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Detection of Pathogenic *Yersinia enterocolitica* by Polymerase Chain Reaction and Digoxigenin-Labeled Polynucleotide Probes

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Yersinia enterocolitica **is widespread in nature, but only a few bioserotypes are involved in human infections. Pigs are considered to be the major reservoirs of pathogenic strains. It is essential to have an accurate and rapid method for the detection of pathogenic yersiniae. To achieve this objective, 19-base synthetic oligonucleotide primers were used in a polymerase chain reaction (PCR) to detect the *ail* gene (which is conserved only in pathogenic strains) in strains of *Y. enterocolitica* and related species originating from pigs or pork products. Digoxigenin-labeled probes derived from the *ail*, *inv*, and *yst* genes were also evaluated on these strains. The PCR amplified a 273-bp fragment of the *ail* gene involved in eukaryotic cell invasion and serum resistance. The PCR detected template DNA only in strains of *Y. enterocolitica* traditionally classified as human pathogens but not in biotype 1A strains and related species. Other members of the family *Enterobacteriaceae* were also negative for the target gene. The digoxigenin-labeled *ail* probe gave identical results to the PCR. By use of this nonisotopic method, *inv*-homologous DNA was detected only among yersiniae, except for *Y. ruckeri*. Although all pathogenic serotypes of *Y. enterocolitica* were positive for the heat-stable enterotoxin *yst* gene, two strains of biotype 1A, one *Y. intermedia* strain, and six other species of the *Enterobacteriaceae* were also positive. Our results support the notion that pigs constitute an important reservoir of pathogenic *Y. enterocolitica* and that the *inv*-homologous sequence is *Yersinia* specific.**

Yersinia enterocolitica is a common human pathogen which causes gastrointestinal syndromes of various severities, ranging from mild diarrhea to mesenteric adenitis evoking appendicitis. Systemic involvement is unusual, but arthritis and erythema nodosum are common complications (5). Recently, *Y. enterocolitica* sepsis associated with transfusion of contaminated erythrocytes, from which there is a high mortality rate, has been reported in several countries (2). *Y. enterocolitica* is heterogeneous, with over 50 serotypes and several biotypes, but only a few of the bioserotypes are pathogenic for humans (4). Swine have been implicated as the principal reservoir of human pathogenic strains (2, 35).

Two genetic loci that confer invasiveness and are necessary for virulence have been identified on the bacterial chromosome. These are the *inv* (invasion) locus of *Y. enterocolitica* and *Y. pseudotuberculosis* and the *ail* (attachment invasion locus) region of *Y. enterocolitica* (14, 24, 25). All isolates of *Y. enterocolitica* that show virulence in humans contain DNA sequences homologous to the *ail* locus, whereas avirulent environmental isolates do not (25). Unlike *ail*-homologous DNA, *inv*-homologous DNA is present in all strains, but the invasion protein is not expressed in environmental isolates (30).

Y. enterocolitica heat-stable enterotoxin is thought to be involved in virulence, although its role is less well defined (28, 29). However, recently the gene (*yst*) for the toxin was cloned and sequenced. On the basis of Southern hybridization studies, it was suggested that *yst* is a virulence determinant (6).

In addition to being associated with selected bioserotypes and with strains carrying specific chromosomal genes, virulence in *Y. enterocolitica* is associated with the carriage of a 67- to 70-kb plasmid (5). The virulence plasmid is unstable and easily lost during laboratory manipulation (24). There is a need for a simple and specific test for detection of pathogenic strains of *Y. enterocolitica* that does not rely on the presence of the virulence plasmid (18).

Because nonpathogens may readily contaminate food, water, or even clinical specimens, it is essential to be able to distinguish true pathogens from their similar but comparatively benign relatives (33). Existing methods to differentiate between pathogenic and nonpathogenic strains are time-consuming and unreliable (39). This is because many isolates of *Y. enterocolitica* give anomalous or contradictory results in the in vitro virulence assays which have been used to infer pathogenicity (33). Many of the assays are also based on the virulence plasmid, which is unstable and is easily lost during laboratory manipulations (26).

The aim of the present study was to evaluate a polymerase chain reaction (PCR) using oligonucleotide primers derived from the *ail* gene and digoxigenin-labeled polynucleotide probes derived from the *ail* and *yst* genes for the detection of pathogenic *Y. enterocolitica*. We were also interested in determining the presence of the *inv* locus in *Y. enterocolitica* and related species. The long-term goal of our studies is to determine the role of swine as a reservoir of human pathogenic strains of *Y. enterocolitica*.

MATERIALS AND METHODS

Bacterial strains. A total of 80 strains of *Y. enterocolitica* and related species isolated from swine and pork products, 12 reference strains (mostly of human origin), and 5 human clinical isolates used in this study have been described

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elsewhere (17, 18). *Y. enterocolitica* 8081C and *Escherichia coli* HB 101(pVM103) and HB 101(pVM101) harboring *ail* and *inv* recombinant plasmids, respectively, were provided by V. L. Miller (University of California, Los Angeles) (24). *E. coli* clone pID10, which contains the *yst* gene, was kindly provided by G. Cornelis (Université Catholique de Louvain, Brussels, Belgium) (6).

DNA techniques. Genomic DNA was isolated as described by Pollard et al. (31), and plasmid DNA was isolated by the miniprep alkaline lysis method (21). Restriction enzymes were used according to the instruction of the manufacturers (Boehringer, Mannheim, Germany; GIBCO/BRL, Burlington, Ontario, Canada; and Sigma Chemical Company, St. Louis, Mo.). Restriction enzyme fragments were purified from agarose gel slices with siliconized glass wool (12), and the DNA was precipitated as described previously (21). For the *ail*- and *inv*-derived probes, the 2.1-kb *Ava*I-*Ava*I and 3.6-kb *Cla*I-*Cla*I fragments were cut from pVM103 and pVM101, respectively (25). The *yst* probe was prepared from a 3.2-kb *Hin*dIII fragment of pID10 (6). The cloned fragments were labeled by the random primer method (10) with digoxigenin-dUTP by using a DIG-DNA labeling and detection kit (Boehringer).

Southern hybridization. Genomic DNA digested with restriction enzyme, following agarose gel electrophoresis, was transferred to a positively charged nylon membrane (Boehringer) by the method of Southern (34) and fixed for about 1 h at 80°C in an oven without the use of a vacuum. Prehybridization and hybridization were done at 68°C as recommended in a mixture of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% (wt/vol) sodium salt of *N*-laurylsarcosine, 0.02% (wt/vol) sodium dodecyl sulfate, and 1% blocking reagent (Boehringer). The hybridization solution contained about 30 to 50 ng of labeled DNA per ml. Hybridization was always carried out overnight in a shaker water bath. Hybrid DNA was visualized by an enzyme immunoassay with antidigoxigenin antibodies conjugated to alkaline phosphatase. The substrates for alkaline phosphatase were nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salts (Boehringer). The color reaction was usually allowed to proceed for 16 to 24 h.

Bacterial colony and DNA dot blot hybridization. Bacteria were spotted and grown on membranes overnight, lysed, and denatured in situ as described by Maniatis et al. (21). Filters were dried at room temperature for about 30 min. DNA released from the cells was fixed by UV light for 3 min. For dot blot hybridization, genomic DNA was denatured by boiling for 10 min, cooled rapidly on ice, spotted onto the membrane, and fixed by UV light. Hybridization and detection of hybrid DNA were carried out as described above. At the beginning of the study, using representative strains of bacteria, we obtained similar results with the three methods (data not shown). Subsequently, detection of homologous DNA in our test strains was done by colony hybridization because of the ease and rapidity of preparing test materials.

Selection of PCR primers. Primers to direct DNA amplification by PCR were generated by a computer program (20) from the open codon reading frame of the published nucleotide sequence of the *Y. enterocolitica ail* gene (23). One pair of 19-base oligonucleotide primers was selected; the primers were designated Ail-a (5′-GAACTCGATGATAACTGGG-3′) and Ail-b (5′-GCAATTCAACCCACTTCAA-3′). The expected product of amplification of the target sequence with these primers was 273 bp long. The primers were synthesized in our department on an Applied Biosystems 380A DNA synthesizer by standard protocols.

PCR amplification. DNA samples (1 μg of nucleic acid) or 1 μl of crude lysate (3) was amplified in a 50-μl reaction mixture containing 200 μM each dATP, dCTP, dGTP, and dTTP; 1× reaction buffer (50 mM KCl, 10 mM Tris-hydrochloride [pH 8.3]); 1 mM $MgCl_2$; 2.5 U of *Taq* polymerase; and a 0.5 μM concentration of each oligonucleotide primer. The samples were overlaid with 100 μl of mineral oil and subjected to 35 cycles of amplification in a DNA Thermal Cycler (Perkin Elmer Cetus). The parameters for the amplification cycles were as follows: denaturation for 1 min at 94°C, annealing of primers for 1 min at 65°C, and primer extension for 1 min at 72°C, with a 15-s autoextension. A negative control with all of the reaction components except template DNA was included with each test run. The crude lysate could be prepared from either broth or plate agar cultures. The PCR-amplified products were analyzed by agarose gel electrophoresis and visualized by being stained with ethidium bromide by a standard technique (21).

RESULTS

One pair of synthetic *ail*-specific oligonucleotide primers targeting a 273-bp fragment of the *ail* gene, which codes for the membrane-associated Ail protein, was used in the PCR. Figure 1 shows the presence of the amplified product in *Y. enterocolitica* and related species. Results of the PCR are summarized in Table 1. The predicted fragment occurred in *Y. enterocolitica* serotypes O:1,2,3, O:3, O:5,27, O:8, and O:9 but not in biotype 1A strains, related species, or *Y. pseudotuberculosis*. Other members of the family *Enterobacteriaceae* were also negative for the target gene. These included *Enterobacter cloacae*, *Edwardsiella tarda*, *E. coli*, a *Salmonella* sp., *Klebsiella pneumoniae*, *Salmonella arizonae*, *Citrobacter freundii*, *Enterobacter hafniae*, *Serratia marcescens*, *Morganella morganii*, and *Proteus vulgaris*, all from the bacterial collection of our department. We also tested the following human clinical isolates (provided by R. Rennie, Royal University Hospital, Saskatoon, Saskatchewan, Canada): *E. coli* (O157:H7), *C. freundii*, *Salmonella typhimurium*, *Providencia stuartii*, *K. pneumoniae*, *M. morganii*, *E. cloacae*, *S. marcescens*, *Proteus mirabilis*, and *Shigella flexneri*. These isolates were also found to be *ail* negative.

On one occasion, we observed the 273-bp product in the single isolate of *Y. kristensenii*, but this result was not reproduced in subsequent tests and thus was considered false positive. In addition to yielding the predicted 273-bp product, serotype O:8 strains yielded a product of about 1.8 kb on most occasions. The recombinant plasmid and the *ail* insert also yielded the 1.8-kb product.

The results of the experiments with digoxigenin-labeled probes are shown in Fig. 2 and summarized in Table 1. Only pathogenic bioserotypes of *Y. enterocolitica* were positive for the *ail* gene; no biotype 1A strains, related species, or other members of the *Enterobacteriaceae* were positive for the *ail* gene. However, very weak hybridization signals were seen with *E. coli* O157:H7 and *S. flexneri* in bacterial colony hybridization tests; these results were considered negative. It was also seen that the intensity of the signal was somewhat higher with *Y. enterocolitica* serotype O:8 than with the other pathogenic bioserotypes.

As expected, *inv*-homologous DNA was present exclusively among *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. enterocolitica*-like species. Also, not unexpectedly, *Y. ruckeri* was negative with the *inv* probe. Although the species related to *Y. enterocolitica* were clearly positive, the

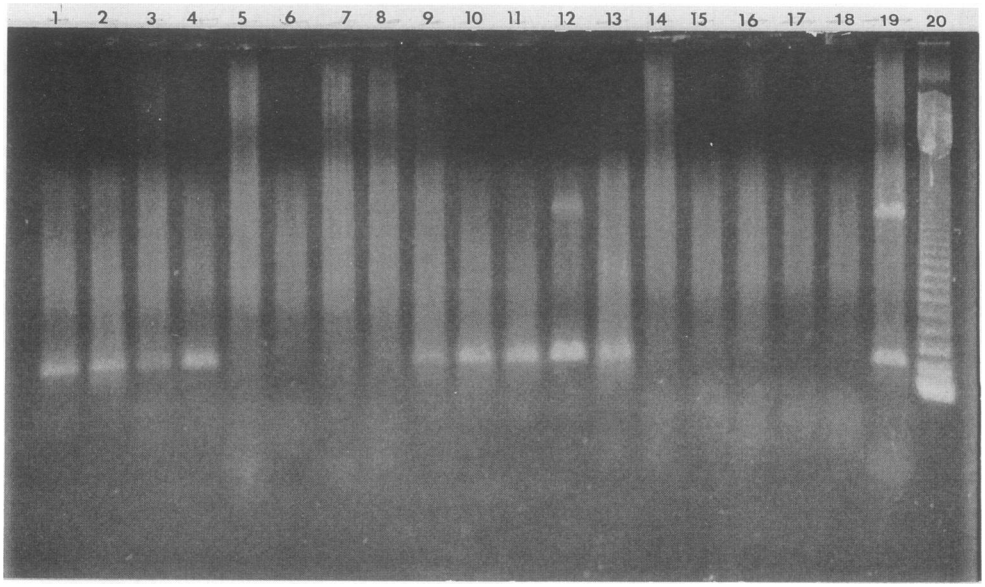

FIG. 1. Occurrence and distribution of the 273-bp amplification fragment of *ail* in the PCR. Lanes 1 to 8, swine isolates of *Y. enterocolitica* O:3, O:5,27, O:1,2,3, O:8, and biotype 1A, *Y. intermedia*, *Y. frederiksenii*, and *Y. kristensenii*, respectively; lane 9, a *Y. enterocolitica* O:3 human clinical strain; lanes 10 to 17, reference strains of *Y. enterocolitica* O:3, O:5,27, O:8, and O:9, *Y. rohdei*, *Y. bercovieri*, *Y. mollaretii*, and *Y. ruckeri*, respectively; lane 18, negative control; lane 19, pVM103; lane 20, a 123-bp DNA ladder (Bethesda Research Laboratories). Note the ca. 1.8-kb product in the reference strain of *Y. enterocolitica* O:8 and pVM103.

hybridization signal was less intense than for *Y. enterocolitica* sensu stricto.

In addition to being conserved among pathogenic bioserotypes of *Y. enterocolitica*, the *yst* gene was present in two strains of biotype 1A *Y. enterocolitica*, one strain of *Y. intermedia* (data not shown), and five other species of the *Enterobacteriaceae* of human origin, namely, *E. coli* (O157:H7), *C. freundii*, *S. typhimurium*, *S. flexneri*, and *E. cloacae*. *E. tarda* from an animal source was also positive. Figure 3 shows that some members of the family *Enterobacteriaceae* are *yst* positive.

DISCUSSION

We have described a PCR protocol for the detection of pathogenic strains of *Y. enterocolitica*. The primers amplified a 273-bp fragment of the *ail* gene involved in eukaryotic cell invasion and complement-mediated serum resistance (9). Also, digoxigenin-labeled polynucleotide probes were evaluated for the detection of *Y. enterocolitica* and related species, including pathogenic strains.

Several nucleic acid-based hybridization techniques have been evaluated for the detection of virulent strains of *Y. enterocolitica* (13, 15, 22, 27, 33). A further improvement of the sensitivity of DNA probes is the amplification of the target DNA by the PCR, which offers the advantages of maximum sensitivity, specificity, and rapidity (37). The PCR has been successfully applied in the detection of virulent strains of various bacterial species, including *Y. enterocolitica* (3, 7, 11, 31, 32, 37–39). However, the previous studies of *Y. enterocolitica* were of a limited nature. Wren and Tabaqchali (39) used primers based on *virF*, which is a key regulatory gene on the virulence plasmid, but plasmid loss resulted in negative outcomes in pathogenic bioserotypes. Feinwick and Murray (11) used a pair of 20-mer primers derived from the *ail* gene to amplify a 359-bp product. However, they used a smaller number of isolates than the present study did, and their strains were derived only from humans. Additionally, our isolates have been well characterized with regard to virulence-associated phenotypes (18).

In the present study, the PCR was specific for virulent strains of *Y. enterocolitica* because template DNA was not detected among biotype 1A strains, related *Yersinia* species, or other members of the family *Enterobacteriaceae*. The technique was rapid because the protocol yielded data in less than 5 h after DNA extraction or preparation of crude lysate. There is a need to evaluate the efficacy of PCR for the detection of virulent strains of *Y. enterocolitica* from enrichment broths, mixed cultures, and even directly from food and clinical specimens.

We cannot explain the presence of an additional amplification product among *Y. enterocolitica* serotype O:8 strains. An attempt was made to rule out gene duplication by digesting the PCR product with restriction enzymes for which the *ail* gene has internal sites. However, this was inconclusive, since we did not have all the restriction enzymes that cut within this gene. Additionally, the presence of this product even when the *ail* insert of pVM103 was used as the target DNA would rule out this possibility, since this clone contains only a single reading frame (23). However, the *ail* sequence and adjacent sequences of serotype O:8 and other American serotypes have been found to differ from non-American pathogenic serotypes such as O:3, O:5,27, O:9, and O:1,2,3 (25).

Digoxigenin-labeled probes have been applied to detection of virulent bacteria (16, 19, 36) but to our knowledge they have not been applied to detection of *Yersinia* species. These authors found that the use of digoxigenin-labeled probes may be comparable to other techniques in terms of specificity and sensitivity (16, 19, 36). The use of the *ail* probe in the digoxigenin technique gave results identical to those of the PCR, but the latter method was more rapid and less labori-

TABLE 1. Results obtained with PCR and digoxigenin-labeled probes for *Y. enterocolitica* and related species

Species or serotype[a]	No. of strains	No. of strains positive with:			
		PCR[b]	Digoxigenin-labeled probe		
			ail	*inv*	*yst*
Isolates from pigs or pork					
Ye O:3 (4)	18	18	18	18	18
Ye O:5,27 (2)	12	12	12	12	12
Ye O:1,2,3 (3)	17	17	17	17	17
Ye O:8 (1B)	1	1	1	1	1
Ye biotype 1A	19	0	0	19	2
Y. intermedia	10	0	0	10	1
Y. frederiksenii	2	0	0	2	0
Y. kristensenii	1	0	0	1	0
Reference strains[c]					
Ye O:8 (1B)	1	1	1	1	1
Ye O:3 (4)	1	1	1	1	1
Ye O:9 (2)	1	1	1	1	1
Ye O:5,27 (2)	1	1	1	1	1
Ye O:3 (3)	1	1	1	1	1
Y. bercovieri	1	0	0	1	0
Y. mollaretii	1	0	0	1	0
Y. rohdei	1	0	0	1	0
Y. ruckeri	1	0	0	0	0
Y. intermedia	1	0	0	1	0
Y. frederiksenii	1	0	0	1	0
Y. kristensenii	1	0	0	1	0
Y. pseudotuberculosis	1	0	0	1	0
Human clinical isolate Ye O:3 (4)	5	5	5	5	5
Other species[d]	13	0	0	0	6

[a] Designations in parentheses are biotypes of the indicated *Y. enterocolitica* (Ye) serotypes.
[b] Number of strains positive for the 273-bp PCR product.
[c] The reference strains are of human origin, except for *Y. ruckeri*, which is from a muskrat.
[d] Thirteen different species of the *Enterobacteriaceae*.

ous. We also noted differences in the intensity of hybridization signals between serotype O:8 and the other pathogenic bioserotypes. The probe was prepared from a wild strain of *Y. enterocolitica* O:8, 8081C (24).

We have found, as have Miller et al. (25), that the *inv* locus is genus specific, being present only among members of the genus *Yersinia*, including *Y. pseudotuberculosis*. Not unexpectedly, however, *Y. ruckeri* was negative for *inv*-homologous sequence. It has been shown that this species is phenotypically and genetically less related to the other species of *Yersinia* (8). Therefore, our finding supports the notion that *Y. ruckeri* does not belong to the genus *Yersinia*.

Delor et al. (6) were able to detect the *yst* gene by Southern hybridization only among pathogenic serotypes of *Y. enterocolitica* and certain serotypes of *Y. kristensenii*. Our results differ somewhat in that we detected *yst*-homologous DNA in 2 of 19 biotype 1A *Y. enterocolitica* strains and 1 of 10 *Y. intermedia* strains but not in our single pork product isolate or the reference strain of *Y. kristensenii*. Not all serotypes of *Y. kristensenii* are *yst*$^+$ or produce the heat-stable enterotoxin (6). Since some biotype 1A *Y. enterocolitica* strains produce Yst (28, 29), it is not inconceivable that they harbor a *yst* homologous sequence. However, this is speculative and has yet to be confirmed. Still, the role of *yst* in *Y. enterocolitica* virulence definitely requires further investigation (6). It also seems that *yst*-homologous DNA is ubiquitous among members of the *Enterobacteriaceae*, since we found 6 of 13 species to be positive by the bacterial colony hybridization method. Several members of the family *Enterobacteriaceae* are known to produce heat-stable enterotoxins that may be similar in structure and properties to the prototype *E. coli* heat-stable enterotoxin (1).

The presence of an *ail*-homologous DNA sequence in a *Y. enterocolitica* strain has been shown to have a perfect correlation with virulence in humans (25). Thus, our findings obtained by both PCR and a digoxigenin-labeled *ail* probe with swine isolates are further evidence that swine constitute

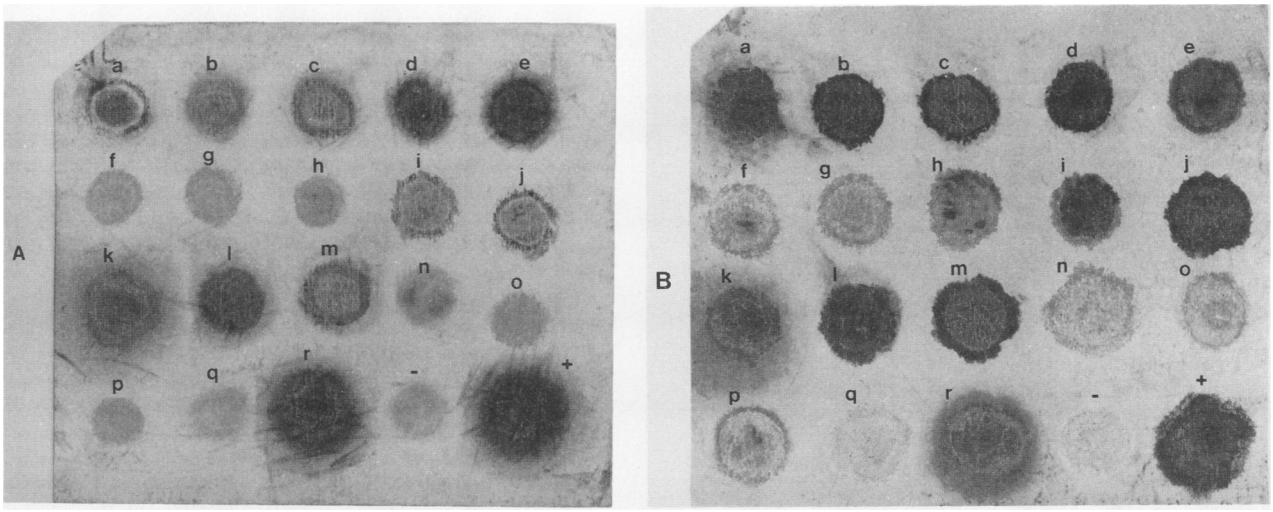

FIG. 2. Bacterial DNA colony hybridization using digoxigenin-labeled DNA probes derived from *ail* (A) and *inv* (B). The strains in colonies a to d (swine origin) are O:3, O:1,2,3, O:5,27, and O:8, respectively; e is O:3 (human clinical isolate, strain 159649); f to h (all swine isolates) are *Y. enterocolitica* biotype 1A, *Y. intermedia*, and *Y. frederiksenii*, respectively; i to q are reference strains of *Y. enterocolitica* serotypes O:9, O:3, O:5,27, O:8, and O:8 (strain 8081C), *Y. rohdei*, *Y. bercovieri*, *Y. mollaretii*, and *Y. ruckeri*, respectively; r is O:3 (human clinical isolate, strain 992206). In both panels, the negative control (−) is *E. coli* HB 101. The positive controls (+) are HB 101(pVM103) harboring the *ail* clone (A) and HB 101(pVM101) harboring the *inv* clone (B).

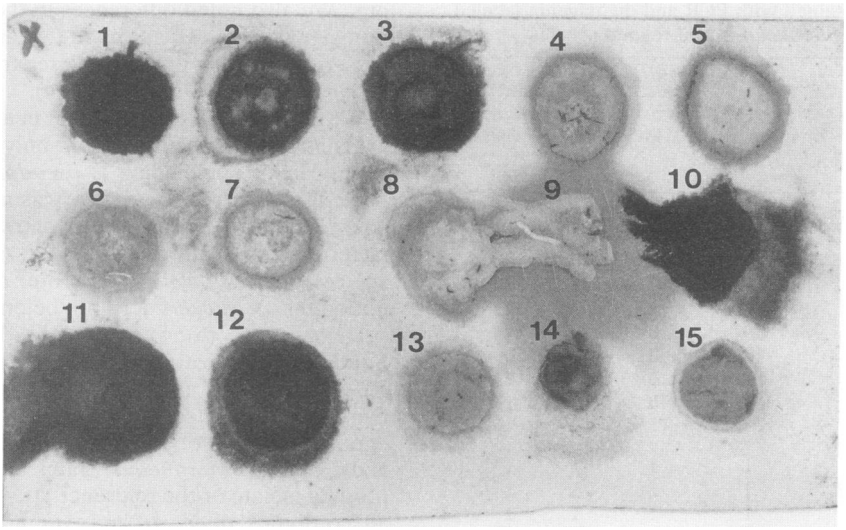

FIG. 3. Bacterial DNA colony hybridization using a digoxigenin-labeled *yst* probe on some members of the family *Enterobacteriaceae*. The species in colonies 1 to 12 are *E. coli* (O157:H7), *C. freundii*, *S. typhimurium*, *P. stuartii*, *K. pneumoniae*, *M. morganii*, *Hafnia alvei*, *S. marcescens*, *P. mirabilis*, *S. flexneri*, *E. cloacae*, and *E. tarda*, respectively (all except *H. alvei* and *E. tarda* are of human origin); colony 13 is a negative control, *E. coli* HB 101 (containing pVM101, an irrelevant clone); colony 14 is *E. coli*(pID10) harboring the *yst* gene; colony 15 is a *yst*$^+$ *Y. enterocolitica* O:3 strain.

an important reservoir of strains pathogenic to humans (2, 18, 35). The use of *ail*-based primers in a PCR or polynucleotide probe is recommended as both a rapid and a specific method for confirmation of virulent cultures of *Y. enterocolitica*. Unlike the virulence plasmid, which is readily lost in culture, chromosomal genes such as *ail* are stably maintained. However, the role of *yst*, which is chromosomally encoded, in virulence remains obscure, and the presence of *yst*-homologous sequence in other species is an area for further studies. The digoxigenin-labeled probe detection system, despite being laborious and less rapid than the PCR, provides an alternative to radiolabeled probe detection methods.

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