JOURNAL OF CLINICAL MICROBIOLOGY, July 1989, p. 1667–1670
0095-1137/89/071667-04$02.00/0

Vol. 27, No. 7

Development and Testing of a Synthetic Oligonucleotide Probe for the Detection of Pathogenic *Yersinia* Strains

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Received 10 November 1988/Accepted 15 March 1989

A 24-base oligonucleotide probe specific for a region of the *Yersinia enterocolitica* virulence plasmid (pYV) associated with HEp-2 cell cytotoxicity and the Sereny reaction was constructed by using sequences flanking critical Tn*phoA* insertions in a subcloned fragment of pYV. This probe, highly specific and sensitive for virulent yersiniae, detected pathogenic *Y. enterocolitica* isolates in artificially inoculated foods.

The association of human illness with consumption of food contaminated with *Yersinia enterocolitica* is well documented (2, 3, 5, 24, 25). Refrigerated foods especially and water sources are potential vehicles for the organisms, since yersiniae grow at low temperatures (15, 19). *Y. enterocolitica* is now recognized as a major pathogen worldwide, with serotype O:3 accounting for most cases (8) and serotype O:9 being the next most common. However, in the United States, most foodborne outbreaks have been associated with strains of serotype O:8 (3, 5, 24, 25). Strains of all serotypes implicated in human disease harbor a plasmid (pYV) of molecular weight ca. 42 megadaltons (22). In the past 5 years there has been an accumulation of evidence documenting the contribution of the plasmid to the pathogenicity of the bacterium (1, 4, 6, 8, 10, 14, 21, 22, 26).

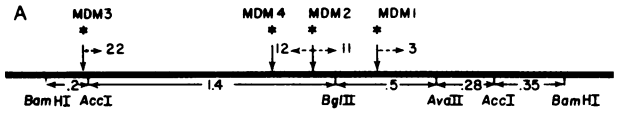
It has been relatively difficult to detect virulent *Y. enterocolitica* in food because of the long enrichment period, the necessity for selective media, and the fact that the plasmid may be lost on prolonged incubation at 37°C (11). Furthermore, once the organism has been isolated from culture and identified, various in vitro and in vivo assays must be performed to check for virulence. This is both time-consuming and, in the case of animal experimentation, expensive and cumbersome. There is a need, therefore, for a simple, quick screening method to detect virulent *Y. enterocolitica*. We describe here the construction of a 24-base synthetic DNA oligomer based on nucleotide sequences of the pYV plasmid and the successful use of this probe as a screening method for the detection of virulent *Y. enterocolitica* in different foods.

(This report was presented in part at the 28th Interscience Conference on Antimicrobial Agents and Chemotherapy, Los Angeles, Calif., 23 to 26 October 1988.)

Construction of oligonucleotide probes. We had previously cloned a 2.6-kilobase (kb) *Bam*HI fragment of pYV from serotype O:8 *Y. enterocolitica* A2635 (5); this fragment has been shown to encode genes associated with production of conjunctivitis in guinea pigs and with cytotoxicity to HEp-2 cells in vitro (M. D. Miliotis et al., submitted for publication). When used as a diagnostic probe, this fragment was found to be both specific and sensitive for plasmid-bearing virulent *Y. enterocolitica*, *Yersinia pestis*, and *Yersinia pseudotuberculosis* (23).

In order to develop a synthetic oligonucleotide probe, regions of the 2.6-kb fragment involved in the cytotoxic phenotype were localized by mutagenesis with the transposon Tn*phoA* (18). Tn*phoA* is a derivative of Tn*5*, which contains a gene for alkaline phosphatase lacking the promoter and signal sequences. In-frame fusion of this gene to genes encoding a secreted protein results in an active alkaline phosphatase enzyme. The 2.6-kb *Bam*HI fragment of pYV, previously cloned into pRK404 (9) to yield pCVD788 (Miliotis et al., submitted), was mutagenized by random insertion of the transposon Tn*phoA* by the method of Manoil and Beckwith (18). Strains containing pCVD 788::Tn*phoA* were selected on the basis of alkaline phosphatase activity on Luria agar containing kanamycin (300 μg/ml), tetracycline (30 μg/ml), and 5-bromo-4-chloro-3-indolyl phosphate (40 μg/ml) (Sigma Chemical Co., St. Louis, Mo.).

Four different Tn*phoA* insertions within the 2.6-kb *Bam*HI fragment were identified (Fig. 1A). Plasmids containing these insertions (designated as pMDM1 through pMDM4) were introduced into plasmid-cured derivatives of *Y. enterocolitica* A2635 and 8081 (designated A2635c and 8081c,



B

SP3 5' - CAA GGG GCG TCC TTA TTC AAG GCA T - 3'

(25-mer, 52% G-C)

SP11 5' - CTG GGC TGC ATG GAG TGT GTC A - 3'

(22-mer, 59% G-C)

SP12 5' - GCC GCT GGG TTG GAA AGG GAA GCC -3'

(24-mer, 67% G-C)

SP22 5' - GCT CCC GAA GCT TCT TGG GTG - 3'

(21-mer, 62% G-C)

FIG. 1. (A) Restriction map of 2.6-kb *Bam*HI fragment showing the four different Tn*phoA* insertion sites. *, Transposon insertions (MDM1, MDM2, MDM3, and MDM4); →, direction sequenced for designated SP3, SP11, SP12, and SP22. (B) Sequences of the synthetic probes.

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TABLE 1. Effect of Tn*phoA* mutations on HEp-2 cytotoxicity

Plasmid[a]	HEp-2 cell cytotoxicity
pYV[b]	+
Control[c]	−
pCVD788[d]	+
pMDM1[e]	+/−[f]
pMDM2	−
pMDM3	+/−
PMDM4	+/−

[a] Introduced into *Y. enterocolitica* A2635 and 8081, from which the virulence plasmid had been cured.
[b] Positive control strains with an intact virulence plasmid.
[c] Negative control strains lacking the virulence plasmid.
[d] Subcloned 2.6-kb *Bam*HI fragment from pYV in pRK404.
[e] pMDM1 through pMDM4 are Tn*phoA* insertions into pCVD788.
[f] +/−, Evidence of cytotoxicity, but results less consistent than those obtained with positive control, including, in many instances, only partial disruption of cell monolayer.

respectively) by filter mating (16) and tested for cytotoxicity to HEp-2 cells in vitro. The insertion in pMDM2 resulted in loss of cytotoxic activity (Table 1).

We utilized the single *Bam*HI site in Tn*phoA* to subclone the *Yersinia* sequences flanking each Tn*phoA* insertion. The four Tn*phoA*-mutagenized plasmids were digested with *Bam*HI and subcloned into pBR325, resulting in eight subclones, each with one end of Tn*phoA* and an adjacent *Yersinia* sequence. Approximately 100 to 150 base pairs of *Yersinia* sequence were determined for each subclone by using the double-stranded sequencing method of Chen and Seeburg (7) and an 18-base primer derived from base pairs 24 to 41 of the IS*50* sequence of Tn*phoA*. Of the eight fragments sequenced, regions from four (Fig. 1) were selected for testing as probes on the basis of the phenotypic results described above. These oligonucleotides were synthesized with β-cyanoethylphosphoramidite chemistry with the DuPont Coder 300 and labeled at the 5′ end by transfer of ^{32}P from [γ-^{32}P]ATP (ICN Radiochemicals, Irvine, Calif.) by using T4 polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, Md.) as described previously (17).

TABLE 2. Results of representative hybridization experiments for food artificially contaminated with *Y. enterocolica*

Food item and plate no.	Aerobic plate count[a]		Dilution of food[b]	Yersiniae added[c]		Plating dilution	Predicted yersinia count/plate[d]	No. of colonies detected with SP12
	PCA	MAC		pYV$^+$	pYV$^-$			
Brie cheese								
1	8×10^6	1×10^4	10^{-2}			Undiluted	0	0
2			10^{-2}	9×10^3		10^{-2}	9×10^0	6×10^0
3			10^{-2}	9×10^3		Undiluted	9×10^2	3×10^2
5			10^{-4}	9×10^3		10^{-1}	9×10^1	6×10^1
6			10^{-4}	9×10^4		10^{-1}	9×10^2	2×10^2
Pork chop								
7	6×10^5	1×10^5	10^{-2}			Undiluted	0	0
8			10^{-2}	9×10^4		Undiluted	9×10^3	+[e]
9			10^{-4}	9×10^3		Undiluted	9×10^2	3×10^2
10			10^{-4}	9×10^4		10^{-1}	9×10^2	1×10^2
Alfalfa sprouts								
11	OG	4×10^7	10^{-4}			Undiluted	0	0
12			10^{-2}	9×10^4		10^{-1}	9×10^2	+
13			10^{-2}		5×10^4	10^{-2}	5×10^1	0
Chocolate milk								
14	6×10^2	NG	10^{-2}	9×10^3		10^{-1}	9×10^1	7×10^1
15			10^{-2}		5×10^4	10^{-1}	5×10^2	0
Tofu								
16	OG	2×10^4	10^{-2}	6×10^2		10^{-1}	6×10^0	3×10^1
17			10^{-2}	6×10^2		Undiluted	6×10^1	2×10^2
Crabmeat								
18	1×10^7	3×10^4	10^{-2}			Undiluted	0	0
19			10^{-2}	8×10^4		10^{-1}	8×10^2	1×10^2
20			10^{-2}	8×10^4		10^{-2}	8×10^1	2×10^1
21			10^{-4}	8×10^4		10^{-1}	8×10^2	1×10^2
Oyster								
22	2×10^7	2×10^5	10^{-3}			Undiluted	0	0
23			10^{-2}	8×10^4		10^{-1}	8×10^2	1×10^2
24			10^{-4}	8×10^3		10^{-1}	8×10^1	3×10^1
25			10^{-4}	8×10^3		10^{-2}	8×10^0	2×10^0

[a] CFU per gram of food. PCA, Plate count agar; MAC, MacConkey agar; OG, overgrowth of highest-dilution plate; NG, no growth.
[b] Includes 10^{-1} dilution required to make homogenate (i.e., 10^{-2} dilution of food = 10^{-1} dilution of food homogenate).
[c] CFU per milliliter. pYV$^+$, Yersinia strain with virulence plasmid; pYV$^-$, yersinia strain cured of plasmid.
[d] Based on 100-μl sample per plate.
[e] +, Positive result, but accurate colony count could not be obtained from autoradiograph.

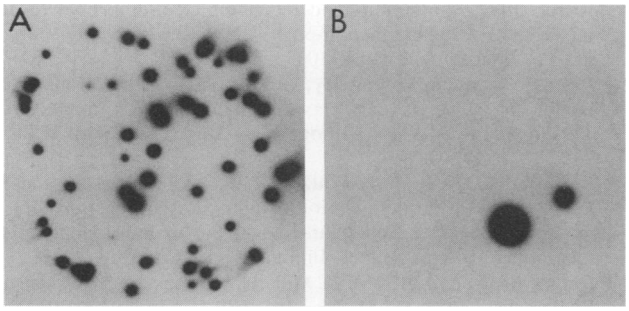

FIG. 2. Autoradiogram of Whatman 541 filters spread with dilutions of food samples artificially infected with virulent *Y. enterocolitica*. (A) Brie cheese: filter with predicted count per plate of 9×10^1 CFU (plate no. 5, Table 2); (B) oyster: filter with predicted count per plate of 8×10^0 CFU (plate no. 25, Table 2).

Testing of probes. Twenty *Yersinia* strains were initially screened for the presence of nucleotide homology with the synthetic probes. Colonies were spotted onto Trypticase soy agar plates, incubated for 24 h at 25°C, transferred to Whatman 541 filters, and hybridized with the probes as previously described (20). Ten of these strains were presumed to be pathogenic because of lethality to mice, calcium dependence, and presence of pYV, and they gave positive results when probed with our original 2.6-kb *Bam*HI fragment and with a 4.2-kb fragment from the calcium dependence region of the plasmid (24). The remaining 10 strains were nonpathogenic by the same criteria and did not hybridize with these two fragments. Of the four synthetic probes tested, only SP12 (derived from DNA flanking one end of the transposon insertion in pMDM2) consistently gave the same results as the 2.6-kb *Bam*HI fragment (results not shown). SP3 and SP11 showed some false-negative results, and SP3 and SP22 revealed one false-positive reaction. The false-positive and false-negative results did not appear to be serotype dependent. Subsequently, we screened a collection of 138 *Yersinia* strains comprising 85 *Y. enterocolitica* (at least 1 strain from each serotype), 8 *Y. intermedia*, 7 *Y. kristensenii*, 6 *Y. frederiksenii*, and 2 *Y. pseudotuberculosis* strains and 30 clinical isolates other than yersiniae (enterotoxigenic, enteropathogenic, enteroinvasive, and enterohemorrhagic *Escherichia coli* and *Citrobacter*, *Kluyvera*, *Aeromonas*, *Hafnia*, *Klebsiella*, and *Enterobacter* isolates). In all instances, the SP12 synthetic probe results were comparable to those of the nonsynthetic 2.6-kb *Bam*HI fragment: all pYV-containing strains of *Y. enterocolitica* and *Y. pseudotuberculosis* hybridized with the probe, and no other strains gave positive results.

Synthetic probe SP12 was therefore chosen to screen food artificially contaminated with either virulent *Y. enterocolitica* A2635 or its plasmid-negative derivative A2635c. By standard methods (12), 50-g food samples were homogenized in 450 ml of Butterfield buffer, and aerobic plate counts were determined on plate count and MacConkey agars (12). Different concentrations of *Y. enterocolitica* were then added to the samples (Table 2) by the methods of Hill and his colleagues (12, 13). Samples (100 μl) were plated out on MacConkey agar and incubated overnight at 30°C. Plates were directly blotted with Whatman 541 filters, and filters were hybridized with our SP12 probe, as described above. On selected plates the identities of probe-positive and probe-negative colonies were confirmed by standard biochemical tests.

A close approximation existed between the predicted yersinia count per plate and the actual number of colonies detected with the SP12 probe (Table 2). Excellent results were obtained with a 10^{-2} food dilution inoculated with 10^2 to 10^4 CFU of *Y. enterocolitica* per ml. This would be equivalent to detection of 10^4 to 10^6 CFU/g of food. While we did not test smaller inocula, we would anticipate no difficulty in using these same techniques to identify smaller numbers of organisms. It is also evident that even when food contains numerous other microorganisms, as was the case with alfalfa sprouts, pork chops, and oysters, virulent yersiniae could be easily identified (Table 2). As few as two colonies on MacConkey agar plates can be detected by SP12 (Fig. 2). None of the food samples inoculated with A2635c (pYV negative) hybridized with SP12 (Table 2).

In summary, this study has demonstrated the sensitivity and specificity of a synthetic defined oligonucleotide probe based on the conjunctivitis- and HEp-2 cell cytotoxicity-associated regions of pYV in identifying virulent *Y. enterocolitica* strains. We have shown that this probe can be successfully used to directly screen cultured food samples; assays utilizing this probe should provide a rapid and accurate means of detecting pathogenic *Y. enterocolitica* strains in a variety of foods and, possibly, in clinical samples. Furthermore, we have shown that random insertion of Tn*phoA* into a gene provides a useful tool not only for mutagenizing the gene, but also for constructing synthetic oligonucleotide probes to detect the gene.

This study was supported in part by contract 223-85-2094 from the U.S. Food and Drug Administration.

LITERATURE CITED

1. **Aulisio, C. C. G., W. E. Hill, J. T. Stanfield, and R. L. Sellers, Jr.** 1983. Evaluation of virulence factors testing and characteristics of pathogenicity in *Yersinia enterocolitica*. Infect. Immun. **40**:330–335.
2. **Aulisio, C. C. G., J. M. Lanier, and M. A. Chappel.** 1982. *Yersinia* enterocolitica 0:13 associated with outbreaks in three southern states. J. Food Prot. **45**:1263.
3. **Aulisio, C. C. G., J. T. Stanfield, S. D. Weagant, and W. E. Hill.** 1983. Yersiniosis associated with tofu consumption: serological, biochemical and pathogenicity studies of *Yersinia enterocolitica* isolates. J. Food Prot. **46**:226–230.
4. **Bakour, R., G. Balligand, Y. Laroche, G. Cornelis, and G. Wauters.** 1985. A simple adult-mouse test for tissue invasiveness in *Yersinia enterocolitica* strains of low experimental virulence. J. Med. Microbiol. **19**:237–246.
5. **Black, R. E., R. J. Jackson, T. Tsai, M. Medvesky, J. C. Feeley, K. I. E. Macleod, and A. M. Wakelee.** 1978. Epidemic *Yersinia enterocolitica* infection due to contaminated chocolate milk. N. Engl. J. Med. **298**:76–79.
6. **Bölin, I., L. Norlander, and H. Wolf-Watz.** 1982. Temperature-inducible outer membrane protein of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* is associated with the virulence plasmid. Infect. Immun. **37**:506–512.
7. **Chen, E. Y., and P. H. Seeburg.** 1985. Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. DNA **4**:165–170.
8. **Cornelis, G., Y. Laroche, G. Balligand, M.-P. Sory, and G. Wauters.** 1987. *Yersinia enterocolitica*, a primary model for bacterial invasiveness. Rev. Infect. Dis. **9**:64–87.
9. **Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X.-W. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski.** 1985. Plasmids related to broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. Plasmid **13**:149–153.
10. **Gemski, P., J. R. Lazere, and T. Casey.** 1980. Plasmid associated with pathogenicity and calcium dependency of *Yersinia enterocolitica*. Infect. Immun. **27**:682–685.
11. **Hill, W. E., and C. L. Carlisle.** 1981. Loss of plasmids during

enrichment for *Escherichia coli*. Appl. Environ. Microbiol. **41**:1046–1048.
12. **Hill, W. E., W. L. Payne, and C. C. G. Aulisio.** 1983. Detection and enumeration of virulent *Yersinia enterocolitica* in food by DNA colony hybridization. Appl. Environ. Microbiol. **46**:636–641.
13. **Jagow, J., and W. E. Hill.** 1986. Enumeration by DNA colony hybridization of virulent *Yersinia enterocolitica* colonies in artificially contaminated food. Appl. Environ. Microbiol. **51**: 441–443.
14. **Lachica, R. V., and D. L. Zink.** 1984. Plasmid-associated cell surface charge and hydrophobicity of *Yersinia enterocolitica*. Infect. Immun. **44**:540–543.
15. **Lee, W. H.** 1979. Testing for the recovery of *Yersinia enterocolitica* in foods and their ability to invade HeLa cells. Contrib. Microbiol. Immunol. **5**:228–233.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 75–96. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Maniatis, T., A. Jeffry, and A. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage lambda. Proc. Natl. Acad. Sci. USA **72**:1184–1188.
18. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82**: 8129–8133.
19. **Meadows, C. A., and B. H. Snudden.** 1982. Prevalence of *Yersinia enterocolitica* in waters of the lower Chippewa river basin, Wisconsin. Appl. Environ. Microbiol. **43**:953–954.
20. **Nishibuchi, M., W. E. Hill, G. Zon, W. L. Payne, and J. B. Kaper.** 1986. Synthetic oligodeoxyribonucleotide probes to detect Kanagawa phenomenon-positive *Vibrio parahaemolyticus*. J. Clin. Microbiol. **23**:1091–1095.
21. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31**:775–782.
22. **Portnoy, D. A., H. Wolf-Watz, I. Bolin, A. B. Beeder, and S. Falkow.** 1984. Characterization of common virulence plasmids in *Yersinia* species and their role in the expression of outer membrane proteins. Infect. Immun. **43**:108–114.
23. **Robins-Browne, R. M., M. D. Miliotis, S. Cianciosi, V. L. Miller, S. Falkow, and J. G. Morris, Jr.** 1989. Comparison of DNA colony hybridization and other techniques for detection of virulence in *Yersinia* species. J. Clin. Microbiol. **27**:644–650.
24. **Shayegani, M., D. Morse, I. DeForge, T. Root, L. M. Parsons, and P. S. Maupin.** 1983. Microbiology of a major foodborne outbreak of gastroenteritis caused by *Yersinia enterocolitica* serogroup O:8. J. Clin. Microbiol. **17**:35–40.
25. **Tacket, C. O., J. P. Narain, R. Sattin, J. P. Lofgren, C. Konigsberg, Jr., R. C. Rendtorff, A. Rausa, B. R. Davis, and M. L. Cohen.** 1984. A multistate outbreak of infection caused by *Yersinia enterocolitica* transmitted by pasteurized milk. J. Am. Med. Assoc. **251**:483–486.
26. **Zink, D. L., J. C. Feeley, J. G. Wells, C. Vanderzant, J. C. Vickery, W. D. Roof, and G. A. O'Donovan.** 1980. Plasmid-mediated tissue invasiveness in *Yersinia enterocolitica*. Nature (London) **283**:224–226.