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ORIGINAL ARTICLE

Novel diagnostic ELISA test for discrimination between infections with *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*

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Abstract

Yersiniosis is a foodborne infection caused by *Yersinia enterocolitica* or *Yersinia pseudotuberculosis*. Although yersiniosis is most often self-limiting, some patients develop chronic infections, such as reactive arthritis, glomerulonephritis, or myocarditis, which require an antibiotic treatment. Whereas early infections can be diagnosed by direct detection of bacteria, chronic infections can only be identified by serological tests. At this point, a serological method for differentiation between infections with the two *Yersinia* species is important since antibiotic susceptibility of these bacteria is different. Traditional immunoassays do not distinguish between infections with *Y. enterocolitica* and *Y. pseudotuberculosis*. The only test that allows for this differentiation is Mikrogen's strip test where discrimination between the two types of infection is based on two recombinant bacterial proteins, MyfA and PsaA (specific for *Y. enterocolitica* and *Y. pseudotuberculosis*, respectively). Here, we show that *Y. enterocolitica* and *Y. pseudotuberculosis*, cultured under the conditions that mimic the natural rout of infection, express surface antigens different from MyfA and PsaA that can also be used in a discrimination test. Further, we describe a new ELISA that is based on the whole bacteria and recombinant MyfA and PsaA as antigens, and that allows the differentiation between infections with *Y. enterocolitica* and *Y. pseudotuberculosis* and simultaneous detection of yersiniosis.

Keywords *Yersinia enterocolitica* · *Yersinia pseudotuberculosis* · Diagnostics · Novel ELISA test · Discrimination between *Yersinia* species

Introduction

Genus *Yersinia* includes three bacteria species that cause human pathology: *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*. Whereas *Y. pestis* causes plague and is transmitted by flea bites or infectious aerosols, *Y. enterocolitica* and *Y. pseudotuberculosis* cause gastroenteritis (so-called yersiniosis) and are mainly transmitted by contaminated food and water. Once in the digestive system, the gastroenteritic *Yersinia* colonize in the intestine-associated lymphatic tissue, where it inhibits phagocytosis and replicates extracellularly [1–3]. In immunocompetent people, yersiniosis is most often self-limiting. However sometimes, the bacteria disseminate to other organs systematically and cause more serious complications, such as reactive arthritis, erythema nodosum, Reiter's syndrome, glomerulonephritis, myocarditis, or sepsis. In general, infections with *Y. pseudotuberculosis* appear more severe and invasive when compared to infections with *Y. enterocolitica* [4], and mesenteric lymph nodes, liver, and spleen are more often affected in the course of *Y. pseudotuberculosis* than *Y. enterocolitica* infection. Importantly, in immunocompromised people (HIV patients or patients undergoing a transplant) or in patients with an underlying disease (as diabetes or liver cirrhosis), the mortality due to *Yersinia* infections can reach up to 50% [2, 5]. Whereas antibiotic treatment may not be necessary during the acute gastroenteritis, but it is the only cure for the secondary complications of *Yersinia* infections. At this point, it seems important to discriminate between infections with *Y. enterocolitica* and *Y. pseudotuberculosis* since the bacteria responds differently to antibiotics. *Y. enterocolitica* produces G-lactamases and is then resistant to penicillin and the first-

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generation of cephalosporins, whereas *Y. pseudotuberculosis* lacks G-lactamase and is susceptible to penicillin [6, 7].

Yersiniosis has been detected on all continents; it is assumed to affect from 0.6 to 2.9% of the population [5, 7]. However, the infection rate is most likely much higher since only serious cases are registered [7], and infections with *Y. pseudotuberculosis* may be underestimated [4]. In the European Union, yersiniosis is the third most common bacterial enteritis [8], with the highest incident in children with age of 1 to 5 years [7]. *Yersinia* are able to grow at low temperatures and its multiplication in refrigerated food is the most important transmission form of pathogenic *Yersinia*. It was estimated that up to 43% of blood donors in Germany and 31% in Finland had specific antibodies to *Yersinia* [9], indicating that these donors had a *Yersinia* infection in the past. Blood transfusion with *Yersinia*-contaminated banked blood may cause septic shock with mortality up to 55% [10].

Surveillance of human *Yersinia* infections is not performed routinely. Differences in reporting methods and tests used for detection of yersiniosis greatly complicate comparisons between countries. Notably, yersiniosis is infrequently monitored in developing countries, where enteric diseases are the major cause of infant and child mortality [5].

Consensus guidelines on the diagnosis of *Yersinia* infections do not exist. At the beginning of infection, during the active gastroenteritis, yersiniosis can be diagnosed with microbiological tests of feces or body fluids (from peritoneum, wounds, or abscesses) where *Yersinia* are identified by a PCR or by a growth on selective media. However, it is very difficult to detect bacteria in body fluids during late yersiniosis, and the only way to diagnose *Yersinia* infection then is to detect anti-*Yersinia* antibodies. *Yersinia* infections induce persistent immune response [11] and several serological tests for detection of yersiniosis have been proposed. The first method, and still in use, was agglutination test in serum. Later methods included ELISA, immunoblotting, and radio-immunotests that were based on lipopolysaccharides (LPS) or whole bacteria. However, these methods gave a high false-positive rate and cross-reactions with other infectious diseases. The newest tests for yersiniosis are based on *Yersinia* outer proteins (YOPs) as antigens and are more specific [12, 13]. However, these tests do not discriminate between infections with *Y. enterocolitica* and *Y. pseudotuberculosis*. The first test (RecomLine immunoblot) for this discrimination has been proposed by Mikrogen Diagnostic where the discrimination is based on two recombinant bacterial proteins—MyfA and PsaA, specific for *Y. enterocolitica* and *Y. pseudotuberculosis*, respectively. These about 17-kDa proteins are structural components of bacterial fibrils and are homologous to the "pH 6 antigen" of *Y. pestis* that is expressed only at 37 °C in acidic pH (pH 6) [2, 14, 15].

Here, we show that *Y. enterocolitica* and *Y. pseudotuberculosis* cultured under the conditions that mimic natural infection route express species-specific surface antigens that can be used in a serological test. Based on this finding, we developed a new ELISA test for detection of yersiniosis and for simultaneous differentiation between infections with *Y. enterocolitica* and *Y. pseudotuberculosis.*

Material and methods

Blood plasma samples

Blood plasma samples from Swedish blood donors and patients diagnosed for rheumatoid arthritis were obtained from the University Hospital in Umeå, Sweden. Blood plasma samples from patients with symptoms of yersiniosis and samples from patients with diagnosed *Borrelia* infection were from the Center of Laboratory Medicine (Dąbrowa Górnicza, Poland, http://www.wielkoszynski.webity.pl/) and from Omnix (St. Petersburg, Russia, http://www.omnix.ru/). All the samples were stored at − 20 °C.

Expression and purification of recombinant MyfA and PsaA expressed in *Escherichia coli*

The cDNAs for MyfA and PsaA (without signal peptides) were synthesized by GenScript Corporation and cloned into pET22a(+) vector using NdeI and XhoI restriction sites. Then, *E. coli* AD494(DE3) (Novagen) was transformed and induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactoside) to produce histidine-tagged MyfA and PsaA.

For purification of MyfA, the bacteria were sonicated in 50 mM Tris-HCl, pH 7.0, with 0.3 M NaCl, and purified on Talon beads (Clontech Laboratories, Inc.) under native conditions according to the manufacturer's instructions.

Bacteria expressing PsaA were lysed in 20 mM Tris-Cl, pH 8, with 20 mM EDTA, 1% Triton X-100, and lysozyme (0.5 mg/ml), and centrifuged. The pellet was dissolved in 10 mM Tris-HCl, pH 8.0, with 50 mM EDTA, 1% Triton X-100, and 0.5 M urea, and the solubilized proteins were renatured by sequential dialyses in 100 mM Tris, pH 8.0, and 0.3 M L-arginine, with step-wise decreasing urea concentrations. After the final dialysis against 50 mM Tris-HCl, pH 7.0, with 0.3 M NaCl, the re-natured PsaA was purified on Talon beads according to the manufacturer's instructions.

The Talon-purified MyfA and PsaA were dialyzed against 25 mM Na-acetate, pH 3.7, and applied on SP-Sepharose columns (GE-Healthcare) equilibrated with 25 mM Na-acetate, pH 4.5. The columns were washed with the equilibration buffer, and proteins were eluted with 25 mM Na-acetate, pH 4.5, with step-wise increasing NaCl (from 0.2 to 2 M). The proteins were then dialyzed against 10 mM phosphate buffer, pH 7.4, with 0.14 M NaCl (PBS) and stored at − 20 °C. The purified MyfA and PsaA were at least 95% pure according to

SDS-PAGE followed by Coomassie blue staining. During the SP-Sepharose purification step, most of *E. coli* LPS (bacterial lipopolysaccharide, measured with Limulus Amebocyte Lysate QCL 1000, Lonza) and contaminating proteins were removed. Removal of the LPS was of particular importance since it can give a high false-positive signal in the ELISA for detection of yersiniosis.

Preparation and inactivation of *Y. enterocolitica* and *Y. pseudotuberculosis*

The virulent strains of *Y. pseudotuberculosis* (strain YPIII/pIB102) and *Y. enterocolitica* (strain W22703; pSW2276; biovar 2, serovar O:9) were kind gifts from Prof. Hans Wolf-Watz [16]. Bacteria were cultivated in brain heart infusion (BHI) medium (Difco Laboratories), pH 7.4, at 26 °C for 18 h (the media for *Y. pseudotuberculosis* were supplemented with 25 μg/ml of kanamycin). The bacteria were then diluted 16-fold with BHI media, pH 6.0, and cultured at 37 °C for 6 h. Finally, the bacteria were inactivated with 4% paraformaldehyde, washed with PBS, and stored in PBS with 0.02% sodium azide at 4 °C.

Yersinia discrimination (YD)-ELISA

In the YD-ELISA, the recombinant MyfA and PsaA as well as the two inactivated *Yersinia* species were used as antigens. MyfA and PsaA were diluted to 5 and 2 μg/ml, respectively, in 100 mM bicarbonate buffer, pH 9.4, and the inactivated *Y. enterocolitica* and *Y. pseudotuberculosis* were diluted in the same buffer to absorbance at 590 nm equal 0.2. Each antigen (100 μl per well) was coated separately on a 96-well Bio-one plate (Greiner) according to a scheme shown in Fig. 1. The plate was incubated at 4 °C for 18 h, then washed three times with PBS-containing 0.1% Tween-20 (PBST) and blocked with 150 μl of 1% bovine serum albumin (BSA, Diagnostic grade K, Millipore) in liquid plate sealer (Candor Bioscience) at room temperature for 4 h. Then, the blocking solution was decanted, the plate was dried at room temperature, and stored at 4 °C. All the plasma samples were diluted 100-fold in PBST supplemented with 0.9% NaCl and 1% BSA, and added in duplicates to the plate (100 μl per well). This indicates that each plasma sample was added to eight wells: to two wells coated with MyfA, two wells coated with PsaA, two wells coated with *Y. enterocolitica*, and two wells coated with *Y. pseudotuberculosis* (see Fig. 1 for an example). The plate was incubated for 1 h at room temperature on a wobbling table (400 rpm) and washed. A 100 μl of antibody against human IgG/IgM/IgA coupled with HRP (Agrisera) diluted in BioStab HRP protector (BioMol Gmbh) were added to each well, and the plate was incubated for 1 h at room temperature on a wobbling table (400 rpm). Following washing, 100 μl of TMB substrate (Agrisera) was added and incubated for 30 min in the dark. Finally, 100 μl of 1 M HCl was added and absorbance at 450 nm (OD) was read. In each plate, a control plasma (pooled plasma from 20 blood donors) was run in triplicate. The cutoff value was calculated as a mean OD for the control plasma plus threefold standard deviation.

Validation of the YD-ELISA

Currently, serological tests used for diagnosis of yersiniosis are based on detection of antibodies for YOP antigens in patient's blood plasma. Therefore, the efficiency of the YD-ELISA to detect yersiniosis was compared with commercial YOP-ELISA (Omnix AB, St. Petersburg, Russia) and YOP-immunoblot tests (Seramun Diagnostica GmbH and Virotech Diagnostics GmbH) that were run according to manufacturer's instructions.

Results and discussion

Preparation of bacterial antigens for *Yersinia* differentiation (YD) ELISA The enteritic *Yersinia* strains undergo a temperature-dependent adaptation during transition from the environment to a host body. *Yersinia* growing at low environmental temperatures have flagella and are motile. However, after entering human body and thus temperature shift to 37 °C, flagella disappear and the bacteria start to produce virulence factors that allow them to invade the host and disseminate [17]. The virulence factors are encoded by so-called pYV plasmid and by chromosomal DNA, and include adhesion proteins (such as Ail and Yad), proteins forming type 3 secretion system (such as outer membrane proteins, YOPs), and lipopolysaccharide (LPS). In addition to the temperature, expression of virulence factors is regulated by pH (for the pH 6 antigens such as MyfA and PsaA) and calcium ion concentration (for YOPs) [2, 14].

Since the route of infection is, to some extent, different for *Y. enterocolitica* and *Y. pseudotuberculosis* [4], we assumed that proteins expressed by the two bacteria species during infection should be, to some extent, different and should induce different antibody responses in the host, allowing for a discrimination for infections with the two bacteria. Therefore, we cultured the *Yersinia* with the temperature and pH shift that resembles natural bacteria propagation and infection, but in the absence of calcium ions. This culturing condition should activate the expression of temperature-regulated virulence factors, but the YOP proteins should not be expressed to high levels [14]. YOPs are highly homologous for both *Y. enterocolitica* and *Y. pseudotuberculosis* and their high expression could hinder the differentiation for infections using the YD-ELISA. As shown below, proteins expressed on bacterial surface under this condition indeed allowed for discrimination between infections by *Y. enterocolitica* and *Y. pseudotuberculosis* in the YD-ELISA.

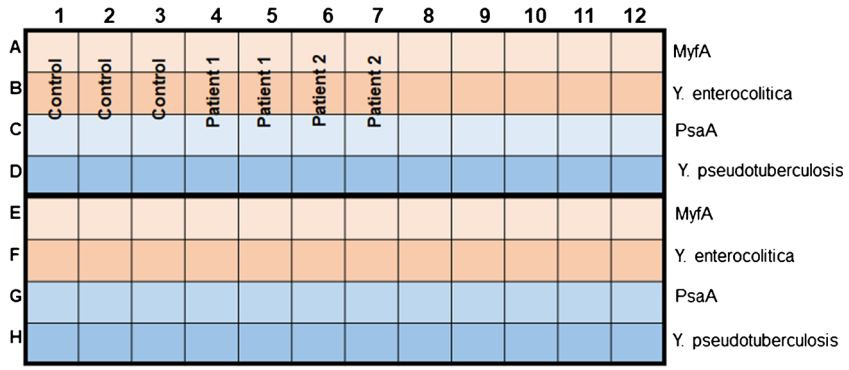

Fig. 1 Scheme for coating and loading of samples for the YD-ELISA. The four antigens used in this ELISA test were coated separately into the 96-well plate. Each patient was tested for all the antigens in duplicates. Pooled plasma from blood donors was used as control for determination of cut-off value

YD-ELISA The YD-ELISA is based on four antigens: the recombinant MyfA and PsaA proteins, and the inactivated *Y. enterocolitica* and *Y. pseudotuberculosis* bacteria. These antigens were coated separately on 96-well plate, and the plate was then incubated with plasma samples from blood donors and patients. In total, 26 plasma samples from healthy blood donors, and 178 plasma samples from patients with symptoms of yersiniosis and/or symptoms for secondary complications after *Yersinia* infections, were analyzed by the YD-ELISA.

The results of YD-ELISA for representative samples from blood donors are shown in Fig. 2a. For each sample, OD values for the four separate antigens are presented. To determine the cutoff value for the ELISA, mean OD value for all donors and antigens and standard deviation were calculated, and the cutoff value was determined as the mean OD value plus threefold standard deviation. From the 26 donor samples analyzed, all were negative for the *Yersinia* antigens with the exception of one donor that most likely was infected by *Y. enterocolitica* earlier in his life (sample number 15 in Fig. 2a). The occurrence of anti-*Yersinia* antibodies was previously reported in German and Finish blood donors [7].

From the 178 patients with symptoms of yersiniosis, 109 patients (61%) were positive for *Yersinia* infection in the YD-ELISA and representative results of the YD-ELISA are shown in Fig. 2b. According to this ELISA, a patient is assumed to be infected with a *Yersinia* when OD value for either of the coated antigens is higher than the cutoff value. A patient is considered infected with *Y. enterocolitica* when OD values for wells coated with MyfA and/or OD values for wells coated with *Y. enterocolitica* are above the cutoff value. In analogy, a patient is considered infected with *Y. pseudotuberculosis* when OD values for wells coated with PsaA and/or OD values for wells coated with *Y. pseudotuberculosis* are above the cutoff value.

Among the 109 *Yersinia*-positive patients, 48% of patients had *Y. enterocolitica* infection and 37% had *Y. pseudotuberculosis* infection (Fig. 3a). Only 15% of the patients had increased OD values for antigens representing both bacteria species (for example patient numbers 1, 4, 6, and 7 in Fig. 2b), and therefore were most likely infected with both bacteria, although not necessary at the same time. For eight patients, repetitive plasma samples were taken for over 1 to 2 years and the OD values from the YD-ELISA were comparable for the different time points, indicating that the levels of anti-*Yersinia* antibodies were stable over this period.

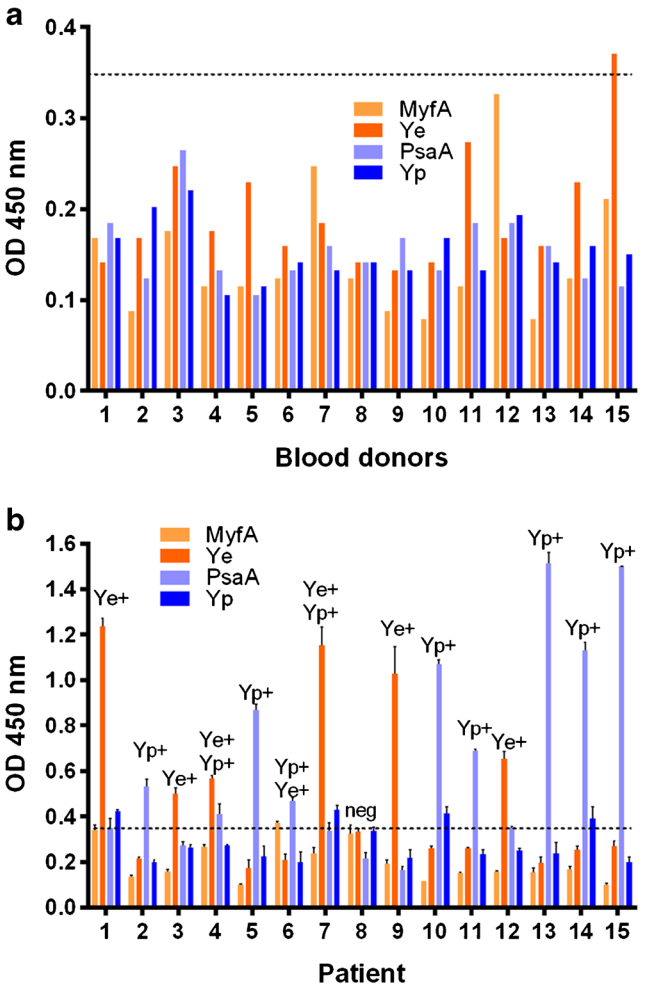

Fig. 2 Representative values obtained in the YD-ELISA for plasma samples from blood donors (**a**) and for plasma samples from patients with symptoms of yersiniosis (**b**). For each sample, OD values for MyfA, PsaA, *Y. enterocolitica* (Ye bacteria) and *Y. pseudotuberculosis* (Yp bacteria) are shown. The OD values represent mean of three separate measurements, and SEM is shown in black for each antigen. The cutoff value is shown as a black dotted line. Ye+ indicates that the patient was diagnosed as infected with *Y. enterocolitica*, and Yp+ indicates that the patient was infected with *Y. pseudotuberculosis*

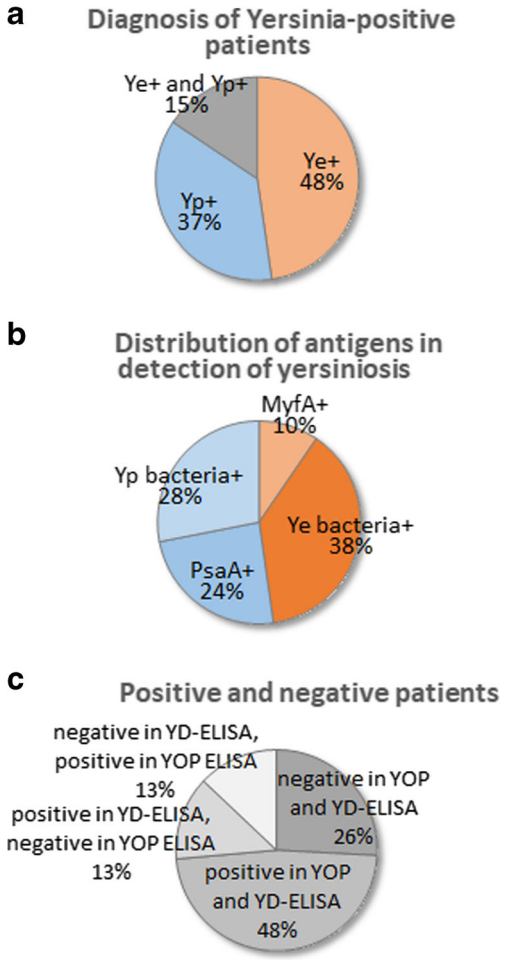

Fig. 3 Characterization of the YD-ELISA. **a** Detection of patients (109 patients tested, assumed as 100%) infected with *Y. enterocolitica* and *Y. pseudotuberculosis* using the YD-ELISA. **b** Response of *Yersinia*-positive patients (109 patients tested, assumed as 100%) to different antigens in the YD-ELISA. **c** Comparison for detection of yersiniosis using the YD-ELISA with commercial YOP tests (178 patients tested, assumed as 100%)

In the *Y. pseudotuberculosis*-infected group, almost the same number of patients had antibodies against PsaA as to against the whole *Y. pseudotuberculosis* bacteria. In the *Y. enterocolitica*-infected group, only 17 patients were positive for recombinant MyfA whereas 68 patients were positive for the whole *Y. enterocolitica* bacteria (Fig. 3b). Recombinant MyfA purified by a single Ni chromatography step was reported to be used in ELISA for detection of *Y. enterocolitica*. In these studies, antibodies to MyfA were detected in as much as 50% of patients positive for YOPs [18]. However, according to our observation, proteins purified by the single chromatography step contain high levels of *E. coli* LPS that gives false-positive results in ELISA.

Table 1 The occurrence of anti-*Yersinia* antibodies in *Borrelia burgdorferi* s.l.-infected patients

Yersinia-positive $N = 7$	*Y. enterocolitica*-positive $N = 4$	MyfA-positive $N = 2$
		Ye-bacteria-positive $N = 2$
	Y. pseudotuberculosis-positive $N = 5$	PsaA-positive $N = 3$
		Yp-bacteria-positive $N = 2$

Between 22 patients with Lyme disease, 7 patients were positive for *Yersinia* infections in the YD-ELISA

Table 2 The occurrence of anti-*Yersinia* antibodies in patients with rheumatoid arthritis

Yersinia-positive $N = 19$ (100%)	*Y. enterocolitica*-positive $N = 11$ (58%)	MyfA-positive $N = 2$ (11%)
		Ye-bacteria-positive $N = 9$ (47%)
	Y. pseudotuberculosis-positive $N = 17$ (90%)	PsaA-positive $N = 7$ (37%)
		Yp-bacteria-positive $N = 17$ (89%)

Between 28 patients with rheumatoid arthritis, 19 patients were positive for *Yersinia* infections in YD-ELISA

Beside the discrimination for infections with the enterocolitic *Yersinias*, the YD-ELISA can also be used for detection of yersiniosis in general. For six patients, feces were tested as positive for *Yersinia* at the clinic and plasma samples from all these patients were also positive for *Yersinia* infection according to the YD-ELISA. Six other patients have been diagnosed with reactive arthritis, and five of them were positive for *Yersinia* infection in the YD-ELISA. In both groups, infections with *Y. enterocolitica* or *Y. pseudotuberculosis* were detected to a similar degree.

The most common serological tests for detection of yersiniosis are YOP-ELISA and YOP-based immunoblot tests. Therefore, we compared the detection of yersiniosis by the YD-ELISA to the results obtained by the commercial YOP-based tests. As shown in Fig. 3c, 26% of the 178 patients tested were negative and 48% were positive for *Yersinia* infections in all the tests, indicating that 74% of samples were identically diagnosed for yersiniosis by the YD-ELISA and YOP tests. About 26% of patients were differently diagnosed the YD-ELISA and YOP tests, with 13% recognized positive only by the YD-ELISA, and 13% recognized as positive by YOP tests only.

Test for cross-reactivity of YD-ELISA with anti-*Borrelia* antibodies Since a high number of plasma samples from patients infected with *Borrelia* recognize the *Yersinia's* YOP proteins in immunoassays, it is generally assumed that there is a cross-reactivity between antibodies produced by patients infected with *Yersinia* species and *Borrelia burgdorferi* s.l. [19, 20]. Here, we analyzed plasma samples from patients diagnosed for Lyme disease (patients that are seropositive for *B. burgdorferi* s.l.) in the YD-ELISA. As shown in Table 1, among the 22 patients tested, 7 patients were *Yersinia*-positive. It is possible that some proteins expressed on the surface of *Borrelia* and *Yersinia* bacteria are similar, which can result in a cross-reactivity in the ELISA. However, we could not find any proteins in *Borrelia* that are

homologous or similar to MyfA and PsaA. Therefore, the positive signal observed in the YD-ELISA for some samples from patients with Lyme disease was most likely due to a *Yersinia* co-infection. In fact, patients infected with *Borrelia* were reported to be co-infected by other bacteria, including *Yersinia* [21].

Occurrence of anti-*Yersinia* antibodies in patients with rheumatoid arthritis (RA) One of the late complications of *Yersinia* infection is reactive arthritis, which sometimes can convert to rheumatoid arthritis [22]. It is known that some patients with RA have antibodies that recognize *Yersinia* antigens [23]. Here, we analyzed plasma samples from 28 patients with RA (patients positive for rheumatoid factor in plasma) using the YD-ELISA. As shown in Table 2, 19 of these patients were *Yersinia*-positive. Almost all *Yersinia*-positive RA patients were positive for *Y. pseudotuberculosis*, and half of them were also positive for *Y. enterocolitica*. This shows that many of the RA patients had antibodies that recognized *Yersinia* antigens and suggests that patients infected with *Y. pseudotuberculosis* have higher probability to develop RA than patients infected with *Y. enterocolitica*.

In conclusion, we provide an evidence that *Y. enterocolitica* and *Y. pseudotuberculosis*, grown in vitro under the conditions that mimic natural infection route, express surface antigens that induce species-specific immune responses in infected people. Therefore, such *Yersinia* cultures can be used for discrimination between infections with the two *Yersinia* species. We also describe a new YD-ELISA where whole *Yersinia* bacteria and two *Yersinia*-specific recombinant proteins (MyfA and PsaA) are used as antigens to detect yersiniosis and to discriminate for infections by *Y. enterocolitica* and *Y. pseudotuberculosis*.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval For this type of study, formal ethical approval is not required.

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