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# Non specific serological reactions in the diagnosis of bovine brucellosis: experimental oral infection of cattle with repeated doses of *Yersinia enterocolitica* O:9

Bruno Garin-Bastuji[a,*], Nathalie Hummel[a], Guillaume Gerbier[b], Christiane Cau[a], Régis Pouillot[b], Marisa Da Costa[a,1], Jean-Jacques Fontaine[c]

[a]*OIE Reference Laboratory for Brucellosis, CNEVA Alfort, BP67, 94703 Maisons-Alfort Cedex, France*
[b]*Epidemiology Unit, CNEVA Alfort, BP67, 94703 Maisons-Alfort Cedex, France*
[c]*Pathology Unit, CNEVA Alfort, BP67, 94703 Maisons-Alfort Cedex, France*

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**Abstract**

Eight heifers were orally infected with $4 \times 10^9$ colony forming units of a field cattle strain of *Yersinia enterocolitica* O:9 in a capsule, 5 days a week, for about 9 weeks (day 0–day 64 (D0–D64)). The faecal shedding of *Y. enterocolitica* O:9 began on D5 for seven out of the eight challenged cattle with a high level of excretion during the first month, followed by a decrease till the day of slaughter (D76). *Y. enterocolitica* O:9 was not isolated from organs collected at slaughter. No clinical symptoms were observed. Hyperplasia of intestinal lymph formations was the sole microscopic lesions observed. Five animals showed a serological reaction against *Brucella* antigens in at least one of the following tests: Rose-Bengal test, complement fixation test, tube agglutination test or indirect ELISA (iELISA) tests. Only one animal showed a high level of serological response and a positive reaction in the dithiothreitol-microagglutination test. The observed variability in terms of individual sensitivity to the *Y. enterocolitica* O:9 infection is in agreement with the low individual prevalence rate and the transient serological reaction and faecal *Y. enterocolitica* O:9 shedding observed in herds showing false positive serological reactions in brucellosis. 

*Keywords*: Cattle-bacteria; *Yersinia enterocolitica*; *Brucella abortus*; Crossreactivity; Experimental infection

* Corresponding author. Tel.: +33-1-49-77-13-00; fax: +33-1-49-77-13-44; e-mail: b.garin@alfort.cneva.fr
[1] Present address: Universidade Federal de Rio Grande do Sul, Instituto de Ciências Básicas de Saúde, Departamento de Microbiologia, Rua Sarmento Leite, 500, 90050-170, Porto Alegre, RS, Brazil.

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## 1. Introduction

Since 1990, the rate of false positive serological reactions (FPSR; Weynants et al., 1996) in bovine brucellosis screening tests increased dramatically in some unvaccinated areas of France and Belgium (Bénet et al., 1991; Dufey, 1992). These reactions are characterised by both a very low individual prevalence rate in the herd (Saegerman et al., 1997; Pouillot et al., 1998) and a rapid decrease of serological titres (Gerbier et al., 1997; Lescoat et al., 1997). Such an increase in the rate of FPSR has been also observed at a lesser degree in Great Britain, The Netherlands, Italy and New Zealand (MacMillan, 1990; Hilbink et al., 1995; Lillini et al., 1997; Olyhoek, personal communication). The serological cross-reactivity between the smooth *Brucella* spp. lipopolysaccharide and various organisms of other genera is well documented (Corbel, 1985). Amongst these, *Yersinia enterocolitica* O:9 shows the most complete similarity to the O-chain of *Brucella* (Caroff et al., 1984). *Y. enterocolitica* O:9 has been identified as a fairly common cause of cross-reactions in pigs and human (Ahvonen and Sievers, 1969; Akkermans and Hill, 1972), and was described as able to induce comparable serological responses in experimentally infected animals (reviewed by Corbel, 1985). *Y. enterocolitica* O:9 was isolated for the first time from cattle, goat, sheep, llama and deer faeces samples once an increased rate of FPSR has been observed in these species (Garin-Bastuji, 1993; Reynaud et al., 1993; Hilbink et al., 1995; Arthur, 1997). Therefore, *Y. enterocolitica* O:9 is considered the most likely agent to induce FPSR in ruminants. However, there is no evidence of the way animals would get naturally infected with this organism. Recent epidemiological studies suggest that it could be widespread in areas of high prevalence of FPSR (Pouillot et al., 1998).

Several experimental infections of cattle with *Y. enterocolitica* O:9 have been reported (Corbel and Cullen, 1970; Mittal and Tizard, 1979; Mittal et al., 1981; Corbel et al., 1984; Kittelberger et al., 1995b; Weynants et al., 1996; Kittelberger et al., 1997), but all used a single parenteral or oral dose. The purpose of this work was to study the response of cattle to a prolonged oral infection with cultures of *Y. enterocolitica* O:9, which might be closer to natural infection, on the assumption that the organism is present in the daily intake of animals. This response was monitored through bacterial cultures of faeces and organs, and serotesting in conventional and indirect ELISA (iELISA) tests.

## 2. Materials and methods

### 2.1. Bacterial cultures

The *Y. enterocolitica* O:9 strain used in this study was isolated from naturally infected cattle (Gerbier et al., 1997). The strain was identified, biotyped and serogrouped by the classical methods (Bercovier et al., 1980; Wauters, 1981; Wauters et al., 1987). This strain harboured the *Yersinia* virulence plasmid pYV, as confirmed by the calcium dependency test (Gemski et al., 1980). During the experiment, the strain was checked regularly for O:9 antigenicity by agglutination with the specific serum (Sanofi Diagnostics Pasteur, France). Capsules containing $4 \times 10^9$ colony forming units (CFU)

of *Y. enterocolitica* O:9 in trypticase-soy broth were prepared, stored at −20°C, and thawed 1 h before each inoculation. Control capsules were prepared the same way, with sterile trypticase-soy broth.

### 2.2. Animals and experimental design

Ten non-pregnant Charolais heifers (aged 18–24 months), non-vaccinated against brucellosis, born in two officially brucellosis-free herds located in an area free of brucellosis and free of FPSR for at least 5 years, entered the experiment. No *Y. enterocolitica* O:9 was isolated from faecal samples 30 days prior to the start of the experiment. Eight heifers (animals #3–#10) were infected orally by drenching with one capsule of *Y. enterocolitica* O:9, 5 days a week, for about 9 weeks (day 0–day 64 (D0–D64)). The two control animals (#1 and #2) were given control capsules. Challenged and control animals were housed in separated units.

Blood and faeces samples were collected twice a week. Faeces samples were collected directly from the rectum of each animal into a plastic flask.

Animals were slaughtered within the 14 days after the last day of experiment (D78). At necropsy, tonsils, retropharyngeal, mesenteric, iliac and submaxillary lymph nodes were collected, as well as samples of liver, spleen, gall and faeces for bacteriological and histological examination. Gall bladder, rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, caecum, colon and rectum were also sampled for histological examination.

### 2.3. Bacteriological examination

Faeces and ground organs were suspended in tryptone water (Merck, France) then seeded on CIN *Yersinia* selective solid medium (Biomérieux, France). Plates were read after 24–48 h of incubation at 30°C. Samples were either plated directly or, in case of negative direct result, after an enrichment incubation of the sample suspensions for 21 days at 4°C (Gerbier et al., 1997). Strains isolated were systematically controlled for the virulence plasmid (Gemski et al., 1980) and colony forming units per gram of faeces were enumerated.

### 2.4. Serological response to Brucella

Serum samples were subjected to the Rose-Bengal test (RBT), the complement fixation test (CFT), the tube agglutination test (SAT), the dithiothreitol-microagglutination test (DTT-MAT) (Antigens purchased from Synbiotics, France) and four different iELISA tests using *Brucella* S-LPS as antigen: iELISA 1 (Chekit Brucellotest sérum, Hoechst Roussel Vet, France), iELISA 2 (Institut Pourquier, France), iELISA 3 (Herd Check Bovine Brucellosis, Idexx, France), and iELISA 4: (Serelisa Brucella Ab mono indirect, Synbiotics, France). CFT was performed on microplates by the cold procedure (Alton et al., 1988; Garin-Bastuji and Trap, 1989). RBT and SAT were performed according to Alton et al. (1988). DTT-MAT was performed according to Thiange et al. (1992). Antigens and iELISA kits were standardised against the International Standard for anti-*Brucella abortus* Serum (ISaBS) according to the EU regulations. Sera were considered as positive

when showing any degree of agglutination in the RBT, 50% or less hemolysis at a dilution of 1/4 or higher in the CFT (i.e. $\geq$20 IU/ml), a titre $\geq$ 30 IU/ml in the SAT or the DTT-MAT. Results in iELISA tests were expressed as either negative, or positive as recommended by the respective kit producer[2]. Doubtful results were considered as negative for the purpose of the study.

## 3. Results

### 3.1. Clinical and pathological examinations

No clinical symptoms were observed except an intermittent diarrhoea in infected animals. Hyperplasia of intestinal associated lymphoid tissue (Peyer's patches and lymphoid follicles) was the sole microscopic lesions observed in infected animals. No other lesions were observed. No symptoms nor lesions were observed in control animals.

### 3.2. Bacteriological examinations

*Y. enterocolitica* O:9 was not isolated from faeces or organs collected from control animals. The results of bacteriological examinations of faecal samples are presented in Fig. 1. First isolations of *Y. enterocolitica* O:9 on infected animals began as early as the first sampling date (D5), for six out of the eight cattle and on the second sampling date (D8) for another animal. One animal (#10) was faeces culture positive only once within the course of the experiment (D36). The level of excretion was high during the first month (maximum shedding between D12 and D29), then decreased progressively until D64, despite a continuous oral inoculation. Two animals were still excreting *Y. enterocolitica* O:9 the day of slaughter (#3 and #8). *Y. enterocolitica* O:9 has never been isolated from all organs collected at slaughter from infected animals. The strain isolated in faeces from the infected animals during the course of the experiment still harboured the *Yersinia* virulence plasmid pYV.

### 3.3. Serological examinations

The results of RBT, CFT, DTT-MAT, SAT, and iELISAs are shown in Fig. 2 and summarised in Table 1. Control animals were negative to all serological tests throughout

[2] Given:

$$\text{Index } I_1 = \frac{\text{O.D.} - \text{mean O.D. negative controls}}{\text{mean O.D. positive controls} - \text{mean O.D. negative controls}} \times 100$$

and

$$\text{Index } I_2 = \frac{\text{O.D.}}{\text{mean O.D. positive controls}} \times 100$$

iELISA 1 is positive if $I_1 \geq 75$; iELISA 2 is positive if $I_2 \geq 110$; iELISA 3 is positive if $I_1 \geq 100$; iELISA 4 is positive if $I_2 \geq 85$.

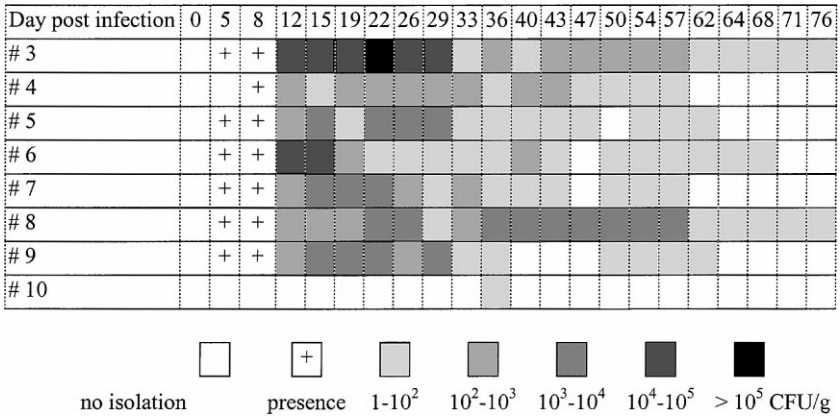


Fig. 1. Faecal excretion of *Y. enterocolitica* O:9 of eight heifers orally infected with $4 \times 10^9$ CFU of *Y. enterocolitica* O:9, 5 days a week, for 9 weeks (from D0 to D64).

the experiment. Three infected animals (#3, #6 and #7) showed positive serological reactions in at least one of the three classical tests, i.e. RBT, CFT or SAT. One animal (#3) showed a high level of response in all tests including MAT-DTT. Its serological response to the RBT and CFT was assessed as positive until the date of slaughter. The other two animals showed a weak, irregular response, variable according to the tests. In RBT, CFT, DTT-MAT and SAT, several reactions were under the official threshold of positivity for two animals (#4 and #8). Two animals (#4 and #7) showed a continuously low level of SAT reaction all along the experiment, generally under the positivity threshold. The response began in all these tests on D22.

Large discrepancies were observed between the different iELISA kits. The animals #3 and #7 were positive at least once in all kits. Animal #9 was positive in three out of the four kits (iELISA 1, iELISA 3 and iELISA 4), while no reaction was observed in RBT, CFT, or SAT for this animal. It showed a continuous low level of reaction in the course of the experiment in iELISA 2 and in iELISA 4. On the other hand, animal #6 was only

Table 1
Number of positive serological results of eight *Y. enterocolitica* O:9 challenged heifers according to the test. Each animal was subjected to 21 tests during the experiment

| Cattle | RBT | CFT | SAT | DTT-MAT | iELISA 1 | iELISA 2 | iELISA 3 | iELISA 4 |
|---|---|---|---|---|---|---|---|---|
| #3 | 16 | 16 | 11 | 4 | 16 | 15 | 16 | 15 |
| #4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| #5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| #6 | 1 | 2 | 0 | 0 | 0 | 0 | 4 | 0 |
| #7 | 0 | 0 | 9 | 0 | 14 | 6 | 15 | 14 |
| #8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| #9 | 0 | 0 | 0 | 0 | 17 | 0 | 1 | 21 |
| #10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

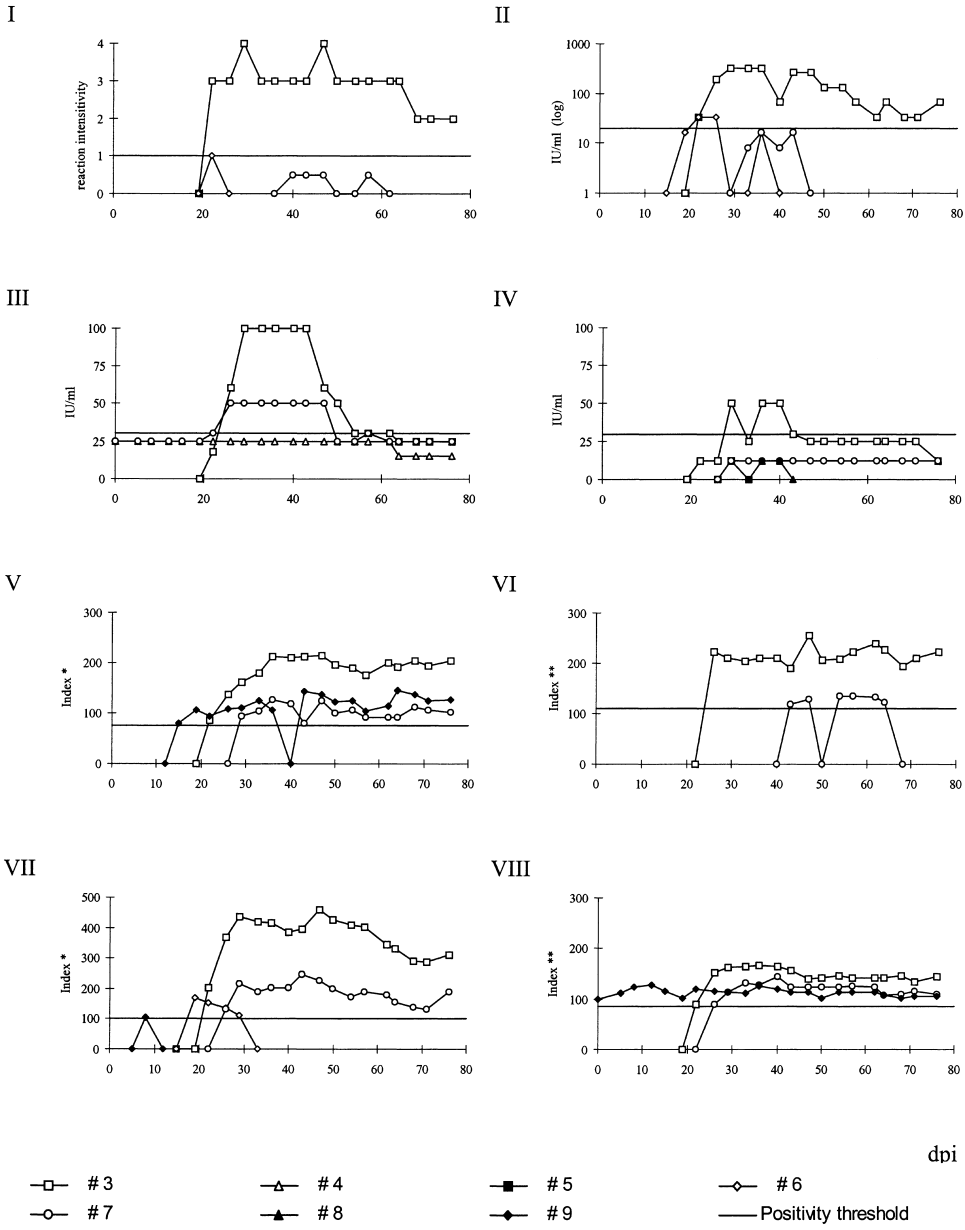


Fig. 2. Serological patterns in eight *Y. enterocolitica* O:9 challenged heifers (#3–#10). I: Rose-Bengal test; II: complement fixation test; III: tube agglutination test; IV: dithiothréitol-microagglutination (DTT-MAT) test; V: iELISA 1; VI: iELISA 2; VII: iELISA 3; VIII: iELISA 4. iELISA patterns are shown only for animals positive at least once in the respective iELISA kit in the course of the experiment.

found positive in the iELISA 3, while it was positive once in the RBT and twice in the CFT.

Four challenged animals (animals #4, #5, #8 and #10) were under the positivity threshold in all tests, throughout the experiment.

## 4. Discussion

This study used for the first time a *Y. enterocolitica* O:9 strain isolated from a naturally infected cattle. *Y. enterocolitica* O:9 was given orally, continuously throughout the experiment, on the assumption that *Yersinia* is present in the daily intake of animal who test FPSR to *B. abortus* (Pouillot et al., 1998). The inoculum was relatively low compared with other studies (Corbel et al., 1984; Weynants et al., 1996). It was enclosed in capsules to ensure a repetitive standard dose to all animals. However, the use of capsules could have led to no or weak contact and infection of tonsils, which could differ from natural oral infection. Heifers were chosen as they appeared to be more sensitive than older animals to the FPSR phenomenon (Saegerman et al., 1997; Pouillot et al., 1998).

The number of bacteria recovered from faeces was much higher than the infection dose for at all but one animals, which revealed a replication of the strain through the digestive tract of all animals. Nevertheless, 46 consecutive oral infections of cattle with a dose of $4 \times 10^9$ CFU of *Yersinia* induce neither obvious clinical symptoms, nor specific lesions. Moreover, while the induced inflammatory reaction of intestinal associated lymphoid tissue revealed a real interaction between the strain and the host, there was no apparent systemic diffusion of the organism, since it could not be isolated from liver, spleen, tonsils or lymph nodes. However, since organs were only collected at D78, it cannot be excluded that bacterial replication in organs occurred earlier. These results confirm the natural resistance of cattle to infection with *Y. enterocolitica* or the low virulence of the strain for cattle (Mollaret and Guillon, 1965; Corbel and Cullen, 1970). Considering the different *Y. enterocolitica* O:9 shedding patterns observed, our study could suggest there is a great individual variation in the natural host resistance to the infection. Moreover, the level of *Y. enterocolitica* O:9 excretion may be related to the level of the anti-S-LPS serological response. On one hand, animal #3 showed a parallel long-lasting serological response and a high level of excretion of *Y. enterocolitica* O:9, throughout the experiment. On the other hand, *Yersinia* was isolated only once from animal #10 which never showed any detectable serological response. It is assumed that this resistance, natural or acquired in the past of the animal, might be due to local immune mechanisms, without any detectable circulating antibody against *Brucella* and *Y. enterocolitica* O:9 S-LPS common epitopes at the time of the experiment. This assumption could be supported by testing the animals in a recently developed iELISA test using *Yersinia* outer membrane proteins (YOP) for the specific detection of *Y. enterocolitica* infection (Weynants et al., 1996).

As a second result, all animals (except #10) seemed to acquire a resistance to the *Y. enterocolitica* O:9 infection during the experiment, since the strain multiplication decreased for all animals after D29, down to almost null at the end of the experiment, despite the continuous oral infection till D64. This resistance occurred in all these animals, but was evidenced by a serological conversion after D20 for some of them only.

It is conceivable that the high cumulative dose used and the subsequent multiplication may be capable of provoking a systemic reaction in some animals, as previously suggested after experimental intramammary infection (Mittal et al., 1981). It would have been of great interest to re-infect these animals some months later, in order to evaluate the length of this acquired resistance.

A great variability is observed amongst the different *Y. enterocolitica* O:9 experimental infections reported in the literature. The comparison between these results is difficult due to the low number of animals used within each study. Moreover, the observed serological patterns are probably variable according to the infection conditions (strain, dose, duration, route). As examples, Weynants et al. (1996) got 1 SAT, 2 CFT and 3 iELISA positive reactions out of four challenged heifers, for at least 45 days, after drenching them with a single dose of $10^{12}$ CFU; Kittelberger et al. obtained a seroconversion in the CFT (up to 80 IU/ml) for 9 to 10 weeks for two calves (aged 5 months) orally infected with a dose of $10^{9}$ CFU (Kittelberger et al., 1995b), but only two seroconversions in the CFT (up to 40 IU/ml) out of 10 calves orally infected with a single dose of $10^{10}$ CFU (Kittelberger et al., 1997). Corbel et al. (1984), inoculating via a stomach tube a high challenge dose of $10^{11}$–$10^{13}$ CFU, reported only one animal out of four with a high serological titre, other animals giving no or low and transient antibody titres. Repeated or single parenteral injections of *Y. enterocolitica* O:9 give long lasting serological responses (Corbel and Cullen, 1970; Mittal and Tizard, 1979; Kittelberger et al., 1997), but certainly do not mimic natural infection. However, great variability is always observed between animals within a given study, which could confirm our hypothesis of a great variability in the individual susceptibility to the infection with *Y. enterocolitica* O:9. The positive results observed in all brucellosis tests were expected; no serological test can actually discriminate *Y. enterocolitica* O:9 from *Brucella* infection (Kittelberger et al., 1995a).

The low number of challenged animals impedes the comparison between tests in this study. Nevertheless, in a quantitative approach, the observed patterns confirm the high level of specificity of the DTT-MAT. Corbel et al. (1984) observed similar results with the 2-mercaptoethanol SAT (2ME-SAT). Due to their neutralizing effect on IgM, MAT-DTT and 2ME-SAT inhibit the serological response to *Y. enterocolitica* O:9, as well as many FPSR (Thiange et al., 1992). The serological reaction to *Yersinia* could be mainly an IgM response (Corbel, 1985). However, the DTT-MAT sensitivity to *Brucella* is lower than the RBT (Pouillot et al., 1997), which impedes its use for brucellosis surveillance.

The specificity of the iELISA kits to *Brucella* appears to be lower than classical brucellosis tests. This is in accordance with results obtained in field conditions, showing more than 1% of false positive reactions in iELISA on cattle negative to classical tests issued from herds located in areas of high FPSR prevalence (Garin-Bastuji, unpublished results). In this experiment, the most specific iELISA kit seems to be the iELISA 2, which is the only kit to include a conjugate consisting of a monoclonal antibody specific to cattle $IgG_1$. The three other kits use a conjugate consisting either of an anti-bovine IgG (H + L) polyclonal serum (iELISA 1), either of a monoclonal anti-bovine IgG antibody (iELISA 3 and iELISA 4), which certainly cross-react more than the monoclonal anti-$IgG_1$ conjugate with IgM (Limet et al., 1988). The fact that cross-reacting antibodies evoked by *Y. enterocolitica* O:9 are mainly of the IgM subclass, especially in those with weak antibody titres could be reinforced by these results. Substantial titres of IgG

(especially of the $IgG_1$ isotype) antibodies could be also produced by some animals (Nielsen, 1990; Weynants et al., 1996), as revealed by the response in the MAT-DTT and the iELISA 2 kit. Further studies should be conducted in *Brucella* infected herds in order to evaluate the respective sensitivity of each iELISA kits to *Brucella* infection.

This experiment reproduced FPSR patterns similar as those observed in the field (Gerbier et al., 1997; Lescoat et al., 1997; Saegerman et al., 1997). Nevertheless, despite the large cumulative size of the inoculum, three animals only would have been considered positive in brucellosis according to the French regulation, i.e. at least positive to either the RBT or the CFT.

Heifers could be classified into three categories in a scale of susceptibility to *Y. enterocolitica* O:9 infection. Susceptible animals shed high doses of *Y. enterocolitica* and respond highly in all official brucellosis serological tests during a long period. Some animals show a weak and transient serological response. Others do not respond in the serological brucellosis tests, indeed even seem to be totally resistant to the infection. These observed patterns could be in accordance with previous observations in field conditions showing (i) a higher prevalence of *Yersinia* culture positive animals than of FPSR (Gerbier et al., 1997), (ii) a low intra-herd prevalence of FPSR observed (Bénet et al., 1991; Gerbier et al., 1997; Saegerman et al., 1997) probably due to a great variability in the host sensibility, (iii) the co-existence within a herd of a majority of transient seroconversions with some long-lasting ones (Lescoat et al., 1997; Saegerman et al., 1997). These results could also support the hypothesis of a large exposure of cattle to *Y. enterocolitica* O:9 in some areas (Pouillot et al., 1998), but with a low level of expression in terms of serological response against *Brucella*.

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