Cross-reactivity in serological tests for brucellosis: a comparison of immune response of Escherichia coli *O157:H7 and* Yersinia enterocolitica *O:9 vs* Brucella *spp.*

Barbara Bonfini[1], Giuseppina Chiarenza[2], Valentina Paci[1], Flavio Sacchini[1], Romolo Salini[1], Gesualdo Vesco[2], Sara Villari[2], Katiuscia Zilli[1] and Manuela Tittarelli[1*]

[1] *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Campo Boario, 64100 Teramo, Italy.*
[2] *Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri", Via Gino Marinuzzi 3, 90129 Palermo, Italy.*

[*] *Corresponding author at: Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G.Caporale", Campo Boario, 64100 Teramo, Italy. Tel.: +39 0861 332431, e-mail: m.tittarelli@izs.it.*

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Brucellosis,
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Cross-reactions,
Immunisation.

Summary
According to European Union (EU) regulations, the serological tests for the eradication of bovine and ovine brucellosis are the Rose Bengal Test, Complement Fixation Test, and i-ELISA. These methods, also recommended by the World Organization for Animal Health (OIE) for international trades, have limitations related to the use of suspensions of smooth *Brucellae* or LPS extracts. Limitations include false-positive serological reactions to brucellosis, which in turn impedes accurate diagnosis in some herds. False positive reactions should be considered carefully during the final stages of an eradication programme and for surveillance purposes in brucellosis-free areas. In this study, we produced specific sera through the experimental infection of sheep with *Y. enterocolitica* O:9 and *E. coli* O157:H7. These are the most important cross-reactive bacteria with *Brucella*. We then evaluated the antibody response of groups of sheep that had been immunised towards homologous antigens and official antigens for brucellosis, in order to identify a differential diagnostic protocol to distinguish cross-reaction in *Brucella*-infected animals.

Reattività crociata nei test sierologici per la brucellosi: una comparazione delle risposte immunitarie di Escherichia coli *O157:H7 e* Yersinia enterocolitica *O:9 vs* Brucella *spp.*

Parole chiave
Brucellosi,
Yersinia enterocolitica O:9,
Escherichia coli O157:H7,
Reattività crociata,
Immunizzazione

Riassunto
In accordo con la normativa dell'Unione Europea, le indagini sierologiche finalizzate all'eradicazione della brucellosi ovina e bovina sono il Rose Bengal Test, il Complement Fixation Test, e l'i-ELISA. Questi metodi, raccomandati anche dal World Organization for Animal Health (OIE) per gli scambi commerciali, hanno limiti correlati all'uso della sospensione di brucella o al LPS estratto che, potendo determinare reazioni sierologiche falso-positive alla *Brucella*, in alcuni casi ostacolano una diagnosi accurata. Le reazioni falso-positive dovrebbero essere considerate con attenzione nelle fasi finali dei programmi di eradicazione e nella sorveglianza nelle aree libere dalla brucellosi. In questo studio si è prodotto un siero specifico infettando sperimentalmente le pecore con *Y. enterocolitica* O:9 e *E. coli* O157:H7, batteri che hanno maggiore reattività crociata con la *Brucella*. Si è valutata la risposta anticorpale di gruppi di ovini che erano stati immunizzati verso antigeni omologhi e antigeni ufficiali per la brucellosi, al fine di identificare un protocollo diagnostico differenziale per distinguere la reazione crociata in animali infetti da *Brucella*.

Introduction

Brucellosis is an infectious and contagious disease caused by bacterial species of the genus *Brucella*. With the only exception of *Brucella ovis*, it is a major zoonosis with direct and indirect negative social and economic impacts. In the European Union, strategies adopted to control and prevent bovine, ovine, and caprine brucellosis aim to eradicate the infection, i.e. eliminate the disease and its aetiological agent from the area[1]. The strategy is based on identification and slaughter of all animals positive to serological or bacteriological tests, the prohibition of vaccination, and the achievement of Brucellosis free status which is a strong incentive for farmers and broader areas of the EU.

According to EU Regulations, serological control is based on flock screening through the Rose Bengal Test (RBT) or indirect ELISA (i-ELISA), followed by the Complement Fixation Test (CFT) or competitive ELISA (c-ELISA) as confirmatory tests. The World Organization for Animal Health (OIE) also recommends these tests for international trade (OIE 2009), although false positive serological reactions (FPSR) can occur causing problems in many countries, especially in Brucellosis-free or almost-free areas. Serological tests are limited by the use of suspensions of smooth *Brucellae* (s-*Brucellae*) as antigens (Alton *et al.* 1988) or lipopolysaccharide (LPS) extracts. A number of other gram-negative bacteria, in particular *Yersinia enterocolitica* O:9, *Escherichia coli* O157:H7, *Salmonella* group N (O:30), and *Vibrio cholerae* O1, may induce antibody responses that cause FPSR in brucellosis tests. Accurate serological diagnosis is crucial either during the final stages of an eradication programme or during a surveillance program in brucellosis-free areas (Mainar-Jaime *et al.* 2005). The serological cross-reaction between s-*Brucellae* and various organisms of other genera is well documented (Corbel 1985). *Yersinia enterocolitica* O:9 shows an O-antigen LPS chain almost identical to that of *Brucella* (Caroff *et al.* 1984).

The RBT and CFT combination, the most widely used serial scheme, has been shown to lack of specificity in differentiating brucellosis-infected animals from cross reacting animals (Gerbier *et al.* 1997, Munoz *et al.* 2005). It is therefore important to identify new diagnostic tools that are capable to discriminate infected from cross-reactive animals in order to control the disease.

In this study, we aim to acquire detailed information on serological cross-reactivity to *Brucella* diagnostic tests and to develop a protocol for differentiating *Brucella* infection from cross-reactions at a serological level. In order to do so we produced cross-reactive specific sera by experimentally infecting sheep with *Y. enterocolitica* O:9 and *E. coli* O157:H7. Using these hyperimmune sera, we then evaluated the antibody response in sheep immunised with *E. coli* O157:H7 and *Y. enterocolitica* O:9 towards homologous antigens and official antigens for brucellosis, in order to identify a differential diagnostic protocol. This protocol would be able to detect FPSR for brucellosis, and is therefore an important instrument to apply in cases in which, in front of serological reactivity, there is no epidemiological evidence to support *Brucella* infection.

Materials and methods

Animals and immunisation

For this study we randomly selected 8 adult cross bred sheep from officially brucellosis-free herds in the Sicily region (Italy). Prior to the experiment, animals were tested for Brucellosis, *Y. enterocolitica*, and *E. coli* by using RBT and CFT, CFT and i-ELISA, respectively. For each cross-reactive pathogen to be examined, 3 sheep were immunised. Animals of group E were injected with a suspension containing 1.5×10^9 colony-forming units (CFU)/ml from a culture of live *E. coli* O157:H7 ATCC reference strain, animals of group Y received 1.5×10^9 colony-forming units (CFU)/ml of the *Y. enterocolitica* O:9 field strain provided by University of Bari (Italy). Group C included 2 sheep which were used as a control.

All animal handling and study procedures undertaken during this experiment complied with current European legislation[2] and the corresponding Italian law[3]. We additionally followed all applicable international, national, and/or institutional guidelines for the care and use of animals.

[1] Commission Decision 984 of 10 December 2008 amending Annex C to Council Directive 64/432/EEC and Decision 2004/226/EC as regards diagnostic tests for bovine brucellosis. *Off J*, **L 352**, 31.12.2008.

[2] Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Off J*, **L 276**, 20.10.2010.

2012/707/EU Commission Implementing Decision of 14 November 2012 establishing a common format for the submission of the information pursuant to Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. *Off J*, **L 320**, 17.11.2012.

2014/11/EU Commission Implementing Decision of 20 December 2013 correcting Annex II to Implementing Decision 212/707/EU establishing a common format for the submission of the information pursuant to Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. *Off J*, **L 10**, 15.01.2014.

[3] Decreto Legislativo 4 marzo 2014, n. 26. Attuazione della direttiva 2010/63/UE sulla protezione degli animali utilizzati a fini scientifici. GU, 61, 14.03.2014..

In order to mimic the natural route of infection, 5 ml of the selected pathogen suspensions were admnistered weekly by oral route (os) until day 99. Starting from day 107, the same suspensions were inoculated subcutaneously (sc). We then doubled the dose (10 ml) from day 289 until the end of the trial. We had to change the administration route as we didn't have any seroconversion by oral route. We hypothesised that frequent oral antigen administrations created a state of immune tolerance or alternatively, that intestinal flora were interfering with the infection. By using the subcutaneous route the enteric tract was bypassed. This enabled us to obtain hyper-immune sera in response to the whole bacterial antigens, without heat-inactivation and with no adjuvants.

None of the animals in the experimental groups showed any clinical sign of disease within the immunisation procedure (e.g. fever or diarrhoea), even after seroconverting.

All animals were bled before the experiment and then weekly for 381 days. For each animal, 40 samples were collected, with the exception of animal 3 in group E (14 samples), which died on day 107 of the study.

Serological tests

All animals in the 3 groups were subjected to weekly serological testing for up to 381 days in order to detect antibodies against *Brucella*, *E. coli* O157:H7, and *Y. enterocolitica* O:9.

Brucellosis

The serological response to *Brucella* spp. was assessed by RBT and CFT, performed according to OIE standard procedures, using antigens derived from strain 99 *Brucella abortus* biovar 1 (AHVLA Weybridge, UK). In addition, sera were also analysed by an i-ELISA produced by IZSAM and calibrated against the International Standard anti-*Brucella melitensis* Serum (McGiven *et al.* 2011) according to the OIE Manual (Year). Briefly, standard 96-well polystyrene plates (PolySorp, NUNCTM, Roskilde, Denmark), coated with purified s-LPS from *B. abortus* produced according to the OIE Manual (year), were incubated for 30 minutes at room temperature (RT) with serum samples, positive and negative controls diluted 1:20 in phosphate buffered saline 0.01M + 0.05% Tween 20, pH 7.2 (PBST). After washing, the plates were incubated for 30 minutes with Protein G-peroxidase (Sigma-Aldrich, St. Louis, Mo) diluted in PBST. We added TMB substrate (3,3',5,5'-tetramethyl-benzidine, Sigma-Aldrich, St. Louis, Mo) and the reaction was stopped with sulfuric acid 0.5N after 30 minutes. The results were expressed as percentage of positivity (PP) of samples with respect to the positive control serum. We considered positive any sera with PP > 35%.

E. coli i-ELISA

To measure antibody response to *E. coli*, we set up an i-ELISA using 10 µg/ml of heat inactivated *E. coli* O157:H7 (IZSAM) adsorbed on PolySorp microplates at 4°C overnight as an antigen. Non-adsorbed material was removed with 1 washing in PBST, and 200 µl of PBST supplemented with 3% skim milk were added for 1 hour at RT. After incubation, we washed the plates 4 times and samples and reference controls were added at 1:50 dilution in PBST and incubated for 30 minutes at RT. We removed the sera and washed the wells 4 times before adding Protein G-peroxidase diluted in PBST for 30 minutes. The plates were then washed 4 times and developed with TMB. We stopped the reaction after 30 minutes with sulfuric acid 0.5N and the results were expressed as percentage of positivity (PP) of samples with respect to the positive control serum. We considered positive sera with PP > 45%.

Y. enterocolitica CFT

Antibody response against *Y. enterocolitica* O:9 was detected by CFT, which we performed using a commercial antigen (deriving from infected cells) and reference sera (Institut Virion\Serion, Germany), according to manufacturer instructions. Sera diluted 1:10 and showing at least 75% inhibition of hemolysis were considered positive.

Statistical analysis

We applied the nonparametric Wilcoxon test for paired samples to *Brucella* and *Y. enterocolitica* CFT results obtained from each of the 6 animals of groups E and Y in order to evaluate possible significant differences of result distribution between the 2 tests at single animal level. P values lower than 0.05 were considered significant (Siegel and Castellan 1988).

Results

At the beginning of the experiment, all animals included in the study were serologically negative to *E. coli*, *Brucella* and *Y. enterocolitica*.

Figures 1, 2, and 3 show the humoral response of the group of animals infected with *E. coli* to homologous and cross-reactive antigens. No *E. coli* O157:H7 antibodies were detected in samples collected until day 91. Between day 99 (Figures 1, 2) and 107 (Figure 3), all animals of the group became positive to homologous i-ELISA, and this positivity

lasted until the end of the trial. A slight and transient antibody reaction to *Yersinia* was also observed until day 91. The titer then increased in all animals, peaking between days 170 and 184. High antibody titres were also recorded from day 310 onwards. The *Brucella* CFT became positive from day 296 (Figures 1, 2) and 303 (Figure 3), 6-13 days after the subcutaneous booster of 10 ml of suspension,

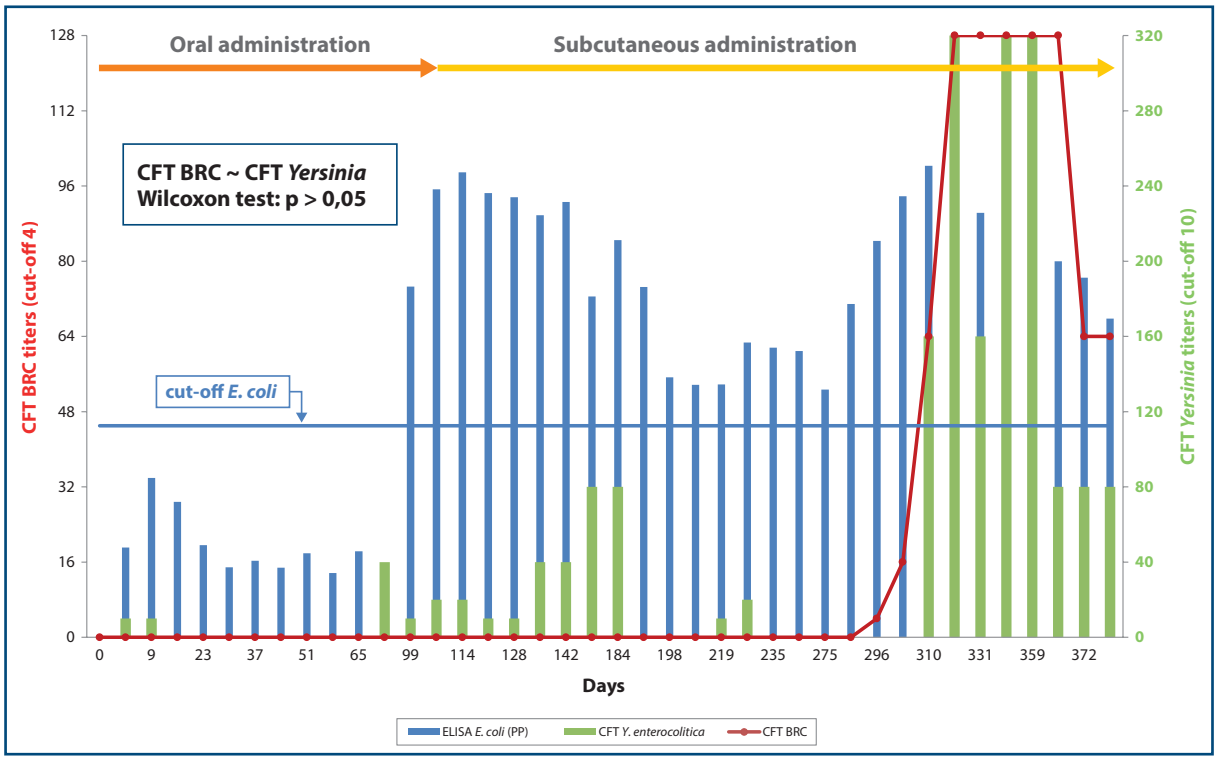

Figure 1. *Results of the serological tests animal 1 Group E (*E. coli*).*

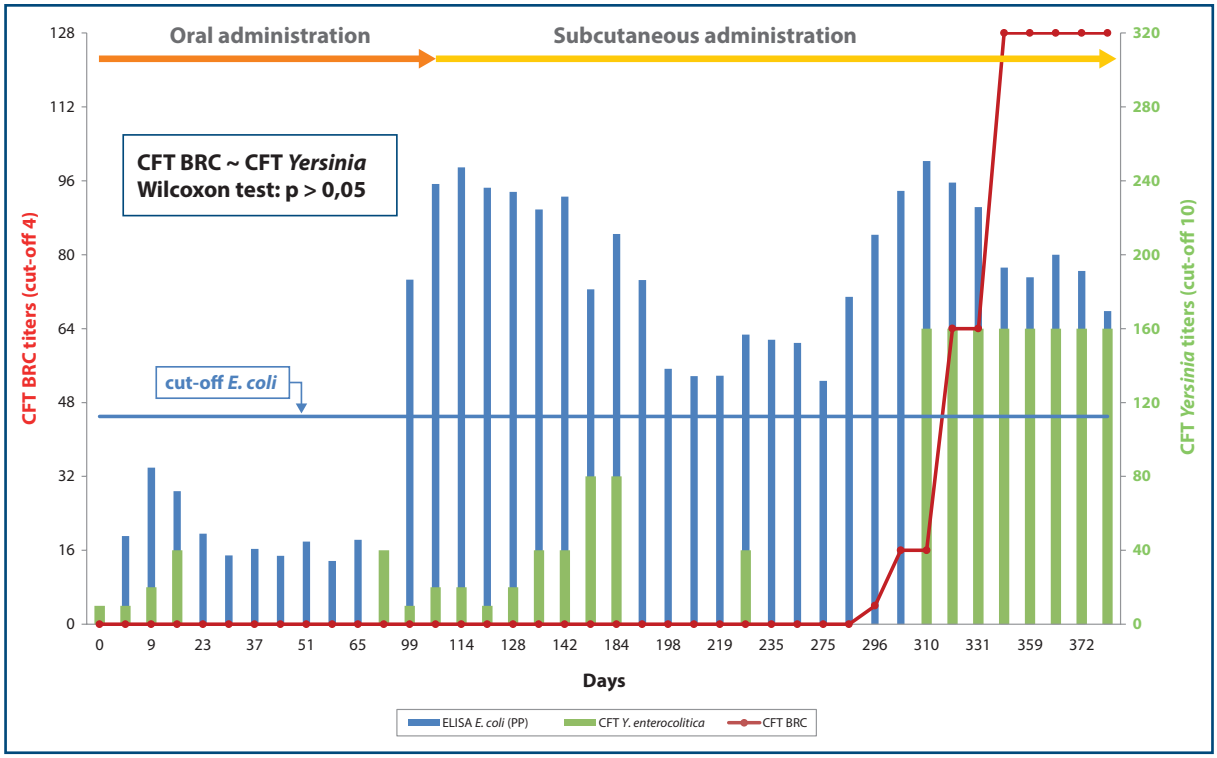

Figure 2. *Results of the serological tests animal 2 Group E (*E. coli*).*

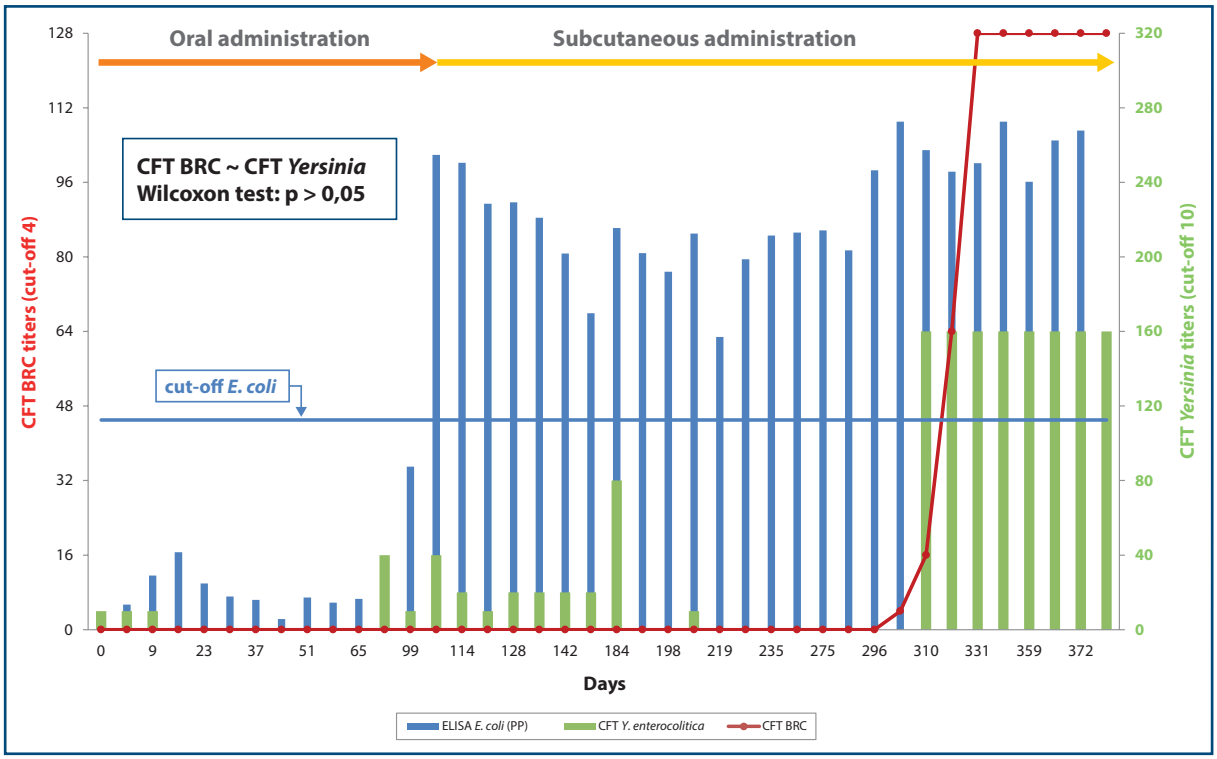

Figure 3. *Results of the serological tests animal 3 Group E (*E. coli*).*

peaking from day 320. *Brucella* and *Yersinia* CFT results were similar ($p > 0.05$).

In Figures 4 and 5 the humoral response of the 2 animals of group Y to homologous and cross-reactive antigens was displayed. The animals were constantly negative to the *E. coli* i-ELISA. Concerning *Y. enterocolitica* O:9 CFT, the trend of the immune response of the 2 surviving animals, was similar. An increased production of antibodies was observed both after oral and subcutaneous immunisation on days 9-23, 91-184 and 198-381. Antibody titres were also detected by the *Brucella* CFT in correspondence to the peaks of *Y. enterocolitica* CFT. The titers however were lower ($p < 0.05$) than those detected by the *Yersinia* CFT.

The third animal of group Y died on day 107. As for the other two animals of the group, *E. coli* ELISA was always negative, while antibody titers were detected by *Y. enterocolitica* CFT between days 9-16 and 91-107. When *Brucella* CFT was used, titers were also detected from day 99. For this animal, a statistical comparison of CFT results between *Brucella* and *Yersinia* did not provide any significant difference ($p > 0.05$).

The 2 sheep used as controls (Group C), consistently recorded negative results to all serological tests.

Table I shows the percentage of cross-reactivity when serum samples from Group E (n = 120), Group Y (n = 94), and Group C (n = 80) were tested by using *Brucella* RBT, CFT, and i-ELISA.

Discussion

Despite being performed on a limited number of animals, this study provided relevant information on the kinetics of antibody response against *E. coli* O157:H7, *Y. enterocolitica* O:9, and on FPSR to brucellosis serological tests.

The results of this study suggest that serological cross-reactivity against *Brucella* spp. mainly occurs following an intense humoral response to *E. coli* O157:H7 and *Y. enterocolitica* O:9, antigens. Interestingly, we observed that immunisation with *E. coli* O157:H7 induced an increased production of homologous antibodies which cross reacted against *Y. enterocolitica* O:9. Conversely, immunisation with *Y. enterocolitica* O:9 determined a specific response against the homologue antigen, but no reactivity against *E. coli* O157:H7. As expected, sera from both *E. coli* O157:H7- and *Y. enterocolitica* O:9-immunised animals showed cross-reactivity against brucellosis serological tests.

A statistical comparison of different diagnostic techniques is difficult to apply and of limited significance. In this study we therefore focused our statistical analysis (non-parametric Wilcoxon test) on results obtained from *Brucella* and *Yersinia* CFT in order to evaluate differences in result distribution rather than the absolute values of antibodies titres between the two tests. In *E. coli*-immunised animals, humoral response against *Yersinia* and *Brucella* cross reacting antigens was similar. Although with different

levels of positivity, *Brucella* cross reactions coincided with positive results to *E. coli* i-ELISA and *Yersinia* CFT. In case of suspected FPSR, performing these tests in parallel can therefore facilitate their interpretation.

In two of the 3 *Y. enterocolitica* O:9-immunised animals the antibody response against *Brucella* cross reacting antigens was different from the humoral response against *Yersinia*. Furthermore,

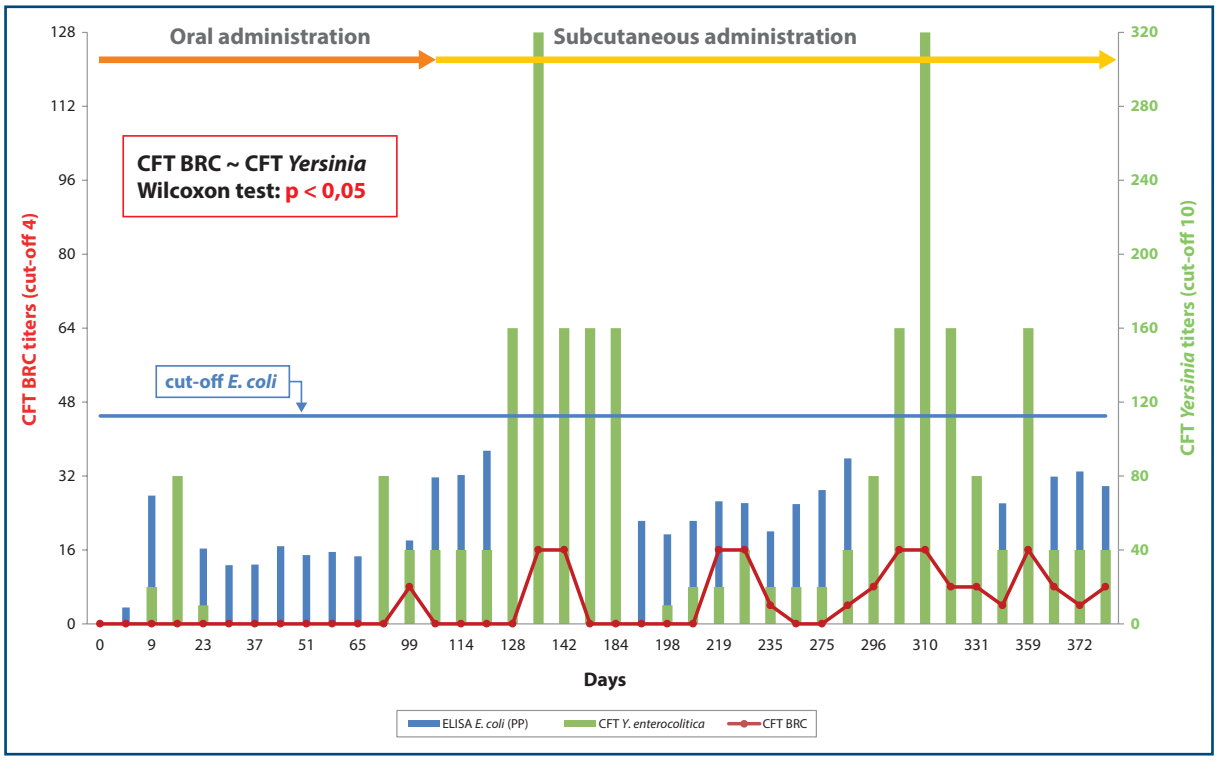

Figure 4. *Results of the serological tests animal 1 Group Y (Y.* enterocolitica*).*

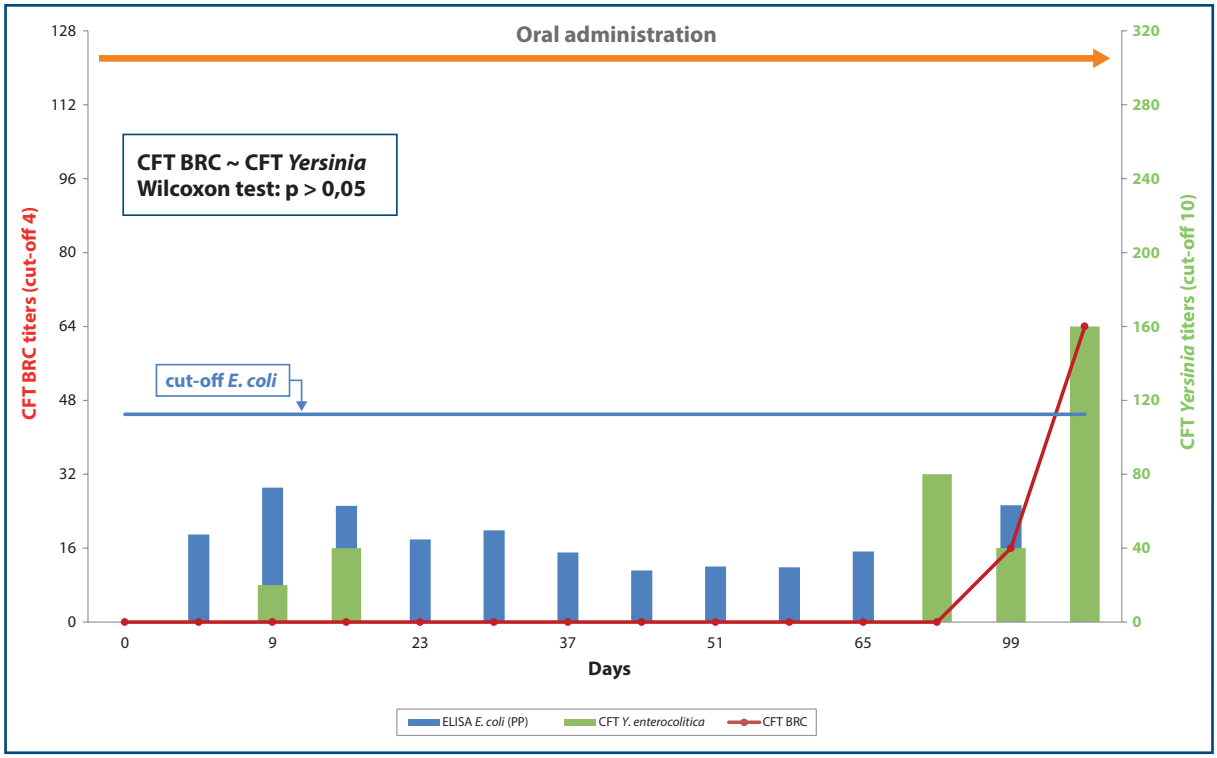

Figure 5. *Results of the serological tests animal 2 Group Y (Y.* enterocolitica*).*

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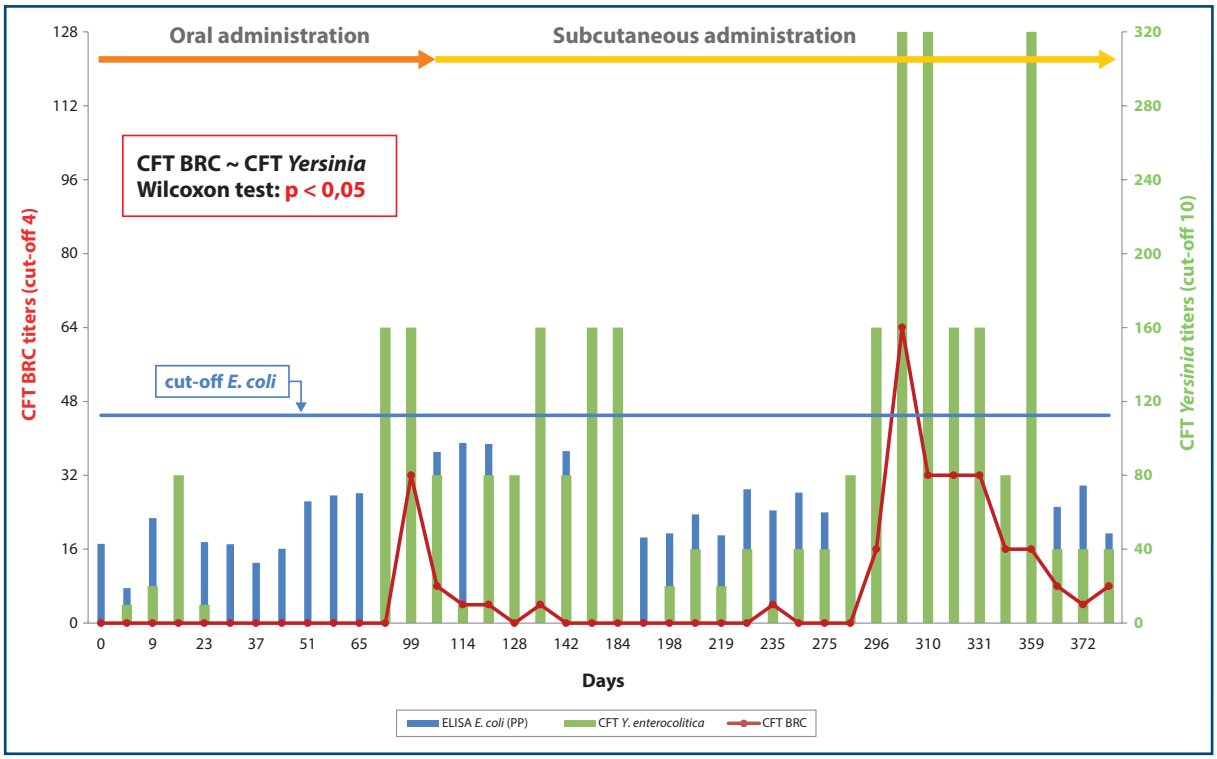

Figure 6. *Results of the serological tests animal 3 Group Y (Y.* enterocolitica*).*

Table I. *Percentage of cross-reactivity for brucellosis serological tests.*

	Group E (*E. coli*)			Group Y (*Y. enterocolitica*)			Group C (control)		
	RBT	CFT	i-ELISA	RBT	CFT	i-ELISA	RBT	CFT	i-ELISA
Tested	120	120	120	94	94	94	80	80	80
Correctly identified as negative	91	91	94	65	59	56	80	80	80
Cross-reactivity (%)	24.2	24.2	21.7	30.9	37.2	40.4	0.0	0.0	0.0

none of the 3 animals showed any positivity to *E. coli* i-ELISA.

According to the results of this study, *Y. enterocolitica* O:9 is the major cause of false positive serological reactions in the diagnosis of brucellosis (Munoz *et al.* 2005).

This animal trial represented a unique opportunity to investigate how the humoral response elicited by major *Brucella* cross-reactive pathogens influences conventional Brucellosis serological tests, leading to false positive serological reactions. These preliminary results suggest the parallel use of serological tests for *Y. enterocolitica* O:9 (CFT), *E. coli* O157:H7 (i-ELISA), and *Brucella* (CFT). This diagnostic protocol should be able to distinguish FPSR from brucellosis and might represent an important tool in all cases where serological reactivity to official tests is not supported by epidemiological evidence of *Brucella* infection. Further studies should be performed to optimise this new protocol and evaluate its effectiveness in field.

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