.5 µg/ml. Monoclonal antibodies, Yps2 and Yps3, showed a marked decrease in ELISA reactivity with the increase in trypsin concentration. This decrease in ELISA reactivity was also observed with the Yps1 monoclonal antibody, but this decrease was not marked when compared with the other 2 clones. The periodate treatment did not affect the reactivity of these monoclonal antibodies. The monoclonal antibody, Yps1 showed a decrease in OD values with the increase in concentration of trypsin sodium-meta-periodate. The ELISA reactivity of this particular antibody was earlier shown to be affected by the trypsin treatment of antigens. Therefore, the antigen recognized by the monoclonal antibody, Yps1 was a glycoprotein.

Specificity testing of monoclonal antibodies

Monoclonal antibody, Yps1 reacted only with *Y. pseudotuberculosis* and *Y. pestis* with soluble antigens in the dot ELISA and Western blot and also in the dot ELISA using the whole cell organisms as antigen. Yps2 cross reacted with soluble antigens of all tested bacteria in the dot ELISA as well as in the Western blot. The reactivity of the monoclonal antibody, Yps3, was restricted to *Y. pseudotuberculosis*, and *Y. pestis* in both the dot ELISA and the Western blot using

soluble antigens. The monoclonal antibodies Yps2 and Yps3 gave no reaction in the dot ELISA with the whole cell antigen preparations of all the *Yersinia* species including *Y. pseudotuberculosis*. The monoclonal antibody, Yps1 recognized a glycoprotein antigen of *Y. pseudotuberculosis* with a reactivity at the 55-75 kDa region, while the Yps2 and Yps3 recognized protein antigens at the 65 kDa and 26-28 kDa molecular weight regions, respectively in the Western blot analysis.

Identification of *Y. pseudotuberculosis* by co-agglutination test

Standard cultures of *Y. pseudotuberculosis* and *Y. pestis* gave a positive co-agglutination test within 2 minutes with the test reagent prepared from Yps1. A weak positive reaction was observed with *Y. fredericksonii*, that appearing after 5 minutes. The reaction with the other tested bacteria remained negative. A positive agglutination reaction was observed with all the 4 isolates of *Y. pseudotuberculosis* and 3 isolates of *Y. pestis* recovered from the surveillance samples. The test reagent prepared from Yps3 gave no visible agglutination with the standard cultures of *Y. pseudotuberculosis* and *Y. pestis*.

Sandwich ELISA for organism identification

The sandwich dot ELISA standardized using the mono-specific polyclonal antibody to *Y. pseudotuberculosis* was utilized as a capture antibody and a monoclonal antibody, and Yps3 as a revealing antibody. Of the different *Yersinia* species, *E. coli*, *K. pneumoniae* and *S. typhi* tested, the antigen-antibody reaction in the form of a dot was observed only with the strains of *Y. pseudotuberculosis*.

DISCUSSION

The incidence of *Y. pseudotuberculosis*, an important enteric pathogen, is on the rise worldwide (Hoogkamp-Korstanje, 1996). However, this being a fastidious and relatively slow growing organism, is not routinely looked for in clinical specimens, as the identification of suspected bacterial colonies would require additional biochemical tests and the bacteriophage lysis tests, which are not being performed in most microbiological laboratories particularly in developing countries.

The present work was undertaken to develop a rapid immuno-detection system for the specific identification of *Y. pseudotuberculosis*. Polyclonal sera raised in rabbits had high titers but showed extensive cross-reactivity with *Yersinia* species and other members of the family enterobacteriaceae. The cross-reactivity of polyclonal sera can be removed by adsorption of antisera with different *Yersinia* species and *S. typhi* and the mono-specific antisera after adsorption, reacted only to *Y. pseudotuberculosis* which was then utilized in the sandwich dot ELISA as a capture antibody.

The monoclonal antibody, Yps1 exhibited cross-reactivity only with *Y. pestis*, with both the soluble and the whole cell antigen preparations appeared to recognize a surface antigen of glycoprotein in nature. Owing to its surface antigen reactivity, this monoclonal antibody can be utilized in a simple co-agglutination test to recognize both *Y. pseudotuberculosis* and *Y. pestis* strains.

The monoclonal antibody, Yps3, also had limited cross-reactivity restricted to the soluble antigen preparation *Y. pestis* did not work in the co-agglutination test. This monoclonal antibody was found suitable for the sandwich ELISA to specifically react to *Y. pseudotuberculosis* in combination with a monospecific polyclonal antibody. In the Western blot, a reaction to both *Y. pseudotuberculosis* and *Y. pestis* was observed with the monoclonal antibody at the 26-28 kDa region. Antigenically and genetically the relationship between *Y. pestis* and *Y. pseudotuberculosis* is considered to be very close. These species share at least 13 antigenic components including the R lipo-polysaccharide (Chen and Meyer, 1966). The DNA hybridization studies have shown that they represent 2 pathotypes of the same single microorganism. The distinction mainly represents differences in the plasmid content (Ben-Gurion and Shafferman, 1981; Feber and Brubaker, 1981). *Y. pseudotuberculosis* shares a nearly 90% DNA sequence homology with *Y. pestis* (Bercovier *et al*, 1980). Since the number of clones generated in the present work was only 3, a *Y. pseudotuberculosis* specific monoclonal antibody was not obtained, may be because of the close homology between the 2 species.

Monoclonal antibodies like Yps3 reacting

specifically to both *Y. pseudotuberculosis* and *Y. pestis* have not been reported so far. Monoclonal antibodies with epitopes shared between *Y. pseudotuberculosis* and *Y. enterocolitica* like 4G1, reactive to the specific heat modifiable protein and not found reactive to the 46 other bacterial strains of enterobacteriaceae tested, have been reported (Ogasawara *et al*, 1985). A monoclonal antibody directed against the plasmid encoded released protein of enteropathogenic *Yersinia* produced by Heeseman and co-workers (1986) was found to be reactive with *Y. pseudotuberculosis* serotypes O:1 and O:3 and with *Y. enterocolitica* serotype O:3, O:8, O:9 and O:5,27 after growth in a calcium deficient medium. Monoclonal antibodies to the invasin protein of *Y. pseudotuberculosis* have been produced and utilized to detect invasins in *Y. pseudotuberculosis*. These monoclonal antibodies were also useful for epitope mapping of the invasin protein (Leong *et al*, 1991).

The application of polyclonal antibodies in the immunoglobulin erythrocytic preparation with the detection limit of 8×10^5 microbial cells per ml and in an agglutination test employing antiserum raised against thermostable *Y. pseudotuberculosis* toxin has been described for the rapid detection of toxin producing *Y. pseudotuberculosis* (Dulatova *et al*, 1992; Venediktov *et al*, 1994). In the reported tests, *Y. pestis*, was not included. Likewise the monoclonal antibodies generated in the present work were also found suitable in simple assays such as the co-agglutination test and the dot ELISA. The co-agglutination test with the monoclonal antibody, Yps1 can identify both species and the sandwich dot ELISA with the Yps3 monoclonal antibody and the mono-specific polyclonal antibody further helped in differentiation of these 2 important pathogens. These reagents may be of value for the direct identification of the organisms from the clinical and the surveillance samples.

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