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Differentiation between serological responses to *Brucella suis* and *Yersinia enterocolitica* serotype O:9 after natural or experimental infection in pigs

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SUMMARY

False-positive serological reactions (FPSR) due to infections with *Yersinia enterocolitica* serotype O:9 (*Ye*O:9) are a problem in tests for brucellosis. In the present study, FPSR in classical and novel tests for brucellosis following experimental infections of pigs with *Ye*O:9 were compared with responses of *B. suis* biovar 2-inoculated pigs. FPSR were limited to 2–9 weeks post-*Ye*O:9 inoculation, while *B. suis*-infected pigs were test-positive throughout the 21-week period of investigation. Although *Ye*O:9-inoculated pigs exhibited FPSR in *Brucella* tests for a limited period of time, the serological responses in a *Ye*O:9-purified O-antigen indirect ELISA did not decrease accordingly. Analysis of available cross-sectional serum samples from pig herds naturally infected with *Ye*O:9 or *B. suis* biovar 2 confirmed that the observed difference in the duration of the serological responses between the two infections could be used to discriminate between herds infected with *B. suis* biovar 2 and *Ye*O:9.

INTRODUCTION

Occurrence of false-positive serological reactions (FPSR) in surveillance and diagnostic testing for animal brucellosis is an important problem in many countries. Most commonly FPSR are caused by infections with *Yersinia enterocolitica* serotype O:9 (*Ye*O:9), as this bacterium possesses an almost identical O-antigen lipopolysaccharide (LPS) chain to that of *Brucella abortus* [1]. *Brucella* LPS contain *Brucella* spp. specific M- and C-epitopes, as well as common A- or C/Y-epitopes that are shared with *Ye*O:9, and a number monoclonal antibodies (mAbs) specifically recognizing these epitopes have been produced [2, 3]. However, although the exact structures of the various epitopes remain unclear, it appears that LPS epitopes span 2–5 overlapping polysaccharide units and that antibodies generated against the common A- or C/Y-epitopes are able to block the specific binding to adjacent M- and C-epitopes in competitive ELISAs [3, 4]. This means that antibodies generated by an infection with *Ye*O:9 will also inhibit binding of *Brucella*-specific mAbs in diagnostic competitive ELISAs. Attempts to overcome the LPS cross-reactions with the use of *Brucella* protein antigens is hampered by the strong humoral immunodominance of smooth LPS in *Brucella* infections [5, 6]. This is an area of continued research and new candidates for antigen preparations continue to be evaluated [7]. Attempts to produce phage-displayed peptide mimotopes of the *Brucella*-specific LPS epitopes have not been successful when used in vaccines or indirect ELISAs [8]. Diagnostic use of cellular-mediated immune responses to discriminate these

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two infections is a potential alternative to serological assays. This approach has been successful in cattle [9, 10] and its application in pigs should be investigated.

In Northern Europe, *B. suis* biovar 2 is the predominant type of *Brucella* infection among pigs. This biovar is endemic in wild boars in Europe and also occurs, albeit more sporadically, in the European hare population [11]. Denmark is officially free of brucellosis. However, in certain regions wild hares have been found to be infected with *B. suis* biovar 2, and in one of these regions, infections with *B. suis* biovar 2 in free-ranging pigs have twice been recorded, in 1994 and 1999 respectively.

The general experience in *Brucella* surveillance laboratories is, that FPSR due to *Ye*O:9 infections are limited in time and that most of these reactions will disappear within weeks, whereas *Brucella* infections result in sustained serological reactions. The aim of the present study was to investigate serological reactions in pigs experimentally infected with *Ye*O:9 or *B. suis* biovar 2 in more detail. Furthermore, based on the results of these experimental studies, cross-sectional samples of pigs in different age groups made it possible to discriminate between herds infected with *B. suis* biovar 2 and *Ye*O:9 by use of an ELISA measuring antibodies against *Ye*O:9 LPS compared with reactions in classical tests for *Brucella*.

METHODS

Bacteriological techniques

Brucella was cultivated on blood agar plates containing 5% cattle blood and on Danish Institute of Food and Veterinary Research (DFVF) *Brucella* agar plates (900 ml Columbia agar, 100 ml horse serum, 10 ml modified *Brucella* selective supplement containing: 5000 IU polymyxin B, 25 000 IU bacitracin, 50 mg natamycin, 5 mg nalidixic acid, 100 000 IU nystatin, 20 mg vancomycin). The plates were incubated in 10% CO_2 at 37 °C for at least 5 days. The isolates were verified as *Brucella* by a genus-specific PCR using primers B4 and B5 as described by Bailey et al. [12].

Examination for *Yersinia* in faeces was performed according to a DFVF standard operating procedure. Briefly, samples of faeces (20 g) were mixed with 180 ml PSB solution (2% sorbitol, 0·15% bile salts and 0·85% sodium chloride) and incubated at 4 °C for 3–5 days. After this incubation, 2 ml of the culture solution was added to 100 ml ITC bouillon (irgasan–ticarcillin–potassium chlorate) and incubated at room temperature for 2–3 days. Then 10 μl of the ITC culture was grown on CIN agar plates (cefsulodin–irgasan–novobiocin) at 28–30 °C overnight and suspicious red colonies were cultured overnight on calf-blood agar plates at room temperature. Isolates were serotyped by monovalent polyclonal *Ye*O:3 and *Ye*O:9 rabbit antisera (Sifin, Berlin, Germany). The presence of the 70-kb virulence plasmid of *Yersinia* (pYV) was confirmed by plasmid profiling.

Inoculation material

B. suis biovar 2 inocula were prepared from a 1994 isolate (DFVF 9405304) from porcine foetal liver isolated from an outbreak of swine brucellosis in a Danish free-ranging pig herd and stored at −80 °C. The isolate was biotyped at the OIE Reference Laboratory at the Veterinary Laboratories Agency (VLA), Weybridge, UK. Inoculation material was prepared by suspending colonies from agar plates in sterile PBS after which the bacterial concentration was adjusted to 3·25 McFarland on a colorimetric scale, corresponding to $\sim 10^9$ c.f.u./ml. Before inoculation this suspension was further diluted 1:10 in sterile PBS.

*Ye*O:9 was prepared from DFVF strain 9913937-1 isolated from the first verified case of *Ye*O:9 in pigs in Denmark in 1999. Cultures were grown overnight at 37 °C in brain heart infusion broth (Difco, Detroit, MI, USA) with 10% sucrose and adjusted to the inoculation dose indicated below immediately prior to inoculation.

Animals

Danish Landrace and Yorkshire crossbred pigs were obtained from two herds that by serological screening were *Yersinia* negative for *Ye*O:3 and *Ye*O:9. Prior to inoculation all pigs included in this study were negative in serological tests for *Brucella*, *Ye*O:3 and *Ye*O:9, and none of them were excreting *Yersinia* by cultivation of faecal samples. The pigs were fed standard pelleted food with *ad libitum* access to water and were housed in groups of 4–5 pigs on straw-bedded concrete floor in appropriate infectious disease containment facilities. All experiments conformed to national guidelines on the use of experimental animals.

Experimental inoculations with *B. suis*

Two groups of five pigs, one consisting of animals aged 8 weeks (3 females and 2 castrated males) and the other aged 16 weeks (1 female and 4 castrated males), were inoculated with 2×10^7 c.f.u. *B. suis* biovar 2 per pig by installing 0·1 ml of a *B. suis* suspension (prepared as described above) into the lower conjunctival sac of the left eye. The inoculum titre was confirmed by colony counts on agar plates. One pig was euthanized due to arthritis (see the Results section) while the remaining nine pigs were killed 21 weeks post-inoculation (p.i.) and samples from tonsils, spleen, liver, kidney and a pool of the retropharyngeal and submandibular lymph nodes were collected for cultivation. From 1 week prior to inoculation and until termination of the experiment at 21 weeks p.i. blood samples were drawn once a week with additional samples at 1½, 2½, and 3½ weeks p.i. using plain venoject tubes (Terumo, Leuven, Belgium), after which serum was produced (with sodium azide added to 0·1% as a safety precaution) and frozen at −20 °C until analysis.

Experimental inoculations with *Ye*O:9

Three *Ye*O:9 inoculation experiments were performed. In all cases the inoculation dose was suspended in 10 ml PBS and either mixed in wet feed on a plate that was subsequently individually fed to the pigs (experiment 1), or slowly administered by a 20-ml syringe fitted with a 15-cm soft plastic tube through a mouth gag to the base of the tongue of the pig. The pigs were fed immediately after inoculation. The bacterial concentrations (c.f.u./ml) in the inoculum were estimated by colony counting on agar plates after serial dilutions.

Experiment 1

Five pigs, ~8 weeks old, were orally inoculated with 10^8 c.f.u. of *Ye*O:9 and five separately housed pigs were kept as non-inoculated controls. All pigs were killed at 11 weeks p.i.

Experiment 2

Five pigs, ~8 weeks old, were inoculated orally five times with increasing inocula sizes from 10^8 to 10^{10} c.f.u. *Ye*O:9 separated by intervals of 1–2 weeks. All pigs were killed 7 weeks after the first inoculation (2 weeks after last inoculation).

Experiment 3

Thirteen pigs, aged 9–13 weeks, were orally inoculated with ~10^{10} c.f.u. *Ye*O:9. Five of these pigs had received an inoculation with pYV-deficient *Ye*O:3 at 1 or 4 weeks prior to the *Ye*O:9 inoculation, and another four pigs were later challenged with pYV containing *Ye*O:3. The *Ye*O:3 inoculations did, however, not result in any differences in the immune responses to the *Ye*O:9 inoculation between pigs in the different groups (data not shown). This is easily explained as the pYV-deficient *Ye*O:3 primary inoculation was non-virulent, and the pYV-containing *Ye*O:3 challenge inoculation was not able to colonize the *Ye*O:9-infected pigs due to cross-protection between serotypes. For the present *Brucella* and *Ye*O:9 analyses these pigs were thus considered as one group. Five pigs remained non-inoculated with *Yersinia* as control pigs. The pigs were killed 5–12 weeks post-*Ye*O:9 inoculation.

In these three experiments faecal samples were taken weekly and cultured for *Yersinia* with subsequent serotyping of isolates. Blood samples were taken once or twice a week for the first 3 weeks p.i and thereafter at weekly or bi-weekly intervals.

Natural infections with *B. suis* biovar 2

In the last two decades there have been two pig herds with confirmed brucellosis in Denmark. In both cases isolates have been determined to be *B. suis* biovar 2 and both herds practised outdoor farming and were located in north-eastern Jutland, on the Danish mainland, where *B. suis* biovar 2 infection occurs in the wild hare population. In the present study, serum samples collected from these outbreaks before depopulation and stored at −20 °C have been analysed to represent samples from verified natural infections with *B. suis* biovar 2. From the first herd (diagnosed in November 1994) 77 serum samples from mature pigs (gilts, sows and boars) were available. From the second herd (diagnosed in October 1999) 44 samples from young pigs and 21 samples from mature pigs were available. In both herds, unresolved reproductive problems had been ongoing for several months before swine brucellosis was diagnosed.

Natural infections with *Ye*O:9

Since *Ye*O:9 was first identified in the Danish pig population in 1999, three indoor breeding herds in the Danish specific pathogen-free (SPF) pig production

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system have been infected. All herds were without any signs of reproductive disorders and were identified by occurrence of false-positive *Brucella* reactions in the serological surveillance prior to animal exports or in quarantine facilities prior to enrolment at AI breeding stations. In the first herd, identified in 1999, the infection was only localized to the sections with young pigs, and this herd was depopulated to prevent *Ye*O:9 from spreading to other Danish pig herds. In the other two herds (both identified in late 2001) no attempts to eradicate the infections have been pursued, but once brucellosis was ruled out and *Ye*O:9 was positively identified by culture, the pattern of serological reactions in the herds has been mapped every 3 months. In total, results of tests for brucellosis and *Ye*O:9 LPS antibodies from 174 and 139 serum samples from young pigs and 168 and 240 serum samples from mature pigs in the two herds respectively, were available.

Serological analyses

Complement fixation test (*CFT*) (performed in 96-well microtitre plates using cold fixation overnight) and *Rose Bengal plate agglutination test* (*RBT*). These tests were performed by standard procedures according to the OIE Manual of Standards [13]. All antigens, complement and haemolysin were obtained from commercial suppliers. Samples with reactions above 20 IU/ml in the CFT were considered positive.

Serum agglutination test (*SAT*) (using 10 mM EDTA in antigen suspension) was performed with commercial concentrated antigen (VLA, Weybridge, UK) according to standard methodology [14]. Samples with reactions above 30 IU/ml were considered positive. (The DFVF is accredited under Danish Accreditation according to DS/EN ISO/IEC 17025 to perform serological analyses by the CFT, SAT and RBT.)

Competitive enzyme-linked immunosorbent assay (*cELISA*). The COMPELISA 400 (VLA) is performed with *B. melitensis* LPS-coated microtitre plates and the *Brucella*-specific monoclonal antibody BM40 as detector antibody [15, 16]. Analyses were performed according to the manufacturer's instructions either at DFVF or VLA. Samples with reactions below 60 OD% of negative control were considered positive.

Fluorescence polarization assay (*FPA*). Analyses were performed at the VLA as previously described [16]. Sample with reactions minus control values >20 millipolarization units (mP)/ml were considered positive.

Yersinia enterocolitica serotype O:9 LPS iELISA. Given the homology between *Brucella* and *Ye*O:9 LPS an indirect ELISA (iELISA) incorporating *Ye*O:9 LPS rather than *Brucella* LPS was used to measure antibody responses to both infections. A liquid culture of the DFVF *Ye*O:9 strain 9913937-1 was grown overnight at 20 °C with slow agitation and LPS was extracted from the phenol phase following hot phenol/water extraction [17] as modified by Hassan et al. [18]. Long O-antigen side-chains were further purified by gel-filtration (HiPrep Sephacryl S100; Amersham Biosciences, Freiburg, Germany) and LPS-containing fractions were identified by silver staining of SDS–PAGE gels. The selected fractions were pooled, dialysed in PBS and stored at 4 °C for a few weeks or in aliquots at −20 °C for longer periods. PolySorp™ microtitre plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with LPS at optimal dilution in 0·5 M HCO_3 buffer (pH 9·6) and subsequently blocked with PBS + 0·05% Tween-20 (PBS-T) + 1% BSA. The plates were washed in PBS-T and serum samples diluted 1:100 in blocking buffer were incubated in the plate in duplicate for 1 h at room temperature. Following the incubation, plates were washed in PBS-T and reacted 1 h with HRP-conjugated rabbit anti-swine Ig (Dako, Glostrup, Denmark), followed by additional washing and OPD substrate reaction. Results were expressed as OD% (of OD = 2·0) following linear regression on a panel of standard sera to eliminate plate-to-plate variation. Sera with values >20 OD% were considered positive.

Brucella LPS iELISA. To investigate possible differences in antigenicity between *Ye*O:9 and *Brucella* LPS in iELISA the reactivity of selected serum samples from pigs infected with *B. suis* or *Ye*O:9 was also analysed in a bovine *B. abortus* LPS ELISA modified to analyse porcine samples. A bovine brucellosis serum ELISA (Institut Pourquier, Montpellier, France) was used according to the manufacturer's instructions, but with the conjugated secondary antibody substituted with HRP-conjugated rabbit anti-swine Ig (Dako).

Unfortunately, there is no common international porcine *Brucella* serum standard available to calibrate species-specific tests. Serial dilutions (in the

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appropriate diluting buffer for each ELISA) of positive sera from both *Brucella*- and *Yersinia*-infected pigs were analysed in parallel with *Brucella* or *Ye*O:9 LPS-coated plates. Results are expressed as the highest pre-dilution of the sample that produced a calibrated OD value $>0{\cdot}4$ when further diluted as prescribed in the tests (1:20 and 1:100 for *Brucella* and *Ye*O:9 iELISA respectively).

RESULTS

Experimental inoculation with *B. suis* biovar 2

Several of the inoculated pigs presented transient vomiting, without accompanying loss of appetite, 1–2 weeks after inoculation. Two pigs in the older age group presented arthritis 4–5 weeks p.i. and were subsequently treated daily by i.m. injection of tetracycline (10 mg/kg). While one pig recovered, the other pig was euthanized and a pure culture of *Acarnobacterium pyogenes* was cultivated from the affected joint and bones. *Brucella* was not isolated from the area or from draining or retropharyngeal lymph nodes or spleen. The euthanized pig was excluded from all analyses. No other clinical signs of disease were observed in any of the pigs inoculated with *B. suis* and all pigs appeared healthy with normal hair coat and weight gain.

The *Ye*O:9 LPS iELISA was effective in detecting antibodies in both the *Ye*O:9 and *B. suis* biovar 2-infected pigs. All iELISA results are from this ELISA except when ELISA titres between the two infections were compared. The first serological evidence of infection appeared 17–21 days p.i and antibody responses remained well above the cut-points for most tests throughout the 21-week experimental period (Figs 1 and 2) in six of the nine inoculated pigs. One pig was *Ye*O:9 iELISA positive, but presented a weaker serological response from day 28 and onwards in classical tests for *Brucella*, while two pigs (one in the 8-week age group and the pig with treatable arthritis from the 16-week age group) never produced responses above the cut-point in any of the serological tests. At autopsy 21 weeks p.i., *B. suis* was isolated from five of the six strongly seropositive pigs and from the weakly seropositive pig while no recovery of *B. suis* was made in the two seronegative pigs. It was concluded that the *B. suis* biovar 2 inoculation resulted in subclinical infections in seven pigs, while the two remaining pigs could not be regarded as *B. suis* infected. Only the *Ye*O:9 iELISA and the FPA were able to correctly identify all the seven *Brucella*-infected pigs in all samples after seroconversion (Fig. 2). The cELISA detected the lowest number of seropositive samples.

Experimental inoculations with *Ye*O:9

The pigs inoculated with *Ye*O:9 excreted the bacteria in their faeces 1–6 weeks p.i. (Fig. 3) and seroconverted in the *Ye*O:9 iELISA (Fig. 2). In addition, between 20% and 80% of the pigs gave positive serological responses in the various tests for brucellosis, depending on the time after inoculation (Fig. 2). However, while anti-*Ye*O:9 LPS antibodies measured in the *Ye*O:9 iELISA remained high throughout the 11-week study period, the false-positive reactions in tests for brucellosis were limited to a maximum of 8–10 weeks p.i. and the levels of responses in *Brucella* tests were of a lower titre compared with the *B. suis*-infected pigs (Fig. 4).

Comparison of reactivity with *B. abortus* and *Ye*O:9 LPS

Serum iELISA titres in microtitre plates coated with LPS from either *B. abortus* or *Ye*O:9 were comparable irrespective of whether the serum originated from a pig infected with *Brucella* or *Yersinia* (Fig. 5). Furthermore, the decrease in time of serological reactions from *Ye*O:9-infected pigs analysed in classical tests for *Brucella* was not correspondingly reflected in decreasing titres in either the *Brucella* or the *Ye*O:9 iELISA.

Field evaluation of serum reactivity patterns in herds with natural infections with *B. suis* biovar 2 or *Ye*O:9

Following the observations of the limited period of *Ye*O:9 false-positive cross-reactivity in tests for *Brucella*, accompanied by a continuous reactivity in *Ye*O:9 iELISA, it was investigated if this was reflected in the pattern of reactions among different age groups in herds infected with *B. suis* biovar 2 or *Ye*O:9 respectively. In herds with verified brucellosis 53–70% of iELISA-positive serum samples were also positive in classical tests for brucellosis irrespective of the age of the animals. In contrast, although equivalent numbers of animals from herds with *Ye*O:9 were positive in the *Ye*O:9 iELISA, the fractions of these that were also *Brucella* RBT- or SAT-positive was restricted to only 7–27% and 1–2%

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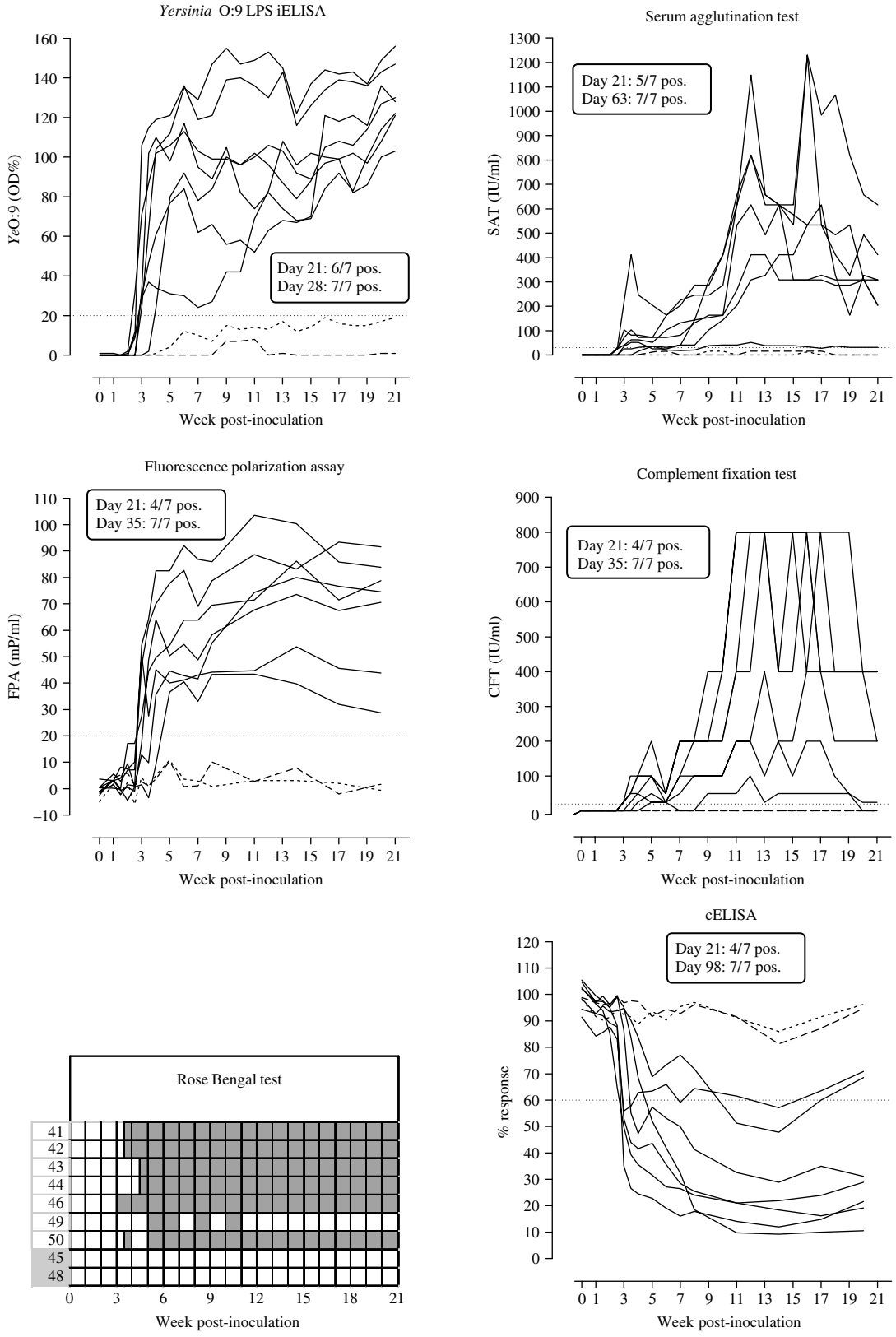

Fig. 1. Individual pig serum antibody responses in different tests for *Brucella* and the *Y. enterocolitica* serotype O:9 iELISA following experimental inoculation with 2×10^7 c.f.u. *B. suis* biovar 2. Serum responses in two pigs that were inoculated, but without evidence of infection are indicated by dotted lines. Horizontal dotted lines indicate cut-points of each test. For the Rose Bengal agglutination test grey shading indicates test-positive results.

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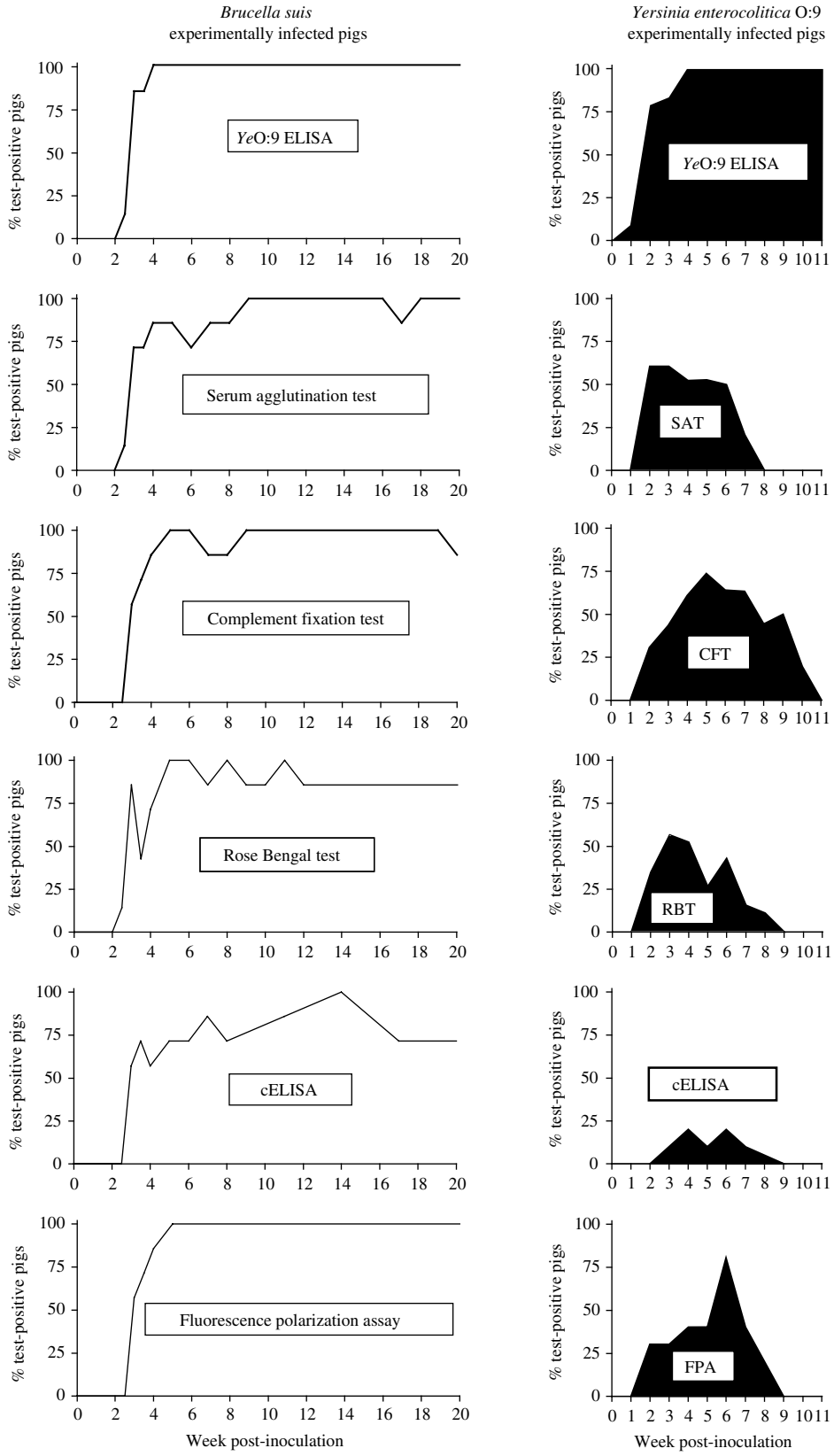

Fig. 2. Relative numbers of test-positive pigs in six different serological tests for *Brucella* and *Yersinia enterocolitica* O:9 iELISA following a subclinical experimental *B. suis* biovar 2 infection (left panel, $n = 7$), or oral inoculation with *Ye*O:9 (right panel; $n =$ varying from 5–23 at different time points). See text for cut-points with each test.

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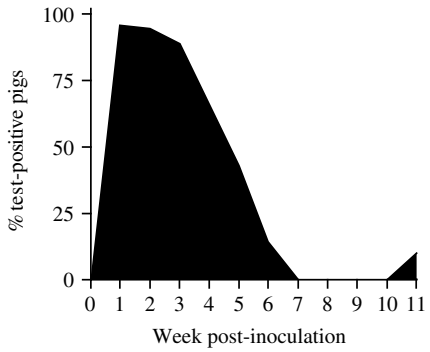

Fig. 3. Relative numbers of *Y. enterocolitica* serotype O:9 (*Ye*O:9) faecal culture-positive pigs following an oral inoculation with *Ye*O:9 (n = varying from 10–23 at different time points).

among young and mature pigs respectively (Fig. 6). All *Ye*O:9 ELISA-negative animals from these herds were also negative by classical tests for brucellosis.

DISCUSSION

The experimental results reported here confirmed previous observations in cattle that *Ye*O:9 infections cause FPSR in all examined serological tests for brucellosis. However, it was also demonstrated that these cross-reactions were limited to a maximal period of 8–10 weeks p.i. in all tests although the anti-*Ye*O:9 LPS response remained high as measured by the *Ye*O:9 iELISA (Fig. 2). The specificity of this ELISA has been determined following its use in a *Ye*O:9 surveillance programme among Danish pig breeding herds during 2002–2003. Of 16 903 samples obtained from consistently negative herds seven samples were tested positive (range 22–56 OD%) resulting in a specificity of 0·9996. Faecal excretion of *Yersinia* was generally found to be of short duration and to precede the development of antibodies, therefore, follow-up by cultivation of faecal samples is not feasible for resolving the cause of serological *Brucella* reactions.

It is known from epidemiological studies of *Ye*O:3 infections in chronically infected pig herds that most weaners are seronegative and that seroprevalence increases with age [19]. There are no biological differences between the serotypes that should indicate different distributions of reactors in herds with *Ye*O:3 and *Ye*O:9 infections. Following the observed limited time-frame of *Brucella* cross-reactivity in *Ye*O:9-infected pigs it was, therefore, hypothesized that the majority of pigs with a recent *Ye*O:9 infection were the animals that cause false-positive *Brucella* seroreactions, and that these pigs are to be found predominantly within the young age groups.

This hypothesis was subsequently verified by investigating the epidemiology of seroreactions in herds with verified *B. suis* biovar 2 or *Ye*O:9 infections. Thus, a pattern of *Brucella* seropositives by classical tests found primarily among young pigs, and a high number of *Ye*O:9 iELISA-positive and *Brucella*-seronegative older pigs, is highly indicative of *Ye*O:9 as the cause of the serological *Brucella* reactions in the herd. In the absence of a reliable diagnostic test to discriminate antibodies raised against these two infections, this information is very useful to resolve suspicions of brucellosis when herds are quarantined due to false-positive individual reactions in surveillance programmes for brucellosis. In such cases an epidemiological investigation in the herd of origin is warranted and required by current European Union legislation [20]. This can be done by serological analysis of a number of sows and fatteners by *Ye*O:9 iELISA and a classical test for *Brucella*. It must be emphasized, however, that in case of a recent introduction of *Ye*O:9 into a herd the described pattern of reactions may differ as the majority of seropositive animals may be within the time-frame of false-positive *Brucella* seroreactions irrespective of their age. It is, therefore, important that research continues in the field of developing tests that discriminates individual animals infected with *Brucella* or *Ye*O:9.

The experimental observation that iELISA titres do not decrease corresponding to the resolving of *Ye*O:9 FPSR indicates that the disappearance of FPSR with time is not reflecting decreasing levels of antibody in serum, but rather a maturation of the antibody response with increasing affinity and specificity in tests with whole cell or crude antigen preparations. However, while the *Brucella* LPS iELISA appeared to be unaffected by this affinity maturation, FPSR in FPA (also using LPS as antigen) were only observed in the period of cross-reactivity observed with classical tests for brucellosis.

Except for the arthritis in two pigs, no pigs in the present experimental infections showed symptoms or pathological findings compatible with brucellosis. The inoculation dose was selected to give a low-grade infection as it is in such cases that a differential diagnosis with *Ye*O:9 is most relevant and it is noteworthy that in spite of the complete lack of symptoms persistent antibody responses were generated in all pigs with evidence of infection. It is well known that

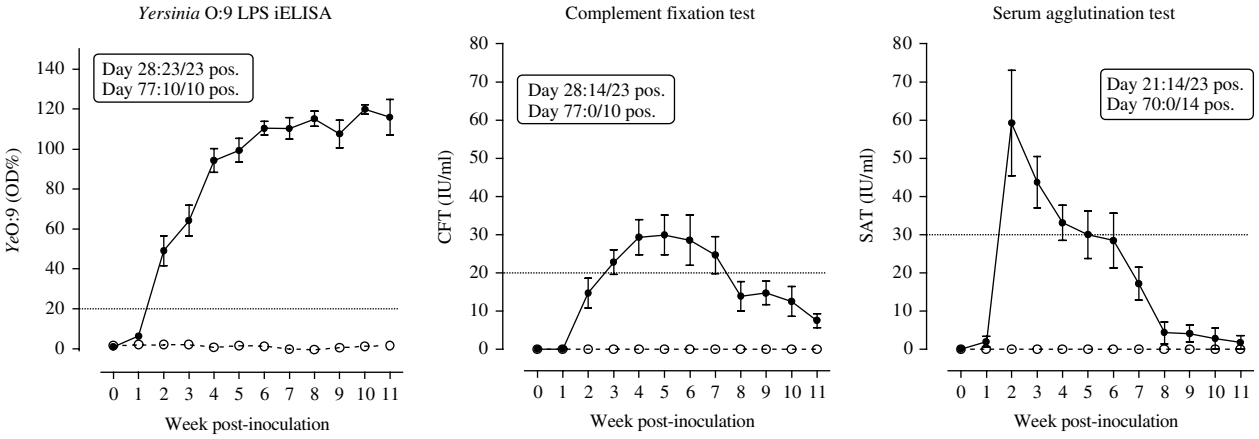

Fig. 4. Mean and S.E.M. of antibody responses in pigs following experimental oral inoculation with *Y. enterocolitica* serotype O:9 (solid lines, $n = 5$–23 at different time points) or non-inoculated controls (dotted lines, $n = 10$). Horizontal dotted lines indicates cut-points of each test. Note that the *y*-axis scale for CFT and SAT is more than 10-fold lower than in Figure 1.

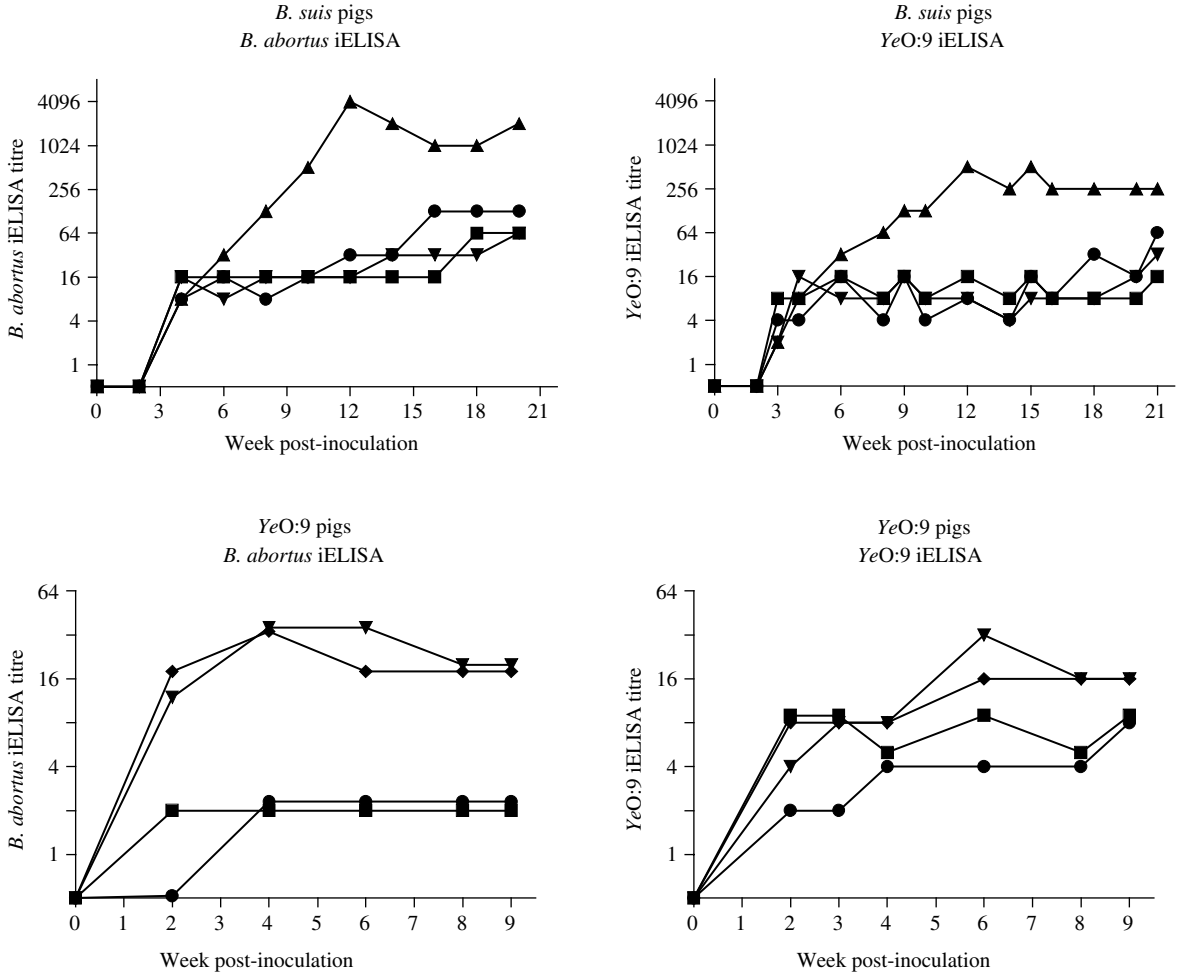

Fig. 5. Levels of serum iELISA titres of pigs in the course of infection with either *B. suis* biovar 2 (top panel) or *Y. enterocolitica* serotype O:9 (bottom panel) using microtitre plates coated with LPS from *B. abortus* (left panel) and *Y. enterocolitica* O:9 (right panel). The titre indicates the highest pre-dilution of sample that was test-positive with subsequent sample dilution. For each infection the symbols are the same for a given pig in both iELISA types.

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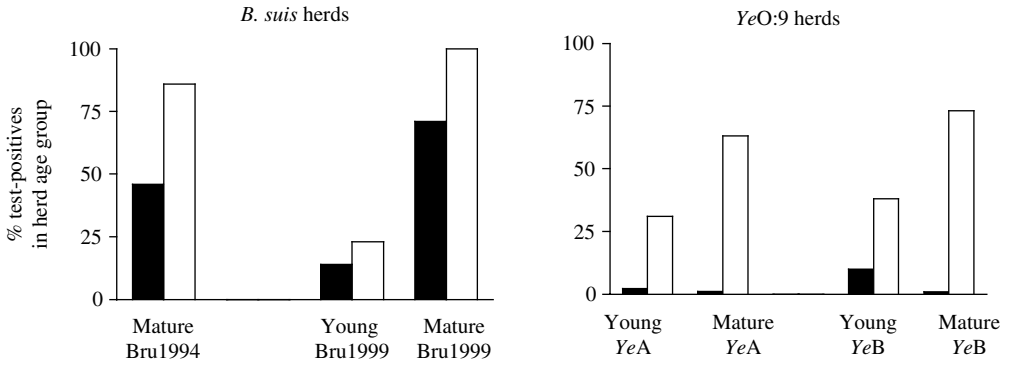

Fig. 6. Prevalence of test-positive pigs for *Brucella* (Serum agglutination test or Rose Bengal test, black bars) and *Ye*O:9 iELISA (white bars) among young and mature pigs from two herds infected with *B. suis* biovar 2 and two herds infected with *Y. enterocolitica* O:9 respectively. See text for numbers of animals in different groups and herd data.

while other *B. suis* biovars are highly pathogenic to humans biovar 2 is only very rarely reported as a zoonosis [11]. The complete lack of symptoms and pathological findings at necropsy in this study may indicate that *B. suis* biovar 2 has a low pathogenicity even in young pigs. It is, therefore, very likely, although not investigated in the present study, that other *B. suis* biovars induce equally persistent infections and antibody responses that allow for resolution of herd infection status by cross-sectional serological analyses.

In terms of epidemiology of the *B. suis* biovar 2 infection it is also noteworthy that two pigs were apparently not infected by the inoculation and that these two pigs (one in each pen) did not seroconvert in spite of their co-habitation with the infected animals throughout the 21-week study period. This lack of horizontal spread of infection would probably have been different if the male pigs used in this study had not been castrated, as the genital tract is a predilection site of *Brucella*. However, in modern pig production boars are often only used as teasers accompanied by an artificial insemination programme, and in such cases the importance of boars in the direct transmission of infection to sows is probably limited.

In addition to its low zoonotic potential, *B. suis* biovar 2 is different from other smooth *Brucella* strains, as it lacks reactivity with the *Brucella* M-epitope specific (and *Ye*O:9 LPS non-reactive) monoclonal antibody BM40 [15]. Nevertheless, antibody responses to *B. suis* biovar 2 infections inhibit binding of the BM40 mAb in the cELISA, as was observed in the present study. This blocking is probably due to overlapping epitope structures, each consisting of a few polysaccharide units [2], similar to the well-known blocking effects of antibody responses to *Ye*O:9 infections [3]. In spite of the blocking reactivity, the lack of specific M-epitope binding antibodies in infections with the *B. suis* biovar 2 is a probable explanation for the relatively lower sensitivity of the cELISA compared with other tests for brucellosis observed in the present study. However, the specificity of the cELISA in relation to *Ye*O:9 FPSR was superior to other investigated serological tests. In the present study a cut-point of 60 OD% was used, although this has only been validated in cattle and it is possible that a better sensitivity and specificity can be obtained with different interpretation criteria for pigs. In addition, the sensitivity of the cELISA may be different with infections of other biovars of *Brucella* where M-epitopes are present.

It is reported that weaner pigs up to 2–3 months may harbour a *Brucella* infection with limited agglutinating antibody response to the infection [13]. In the present study pigs were inoculated at the age of 8 or 16 weeks respectively, and no obvious differences in the serological responses were observed. In addition, the pigs that did not develop antibody responses could not be verified as being infected.

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DECLARATION OF INTEREST

None.

REFERENCES

1. **Caroff M, Bundle DR, Perry MB.** Structure of the O-chain of the phenol-phase soluble cellular lipopolysaccharide of *Yersinia enterocolitica* serotype O:9. Eur J Biochem 1984; **139**: 195–200.
2. **Bundle DR, Cherwonogrodzky JW, Gidney MA, Meikle PJ, Perry MB, Peters T.** Definition of *Brucella* A and M epitopes by monoclonal typing reagents and synthetic oligosaccharides. Infect Immun 1989; **57**: 2829–2836.
3. **Weynants V, Gilson D, Cloeckaert A, et al.** Characterization of smooth lipopolysaccharides and O polysaccharides of *Brucella* species by competition binding assays with monoclonal antibodies. Infect Immun 1997; **65**: 1939–1943.
4. **Kittelberger R, Bundesen PG, Cloeckaert A, Greiser-Wilke I, Letesson JJ.** Serological cross-reactivity between *Brucella abortus* and *Yersinia enterocolitica* O:9: IV. Evaluation of the M- and C-epitope antibody response for the specific detection of *B. abortus* infections. Vet Microbiol 1998; **60**: 45–57.
5. **Kittelberger R, Hilbink F, Hansen MF, et al.** Serological crossreactivity between *Brucella abortus* and *Yersinia enterocolitica* O:9 I immunoblot analysis of the antibody response to *Brucella* protein antigens in bovine brucellosis. Vet Microbiol 1995; **47**: 257–270.
6. **Spencer SA, Broughton ES, Hamid S, Young DB.** Immunoblot studies in the differential diagnosis of porcine brucellosis: an immunodominant 62 kDa protein is related to the mycobacterial 65 kDa heat shock protein (HSP-65). Vet Microbiol 1994; **39**: 47–60.
7. **Erdenebaatar J, Bayarsaikhan B, Watarai M, Makino Si, Shirahata T.** Enzyme-linked immunosorbent assay to differentiate the antibody responses of animals infected with *Brucella* species from those of animals infected with *Yersinia Enterocolitica* O9. Clin Diagn Lab Immunol 2003; **10**: 710.
8. **Mertens P, Walgraffe D, Laurent T, Deschrevel N, Letesson J-J, De Bolle X.** Selection of phage-displayed peptides recognised by monoclonal antibodies directed against the lipopolysaccharide of *Brucella*. Inter Rev Immunol 2001; **20**: 181–99.
9. **Godfroid J, Saegerman C, Wellemans V, et al.** How to substantiate eradication of bovine brucellosis when aspecific serological reactions occur in the course of brucellosis testing. Vet Microbiol 2002; **90**: 461–77.
10. **Weynants V, Godfroid J, Limbourg B, Saegerman C, Letesson JJ.** Specific bovine brucellosis diagnosis based on in vitro antigen-specific gamma interferon production. J Clin Microbiol 1995; **33**: 706–712.
11. **Godfroid J.** Brucellosis in wildlife. Rev Sci Tech 2002; **21**: 277–286.
12. **Baily GG, Krahn JB, Drasar BS, Stoker NG.** Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. J Trop Med Hyg 1992; **95**: 271–275.
13. **MacMillan AP, Starck J.** Manual of standards for diagnostic tests and vaccines, 4th edn., Chapters 2.3.1 and 2.6.3. Paris: OIE, 2000.
14. **Brinley Morgan WJ, MacKinnon DJ, Gill KPW, Gower SGM, Norris PIW.** Brucellosis diagnosis – standard laboratory techniques, 2nd edn. Alnwick: MAFF Publications, 1978.
15. **Greiser-Wilke I, Moennig V.** Monoclonal antibodies and characterization of epitopes of smooth *Brucella* lipopolysaccharides. Ann Inst Pasteur Microbiol 1987; **138**: 549–560.
16. **McGiven JA, Tucker JD, Perrett LL, Stack JA, Brew SD, MacMillan AP.** Validation of FPA and CELISA for the detection of antibodies to *Brucella abortus* in cattle sera and comparison to SAT, CFT, and IELISA. J Immunol Methods 2003; **278**: 171–178.
17. **Westphal O, Lüderitz O, Bister F.** Concerning extraction from bacteria ith phenol/water [in German]. Z Naturforsch Teil B 1952; **76**: 148–155.
18. **Hassan JO, Barrow PA, Mockett AP, Mcleod S.** Antibody response to experimental *Salmonella* Typhimurium infection in chickens measured by ELISA. Vet Rec 1990; **126**: 519–522.
19. **Wingstrand A, Nielsen B.** Cross-sectional investigation of pig herds for *Yersinia enterocolitica* O:3. Proceedings of the 14th IPVS Congress, Bologna, Italy 1996.
20. **European Community Council Directive.** 1990; 90/429/EEC.