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PCR Detection of Virulence Genes in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* and Investigation of Virulence Gene Distribution

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PCR-based assays were developed for the detection of plasmid- and chromosome-borne virulence genes in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, to investigate the distribution of these genes in isolates from various sources. The results of PCR genotyping, based on 5 virulence-associated genes of 140 strains of *Y. enterocolitica*, were compared to phenotypic tests, such as biotyping and serotyping, and to virulence plasmid-associated properties such as calcium-dependent growth at 37°C and Congo red uptake. The specificity of the PCR results was validated by hybridization. Genotyping data correlated well with biotype data, and most biotypes resulted in (nearly) homogeneous genotypes for the chromosomal virulence genes (*ystA*, *ystB*, and *ail*); however, plasmid-borne genes (*yadA* and *virF*) were detected with variable efficiency, due to heterogeneity within the bacterial population for the presence of the virulence plasmid. Of the virulence genes, only *ystB* was present in biotype 1A; however, within this biotype, pathogenic and apathogenic isolates could not be distinguished based on the detection of virulence genes. Forty *Y. pseudotuberculosis* isolates were tested by PCR for the presence of *inv*, *yadA*, and *lcrF*. All isolates were *inv* positive, and 88% of the isolates contained the virulence plasmid genes *yadA* and *lcrF*. In conclusion, this study shows that genotyping of *Yersinia* spp., based on both chromosome- and plasmid-borne virulence genes, is feasible and informative and can provide a rapid and reliable genotypic characterization of field isolates.

Yersinia enterocolitica and *Y. pseudotuberculosis*, both members of the family *Enterobacteriaceae*, are comprised of strains with different degrees of pathogenicity. Both pathogenic and nonpathogenic strains are frequently isolated from various animals (birds, mammals, and reptiles) as well as from the environment (water and soil). Rodents (mice and rats), hares, rabbits, and birds serve as reservoirs for *Y. pseudotuberculosis* (1). Pathogenic strains of *Y. enterocolitica* and *Y. pseudotuberculosis* are frequently present in pigs without normally causing disease in these animals. Other food-producing animals, such as cattle, harbor mostly nonpathogenic strains of *Y. enterocolitica*.

In humans, *Y. enterocolitica* and *Y. pseudotuberculosis* are well-known food-borne pathogens and are mainly transmitted through ingestion of contaminated pork, milk, or water. Yersiniosis frequently occurs in young children as enterocolitis with fever, diarrhea, and abdominal cramps. Although the disease is usually self-limiting, complications (e.g., septicemia) are not uncommon in immunocompromised hosts. Furthermore, sequelae, such as reactive arthritis, have been reported (21).

The identification and further typing of subspecies, aiming at recognition of pathogenic strains of *Yersinia* spp., are traditionally based on phenotypic tests. *Y. enterocolitica* can be classified into biotype 1A, generally regarded as nonpathogenic (9), and the pathogenic biotypes 1B, 2, 3, 4, and 5. Both species can also be divided into serotypes with predictive values for pathogenicity. Serological and biochemical classification, however, are time consuming and are not generally available in routine laboratories. Alternative phenotypical tests, such as calcium-dependent growth at 37°C, Congo red binding (26), pyrazinamidase testing (16), autoagglutination testing, and serum resistance testing (2, 4, 5, 6, 13, 28) all have limited predictive value for the pathogenicity of *Y. enterocolitica* and *Y. pseudotuberculosis*. The tests are frequently ambiguous to read, and their outcome may be unreliable, since they depend on the presence and expression of (plasmid-borne) virulence genes and the virulence plasmid pYV can easily be lost depending on the culture conditions. Therefore, differentiation of pathogenic strains should not rely solely on the expression or detection of the virulence plasmid but also on the detection of chromosomal virulence factors.

The aim of this study was to develop PCR assays for the detection of plasmid- and chromosome-borne virulence genes of *Y. pseudotuberculosis* and *Y. enterocolitica*. The obtained results were compared to classical phenotypic subtyping methods. The presence or absence of various virulence genes was compared in strains isolated from human patients, food, (food-producing) animals, and the environment. The following chromosomal virulence genes were included in the analysis: *ail*, the *Y. enterocolitica* attachment invasion locus gene, reported to be present in pathogenic strains only (22, 23); *ystA*, which is responsible for the production of a heat-stable enterotoxin in *Y. enterocolitica* (12); *ystB*, which has been observed to encode an enterotoxin present mainly in biotype 1A strains of *Y. enterocolitica* (27, 29, 33); and *inv*, which is present in pathogenic *Y.*

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TABLE 1. Field bacterial strains used in this study

Species	Source	No. of isolates	No. of isolates of serotype:		
			O:3	O:9	Other
Y. enterocolitica	Animal	53	8	33	12
Y. enterocolitica	Human	42	16	11	15
Y. enterocolitica	Food	25	3	2	20
Y. enterocolitica	Environment	8	1	0	7
Y. pseudotuberculosis	Animal	34			
Y. pseudotuberculosis	Human	5			
Y. intermedia	Animal	1			
Y. intermedia	Environment	7			
Y. frederiksenii	Environment	3			
Y. kristensenii	Environment	1			

pseudotuberculosis (15). The plasmid-borne virulence genes analyzed are *yadA*, whose product is involved in autoagglutination, serum resistance, and adhesion (30), and *virF* or *lcrF* (for *Y. enterocolitica* and *Y. pseudotuberculosis*, respectively), which encodes transcriptional activators of the *yop* regulon (8, 11, 31). The results were compared to phenotypic tests with predictive values for pathogenicity.

MATERIALS AND METHODS

Bacterial strains and culture conditions. In this study, *Y. enterocolitica* (140 strains), *Y. pseudotuberculosis* (40 strains), *Yersinia kristensenii* (1 strain), *Yersinia frederiksenii* (4 strains), and *Yersinia intermedia* (8 strains) were analyzed. These included reference strains, human clinical isolates, animal strains, food strains, and environmental strains (Tables 1 and 2). In addition, the following species were used to test the specificity of the PCR assays: 15 strains comprising 11 *Aeromonas* spp., 3 *Vibrio* spp., 2 *Campylobacter* spp., 2 *Staphylococcus* spp., 3 type strains of *Clostridium perfringens*, 3 type strains of *Escherichia coli*, and type strains of *Bacillus cereus*, *Bacteroides coagulans*, *Candida albicans*, *Citrobacter freundii*, *Enterococcus faecalis*, *Lactobacillus acidophilus*, *Listeria monocytogenes*, *Micrococcus luteus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, and *Streptococcus agalactiae*.

Phenotyping. The isolates of *Y. enterocolitica* were grouped by biotyping with discriminatory tests (lipase, esculin, salicin, indole, xylose, and trehalose) described previously (32) and serotyped by using commercial serum agglutinant anti-*Y. enterocolitica* O:3 and anti-*Y. enterocolitica* O:9 (Bio-Rad, Marnes-la-Coquette, France). The calcium dependency of all *Y. enterocolitica* and *Y. pseudotuberculosis* strains was tested with magnesium oxalate (MOX) agar as described by Prpic et al. (26) in order to differentiate between plasmid-bearing (resulting in typical pinpoint growth at 37°C) and plasmidless strains. In addition, the ability of *Y. enterocolitica* and *Y. pseudotuberculosis* strains to bind Congo red was used to distinguish plasmid-bearing strains (forming small red colonies) from plasmidless strains. The test was performed according to the method described in reference 26 by using CRAMP agar (Sigma, St. Louis, Mo.) incubated at 32°C for 72 h. Moreover, the pyrazinamidase test was used to distinguish potential pathogenic strains from nonpathogenic strains of *Y. enterocolitica* and performed according to the method described in reference 16.

DNA isolation. DNA used for PCR was isolated by using the InstaGene matrix (Bio-Rad Laboratories AG). Briefly, 100 μl of cultivated tryptone soy broth was added to 1 ml of sterile double-distilled water and centrifuged for 2 min at 15,300 × *g* at 4°C. Two hundred microliters of the InstaGene matrix was added to the cell pellet. The mixture was incubated at 56°C for 30 min and then vortexed at high speed for 10 s. Following a boiling water bath for 8 min, lysed cells were mixed and spun at 15,300 × *g* for 3 min. Five microliters of the resulting supernatant was used as a template for each 50-μl PCR mixture. The remaining supernatant was stored at −20°C for future use.

DNA used for hybridization (dot blot and Southern blot analysis) was isolated by the guanidium thiocyanate extraction method for genomic DNA (25). DNA of gram-positive bacteria was isolated by using the E.Z.N.A. bacterial DNA kit (peqlab Biotechnologie GmbH, Erlangen, Germany) and following the instructions of the manufacturer. If necessary, the DNA was concentrated by using the protocol of the manufacturer to concentrations of at least 50 ng of DNA/μl.

Primers and PCR conditions. Primers specific for the *ail*, *ystA*, and *ystB* genes of *Y. enterocolitica*, the *yadA* and *virF*/*lcrF* genes of *Y. enterocolitica* and *Y. pseudotuberculosis*, and the *inv* gene of *Y. pseudotuberculosis* were designed with Primer Designer software, versions 2.01 and 3.0 (Scientific & Educational Software, Durham, N.C.), and are listed in Table 3. PCRs were performed in 50-μl volumes containing 5 μl of DNA template, 0.2 mM concentrations of deoxynucleoside triphosphates, 5 μl of 10× PCR buffer II (Perkin Elmer, Rotkreuz, Switzerland), 3 mM $MgCl_2$, 1 μM concentrations of each forward and reverse primer, 1.25 U of Ampli*Taq* Gold (Perkin Elmer), and 2% Tween 20. The thermal cycling conditions performed with a GeneAmp 9600 from Perkin Elmer were as follows: 1 cycle of denaturation at 95°C for 10 min; 25 cycles of melting at 95°C for 15 s, annealing at various temperatures depending on the primer pair used (Table 3) for 30 s, and elongation at 72°C for 30 s; and a final extension at 72°C for 10 min. Afterwards, 10 μl of amplicon was analyzed by electrophoresis on a 2.5% agarose gel. For the production of probes, the PCR was performed as described above with the addition of 40 μM Dig-11-dUTP (Roche Diagnostics, Mannheim, Germany). Labeled PCR amplicons were used as probes for hybridization.

Hybridization techniques. Hybridization experiments were performed by dot blot and Southern blot analyses. For dot blots, the template DNA isolated by the miniprep method for genomic DNA was quantified by visual comparison after gel electrophoresis with a DNA standard (100 ng/μl). DNA was denatured in 0.4 M NaOH–10 mM EDTA, and 100 ng of denatured DNA was spotted onto a membrane (Zeta-Probe GT genomic tested blotting membranes; Bio-Rad Lab-

TABLE 2. Reference bacterial strains used in this study

Species	Strain designation(s)[a]	Biotype[b]	Serotype(s)	Source (country)
Y. enterocolitica	CIP 80.27 and ATCC 9610	1	O:8	Face (glanders-like infection) (France)
Y. enterocolitica	ATCC 23715	1	O:8	Blood (not specified)
Y. enterocolitica	IP Ye 1105	(4)	O:8	Not specified (not specified)
Y. enterocolitica	CCTM 3247	(3)	O:5, O:27	Not specified (not specified)
Y. enterocolitica	LMG 15558 and CCUG 8233	4	O:3	Mesenteric lymph node of a human with acute terminal ileitis (Sweden)
Y. enterocolitica	IP Ye 134	(4)	O:3	Not specified (not specified)
Y. enterocolitica	CIP 81.42	2	O:9	Human feces (Belgium)
Y. enterocolitica	ATCC 55075	(2)	O:9	Not specified (Canada)
Y. enterocolitica	IP Ye 21991	(1A)	O:5	Not specified (not specified)
Y. enterocolitica	IP Ye 102	(1A)	O:6, O:30	Not specified (not specified)
Y. enterocolitica	IP Ye 106	(1A)	O:7, O:8	Not specified (not specified)
Y. enterocolitica	IP Ye 1501	(1A)	O:34	Not specified (not specified)
Y. pseudotuberculosis	CIP 55.85			Turkey (Sweden)
Y. frederiksenii	ATCC 33641			Sewage (Denmark)

[a] Culture collection designations: ATCC, American Type Culture Collection; CIP, Collection de l'Institut Pasteur; IP, Institut Pasteur; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; CCTM, Centre de Collection de Types Microbiens, Université de Lausanne, Lausanne, Switzerland.

[b] The biotypes in parentheses were typed in this study. The biotypes not in parentheses were obtained from the literature or strain collection data.

TABLE 3. Primers used in this study

Target gene and primer direction	Sequence (5′→3′)	GenBank accession no.	Location (nucleotide)	Amplicon length (bp)	Annealing temp (°C)
ail					
Forward	TAATGTGTACGCTGCGAG	M29945	00544–00894	351	57
Reverse	GACGTCTTACTTGCACTG				
ystA					
Forward	ATCGACACCAATAACCGCTGAG	X65999	00093–00171	79	61
Reverse	CCAATCACTACTGACTTCGGCT	U09235	01181–01259	79	
ystB					
Forward	GTACATTAGGCCAAGAGACG	D88145	00143–00288	146	61
Reverse[a]	GCAACATACCTCACAACACC				
inv					
Forward	CGGTACGGCTCAAGTTAATCTG	M17448	00923–01105	183	61
Reverse	CCGTTCTCCAATGTACGTATCC				
yadA		X13882	01024–01872	849	
Forward	CTTCAGATACTGGTGTCGCTGT	AF102990	00465–01313	849	
Reverse	ATGCCTGACTAGAGCGATATCC	AF056092	56159–57007	849	60
		X13881[b]	00749–01507	759	
		X13883[c]	00868–01548	681	
virF/lcrF					
Forward	GGCAGAACAGCAGTCAGACATA	AF102990	32162–32722	561	63
Reverse	GGTGAGCATAGAGAATACGTCG	M86690[d]	00874–00314	561	

[a] From Ramamurthy et al. (27).
[b] *Y. enterocolitica* serotype O:8.
[c] *Y. pseudotuberculosis.*
[d] *Yersinia pestis lcrF* gene.

oratories AG). After 30 min of air drying, the membrane was rinsed in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The damp membrane was cross-linked in the GS gene linker UV chamber (Bio-Rad Laboratories AG). Hybridization was performed as described previously (18) at a temperature of 68°C, and washing steps were two times for 5 min in 2× SSC–0.1% sodium dodecyl sulfate at room temperature and two times for 15 min in 0.2× SSC–0.1% sodium dodecyl sulfate at 68°C. Detection of the signals was done by using chromogenic substrates (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate; Roche Diagnostics) according to the manufacturer's instructions.

For Southern blots, approximately 100 ng of template DNA was digested with *Eco*RV at 37°C overnight and separated on a 0.7% agarose gel. Southern blotting was done by alkaline transfer of DNA onto positively charged nylon membranes (Zeta-Probe GT genomic tested blotting membranes; Bio-Rad Laboratories AG) with an LCB 2016 VacuGene vacuum blotting pump (Amersham Pharmacia Biotech, Uppsala, Sweden). Gels were treated according to standard protocols (3). Hybridization was carried out as described above.

Statistical calculations. The statistical significance of the correlation of *Y. enterocolitica* biotypes and their origins was calculated by using two-by-two tables and evaluated by Fisher's exact test by using the software NCSS 2000.

RESULTS AND DISCUSSION

The biotypes of 140 *Y. enterocolitica* strains included in this study were determined; the distribution of the obtained biotypes for different origins of isolation is shown in Fig. 1. Strains of the pathogenic biotypes 1B, 2, 3, and 4 were significantly more-frequently isolated from humans and animals than from food and environmental sources in comparison to the biotype 1A strains ($P < 0.001$), indicative of the less-pathogenic nature of biotype 1A. Of 53 animal isolates of *Y. enterocolitica,* 46 strains (87%) were isolated from pigs, which mainly belonged to the pathogenic biotypes. The other animal isolates were a biotype 2 from a goat, a biotype 4 from a dog, and 5 biotype 1A's from 3 birds, 1 cow, and 1 dog. All field strains were further serotyped for O:3 and O:9, which were shown to be the most important pathogenic serotypes in Switzerland (Tables 1 and 2). Of isolates from human and animal sources, 68 out of 95 (71%) belonged to these serogroups, compared to 6 out of 33 (18%) isolates from food and the environment.

Gene-specific PCR tests were developed for 5 well-characterized virulence genes of *Y. enterocolitica*: the plasmid-borne genes *virF* and *yadA* and the chromosomal genes *ystA*, *ystB*, and *ail*. For *Y. pseudotuberculosis*, *yadA* and *lcrF* represented plas-

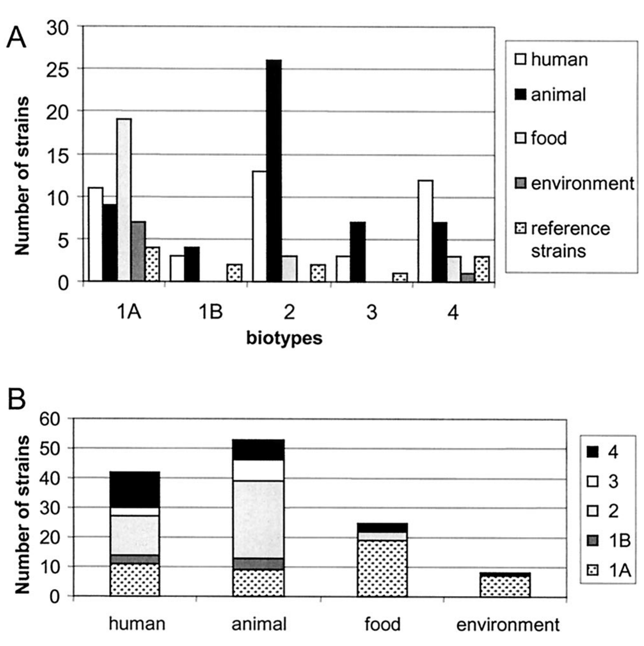

FIG. 1. Diagram of the distribution of *Y. enterocolitica* strains included in this study. (A) Strains sorted by biotype and source. (B) Strains stacked by genotype for each isolation source. Reference strains are not included.

TABLE 4. Genotyping of *Y. enterocolitica* and *Y. pseudotuberculosis* by PCR detection of virulence genes

Species and biotype (*n*)	No. of strains with chromosome-borne gene:				No. of strains with plasmid-borne gene:		
	ystA	*ystB*	*ail*	*inv*	*yadA*	*virF*	*lcrF*
Y. enterocolitica							
1A (50)	2	43	2		1	3	
1B (9)	9	0	8		0	0	
2 (44)	44	0	44		29	29	
3 (11)	11	0	11		8	9	
4 (26)	26	3[a]	26		11	12	
Y. pseudotuberculosis (40)				40	35		35

[a] Weak signal only.

mid-borne genes and *inv* represented a chromosomal gene. The developed PCR tests did not result in a detectable product with any of the control species (results not shown). The PCR results for *Y. enterocolitica* (grouped with regard to biotype) and *Y. pseudotuberculosis* are summarized in Table 4. The predominant genotype (40 out of 50, 80%) of biotype 1A strains was *ystB*$^+$ (lacking *ystA*, *ail*, *yadA*, and *virF*), showing that these strains are devoid of the virulence plasmid. Six biotype 1A strains isolated from food (3 isolates), 1 reference strain, 1 environmental isolate, and 1 pig isolate lacked both *ystA* and *ystB*. Additionally 1 reference strain had a negative result for *ystB* when analyzed by PCR but a positive result when analyzed by hybridization (Table 5). Eleven human clinical isolates belonged to biotype 1A, suggesting that this biotype is not completely nonpathogenic. All human isolates of biotype 1A contained *ystB*, and although 3 of the 19 food isolates, 1 of the 9 pig strains, and 1 of the 7 environmental strains lacked *ystB*, this difference is not statistically significant. The predominant genotype for 1B strains was *ystA*$^+$ *ail*$^+$ (lacking *ystB*, *yadA*, and *virF*) (8 out of 9, 89%, the exception was an *ail*-negative strain). Thus, all biotype 1B strains lacked plasmid-borne virulence genes.

The predominant genotype for biotype 2 strains was *ystA*$^+$ *ail*$^+$ *yadA*$^+$ *virF*$^+$ (lacking *ystB*) (29 out of 44, 66%). The same genotype was also found in biotype 3 (8 out of 11, 73%). In addition, one biotype 3 strain tested positive for *virF* but not for *yadA*. Variation in plasmid content was also found in biotype 4, with a predominant genotype of *ystA*$^+$ *ail*$^+$ (lacking *ystB*, *yadA*, and *virF*) (12 out of 26, 46%). Three strains of biotype 4 were weakly positive for *ystB* (Table 5).

Our findings indicate that isolates should always be screened for the presence of the virulence plasmid (by PCR detection of *yadA*) as well as for at least one virulence gene located on the chromosome, in order to avoid the possibility that potentially pathogenic strains will be classified as apathogenic as a result of plasmid loss. We have observed that the virulence plasmid can easily be lost when the strains are subcultivated at temperatures higher than 30°C, if they are repeatedly subcultivated, or if they are stored over time. Even reference strains which are cultivated only at 30°C may lose the plasmid.

The predominant genotype for *Y. pseudotuberculosis* was *inv*$^+$ *yadA*$^+$ *lcrF*$^+$ (35 out of 40 strains, 88%). It should be noted that 87.5% of the examined *Y. pseudotuberculosis* strains were plasmid-harboring strains. Absence of the virulence plasmid was less-frequently observed than with *Y. enterocolitica*, where the percentage of plasmid-harboring strains ranged from 0 to 72.73%, depending on the biotype. Furthermore, phenotypic tests screening for the presence of virulence plasmid, such as calcium-dependent growth at 37°C and Congo red uptake, were easier to read when screening *Y. pseudotuberculosis* than *Y. enterocolitica*. These results indicate that the plasmid is less-frequently lost in *Y. pseudotuberculosis* under the given culture conditions.

Y. enterocolitica strains of serotypes O:3 ($n = 30$) and O:9 ($n = 48$) were next compared for genotype and biotype (Fig. 2). The predominant genotype was *ystA*$^+$ *ail*$^+$ *yadA*$^+$ *virF*$^+$ (lacking *ystB*) for serotype O:9 (32 out of 48, 67%) as well as for serotype O:3 (14 out of 30, 47%). Three strains of serotype O:3 had weakly positive results for *ystB* (Table 5), and one strain of serotype O:3 was positive for *virF* but not for *yadA*. Thus, strains of the most common pathotypes in Switzerland, serotypes O:3 and O:9, did not differ in the distribution of their virulence factors.

For each gene-specific PCR test, one PCR product was sequenced for confirmation and these products were used as

TABLE 5. *Y. enterocolitica* strains with conflicting data from PCR and blotting

Our strain collection no.	Serotype(s)	Biotype	Result[a] for gene:									
			yadA		*virF*		*ail*		*ystA*		*ystB*	
			PCR	Blotting	PCR	Blotting	PCR	Blotting	PCR	Blotting	PCR	Blotting
22[c]	O:5	1A	−	−	+	−	(+)	−	(+)	−	−	+
38	ND[b]	1A	−[e]	−	+	−	−	−	(+)	−	+	+
122	ND	1A	+	+	+	+	−	−	−	−	+	+
20[d]	O:5, O:27	3	−	−	(+)	−	+	+	+	+	−	−
32	O:3	4	−[e]	−	+	−	+	+	+	+	−	−
54	O:3	4	−	−	−	−	+	+	+	+	(+)	−
69	O:3	4	+	+	+	+	+	+	+	+	(+)	−
86	O:3	4	−	−	−	−	+	+	+	+	(+)	−
75	ND	1A	−	−	−	−	−	−	−	−	+	−

[a] −, negative; +, positive; (+), weak positive.
[b] ND, tested as non-O:3 and non-O:9.
[c] Reference strain IP Ye 21991.
[d] Reference strain CCTM 3247.
[e] Red colonies from CRAMP agar were *yadA*$^+$ by PCR.

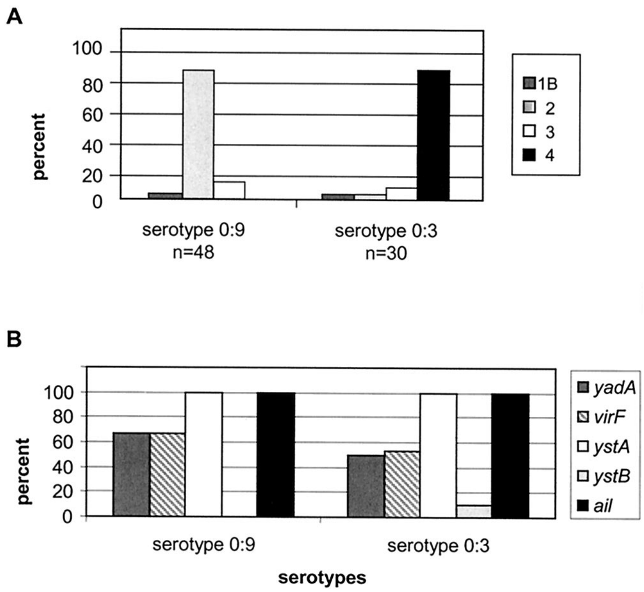

FIG. 2. Distribution of virulence genes and biotypes in serotypes O:3 and O:9 of *Y. enterocolitica*. (A) Distribution of the virulence genes in both serotypes. (B) Distribution of biotypes for the two serotypes

probes in hybridization experiments in which ambiguous strains were further analyzed (Table 5). As a negative control, nonyersiniae and *Yersinia* spp. other than *Y. pseudotuberculosis* or *Y. enterocolitica* were included in the Southern blots. All negative-control lanes were negative (results not shown). PCR testing for *ystA* demonstrates an agreement of PCR results and hybridization of 97.1%. The presence of *ystA* or *ystB* was found to be mutually exclusive in most cases. In four cases, PCR results for *ystB* were positive but hybridization results were not, and the results were reversed in one case. Table 5 lists some *Y. enterocolitica* strains that were checked by hybridization because their by PCR results did not fit a typically expected genotype or because the obtained PCR products were weak. For some of these genes, no hybridization signal was obtained, suggesting that the PCR product was nonspecific. In other cases, ambiguous PCR results were obtained due to differences in the sensitivity of PCRs for presumably heterogeneous populations, for instance, in two cases where a *virF* PCR product but not a *yadA* product was obtained (both genes are plasmid borne). One such example was biotype 4 strain 32; it was further examined since it was heterogeneous on agar plates, showing a minority of red colonies on CRAMP agar. Such red colonies were genotyped again and were found to have *yadA* and *virF* by PCR. This suggests that the PCR for *virF* displayed a higher sensitivity than that for *yadA*, since in the heterogeneous population, the latter could not be detected by PCR. In general, strains giving weak PCR signals for *virF*, *ail*, *ystA*, and *ystB* resulted in negative hybridization, suggesting that such weak PCR bands can be ignored; however, biotype 1A strain 75 was convincingly PCR positive for *ystB* but negative by hybridization. Unexpectedly, reference strain ATCC 9610 (equivalent to CIP 80.27) was devoid of the *ail* gene, as shown by PCR and hybridization. In accordance with this result, Blais and Phillippe (7) classify this strain as avirulent because they also found the strain to lack *yadA* and *ail.* All other strains had

TABLE 6. Correlation of *Y. enterocolitica* biotypes and results of pyrazinamidase testing

Y. enterocolitica biotype (*n*)	No. of strains with pyrazinamidase result			No. of strains not tested
	Positive	Negative	Intermediate	
1A (50)	42	3[a]	4[b]	1
1B (9)	0	7	0	2
2 (44)	2[c]	39	3[d]	0
3 (11)	0	11	0	0
4 (26)	0	23	0	3

[a] *ystB* positive.
[b] All but one strain was *ystB* positive.
[c] One strain was *ystA*$^+$ *ail*$^+$ *yadA*$^+$ *virF*$^+$ (lacking *ystB*), and one strain was *ystA*$^+$ *ail*$^+$ (lacking *ystB, yadA,* and *virF*).
[d] Two strains were *ystA*$^+$ *ail*$^+$ *yadA*$^+$ *virF*$^+$ (lacking *ystB*), and one strain was *ystA*$^+$ *ail*$^+$ (lacking *ystB, yadA,* and *virF*).

matching data for PCR and hybridization, so that the correlation of PCR and hybridization was 97.1% for *yadA*, 100% for *inv*, 94.5% for *virF*/*lcrF*, 98.6% for *ail*, 97.1% for *ystA*, and 92.9% for *ystB*.

We next compared the obtained PCR results with phenotypic analysis. Out of 140 strains of *Y. enterocolitica*, 134 were tested for pyrazinamidase activity (Table 6). Three strains of biotype 1A were incorrectly typed as pyrazinamidase negative (all biotype 1A strains should be positive), and two biotype 2 strains were incorrectly found positive. Seven strains showed an intermediate reaction; 4 of them were biotype 1A strains, which were expected to give a positive result, and 3 of them were of biotype 2, expected to react negatively for pyrazinamidase activity. The pyrazinamidase test was thus found to be less conclusive and more ambiguous than PCR.

The correlation of MOX agar with the plasmid-specific PCR for *yadA* and *lcrF* was 100% in the case of the 40 *Y. pseudotuberculosis* strains; however, the two assays correlated less well for *Y. enterocolitica*. Seven *Y. enterocolitica* strains were read as MOX positive but were negative in both plasmid-specific PCR assays. Ten strains of *Y. enterocolitica* did not show pinpoint colonies or had only very few pinpoint colonies but were positive in both plasmid-specific PCR assays, and 4 more strains were negative on MOX agar but reacted in PCR (confirmed by Southern blot) as *yadA*-lacking and *virF*$^+$. Strains reacting positively on MOX agar were often a mixture of plasmid-positive and plasmid-negative colonies, and the number of pinpoint colonies varied between strains.

As with MOX agar, the correlation of CRAMP agar with the plasmid-specific PCR assays was good for *Y. pseudotuberculosis*. Only one *Y. pseudotuberculosis* strain (lacking *yadA* and *lcrF*) gave rise to a few positive colonies on CRAMP agar. With regard to *Y. enterocolitica* strains, no false-negative reactions were seen; however, 19 strains positive on CRAMP agar had a genotype lacking *yadA* and *virF*. These consisted of 12 biotype 1A strains and 7 strains of biotype 2 (n = 3), biotype 3 (n = 1), biotype 4 (n = 2,) and biotype 1B (n = 1). Positive CRAMP reactions were hard to read, since intermediate results such as rosa colonies or only a few red colonies were commonly formed. Variation in plasmid carriage is a known problem with *Yersinia* spp. Robins-Browne et al. (29) found that calcium dependence was the least-sensitive and -specific of the assays for plasmid carriage. Koeppel et al. (17) report that the proportion of microcolonies (plasmid-bearing cells) can range from 5 to over 95%. Plasmid-harboring strains on CRAMP agar in our hands frequently gave rise to an intermediate reaction, probably also due to the loss of a plasmid. Twelve *Y. enterocolitica* strains of biotype 1A were falsely positive by testing the Congo red uptake. Kwaga et al. (19) and Lewin et al. (20) found *Y. enterocolitica* strains of biotype 1A with plasmids of various sizes. Some of those strains showed positive reactions in the virulence assay. It was reported (20) that after 16 h all strains had at least a weak-positive reaction for Congo red uptake, but none were positive for low calcium response. Although genotyping is not completely devoid of false-positive or false-negative reactions, it performs at least as well as, and frequently better than, phenotyping. Moreover, the outcome is less subjective than that of phenotyping.

The predictive value of the obtained genotyping data for virulence was assessed. For all strains investigated, *Y. enterocolitica* strains of pathogenic biotypes (1B and 2 to 4) harbor the chromosome-borne virulence genes *ystA* and *ail* independent of their origin of isolation. Thus, a clear correlation exists between chromosome-borne virulence genes and biotype for these biotypes. As expected, the findings for plasmid-borne genes are less consistent due to the possible loss of a plasmid. Gene *ystB* is exclusively found in biotype 1A strains, independent of the source of isolates. This genotyping test thus performs at least as well as the pyrazinamidase testing, which allows differentiation between the pathogenic biotypes and biotype 1A strains but is difficult to read (Table 6). It is important to note, however, that biotype 1A strains isolated from clinical cases did not differ significantly in their virulence gene content (as detected by PCR) compared to 1A strains isolated from other sources. The genotypic characterization, therefore, has less predictive value for virulence in this group of strains. All *Y. pseudotuberculosis* isolates harbored the *inv* gene, and most isolates contained plasmid-borne genes (Table 4). However, lack of discrimination by genotyping based on *yadA*, *lcrF*, and *inv* resulted in little predictive value for the pathogenicity of *Y. pseudotuberculosis*.

The results of genotyping disseminated to human and nonhuman isolates are presented in Fig. 3. Although there is no striking difference between human and nonhuman isolates, the most remarkable observation is the absence of *ystB* and the presence of *ystA* in strains of serotypes 1B and 2 to 4. Human and nonhuman isolates of serotype 1A did not differ in genotype.

The genotyping scheme used in this study clearly separated biotype 1A from the others. However, it did not differentiate between pathogenic and apathogenic strains within this serotype. Our results indicate that the best predictive value for the pathogenicity of the classical pathogenic serotypes of *Y. enterocolitica* is the genotype $ystA^+$ (lacking *ystB*). If only these genes were tested, 96% of the strains belonging to biotypes 1B and 2 to 4 would be characterized correctly. However, 26% of human isolates (all biotype 1A) were $ystB^+$ (lacking *ystA*), which is the predominant genotype for that serotype in our findings. One study reported a frequency of *ystB* presence in biotype 1A strains of 85% (14); another study (27) described 100% of biotype 1A strains as *ystB* positive. From our data, the PCR results for 6 strains of biotype 1A (out of 50) were negative for *ystA* and *ystB*; none of these strains were of human origin. It is

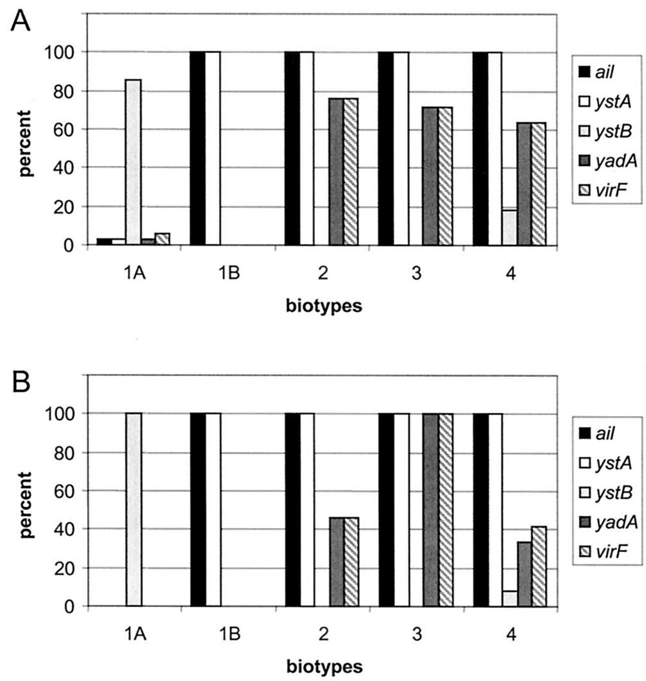

FIG. 3. Diagram of the distribution of *Y. enterocolitica* genotypes, as determined by PCR, in different biotypes. (A) Distribution of genotypes in biotypes isolated from human sources. (B) Distribution of genotypes in biotypes isolated from nonhuman sources.

possible that these isolates do not have a gene for enterotoxin or that these genes differ substantially in nucleotide sequence. Ramamurthy et al. (27) described an additional, rare subtype gene, *ystC*; 16.5% of their strains remained negative with all three probes. Similarly, Grant et al. (14) found 6 strains of *Y. enterocolitica* biotype 1A which were enterotoxin-producing strains but did not anneal to probes for *ystA*, *ystB*, and *ystC*. In our study, all human biotype 1A strains were positive for *ystB*, but 80% of the animal, food, and environmental strains were also positive and biotype 1A strains are overrepresented in food and environmental strains. The absence of a striking marker for virulence in biotype 1A strains isolated from human clinical cases may be illustrative of the intrinsic weak pathogenicity of this biotype. The clinical outcome is more likely determined by host factors than by bacterial virulence factors in this case. It has been said that biotype 1A strains, classified as avirulent *Y. enterocolitica*, are able to evoke clinical disease symptoms similar to those strains belonging to classical pathogenic bioserotypes (10, 24).

In conclusion, PCR-dependent detection of virulence genes results in a rapid characterization of *Y. enterocolitica* and *Y. pseudotuberculosis* isolates. These tests can now be further evaluated and refined for their potential to predict pathogenicity, even after loss of the virulent plasmid.

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REFERENCES

1. **Aleksic, S., and J. Bockemühl.** 1999. *Yersinia* and other enterobacteriaceae, p. 483–496. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 7th ed. American Society for Microbiology, Washington, D.C.
2. **Aulisio, C. C., W. E. Hill, J. T. Stanfield, and R. L. Sellers, Jr.** 1983. Evaluation of virulence factor testing and characteristics of pathogenicity in *Yersinia enterocolitica*. Infect. Immun. **40:**330–335.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1999. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
4. **Bhaduri, S., C. Turner-Jones, M. M. Taylor, and R. V. Lachica.** 1990. Simple assay of calcium dependency for virulent plasmid-bearing clones of *Yersinia enterocolitica*. J. Clin. Microbiol. **28:**798–800.
5. **Bhaduri, S., C. Turner-Jones, and R. V. Lachica.** 1991. Convenient agarose medium for simultaneous determination of the low-calcium response and Congo red binding by virulent strains of *Yersinia enterocolitica*. J. Clin. Microbiol. **29:**2341–2344.
6. **Bhaduri, S., and B. Cottrell.** 1997. Direct detection and isolation of plasmid-bearing virulent serotypes of *Yersinia enterocolitica* from various foods. Appl. Environ. Microbiol. **63:**4952–4955.
7. **Blais, B. W., and L. M. Phillippe.** 1995. Comparative analysis of *yadA* and *ail* polymerase chain reaction methods for virulent *Yersinia enterocolitica*. Food Control **6:**211–214.
8. **Bölin, I., A. Forsberg, L. Norlander, M. Skurnik, and H. Wolf-Watz.** 1988. Identification and mapping of the temperature-inducible plasmid-encoded proteins of *Yersinia* spp. Infect. Immun. **56:**343–348.
9. **Bottone, E. J.** 1997. *Yersinia enterocolitica*: the charisma continues. Clin. Microbiol. Rev. **10:**257–276.
10. **Burnens, A. P., A. Frey, and J. Nicolet.** 1996. Association between clinical presentation, biogroups and virulence attributes of *Yersinia enterocolitica* strains in human diarrhoeal disease. Epidemiol. Infect. **116:**27–34.
11. **Cornelis, G., C. Sluiters, C. L. de Rouvroit, and T. Michiels.** 1989. Homology between VirF, the transcriptional activator of the *Yersinia* virulence regulon, and AraC, the *Escherichia coli* arabinose operon regulator. J. Bacteriol. **171:**254–262.
12. **Delor, I., A. Kaeckenbeeck, G. Wauters, and G. R. Cornelis.** 1990. Nucleotide sequence of *yst*, the *Yersinia enterocolitica* gene encoding the heat-stable enterotoxin, and prevalence of the gene among pathogenic and nonpathogenic yersiniae. Infect. Immun. **58:**2983–2988.
13. **Farmer, J. J., G. P. Carter, V. L. Miller, S. Falkow, and I. K. Wachsmuth.** 1992. Pyrazinamidase, CR-MOX agar, salicin fermentation-esculin hydrolysis, and D-xylose fermentation for identifying pathogenic serotypes of *Yersinia enterocolitica*. J. Clin. Microbiol. **30:**2589–2594.
14. **Grant, T., V. Bennett-Wood, and R. M. Robins-Browne.** 1998. Identification of virulence-associated characteristics in clinical isolates of *Yersinia enterocolitica* lacking classical virulence markers. Infect. Immun. **66:**1113–1120.
15. **Isberg, R. R., D. L. Voorhis, and S. Falkow.** 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50:**769–778.
16. **Kandolo, K., and G. Wauters.** 1985. Pyrazinamidase activity in *Yersinia enterocolitica* and related organisms. J. Clin. Microbiol. **21:**980–982.
17. **Koeppel, E., R. Meyer, J. Luethy, and U. Candrian.** 1993. Recognition of pathogenic *Yersinia enterocolitica* by crystal violet binding and polymerase chain reaction. Lett. Appl. Microbiol. **17:**231–234.
18. **Kuhnert, P., J. Hacker, I. Mühldorfer, A. P. Burnens, J. Nicolet, and J. Frey.** 1997. Detection system for *Escherichia coli*-specific virulence genes: absence of virulence determinants in B and C strains. Appl. Environ. Microbiol. **63:**703–709.
19. **Kwaga, J., J. O. Iversen, and V. Misra.** 1992. Detection of pathogenic *Yersinia enterocolitica* by polymerase chain reaction and digoxigenin-labeled polynucleotide probes. J. Clin. Microbiol. **30:**2668–2673.
20. **Lewin, A., E. Strauch, S. Hertwig, B. Hoffmann, H. Nattermann, and B. Appel.** 1996. Comparison of plasmids of strains of *Yersinia enterocolitica* biovar 1A with the virulence plasmid of a pathogenic *Y. enterocolitica* strain. Zentbl. Bakteriol. **285:**52–63.
21. **Lindsay, J. A.** 1997. Chronic sequelae of foodborne disease. Emerg. Infect. Dis. **3:**443–452.
22. **Miller, V. L., J. J. Farmer III, W. E. Hill, and S. Falkow.** 1989. The *ail* locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. Infect. Immun. **57:**121–131.
23. **Miller, V. L., J. B. Bliska, and S. Falkow.** 1990. Nucleotide sequence of the *Yersinia enterocolitica ail* gene and characterization of the Ail protein product. J. Bacteriol. **172:**1062–1069.
24. **Morris, J. G., Jr., V. Prado, C. Ferreccio, R. M. Robins-Browne, A.-M. Bordun, M. Cayazzo, B. A. Kay, and M. M. Levine.** 1991. *Yersinia enterocolitica* isolated from two cohorts of young children in Santiago, Chile: incidence of and lack of correlation between illness and proposed virulence factors. J. Clin. Microbiol. **29:**2784–2788.
25. **Pitcher, D., N. Saunders, and R. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. **8:**151–156.
26. **Prpic, J. K., R. M. Robins-Browne, and R. B. Davey.** 1983. Differentiation between virulent and avirulent *Yersinia enterocolitica* isolates by using Congo red agar. J. Clin. Microbiol. **18:**486–490.
27. **Ramamurthy, T., K. I. Yoshino, X. Huang, G. Balakrish Nair, E. Carniel, T. Maruyama, H. Fukushima, and T. Takeda.** 1997. The novel heat-stable enterotoxin subtype gene (*ystB*) of *Yersinia enterocolitica*: nucleotide sequence and distribution of the *yst* genes. Microb. Pathog. **23:**189–200.
28. **Riley, G., and S. Toma.** 1989. Detection of pathogenic *Yersinia enterocolitica* by using Congo red-magnesium oxalate agar medium. J. Clin. Microbiol. **27:**213–214.
29. **Robins-Browne, R. M., T. Takeda, A. Fasano, A. M. Bordun, S. Dohi, H. Kasuga, G. Fang, V. Prado, R. L. Guerrant, et al.** 1993. Assessment of enterotoxin production by *Yersinia enterocolitica* and identification of a novel heat-stable enterotoxin produced by a noninvasive *Y. enterocolitica* strain isolated from clinical material. Infect. Immun. **61:**764–767.
30. **Skurnik, M., and H. Wolf-Watz.** 1989. Analysis of the *yopA* gene encoding the Yop1 virulence determinants of *Yersinia* spp. Mol. Microbiol. **3:**517–529.
31. **Straley, S. C.** 1991. The low-Ca^{2+} response virulence regulon of human-pathogenic *yersiniae*. Microb. Pathog. **10:**87–91.
32. **Wauters, G., K. Kandolo, and M. Janssens.** 1987. Revised biogrouping scheme of *Yersinia enterocolitica*. Contrib. Microbiol. Immunol. **9:**14–21.
33. **Yoshino, K., H. Xiaozhe, M. Miyachi, Y. Hong, T. Takao, H. Nakao, T. Takeda, and Y. Shimonishi.** 1994. Amino acid sequence of a novel heat-stable enterotoxin produced by a *yst* gene-negative strain of *Yersinia enterocolitica*. Lett. Pept. Sci. **1:**95–105.