***Mycoplasma synoviae* surface protein MSPB as a recombinant antigen in an indirect ELISA**

Amir H. Noormohammadi,[†] Philip F. Markham, Jillian F. Markham, Kevin G. Whithear and Glenn F. Browning

Author for correspondence: Amir H. Noormohammadi. Tel: +61 3 9345 2475. Fax: +61 3 9347 0852.
e-mail: amir_h@wehi.edu.au

School of Veterinary
Science, The University of
Melbourne, Parkville,
Victoria, Australia 3052

***Mycoplasma synoviae* is a poultry pathogen causing respiratory disease and synovitis. A number of serological assays have been developed for diagnosis of *M. synoviae* infection; however, they lack sensitivity and/or are prone to false-positive reactions. Using a combination of PCR and expression cloning, four overlapping regions (regions 1–4) of the surface antigen MSPB of *M. synoviae* WVU-1853 were expressed in a bacterial expression system. Immunostaining of the resultant polypeptides with chicken sera raised against different *M. synoviae* strains, or *Mycoplasma gallisepticum* S6, suggested that region 4 contained a highly antigenic and species-specific domain (amino acids 178–213) of MSPB. A fusion protein of region 4 was expressed in the pMAL expression system and purified from cold-osmotic-shock fluids of *Escherichia coli* cells for use in an indirect ELISA. The potential of the purified antigen for detection of *M. synoviae* antibodies was assessed with sera obtained from chickens experimentally infected with different strains of *M. synoviae* or *M. gallisepticum*, or from uninoculated chickens. All the sera from *M. synoviae*-inoculated chickens provided higher absorbance values than those from *M. gallisepticum*-inoculated or uninoculated chickens. Chickens inoculated with *M. synoviae* 86079/7NS had detectable increases of serum anti-MSPB immunoglobulins at day 7 after inoculation. These studies have identified the most antigenic region of one of the major species-specific surface proteins of *M. synoviae*, and shown the potential of this protein as a serodiagnostic reagent.**

Keywords: *Mycoplasma synoviae*, surface antigens, MSPB, ELISA, serology

INTRODUCTION

Mycoplasma synoviae is an important cause of chronic respiratory disease and synovitis in chicken, and causes a major economic loss in the poultry industry throughout the world. Control of this pathogen is highly dependent on serological assays to detect infected flocks; however, these assays suffer from limited specificity and sensitivity. The low specificity of current serological assays is mainly attributed to cross-reactions with *Mycoplasma gallisepticum* (Kleven, 1997).

[†]**Present address:** WEHI, Post Office, Royal Melbourne Hospital, VIC 3050, Australia.

Abbreviations: HRP, horseradish peroxidase; RSA, rapid serum agglutination; RU, relative units; SPF, specific-pathogen-free.

The GenBank accession number for the *v1hA* gene sequence is AF035624.

Several ELISAs have been developed to detect antibodies against *M. synoviae*, but they have generally been based on poorly defined membrane components. A number of earlier studies of *M. synoviae* ELISAs used crude membrane preparations (Opitz *et al.*, 1983; Patten *et al.*, 1984; Higgins & Whithear, 1986), produced by either osmotic lysis or sonication of whole cells, or Triton X-100-solubilized membrane proteins (Opitz & Cyr, 1986) of *M. synoviae*, as antigens. These studies suggested that ELISA may serve as a sensitive assay for detection of *M. synoviae* infection. Using SDS-PAGE, Avakian & Kleven (1990) purified three membrane antigens of *M. synoviae* F10-2AS (p53, p41 and p22) and used them in a dot-ELISA. Whilst assays using p53 and p22 were not sensitive, that based on p41 was more sensitive than the haemagglutination inhibition test in detecting antibodies to *M. synoviae*. In a recent study (Gurevich *et al.*, 1995) directed at improving the specificity and sensitivity of

the *M. synoviae* ELISA, a protein fraction highly enriched in an immunodominant cluster of *M. synoviae* WVU-1853 proteins (p46–52) was extracted from the Triton X-114 detergent phase of this organism and further purified by ion-exchange chromatography. Results of an indirect ELISA, based on the purified fraction as antigen, showed a strong correlation ($r = 0.776$) with results of the rapid serum agglutination (RSA) test on individual sera.

Although these ELISA systems are rapid and sensitive, they are relatively expensive to produce and, in some cases, have limited specificity (Kleven & Yoder, 1989). In addition, studies on *M. synoviae* surface proteins (Noormohammadi *et al.*, 1997) have highlighted the variable expression of major antigens of this organism and thus suggested that it may be difficult to ensure consistency of antigen preparations from cultures of *M. synoviae*.

A recent study in our laboratory (Noormohammadi *et al.*, 1997) revealed that the immunodominant 42–56 kDa cluster of *M. synoviae* WVU-1853 consists of two major membrane antigens, MSPA and MSPB, and that MSPB was expressed in all *M. synoviae* strains tested. These results suggested the potential of MSPB as a diagnostic antigen (Noormohammadi *et al.*, 1997). Subsequently, the DNA sequence of the gene (*vlhA*) which encodes both MSPB and MSBA was determined (Noormohammadi *et al.*, 1998).

The aim of this study was to determine the most antigenic region of MSPB and to assess the potential of a serological test based on this antigen, in comparison to the RSA.

METHODS

Bacterial strains and growth media. *Escherichia coli* strain DH5 α or JM109 was electrottransformed with recombinant plasmid and grown at 37 °C in rich broth containing 50 μ g ampicillin ml⁻¹ (Sambrook *et al.*, 1989). Rich broth contained (l⁻¹) 10 g tryptone (Oxoid), 5 g yeast extract (Oxoid), 5 g NaCl and 2 g glucose.

M. synoviae strain WVU-1853, modified live vaccines MS-H (Vaxsafe MS) and BC-MS, and field isolates 69808 and 86079/7NS (Gurevich *et al.*, 1995), and *M. gallisepticum* strain S6, field isolates NS-2, MGL (80083) and AP3AS (Soeripto *et al.*, 1989), and modified live vaccine ts-11 (Vaxsafe MG) (Whithear *et al.*, 1990) were grown in mycoplasma broth to the late-exponential growth phase (Whithear, 1993) and used for inoculation of chickens. Identity of each species was confirmed by immunofluorescence or growth inhibition test, as described previously (Whithear, 1993).

PCR. On the basis of the *vlhA* gene sequence, a single forward oligonucleotide primer, F₁, was used in separate PCR reactions with either of the oligonucleotide primers R₁, R₂, R₃ or R₄, to amplify four overlapping 5' regions of the *M. synoviae* WVU-1853 *vlhA* gene that encode part of MSPB (Noormohammadi *et al.*, 1998). Table 1 describes the oligonucleotide primers used for PCR amplification, and their target sites within the *vlhA* gene. PCR was performed using Taq DNA polymerase (Promega) as described previously (Noormohammadi *et al.*, 1998).

Expression cloning of fragments spanning the MSPB gene.

For expression of polypeptides from regions 1–4 of the *vlhA* gene, the relevant PCR product was first purified using Bio-Spin 6 chromatography columns (Bio-Rad) and ligated to the plasmid vector PinPoint Xa1 T (Promega) as instructed by the manufacturer. Electroporation of *E. coli* JM109 cells with the ligation mixture was performed as described previously (Sambrook *et al.*, 1989), and the resultant colonies were inoculated into rich broth and screened for expression of novel polypeptides by SDS-PAGE of their whole-cell proteins followed by Coomassie blue staining, or by Western blotting and probing with antibodies specific for MSPB (see immunoblotting section). Attempted purification of the biotinylated fusion protein of region 4 was performed by affinity chromatography using streptavidin beads (Promega) as instructed by the manufacturer.

For expression of region 4 in the pMAL-p2 expression system (New England BioLabs), an 853 bp fragment of *vlhA* was obtained by digestion of a PinPoint Xa1 T plasmid containing the 5' end of the gene (Noormohammadi *et al.*, 1998) with two restriction endonuclease enzymes, *Bam*HI and *Hind*III. The resultant fragments were subjected to electrophoresis through a 0.8% agarose gel as described before (Sambrook *et al.*, 1989); the 853 bp fragment was excised from the gel, purified using the Wizard DNA clean-up system kit (Promega) and ligated to the compatibly digested plasmid vector pMAL-p2 as instructed by the manufacturer. The ligated plasmid was used to transform *E. coli* DH5 α cells and the resultant colonies were screened for expression of the polypeptide as described above. Using amylose resin beads (New England BioLabs) as instructed by the manufacturer, the resultant fusion protein was purified from cold-osmotic-shock fluids from a culture of cells containing the recombinant plasmid.

SDS-PAGE and immunoblotting. *E. coli* whole-cell proteins and purified MSPB fusion protein were analysed by SDS-PAGE or immunoblotting as described previously (Gurevich *et al.*, 1995). Mouse mAbs 97 and 334, rabbit anti-MSPB polyclonal serum and chicken anti-*M. gallisepticum*, anti-WVU-1853, anti-7NS and anti-BC-MS sera were used in immunostaining experiments. The origin of the mAbs 97 and 334 has been described previously (Gurevich *et al.*, 1995). Monospecific polyclonal antisera to MSPB were produced by immunizing rabbits with proteins excised from nitrocellulose membranes as described previously (Noormohammadi *et al.*, 1997). Anti-*M. gallisepticum* was a pool of sera from specific-pathogen-free (SPF) chickens that had been infected with *M. gallisepticum* S6. Anti-WVU, anti-7NS and anti-BC-MS were from SPF chickens infected with *M. synoviae* WVU-1853, 86079/7NS or BC-MS, respectively. Normal serum was from a single uninoculated SPF bird that had been found negative for *M. gallisepticum* and *M. synoviae* by RSA.

Test sera. Thirteen groups of chicken sera (named groups 1–13) were used in ELISA experiments, all of which were obtained from SPF Hybrid White Leghorn-type chickens (Commonwealth Scientific and Industrial Organization, Animal Health Division, Victoria, Australia). Sera against *M. synoviae* (groups 1–5 and 8–13) or *M. gallisepticum* (group 6) strains/isolates were raised by eye-drop inoculation with mycoplasma cultures.

Groups 1–5, each consisting of five to seven serum samples, were from chickens infected with *M. synoviae* BC-MS, 86079/7NS, MS-H, WVU-1853 or 69808, respectively (Table

Table 1. Sequence, target site and orientation of the PCR oligonucleotide primers

Name	Orientation*	Target site†	Sequence (5'–3')
F	Forward	78–103	TGGATCCCAAACCTCCAGCACCTGAAC
R1	Reverse	409–392	CGCTTAATGCTTTTACAG
R2	Reverse	529–511	CATCTTCTAGTAATGCTGT
R3	Reverse	636–618	TGGTTTAACTGCTGCAACT
R4	Reverse	1045–1026	GTTTGAATTCTGATTGTCT

* Forward and reverse refer to orientation of the coding and complementary strands, respectively.

† Positions of bases correspond to the nucleotide sequence of the *vlhA* gene and are relative to the ATG initiation codon.

Table 2. Statistical analysis of RU values of sera groups 1–7

Group	Inoculum*	n†	Mean	SE	Range
1	<i>M. synoviae</i> BC-MS	6	2013.00	640.00	289–3862
2	<i>M. synoviae</i> 86079/7NS	5	448.40	95.30	179–691
3	<i>M. synoviae</i> MS-H	7	273.60	77.20	112–691
4	<i>M. synoviae</i> WVU-1853	6	1316.00	763.00	229–5092
5	<i>M. synoviae</i> 69808	6	256.10	73.60	56–479
6	<i>M. gallisepticum</i> strains	10	27.60	7.39	3–77
7	—	10	8.10	1.66	2–18

* *Mycoplasma* strain/isolate used for raising antibodies in chickens of each group.

† Number of serum samples in each group.

2). Group 6 consisted of 10 serum samples from five pairs of chickens infected with *M. gallisepticum* NS-2, AP3AS, MGL, ts-11 or S6. Sera from groups 1–6 were all collected 4 weeks post-inoculation. Group 7 contained 10 serum samples from uninoculated chickens that were kept as uninfected controls. Groups 8–13 each consisted of nine or ten serum samples from chickens infected with *M. synoviae* 86079/7NS. Serum samples were collected immediately before inoculation (group 8) and at days 7 (group 9), 10 (group 10), 17 (group 11), 24 (group 12) and 28 (group 13) post-inoculation, respectively. All of the sera had been previously tested for *M. synoviae* and *M. gallisepticum* antibodies using the RSA assay as described previously (Whithear, 1993).

The *M. synoviae*-positive control serum was obtained from an SPF chicken inoculated with *M. synoviae* WVU-1853 as described above, and the *M. synoviae*-negative control serum was from an uninoculated chicken. These sera were examined for anti-*M. synoviae* antibodies by RSA, immunoblotting and ELISA assays.

Before use in ELISA, all serum samples were thawed, centrifuged at 16000 g for 30 min at 4 °C and diluted appropriately in ELISA buffer [0.1 M Tris, pH 7.4 (HCl); 0.5 M NaCl; 1 mM Na₂EDTA; 2% (w/v) BSA; 3% (v/v) Triton X-100; and 3% (v/v) Tween 20].

ELISA protocol. Wells of a 96-well flat-bottom plate (MaxiSorp; Nunc Inter Med) were coated with 100 µl antigen (purified MSPB fusion protein) diluted in carbonate buffer (0.032 M Na₂CO₃, 0.068 M NaHCO₃, pH 9.6) and the plate

was incubated overnight at 4 °C. The wells were washed three times with 200 µl 0.05% Tween 20 in PBS and incubated for 1 h at room temperature with 200 µl per well of 1% (w/v) BSA in PBS. The wells were washed as before and incubated for 3 h at room temperature with 100 µl per well of each dilution of test serum. After washing (as before) 100 µl horseradish-peroxidase (HRP)-conjugated rabbit anti-chicken IgG (Chemicon) diluted in ELISA buffer was added to each well and the plate was incubated for 1 h at room temperature. The wells were washed as before, 100 µl substrate [1 mg 3,3',5,5'-tetramethylbenzidine dihydrochloride (Sigma) in 10 ml of the buffer recommended by the manufacturer with 0.006% (v/v) H₂O₂] was added to each well and the plate was incubated for 5 min at room temperature. The enzymic reaction was stopped after 5 min with the addition of 25 µl 2 M H₂SO₄ to each well and the A₄₅₀ of the contents of each well was measured. The *M. synoviae*-positive serum, the *M. synoviae*-negative serum and a conjugate control (containing antigen and conjugated antibody only) were included in each plate.

Determination of test sera reactivity. Analysis of ELISA results was performed using the program Delta Soft 3 (Dr E. Bechtold and BioMetallics). Each serum was tested at a 1/800 dilution. If the difference in absorbance of duplicate wells was greater than 10% of their respective mean value, the serum was assayed again. The mean absorbance of the duplicate conjugate control was subtracted from the absorbances of the assayed sera. The *M. synoviae*-positive serum was assayed in duplicate at 1/800 and serial twofold dilutions to 1/204800, to provide a series of standards on each plate. The four-

parameter curve fit method was applied to the series of standards to obtain a standard curve, and means of the absorbances of duplicate serum dilutions were interpolated on the standard curve to express the reactivity of the test sera in units relative to the *M. synoviae*-positive serum. These calculations were performed to eliminate minor variations in conditions of the ELISA procedure between different plates. The reactivity of the *M. synoviae*-positive serum was defined as 1600 relative units (RU) of IgG μl^{-1} . Sera with out-of-range absorbance values (values within $\pm 1\%$ of the maximum absorbance value for the *M. synoviae*-positive serum or more, and values within $\pm 1\%$ of the minimum absorbance value for the *M. synoviae*-positive serum or less) were re-tested at three lower or higher twofold serial dilutions.

RSA test. The RSA tests for antibodies to *M. gallisepticum* and *M. synoviae* were performed using commercial reagents (Intervet International) as described previously (Whithear, 1993).

RESULTS

Determination of the most antigenic region of MSPB

To assess the antigenicity of different regions of the MSPB, and their reaction with *M. gallisepticum*-infected chicken sera, MSPB was expressed as a panel of smaller fusion proteins. Four pairs of oligonucleotide primers were used to amplify four overlapping regions of the *vlbA* gene which did not contain TGA codons at their 5' end (TGA encodes tryptophan in mycoplasmas). The resultant PCR products were ligated to the expression vector PinPoint Xa1 T, and this was used to transform *E. coli* JM109 cells. Regions 1–4 (Fig. 1a) were predicted to encode polypeptides of 110 (amino acids 27–137), 150 (amino acids 27–177), 185 (amino acids 27–212) and 290 (amino acids 27–317) amino acids, respectively, in *E. coli* cells. SDS-PAGE of the whole-cell proteins of *E. coli* cells containing the recombinant plasmids demonstrated the expression of fusion proteins of approximately 32, 35, 38 and 50 kDa from the plasmids containing regions 1–4, respectively. Immunostaining of Western blots of the whole-cell proteins of the clones expressing polypeptides from regions 1–4 (Fig. 1b) showed that anti-*M. gallisepticum* or normal chicken sera detected a number of *E. coli* proteins, but not any of the fusion proteins. Anti-7NS and anti-BC-MS chicken sera similarly bound a number of *E. coli* proteins, but also the 50 kDa fusion protein, and weakly bound the 38 kDa fusion protein. No reaction could be detected between these sera and the 32 and 35 kDa fusion proteins. In contrast, sera from chickens infected with *M. synoviae* WVU-1853 bound intensely to all four fusion proteins. Similar experiments using MSPB-specific mAbs 97 and 334 revealed that they bound only the 50 kDa fusion protein (results not shown).

Purification of the highly antigenic region of MSPB

An attempt was made to purify the fusion protein of region 4 using the PinPoint Xa protein purification system; however, due to the low yield of the purified fusion protein and the presence of persistent con-

taminants, an alternative expression and purification system, pMAL, was used to obtain a purified fusion protein (containing the polypeptide expressed from region 4) in sufficient quantity for use in an ELISA. An 853 bp fragment of the *vlbA* gene was obtained as described in Methods and polypeptide was expressed from it in *E. coli* cells using the pMAL expression vector (Fig. 2, lane 2). The resultant fusion protein was partially purified by cold-osmotic-shock lysis of *E. coli* cells (lane 3) and further purified by affinity chromatography using amylose resin beads (lane 4). Approximately 25 μg pure fusion protein was obtained per ml of culture used. Immunostaining of Western blots of the affinity-purified fusion protein did not reveal any reactivity with anti-*M. gallisepticum* or preinoculation chicken sera, but reacted with anti-WVU-1853, anti-7NS and anti-BC-MS chicken sera (results not shown).

Optimization of antigen concentration, test serum and anti-chicken conjugate antibody dilutions for an indirect ELISA (MSPB-ELISA)

The recombinant fusion protein was used at a concentration of 16 $\mu\text{g ml}^{-1}$, and at 10 twofold dilutions to 0.03 $\mu\text{g ml}^{-1}$, to coat duplicate wells of a microtitre ELISA plate. The bound antigen was tested with the *M. synoviae*-positive and -negative sera diluted at 1/1000. Chicken IgG bound to the antigen was detected with HRP-conjugated rabbit anti-chicken IgG diluted 1/500. The antigen concentration that provided the lowest non-specific binding of the *M. synoviae*-negative sera and maximum absorbance with the *M. synoviae*-positive serum was determined to be approximately 4 $\mu\text{g ml}^{-1}$ (Fig. 3a).

The *M. synoviae*-positive and -negative sera were assayed at 1/50 and at serial twofold dilutions to 1/819200 and 1/12800, respectively, using the antigen at optimal concentration and conjugate at 1/500 dilution. The maximum absorbance of the *M. synoviae*-positive serum and the minimum non-specific reaction of the *M. synoviae*-negative serum were obtained at 1/800 and 1/1600 serum dilutions (Fig. 3b).

To determine the optimal dilution of the HRP-conjugated rabbit anti-chicken IgG, the assay was performed with antigen at a concentration of 4 $\mu\text{g ml}^{-1}$, the *M. synoviae*-positive and -negative sera at a dilution of 1/800, and the conjugate at five serial twofold dilutions, from 1/200 to 1/3200 (Fig. 3c). The optimal dilution of the conjugate was determined to be 1/800.

Differentiation of sera from *M. synoviae*-infected, *M. gallisepticum*-infected and uninoculated chickens by MSPB-ELISA

Using the optimal conditions for antigen and conjugate described above, sera from groups 1–7 were examined at three dilutions, 1/800, 1/1600 and 1/3200, and the amount of MSPB antibodies present in these sera was interpolated from absorbance values using the standard

Fig. 1. Expression and detection of the four overlapping regions of the *v/hA* gene. (a) The *v/hA* gene product (large arrow) and regions 1–4 (small arrows below) expressed in *E. coli* cells using the plasmid vector PinPoint Xa1 T. The scale on the top indicates the number of amino acid residues. (b) Immunoblots of whole-cell proteins of the *E. coli* cells expressing fusion proteins of regions 1–4 (lanes 1–4 in each panel, respectively) from recombinant plasmid PinPoint Xa1 T. Each panel was probed with serum from uninoculated chickens or with sera from chickens inoculated with *M. gallisepticum* S6 (MG), *M. synoviae* WVU-1853, 86079/7NS or BC-MS. The lane marked M shows the prestained broad range protein molecular mass markers (New England BioLabs).

Fig. 2. SDS-PAGE followed by Coomassie brilliant blue staining of the purification steps of the MSPB fusion protein. *E. coli* cells containing the recombinant plasmid pMAL (lane 1) were induced to express the MSPB fusion protein (lane 2) and then subjected to cold-osmotic-shock lysis. The fluid resulting from cold-osmotic-shock lysis was extracted (lane 3) and the fusion protein was affinity-purified (lane 4) using amylose resin beads. The lane marked M shows the prestained broad range protein molecular mass markers (New England BioLabs).

curve. A typical standard curve derived from the *M. synoviae*-positive serum is shown in Fig. 3(d). The mean, standard error and range of RU for each group

are shown in Table 2. The mean RU of groups 1–5 (obtained from *M. synoviae*-infected chickens) was 710. Groups 1 and 4 had the highest RU while groups 3 and 5 had the lowest RU. The mean RU of each of groups 1–5 was higher than that of group 6 (obtained from *M. gallisepticum*-infected chickens) or 7 (obtained from uninoculated chickens). All 30 individual sera from groups 1–5, except for one sample from group 5 with 56 RU, contained 95 RU or more. In contrast, all 10 individual sera from group 7 contained 18 RU or less. Sera from group 6 had a mean of 28 RU and tended to give higher RU values than those from uninoculated chickens. Nine out of 10 serum samples tested from group 6 had 52 RU or less with one serum sample containing 77 RU. Fig. 4 compares the upper and lower limits of interquartile ranges and confidence intervals for sera groups 1–7.

The Spearman's rank coefficient of correlation between the RU values and RSA scores of the individual sera from groups 1–7 was 0.803, indicating a strong correlation between the two tests for these groups.

Detection of seroconversion in *M. synoviae*-infected birds by MSPB-ELISA

To examine the kinetics of development of antibodies against MSPB after infection with *M. synoviae*, sera from groups 8–13 were tested in the MSPB-ELISA as described above. The mean, standard error and range of RU for each group are shown in Table 3. Comparison of the RU values of the sera from these groups revealed an increase in MSPB antibodies at day 7 post-inoculation

Fig. 3. Optimization of MSPB-ELISA parameters. (a) Optimization of antigen concentration. The *M. synoviae*-positive and -negative sera were diluted to 1/1000 and incubated with the titrated MSPB fusion protein. Reactivity of serum IgG was detected by indirect ELISA using HRP-conjugated rabbit anti-chicken IgG diluted 1/500. (b) Optimization of test serum dilution. The *M. synoviae*-positive and -negative sera were assayed in serial twofold dilutions with the antigen at a concentration of $4 \mu\text{g ml}^{-1}$, and conjugate at a dilution of 1/500. (c) Optimization of the conjugate. Antigen (at a concentration of $4 \mu\text{g ml}^{-1}$) and the *M. synoviae*-positive and -negative sera (diluted 1/800) were titrated against five serial twofold dilutions of the conjugate. (d) An example of the standard curve obtained for each microtitre plate from serial twofold dilutions of the *M. synoviae*-positive serum. ■, Positive serum; ▲, negative serum.

Table 3. Statistical analysis of RU values of sera groups 8–13

Group	PI (d)*	n†	Mean	SE	Range
8	0	8	6.25	1.28	3–14
9	7	8	63.60	11.40	27–107
10	10	9	487.60	43.20	275–675
11	17	9	1076.00	284.00	200–3009
12	24	9	1113.00	397.00	127–3927
13	28	9	1403.00	404.00	151–3190

* Post-inoculation (d).

† Number of serum samples in each group.

and a sharp rise at days 10 and 17 post-inoculation. Fig. 5 compares the upper and lower limits of interquartile ranges and confidence intervals for sera groups 8–13.

The Spearman's rank coefficient of correlation was 0.797, indicating a strong correlation between the two tests for these sera groups.

DISCUSSION

Several studies have reported increased sensitivity of *M. synoviae* ELISAs using different antigen preparations (Opitz *et al.*, 1983; Patten *et al.*, 1984; Higgins & Whithear, 1986; Opitz & Cyr, 1986; Avakian & Kleven, 1990; Gurevich *et al.*, 1995); however, specificity continues to be a problem associated with *M. synoviae* ELISAs.

Our previous study (Noormohammadi *et al.*, 1997) suggested the potential of MSPB as a diagnostic reagent to detect infection caused by different *M. synoviae* strains. In this study, immunostaining of four different regions of MSPB revealed that only the polypeptide expressed from region 4 was detected by all sera from

Fig. 4. Boxplots of the RU values of sera from chickens inoculated with *M. synoviae* BC-MS (group 1), 86079/7NS (group 2), MS-H (group 3), WVU-1853 (group 4) or 69808 (group 5), or with *M. gallisepticum* (group 6), or from uninoculated chickens (group 7) measured in the MSPB-ELISA. Rectangles represent the interquartile range (the range of the middle 50% of the data) of each group. The 95% confidence interval of each group is shown by lines extended from the sides of the rectangles.

Fig. 5. Boxplots of the RU values of sera from chickens inoculated with *M. synoviae* 86079/7NS and collected at days 0, 7, 10, 17, 24 or 28 post-inoculation. Rectangles represent the interquartile range of each group. The 95% confidence interval of each group is shown by lines extended from the sides of the rectangles.

chickens infected with the *M. synoviae* strains used, while the polypeptides from regions 1, 2 and 3 were only detected by serum raised against the homologous strain used for DNA cloning (WVU-1853). The difference observed in reactivity of MSPB from *M. synoviae* WVU-1853 with sera from birds infected with different *M. synoviae* strains could be related to the phenomenon of antigenic variation described previously (Noormohammadi *et al.*, 1997). Also it was shown that region 4, but not regions 1, 2 or 3, contained epitopes for the MSPB-specific mAbs 97 and 334. These observations suggest that the region between amino acids 212 and 317

is highly antigenic and contains the epitopes which are most cross-reactive between different *M. synoviae* strains and best recognized by chickens during the course of infection. Whilst the fusion protein of region 4 was found to be highly antigenic, it did not bind anti-*M. gallisepticum* or normal chicken sera, suggesting that a polypeptide expressed from region 4 can serve as a species-specific antigen.

The potential of the MSPB fusion protein, expressed from the *vlhA* gene in *E. coli* cells, had to be further assessed in an ELISA using a panel of sera from chickens infected with different strains of *M. gallisepticum* and *M. synoviae*. Hence, an indirect ELISA (MSPB-ELISA) was devised and optimized in terms of antigen, conjugate and test serum concentrations. A battery of sera from chickens experimentally infected with *M. synoviae* or *M. gallisepticum*, and from uninoculated chickens, were examined in the MSPB-ELISA. The results were encouraging from two points of view. Firstly, with a margin of 40 RU, all but one of the *M. synoviae*-inoculated chicken sera had higher RU than sera from uninoculated and *M. gallisepticum*-infected chickens. The only exception was a single serum from a chicken infected with *M. synoviae* 69808 which had 56 RU. As this serum had an RSA score of one, the low RU value seen in the MSPB-ELISA could well be due to an exceptionally low *M. synoviae* antibody titre in this particular serum. Secondly, unlike some other prokaryotic purification tags, such as the glutathione S-transferase protein used in the pGEX expression and purification system (Pharmacia Biotech) (Browning, 1994), the maltose-binding protein (expressed with the MSPB fusion protein as a purification tag) did not bind non-specifically to antibodies from chicken sera.

Lower levels of MSPB antibodies were seen in chickens inoculated with *M. synoviae* 69808 and MS-H. Given that these groups also had lower RSA values, this observation may reflect the limited ability of *M. synoviae* 69808 and MS-H to elicit antibodies, at least in the serum of the experimentally infected chickens.

In order to study the kinetics of seroconversion of chickens infected with *M. synoviae*, sera from chickens were collected at different time points after inoculation with *M. synoviae* 86079/7NS, and tested in the MSPB-ELISA. Comparison of the RU values of these sera showed that MSPB antibodies could be detected by day 7 post-inoculation, with the highest level in this experiment observed around day 28 post-inoculation. There was a relatively strong correlation between the RSA scores and RU values of the individual sera from these sera. These results revealed that the MSPB-ELISA could be as sensitive as the RSA in its ability to detect *M. synoviae* antibodies in chicken sera early in infection.

The results from the study presented here suggest that the MSPB-ELISA could be a reliable substitute for antigens used in current *M. synoviae* ELISAs. The MSPB fusion protein has several advantages over previously described *M. synoviae* -ELISA antigens, including reproducibility (as it overcomes the problem of phase

variation which affects the yield and/or immunoreactivity of the *M. synoviae* native antigen), cost and ease of preparation. Additionally, it provides specific and relatively sensitive detection of *M. synoviae* antibodies.

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Received 2 February 1999; revised 16 April 1999; accepted 20 April 1999.