 Comparative Study of a DNA Hybridization Method and Two Isolation Procedures for Detection of Yersinia enterocolitica O:3 in Naturally Contaminated Pork Products

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We compared a DNA-DNA hybridization assay, using a synthetically produced oligonucleotide probe, and two conventional isolation procedures (methods A and B) with regard to their relative efficiency in detecting Yersinia enterocolitica O:3 in naturally contaminated pork products. Method A was as described by Wauters et al. (Appl. Environ. Microbiol. 54:851-854, 1988). Method B has been recommended by the Nordic Committee on Food Analysis (method no. 117, 1987). The genetic probe was used in a colony hybridization assay to detect virulent yersiniae at each of the isolation steps which composed methods A and B. A total of 50 samples of raw pork products obtained from 13 meat-processing factories in Norway were examined. Y. enterocolitica serogroup O:3, biolvar 4, was isolated from altogether 9 (18.0%) of the samples by using the two isolation procedures. In contrast, colony hybridization using the genetic probe indicated that 30 (60.0%) of the samples contained virulent yersiniae. All samples which were positive on cultivation were also positive by hybridization. The results indicate that the occurrence of pathogenic Y. enterocolitica in Norwegian pork products is substantially higher than previously demonstrated and, therefore, reinforce our suggestion that pork products represent an important potential source of human infection in Norway. The results also indicate that the use of conventional isolation procedures may lead to considerable underestimation of pathogenic Y. enterocolitica in pork products.

There is strong indirect evidence that swine constitute an important reservoir for human infection with Yersinia enterocolitica (5, 7, 14, 23). Numerous surveys have shown that swine may be healthy carriers of serogroups O:3 and O:9, belonging to the same biovars and phagevars as those involved in human disease (14, 23). The pig is the only food animal which regularly harbors pathogenic Y. enterocolitica. In contrast, serogroup O:8, the predominant human pathogen in the United States, appears to be rare in swine. O:8 may have an entirely different reservoir(s) and ecology (23). In addition to their function as fecal commensals, serogroups O:3 and O:9 also inhabit the oral cavities of swine, especially the tongue and tonsils. The bacteria are also frequently encountered as surface contaminants on freshly slaughtered pig carcasses. In Belgium, which is the country with the highest reported incidence of Y. enterocolitica infection, a case-control study has shown that the infection is strongly associated with the consumption of raw pork (25). It has not been possible to demonstrate any phenotypic or genotypic differences between human and porcine isolates (7, 14, 23). The finding that porcine and human isolates harbor virulence plasmids with identical restriction endonuclease cleavage patterns provides additional evidence that the pig plays a role in the epidemiology of human Y. enterocolitica infection (19). Restriction enzyme analysis of chromosomal DNA has further supported this suggestion (9).

In contrast to the frequent occurrence of the bacteria in live pigs and on freshly slaughtered carcasses, pathogenic

yersiniae have only occasionally been recovered from pork products at the stage of retail sale, with the exception of fresh tongues (11, 23, 30). This phenomenon might be explained by the lack of proper selective methodology for the isolation of pathogenic strains. The development of isolation procedures which clearly differentiate pathogenic from nonpathogenic variants has proven to be problematical. Recently, however, Wauters et al. (28) described a new enrichment method with allows Y. enterocolitica O:3 to be recovered from a relatively high percentage of pork samples. Although a number of poly- and oligonucleotide probes have been described for Y. enterocolitica (2, 4, 6, 8, 13, 21), as far as we know, none of these have been compared with conventional culture methods with regard to their ability to demonstrate bacteria in naturally infected foods. In a recent report (8), we described the development and testing of a synthetically produced oligonucleotide probe which proved to be an efficient tool for the detection and enumeration of virulent Y. enterocolitica in artificially contaminated foods. The purpose of the present investigation was therefore to compare a DNA-DNA hybridization assay, using this probe, and two conventional isolation procedures, including the new method described by Wauters et al. (28), with regard to their relative efficiency in detecting virulent Y. enterocolitica in naturally contaminated pork products.

MATERIALS AND METHODS

Food samples. A total of 50 samples of raw pork products were examined during the periods October through November 1988 (26 samples) and May through June 1989 (24

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samples). The samples originated from 13 meat-processing establishments which were located in 12 of the 19 counties in Norway. The pork products examined comprised (i) fresh pig tongues (5 samples), (ii) pork cuts (12 samples), and (iii) pork sausage meat (33 samples). The tongues were collected immediately after evisceration of the pigs, placed in sterile polystyrene bags, and transported at ambient temperature to the laboratory, where cultivation was initiated within 2 h after collection. The remaining samples were placed in sterile polystyrene bags and kept frozen at −20°C for up to 2 weeks. Before cultivation, the samples were thawed at 4°C overnight.

All samples were examined for the presence of Y. enterocolitica serogroup O:3, biovar 4, by two isolation procedures (methods A and B) and one DNA-DNA colony hybridization method, as described below. Prior to examination, a 90-cm² area, comprising epithelium, mucosa, and up to a 0.5-cm layer of muscle, was excised from the upper surface of each tongue. Each tongue was then cut into two along the midline, yielding two similar samples, each of 45 cm². One of these was examined by method A, and the other was examined by method B.

**Method A.** The isolation procedure known as method A was as described by Wauters et al. (28). Each tongue sample (45 cm²) was homogenized with 100 ml of phosphate-buffered saline (PBS) (pH 7.6). Five-gram samples of pork cuts and pork sausage meat were homogenized with 20 ml of PBS in a Colworth 400 stomacher (A. J. Seward, London, England). From each homogenate, 2-ml portions were transferred into tubes containing 100 ml of igeran-ticarcillin-potassium chloride (ITC) enrichment broth (28). Wauters (27) recommended the transfer of 2 ml of homogenate instead of the 1-ml volume which is described in the original procedure (28). After incubation for 2 days at 24°C, 0.1 ml was plated out onto modified Salmonella-Shigella-deoxycholate-calcium (SSDC) agar (28) (Yersinia agar; E. Merck AG, Darmstadt, Federal Republic of Germany). If no growth was detected, the ITC enrichments were incubated further and plated after 3 days. All plates were incubated at 30°C and read after 24 h. From each enrichment, an additional 0.1 ml was spread plated onto a SSDC plate covered with a nylon membrane filter (NEF-978; Du Pont, NEN Research Products, Boston, Mass.). These plates were incubated at room temperature (20 to 25°C) for 48 h.

**Method B.** Isolation method B was as recommended by the Nordic Committee on Food Analysis (20). Tongue samples (45 cm²) and meat samples (25 g) were homogenized in a stomacher with 225 ml of PBS-sorbitol-bile (PSB) enrichment medium, which consisted of PBS (pH 7.6) with 1.0% sorbitol and 0.15% bile salts (no. 3; Oxoid Ltd., Basingstoke, Hampshire, England). The following procedure consisted of three isolation steps.

(i) **Resuscitation.** Samples homogenized in PSB were incubated at room temperature for 3 h, and 0.1-ml portions were plated out onto cefsulodin-igrasan-novobiocin (CIN) agar (CM653 and SR109; Oxoid Ltd.).

(ii) **Selective enrichment.** The PSB cultures were incubated at 4°C for 8 days, and 0.1 ml was then transferred to tubes with 10 ml of a modified Rappaport broth (MRB) (no. 15209; E. Merck AG), containing 80.0 g of MgCl₂ per liter. Carbenicillin was omitted from MRB, as recommended by Schie mann (22). Selective enrichment in MRB proceeded for 4 days at room temperature, after which 0.1 ml was plated onto CIN agar.

(iii) **Cold enrichment.** Three weeks of cold enrichment was accomplished by further incubation of the PSB cultures at 4°C for another 2 weeks, and two loopfuls (0.02 ml) were finally plated onto CIN agar.

At each isolation step, an additional 0.1 ml (0.02 ml at step 3) was spread plated onto a CIN agar plate covered with a nylon membrane filter and was incubated at room temperature for 48 h. Plates without membranes were incubated at 28°C and read after 18 to 22 h.

**Colonel hybridization.** The genetic probe used in this study consisted of a 19-mer oligonucleotide which was selected on the basis of sequence analysis of the yadA gene (previously designated yopA gene) located on the 40- to 50-MDa virulence plasmid of Yersinia spp. (8, 24). The development and testing of the probe have been described in a previous report (8). The oligonucleotide was chemically synthesized by using an automatic DNA synthesizer (8) and labeled at the 5' end with [δ3P]ATP (Amersham International plc, Buckinghamshire, England) with bacteriophage T4 polynucleotide kinase (Amersham), as described by Mammatis et al. (12). Labeled probes were separated from unincorporated nucleotides by running the samples through a Sephadex G-50 spin column (12).

Before hybridization, nylon membranes with bacterial colonies were removed from the agar plates, the bacteria were lysed, and their DNA was denatured and fixed to the membranes as described previously (8). Membranes were stored for up to 2 months before hybridization. Prehybridization, hybridization, stringent washing, and autoradiography were also carried out as described previously (8).

**Colonel hybridization after direct plating.** In order to investigate the efficacy of colony hybridization after direct plating without prior enrichment or resuscitation steps, 24 of the samples (5 g of each) were homogenized in 20 ml of PBS, and 0.1 ml was spread plated on CIN and SSDC agar with nylon membranes. Hybridization was carried out as specified above.

**Identification and typing of isolates.** Suspect colonies on SSDC or CIN agar were subcultured on lactose bromothymol-blue agar for further morphological inspection. Cultures resembling Yersinia spp. were then subjected to a primary biochemical screening using the three-tube method described by Lassen (10), followed by more extensive biochemical and cultural characterization as described elsewhere (18). Y. enterocolitica was identified according to established criteria (1). All isolates identified as Y. enterocolitica were biotyped by the methods and criteria of Wauters (29). Serological screening of the isolates was carried out by slide agglutination against an absorbed rabbit antiserum representing O antigen factor 3 (26). Only isolates belonging to Y. enterocolitica serogroup O:3, biovar 4, were considered in this work.

**Statistical analyses.** Statistical analyses were performed by using the Mantel-Haenszel corrected chi-square test by means of the data program package EpilInfo (Centers for Disease Control, Atlanta, Ga.).

**RESULTS**

**Isolation.** Y. enterocolitica serogroup O:3, biovar 4, was isolated from altogether 9 (18.0%) of 50 pork products by using the two conventional isolation procedures included in the present investigation (Table 1). The highest number of positive samples were detected with method A, which demonstrated the pathogenic serogroup in 8 (16.0%) of the samples, while 5 (10.0%) of the samples were positive by the more time-consuming method B (P > 0.05). Of the nine positive samples, four were positive by both methods, four
TABLE 1. Comparison of colony hybridization and two isolation procedures for detection of *Y. enterocolitica* O:3 in naturally contaminated pork

<table>
<thead>
<tr>
<th>Procedure</th>
<th>No. of positive samples from pork product</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Tongues (n = 5)</td>
</tr>
<tr>
<td>Method A*</td>
<td></td>
</tr>
<tr>
<td>Isolation</td>
<td>1</td>
</tr>
<tr>
<td>Hybridization</td>
<td>2</td>
</tr>
<tr>
<td>Both methods</td>
<td>2</td>
</tr>
<tr>
<td>Method B*</td>
<td></td>
</tr>
<tr>
<td>Isolation</td>
<td>1</td>
</tr>
<tr>
<td>Hybridization</td>
<td>2</td>
</tr>
<tr>
<td>Both methods</td>
<td>2</td>
</tr>
<tr>
<td>Method A plus method B</td>
<td></td>
</tr>
<tr>
<td>Isolation</td>
<td>1</td>
</tr>
<tr>
<td>Hybridization</td>
<td>2</td>
</tr>
<tr>
<td>Both methods</td>
<td>2</td>
</tr>
</tbody>
</table>

* Described by Wauters et al. (28).
* Described by the Nordic Committee on Food Analysis (20).

were positive only by method A, and one sample was positive only by method B.

**Colony hybridization.** Colony hybridization using the synthetic oligonucleotide probe indicated that 30 (60.0%) of the 50 samples examined were contaminated with virulent *yersinia* (Table 1). All samples which were positive on cultivation were also positive by hybridization, while positive results obtained from 21 samples by hybridization could not be confirmed by isolation of *Y. enterocolitica* from associated culture media. Our ability to confirm hybridization positive results differed significantly depending on the number of hybridization signals detected. For 10 of the 47 membranes which were positive by hybridization, the number of signals were too numerous to count. With one exception, all of the positive results (90.0%) could be confirmed by isolation (Fig. 1). The single result that could not be confirmed was for a membrane that was completely overgrown by the background flora. The remaining 37 hybridization-positive membranes exhibited fewer than 15 signals in the autoradiograms (mean, 3.0; median, 1; range, 1 to 14). Only two (5.4%) of these could be confirmed by isolation (*P* < 0.0001; 9 of 10 versus 2 of 37). The results indicate that colony hybridization was essential for detection of virulent *yersinia* from samples which produced only a few such colonies on the agar media.

DNA hybridization based on method A detected virulent *yersinia* in 19 (38.0%) of the samples, while 24 (48.0%) were positive when hybridization was carried out with membranes prepared from method B (Table 1), despite the fact that method A isolated *Y. enterocolitica* from more samples than method B (see above). For both methods, however, DNA hybridization resulted in a detection rate which was significantly higher than the isolation rate achieved (method A, 19 of 50 versus 8 of 50, *P* = 0.01; method B, 24 of 50 versus 5 of 50, *P* < 0.0001). Of altogether 30 samples which were positive with hybridization, 6 were positive only by method A, 11 were positive only by method B, and 13 were positive by both methods. The hybridization signal count was on average lower on membranes prepared from method B, with 26 (92.9%) of the 28 positive membranes showing fewer than 15 signals, compared with method A, by which 11 (57.9%) of 19 positive membranes showed fewer than 15 signals (*P* = 0.004).

A comparison of DNA hybridization and isolation methods for the three different steps composing method B is presented in Table 2. It is especially noteworthy that *Y. enterocolitica* O:3 was not isolated from any of the samples after 3 weeks of cold enrichment (step 3), while DNA hybridization gave positive results for seven samples after this enrichment procedure. This discrepancy may be explained by (i) the generally high number of background flora on membranes prepared after step 3 and by (ii) the low number of *yersinia* present, as evidenced by the low number of hybridization signals on the membranes concerned (mean, 2.4; median, 1; range, 1 to 8). None of these seven samples were positive with colony hybridization after steps 1 and 2, and only one was positive after cultivation (step 2).

**Colonial hybridization after direct plating.** Positive hybridization signals were obtained from altogether 11 (45.8%) of 24 samples which were examined after direct plating without prior resuscitation or enrichment. Nevertheless, when the same samples were examined by hybridization after enrichment in methods A and B, 11 more positive samples were detected, all of which were negative after direct plating. Only a single sample was positive after direct plating but negative after the two other methods.

**Enumeration.** The results achieved with colony hybridization after direct plating allowed the number of virulent *Yersinia* bacteria per gram in the original food product to be estimated by multiplying the number of hybridization signals by 50 (the dilution factor). In this way, it was estimated that the count of virulent *yersinia* in eight samples of pork sausage meat varied from 50 to 2,500/g (average, 439 CFU/g). In three samples of pork cuts, the counts were found to range between 50 and 300/g (average, 200 CFU/g).

**DISCUSSION**

Of the two conventional isolation procedures compared in this study, the highest number of positive samples was detected with method A. Wauters et al. (28) compared this method with a number of one- or two-step enrichment procedures and, in agreement with our results, found method A to be by far the most sensitive for recovery of *Y. enterocolitica* O:3; especially from ground pork and mas- seter muscle samples. Even though we could not demonstrate any statistically significant difference between methods A and B as regards isolation rate, there are, nevertheless, several reasons for preferring method A. First, while all samples which were positive with method B were also positive with method A, with only one exception, method A detected four positive samples which were negative with method B. Furthermore, method B is much more time-consuming than method A, a factor of considerable practical significance.

It is well known that the different serogroups within *Y. enterocolitica* vary in their tolerance to selective components and other factors during the isolation process (23). As these serogroups have different geographic distributions, a method which functions well in one part of the world may not necessarily be optimal for use in other countries. Both the agar medium (SSDC) and the enrichment medium (ITC) included in method A are specifically designed for isolation of the pathogenic serogroup O:3, which is by far the most important causal agent of human *Y. enterocolitica* infection.
FIG. 1. Autoradiograms of colony hybridization membranes inoculated with one sample of naturally contaminated pork product. The membranes were prepared after different isolation steps: A, direct plating on SSDC agar; B, direct plating on CIN agar; C, enrichment in ITC broth (2 days at 24°C) followed by plating on SSDC agar (method A); D, resuscitation in PSB medium (3 h at room temperature) (method B, step 1); E, preenrichment in PSB (8 days at 4°C) followed by selective enrichment in MRB (4 days at room temperature) and plating on CIN agar (method B, step 2); F, cold enrichment in PSB (3 weeks at 4°C) followed by plating on CIN agar (method B, step 3). Results C and E were confirmed by isolation from the corresponding culture media. Results D and F were negative by isolation. Isolation was not attempted from culture media corresponding to A and B.
TABLE 2. Comparison of colony hybridization and the three different isolation steps of method B

<table>
<thead>
<tr>
<th>Procedure</th>
<th>No. of positive samples after isolation step:</th>
<th>1(^{st})</th>
<th>2(^{nd})</th>
<th>3(^{rd})</th>
<th>All steps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation</td>
<td></td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Hybridization</td>
<td></td>
<td>9</td>
<td>12</td>
<td>7</td>
<td>24</td>
</tr>
<tr>
<td>Both methods</td>
<td></td>
<td>10</td>
<td>13</td>
<td>7</td>
<td>24</td>
</tr>
</tbody>
</table>

* a Described by the Nordic Committee on Food Analysis (20).
* b Resuscitation (3 h at room temperature in PSB).
* c Preenrichment in PSB (8 days at 4°C) followed by selective enrichment in MRB (4 days at room temperature).
* d Cold enrichment in PSB (3 weeks at 4°C).

in Europe. Both ITC and SSDC will to some extent hinder the growth of the serogroups O:8 and O:5,27, which dominate in the United States, and these media will therefore tend to underestimate such variants, although they are excellent for the recovery of O:3 and probably also O:9 (28). Method B, on the other hand, includes media which permit the isolation of all pathogenic serogroups, including O:8 and O:5,27. Neither method B nor other multivalent methods so far described, however, allow optimal recovery of all the relevant serogroups (23, 25).

The genetic probe employed in our study is based on a plasmid-encoded virulence determinant which occurs in all pathogenic Y. enterocolitica serogroups (8). We have previously demonstrated that this probe is able to identify a number of pathogenic serogroups equally effectively (8). The probe detected virulent Y. enterocolitica in artificially contaminated food samples. However, negative hybridization results were obtained for uninoculated pork products with a large background of indigenous bacteria, indicating that the probe does not share DNA sequence homology with bacteria normally present in this kind of food. Furthermore, the probe was able to differentiate pathogenic variants from a broad spectrum of nonpathogenic yersiniae. This is an important characteristic, as such environmental yersiniae are frequently encountered in many foods, including pork products (4, 7, 11, 23, 30). It has been claimed that such bacteria may conceal the presence of pathogenic variants because of considerable morphological similarity, with underestimation of the latter as a likely consequence (11, 23, 30). Our results support the assumption that the use of conventional cultivation methods may lead to considerable underestimation of pathogenic Y. enterocolitica in pork products. By combining colony hybridization with the two isolation procedures, we achieved a significantly higher detection rate than was achieved by using the isolation methods alone. Although we cannot completely rule out the possibility of false-positive hybridization signals, the data referred to above suggest that the probe is highly specific. Furthermore, with one exception, all hybridization-positive membranes that could not be confirmed by isolation showed only a few signals in the autoradiograms (mean, 2.8; median, 1; range, 1 to 14). It is conceivable that such a low number of Yersinia colonies may be difficult to detect by conventional isolation methods.

The difference between isolation and hybridization was greatest for method B, by which hybridization led to a 380% increase in detection rate, the corresponding increase for method A being 138%. Colony hybridization based on method B was the procedure which achieved the highest detection rate, despite the fact that method B was inferior as an isolation method per se. Furthermore, colony hybridization indicated that the number of colonies (observed as dark spots on the autoradiograms) was, on average, higher on the SSDC plates from method A than on the CIN plates from method B. Our results therefore suggest that method B allowed growth of Y. enterocolitica from more samples than method A, though the number of colonies on each plate was relatively low. Method A, on the other hand, seems to allow better multiplication of O:3, such that the bacteria are easier to isolate.

Colony hybridization based on method B gave a positive result for a number of samples which were negative with method A, though the converse was true in some cases. Optimal detection was achieved when the two methods were combined, a procedure which is, however, much too costly and time-consuming for most laboratories. Which of the two enrichment procedures one chooses to combine with hybridization will depend on (i) the serogroups which one expects to find, (ii) the requirements which one sets for sensitivity, (iii) the importance of obtaining a rapid result, and (iv) the economic resources available. Though, ideally, it would be desirable to be able to detect the bacteria without prior enrichment, this procedure gave a negative result in 50% of the samples which proved positive after enrichment.

The DNA probe employed cannot differentiate between pathogenic Y. enterocolitica, Y. pseudotuberculosis, or Y. pestis, as it is based on a DNA sequence which is conserved in all these species (8, 24). This scarcely represents a problem in the context of the present investigation, as we have never been able to demonstrate virulent Y. pseudotuberculosis (or Y. pestis) in Norwegian pork products or on pig carcasses (15–18). Y. enterocolitica O:3 is the only virulent Yersinia variant which we have isolated from these sources. Although single isolates of both Y. pseudotuberculosis O:1 and Y. enterocolitica O:5,27 have been obtained, both these isolates lacked the virulence plasmid (18). It is therefore highly probable that the positive samples detected by DNA hybridization in this study actually contained O:3 and not the other pathogenic serogroups, even though this possibility cannot be entirely excluded.

In previous investigations, we showed that Y. enterocolitica O:3 occurred in large numbers in the oral cavity in a high proportion of the slaughter pigs which were examined (15, 16, 18). The bacterium was also a common surface contaminant on fresh carcasses (16). In contrast, Y. enterocolitica O:3 was only isolated from one of 127 raw pork products at the stage of retail sale (17). The results of the present investigation indicate that the occurrence of pathogenic Y. enterocolitica is substantially higher than previously demonstrated and, therefore, reinforce our hypothesis that pork products represent an important potential source of human infection in Norway. Even though the estimated bacterial counts per gram were not notably high, the presence of pathogenic Y. enterocolitica must always be considered to represent a potential health hazard, as the bacterium is able to survive and multiply in properly refrigerated foods (3).

Further studies are necessary to ascertain more precisely the occurrence of Y. enterocolitica O:3 in various types of products and to trace contamination at the various stages in the food production chain. DNA hybridization should prove to be a useful tool in this connection.

ACKNOWLEDGMENTS

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27. Wauters, G. Personal communication.

