Research Note

Enrichment, Isolation, and Virulence of Freeze-Stressed Plasmid-Bearing Virulent Strains of Yersinia enterocolitica on Pork†

SAUMYA BHADURI*

Microbial Food Safety Research Unit, U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038, USA

ABSTRACT

The influence of freeze stress at -20°C on the enrichment, isolation, detection, presence of virulence plasmid, and expression of virulence of plasmid-bearing Yersinia enterocolitica (YEP+) inoculated on pork chop medallions was assessed. Pork chop medallions (10 cm²) artificially contaminated with 10, 1, and 0.5 CFU/cm² of YEP+ strains (serotype O:3) were placed in sterile petri dishes at -20°C for 24 h. The medallions were swabbed when frozen, after thawing at room temperature for 1.5 h and after thawing at 4°C for 18 h. Swabs were enriched and YEP+ were detected and isolated using the Congo red-binding and low-calcium-response assays. The YEP+ were isolated under all conditions on pork chop medallions inoculated with 10 CFU/cm² and at a level of 1 CFU/cm² when thawed at room temperature and at 4°C but not from frozen pork chop medallions. The YEP+ were not isolated from pork chop medallions inoculated with 0.5 CFU/cm² and then frozen, whereas YEP+ were recovered when inoculated at this level from pork chop medallions not subjected to freezing. Virulence of the strains isolated from frozen pork chop medallions was confirmed by PCR and the expression of plasmid-associated phenotypes. These results indicate that YEP+ subjected to freezing on pork are potentially capable of causing foodborne illness and that freezing is not a substitute for safe handling and proper cooking of pork.

An estimated 96,000 individuals suffer from yersiniosis in the United States annually (16, 17). Ninety percent of the cases are the result of foodborne transmission of pathogenic Yersinia enterocolitica (1, 16, 17). Swine are the principal reservoir for this organism and Yersinia infection in the United States is generally transmitted to humans by pork products during industrial processing (10). The estimated carrier rate of pathogenic Y. enterocolitica ranges from approximately 35 to 70% in swine herds (10, 16). This bacterium is most frequently isolated from pig tongues and tonsil, but has also been found on carcasses, pork products, and feces by culture methods (1, 3, 5-9, 11, 12). The epidemiological evidences indicate that pork products transmit pathogenic strains to humans and cause yersiniosis (13-15). Since plasmid-bearing virulent Y. enterocolitica (YEP+) strains are cold-tolerant, pork samples are often frozen prior to their analysis to avoid skewing the results if enumerating YEP+. It is generally recognized that the organism can withstand freezing (4, 18); however, quantitative data on its isolation and detection in pork samples contaminated with a low number of cells after freezing are lacking. Moreover, freezing may impair the isolation of physiologically injured cells and/or detection of low levels of the target organism. Thus, the purpose of the present study was to determine the effect of freeze stress on the enrichment, isolation, and detection of YEP+ from pork chop medallions artificially contaminated at low levels using a method previously developed in our laboratory (5, 6). The information obtained will help to determine the effect of freezing on the isolation of low levels of YEP+ on pork.

MATERIALS AND METHODS

Bacteria and preparation of media. It was previously reported that serogroup O:3 is the most dominant virulent serogroup associated with pork and yersiniosis in the United States (1, 5-8, 13-15). A clinical isolate of serotype O:3 YEP+ strain GER and four serogroup O:3 porcine tongue isolates (SB1, SB7, SB9, and SB10) were used in the current study. Brain heart infusion (BHI) broth, brain heart infusion agar (BHA), and 0.1% peptone water were prepared as recommended by the supplier (Difco Laboratories, Detroit, Mich.). Modified Trypticase soy broth (MTSB; Difco Laboratories) containing bile salts #3 (Difco) and low-calcium (238 μM) Congo red (CR; Sigma Chemical Co., St. Louis, Mo.) BHI agarose (CR-BHO; Gibco BRL, Gaithersburg, Md.) were prepared as described previously (5, 7).

Inoculation of pork chop medallions. The YEP+ cultures were separately grown in BHI broth for 18 h at 28°C with shaking to a population density of approximately 10⁸ CFU/ml. The cultures were diluted in 0.1% peptone water prior to inoculating pork chop medallions with each strain individually. Ten-square-centimeter medallions of pork chops placed in sterile petri dishes were
Results and Discussion

Food tested for pathogenic bacteria is often subjected to freezing prior to analysis, which may impair the isolation of low levels of the target organism. In this work, pork chop medallions were artificially surface contaminated with five serotypes of O:3 strains of YEP+ at concentrations of 10, 1, and 0.5 CFU/cm² to evaluate the effect of freeze stress at -20°C and subsequent swabbing at frozen, after thawing at RT for 1.5 h, and after thawing at 4°C for 18 h.

Enrichment, isolation, and detection. Each swab was placed in a sterile Whirl Pak bag containing 90 ml of MTSB and enriched in a shaking incubator (100 rpm) at 12°C for 48 h as described previously (7). Selectively enriched samples were diluted and plated onto CR-BHO. All plates were incubated at 37°C for 24 h. The YEP+ strains appeared as red pinpoint colonies on CR-BHO showing CR binding and low calcium response (LCR) (7). As a control, fresh pork chop medallions were artificially contaminated separately with the same five strains, swabbed, enriched, and isolated as described above without freezing (Table 1).

Virulence characteristics of YEP+ isolated from freeze-stressed pork chop medallions. The identification of YEP+ was further confirmed by multiplex PCR targeting the chromosomal ail gene (attachment-invasion locus) and the virF gene (transcriptional activator for the expression of pYV-encoded outer membrane protein Yop51) (3). The phenotypic virulence characteristics including colonial morphology, crystal violet (CV) binding, LCR, CR binding, hydrophobicity (HP), and autoagglutination (AA) were performed as described previously (5, 7).

TABLE 1. Isolation of YEP+ from pork chop medallions separately contaminated with strains of GER, SB1, SB7, SB9, and SB10 and freeze-stressed at -20°C for 24 h, swabbed, and subjected to enrichment for 48 h.

<table>
<thead>
<tr>
<th>Contamination level, CFU/cm²</th>
<th>Swabbing conditions</th>
<th>Fresh</th>
<th>Frozen</th>
<th>Thawed at RT</th>
<th>Thawed at 4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.5</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Detected by CR binding and LCR techniques. The results are based on three individual experiments.

Surface contaminated as described previously (7) at 10, 1, and 0.5 CFU/cm² and kept at -20°C for 24 h.

Swabbing of pork chop medallions. Since thawing may cause lysis/injury of cells, the pork chop medallions were swabbed with a sterile cellulose sponge (5 by 1.25 cm) moistened in 10 ml of MTSB (7) after freezing, after thawing at RT for 1.5 h, and after thawing at 4°C for 18 h.

The possible reason is that some cells will not survive or are injured during freezing regardless of the number of cells present in food (4, 18) and/or that a minimum number of target cells are required for enrichment. Thus, pork chop medallions contaminated with an initial level of 0.5 CFU/cm² did not contain a minimum of viable or injured cells required for enrichment. Results were the same for all five strains tested. These results showed that freezing of contaminated pork chop medallions inhibited the isolation of YEP+ at a very low initial level of contamination, and sampling should be done prior to freezing to avoid false-negative results.

In previous studies the presence of ail and VirF genes, the plasmid-encoded phenotypic virulence factors, were correlated with mouse pathogenicity (5, 7) and thus were used as direct markers for pathogenicity for YEP+. The YEP+ recovered from frozen pork chop medallions in the present study were confirmed as potentially virulent by a multiplex PCR assay targeting ail and virF genes and by the expression of plasmid-associated phenotypic virulence characteristics, including colonial morphology, CV binding, LCR, CR uptake, HP, and AA.

In summary, the present study is the first one to show the effect of freeze stress on the enrichment and isolation of YEP+ when pork is contaminated with low inoculum levels of this pathogen. The YEP+ can survive freeze stress when inoculated onto pork at 10 and 1 CFU/cm² and can be enriched and isolated after thawing at RT and at 4°C. However, the organism could not be isolated using an inoculum level of 0.5 CFU/cm². Of greater significance is that YEP+ subjected to freezing on pork retained the pathogenic traits and are still capable of causing human diseases. The present study indicates that freezing alone will not add a significant margin of safety with respect to this pathogen and cannot replace sanitary production and handling. Pork contaminated with YEP+ can lead to infection of humans if it is not properly handled and sufficiently cooked, even if the meat has been previously frozen.

Acknowledgments

I thank Bryan Cottrell and Ms. Kenyetta Chaney of the Microbial Food Safety Research Unit at the USDA (Eastern Regional Research Center, Wyndmoor, Pa.) for their technical assistance in this study.
REFERENCES


