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Fast and Sensitive Detection of Enteropathogenic *Yersinia* by Immunoassays

Jérôme Laporte,[a] Cyril Savin,[b] Patricia Lamourette,[a] Karine Devilliers,[b] Hervé Volland,[a] Elisabeth Carniel,[b] Christophe Créminon,[a] Stéphanie Simon[a]

CEA Saclay, iBiTec-S, Service de Pharmacologie et d'Immunoanalyse, Laboratoire d'Etudes et de Recherches en Immunoanalyse, Gif sur Yvette, France[a]; Institut Pasteur, Yersinia Research Unit, National Reference Laboratory and WHO Collaborating Center, Paris, France[b]

***Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, the two *Yersinia* species that are enteropathogenic for humans, are distributed worldwide and frequently cause diarrhea in inhabitants of temperate and cold countries. *Y. enterocolitica* is a major cause of foodborne disease resulting from consumption of contaminated pork meat and is further associated with substantial economic cost. However, investigation of enteropathogenic *Yersinia* species is infrequently performed routinely in clinical laboratories because of their specific growth characteristics, which make difficult their isolation from stool samples. Moreover, current isolation procedures are time-consuming and expensive, thus leading to underestimates of the incidence of enteric yersiniosis, inappropriate prescriptions of antibiotic treatments, and unnecessary appendectomies. The main objective of the study was to develop fast, sensitive, specific, and easy-to-use immunoassays, useful for both human and veterinary diagnosis. Monoclonal antibodies (MAbs) directed against *Y. enterocolitica* bioserotypes 2/O:9 and 4/O:3 and *Y. pseudotuberculosis* serotypes I and III were produced. Pairs of MAbs were selected by testing their specificity and affinity for enteropathogenic *Yersinia* and other commonly found enterobacteria. Pairs of MAbs were selected to develop highly sensitive enzyme immunoassays (EIAs) and lateral flow immunoassays (LFIs or dipsticks) convenient for the purpose of rapid diagnosis. The limit of detection of the EIAs ranged from 3.2×10^3 CFU/ml to 8.8×10^4 CFU/ml for pathogenic serotypes I and III of *Y. pseudotuberculosis* and pathogenic bioserotypes 2/O:9 and 4/O:3 of *Y. enterocolitica* and for the LFIs ranged from 10^5 CFU/ml to 10^6 CFU/ml. A similar limit of detection was observed for artificially contaminated human feces.**

The genus *Yersinia* belongs to the family of *Enterobacteriaceae* and is composed of three human-pathogenic species: *Yersinia pestis*, the causative agent of the plague, and *Yersinia enterocolitica* and *Y. pseudotuberculosis*, responsible for human enteric yersiniosis. Enteric yersiniosis is a foodborne disease caused by consumption of contaminated food or water (1) and can be transmitted between humans through the fecal-oral route. The disease is usually characterized by a self-limiting acute infection beginning in the intestine and is often limited to the ileocecal junction for *Y. enterocolitica*. In contrast, *Y. pseudotuberculosis* often disseminates deeply to the mesenteric lymph nodes. Clinical presentation is characterized by enterocolitis (diarrhea, abdominal pain, fever, and sometimes vomiting) (2), which predominates in young children and is often self-limiting. However, diarrhea is a predominant symptom of *Y. enterocolitica* infection whereas abdominal pain is more usual in *Y. pseudotuberculosis* infection. Moreover, *Y. pseudotuberculosis* can also cause different clinical symptoms such as scarlatinoid rash, conjunctivitis, acute organ failure, and toxic shock syndrome often reported in Far East (3). For both enteropathogenic *Yersinia* species, more-serious infections and sepsis can also occur, particularly in new-born, elderly, and immunocompromised patients. Sometimes, the infection appears as a pseudoappendicular syndrome in which mesenteric lymph nodes are involved, thus possibly leading to unnecessary appendectomies (4). Some secondary complications such as reactive arthritis and erythema nodosum are sometimes observed (5, 6). Rarely, *Y. enterocolitica* is responsible for a serious sepsis incident after transfusion of contaminated red blood cell preparations (7).

Y. enterocolitica and *Y. pseudotuberculosis* are widespread worldwide, with a higher incidence in cold and temperate regions. Most *Y. enterocolitica* strains associated with human yersiniosis belong to bioserotypes 2/O:9, 4/O:3, 2/O:5,27, 3/O:3, and 1B/O:8 (8). In France and worldwide, serotypes 2/O:9 and 4/O:3 and *Y. pseudotuberculosis* serotypes I and III are the prevailing isolated strains (9). The incidence of human enteric yersiniosis has been estimated to be 16, 1.65, and 0.35 per 100,000 inhabitants in France (10), Europe (11), and the United States (12), respectively, but is probably largely underestimated for many reasons. *Y. enterocolitica* is the third greatest causative agent of diarrhea of bacterial origin in France and Europe after *Campylobacter* and *Salmonella* (11). Even when the incidence of *Y. pseudotuberculosis* is lower, it represents a major public health problem in some countries such as Japan or Russia, where it causes a particular and severe infection known as Far East scarlet-like fever or Izumi fever (13, 14), and in Finland, where multiple outbreaks were observed (15). In France, a sudden onset of *Y. pseudotuberculosis* infections was reported between 2004 and 2005 (16).

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Address correspondence to Stéphanie Simon, stephanie.simon@cea.fr.

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Nowadays, diagnosis of enteric yersiniosis is performed by a direct isolation of enteropathogenic *Yersinia* from stool cultures together with an enrichment in a specific broth before isolation on a semiselective medium known as cefsulodin-irgasan-novobiocin medium (CIN). Since *Yersinia* strains differ by a lower growth rate and a different optimal growth temperature (28°C instead of 37°C) from other enterobacteria, stool cultures performed at 37°C for 24 h (optimal conditions for most enterobacteria) are not efficient for recovering *Yersinia* colonies in the commensal flora. Moreover, isolation, even performed on selective media, needs time-consuming enrichment steps and is poorly successful for *Y. pseudotuberculosis* (17). Finally, detection of enteropathogenic bacteria is generally not required by physicians due to the lack of knowledge about these pathogens. However, personnel in clinical laboratories are becoming more and more conscious of the enteropathogenic *Yersinia* issues and are disposed to perform systematic analysis on feces samples.

After a bacterial colony is isolated, identification of the *Yersinia* species is achieved by a biochemical characterization with commercial systems such as API 20E or 50CH (bioMérieux). For *Y. enterocolitica*, determination of the biotype is necessary to assess the pathogenicity of the strain and is based on a biotype scheme involving supplementary biochemical characterizations (18). For *Y. pseudotuberculosis*, as all strains are considered pathogenic, determination of the biotype is not done. Furthermore, the characterization of serotypes for both enteropathogenic *Yersinia* species can be achieved by seroagglutination of strains. However, this technique is available only in specialized laboratories and serotypes are not necessary related to the pathogenicity of *Y. enterocolitica* (19). Some molecular techniques such as DNA colony hybridization, PCR, real-time PCR, multilocus sequence typing (MLST), and pulsed-field gel electrophoresis (PFGE) have been developed, but only PCR techniques are used for detection (20). However, most of these techniques need isolation of the bacteria or an enrichment step to avoid inhibition due to the complex composition of stool samples (21) and may also require specific devices possibly not present in clinical laboratories (8, 22). Until now, there has been no available standard procedure for identification of all enteropathogenic *Yersinia* enterobacteria.

New diagnostic tools are needed to complete the currently gold standard tests, i.e., the microbiological methods that are quite time-consuming. Ideally, these tests should be rapid, sensitive, specific, efficient when performed on fecal samples, inexpensive, and user-friendly. Among the analytical methods, immunoassays, particularly lateral flow immunoassays, are simple to perform. Some lateral flow immunoassays have already been successfully developed for *Yersinia pestis* (23, 24), *Vibrio cholerae* (25), and other enterobacteria such as *Shigella* spp. (26, 27). The aim of this study was thus to develop sensitive and specific immunoassays using either the enzyme immunoassay (EIA) format or the lateral flow immunoassay (LFI or dipstick) format, targeting enteropathogenic *Yersinia*, for rapid and simple detection in human samples. Monoclonal antibodies (MAbs) were thus raised against two *Y. enterocolitica* bioserotypes (2/O:9 and 4/O:3) and two *Y. pseudotuberculosis* serotypes (I and III).

MATERIALS AND METHODS

Ethics statement. All experiments were performed in compliance with the French and European regulations on care and protection of laboratory animals (European Community [EC] Directive 86/609, French Law 2001-486, 6 June 2001) and with agreement no. 91-416 delivered to S. Simon by the French Veterinary Services and CEA agreement D-91-272-106 from the Veterinary Inspection Department of Essonne (France).

No ethics approval or written consent was necessary because the human sample was recovered from the first author of the article. Verbal informed consent was obtained from the first author.

Reagents. Biotin N-hydroxysuccinimide ester, streptavidin, gold chloride solution, and N-succinimidyl-S-acetyl-thioacetate (SATA) were from Sigma-Aldrich. Goat anti-mouse IgG and IgM polyclonal antibodies were from Jackson ImmunoResearch. Proteinase K was from Bio-Rad. Stabilized goat anti-mouse IgG (H+L)-conjugated horseradish peroxidase (HRP) was from Thermo Fisher. The polyvinylidene difluoride (PVDF) membrane for Western blotting was from Amersham Biosciences. Luminata Forte Western HRP substrate and protein A Sepharose were from Millipore (ProsepA). EIA was performed with MaxiSorp 96-well microtiter plates (Nunc), and all reagents were diluted in EIA buffer (0.1 M phosphate buffer [pH 7.4] containing 0.15 M NaCl, 0.1% bovine serum albumin [BSA], and 0.01% sodium azide). Plates coated with proteins were saturated in EIA buffer (18 h at 4°C) and washed with washing buffer (0.01 M potassium phosphate [pH 7.4] containing 0.05% Tween 20).

Strains and growth conditions. The bacterial strains used in this study are listed in Table 1 and Table 2. All enterobacteria (*Yersinia*, *Escherichia coli*, *Shigella*, and *Salmonella*) were grown in Luria Bertani broth (LB), and *Brucella* species were grown in Trypticase soy agar (TSA) with 5% sheep blood (bioMérieux). Growth temperatures were 28°C or 37°C for *Yersinia* strains and 37°C for other bacteria. Before the assays were performed, all strains were grown overnight in 5 ml LB broth using agitation at 28°C or 37°C. Bacteria were subcultured with 1:100 of the first culture for 4 h under the same conditions.

Production of monoclonal antibodies. Ten-week-old female Biozzi mice were immunized monthly for 3 months by injection of 10^9 heat-inactivated *Yersinia* enterobacteria cultured at 37°C into the footpad to simulate the temperature conditions before an infection. Mice were bled before the first immunization (S0, used as the negative control) and 2 weeks after each injection (S1, S2, and S3). The immune polyclonal response was evaluated by enzyme-linked immunosorbent assay (ELISA) using enteropathogenic *Yersinia* strains as coated antigens (see "Enzyme immunoassays" below). The two mice presenting the highest ELISA titer were selected for preparation of MAbs and given two intravenous booster injections of 10^9 CFU of heat-killed bacteria 2 months after the last immunization. Two days after the last boost, spleen cells from mice were fused with myeloma NS1 cells as previously described (28). The hybridoma culture supernatants were screened for the presence of anti-*Yersinia* antibodies by ELISA (see "Enzyme immunoassays" below). Selected hybridomas were subsequently cloned by limiting dilution, and MAbs were obtained after inducing ascitic fluid in BALB/c mice. MAbs were further purified by affinity chromatography using protein A and dialyzed in 0.05 M phosphate buffer (pH 7.4). Purity was assessed by SDS-PAGE and Coomassie blue staining.

In this study, MAbs obtained after immunization with *Y. enterocolitica* bioserotype 2/O:9 or bioserotype 4/O:3 were called Ye, and MAbs obtained after immunization with *Y. pseudotuberculosis* serotype I or serotype III were called Yps. MAbs called Yp were obtained from a previous study (unpublished work) after immunization with *Y. pestis*.

Enzyme immunoassays. (i) Evaluation of polyclonal response and screening of MAbs in hybridoma supernatants. Anti-*Yersinia* antibodies were detected in sera of immunized mice or hybridoma culture supernatants using ELISA. Briefly, 50 μl of 2×10^8 CFU/ml of enterobacteria in sterile water was added to each well of microtiter plates and allowed to dry overnight (ON) at room temperature (RT). The plates were then saturated with 300 μl/well of EIA buffer ON at 4°C. After a washing cycle was performed with the washing buffer, the plates were incubated with 100 μl/well of each hybridoma culture supernatant or serial dilutions of mouse sera for 2 h at RT. The plates were then washed before the addition

TABLE 1 Specificity and comprehensiveness of the lateral flow immunoassay for *Yersinia enterocolitica* detection[a]

Species	Bioserotype or characteristic(s)	Tested concn (CFU/ml)	Country	Origin	Strain no.	2/O:9 LFI result	4/O:3 LFI result
Yersinia enterocolitica	2/O:9	5×10^6	Belgium	Clinical	IP00383	+	−
	2/O:9	5×10^6	France	Clinical	IP28114	+	−
	2/O:9	5×10^6	France	Clinical	IP29717	+	−
	2/O:9	5×10^6	France	Clinical	IP29193	+	−
	2/O:9	5×10^6	France	Clinical	IP29476	+	−
	2/O:9	5×10^6	France	Clinical	IP29523	+	−
	2/O:9	5×10^6	France	Clinical	IP29944	+	−
	2/O:9	5×10^6	France	Clinical	IP33498	+	−
	2/O:9	5×10^6	France	Clinical	IP33617	+	−
	2/O:9	5×10^6	France	Animal	IP33949	+	−
	2/O:9	5×10^6	France	Clinical	IP34070	+	−
	2/O:9	5×10^6	Netherland	Animal	Ye21	+	−
	2/O:9	5×10^6	Belgium	Clinical	IP4294	+	−
	4/O:3	10^6	Sweden	Clinical	IP00134	−	+
	4/O:3	10^6	Greece	Clinical	IP08896	−	+
	4/O:3	10^6	France	Clinical	IP10393	−	+
	4/O:3	10^6	France	Clinical	IP28096	−	+
	4/O:3	10^6	France	Clinical	IP28164	−	+
	4/O:3	10^6	France	Clinical	IP28983	−	+
	4/O:3	10^6	France	Clinical	IP29001	−	+
	4/O:3	10^6	France	Clinical	IP29310	−	+
	4/O:3	10^6	France	Clinical	IP29534	−	+
	4/O:3	10^6	France	Clinical	IP29610	−	+
	4/O:3	10^6	France	Clinical	IP33526	−	+
	4/O:3	10^6	France	Clinical	IP33550	−	+
	4/O:3	10^6	France	Clinical	IP33563	−	+
	4/O:3	10^6	France	Clinical	IP34075	−	+
	2/O:5	5×10^6	France	Clinical	IP34120	−	−
	1A/NAG	5×10^6	France	Clinical	IP25166	−	−
	1A/O:7,8-8-8,19	5×10^6	France	Animal	IP26014	−	−
	1A/O:10,34	5×10^6	India	Environment	IP26309	−	−
	1A/O:5	5×10^6	Italy	Food	IP26618	−	−
	1A/O:6,30-6,31	5×10^6	France	Clinical	IP29463	−	−
	1A/O:41,42-41,43	5×10^6	France	Clinical	IP29465	−	−
	1A/O:12,25-12,26-25,35-35	5×10^6	France	Clinical	IP29469	−	−
	1A/NAG	5×10^6	France	Clinical	IP29845	−	−
	1A/O:12,25-12,26	5×10^6	France	Clinical	IP33764	−	−
	1A/O:3	5×10^6	France	Clinical	IP27875	−	+
	1A/NAG	5×10^7	France	Clinical	IP33592	−	−
	1B/O:8	5×10^7	USA	Clinical	CIP80.27	−	−
	2/O:5,27	5×10^7	United Kingdom	Animal	CIP106676	−	−
Yersinia pseudotuberculosis	I	5×10^7	Cuba	Animal	IP31629	−	−
	III	5×10^7	France	Clinical	IP33434	−	−
	V	5×10^7	Sweden	Animal	CIP55.88	−	−
Escherichia coli	BL21(DE3)	5×10^7		Invitrogen	NA	−	−
	DH5 alpha	5×10^7		Invitrogen	NA	−	−
	Serotype O26	5×10^7			CIP52.172	−	−
	Serotype O55	5×10^7			CIP52.170	−	−
Shigella sonnei	Lysotype 4	5×10^7			CIP67.63	−	−
Shigella flexneri	Serotype 2	5×10^7			CIP106236	−	−
Salmonella enterica	Serotype Typhimurium	5×10^7			CIP104474	−	−
	Serotype Enteritidis	5×10^7			CIP105150	−	−
	Serotype Paratyphi A	5×10^7			CIP55.155	−	−
	Serotype Senftenberg	5×10^7			CIP105343	−	−

(Continued on following page)

TABLE 1 (Continued)

Species	Bioserotype or characteristic(s)	Tested concn (CFU/ml)	Country	Origin	Strain no.	2/O:9 LFI result	4/O:3 LFI result
Erwinia pyrifoliae		5×10^7			CIP106111	−	−
Brucella abortus	Biotype 4	5×10^7			NCTC10503	−	−
Brucella melitensis	Biotype 2	5×10^7			NCTC10508	−	−
	Biotype 3	5×10^7			NCTC10509	−	−

[a] The bacterial suspensions were grown at 28°C for *Yersinia* or 37°C for other bacteria. +, positive test line estimated by eye; −, negative test line estimated by eye; NA, not applicable; NAG, nonagglutinable; IP, strains from the collection of the Yersinia Research Unit/National Reference Laboratory; CIP, strains from the Collection of the Institut Pasteur; NCTC, strains from the National Counterterrorism Center.

of 100 μl of acetylcholinesterase (AChE; EC 3.1.1.7)-labeled anti-mouse IgG and IgM conjugate (2 Ellman units [EU]/ml [29]) to each well (for AChE labeling details, see below). After 2 h of incubation at RT followed by three washing cycles, 200 μl of Ellman's reagent (30) was added, and the absorbance was measured at 414 nm after 30 min of reaction time.

(ii) Labeling with biotin. A mixture of 0.67 nmol of MAb and 400 μl of borate buffer (0.1 M; pH 8.5) was incubated at a 1:20 molar ratio with biotin-N-hydroxysuccinimide ester (13.3 nmol) dissolved in 6 μl of anhydrous dimethylformamide (DMF). The reaction was stopped after 30 min at RT by adding 100 μl of 1 M Tris-HCl (pH 8) for 30 min. Finally, 500 μl of EIA buffer was added and the preparation was stored frozen at −20°C until use.

(iii) Labeling with AChE. The labeling was performed as described in reference 31. In this procedure, MAbs of the IgG1 isotype (Ye18 and Yps104) were fragmented and reduced to Fab′ fragments in order to make thiol groups accessible, and MAbs of the IgG2b isotype (Ye300 and Yps2) were thiolated with SATA (N-succinimidyl-S-acetyl-thioacetate). Maleimide groups were introduced into the tetrameric form (G4) of AChE by reaction with SMCC [N-succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate]. MAbs were then coupled covalently to acetylcholinesterase (AChE) by the reaction of the thiol groups with the maleimide groups.

(iv) Evaluation of the best MAb pairs. To select the best MAb pairs to develop two-site immunometric tests, a combinatorial analysis was carried out using each MAb as either a capture or a tracer MAb. Immobilization of the capture MAb in microtiter plates was performed overnight at RT after distributing 120 μl/well of the MAb at a concentration of 10 μg/ml in potassium phosphate buffer (0.05 M; pH 7.4). The plates were then emptied, saturated with 300 μl/well of EIA buffer, and kept at 4°C until use (within 1 year). Before use, saturated plates were washed once with washing buffer. Overnight cultures of *Yersinia* and other enterobacteria were adjusted to an optical density of 1 at 600 nm (corresponding to approximately 5×10^8 CFU/ml) in EIA buffer. Then, 50 μl/well of serial dilutions of bacteria and 50 μl/well of biotin-labeled MAb as a tracer (500 ng/ml) were distributed in duplicate in the microtiter plates coated with the various capture antibodies to be tested. EIA buffer was used as a negative control. After an ON incubation at 4°C and three washing cycles, 100 μl/well of AChE-labeled streptavidin (2 Ellman units/ml) was added for 2 h at RT. Plates were then washed 3 times before the addition of 200 μl/well of Ellman's reagent and measurement of the absorbance at 414 nm after 30 min at RT. For the following EIA, 100 μl of the bacterial sample and 100 μl of AChE-labeled MAb (2 or 5 EU/ml final concentration) were distributed in duplicate into the wells in order to obtain better sensitivity. Microtiter plates were optionally centrifuged for 5 min at 1,000 × *g* and incubated for 30 min or 3 h at RT. After three washing cycles, 200 μl/well of Ellman's reagent was added and the plates were kept at RT for 30 min before measurement of the absorbance at 414 nm was performed. Each measurement was independently performed three times. The five-parameter logistic fit (5-PL) function (GraphPad Prism 5) was used to fit the standard curve. The limit of detection (LoD) was calculated as the concentration providing a signal corresponding to the average background signal (as measured for 8 wells) plus 3 standard deviations.

Statistical analysis. Statistical significance was assessed using an unpaired *t* test for comparisons between values of signals. Results were considered statistically significant when *P* values of <0.05 were obtained.

Western blot experiments. Bacterial cultures of *Yersinia* were grown at 28°C to obtain similar immunogenicities of the *in vivo* lipopolysaccharide (LPS) (32). Cells were suspended in Laemmli buffer containing 2% SDS and separated into two samples. One sample was treated with proteinase K (80 μg/ml) during 30 min at 37°C. All samples were then denatured for 5 min at 95°C, and 10^7 to 10^8 CFU per well was subjected to SDS-PAGE for 1 h 30 min at 150 V in a 16% gel. Transfer onto a PVDF membrane was performed ON at RT at 25 V. For the saturation step, the membrane was blocked with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) with 5% skimmed dry milk during 30 min at RT. After two washes in TBST, specific MAbs (4 μg/ml in TBST containing 1% skimmed dry milk) were incubated for 30 min at RT with the membrane. After three washes in TBST, the membrane was incubated for 20 min at RT with HRP-labeled polyclonal goat anti-mouse immunoglobulins diluted to 1:5,000 in TBST containing 3% skimmed dry milk. After three washes in TBST and a brief wash in TBS, bands were detected by chemiluminescence.

Lateral flow immunoassays. The colloidal-gold-labeled MAb was prepared by adding 25 μg of MAb to 1 ml of colloidal gold before mixing with 100 μl of 0.02 M borax buffer (pH 9.3) was performed. The reaction mixture was incubated for 1 h in the dark at RT. Then, 100 μl of 0.02 M borax buffer (pH 9.3) containing 1% BSA was added and the mixture was centrifuged at 15,000 × *g* for 50 min at 20°C. The supernatant was discarded, and the pellet was suspended in 250 μl of 0.002 M borax buffer (pH 9.3) containing 1% BSA, sonicated for a few seconds, and stored at 4°C in the dark until use. The strips (0.5 cm in width and 4.5 cm in length) were composed of 3 parts (33), (i) a sample pad (Standard 14; Whatman) (0.5 cm in length), (ii) a nitrocellulose membrane (Prima 40 or Hi-Flow Plus 75; Whatman or Millipore, respectively) (2.5 cm in length), and (iii) an absorption pad (Cellulose grade 470; Whatman) (1.5 cm in length), all attached to a backing card. The detection zone contained immobilized goat anti-mouse antibodies as a control line and an anti-*Yersinia* MAb as a test line (1 or 2 mg/ml in 0.05 M sodium phosphate buffer; pH 7.4) dispensed at 1 μl/cm using an automatic dispenser (Airjet XYZ 3050; BioDot). After drying for 1 h at 40°C in an air oven, the membrane was incubated with a blocking solution (PBS [pH 7.4] containing 0.5% BSA) for 30 min at RT. The membrane was washed twice with deionized water, incubated for 20 min at RT in a preserving solution (PBS containing 0.1% Tween 20 and 7.5% glucose), and then dried for 20 min at 40°C in an air oven. After the absorption pad and the sample pad were fixed to the top and the bottom of the membrane, respectively, the card was cut into strips 5 mm in width using an automatic programmable cutter (CM4000 Guillotine cutting system; BioDot). During the experiment, 100 μl of bacterial suspensions in analysis buffer (EIA containing 0.5% Tween 20) was mixed with 10 μl of colloidal-gold-labeled antibodies (20 μg/ml) in the wells of a 96-well microtiter plate. After 10 min of incubation of the mixture at RT, the strips were inserted into the wells. The capillary migration from the bottom of the sample pad to the absorption pad in the upper position

TABLE 2 Specificity and comprehensiveness of the lateral flow immunoassay for *Yersinia pseudotuberculosis* detection[a]

Species	Serotype or characteristic(s)	Tested concn (CFU/ml)	Country	Origin	Strain no.	Yps LFI result
Yersinia pseudotuberculosis	I	10^6	Morocco	Animal	IP30636	+
	I	10^6	Tunisia	Animal	IP30642	+
	I	10^6	Cuba	Animal	IP31629	+
	I	10^6	Poland	Animal	IP32080	+
	I	10^6	Hungary	Animal	IP32414	+
	I	10^6	former Czechoslovakia	Animal	IP32575	+
	I	10^6	Russia	Animal	IP33178	+
	I	10^7	Hungary	Animal	IP30842	+
	I	10^7	Cuba	Animal	IP31630	+
	I	10^7	Chile	Clinical	IP32654	+
	I	10^7	former Yugoslavia	Animal	IP32665	+
	I	10^7	England	Animal	IP32670	+
	I	10^7	Switzerland	Clinical	IP32730	+
	I	10^7	Switzerland	Clinical	IP32907	+
	I	10^7	France	Clinical	IP32953	+
	I	10^7	Switzerland	Clinical	IP32989	+
	I	10^7	Russia	Animal	IP33242	+
	I	10^7	Russia	Clinical	IP33247	+
	I	10^7	France	Animal	IP33424	+
	I	10^7	France	Clinical	IP33438	+
	I	10^7	former Yugoslavia	Animal	IP32651	−
	I	10^7	France	Clinical	IP32777	−
	I	10^7	Italy	Animal	IP32784	−
	I	10^7	Italy	Animal	IP32800	−
	I	10^7	Switzerland	Clinical	IP32906	−
	I	10^7	France	Clinical	IP32950	−
	I	10^7	France	Clinical	IP32953	−
	I	10^7	USA	unknown	IP33035	−
	I	10^7	France	Clinical	IP33053	−
	I	10^7	France	Clinical	IP33109	−
	I	10^7	France	Clinical	IP33285	−
	I	10^7	France	Animal	IP33427	−
	II	10^7	France	Clinical	IP32554	+
	II	10^7	Spain	Animal	IP32584	+
	II	10^7	New Zealand	Clinical	IP32589	+
	II	10^7	France	Clinical	IP32598	+
	II	10^7	France	Animal	IP32870	+
	II	10^7	France	Clinical	IP32951	+
	II	10^7	France	Clinical	IP33006	+
	II	10^7	France	Clinical	IP33047	+
	II	10^7	France	Animal	IP33098	+
	II	10^7	France	Clinical	IP33306	+
	III	5×10^6	Spain	Clinical	IP32666	+
	III	5×10^6	Italy	Animal	IP32787	+
	III	5×10^6	Argentina	Animal	IP32976	+
	III	5×10^6	Australia	Animal	IP32990	+
	III	5×10^6	France	Animal	IP33049	+
	III	5×10^6	Argentina	Animal	IP33104	+
	III	5×10^6	Russia	Clinical	IP33185	+
	III	5×10^6	Russia	Clinical	IP33250	+
	III	5×10^6	Argentina	Animal	IP33297	+
	III	5×10^6	France	Clinical	IP33377	+
	III	5×10^6	France	Clinical	IP33434	+
	IV	5×10^6	Japan	Animal	Ryster	+
	IV	5×10^6	Japan	Animal	IP30103	+
	IV	5×10^6	former USSR	Animal	IP30290	+
	IV	5×10^6	former USSR	Animal	IP30291	+
	IV	5×10^6	England	Clinical	IP30298	+
	IV	5×10^6	Denmark	Animal	IP31411	+
	IV	5×10^6	England	Clinical	IP31830	+

(Continued on following page)

TABLE 2 (Continued)

Species	Serotype or characteristic(s)	Tested concn (CFU/ml)	Country	Origin	Strain no.	Yps LFI result
	IV	5×10^6	England	Animal	IP31833	+
	IV	5×10^6	France	Animal	IP32687	+
	IV	5×10^6	Russia	Animal	IP33234	+
	V	10^7	Sweden	Animal	CIP55.88	+
	V	10^7	Switzerland	Animal	IP32463	+
	V	10^7	France	Animal	IP32699	+
	V	10^7	France	Animal	IP32727	+
	V	10^7	Japan	Unknown	IP32814	+
	V	10^7	Japan	Unknown	IP32817	+
	V	10^7	France	Clinical	IP32821	+
	V	10^7	France	Clinical	IP32843	+
	V	10^7	Germany	Animal	IP33061	+
	V	10^7	Russia	Clinical	IP33278	+
	V	10^7	France	Clinical	IP33397	+
Yersinia similis	O:1c	10^7	Japan	Environment	Kuratani-2	−
	O:6	10^7	Germany	Animal	CIP109846	−
	O:6	10^7	Japan	Animal	R116	−
	O:7	10^7	Japan	Animal	R2091-2	−
	O:11	10^7	Japan	Animal	R2031	−
Yersinia wautersii	O:4a	10^7	Japan	Clinical	#51	−
	O:4a	10^7	Germany	Animal	Y428	−
	O:11	10^7	Korea	Environment	WP-930601	−
	O:11	10^7	Korea	Environment	WP-931205	−
	O:15	10^7	Korea	Clinical	12-219N1	−
Yersinia enterocolitica	2/O:9	5×10^7	Belgium	Clinical	IP00383	−
	4/O:3	5×10^7	Sweden	Clinical	IP00134	−
	1A/NAG	5×10^7	France	Clinical	IP33592	−
	1B/O:8	5×10^7	USA	Clinical	CIP80.27	−
	2/O:5,27	5×10^7	United Kingdom	Animal	CIP106676	−
Escherichia coli	BL21(DE3)	5×10^7		Invitrogen	NA	−
	DH5 alpha	5×10^7		Invitrogen	NA	−
	Serotype O26	5×10^7			CIP52.172	−
	Serotype O55	5×10^7			CIP52.170	−
Shigella sonnei	Lysotype 4	5×10^7			CIP67.63	−
Shigella flexneri	Serotype 2	5×10^7			CIP106236	−
Salmonella enterica	Serotype Typhimurium	5×10^7			CIP104474	−
	Serotype Enteritidis	5×10^7			CIP105150	−
	Serotype Paratyphi A	5×10^7			CIP55.155	−
	Serotype Senftenberg	5×10^7			CIP105343	−
Erwinia pyrifoliae		5×10^7			CIP106111	−
Brucella abortus	Biotype 4	5×10^7			NCTC10503	−
Brucella melitensis	Biotype 2	5×10^7			NCTC10508	−
	Biotype 3	5×10^7			NCTC10509	−

[a] The bacterial suspensions were grown at 28°C for *Yersinia* or 37°C for other bacteria. +, positive test line estimated by eye; −, negative test line estimated by eye; NA, not applicable; NAG, nonagglutinable; IP, strains from the collection of the Yersinia Research Unit/National Reference Laboratory; CIP, strains from the Collection of the Institut Pasteur. *Y. wautersii* strain Y428 was kindly provided by the Thüringer Landesamt für Verbraucherschutz (TLV), Bad Langensalza, Germany. Other *Y. similis* and *Y. wautersii* strains were from the collection of the Shimane Prefectural Institute of Public Health and Environmental Science, Matsue, Japan.

lasted for about 30 min. The signal intensities of the test and control lines were visually estimated.

Detection in artificially spiked stool cultures. A pea-size sample, corresponding to approximately 1 g of feces from a healthy individual, was diluted in 10 ml of EIA buffer for the enzyme immunoassay or in 10 ml of EIA buffer containing 0.5% Tween 20 (feces buffer) for the LFI. Overnight cultures of enteropathogenic *Yersinia* and other enterobacteria grown in LB broth at 28°C or 37°C were adjusted (based on optical density at 600

nm) to 5×10^7 CFU/ml in feces buffer and serially diluted in the same buffer either for EIA or for LFI just before analysis was performed using the conditions described above. The exact concentration of bacteria was then determined by enumeration of serial dilutions in EIA buffer.

RESULTS

Production and selection of anti-*Yersinia* MAbs. After fusion of spleen cells with myeloma cells, hybridomas were screened by ELISA for the presence of specific antibodies directed against bioserotypes 2/O:9 and 4/O:3 of *Y. enterocolitica* and serotypes I and III of *Y. pseudotuberculosis*. As the aim of this study was to obtain antibodies specific for each of the prevailing species and bioserotypes of *Yersinia* strains, selection of antibodies was performed by differential-screening ELISA, using plates coated with different bacterial strains. A first selection of antibodies was achieved using a species/bioserotype screening (for example, *Y. enterocolitica* 2/0:9 was screened against *Y. enterocolitica* 4/O:3 and *Y. pseudotuberculosis* I and III) and a second one using a genus screening (against other Gram-negative enterobacteria: *E. coli*, *Shigella sonnei*, and *Salmonella enterica* serovar Typhimurium). These screenings led to the selection of 4 series of 15, 14, 14, and 17 specific MAbs against *Y. enterocolitica* 2/O:9, *Y. enterocolitica* 4/O:3, *Y. pseudotuberculosis* I, and *Y. pseudotuberculosis* III, respectively.

Combinatorial analyses were performed using biotinylated antibodies as tracer antibodies in a two-site immunometric format by testing (i) their specificities for the genus *Yersinia* compared to other enterobacteria (*E. coli*, *S. sonnei*, and *S. enterica*), (ii) their specificities in comparisons between the two enteropathogenic *Yersinia* species (*Y. enterocolitica* and *Y. pseudotuberculosis*), and (iii) their specificities in comparisons between the different bioserotypes of *Y. enterocolitica* strains (2/O:9 or 4/O:3 against 1A, 1B, and 2/O:5,27) and different serotypes of *Y. pseudotuberculosis* strains (I, III, and V). As shown in Table S1 in the supplemental material, among the 225 combinations tested, 56 detected *Y. enterocolitica* 2/O:9 but 8 appeared to lack specificity, with detection of *E. coli* also. For *Y. enterocolitica* 4/O:3 (as shown in Table S2 in the supplemental material), only a few (11 of 169) combinations were positive, all specific for the expected strain. As shown in Table S3 in the supplemental material, among the 196 *Y. pseudotuberculosis* I tests, 52 were shown to detect the strain but 13 exhibited cross-reactivity (CR) with unrelated strains such as other enterobacteria tested and *Y. enterocolitica* bioserotype 4/O:3. Finally, for *Y. pseudotuberculosis* III (shown in Table S4), most (265) of the 289 combinations strongly detected the expected strain and 16 of those were devoid of specificity, with nonspecific detection of the other enterobacteria tested. A second round of testing was performed for the positive and specific combinations by evaluating their sensitivity using serial dilutions of the related *Yersinia* strains (data not shown). For each MAb series (anti-*Y. enterocolitica* 2/O:9 anti-*Y. enterocolitica* 4/O:3, anti-*Y. pseudotuberculosis* I, and anti-*Y. pseudotuberculosis* III), the MAb tracers allowing the best sensitivity (Ye18*, Ye300*, Yps2*, and Yps104*) were chosen for further development.

Optimization of the EIA. The 4 MAbs selected for tracer preparation were labeled with AChE to reduce the number of enzyme immunoassay (EIA) steps and to possibly improve the sensitivity of the tests. Combinatorial analyses using serial dilutions of the related *Yersinia* strains were performed to determine which capture MAbs provided the best sensitivity in association with these AChE-labeled MAbs. Four MAb pairs were finally retained: Ye4 and Ye18* for *Y. enterocolitica* 2/O:9, Ye300 and Ye300* for *Y. enterocolitica* 4/O:3, Yps2 and Yps2* for *Y. pseudotuberculosis* I, and Yps108 and Yps104* for *Y. pseudotuberculosis* III. The influence of different assay parameters (temperature, centrifugation step at the beginning of the incubation step, length of incubation) was evaluated, finally leading to an optimized assay that was performed for 3 h 30 min and included a centrifugation step compared to the initial 20-h format.

The specificity of the immunoassays was checked using a high concentration of enterobacteria (5×10^7 CFU/ml) cultured under their optimal growth conditions by comparing the results determined with their related *Yersinia* species to the results determined with other genera (Fig. 1A) and other species and bioserotype (Fig. 1B). As observed (Fig. 1), only the *Y. pseudotuberculosis* I EIA exhibited faint cross-reactivity (CR; calculated as the ratio of the absorbance unit values obtained for nonspecific and specific bacteria at the same concentration $\times$ 100) with unrelated *E. coli* strains (CR = <3%) and some *Y. enterocolitica* and *Y. pseudotuberculosis* strains (CR = <5%). The 3 other EIAs appeared to be totally specific, even though *Y. enterocolitica* 4/O:3 EIA provides a limited signal for this important concentration compared to the other EIAs, possibly indicating a difference of sensitivity. The optical signal was obtained for all MAb pairs selected.

To evaluate both the sensitivity of these EIAs and the possible influence of the bacterial growth temperature, serial dilutions of the related *Yersinia* strain cultured at two temperatures (28°C and 37°C) were assayed. All the EIAs allowed sensitive detection in the 10^4 to 10^5 CFU/ml range. On the other hand, as expected, better signals were obtained with a growth temperature of 28°C for *Y. enterocolitica* 2/O:9 and *Y. pseudotuberculosis* III, while, surprisingly, 37°C appeared more favorable for *Y. pseudotuberculosis* I (Fig. 2). The signals were not significantly different for *Y. enterocolitica* 4/O:3 at the two temperatures. However, calculated LoD values were close for each of the targets, whatever the growth temperature, as illustrated in Table 3. It is worth noting that for the 37°C temperature setting, the optimized procedure provided an increase in sensitivity of at least 14-fold compared to the EIA performed under the initial conditions.

Enzyme immunoassay with artificially contaminated stool cultures. To validate these new EIAs, artificially contaminated stool samples were tested. The genus, the species, and the bioserotype specificities were checked as described above (see "Optimization of the EIA" above) using various *Yersinia* strains (*Y. enterocolitica* 2/O:9, 4/O:3, 1A 1B, and 2/O:5,27 and *Y. pseudotuberculosis* I, III, and V) and other enterobacteria (*E. coli* BL21, O26, and O55, *S. sonnei*, *S. flexneri*, *S. enterica* serovars Typhimurium, Enteritidis, Paratyphi A, and Senftenberg, and *Erwinia pyrifoliae*). As previously obtained with bacteria in simple EIA buffer, a specific signal was recovered for *Y. enterocolitica* 2/O:9, *Y. enterocolitica* 4/O:3, *Y. pseudotuberculosis* I, and *Y. pseudotuberculosis* III with MAb pairs Ye4 and Ye18*, Ye300 and Ye300*, Yps2 and Yps2*, and Yps108 and Yps104*, respectively.

Analyzing the signal intensity and the LoDs, the same influence of growth temperature was observed for the stool samples as was observed previously with samples in EIA buffer. As shown in Table 3, no clear change in sensitivity was induced by the stool medium, thus indicating the absence of an important matrix effect for the EIAs, except for the *Y. enterocolitica* 2/O:9 and *Y. pseudotuberculosis* I EIAs, for which the LoDs increased 20-fold and 35-fold, respectively, when bacteria were grown at 37°C.

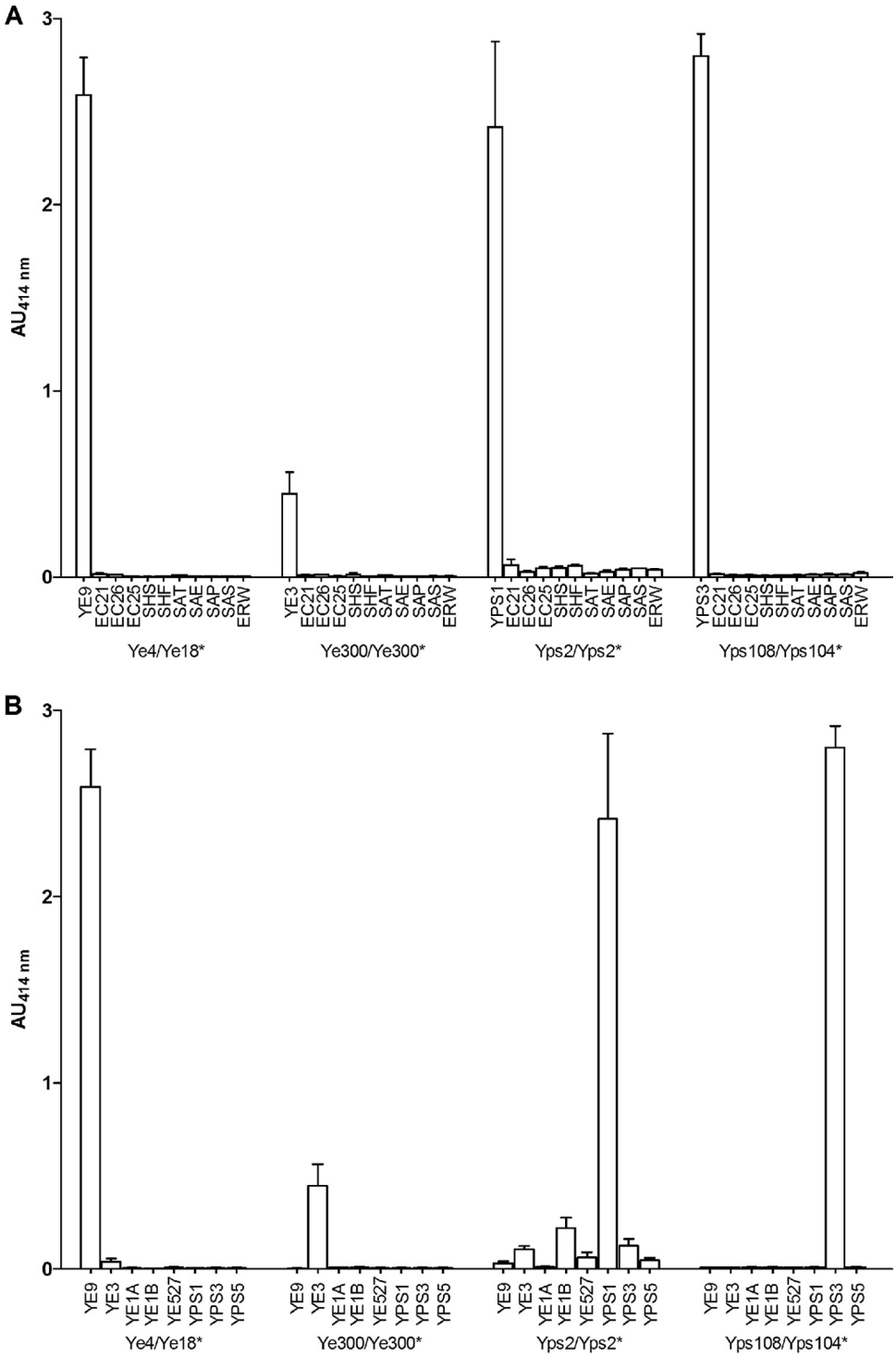

FIG 1 Specificity of *Yersinia*-optimized enzyme immunoassays. *Yersinia* enterobacteria were grown at 28°C for EIAs using Ye4/Ye18*, Ye300/Ye300*, and Yps108/Yps104* and at 37°C for an EIA using Yps2/Yps2* (the * indicates the tracer antibody). Other enterobacteria were grown at 37°C. Each strain was used at the concentration of 5×10^7 CFU/ml and was incubated during 3 h. Absorbance was measured at 414 nm after 30 min of incubation with Ellman's reagent. YE9, *Y. enterocolitica* 2/O:9; YE3, *Y. enterocolitica* 4/O:3; YE1A, *Y. enterocolitica* 1A; YE1B, *Y. enterocolitica* 1B; YE527, *Y. enterocolitica* O:5,27; YPS1, *Y. pseudotuberculosis* I; YPS3, *Y. pseudotuberculosis* III; YPS5, *Y. pseudotuberculosis* V; EC21, *E. coli* BL21; EC26, *E. coli* O26; EC25, *E. coli* O55, SHS, *Shigella sonnei*; SHF, *Shigella flexneri*; SAT, *Salmonella enterica* Typhimurium; SAE, *Salmonella enterica* Enteritidis; SAP, *Salmonella enterica* Paratyphi A; SAS, *Salmonella enterica* Senftenberg; ERW, *Erwinia pyrifoliae*. (A) Genus specificity of *Yersinia* compared to other enterobacteria. AU, absorbance units. (B) Species and biotype/serotype specificities compared to *Y. enterocolitica* strains (2/O:9, 4/O:3, 1A 1B, 2/O:5,27) and *Y. pseudotuberculosis* strains (I, III, V).

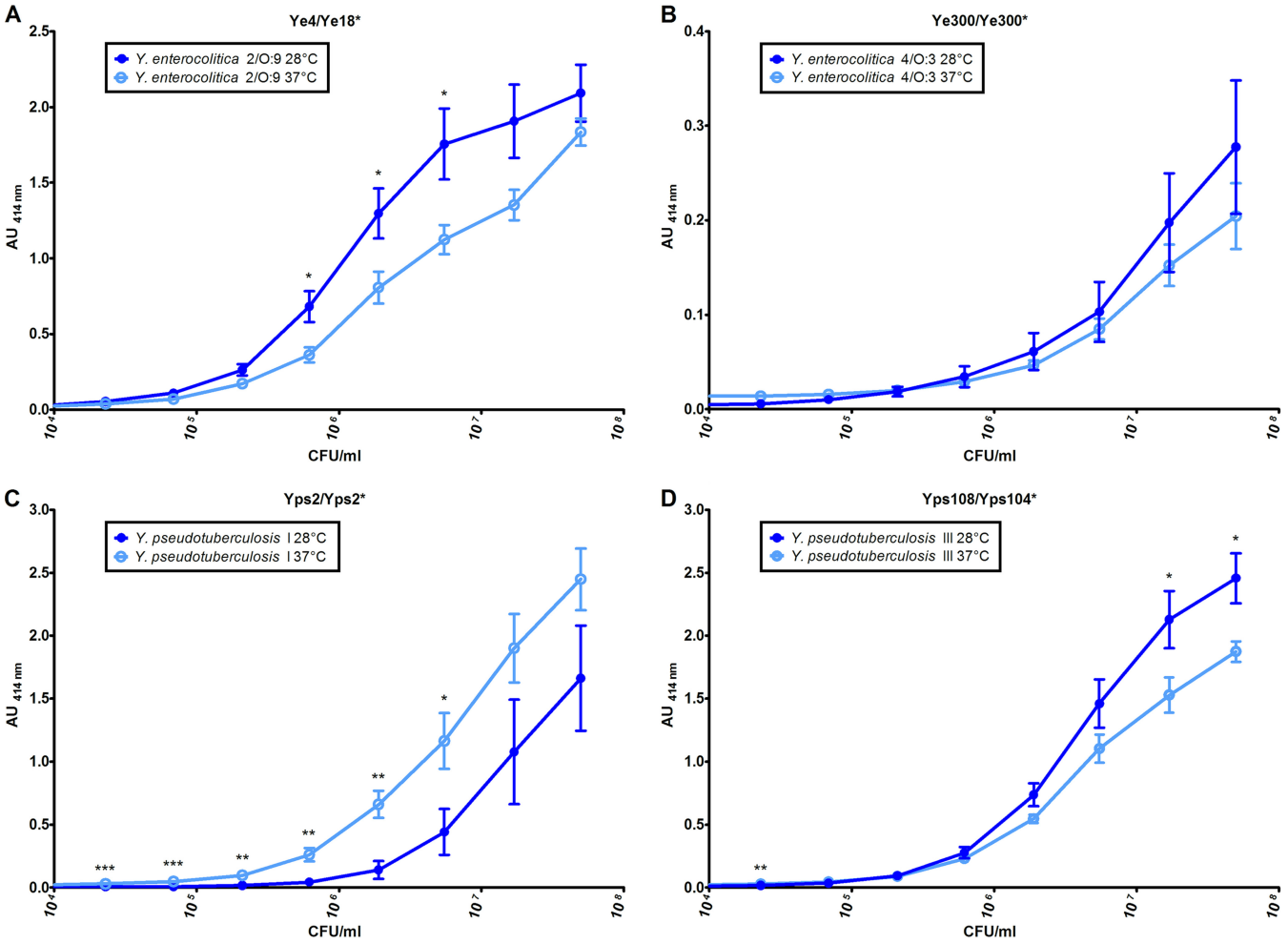

FIG 2 Impact of the growth temperature on detection of *Yersinia* by optimized enzyme immunoassays. *Yersinia* species were grown at 28°C (dark-blue curve) and 37°C (light-blue curve), and 10-fold serial dilutions were performed before a 3-h incubation. (A) EIA Ye4/Ye18* for detection of *Y. enterocolitica* 2/O:9. (B) EIA Ye300/Ye300* for detection of *Y. enterocolitica* 4/O:3. (C) EIA using Yps2/Yps2* for detection of *Y. pseudotuberculosis* I. (D) EIA using Yps108/Yps104* for detection of *Y. pseudotuberculosis* III. Asterisks indicate values that are significantly different. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (for comparisons between signals).

Analysis of the nature of the antigens recognized by the antibodies by the use of Western blotting. In order to determine if the recognized antigens were proteins, samples were treated or not with proteinase K prior to Western blot analysis (Fig. 3). The immunoblot analysis showed that only MAb Yps2 on the *Y. pseudotuberculosis* I extract recognized a band of approximately 70 kDa that was sensitive to proteinase K treatment, indicating that

TABLE 3 Limits of detection of the enzyme immunoassays for *Yersinia* detection in EIA buffer or in artificially contaminated stool samples[a]

Strain	Limit of detection (CFU/ml)			
	EIA buffer ($n = 3$)		Stool samples ($n = 3$)	
	28°C	37°C	28°C	37°C
Y. enterocolitica 2/O:9	3.4×10^3	4.9×10^3	3.4×10^4	1.3×10^4
Y. enterocolitica 4/O:3	7.8×10^4	1.4×10^5	5.2×10^4	7.5×10^4
Y. pseudotuberculosis I	2.0×10^5	1.2×10^3	5.4×10^6	2.6×10^5
Y. pseudotuberculosis III	1.9×10^4	1.1×10^4	4.7×10^4	3.3×10^4

[a] *Yersinia* enterobacteria were grown at 28°C and 37°C.

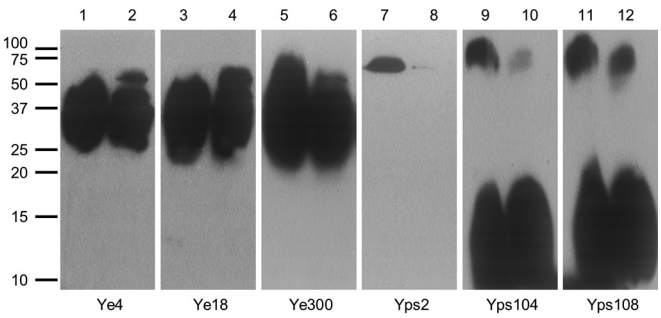

FIG 3 Recognition of protein and LPS epitopes with anti-*Yersinia* MAbs. Western blotting was performed with Ye4 (lanes 1 and 2) and Ye18 (lanes 3 and 4) against *Y. enterocolitica* 2/O:9 (10^7 CFU/well), with Ye300 against *Y. enterocolitica* 4/O:3 (10^7 CFU/well) (lanes 5 and 6), with Yps2 against *Y. pseudotuberculosis* I (10^7 CFU/well) (lanes 7 and 8), and with Yps 104 (lanes 9 and 10) and Yps108 (lanes 11 and 12) against *Y. pseudotuberculosis* III (10^8 CFU/well). Bacterial samples in lanes 2, 4, 6, 8, 10, and 12 were treated with proteinase K. Numbers on the left indicate the molecular mass markers (in kDa).

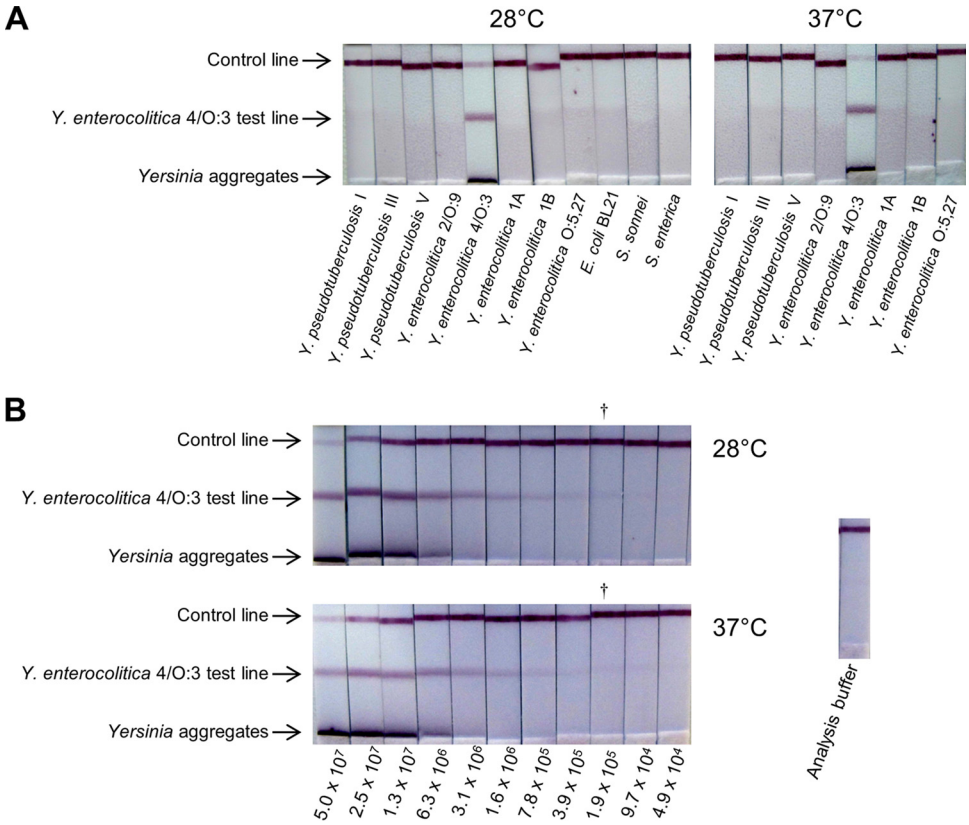

FIG 4 Ye4O/3 lateral flow immunoassay. Bacteria were grown at 28°C and 37°C. The bacterial suspensions were incubated for 10 min with colloidal gold-labeled Ye300* MAb, and the Ye300 dipsticks were then dipped for 30 min into 100 μl of the bacterial suspensions for upward migration of the liquid. (A) Specificities of the Ye4/O:3 LFI. Each strain was used at the concentration of 5×10^7 CFU/ml. (B) Sensitivities of the Ye4/O:3 LFI for serial dilution of *Y. enterocolitica* 4/O:3. Numbers below the dipsticks indicate the number of CFU/ml. †, the last dipstick with a visible signal estimated by eye.

the MAb Yps2 bound protein epitope (lanes 7 and 8). For the other MAbs, Ye4 and Ye18 on *Y. enterocolitica* 2/O:9 extract and Ye300 on *Y. enterocolitica* 4/O:3 extract, proteinase K treatment did not induce any clear modification of the recognition results. For MAbs Yps104 and Yps108 on *Y. pseudotuberculosis* III extract, only the high molecular band around 100 kDa appeared partially sensitive to proteinase K treatment, with a signal diminution and a shift of the molecular mass to approximately 80 kDa (lanes 9 to 12). Moreover, a typical pattern profile of long-chain LPS without a ladder was observed on the immunoblot analysis using MAbs Ye4, Ye18, and Ye300 (lanes 1 to 6) (34). For MAbs Yps104 and Yps108, another typical pattern was observed, with signal for the core LPS and less signal on the long-chain LPS (lanes 9 to 12) (35). Therefore, all these samples presented a characteristic smear pattern profile of LPSs, indicating that these MAbs presumably interacted with LPSs of their related *Yersinia* strains.

Development of LFIs. To set up an even faster and easier handling test, i.e., a lateral flow immunoassay (LFI), all MAb pairs displaying a specific signal for enteropathogenic *Yersinia* detection in the previous immunoenzymatic assay were reevaluated during the LFI development. As the format of the test can influence the specificity, we first checked the genus/species/bioserotype specificities, using 5×10^7 CFU/ml (5×10^6 CFU/dipstick) of various *Yersinia* and other enterobacterial species (*E. coli* BL21, O26, and O55, *S. sonnei*, *S. flexneri*, *S. enterica* serovars Typhimurium, Enteritidis, Paratyphi A, and Senftenberg, and *Erwinia pyrifoliae*). Then, the sensitivity of the selected specific LFI was evaluated using serial dilutions of the related *Yersinia* strains. The best MAb combinations for detecting *Y. enterocolitica* 2/O:9 and *Y. enterocolitica* 4/O:3 were the Ye4 and Ye18* pair (2/O:9 LFI) and the Ye300 and Ye300* pair (4/O:3 LFI), respectively. MAb pair Yp7 and Yp7* recognizing serotypes I, II, IV, and V of *Y. pseudotuberculosis* and MAb pair Yps103 and Yps104* recognizing serotype III were combined to achieve a single LFI allowing *Y. pseudotuberculosis* detection for serotypes I, II, III, IV, and V (Yps LFI). Since the results were similar for all the different LFIs, the results obtained for the 4/O:3 LFI are presented as an illustration (Fig. 4). The different LFIs proved to be as specific as the optimized EIA, and no cross-reactivity was observed with enterobacteria other than the related enteropathogenic *Yersinia* strains (see Fig. 4A for Ye4/O:3 LFI). Although *Y. enterocolitica* O:9 LPS exhibits a strong similarity to *Brucella* LPS (35), no cross-reactivity was observed for the *Y. enterocolitica* 2/O:9 LFI with *Brucella* strains (*B. abortus* and *B. melitensis*). The Ye4/O:3 LFI and Yps LFI were also tested with *Brucella* strains and provided the same negative result.

The sensitivities obtained for the different LFIs were 5×10^5 CFU/ml and 10^5 CFU/ml for detection of *Y. enterocolitica* 2/O:9 and *Y. enterocolitica* 4/O:3, respectively (see Fig. 4B for Ye4/O:3 LFI). The LoDs of the Yps LFI for *Y. pseudotuberculosis* detection were close to 10^5 CFU/ml for serotypes I, 5×10^5 CFU/ml for serotypes III and IV, and 10^6 CFU/ml for serotypes II and V. As expected, the LFIs appeared less sensitive than optimized EIA but

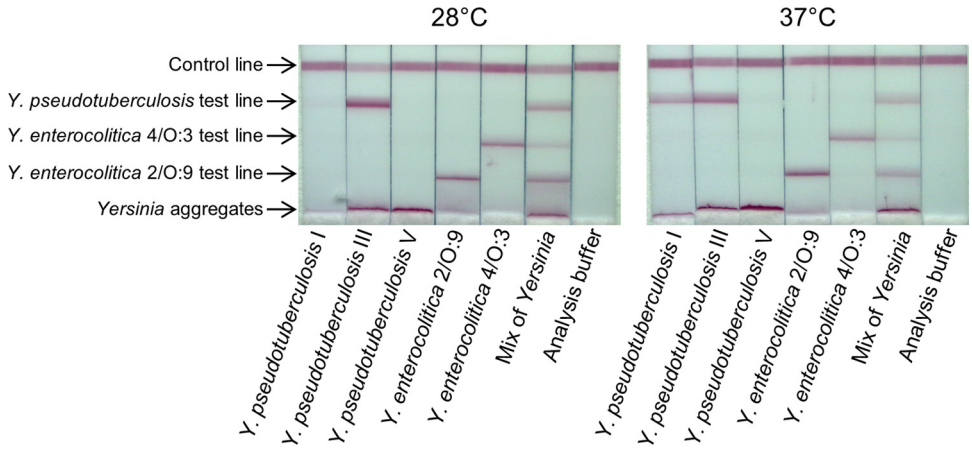

FIG 5 Multiplex lateral flow immunoassay. Bacteria were grown at 28°C and used at 10 times the limit of detection. The bacterial suspensions were incubated for 10 min with a mix of colloidal gold-labeled Ye18*, Ye300*, Yps 104*, and Yp7* MAbs, and the LFI dipsticks were then dipped for 30 min into 100 μl of the bacterial suspensions for upward migration of the liquid. The Ye4 and Ye18 MAbs were used for the test line of *Y. enterocolitica* 2/O:9 and *Y. enterocolitica* 4/O:3, respectively. A mix of Yps103 and Yp7 was used for the test line of *Y. pseudotuberculosis*.

no difference of sensitivity between the *Yersinia* strains grown at 28°C and those grown at 37°C was observed. It is worth noting that for all LFIs, a specific band was detected for high bacterial concentrations, in the lower part of the membrane, resulting from the presence of bacterial aggregates that were still recognized by the tracer MAbs but were too large to migrate along the membrane. At lower bacterial concentrations, the intensity of this band decreased (Fig. 4B).

All LFI were then tested with a large number of strains, including related *Yersinia* strains along with strains of unrelated *Yersinia* and other bacteria. All the related strains were tested at a concentration of 10× the LoD, and unrelated bacteria were tested at concentrations between 5×10^6 and 5×10^7 CFU/ml. For the 2/O:9 LFI, all 13 of the tested strains from bioserotype 2/O:9 were detected at 10× the LoD (5×10^6 CFU/ml), while there was no detection of 14 tested strains from biotype 4/O:3 (Table 1). For the 4/O:3 LFI, all 14 of the tested strains from bioserotype 4/O:3 proved to give positive results at 10^6 CFU/ml (10× the LoD), in contrast to the 13 tested strains from bioserotype 2/O:9 (Table 1). Except for *Y. enterocolitica* 1A/O:3, none of the other unrelated bacteria were recognized with the 4/O:3 LFI (Table 1). The biotype and serotype of this 1A/O:3 strain were verified before and after the experiment to confirm its characteristics. For *Y. pseudotuberculosis*, the Yps LFI detected 7 of 32 tested strains of serotype I at 10^6 CFU/ml (10× the LoD), and this number increased to 20 of 32 for a 10^7 CFU/ml concentration (Table 2). All the strains of serotypes II (10 strains), III (11 strains), IV (10 strains), and V (11 strains) were detected. Moreover, none of the other unrelated bacteria, including *Y. enterocolitica*, *Yersinia similis*, and *Yersinia wautersii*, were detected by the Yps LFI.

A multiplex LFI involving all the MAbs previously selected was designed with the aim to provide simultaneous detection of enteropathogenic *Yersinia* strains (Fig. 5). The sensitivity obtained with the multiplex LFI for the different bacterial strains proved to be similar to those described with the single LFIs.

Lateral flow immunoassays with artificially contaminated stool cultures. As was performed for the EIA, LFIs were tested with the different bacteria spiked in feces and diluted in buffer before the analysis. The specificities and sensitivities of the different LFIs in feces were very similar to the results obtained in buffer. The LoD of the 2/O:9 LFI and 4/O:3 LFI was 5×10^5 CFU/ml for detection of *Y. enterocolitica* 2/O:9 and *Y. enterocolitica* 4/O:3, respectively. The LoDs of the Yps LFI for *Y. pseudotuberculosis* detection were close to 10^5 CFU/ml for serotype I, 5×10^5 CFU/ml for serotype III, and 10^6 CFU/ml for serotype V. Serotypes II and IV were not tested with artificially contaminated stool cultures. Before the LFI, bacterial samples in feces buffer were diluted 1:2 in EIA buffer containing 0.5% Tween 20.

DISCUSSION

The incidence of enteric yersiniosis caused by *Y. enterocolitica* and *Y. pseudotuberculosis* enteropathogenic bacteria is probably largely underestimated all around the world. One of the main reasons for this underestimation is the difficulty of recovery of *Yersinia* strains from bacterial flora present in stool samples. Because of their optimal growth temperature of 28°C, the conditions of culture suitable for most enterobacteria performed using nonselective medium at 37°C for 24 h do not allow optimal detection of *Yersinia* strains. This detection can be improved using enrichment procedures, but this requires additional time, money, and work. Even when some *Yersinia*-selective media were developed, allowing improvement of the isolation of *Y. enterocolitica* strains, the growth of *Y. pseudotuberculosis* was inhibited or not tested (36–39). Moreover, these selective media are not yet marketed and consequently cannot be routinely used by clinical laboratories. Nowadays, CIN can be used for isolation of *Yersinia* species but only at their optimal growth temperature of 28°C (40), thus requiring an additional device(s) for a clinical laboratory. Although CIN is marketed, this medium is not consistently used because of its additional cost. Moreover, CIN inhibits the growth of some *Y. pseudotuberculosis* strains while allowing the growth of nonpathogenic *Yersinia*. Given the lack of rapid and simple methods for detection of enteropathogenic *Yersinia* in human samples, the aim of this study was to develop two sensitive and specific immunoassay formats for the detection of the most frequent bioserotypes of *Y. enterocolitica* and *Y. pseudotuberculosis*.

We first produced a large set of MAbs against *Y. enterocolitica* and *Y. pseudotuberculosis* and screened them for their specificity

and sensitivity. This selection was achieved for specific detection of each of the four prevailing isolated strains: *Y. enterocolitica* bioserotypes 2/O:9 and 4/O:3 and *Y. pseudotuberculosis* serotypes I and III. These MAbs led to the development of a one-step, 3-h-30-min EIA with excellent sensitivity and specificity and of a low-cost and user-friendly LFI with good sensitivity and specificity.

Compared to methods of isolation and characterization protocol involving CIN, these two immunoassays offer a great improvement with respect to the duration of *Yersinia* detection. Indeed, the LFI and the EIA allow characterizations of enteropathogenic *Yersinia* in 40 min and 3 h 30 min, respectively, while the CIN requires 48 h for isolation of bacterial colonies without further characterization. Moreover, LFI and EIA do not require complex sample preparation and are thus well suited for clinical laboratory applications. It has been shown that CIN is permissive for the growth of some bacterial species found in feces samples such as *Acinetobacter*, *Aeromonas*, *Citrobacter*, *Enterobacter*, *Morganella*, *Pseudomonas*, and *Serratia* (40, 41). Usually, the morphologies of the colonies of *Y. enterocolitica* and other bacteria can be distinguished, but colonies of *Citrobacter freundii*, *Enterobacter agglomerans*, and *Serratia liquefaciens* look similar to those of *Y. enterocolitica*, leading to possible misidentifications. Regarding the specificity of our immunoassays, none of the enteropathogenic bacteria such as *S. sonnei* and *S. enterica* serovars Typhimurium, Enteritidis, Paratyphi A, and Senftenberg usually searched for in stools of patients were detected. Moreover, the tests are robust enough to retain their sensitivity in complex matrices such as feces.

We developed a highly sensitive EIA with LoDs ranging from 1.2×10^3 to 2.0×10^5 CFU/ml for *Y. enterocolitica* bioserotypes 2/O:9 and 4/O:3 and *Y. pseudotuberculosis* serotypes I and III. For the *Y. enterocolitica* 2/O:9, *Y. enterocolitica* 4/O:3, and *Y. pseudotuberculosis* III EIAs, better signals were obtained when bacteria were grown at 28°C rather than 37°C. This result can be explained by the fact that the MAbs used in these EIA recognize LPS, production of which is higher at 28°C *in vitro* (42). For the *Y. pseudotuberculosis* serotype I EIA, the signal and the LoD were better for bacteria grown at 37°C. Since the MAb used in this EIA was targeting a protein, this higher temperature possibly enhanced its expression at 37°C *in vitro*. When stool samples were used to mimic clinical samples, the LoDs were slightly modified or left unmodified, except for the LoDs of the *Y. enterocolitica* 2/O:9 and *Y. pseudotuberculosis* I EIAs, which were 20 to 35 times higher when the bacteria were grown at 37°C.

Validation of LFI with a large number of bacteria showed 100% detection for *Y. enterocolitica* (bioserotypes 2/O:9 and 4/O:3) and for *Y. pseudotuberculosis* (serotypes II, III, IV, and V). For serotype I, 62.5% of the strains were detected at 10^7 CFU/ml. It is worth noting that CIN has been shown to exert an inhibitory effect on the growth of some *Y. pseudotuberculosis* strains (17); thus, our immunoassays could lead to better detection of this species from biological samples.

It has been previously reported that colonies of *Yersinia* exhibit similar aspects after isolation on CIN (17), making it impossible to distinguish between pathogenic and nonpathogenic strains. We thus tested a large number of nonpathogenic strains of *Y. enterocolitica* biotype 1A, which is ubiquitous in the environment and is the predominant biotype of *Y. enterocolitica* detected among *Yersinia* isolates from human clinical stool samples in some European countries (43, 44). We observed that only a single strain among 11 was detected with the 4/O:3 LFI. It appeared that this positive strain was of serotype O:3, which possesses the same LPS as *Y. enterocolitica* 4/O:3. This cross-reactivity can be explained by the epitope recognized by MAb Ye300, which involved the LPS. It is noteworthy that recovery of bioserotype 1A/O:3 from clinical samples is quite unusual (45), and only 4 strains from this exceptional bioserotype were present among 2,722 in the collection of the French National Reference Laboratory.

Although serotype O:9 shares epitopes with the O-antigen of *Brucella* (35), none of the three *Brucella* strains tested was detected by the 2/O:9 LFI, and the other LFIs gave similarly negative results. Our Yps LFI was also tested with *Y. similis* and *Y. wautersii*, two species that were recently described and are closely related to *Y. pseudotuberculosis* and (in the case of *Y. wautersii*) presumably pathogenic for human (46, 47), and gave negative results.

The LoDs of the LFIs ranged from 10^5 to 10^6 CFU/ml for detection of the four prevailing isolated strains: *Y. enterocolitica* bioserotypes 2/O:9 and 4/O:3 and *Y. pseudotuberculosis* serotypes I and III. Moreover, our Yps LFI was able to detect *Y. pseudotuberculosis* serotypes II, IV, and V. Thanks to the high LFI robustness, the corresponding LoDs were identical with those determined for the artificially contaminated stool samples.

To our knowledge, only one study has previously reported that the loads of *Y. enterocolitica* in stools of infected patients range from 10^5 to 10^8 CFU/g of feces (22). However, no experiment was done to support this affirmation. Using a procedure common for clinical laboratories, i.e., one pea-size sample of feces (corresponding to approximately 1 g, resuspended in 10 ml of buffer), the present EIA should clearly be useful, presenting a LoD value below that range. If this load of *Y. enterocolitica* in stools is correct, the LoDs of LFIs are above the lowest limit of 10^5 CFU/g of feces, but the tests should work properly in a large part of the concentration range expected in biological samples. Because we used stool samples from a healthy individual, dilution was necessary to obtain liquid samples. It could be interesting to determine if patient stool samples without or with a lower dilution can improve these LoDs. Compared to more-selective methods such as the use of CIN or real-time PCR with a LoD of $\geq 10^3$ CFU/g of feces, our EIAs and LFIs provide very fast and economical detection and can be used by minimally trained personnel without additional devices being required. Moreover, our LFIs would be a useful tool to determine the exact load of enteropathogenic *Yersinia* in stools of diarrheal patients. Real-time PCR needs an enrichment step that is as long in duration as the isolation step on CIN and also requires the acquisition of expensive devices. The time saving of the approach described here can easily circumvent unadapted antibiotic treatments or unnecessary appendectomies.

Furthermore, it appears that the LFI can be easily handled under field conditions and consequently can be used for veterinary applications. First, serotype O:9 of *Y. enterocolitica* is often naturally isolated from cows, goats, sheep, or pigs but their antibodies against serotype O:9 cross-react with *Brucella* O-antigen and cause false-positive reactions in brucellosis serological diagnosis tests (48, 49). This represents a major economic problem for stock farmers because suspicion of brucellosis leads to the elimination of the animals in accordance with public health regulations. Our LFI can be used to discriminate between a *Y. enterocolitica* infection and a *Brucella* infection. Second, bioserotype 4/O:3 of *Y. enterocolitica* is frequently isolated from pigs on farms or in slaughterhouses, and consumption of pork meat is associated with *Y.*

enterocolitica infections (50, 51). Our LFI can be used by the meat industry at different levels: on livestock, to control the absence of *Y. enterocolitica* and prevent contamination from positive herds, and in slaughterhouses, to exclude contaminated pig carcasses or for epidemiological studies. Additionally, infection by *Y. pseudotuberculosis* appears to be a recurrent veterinary issue with a significant economic burden in livestock and zoo animals (52). Our Yps LFI can be easily handled under field conditions and would be useful as a control test for the presence of the pathogen.

In conclusion, this report presents the first description of a very sensitive EIA and of a rapid LFI test suitable for detection of enteropathogenic *Yersinia* in stool samples which can be helpful to physicians for the diagnosis of patients. The EIA or the LFI would be useful as a first-line rapid test in clinical laboratories. They can be a great complementary tool to help clinical laboratories to focus the research of enteropathogenic *Yersinia* on positive stools before performing the gold standard test for confirmation, i.e., isolation of *Yersinia* with traditional culture methods. Therefore, we need to evaluate our immunoassays with pathogenic stool samples under the conditions encountered in clinical laboratories. Furthermore, both immunoassays, especially the easy-to-use LFI, have the potential to be used for veterinary applications for detection of *Y. enterocolitica* and *Y. pseudotuberculosis*.

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