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Detection of pYV$^+$ *Yersinia enterocolitica* Isolates by P1 Slide Agglutination

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Rabbit polyclonal antisera were raised against the pYV-encoded outer membrane protein P1 of five *Yersinia enterocolitica* strains belonging to serogroups O:3, O:5,27, O:8, and O:9. Analysis of these strains with the sera showed that P1 presented at least six different antigenic factors. Two of the serum specimens were chosen to test the P1 agglutinability of 797 strains isolated from various sources. This technique appeared to be more reliable than autoagglutination and Ca^{2+} dependency to monitor the presence of the pYV plasmid. Hence, we propose this P1-mediated agglutination as a new and easy virulence test.

The species *Yersinia enterocolitica* is subdivided into five main biogroups containing several serogroups (37). A 70-kb pYV plasmid conditions the pathogenicity of a number of *Yersinia* strains. So far, this plasmid has been found in strains from serogroups O:4, O:8, O:13a,13b, and O:21, which are generally referred to as American strains (biogroup 1^B), as well as in strains belonging to serogroups O:9 (biogroup 2), O:5,27 (biogroup 2 or 3), O:1,2,3 (biogroup 3), O:3 (biogroup 3 or 4), and O:2,3 (biogroup 5).

Besides a Ca^{2+} requirement for growth at 37°C, the virulence plasmid of *Y. enterocolitica* encodes a set of released proteins (14, 28) termed Yops, at least one outer membrane protein called P1 (4, 5), and a lipoprotein called YlpA (B. China, T. Michiels, and G. R. Cornelis, Mol. Microbiol., in press). P1 production occurs only at 37°C and, unlike Yops production, occurs irrespective of the presence of Ca^{2+} ions (for a review, see reference 11). This protein is known as a ±200-kDa polymer composed of subunits of about 50 kDa (34, 38). It forms a fibrillar structure at the surface of *Y. enterocolitica* (19, 22, 38) and has been shown to be involved in several membrane-associated properties such as autoagglutination (1, 34), surface hydrophobicity (27), hemagglutination (18), collagen binding (13), and adherence to HEp-2 cells (15). The P1 synthesis during infection (26, 33) is thought to contribute to resistance to the bactericidal activity of human serum (1) and to favor colonization of the ileum (20). It is currently considered as the *Y. enterocolitica* adhesin.

In this study, we used polyclonal rabbit anti-P1 sera in a slide agglutination test to analyze the presence of pYV in many *Y. enterocolitica* strains. Despite the antigenic diversity between P1 protein in different bio- and serogroups, two antisera agglutinated all the pYV$^+$ *Y. enterocolitica* strains, including the so-called American strains (biogroup 1^B). This rapid and easy test appeared to be more reliable than autoagglutination and Ca^{2+} dependency.

MATERIALS AND METHODS

Bacterial strains. Strain W227 (serogroup O:9) has been thoroughly characterized (1, 2, 7, 8, 29). The presence of the pYV plasmid in the four other strains was monitored by plasmid DNA purification and restriction as described elsewhere (1). Strain WE261/87 (serogroup O:3) was shown to contain a typical pYVeO:3 plasmid (16, 24, 30, 32). Strain WA289 (serogroup O:8) was found to contain a plasmid resembling pYV8081, the archetype of pYV plasmids from serogroup O:8 strains (3). According to the size and number of the restriction fragments, strains WA375 (serogroup O:5,27, biogroup 3) and WE480/88 (serogroup O:5,27, biogroup 2) contain different pYV plasmids that resemble pYVeO:9 and pYVeO:3.

Since plasmids of strains from serogroup O:5,27 have not been characterized as well as the others have, we checked for the presence of the gene *yadA* (for *Yersinia* adhesin) that encodes P1 by DNA-DNA hybridization. We selected an oligonucleotide probe in a conserved region of the *yadA* sequence of pYVeO:3 and pYV8081 (35) and hybridized it with Southern blots of plasmid digests using standard procedures (25). The probe clearly hybridized with a fragment of the pYV plasmid of the five strains selected for the study (data not shown). Hence, we concluded that all these strains contain a *yadA* gene.

In accordance with these genetic data, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis showed that all these strains produced a protein of about 200 kDa. This protein was produced by the pYV$^+$ strain grown at 37°C in brain heart infusion supplemented with 0.02 M sodium oxalate or 0.005 M $CaCl_2$ (see below), and it disappeared when the whole-cell protein preparations were treated with urea before they were loaded onto the gel (data not shown). This protein thus had the known properties of P1: it was encoded by pYV, it was produced at 37°C independently of the presence of Ca^{2+}, and it formed a polymeric structure that was denatured by a strong denaturing agent (38). Its apparent molecular weight varied between the strains tested (Fig. 1A).

pYV$^-$ isogenic strains were derived from each strain and checked by plasmid DNA analysis.

Growth conditions. Bacteria were routinely grown in tryptic soy broth (Difco) and on tryptic soy agar (TSA; Difco). Ca^{2+} dependency was tested on TSA supplemented with 0.02 M sodium oxalate and 0.02 M $MgCl_2$. pYV$^-$ variants were selected on TSA supplemented with 0.02 M sodium oxalate and 0.02 M $MgCl_2$.

Autoagglutination test. The autoagglutination test was done as described by Laird and Cavanaugh (23).

Preparation of antisera and P1 slide agglutination test.

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TABLE 1. Bacterial strains used in this study

Strain	Biogroup	Serogroup	Origin
WE261/87	4	O:3	Human clinical isolate, Belgium
W227	2	O:9	Human clinical isolate, Belgium
WE480/88	2	O:5,27	Human clinical isolate, Belgium
WA375	3	O:5,27	Swine isolate, United States
WA289	1[B]	O:8	Human clinical isolate, United States

Antisera against P1 were raised in rabbits by using the pYV^+ variants grown in tryptic soy broth for 24 h at 37°C. After washing, bacterial suspensions were adjusted to approximately 3×10^9 cells ml^{-1}, and 1% Formalin was added. Rabbits were injected four times intravenously: once each with 0.5 and 1 ml and twice with 2 ml at 4-day intervals. They were bled 9 days after the last injection. Absorption of the sera was done with the pYV^- variant of the homologous strain grown at 37°C on TSA. In highly motile strains (strains from serogroups O:8 and O:9), additional absorption with the same strain grown at 22°C was necessary to remove the H agglutinins present in the antisera.

Slide agglutination was done with a 1/10 serum dilution on strains grown at 22, 29, and 37°C on TSA. The titers of the antisera before and after heterologous absorption were determined by tube agglutination by using a 24-h culture on TSA at 37°C. A bacterial suspension corresponding to a density of a 1 to 2 McFarland standard (about 5×10^8 cells ml^{-1}) was made in saline with 0.02% (wt/vol) merthiolate and added to an equal volume of serial serum dilutions.

P1 protein analysis. A fresh culture was diluted to an optical density at 600 nm of 0.1 in brain heart infusion (Difco) containing 0.4% glucose. This medium was supplemented with 0.02 M $MgCl_2$ and with either 0.02 M sodium oxalate or 0.005 M $CaCl_2$. Bacteria were grown with shaking for 2 h at 22°C, and expression of the plasmid genes was induced by incubation for 4 h at 37°C (10). Bacteria were harvested and lysed by boiling in the sample buffer used for SDS-PAGE. Proteins from about 3×10^8 bacteria were loaded onto the gel (gradient, 5 to 20%). SDS-PAGE and immunoblotting were done as described previously (36).

RESULTS

Control of anti-P1 polyclonal sera. We selected five *Y. enterocolitica* reference strains (Table 1) representative of the major pathogenic serogroups, and we ensured by DNA analysis that they carried a typical pYV plasmid, including the *yadA* gene that codes for P1 (see above). These strains were WE261/87 (serogroup O:3), W227 (serogroup O:9), WE480/88 (serogroup O:5,27, biogroup 2), WA375 (serogroup O:5,27, biogroup 3), and WA289 (serogroup O:8). Rabbit polyclonal antisera against P1 of these strains were prepared by injection of pYV^+ bacteria grown at 37°C and absorbed by the pYV^- derivatives (see above). Immunoblot analysis of total cell proteins showed that each serum specimen reacted with the 200-kDa protein P1 (data not shown). A protein of about 50 kDa was also detected. This protein was assumed to be the P1 monomer since its production appeared to be pYV and temperature dependent but independent of the Ca^{2+} concentration. All the antisera reacted with the protein P1 not only of the homologous strains but also of the heterologous strains. The cross-reaction between P1 from strain WA289 (serogroup O:8) and serum against strain W227 (serogroup O:9) was, however, very weak (Fig. 1B).

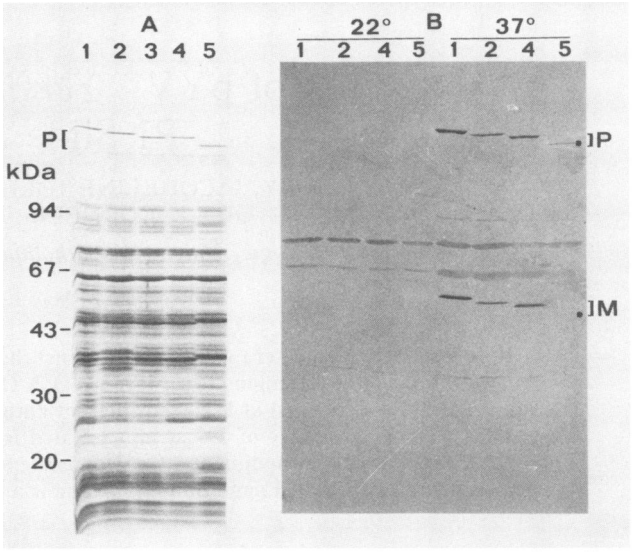

FIG. 1. (A) SDS-PAGE analysis of whole-cell proteins of *Y. enterocolitica* grown at 22°C for 2 h and incubated at 37°C for 4 h. Lane 1, strain WE261/87 (serogroup O:3); lane 2, strain WE480/88 (serogroup O:5,27, biogroup 2); lane 3, strain WA375 (serogroup O:5,27, biogroup 3); lane 4, strain W227 (serogroup O:9); lane 5, strain WA289 (serogroup O:8). The molecular masses of the standard markers are noted in kilodaltons. The polymeric protein P1 is indicated by P. (B) Immunoblot analysis of whole-cell proteins of *Y. enterocolitica* grown at 22°C for 6 h or at 22°C for 2 h and incubated at 37°C for 4 h. Serum directed against P1 of strain W227 was used. P1 and its monomeric form are indicated by P and M, respectively. Asterisks indicate protein P1 of strain WA289 and its subunit. Lane numbers are as in panel A.

Analysis of P1 from the reference strains by slide agglutination. The five strains grown at 37°C were agglutinated by the homologous anti-P1 serum. Titers, which were determined by tube agglutination with the homologous strain, ranged from 1/1,600 to 1/6,400 (Table 2). None of the strains that were grown at 22°C was agglutinated, and after growth at 29°C, only strain WA289 (serogroup O:8) reacted.

In order to ensure that the agglutination did not involve Yops, we tested a few mutants of W227 that were altered either in P1 production or in Yops production. W227(pGB08) and W227(pGB910) are mutated in *yadA* (1). They do not express P1 but they still code for Yops. W227(pGC633) is a mutant that produces P1 but not Yops (9). W227(pGC565), which expresses both the *yop* genes and *yadA* (10), was selected as a control. The sera used in this experiment were those obtained against strains WE261/87 (serogroup O:3) and W227 (serogroup O:9) and absorbed with the pYV^- isogenic strains incubated at 22 and 37°C. These sera were found to agglutinate W227(pGC565) and W227(pGC633). However, W227(pGB08) and W227(pGB910) were not agglutinated. Hence, the agglutination was P1 specific.

When sera were tested against heterologous strains, agglutination titers varied to large extents, suggesting differences in the antigenic patterns of P1 from the various strains. For example, heterologous strains were only slightly agglutinated by serum against strain WA289 (serogroup O:8). Likewise, this strain was agglutinated at a low titer only by serum against strain W227 (serogroup O:9) (Table 2).

TABLE 2. Agglutination titers of P1 antisera against homologous and heterologous *Y. enterocolitica* strains before and after cross-absorption

Strain used to raise serum	Strain used to absorb serum	Agglutination titer				
		WE261/87	W227	WA375	WE480/88	WA289
WE261/87	None	3,200	800	400	1,600	0
	W227	1,600	0	0	1,600	0
	WE480/88	400	800	400	0	0
	W227 + WE480/88	0	0	0	0	0
W227	None	6,400	6,400	6,400	800	200
	WE261/87	0	3,200	6,400	0	0
	WE480/88	6,400	6,400	6,400	0	0
	WA289	6,400	6,400	6,400	0	0
	WA375	0	0	0	0	0
WA375	None	3,200	3,200	3,200	1,600	0
	W227	0	0	0	0	0
WE480/88	None	1,600	200	200	1,600	0
	WE261/87	0	0	0	1,600	0
	W227	1,600	0	0	1,600	0
WA289	None	400	800	200	200	1,600
	WE261/87	0	0	0	0	1,600
	W227	0	0	0	0	1,600
	WE480/88	0	0	0	0	800

TABLE 3. Antigenic composition of P1

Strain	Serogroup	P1 epitope
WE261/87	O:3	A, B, (D)
W227	O:9	B, C, (D)
WE480/88	O:5,27	A, (D), E
WA375	O:5,27	B, C, (D)
WA289	O:8	(D), F

In order to analyze these differences, the five anti-P1 sera were cross-absorbed with each of the heterologous strains and tested again against the five reference strains by P1 agglutination. This analysis, results of which are presented in detail in Table 2, allowed us to determine different antigenic patterns based on the combination of six antigenic factors called A, B, C, D, E, and F (Table 3). Single-factor sera were obtained for all antigenic determinants, except for factor D, which was common to all the strains tested. However, factor D reacted weakly with sera and, therefore, was put in parentheses in Table 3. Antisera from strains W227 (serogroup O:9) and WA375 (serogroup O:5,27, biogroup 3) were totally cross-absorbed. Hence, the P1 antigenic pattern of these two strains were considered to be identical. To confirm these results, the single-factor sera were tested in immunoblots and shown to react with the P1 proteins containing the corresponding determinants (Fig. 2).

P1 slide agglutination as a marker of pYV$^+$ *Y. enterocolitica*. Since factors A and B are well represented in the four

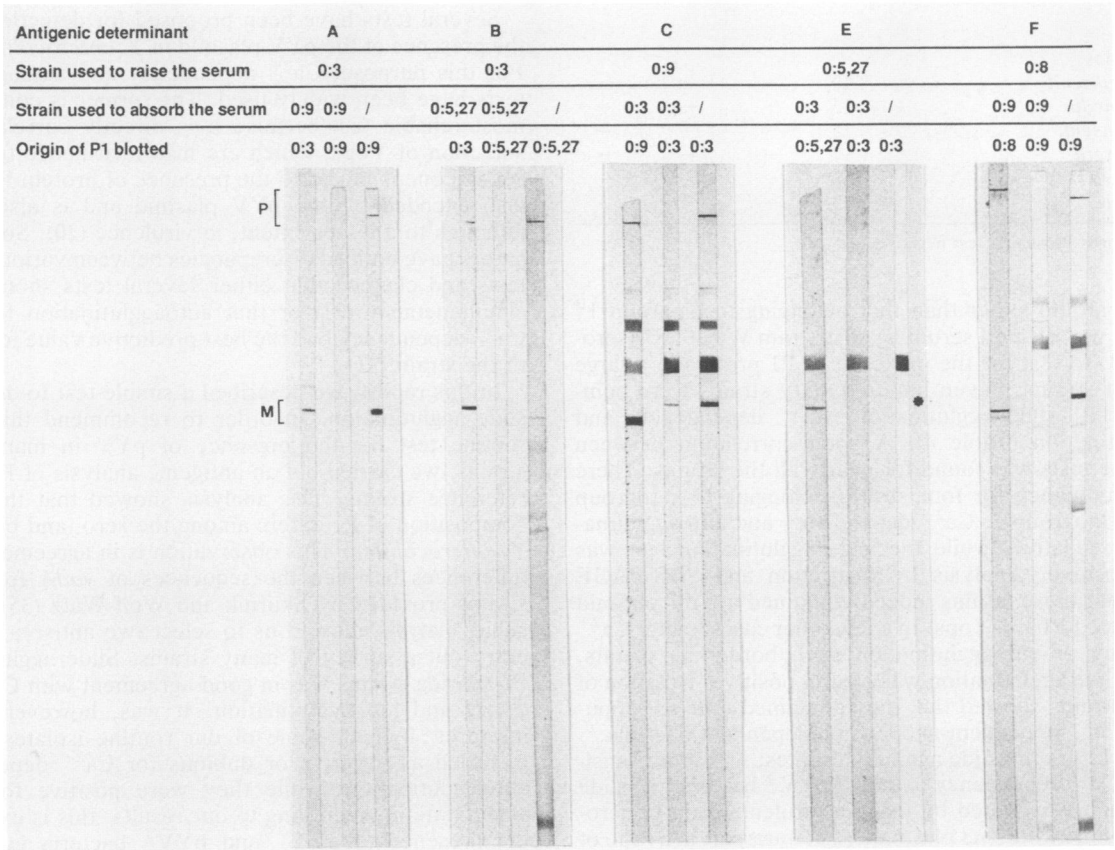

FIG. 2. Reaction of P1 with single-factor sera. Whole-cell proteins of strains of serogroups O:3 (WE261/87), O:9 (W227), O:5,27 (WE480/88), and O:8 (WA289) were analyzed with absorbed and unabsorbed sera. P1 (P) and its monomeric form (M) are indicated. The asterisk indicates a faint band that was present on the membrane but not visible on the photograph.

TABLE 4. Comparison of P1 slide agglutination, Ca^{2+} dependency, and autoagglutination

Yersinia species and biogroup	Serogroup	No. of strains tested	P1 slide agglutination	Ca^{2+} dependency	Autoagglutination
Y. enterocolitica					
4	O:3	398	+	+	+
		10	+	±	±
		7	+	±	+
		42	−	−	−
2	O:9	47	+	+	+
		2	+	+	±
		12	−	−	−
2	O:5,27	20	+	+	+
		1	+	+	±
		4	+	−	−
		11	−	−	−
3	O:3 (VP^{-a})	2	+	+	+
		3	−	−	−
3	O:1,2,3	5	+	+	+
		6	−	−	−
3	O:5,27	2	+	+	+
		1	−	−	−
5	O:2,3	4	+	1+, 3±	+
		10	−	−	−
1^A	Different serogroups	88	−	−	−
Other species					
Y. frederiksenii		19	−	−	−
Y. intermedia		15	−	−	−
Y. kristensenii		15	−	−	−
Y. mollaretii		17	−	−	−
Y. bercovieri		16	−	−	−
Y. rohdei		3	−	−	−

[a] VP^-, Voges-Proskauer test negative.

reference strains other than that belonging to biogroup 1^B (Table 3), we selected serum against strain WE261/87 (serogroup O:3) to test for the presence of P1 protein in a large number of strains. Seven hundred sixty strains were compared for P1 slide agglutination, Ca^{2+} dependency, and autoagglutination (Table 4). A good correlation between these three tests was found for nearly all the strains. There was a discrepancy for four strains belonging to serogroup O:5,27 of biogroup 2: Ca^{2+} dependency and autoagglutination were negative, while the slide agglutination test was clearly positive. Analysis by restriction and SDS-PAGE showed that these strains indeed contained a pYV plasmid and produced P1 and Yops. In a few other cases, either Ca^{2+} dependency or autoagglutination gave borderline results, while P1 slide agglutination was clearly positive. Isolation of all these strains showed that they contained a mixed population of Ca^{2+}-dependent and Ca^{2+}-independent bacteria.

To check that the slide agglutination test was more sensitive than Ca^{2+} dependency to detect pYV^+ bacteria, P1 slide agglutination was tested by using a virulent strain of serogroup O:3 mixed with its plasmidless counterpart in a ratio of 1/3. As expected, P1 slide agglutination was clearly positive, whereas Ca^{2+} dependency and autoagglutination was negative or dubious.

TABLE 5. Comparison of P1 slide agglutination, Ca^{2+} dependency, and autoagglutination in strains of biogroup 1^B

Serogroup	No. of strains tested	P1 slide agglutination	Ca^{2+} dependency	Autoagglutination
O:8	6	+	+	+
	9	−	−	−
O:4	3	+	+	+
	1	−	−	−
O:18	1	−	−	−
O:20	2	−	−	−
O:21	2	+	+	+
	2	−	−	−
O:13a,13b	4	+	+	+
	1	+	±	+
	6	−	−	−

To analyze the strains of biogroup 1^B, we used the serum raised against strain WA289 (serogroup O:8). Thirty-seven strains from our collection were tested. As shown in Table 5, there was a good agreement between P1 slide agglutination, Ca^{2+} dependency, and autoagglutination, suggesting that this serum could be used to monitor the presence of pYV in the strains of biotype 1^B.

DISCUSSION

Several tests have been proposed for detecting routinely the presence of the pYV plasmid in *Y. enterocolitica* strains. For this purpose, Ca^{2+} dependency and autoagglutination tests have been widely used. The former is considered the most reliable test because it is directly correlated to the secretion of Yops, which are major virulence factors. The second one is linked to the presence of protein P1, which is also encoded by the pYV plasmid and is also involved, although to a lesser extent, in virulence (20). Some investigators have noticed discrepancies between various virulence tests and claimed that either several tests should be done simultaneously (21) or that autoagglutination followed by Ca^{2+} dependency had the best predictive value for virulence of the strain (31).

In this report, we described a simple test to detect P1 by slide agglutination. In order to recommend this test as a routine test for the presence of pYV in many different strains, we carried out an antigenic analysis of P1 from five reference strains. This analysis showed that the antigenic composition of P1 differs among the sero- and biogroups of *Y. enterocolitica*. This observation is in agreement with the differences between the sequences of *yadA* from various strains provided by Skurnik and Wolf-Watz (35). Our antigenic analysis allowed us to select two antisera in order to carry out a survey of many strains. Slide agglutination of P1-bearing strains was in good agreement with Ca^{2+} dependency and autoagglutination. It was, however, easier to interpret. Indeed, some of our routine isolates were first recorded as negative or dubious for Ca^{2+} dependency or autoagglutination, while they were positive for P1 slide agglutination. According to our results, this is explained by the presence of pYV^- and pYV^+ bacteria in the initial culture.

Doyle et al. (12) described the use of an antiserum raised against a strain of serogroup O:8 for agglutination in micro-

dilution plates. This serum was obtained by intravenous injection of viable bacteria to rabbits and was absorbed by the pYV^- derivative of this strain. According to the results of Chang and Doyle (6), this antiserum was directed against several outer membrane proteins, including P1, and was able to agglutinate virulent strains of different serogroups. Our results are in good agreement with those of Chang and Doyle (6), and they show that the agglutination is due to P1 and not Yops. They also explain the low titers obtained by those investigators with strains of serogroups O:3, O:9, and O:5,27. This was due to the weak antigenic relationship of P1 from strains of biogroup 1^B and other biogroups.

Recently, Kaneko and Maruyama (17) proposed an enzyme immunoassay to discriminate pYV^+ from pYV^- strains. This test seems to be based essentially on the secreted Yops rather than on P1. It thus represents a different and rather complementary approach than ours. The P1 slide agglutination test is faster and easier to perform. On the other hand, since Yops are immunologically related between the different *Yersinia* species, the enzyme immunoassay can be applied to *Y. pseudotuberculosis* strains.

In conclusion, we propose a rapid P1 slide agglutination test to monitor the presence of pYV in clinical isolates. Two antisera are required: one for strains of biogroup 1^B and another one for strains of other biogroups. This test is more sensitive than Ca^{2+} dependency and autoagglutination. According to our results, in all cases, the pYV plasmid carries both a P1 gene and a functional *yop* regulon. Hence, P1 slide agglutination seems to be a reliable clue for the presence of a functional pYV plasmid. However, it must be kept in mind that Ca^{2+}-independent laboratory mutants lacking Yops but producing P1 have been isolated in vitro (1, 9) and, hence, that expression of P1 does not necessarily correlate with expression of the *yop* regulon.

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