APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1981, p. 661-666
0099-2240/81/100661-06$02.00/0

Vol. 42, No. 4

# Isolation of Virulent *Yersinia enterocolitica* from Porcine Tongues

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Received 23 March 1981/Accepted 1 July 1981

Thirty-one tongues from apparently normal, freshly slaughtered pigs were assayed for the presence of *Yersinia enterocolitica* by different enrichment and postenrichment techniques. Sixteen different isolates were recovered, including six of serotype O:8, four of serotype O:6,30, two of serotype O:3 phage type IXb, and one each of serotypes O:13,7, O:18, and O:46. One isolate was not typable. Cold enrichment in phosphate-buffered saline followed by treatment with dilute KOH or subsequent enrichment in modified Rappaport broth recovered 12 and 7 isolates, respectively. Four of the same isolates were recovered from the same tongues by both procedures. Cold enrichment without a selective postenrichment treatment recovered two isolates. Direct enrichment in modified Rappaport broth or modified selenite broth was ineffective in recovering yersiniae, as no isolates were obtained by either method. All of the serotype O:8 isolates were virulent to mice, causing the death of adults after oral challenge. This is the first report that associates *Y. enterocolitica* serotype O:8 with a natural reservoir.

*Yersinia enterocolitica* has been a recognized human pathogen for nearly 50 years (4, 12, 28); however, its significance as a food-borne pathogen has been only recently realized. The first definitive food-associated outbreak of yersiniosis occurred in 1976 in Oneida County, New York, where over 220 individuals, primarily school children, were stricken with an acute intestinal illness (3). Several children were hospitalized and diagnosed as having appendicitis. Sixteen children had appendectomies before the responsible agent, *Y. enterocolitica* serotype O:8, Wauters biotype 1, was identified. This incident is the only firm linkage between *Y. enterocolitica* in food and human infections. Lee et al. (19) suggested that a reason why this organism has not been widely traced to foods is that current enrichment procedures fail to recover low levels of clinical strains from foods. Many clinical strains, including those of serotype O:8, are sensitive to selective agents commonly used to isolate *Y. enterocolitica*.

Several methods have been proposed to isolate *Y. enterocolitica* from clinical and environmental samples. Most involve enrichment of the sample followed by plating onto selective agar. Several different enrichment procedures have been proposed. Enrichment in phosphate-buffered saline (PBS) at 4°C for 14 or 21 days has been successfully used by many investigators (7, 13, 16, 22, 35, 36, 40). Wauters recommended that selective enrichment in modified Rappaport broth (MRB) incubated at room temperature for 2 days could be used to isolate strains of serotypes O:3 and O:9 (37, 38). An enrichment recommended by the American Public Health Association includes cold enrichment in PBS followed by selective enrichment in MRB (10). Recently, Lee et al. (19) developed two modified selenite enrichment broths (MS) and proposed that they be used to recover sensitive clinical strains, such as serotype O:8, of *Y. enterocolitica* from foods. They determined that enrichment in MS is more effective in recovering both resistant and sensitive clinical strains of *Y. enterocolitica* from meats than is enrichment in either MRB or PBS.

Although the purpose of enrichment is to promote the growth of *Y. enterocolitica*, other microbial competitors may also proliferate. Hence, a postenrichment medium or treatment is needed to select for *Y. enterocolitica*. Different strains of *Y. enterocolitica* vary in their ability to grow on various enteric selective media; however, most can grow on MacConkey agar (9, 10). Unfortunately, many enteric bacteria that have survived enrichment can also grow on MacConkey agar. Aulisio et al. (1) observed that many strains of *Y. enterocolitica* are tolerant to dilute alkali and subsequently used this principle to develop a postenrichment treatment to select for yersiniae. They exposed samples of cultures grown in enrichment broth to 0.5% KOH before plating onto MacConkey agar. The treatment

successfully eliminated most background contaminants and allowed a fourfold increase in the recovery of *Y. enterocolitica*.

Studies done in Europe, Canada, and Japan have shown that swine are an important reservoir of *Y. enterocolitica* (6, 8, 16, 23–25, 27, 31, 33, 38, 42). This is particularly true of strains of serotypes O:3 and O:9, which are the predominant cause of yersiniosis in those areas. In the United States, Hanna et al. (14) isolated serotype O:5 from 8 of 27 porcine tonsils, and Stern (29) isolated a nontypable strain of *Y. enterocolitica* from a porcine tongue. Stern suggested that this organism was potentially virulent based on its ability to autoagglutinate when grown in tissue culture medium at 37°C, but not at 22°C (17, 29). However, to date, no one has identified a natural reservoir for serotype O:8 which is one of the most common causes of yersiniosis in the United States (2, 3, 39; E. Christenson, personal communication).

This paper contributes additional information regarding the presence of clinically important strains of *Y. enterocolitica* in swine in the United States and illustrates the need for cold enrichment for recovering these organisms from pork samples.

## MATERIALS AND METHODS

**Samples.** Thirty-one tongues were obtained from apparently healthy market-weight hogs that were butchered by a local meat processor using normal slaughtering practices. All tongues were refrigerated and assayed within 24 h after removal from animals.

**Isolation methods.** Portions (25 g) of tongue were blended for 2 min with 225 ml of 0.067 M disodium-PBS (pH 7.6) (10) and MRB (10, 37). Inoculated PBS (2 ml) was added to 100 ml of MS such that 0.2 g of meat suspension was present in 100 ml of enrichment broth (19). PBS was incubated at 4°C for 21 days (10), MRB was incubated at room temperature for 7 days (27), and MS was incubated at 22°C for 2 and 3 days (19). After incubation, a 1.0-ml sample of PBS enrichment was added to 10 ml of MRB and incubated at 27°C for 2 days (10, 27).

After incubation, loopfuls of enrichment broths were both streaked directly onto MacConkey agar (9, 10) and given an alkali treatment (KOH) before being streaked onto MacConkey agar (1). Alkali treatment included adding 0.5 ml of enrichment broth to 4.5 ml of 0.5% KOH in saline, agitating with a Vortex mixer, and streaking onto agar within 1 min. Agar plates were incubated at 27°C for 2 days. Five translucent colonies having characteristics of *Y. enterocolitica* were transferred from each plate to triple-sugar-iron agar slants. Typical isolates, $A/A^-$ or $K/A^-$, were tested on Christiansen urea agar. Urea-positive isolates were biochemically characterized and then serotyped and phage typed by S. Toma of the Canadian National References Center for *Yersinia*, Ontario Ministry of Health, Toronto, Canada.

**Virulence testing.** A modification of the method described by Laird and Cavanaugh (17) was used to identify isolates that could autoagglutinate. Isolates were grown in Trypticase soy broth (BBL Microbiology Systems) for 18 h at 22°C, concentrated by centrifugation, and suspended in 1% peptone water-48.5% glycerol. These cultures were maintained at −20°C until used, at which time 0.1 ml was inoculated into each of two test tubes containing 2 ml of Trypticase soy broth. One tube was incubated at 37°C and the other was incubated at 22°C, each for 18 h. As defined by Laird and Cavanaugh (17), growth of autoagglutination-positive strains consisted of an irregularly edged layer of agglutinated bacteria that formed a flocculate covering the bottom of the tube. The medium in such tubes was usually clear. Growth of nonagglutinating strains generally remained in suspension, creating a turbid medium. Isolates were considered autoagglutination positive only if they autoagglutinated at 37°C, but not at 22°C.

Virulence was determined by orally feeding mice as described by Laird and Cavanaugh (17). Groups of three Swiss albino mice (Sprague-Dawley-ICR), weighing 15 to 20 g, were deprived of water for 18 h. Subsequently, mice were allowed to drink ad libitum from a 50-ml water suspension of each strain grown at 25°C containing $\sim 10^9$ bacteria per ml. Mice were examined twice daily for death. Studies were terminated after 14 days. Spleens and blood from the hearts of dead animals were cultured by standard bacteriological techniques to detect the presence of challenge organisms.

## RESULTS AND DISCUSSION

**Characteristics of isolates.** Twenty-one isolates of *Y. enterocolitica* were obtained from 31 porcine tongues. Of these, three isolates with the same serotype and biotype were recovered from the same tongues by two different procedures and one isolate with identical characteristics was recovered from the same tongue by three different procedures. Hence, 16 distinct isolates were recovered from the tongues (Table 1).

Strains of serotype O:8 were most frequently isolated. These were isolated from six different tongues. Interestingly, strains of this serotype are among those types of *Y. enterocolitica* most frequently associated with human infections in the United States. Weaver and Jordan (39) reported that, of 27 isolates of *Y. enterocolitica* that were received by the Centers for Disease Control from October 1969 through March 1972 from 13 states and the District of Columbia, 14 were serotype O:8. Similar observations were made by Bissett (2), who reported that 6 of 24 human isolates of *Y. enterocolitica* submitted to the California Department of Health from 1968 through 1975 were serotype O:8. Serotype O:8 is also prevalent among the types of *Y. enterocolitica* isolated from humans in the state of Wis-

TABLE 1. *Types of Yersinia enterocolitica isolated from porcine tongues*

| Serotype | Biotype[a] | Phage type | No. of strains isolated |
|---|---|---|---|
| O:3 | 4 | IXb | 2 |
| O:6,30 | 1 | | 4 |
| O:8 | 1 | | 6 |
| O:13,7 | 2 | | 1 |
| O:18 | 1 | | 1 |
| O:46 | 1 | | 1 |
| NT[b] | 2 | | 1 |

[a] Based on the scheme of Wauters (10).
[b] NT, Not typeable.

consin (E. Christenson, personal communication). Additionally, *Y. enterocolitica* serotype O:8 was the cause of the chocolate milk outbreak in Oneida County, New York (3).

The second most frequently isolated type of *Y. enterocolitica* recovered from the tongues was serotype O:6,30. *Y. enterocolitica* of this serotype was isolated from four different tongues. Although strains of *Y. enterocolitica* belonging to this serotype are among those most frequently isolated from humans in the state of Wisconsin (E. Christenson, personal communication) and a strain of serotype O:6,30 was associated with one of six cases of human yersiniosis recently reported in Missouri (40), Pai and DeStephano (Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B52, p. 23) observed that many strains of this serotype are avirulent in animal models.

*Y. enterocolitica* serotype O:3, biotype 4, is the most frequent cause of human yersiniosis in Canada (32) and most of Europe (21); however, in the United States, *Y. enterocolitica* of this serotype is seldom associated with human infections (2, 39; E. Christenson, personal communication). In Canada (27, 31), Europe (6, 8, 16, 24–26, 38), and Japan (33, 42), swine were found to be the only animal species that consistently harbors *Yersinia* sp. of this serotype. In Belgium, the incidence of *Y. enterocolitica* serotype O:3 is so great among swine that it is considered to be part of the normal flora of the oral cavity (38). Results from this study indicate that *Y. enterocolitica* serotype O:3 may be present as part of the oral flora of swine in Wisconsin; however, its incidence appears to be less than that of serotypes O:8 and O:6,30. Interestingly, the phage type of the O:3 isolates was IXb. This conforms with the specific phage type of *Y. enterocolitica* O:3 strains isolated in Canada and differs from the serotype O:3, phage type VIII, strains that predominate in Europe and Japan (31).

Other serotypes of *Y. enterocolitica* recovered during this study included one isolation each of serotypes O:13,7, O:18, and O:46. One strain was nontypable.

**Efficacy of recovery procedures.** Four different enrichment procedures were evaluated for their ability to recover naturally occurring *Y. enterocolitica* from porcine tongues. Two procedures, PBS and PBS-MRB, involved cold enrichment in which samples were incubated at 4°C for 21 days before being subjected to selective agents. The other two procedures, MRB and MS, were selective enrichments in which samples were subjected to selective agents during enrichment. Samples receiving selective enrichments were incubated at room temperature, which approaches the optimum temperature for growth of *Y. enterocolitica* (41). After enrichment, samples were directly plated or treated with dilute alkali before being plated onto MacConkey agar plates.

The efficacy of the different isolation procedures is shown in Table 2. The organism was only recovered from samples that received cold enrichment. Postenrichment treatment with dilute alkali recovered the most *Y. enterocolitica* isolates. Twelve distinct isolates were recovered by this method, including four of serotype O:8, four of O:6,30, one of O:3, one of O:18, one of O:13,7, and one of O:46. Selective enrichment in MRB after cold enrichment in PBS yielded seven successful isolations of *Y. enterocolitica*; however, in comparison to the PBS-KOH treatment, only three isolations of serotype O:8, two of O:6,30, one of O:3, and one nontypable were made. Two of the O:8 and one of the O:6,30 isolates were recovered from the same tongue by both procedures. Only two isolations were made, one of O:8, and one of O:6,30, from samples plated directly onto MacConkey agar after cold enrichment in PBS. The O:6,30 isolate was re-

TABLE 2. *Comparison of isolation procedures used to recover Y. enterocolitica from porcine tongues*

| Isolation procedure | No. of *Y. enterocolitica* isolated/no. of tongues samples |
|---|---|
| PBS | 2/31[a] |
| PBS-KOH | 12/31[a,b] |
| PBS-MRB | 7/31[a,b] |
| MRB | 0/31 |
| MRB-KOH | 0/31 |
| MS | 0/31 |
| MS-KOH | 0/31 |

[a] One strain having identical characteristics was isolated from the same tongue by these procedures.
[b] Three strains having identical characteristics were isolated from the same tongues by these procedures.

covered from the same tongue by all three of the aforementioned procedures.

Surprisingly, neither of the selective enrichment procedures, i.e., MRB or MS, recovered *Y. enterocolitica* from the tongues. It is generally accepted that enrichment in MRB is a reliable procedure for recovering strains of serotype O:3 from food and feces (26, 27, 35, 37, 38). Schiemann has shown that as few as 35 cells of serotype O:3 can be recovered from inoculated pork and beef after enrichment in MRB for up to 7 days at room temperature (26). In a later study, Schiemann observed that direct enrichment in MRB recovers more *Y. enterocolitica*, primarily serotype O:3, from pork products which include tongues than does cold enrichment in PBS followed by selective enrichment in MRB (27). Similar observations were made by Wauters (38). Since two isolations of serotype O:3 were made by cold enrichment in this study, it is surprising that organisms of this serotype were not recovered by enrichment in MRB.

Several investigators have observed that isolates of serotype O:8 are more sensitive to selective agents than many other types of *Y. enterocolitica*; hence, they are not readily recovered from foods by selective enrichment (7, 27, 34, 35). VanNoyen et al. (35) reported that cold enrichment enhances the recovery of strains belonging to biotype 1, which generally includes serotype O:8, but does not enhance recovery of serotype O:3, biotype 4, or serotype O:9, biotype 2. However, Lee (18) and Mehlman et al. (20) reported that cold enrichment in PBS results in poor recovery of clinical strains of both serotypes O:3 and O:8 from inoculated pork and oysters. Lee et al. (19) subsequently developed two MS that were effective in recovering sensitive clinical strains of serotype O:8 from inoculated pork and beef. With these enrichment media, Stern and Oblinger (30) were successful in approximately 50% of their attempts to recover serotype O:8 from inoculated surfaces of beef hearts and livers. However, in our study, enrichment in MS, with or without an alkali postenrichment treatment, proved to be unsuccessful for isolating any serotype O:8 or other strains of *Y. enterocolitica* that were naturally present on porcine tongues.

**Virulence of isolates.** Two methods were used to assess virulence of the different strains of *Y. enterocolitica* isolated from porcine tongues: (i) the autoagglutination assay of Laird and Cavanaugh (17) and (ii) lethality of adult mice by oral feeding. Laird and Cavanaugh recently reported that a strong correlation exists between mouse-virulent strains of *Y. enterocolitica* and their ability to autoagglutinate in tissue culture medium when grown at 36°C but not at 26°C. During the course of our studies, we observed that tissue culture medium was not necessary to obtain an autoagglutination response by strains of *Y. enterocolitica* known to be autoagglutination positive. Growth in Trypticase soy broth at temperatures of 37 and 22°C yielded the same response. Hence, Trypticase soy broth was used as the substrate for our autoagglutination studies (Table 3). All of the serotype O:3 and O:8 isolates were autoagglutination positive, whereas all other isolates were negative. Similarly, all control strains, which were also serotype O:3 or O:8, were autoagglutination positive. These strains were originally isolated from infected humans and are therefore apparently virulent. These results conform with the observations of Laird and Cavanaugh, i.e., that strains of *Y. enterocolitica* virulent for mice autoagglutinate, and also suggest that the autoagglutination-positive strains isolated from the porcine tongues of this study might have been virulent. However, it is not conclusively known whether strains of *Y. enterocolitica* that are virulent for mice are virulent for humans.

Additional evidence supporting the virulence of the serotype O:8 porcine isolates is their effect

TABLE 3. *Virulence of strains of Y. enterocolitica isolated from porcine tongues*

| Isolate no. | Recovery procedure | Serotype | Autoagglutination | Death of mice |
|---|---|---|---|---|
| FRI-YE 1 | PBS-KOH | O:8 | + | 3/3 |
| FRI-YE 2 | PBS-KOH | O:18 | − | 0/3 |
| FRI-YE 3[a] | PBS-KOH | O:8 | + | 3/3 |
| FRI-YE 3[a] | PBS-MRB | O:8 | + | 3/3 |
| FRI-YE 4 | PBS-KOH | O:6,30 | − | 0/3 |
| FRI-YE 5 | PBS-KOH | O:8 | + | 3/3 |
| FRI-YE 6 | PBS-KOH | O:46 | − | 0/3 |
| FRI-YE 8 | PBS-KOH | O:13,7 | − | 0/3 |
| FRI-YE 9[b] | PBS-MRB | O:8 | + | 3/3 |
| FRI-YE 9[b] | PBS | O:8 | + | 3/3 |
| FRI-YE 10 | PBS-KOH | O:8 | + | 3/3 |
| FRI-YE 11 | PBS-MRB | O:3 | + | 0/3 |
| FRI-YE 12 | PBS-KOH | O:6,30 | − | 0/3 |
| FRI-YE 13 | PBS-MRB | O:8 | + | 3/3 |
| FRI-YE 14[c] | PBS-KOH | O:6,30 | − | 0/3 |
| FRI-YE 14[c] | PBS-MRB | O:6,30 | − | 0/3 |
| FRI-YE 14[c] | PBS | O:6,30 | − | 0/3 |
| FRI-YE 16 | PBS-KOH | O:3 | + | 0/3 |
| FRI-YE 17[d] | PBS-KOH | O:6,30 | − | 0/3 |
| FRI-YE 17[d] | PBS-MRB | O:6,30 | − | 0/3 |
| FRI-YE 18 | PBS-MRB | NT | − | 0/3 |
| WA | Control | O:8 | + | 6/6 |
| C122-76 | Control | O:3 | + | 0/3 |
| 700 | Control | O:3 | + | 0/3 |
| 6806 | Control | O:3 | + | 0/3 |
| C108-76 | Control | O:3 | + | 0/3 |

[a,b,c,d] Each letter indicates an isolate was recovered from the same tongue by different procedures. Different letters represent isolates recovered from different tongues.

[c] NT, Not typable.

on mice after oral ingestion. All of these isolates, including the control (strain WA), caused death of adult mice. This conforms with the observations of Gemski et al. (11), Carter et al. (5), and Laird and Cavanaugh (17) who also reported death of adult mice after peroral administration of virulent strains of *Y. enterocolitica* serotype O:8.

**Implications of the results.** Although *Y. enterocolitica* serotype O:8 is one of the most common causes of yersiniosis in humans in the United States, relatively little is known about the epidemiology of this bacterium. We found an important piece to this puzzle, having identified the oral cavity of swine to be a natural reservoir for strains of this serotype. Furthermore, all of the serotype O:8 strains found to be associated with swine were determined to be virulent for mice. This finding has important ramifications, because *Y. enterocolitica* can grow and develop large populations on pork tissue during refrigerated storage (15). It is reasonable to speculate that cuts of meat prepared from pigs harboring virulent strains of this microorganism may be contaminated by such bacteria. This is supported by the observations of Schiemann (27), who isolated a strain of *Y. enterocolitica* O:8 from a sample of ground pork. Should such meat be improperly cooked or allowed to come in contact with foods that do not receive a heat treatment, their consumption may result in outbreaks of yersiniosis.

## ACKNOWLEDGMENTS

We thank S. Toma for serotyping and phage typing bacteria isolated as part of this study. Some cultures used in this study were provided by I. J. Mehlman, Food and Drug Administration, Washington, D.C.; C. H. Pai, McGill University-Montreal Children's Hospital Research Institute, Montreal, Quebec, Canada; R. M. Robins-Browne, University of Witwatersrand, Johannesberg, South Africa; and T. Caprioli, Ministère des Affaires Sociales du Quebec, Quebec, Canada, to whom we are most grateful.

This work was supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and by contributions to the Food Research Institute.

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