To the Graduate Council:

I am submitting herewith a thesis written by Joo-Sung Kim entitled "Evaluation of Enrichment Methods for Recovery of *Yersinia enterocolitica* O:3 and O:8 from Swine Feeds and Its Transformation with pGFPuv Vector." I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

F. Ann Draughon

F. Ann Draughon, Major Professor

We have read this thesis
And recommend its acceptance:

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Evaluation of Enrichment Methods for Recovery of *Yersinia enterocolitica* O:3 and O:8 from Swine Feeds and Its Transformation with pGFPuv Vector

A Thesis Presented for the Master of Science Degree

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Joo-Sung Kim

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LIST OF ABBREVIATIONS

BHI : Brain heart infusion

BOS : Bile-oxalate-sorbose

CAL : Cellobiose-arginine-lysine

CIN : Cefsulodin-irgasan-novobiocin

CR-BHO : Low-calcium congo red – brain heart infusion agarose

GFP : Green fluorescent protein

ITC : Irgasan, ticarcillin, and potassium chlorate

MAC : MacConkey

MRB : Modified rappaport broth

MS : Modified selenite enrichment broth

PBS : Phosphate buffered saline

PCR : Polymerase chain reaction

PSB : Phosphate-buffered saline with 1% sorbitol and 0.15% bile salts

SS : Salmonella-shigella

SS-D : Salmonella-shigella deoxycholate

SSDC : Salmonella-shigella deoxycholate calcium

TSB : Tryptic soy broth or Trypticase soy broth

TSBPN : TSB with Polymyxin B (5 IU/ml) and Novobiocin (10 μg/ml)

TSI : Triple sugar iron

VYE : Virulent *Yersinia enterocolitica*

YER : Yeast extract – rose bengal

Part I:

Literature review

History

The genus *Yersinia* is Gram-negative coccobacilli and contains three well-recognized human pathogens including *Y. enterocolitica, Y. pestis* (the plague bacillus), and *Y. pseudotuberculosis*. The name of *Yersinia* is after the French bacteriologist Alexandre Yersin who first described the plague bacillus (Bottone, 1999).

In 1934, the first recognition of *Y. enterocolitica* was made in US under the name *Flavobacterium pseudomallei*, with the description that a small gram negative coccobacillus was isolated from two facial boil of a 53-year-old farm dweller. It was also isolated from the cervical lymph nodes (McIver and Pike, 1934). In 1939, based on microbiologic properties and origins, the unidentified microorganism was named *Bacterium enterocoliticum* which was proposed by Schleifstein and Coleman working at the New York State Department of Health (Schlelfstein and Coleman, 1939). In the US, *Y. enterocolitica* serotype O:8 presumptive strains which were isolated from patients with enterocolitis were also named *Bacterium enterocoliticum* based on biochemical reactions in 1943 (Bottone, 1999). In 1944, the change of *Pasteurella pestis* and *P. pseudotuberculosis* to newly defined *Yersinia* was proposed by Van Loghem (Aleksic and Bockemühl, 1999). During the periods that *Yersinia* was called *Pasteurella*, strains that differed from *Pasteurella pseudotuberculosis* in biochemical reactions, susceptibility to phage lysis, and pathogenicity for guinea pigs and rabbits were suggested to be classified as a new species of *Pasteurella*. It was later called *Pasteurella* "X" (Schiemann, 1989).

Y. enterocolitica was first designated by Frederiksen in 1964. The first major reported foodborne outbreak from *Y. enterocolitica* occurred among 36 children who had consumed chocolate milk contaminated with *Y. enterocolitica* O:8 in New York State in 1976 (Black et al., 1978). This outbreak created scientific interest in this neglected bacterial species (Bottone, 1999).

Characteristics

Y. enterocolitica is a Gram-negative and facultatively anaerobic coccobacillus under the genus *Yersinia* in the *Enterobacteriaceae* family. Because it is recognized as a food-borne pathogen (Lee et al., 1981) with growth at refrigerator temperature, it is called a psychrotrophic pathogen. Refrigerated foods are potential vehicles for foodborne illness for the organism (Bhaduri, 1999; Fukushima and Gomyoda, 1986a; Lee et al., 1980).

It is a major foodborne pathogen in the United States, which is associated with human gastroenteritis, pseudoappendicitis, mesenteric lymphadenitis, terminal ileitis, reactive arthritis, peritonitis, colon and neck abscesses, and cholecystis (Jay, 2000). Yersiniosis occurs primarily in infants or young children (Metchock et al., 1991). Fever, abdominal pain, and diarrhea are the most common symptoms (Schiemann, 1989). Watery, sometimes bloody stool is characteristic of *Y. enterocolitica*. Bloody diarrhea is observed mostly in adults (Aleksic and Bockemühl, 1999).

Growth of *Y. enterocolitica* has been identified over the temperature range –2°C to 45°C (Stern et al., 1980), but optimum temperature has been found ranging from 32°C to 35°C (Schiemann, 1980b). The minimum growth temperature can be raised by the addition of NaCl. Growth was observed at 3°C over the pH range 4.6-9.0 (Stern et al.,

1980). It is destroyed in 1-3 minutes at 60°C (Hanna et al., 1977), but is rather resistant to freezing (Jay, 2000).

Y. enterocolitica is distributed worldwide (Aleksic and Bockemühl, 1999) and is a genotypically, phenotypically and ecologically heterogeneous species (Aulisio et al., 1980; Kapperud, 1991; Stock and Wiedemann, 1999). The sugar content of the lipopolysaccharides (LPS) of the cell wall varies among strains of *Y. enterocolitica* and makes serotyping possible (Schiemann, 1989). A number of biotyping schemes have been proposed based on biochemical tests (Schiemann, 1989).

Strain-to-strain variation exists in the pathogenicity of *Y. enterocolitica*. The presence of a 70-75 kbp plasmid is directly involved with the virulence (Bhaduri, 1999), and is also related to calcium dependency of this microorganism (Gemski et al., 1980). The physiological characteristics related to the virulence plasmid are only expressed at 37°C, but this temperature also facilitates the loss of the plasmid which means the disappearance of the associated phenotypic virulence characteristics (Bhaduri, 1999). The invasiveness of *Y. enterocolitica* is also related to two chromosomal loci, *inv* and *ail* (Miller and Falkow, 1988).

The enterotoxin produced by *Y. enterocolitica* is heat-stable and survives 100°C for 15 min (Pai and Mors, 1978).

Isolation from the environment and epidemiology

Many studies were conducted to isolate *Y. enterocolitica* from swine and the environment and they validate that swine is a major reservoir of pathogenic *Y. enterocolitica*.

Yersinia spp. were isolated from the tonsils of 200 (43.4%) of the 461 slaughtered pigs tested in Norway in 1984 (Nesbakken and Kapperud, 1985). Among the total isolates, 92.7% were identified as *Y. enterocolitica* and 70.9% were *Y. enterocolitica* O:3. Serotype O:3/biotype 4 was found in 31.7% of all pigs, which is typical in European countries (Nesbakken and Kapperud, 1985).

Y. enterocolitica was also isolated from 25% of the pigs in Danish herds during an investigation from 1982 to 1983 (Andersen et al., 1991). *Y. enterocolitica* was isolated from water from water nipples and swabs of pigpen floors at swine farms, as well as from pork at swine processing rooms in Korea (Rho et al., 2001).

The prevalence of *Y. enterocolitica* among swine is dependent on seasons. The prevalence in winter was significantly higher than summer (Letellier et al., 1999).

In the US, some studies revealed that swine harbor *Y. enterocolitica*. Twenty-one *Y. enterocolitica* isolates were obtained from 31 swine tongues and serotype O:8 was most frequent (Doyle et al., 1981). This was the first finding of an association between O:8 and a natural reservoir. *Y. enterocolitica* was found in 9 tongues out of 25 swine tongues in another study (Doyle and Hugdahl, 1983). Among 103 lots of market swine located in midwestern area, *Y. enterocolitica* was found in ninety-five lots (92.2%), with serotype O:5 (89.7%) and O:3 (3.7%) found in the 107 pathogenic strains (Funk et al., 1998).

In other study, pathogenic strains were also found in tonsils, mesenteric nodes and tongues of pigs at a slaughterhouse in Rome, Italy, with 47.2%, 15.7%, and 4.7%, respectively, among samples (De Giusti et al., 1995).

In Canada, *Y. enterocolitica* was recovered from cecal materials of 21% of finished swine. A high percentage (85.5%) of isolates was serotype O:3 (Letellier et al., 1999).

Wild animals, environmental water, and swine feeds are also contaminated with *Y. enterocolitica* (Fukushima et al., 1984; Jay, 2000). In Scandinavia, *Y. enterocolitica* was found in the environment and in wild animals including fish, birds, small rodents, shrews and foxes (Kapperud, 1981). In Denmark, *Y. enterocolitica* O:3 was isolated from pigs, pork, dogs, sewage of private households, slaughterhouses, and drinking water (Christensen, 1987). In Bulgaria, O:3 was found in many wild animals (Nikolova et al., 2001). The World Health Organization (WHO) reported many possible origins of *Y. enterocolitica*, including domestic and wild animals, rodents, birds and freshwater fish (Mollaret et al., 1979). In Japan, one strain of *Y. enterocolitica* 4/O3 was isolated from swine formula feeds (Fukushima et al., 1984).

Hake fillet samples were found to harbor pathogenic *Y. enterocolitica* in Argentina (Velázquez et al., 1996). Oysters (13%), shrimp (4%), and crab (21%) were also found to harbor *Y. enterocolitica* (Peixotto et al., 1979).

Infection of *Y. enterocolitica* may be due to consumption of contaminated food or water, also rarely, person-to-person transmission (Aleksic and Bockemühl, 1999).

Y. enterocolitica O:3, O:5,27, O:8, and O:9 are the most common serotypes associated with human infections (Jay, 2000). Jay (2000) reported that O:8 (biovars 2 and 3) is the most commonly isolated pathogenic strain in the United States, but it should be noted that the isolation of O:3 has increased significantly and it has emerged as the predominant serotype in California (Bissett et al., 1990). Serotype O:3 was also predominant in Georgia (Metchock et al., 1991). Serovar O:3 (biovar 4) is the most common serovar in Europe (Nesbakken and Kapperud, 1985), Canada, Africa, and Japan

(Zen-Yoji et al., 1973). The second most common in Europe and Africa is serotype O:9 (Ahvonen, 1972; Jay, 2000) .

Swine is widely believed to constitute the single most common source of *Y. enterocolitica* for humans (Jay, 2000; Kapperud 1991; Nesbakken and Kapperud, 1985; Schiemann 1980a). Pigs and food products from pigs are highly associated with human infection with *Y. enterocolitica* O:3 and O:9 based on strong indirect evidence, but the linkage with O:8 is not clear (Kapperud, 1991). In Norway, a case-control study suggested a link between yersiniosis and consumption of raw or rare pork and untreated water (Ostroff et al., 1994). But, *Y. enterocolitica* serovars isolated from other species of animals, water and foods are normally different from ones associated with human infection in biochemical and serological traits (De Boer, 1992; Schiemann and Toma, 1978; Schiemann, 1979a).

It was reported that half of raw retail pork and 7% of processed pork contained *Y. enterocolitica* in Canada (Schiemann, 1980a). Isolates from raw pork were mostly from tongues. Vacuum packed meat under refrigeration can also be a source of *Y. enterocolitica* infection (Hanna et al., 1976). In Denmark, *Y. enterocolitica* O:3 was detected in minced pork and minced beef collected at 10 different retail butcher's shops (Andersen et al., 1991). Interestingly all positive beef samples were collected from butcher's shops where minced pork was found positive. This indicates possible cross contamination at the butcher's shops (Andersen et al., 1991).

Isolation of *Y. enterocolitica* from swine varies from herd to herd, and this may be attributed to the fact that it spreads on a pen basis (Andersen et al., 1991).

Besides intestinal inhabitation, serotype O:3 and O:9 also inhabit the oral cavity of swine, particulary tongue and tonsil (Bhaduri and Cottrell, 1997; Fredriksson-Ahomaa et al., 2000; Kapperud, 1991; Nesbakken and Kapperud, 1985). The incidence of *Y. enterocolitica* O:3 in throat flora of swine was investigated in Ontario, Canada (Schiemann, 1981). The strains were found in 20%, 50%, and 55% of tonsils, throats, and tongues, respectively. Human incidence with the same serotype comprised 81% among human isolates in eastern Canada. It is believed that the bacteria common in the throat of swine at the age of slaughter may be spread to pork products during slaughter and processing, similar to *salmonella* (Fredriksson-Ahomaa et al., 2001; Shiemann, 1989).

To reduce fecal contamination of swine carcasses, 'bagging technique' (enclosure of anus and rectum in a plastic bag during the slaughter of pigs) has been gradually introduced in Norway, and the incidence of pathogenic *Y. enterocolitica* in pork products has decreased (Johannessen et al., 2000).

Water has been also occasionally associated with yersiniosis for a small number of cases (Schiemann, 1989). Tofu associated yersiniosis in US was attributed to contaminated water during the manufacture (Tacket et al., 1985). Some *Y. enterocolitica* strains, including O:13 pathogenic strains were isolated from drinking-water systems such as wells and waterworks (Lassen, 1972). A few drinking water supplies such as wells and springs in Turkey were found to be contaminated with *Y. enterocolitica* (Gönül and Karapinar, 1991).

Milk is another source which has caused foodborne outbreaks linked to *Y. enterocolitica*. Raw milk has commonly been involved in *Y. enterocolitica* (Kushal and Anand, 2001; Schiemann and Toma, 1978; Schiemann, 1989). *Y. enterocolitica* from

pasteurized milk is likely to be from postpasteurization contamination (Ackers et al., 2000). Insanitary conditions and temperature abuse of raw milk allow for the presence of *Y. enterocolitica* and their multiplication (Schiemann and Toma, 1978). Yersiniosis among patients in one hospital was traced to contaminated pasteurized milk supplied to this hospital (Greenwood and Hooper, 1990). Most of raw milk samples (81%) were implicated in *Y. enterocolitica* in France (Vidon and Delmas, 1981).

Yersiniosis in the United States has been normally associated with consumption of foods such as milk and tofu and serotype O:8 has been more common (Ackers et al., 2000; Black et al., 1978; Morse et al., 1984; Tacket et al., 1985) than O:3 (Lee et al., 1990).

Methodology

The use of good and appropriate isolation methods is very important to isolate *Y. enterocolitica*, and the ability of *Y. enterocolitica* to grow on isolation media varies depending on the type of media(Head et al., 1982). Also, improper selection of media makes the detection of *Y. enterocolitica* difficult from clinical and environmental samples (Bercovier et al., 1984).

The use of enrichment before plating facilitates the successful isolation of *Y. enterocolitica* (De Boer, 1992; Jiang et al., 2000; Schiemann, 1979a; Schiemann, 1982), because foods normally have small numbers of *Y. enterocolitica* and large numbers of contaminating bacteria (Thippareddi et al., 1995; Toora et al., 1994b; Wauters et al., 1988). Furthermore, the growth of *Y. enterocolitica* is normally masked by background microflora (Aulisio et al., 1980; Escudero et al., 1995; Lee et al., 1980). The use of two-step enrichment can be beneficial because pre-enrichment can be less selective, thus

allowing for growth of small inocula of *Y. enterocolitica* and repair of injured cells. Subsequent selective enrichment can be highly selective because it has high inocula and healthy cells (Schiemann, 1982).

Isolation of *Y. enterocolitica* from foods is normally more difficult than from human feces because the number of *Y. enterocolitica* may be smaller, and the number of background flora is likely to be higher (Schiemann, 1982). Furthermore, antagonism by certain gram-negative bacteria in foods at high temperature makes the development of isolation methods more difficult (Schiemann, 1983). Serotype O:8 is known to be more difficult to recover from foods than other human serotypes (Lee et al., 1980).

During the 1970s, enrichment methods to recover *Y. enterocolitica* were primarily based on cold enrichment (Eiss, 1975) to take advantage of the psychrotrophic nature of *Y. enterocolitica* and to suppress the growth of background microflora (Bhaduri et al., 1997). But, there have been many efforts to shorten the incubation time since then, therefore selective and effective enrichment methods with shorter incubation time and higher incubation temperature have been developed (Bhaduri et al., 1997; Doyle and Hugdahl, 1983; Landgraf et al., 1993; Toora et al., 1994b; Wauters, 1973; Wauters et al., 1988). Because *Y. enterocolitica* varies in growth characteristics among serotypes, the methods for recovery have been mostly limited to specific serotypes or environmental strains (Toora et al., 1994b). The efficiency of *Y. enterocolitica* recovery also varies depending on foods being tested (Bhaduri and Cottrell, 1997).

Improved isolation methods and DNA hybridization methods with gene probes have indicated that the prevalence of pathogenic *Y. enterocolitica* in pork is higher than previously reported (Kapperud, 1991). For example, *Y. enterocolitica* O:3 was detected

from 60% of raw pork samples in Norway by using DNA-DNA hybridization assay while only 18% of samples by conventional procedures like irgasan, ticarcillin, and potassium chlorate (ITC) enrichment (Nesbakken et al., 1991) were positive.

Polymerase chain reaction (PCR) methods recovered slightly more pathogenic bioserotype 4/O:3 strains than did cultural methods, including tryptic soy broth (TSB) and modified rappaport broth (MRB) from pig tonsils in Finland (Fredriksson-Ahomaa et al., 2000). PCR gave higher positive samples (17%) harboring *Y. enterocolitica* O:3 than traditional culture method (2%), when compared to Phosphate-buffered saline with 1% Sorbitol and 0.15% Bile salts (PSB) and MRB, out of 300 pork samples. But the superiority of PCR methods is still uncertain (Johannessen et al., 2000). Pulsed-field gel electrophoresis (PFGE) is an efficient tool for subtyping isolates belonging to bioserotype 4/O:3 (Fredriksson-Ahomaa et al., 2000).

Compared to cultural methods, the advantage of PCR is in its increased sensitivity and higher specificity while the disadvantage is possible false positive or inability to differentiate between viable and non-viable bacteria (Lambertz et al., 1996).

The development of an effective method should be related to its ability to recover all pathogenic forms with equal reliability and within a reasonable time frame (Schiemann, 1983).

Plating media

The isolation medium, BABY4 developed by Bercovier et al. (1984) is intended for environmental samples such as water and vegetables (Table 1). It is designed to have high selectivity for *Y. enterocolitica* because it has potassium chlorate which inhibits the growth of a majority of *Enterobacteriaceae* with a type A nitrate reductase. Sorbose

added to the agar differentiates *Y. enterocolitica* from other sorbose-negative bacteria. This agar method isolated more *Y. enterocolitica* strains compared to Salmonella-shigella (SS) agar from stools and drinking water samples (Bercovier et al., 1984).

Cefsulodin-irgasan-novobiocin (CIN) agar was developed by Shiemann (Schiemann, 1979b) (Table 1). It is a highly selective medium based on colony morphology and mannitol fermentation, which makes it possible to differentiate *Y. enterocolitica* from most Gram-positive and Gram-negative bacteria. The average colony size measures 1.35 mm at 32°C and 24 hours (h), which is larger than colonies on MacConkey (MAC) agar. The colony appearance is distinctive. It has a deep red center with either entire or irregular border which is usually translucent. The deep red pigment is from mannose fermentation. This agar is especially inhibitory to *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* which are competitors in the isolation of *Y. enterocolitica* from both clinical and environmental samples. CIN agar showed higher recovery of *Y. enterocolitica* from feces than Salmonella-shigella (SS) and MAC agars for both direct plating and after cold enrichment (Schiemann, 1979b). CIN agar can significantly reduce the amount of work to isolate *Y. enterocolitica* with specific colony morphology and the highest isolation rate compared to SS, MAC, desoxycholate hydrogen sulphide lactose (DHL), bismuth sulfite, and cellobiose-arginine-lysine (CAL) agar (Schiemann, 1979b; Schiemann, 1983). But, the difference of colony morphology between pathogenic and non-pathogenic *Yersinia* spp. is small (Johannessen et al., 2000). Other minor drawbacks are that colonies of *Citrobacter freundii*, *Serratia liquefaciens*, and *Enterobacter agglomerans* and those of environmental *Yersinia* spp., especially

Yersinia intermedia may not be differentiated from those of *Y. enterocolitica* (Fukushima, 1987; Head et al., 1982).

MacConkey (MAC) agar supports the growth of all strains of *Y. enterocolitica* (Table 1), but drawbacks lie in a low selectivity and the absence of differential reaction (Lee, 1977a; Schiemann, 1979b). The use of MAC agar can be troublesome because foods have high amounts of microorganisms that will resemble *Y. enterocolitica* on MAC agar (Schiemann, 1979b). MAC agar had a higher recovery rate compared to SS, hektoen, Levine eosin methylene blue (EMB), and bismuth sulfite agars that are no longer commonly used to isolate *Y. enterocolitica* (Mehlman et al., 1978).

In another study for recovery rate of *Y. enterocolitica*, both MAC and CIN agars were better by 75 and 85%, respectively, compared to other media, including SS, pectin, and CAL agars (Head et al., 1982). When inoculated fecal materials were tested, the recovery rate of CIN agar was 100%, whereas the rates of CAL and SS agars were both 0% (Head et al., 1982). Thus, CIN agar was by far better than other plating media.

When the efficiency of CIN and MAC agars were compared for isolation from the swine tongues, MAC agar isolated more *Y. enterocolitica* strains from same samples than did CIN agar (Doyle and Hugdahl, 1983). However, CIN agar has an advantage in that the colonies of *Y. enterocolitica* are more easily identified because they have characteristic "bull's eye" characteristics (Doyle and Hugdahl, 1983; Fredriksson-Ahomaa et al., 2000).

Modified MAC agar with Tween 80 is used to differentiate *Y. enterocolitica* colonies from other lactose-negative colonies (Table 1). Tween 80-positive *Y. enterocolitica*

Table 1. Plating media to isolate *Y. enterocolitica*

Plating media	Incubation time and temperature	Reference
BABY4	48 h under anaerobic conditions, then 24 h under aerobic conditions, 35°C.	Bercovier et al., 1984
CIN[a]	24 h at 32°C or 48 h at 22°C	Schiemann, 1979
MAC[b]	48 h, 26°C	Mehlman et al., 1978
MAC[b] with Tween 80	48 h, 25°C	Lee, 1977b
SS[c]	Overnight at 37°C, then 24 h at 22°C 48 h, 25°C	Van Noyen et al., 1980 Eiss, 1975
SS-D[d]	20 h, 29°C	Wauters, 1973
SSDC[e]	24 h, 30°C	Wauters et al., 1988
CR-BHO[f]	18 to 24 h, 37°C	Bhaduri et al., 1997
VYE[g]	24 h, 37°C	Fukushima, 1987
KV202	24 h, 32°C	Jiang et al., 2000

[a] Cefsulodin-irgasan-novobiocin.

[b] MacConkey.

[c] Salmonella-shigella.

[d] Salmonella-shigella deoxycholate.

[e] Salmonella-shigella deoxycholate calcium.

[f] Low-calcium congo red – brain heart infusion agarose.

[g] Virulent *Yersinia enterocolitica*.

colonies are characterized by a surrounding zone of sheen. Even though some strains are negative, the agar recovered *Y. enterocolitica* better than did SS agar from meat and oysters (Lee, 1977b).

SS agar was compared to MAC agar for clinical specimens (Table 1)(Van Noyen et al., 1980). SS agar resulted in much higher efficiency of isolation of *Y. enterocolitica*, especially O:3 and O:9, compared to MAC agar. However, decreased isolation at 37°C has been reported (Chester and Stotzky, 1976).

Salmonella-shigella deoxycholate (SS-D) agar was developed by Wauters (1973) to isolate O:3 and O:9 (Table 1). It is modified from SS agar by adding 2% of sodium deoxycholate, since *Y. enterocolitica* is highly tolerant of that chemical. It had higher recovery of O:3 than did SS agar. The colony is granular and not iridescent. A short incubation time of 20 h was achieved (Wauters, 1973).

Salmonella-shigella deoxycholate calcium (SSDC) agar was used by Wauters et al. (1988) (Table 1). Calcium (0.1%) was added to original SS-D agar, and it enhanced the growth of O:3. The agar, however, was not appropriate for other pathogenic and nonpathogenic *Yersinia* strains (Wauters et al., 1988).

Low-calcium Congo red (CR) – brain heart infusion agarose (CR-BHO) was developed by Bhaduri et al. (1997) to isolate plasmid-bearing virulent *Y. enterocolitica* (Table 1). Its formulation is based on the principle that agarose contains less calcium than agar (Bhaduri et al., 1990). It produces red and "pinpoint" colonies because CR binding contributes to red color and low-calcium response contributes to the pinpoint colonies with 0.36 mm diameter at 37°C after 24 h. However, plasmidless strains (P-) produce large colonies, irrespective of calcium level. Its combination with MacConkey agar

permitted isolation of virulent *Y. enterocolitica*, whereas CIN agar did not (Berche and Carter, 1982; Bhaduri et al., 1997).

Virulent *Yersinia enterocolitica* (VYE) agar was developed to isolate virulent *Yersinia enterocolitica* from environmental or clinical samples (Fukushima, 1987) (Table 1). The isolates of virulent *Y. enterocolitica* are easily differentiated. They have red colonies as a result of mannitol fermentation and esculin nonhydrolysis, whereas most environmental *Yersinia* organisms (*Y. enterocolitica*, *Y. intermedia*, and *Y. frederiksenii*) and other gram negative microorganisms have pink or dark-red colonies or transparent colonies with a dark edge. This agar contains four antibiotics, which include cefsulodin, irgasan, josamycin, and oleandomycin (Fukushima, 1987). It is especially effective for *Y. enterocolitica* biotype 3B serotype O:3, which is inhibited on CIN agar (Fukushima and Gomyoda, 1986b). But, other studies showed that the use of josamycin and oleandomycin is unsatisfactory to inhibit irgasan-resistant Gram-negative bacteria (Toora et al., 1994a).

CAL and bismuth sulfite agars were not productive in recovery of *Y. enterocolitica* (Schiemann, 1983).

The performances of CIN and KV202 agar plates (Table 1) in isolating *Y. enterocolitica* from ground beef samples were compared (Jiang et al., 2000). More suspect colonies were found on CIN plates than KV202, but the number of positive colonies was similar between CIN (23%) and KV202 (20%). CIN agar had more false-positive colonies (Jiang et al., 2000). Compared to CIN plates, which can not differentiate *Salmonella*, *Serratia liquefaciens*, *Citrobacter freundii*, and *Enterobacter agglomerans*, KV202 plates easily differentiate *Serratia*, *Enterobacter*, and *Salmonella*. *Y. enterocolitica* has light yellow colonies without a black zone (Jiang et al., 2000).

Potassium hydroxide (KOH) treatment

Y. enterocolitica is generally more tolerant to potassium hydroxide (KOH) treatments than other Gram-negative bacteria (Aulisio et al., 1980; Fukushima, 1985; Schiemann, 1982). The treatment provides a simple, sensitive and rapid tool to recover *Y. enterocolitica* from mixed cultures (Aulisio et al., 1980).

The exposure of a 0.5 ml *Y. enterocolitica* culture to 4.5 ml of 0.5% KOH led to decreases of 2 to 3 logs, whereas 7 to 8 log decreases occurred for other *Enterobacteriaceae* such as *E. coli* and *Salmonella*. Two days of incubation at 26°C rather than 1 day, and 13 days of incubation at 4°C rather than 7 days, by enrichment in phosphate buffered saline (PBS) led to more effective KOH treatment (Aulisio et al., 1980). Regarding isolation of *Yersinia* from foods, 80% were recovered by alkali treatment, but only 20% were recovered by saline treatment (Aulisio et al., 1980). Direct KOH treatment was very effective in recovering *Y. enterocolitica* from fresh water (Fukushima, 1992). In another study, direct KOH treatment to pork, which was artificially contaminated from 10 to 10^4 cells/g, effectively recovered all strains tested from 10^2 cells/g, whereas KOH post-enrichment treatment after PBS was ineffective (Fukushima, 1985). KOH treatment of 0.5% in 0.5% NaCl resulted in better selectivity compared to only 0.5% NaCl treatment to isolate *Y. enterocolitica* from milk (Kushal and Anand, 2001).

The effects of different KOH treatments on *Y. enterocolitica* were evaluated. *Y. enterocolitica* was more sensitive to 0.5% KOH for 15 seconds (s) than 0.35% KOH for 30 s and 0.25% KOH for 2 minutes (min) treatment (Doyle and Hugdahl, 1983).

Enrichment methods

Cold enrichment has been used frequently by many researchers to recover *Y. enterocolitica* from foods or swine by the use of 4°C temperature and several weeks of incubation (Andersen et al., 1991; Doyle et al., 1981; Eiss, 1975; Funk et al., 1998; Jiang et al., 2000; Schiemann, 1983; Van Noyen, 1980). From a clinical standpoint, however, cold enrichment for extended incubation was not effective to recover human pathogenic serotype (Hoorfar and Holmvig, 1999; Van Noyen et al., 1980). In another study, however, this method increased isolation of O:3 and O:5 compared to rappaport broth for 48 h at 25°C from stool specimens (Eiss, 1975).

Phosphate buffered saline (PBS) has been most commonly used for cold enrichment (Funk et al., 1998; Schiemann, 1983), and its effect was maximal after three weeks of incubation (Van Pee and Stragier, 1979).

PBS at 4°C was used to isolate *Y. enterocolitica* from cecal materials of swine (Letellier et al., 1999) (Table 2). It recovered various serotypes including O:3, O:5 and O:9.

Cold enrichment using PBS for 21 days at 4°C followed by 0.5% KOH post-enrichment treatment was successful for isolating virulent *Y. enterocolitica* from porcine tongues (Doyle et al., 1981) (Table 2). But, modified rappaport broth (MRB) and modified selenite enrichment broth (MS) were not effective.

PBS at 25 °C – KOH post-enrichment method was found to be effective as a 14 to 21 day cold enrichment method to recover *Y. enterocolitica* from meats and swine tongue (Doyle, 1983) (Table 2). KOH treatment was effective to screen out other background

microflora. This method had shortened incubation times of 1 to 3 days. The inoculum ranging from 1.8 to 12 cells/g meat was great enough for this method to recover *Y. enterocolitica* with the aerobic plate count from 4.8×10^5 to 7.1×10^7 CFU/g of meat.

PBS with 1% sorbitol and 0.15% bile salts (PSB) was found to be 10-fold more sensitive than only PBS to recover *Y. enterocolitica* from pork. Recovery was obtained with the inoculum level of 1000 CFU/ml (Mehlman et al., 1978) (Table 2). *Yersinia* strains tolerate 0.15% bile salts at 4°C (Mehlman et al., 1978). This method was also used to isolate *Y. enterocolitica* from ground beef and pork samples (Jiang et al., 2000) and there was no statistical difference between 4°C/3 week, 4°C/2 week, and 25°C/24 h. However, long incubation times and high number of background microorganisms are disadvantagious in this PSB enrichment (Hoorfar and Holmvig, 1999). PSB in combination with 0.5% KOH post-enrichment treatment was used to isolate *Y. enterocolitica* from hake fillets (Velázquez et al., 1996).

Phosphate buffer M/15, pH 7.6 is not as useful when the nutrients of foods are not provided, such as swabs (Feeley and Schiemann, 1984). Rather than phosphate buffer M/15, nutritional medium such as TSB or brain heart infusion (BHI) broth for cold enrichment was recommended due to their better enrichment effect (Van Pee and Stragier, 1979). Several researches have indicated that more nutrient media than inorganic buffers can reduce the time for cold enrichment (Feeley and Schiemann, 1984; Schiemann, 1982).

Modified rappaport broth (MRB) was proposed by Wauters (1973) (Table 2). This enrichment method can be applied only to recover serotypes O:3 and O:9. Certain

Table 2. Enrichment media to isolate *Y. enterocolitica*

Enrichment	Incubation time and temperature	Plating media	Reference
PBS	6 weeks, 4°C	CIN	Letellier et al., 1999
PBS – KOH post-enrichment treatment	21 days, 4°C	MAC	Doyle et al., 1981
PBS (pH 7.2) – KOH post-enrichment treatment	1 to 3 days, 25°C	CIN, MAC	Doyle and Hugdahl, 1983
PBS with 1% sorbitol and 0.15% bile salts (PSB)	4 to 21 days, 4°C	MAC, 48 h, 26°C	Mehlman et al., 1978
MRB	2 days, 23°C	MAC with Twin 80; SS	Schiemann, 1979a; Van Noyen et al., 1980
MRB	7 days, 22°C	MAC	Schiemann, 1980a
Phosphate buffer (PB) as pre-enrichment and MRB as selective enrichment	PB : 14 days, 4°C MRB : 5 days, 23°C	MAC with Tween 80	Schiemann and Toma, 1978
Cooked meat media - MRB	Cooked meat: 14 days, 4°C MRB: 2 days, 23°C	MAC with Twin 80	Schiemann, 1979a
Modified selenite media	2 and 3 days, 22°C	MAC with Twin 80	Lee et al., 1980

Table 2. Continued.

Enrichment	Incubation time and temperature	Plating media	Reference
YER – BOS	YER: 3 days, 10°C BOS: 3-5 days, 22°C	CIN	Schiemann, 1982
TSB - BOS	TSB: 7 days, 2°C BOS: 5 days, 22°C	CIN	Schiemann, 1983
ITC enrichment	2 or 3 days, 24°C	SSDC at 30°C,24 h	Wauters et al., 1988
TSB plus Polymyxin B and Novobiocin	3 days, 18°C	CIN MAC	Landgraf et al., 1993
TSB including yeast extract and bile salts as first enrichment, then the addition of irgasan for second enrichment	3 days, 10°C – first enrichment 3 days, 22°C – second enrichment	CIN	Toora et al., 1994b
TSB including yeast extract and bile salts as pre-enrichment, and the addition of irgasan for selective enrichment	24 h, 12°C as pre-enrichment, then 48 h, 12°C	MAC, then CR-BHO	Bhaduri et al., 1997
TSB containing bile salts, then another 24 h after the addition of irgasan	24 h, 12°C, then 24 h, 12°C after the addition of irgasan	CR - BHO	Bhaduri and Cottrell, 1997
Luria-Bertani-Bile Salts Irgasan (LB-BSI) broth	24 h, 12°C and re-incubate at 12°C for 48 h after the addition of irgasan	CIN	Hussein et al., 2001

serotypes, such as O:8, which is common in North America, and O:5, 27, can not be recovered by this method (Schiemann, 1983). This method was modified by the addition of more magnesium chloride and less malachite green than original rappaport broth (Wauters, 1973). This medium showed better recovery of O:3 from ground beef and pork (Schiemann, 1979a) (Table 2) than other methods, such as PBS with 1.0% mannitol (Christenson and Jansen, 1977), cooked meat broth and butterfield's phosphate buffer. This medium was effective enough to recover 35 cells. MRB recovered O:3 and O:9 successfully from clinical specimen in combination with SS agar (Van Noyen et al., 1980).

MRB, when incubated for 7 days at 22°C, gave higher percentages of *Y. enterocolitica* isolation from raw pork than two-step enrichment with PBS at 4°C for 21 days followed by MRB at room temperature for 2 days (Schiemann, 1980a) (Table 2). But, in another study, MRB enrichment alone was inferior to two-step enrichments of PSB at 4°C for 14 days and subsequent MRB at 22°C for 4 days to isolate *Y. enterocolitica* from pork tongues (Schiemann, 1982).

Phosphate buffer – MRB showed increased isolation of *Y. enterocolitica* from raw milk samples (Schiemann and Toma, 1978) (Table 2). The isolation rate of this method was much higher than rappaport broth, phosphate buffer, and cooked meat medium.

Three methods (direct plating, PSB-MRB, and cold enrichment in PSB for three weeks) were compared to isolate *Y. enterocolitica* from pig tonsils (Nesbakken and Kapperud, 1985). Cold enrichment in PSB was superior to other methods.

A two-step enrichment procedure with cooked meat medium at 4°C for 14 days and subsequent MRB at 23°C for 2 days (Table 2) showed higher recovery than other methods, including buffer methods or only cooked meat for isolation of *Y. enterocolitica* from raw milk (Schiemann, 1979a).

Two modified selenite media, sel (lower selenite concentration) and SEL (higher selenite concentration), were developed by Lee et al. (1980) (Table 2). They include phosphate buffer with Na_2SeO_3 and carbenicillin. In the recovery of O:9 from pork, the SEL medium was more effective at an inoculum level of 200 CFU/g than MRB by Wauter (1973). Serotype O:8 was also recovered from beef and pork in selenite media, but not in MRB. PBS – KOH treatment was inferior to either selenite media or selenite – KOH at 200 CFU/g inoculum. KOH treatment resulted in more pure *Y. enterocolitica* culture on the plates (Lee et al., 1980).

Another two-step enrichment method of yeast extract – rose bengal (YER) enrichment at 10 °C for 3 days as pre-enrichment and bile-oxalate-sorbose (BOS) enrichment at 22°C for 3 to 5 days as the selective second enrichment was developed by Schiemann (1982) (Table 2). The recovery of *Y. enterocolitica* from vegetables-chicken baby food by these enrichments was better than by PSB at 4°C for 14 days –MRB at 22°C for 3 days. But, YER-BOS method is not effective for serotype O:5,27, compared to other pathogenic serotypes (Schiemann, 1982; Schiemann, 1983). A PSB-BOS procedure was also effective, but it takes more incubation time up to 19 days. Serotype O:8 was recovered by the YER-BOS method at an inoculum level of 80 to 90 CFU/g food. Increasing incubation time of BOS to 5 days was more effective. The use of YER at

4°C for 9 days was also effective for pork tongues (Schiemann, 1982). Even though various strains can be recovered, this method is not commonly used because of its complexity (Toora et al., 1994b).

TSB (2°C for 7 days as a pre-enrichment) with BOS (22°C for 5 days as a selective enrichment) (Table 2) was effective for recovery of serotype O:3, and the same enrichment except for 3 day incubation for BOS instead of 5 day was effective for recovery of O:8 from inoculated beef stew (Schiemann, 1983).

Irgasan-ticarcillin-potassium chlorate (ITC) enrichment derived from the MRB was developed by Wauters et al. (1988) (Table 2). Irgasan, ticarcillin, and potassium chlorate contribute to the selectivity of the medium (Wauters et al., 1988). But, this method may be efficient only for O:3 (Wauters et al., 1988). Recovery of O:9 from minced pork by ITC enrichment was unsatisfactory (De Zutter et al., 1994). Serotype O:9 is much more sensitive to potassium chlorate than serotype O:3. The addition of $MgCl_2$ and malachite green also inhibits the growth of O:9, compared to serotype O:3 which is unaffected (De Zutter et al., 1994).

ITC enrichment for 2 or 3 days at 24°C followed by plating on SSDC agar gave increased recovery of *Y. enterocolitica* O:3 from pork tongues compared to two-step and cold enrichments. But, it was less effective for nonpathogenic strains from ground pork. The use of SSDC agar for ITC enrichment was better for recovery than the use of CIN agar (Wauters et al., 1988). Harshness of irgasan present in both ITC enrichment and CIN agar may be the reason for ineffective recovery on CIN plates (Hoorfar and Holmvig, 1999).

In another study, the ITC enrichment also gave much higher recovery of O:3 strains from swine tonsils and mesenteric nodes than other methods containing ITC enrichment for 5 days, KOH-direct plating, and cold enrichment (De Giusti et al., 1995). The addition of supernatant of filtrate of a pork homogenate to ITC enrichment was found to be more effective for isolating O:3 strains than the direct addition of meat. Also, the addition of 10 g pork samples to 100 ml ITC enrichment resulted in higher isolation of *Y. enterocolitica* O:3 than the addition of 1 g (De Zutter et al., 1995). ITC enrichment was superior to PSB and YER – BOS enrichments (De Zutter et al., 1994). ITC enrichment is also well known in the isolation of *Y. enterocolitica* from swine faecal samples (Hoorfar and Holmvig, 1999). In a study to isolate *Y. enterocolitica* from swine faecal samples, PSB, PSB – MRB, and PSB - ITC enrichments were compared. PSB - ITC enrichment gave the highest proportion of positive samples (24%), while PSB resulted in lower recovery rate. Shorter incubation time (2 days) of ITC enrichment gave higher recovery than longer incubation time (10 and 24 days) (Hoorfar and Holmvig, 1999). However, the recovery of all pathogenic serotypes is still impossible by either YER-BOS or ITC enrichment (Landgraf et al., 1993).

TSB plus polymyxin (5 IU/ml) and novobiocin (10 μg/ml) (TSPN) was proposed by Landgraf et al. (1993) in the isolation of *Y. enterocolitica* from raw milk (Table 2). On the use of TSPN, *Y. enterocolitica* O:3, O:5, O:8, and O:9 were recovered even at 10 CFU/ml milk from raw milk samples. This method was better than sorbitol bile salts (SB) broth. The TSPN method also gave higher recovery for pasteurized milk.

The enrichment developed by Toora et al. (1994b) is a modified TSB including 0.25% yeast extract and 0.2% bile salts incubated at 10°C for 3 days and then, the

addition of irgasan (4 μg/ml) followed by another 3 days of incubation at 22°C (Table 2). Non-pathogenic *Y. enterocolitica* biotype 1A was isolated from ready-to-eat foods such as pasteurized chocolate milk, salad, and sandwich, and pathogenic O:3 from pork samples by this method, whereas none were isolated by cold enrichment of TSB with yeast extract for 14 days (Toora et al., 1994b).

TSB containing yeast extract, bile salts, and irgasan with the use of CR-BHO as plating media was developed by Bhaduri et al. (1997). TSB containing yeast extract and bile salts was incubated at 12°C for 24 h, then it was followed by another 48 h incubation at 12°C after the addition of irgasan (4 μg/ml) (Bhaduri et al., 1997) (Table 2). This temperature is used as a compromise between the suppression of other background microflora by using the low temperature and the allowance for rapid growth of *Y. enterocolitica* compared to 4°C (Hussein et al., 2001). Presumptive *Y. enterocolitica* colonies were picked from MAC agars and then transferred on CR-BHO. Presumptive virulent *Y. enterocolitica* colonies were identified on CR-BHO. Plasmid-bearing virulent *Y. enterocolitica* was recovered in this enrichment at the inoculum level of 9 CFU/g pork samples. Eleven virulent O:3 strains were isolated from 30 naturally contaminated porcine tongues. The advantage of this method lies in recovering plasmid-bearing virulent *Y. enterocolitica* effectively from pork samples (Bhaduri et al., 1997).

The previous method was modified to shorten the procedure and time in the isolation of *Y. enterocolitica* from food surfaces (Bhaduri and Cottrell, 1997). TSB containing only bile salts was incubated at 12°C for 24 h. It was followed by another 24 h incubation after the addition of irgasan (Table 2). It eliminated presumptive colony

isolation step on MAC or CIN agars to prevent virulent plasmid loss. Virulent *Y. enterocolitica* colonies were easily differentiated on CR-BHO. They can be recovered at an initial inoculum level of 10 and 1 CFU/cm^2 on food surfaces such as pork chops, ground pork, cheese, and zucchini (Bhaduri and Cottrell, 1997).

Luria-Bertani-Bile Salts Irgasan (LB-BSI) broth is based on the use of cefsulodin, novobiocin, and irgasan with incubation temperature at 12°C (Hussein et al., 2001) (Table 2). LB-BSI significantly increased the recovery of *Y. enterocolitica* from oral and rectal swabs of pigs compared with cold enrichment in M/15 PBS and direct plating.

Selective agents

Y. enterocolitica shows high tolerance to surface active agents such as bile salts, sodium desoxycholate, sodium taurocholate, Teepol 610, and Tergitol 7 (Schiemann, 1980b). Irgasan (2, 4, 4′-trichloro-2-hydroxydiphenyl ether), also known as Triclosan is very commonly used to selectively recover *Y. enterocolitica* from heavy background microorganisms (Fukushima, 1987; Schiemann, 1982; Wauters et al., 1988) and its selectivity was confirmed by Toora et al. (1994a). Irgasan is tolerated by *Y. enterocolitica* at the concentration which is inhibitory to some other members of *Enterobacteriaceae* like *E. coli*(Schiemann, 1980b). It is sometimes added after pre-enrichment because it can inhibit growth (Bhaduri et al., 1997). *Y. enterocolitica* is also relatively resistant to carbenicillin, novobiocin, and cefsulodin (Schiemann, 1980b). It is also highly tolerant to magnesium chloride. Selenite, the ingredient in selenite broth is tolerated at 1.0% (Schiemann, 1980b). Calcium chloride enhances the growth of *Y. enterocolitica* (Bhaduri et al., 1990; Toora et al., 1994a).

Each selective agent or its combinations of cefsulodin, irgasan, and novobiocin affects the growth of *Y. enterocolitica* O:3 differently (Logue et al., 2000). The lag phase duration for growth of *Y. enterocolitica* O:3 was generally longer in the presence of cefsulodin, irgasan, or novobiocin in *Yersinia*-selective broth base (CIN NA). Also, the lag phase of plasmid-bearing O:3 (P+) was longer than plasmidless O:3 (P-) in CIN NA with both cefsulodin and novobiocin, but vice versa in CIN NA with irgasan or full supplements. When the growth rates were compared at 37°C, P- grew significantly faster than P+ in CIN NA with most combinations of cefsulodin, irgasan, and novobiocin except that P+ grew faster than P- in CIN NA with CIN supplement (Logue et al., 2000). Irgasan has been normally added in the second step during two-step enrichments (Bhaduri et al., 1997; Schiemann, 1982; Toora et al., 1994b), but interestingly the addition of cefsulodin, irgasan, or novobiocin to enrichment was found to increase the growth rate of *Y. enterocolitica* (Logue et al., 2000).

Green fluorescent protein

Green fluorescent proteins (GFPs) are from a Pacific Northwest jellyfish, *Aequorea victoria*. *Aequorea* GFP is a protein of 238 amino acid residues. It has received attention because it was the first general method to create strong visible fluorescence, which emits green fluorescence in that excitation is at 395 nm with an emission maximum at 508 nm (Cubitt et al., 1995).

It has been used as a reporter of gene expression, tracers of cell lineage or a protein tag or to monitor protein-protein interactions (Cubitt et al., 1995). GFP was also first used to visualize dynamic cellular events. Another advantage of GFP is that no factors or

substrates for the generation of its green light are required and no considerable toxicity of GFP was observed. High throughput screens for GFP become possible by simple visual inspection (Misteli and Spector, 1997). Chromophore formation is not species-specific.

On expression of GFP in *E. coli*, fluorescent bacteria were detected on plates containing the inducer isopropyl-β-D-thiogalactoside (IPTG), upon illumination with a longwave UV source (Chalfie et al., 1994).

Transformed microorganisms with GFP plasmid have been used to evaluate antimicrobials (Ajjarapu and Shelef, 1999), the infiltration of bacteria to food matrix (Burnett et al., 2000; Pao et al., 2001), or spatial location of them on foods (Gandhi et al., 2001). However, transformation of *Y. enterocolitica* with GFP plasmid has not been performed for these purposes. This is a useful method which should be given increased use in the food microbiology laboratory.

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Part II:

Evaluation of enrichment methods for recovery of *Yersinia enterocolitica* O:3 and O:8 from swine feeds

Abstract

Swine are major reservoirs of *Yersinia enterocolitica* and pigs are easily colonized through oral infections. The objective of this study was to evaluate five different enrichment methods to recover *Y. enterocolitica* from swine feeds since optimal methods for isolation of *Y. enterocolitica* are not available for farm samples having a heavy background microflora. Recovery of *Y. enterocolitica* from swine feeds using enrichment was evaluated with an inoculum level of 1000, 100, and 10 CFU/g feed. Enrichment media evaluated included: Phosphate Buffered Saline (PBS) at 4°C, PBS with sorbitol (1%) and bile salts (0.15%) (PSB) at 4°C and 21°C, ITC enrichment at 22°C and tryptic soy broth with polymyxin B(5 IU/ml) and novobiocin (10 μg/ml) (TSBPN) at 18°C. Incubation periods for enrichment were evaluated from 24 h up to 6 weeks. CIN, MacConkey (MAC) and SSDC agar plates were used for differential plating. KOH treatments at concentrations of 0.25% to 0.13% were evaluated. PBS at 4°C and PSB at 4°C or 21°C were not effective for recovery of *Y. enterocolitica* O:3 and O:8 from swine feeds. KOH treatment did not generally improve recovery of *Y. enterocolitica* in the methods studied except for recovery of O:8 from MAC agars after two day enrichment in TSBPN. TSBPN at 18°C with 24-h incubation was better than any other methods evaluated for recovery of both O:3 and O:8, which showed good recovery up to 10 and 1000 CFU/g feed for O:3 (89%) and O:8 (100%), respectively. ITC enrichment gave good recovery (56%) of serotype O:3 up to 10 CFU/g feed, but was not effective for recovery of O:8. CIN agar plates were preferred in this study due to the distinctive colony morphology and high selectivity. Overall, optimal recovery of *Y. enterocolitica* from

swine feeds, based on these data, was achieved using TSBPN enrichment at 18°C with incubation for 24 h followed by differential plating on CIN agar at 30°C for 24 h.

Introduction

Foodborne illness caused by *Yersinia enterocolitica* is estimated at 96,000 cases in the United States annually (Mead et al., 1999). It is characterized as a psychrotrophic bacterium (Jay, 2000). *Y. enterocolitica* has been isolated from a wide range of foods such as raw milk, pork, chicken, oysters, shrimp, and crab (De Boer et al., 1982; De Boer and Nouws, 1991; Peixotto et al., 1979; Schiemann and Toma, 1978). Swine is known as a major source of pathogenic *Y. enterocolitica* in human, while the majority of environmental *Y. enterocolitica* isolates are nonpathogenic (Bottone, 1997; Jay, 2000).

Monitoring the environment of swine farms should be important to find out the sources for *Y. enterocolitica* infection of swine. These data are necessary to begin control programs for the foodborne pathogen on the farm with the goal of eventually safeguarding pork products from being contaminated by the pathogen.

Swine feeds may be a source of infection to swine because swine are normally orally infected and swine tongue is a major source of *Y. enterocolitica* (Doyle and Hugdahl, 1983).

Since foods have normally small number of *Y. enterocolitica* and large number of contaminating bacteria (Thippareddi et al., 1995) and the growth of *Y. enterocolitica* is normally masked by background microflora (Aulisio et al., 1980), the development of efficient enrichment methods has been difficult. Several isolation methods have been produced, however, no simple, effective and single method has still been found in recovery of a wide range of *Y. enterocolitica* from swine feeds.

In this study, five different enrichment methods and three different agar plates were evaluated in the isolation of *Y. enterocolitica* O:3 and O:8, which are most prevalent in Europe, Canada, or the United States from swine feeds.

Materials and Methods

***Yersinia enterocolitica* culture**

Two *Y. enterocolitica* strains were used in this study. *Y. enterocolitica* serotype O:8, ATCC 9610 was obtained from American Type Culture Collection (Manassas, VA, USA). *Y. enterocolitica* GER-C, serotype O:3 P+ was supplied by Dr. S. Bhaduri (USDA, ARS, ERRC, Philadelphia, PA, USA). The stock cultures were subcultured onto brain heart infusion (BHI) agar slants and maintained in the refrigerator at 4°C.

Swine feed samples

Swine feed samples, grower finisher feeds freshly made were collected from either the Blount unit (Manager : Danny Sutton) at Knoxville station, Tennessee Agricultural Experimental Station or JARTU (Johnson Animal Research & Teaching Unit) at Knoxville, TN. Upon arrival, the feed samples were refrigerated. The grower finisher feeds were composed of corn (72.2%), soybean meal (SBM) (19.9%), 540 base mix (2.5%), 95555 lean pak (lysine) (5.0%), medication aprolon apromic (0.2%), and tylan 10 (0.2%). In this study, no antibiotics were added to the feeds sampled.

Preparation of inocula and inoculation of samples

One loopful of *Y. enterocolitica* cultures, either serotype O:3 or O:8 on BHI agar slants was inoculated into 10 ml BHI broth, and incubated at 21°C for 21 to 24 h to provide a cell density of 10^8 CFU/ml. Afterwards, ten fold serial dilutions were made in

0.85% saline subsequently to make an inoculum of 10000 CFU/ml, 1000 CFU/ml or 100 CFU/ml saline. One ml of the inoculum, either O:3 or O:8, was spiked to 10 g swine feed samples, which resulted in the inoculum level of 1000 CFU/g feed, 100 CFU/g feed, or 10 CFU/g feed, respectively. For 25 g feed samples, 2.5 ml of the inoculum saline was spiked to maintain same conditions.

In the first study, five different enrichment procedures were evaluated with inoculum level of 1000 CFU/g feed on each serotype O:3 and O:8. Then, only enrichment procedures which showed good recovery were tested for reduced inoculum level of 100 CFU/g feed. Similarly, only procedures giving good recovery at higher inoculation were evaluated at 10 CFU/g feed. During this research, three feed samples in triplicate were used for each treatment.

Enrichments

Phosphate buffered saline (PBS) (0.119 mol/l) (Letellier et al., 1999) (Fisher scientific, Fair Lawn, NJ 07410), PBS with 1.0% sorbitol and 0.15% bile salts (PSB) (Nesbakken et al., 1991), ITC enrichment (Wauters et al., 1988), and TSB (BBL, Cockeysville, MD 21030) plus Polymyxin B Sulfate (5 IU/ml) (Sigma, St. Louis, MO 63178) and Novobiocin (10 μg/ml) (Sigma, St. Louis, MO 63178) (TSBPN) (Landgraf et al., 1993) were used in this study. Reasonable modifications were tried for better recovery. Each five different enrichment method, based on mentioned enrichments, was spiked with specific inoculum of *Y. enterocolitica*, ranging from 1000 CFU/g feed to 10 CFU/g feed of either O:3 or O:8 serotype and compared for recovery. Whirl-pak plastic bags (Nasco) fitted with nylon mesh filters were used as enrichment containers. Five different enrichment procedures are outlined in Table 3. For methods 2 and 5, two

Table 3. Experimental enrichment and isolation methods for *Y. enterocolitica*

No.	Enrichment	Plates	Confirmation
1	10 g sample is put in 90 ml PBS (0.119 mol/l) - pH 7.6. Incubate at 4°C up to 6 weeks	CIN agar [a]	TSI agar, Sucrose utilization, Rhamnose utilization, Urease, Simmons citrate, ONPG, Oxidase
2	(1) One-step: 10 g sample is put in 90 ml ITC enrichment. Incubate up to 10 days at 22°C. (2) Two-step: Incubate pre-ITC enrichment[c] for 15 h. Transfer 1 ml to ITC enrichment and reincubate.	CIN agar [a] SSDC agar at 21°C for 24 h	
3	25 g sample is put in 225 ml PBS with 1.0% sorbitol and 0.15% bile salts (PSB). Incubate at room temperature (21±1°C).	CIN agar [a]	
4	25 g sample is put in 225ml PSB. Incubate at 4°C up to 6 weeks.	CIN agar [a] MAC agar [b]	
5	(1) One-step: 10 g sample is put in 90 ml TSB plus Polymyxin B Sulfate(5 IU/ml) and Novobiocin(10 μg/ml) – TSBPN. Incubate at 18°C up to 4 days. (2) Two-step: Incubate TSB for 15 h. Transfer 1 ml to TSBPN and reincubate.	CIN agar [a] MAC agar [b]	

[a] Incubation at 30 to 32°C for 24 h.

[b] Incubation at 35 ± 2°C for 20 to 24 h.

[c] ITC enrichment omitting irgasan, ticarcillin, and potassium chlorate.

different enrichment procedures, one-step and two-steps were conducted based on the advice of Dr. S. Bhaduri (Personal communication) to prevent selective ingredients from inhibiting the onset of growth of *Y. enterocolitica* in enrichments. One positive and one negative control were accompanied in most case. Positive control included both enrichment and inoculum without feed samples, and negative control included both feed samples and enrichment without inoculum.

KOH post-enrichment treatment

KOH post-enrichment treatments were done in some experiments to compare its effectiveness for better recovery. Potassium hydroxide (KOH) pellets were dissolved in 0.5 or 0.85% NaCl. The treatments included 0.5 ml enrichment broth in 0.25% KOH of 4.5 ml for 20 and 30 s, 0.5 ml enrichment in 0.13% KOH of 4.5 ml for 30 s, and 0.5 ml enrichment in 0.5% KOH of 0.5 ml for 30 s.

Plating media

After incubation of enrichment, each loopful of enrichment was streaked onto each plate to isolate *Y. enterocolitica* colonies, then the plates were incubated.

The following plating media were used in this study.

(1) CIN agar : *Yersinia* selective agar base (Difco, Sparks, MD 21152) with *Yersinia* antimicrobic supplement CN (Difco, Sparks, MD 21152).

(2) MAC agar (Difco, Sparks, MD 21152).

(3) SSDC agar : Salmonella-shigella agar (BBL, Cockeysville, MD 21030) with 1% sodium deoxycholate (Acros organics, NJ) and 0.1% calcium chloride (Fisher scientific, Fair lawn, NJ 07410).

(4) Modified CIN agar : CIN with Polymyxin B Sulfate (5 IU/ml).

Confirmation of presumptive colonies

Biochemical tests were done to confirm presumptive colonies of *Y. enterocolitica*. Typical colonies on plates were inoculated onto triple sugar iron (TSI) media (BBL, Cockeysville, MD 21030), sucrose and rhamnose purple broth agar (purple broth with 0.8% sugar and 1.5% agar) to confirm *Y. enterocolitica* colonies. For colonies which showed typical biochemical reactions of *Y. enterocolitica*, additional tests were conducted such as urease, simmons citrate, o-Nitrophenyl-beta-D-galactopyranoside (ONPG), and oxidase tests. Typical biochemical reactions of *Y. enterocolitica* serotype O:8, ATCC 9610 and *Y. enterocolitica* GER-C, serotype O:3 are listed in Table 4.

Statistical analysis

A chi-square analysis (alpha = 0.05) was used to find differences in recovery or isolation rate of *Y. enterocolitica* over treatment effects such as enrichments, plates, and potassium hydroxide. The presence or absence of *Y. enterocolitica* colonies on each plate was calculated as categorical data. SAS Proc FREQ (SAS 8.2, Cary, NC 27513) was used. The only 2×2 contingency table was used for the relationship between 2 variables throughout the study (Kachigan, 1986).

The chi-square (χ^2) statistic was calculated to assess how much the observed distribution of frequencies in the cells in the contingency table differs from that which would be expected (Orr, 1995).

$$\chi^2 \text{ statistic} = \Sigma\, (f - F)^2/F, \text{ taken over all cells.}$$

f is the observed frequency in the cell, and F is the expected frequency in the cell.

Table 4. Biochemical characteristics of *Y. enterocolitica* serotype O:8, ATCC 9610 and *Y. enterocolitica* GER-C, serotype O:3

Tests	Reactions
TSI	A/A, no gas
Sucrose	+
Rhamnose	-
Urease	+
Simmons citrate	-
ONPG	+
Oxidase	-

The value of $\chi^2 = 3.841$ for degrees of freedom ($df = 1$) and for significance level ($P = 0.05$) was used to reject the null hypothesis (Kachigan, 1986). When the data set was not large enough for chi-square tests, the Fisher's exact test was performed.

Results

Inoculum level of 1000 CFU/g feed

Five different enrichment procedures were compared at this inoculum level for each serotype O:3 and O:8 in separate enrichments.

Method 1, 10 g sample and 90 ml PBS at 4°C did not recover *Y. enterocolitica* O:3 and O:8 (Table 5). Except sample 3 inoculated with serotype O:8 on day 35, no *Y. enterocolitica* was recovered in up to 42 days. Also, KOH post-enrichment treatment was added before plating to reduce background microflora on the plates based on alkali resistant characteristics of *Y. enterocolitica*, but it was not successful (Table 5).

ITC enrichment method as method 2 was successful in recovery of serotype O:3 (Table 6). All samples were positive for the detection of *Y. enterocolitica* up to day 3, and day 1 resulted in significantly higher recovery rate than day 3 ($\chi^2_1 = 18.0000$, $P < 0.05$). KOH post-enrichment treatment did not improve recovery. Furthermore, KOH had a negative effect on recovery ($\chi^2_1 = 7.1111$, $P < 0.05$). No difference was found in isolation of *Y. enterocolitica* between CIN and SSDC plates up to day 3 ($\chi^2_1 = 0.5000$, $P > 0.40$). There was a large difference in recovery efficiency of ITC enrichment between O:3 and O:8. Serotype O:8 did not have good recovery in ITC enrichment (Table 7). KOH treatment was ineffective also. *Y. enterocolitica* O:8 was recovered from only one sample

Table 5. Recovery of *Y. enterocolitica* O:3 and O:8, 1000 CFU/g feed from swine feeds using 10 g sample and 90 ml PBS at 4°C with KOH[a] and without KOH post-enrichment treatment followed by plating on CIN agar

Sample	Recovery (%)[b]									
	Day 7	Day 14		Day 21		Day 28		Day 35		Day 42
	O:8	O:3[c]	O:8[c]	O:3	O:8	O:3	O:8	O:3	O:8	O:8
# 1	0	0	0	0	0	0	0	0	0	0
# 2	0	0	0	0	0	0	0	0	0	0
# 3	0	0	0	0	0	0	0	0	33	0
+ control	N/A	0	N/A	0	N/A	0	N/A	0	N/A	N/A
- control	N/A	0	N/A	0	N/A	0	N/A	0	N/A	N/A

[a] No *Y. enterocolitica* O:3 was detected from day 14 to 35 from 0.5 ml enrichment gently mixed in 0.13% KOH of 4.5 ml for 30 s.

No *Y. enterocolitica* O:8 was detected from day 7 to 42 from 0.5 ml enrichment gently mixed in 0.25% KOH of 4.5 ml for 20 s.

[b] No KOH treatment applied.

[c] Recoveries of O:3 and O:8 are separate experiments.

Table 6. Recovery of *Y. enterocolitica* O:3, 1000 CFU/g feed from swine feeds using 10 g sample and 90 ml ITC enrichment at 22°C and KOH post-enrichment treatment

Sample	Recovery (%)								
	Day 1			Day 3			Day 5		
	CIN		SSDC	CIN		SSDC	CIN		SSDC
	No KOH	KOH[a]	No KOH	No KOH	KOH[a]	No KOH	No KOH	KOH[a]	No KOH
# 1	100	33	100	67	100	33	0	0	33
# 2	100	0	100	33	0	0	0	0	0
# 3	100	33	100	33	0	33	33	33	0
+ control	0	N/A	0	100	N/A	100	100	N/A	100
- control	0	N/A	0	0	N/A	0	0	N/A	0

[a] KOH post-enrichment treatment, 0.5 ml enrichment was gently mixed in 0.13% KOH of 4.5 ml for 30 s.

Table 7. Recovery of *Y. enterocolitica* O:8, 1000 CFU/g feed from swine feeds using 10 g sample and 90 ml ITC enrichment at 22°C and KOH post-enrichment treatment followed by plating on SSDC agar

Sample	Recovery (%)								
	Day 1	Day 2		Day 5		Day 8		Day 11	
	No KOH	No KOH	KOH[a]	No KOH	KOH[a]	No KOH	KOH[a]	No KOH	KOH[a]
# 1	0	0	0	0	0	0	0	0	0
# 2	0	0	0	0	0	0	0	0	0
# 3	0	48	0	0	0	0	0	0	0

[a] KOH post-enrichment treatment, 0.5 ml enrichment was gently mixed in 0.25% KOH of 4.5 ml for 30 s.

on day 2. However, O:8 recovery did not depend on incubation day ($\chi^2_1 = 2.2500$, $P > 0.05$).

Method 3, 25 g sample and 225 ml PSB at 21°C was unreliable for recovery of serotype O:3 (Table 8). Serotype O:3 was only recovered from one sample on day 4. KOH (0.13%) post-enrichment treatment was also conducted but it was unsuccessful. The modified CIN agar with polymyxin B was also unsuccessful. The same enrichment procedure with slightly different days and KOH (0.25%) treatment was applied to serotype O:8 (Table 9) and was also unreliable.

PSB at 4°C (method 4) for recovering both *Y. enterocolitica* O:3 (Table 10) and O:8 (Table 11) up to day 41 was also ineffective.

TSBPN, method 5 resulted in good recovery for both serotype O:3 and O:8 on both CIN and MAC agar plates (Table 12). One day incubation showed 100% recovery for both serotypes. Day 3 for O:3 resulted in no recovery and day 1 was significantly better than day 3 to recover O:8 ($\chi^2_1 = 11.4545$, $P < 0.05$). Recovery of O:8 after 1 day incubation of TSBPN (Table 12) was significantly better than after 2 day incubation by using ITC enrichment (Table 7) ($P < 0.05$). Recovery of O:8 was compared between CIN and MAC agar plates from day 2 to day 4. The use of CIN plates gave significantly higher isolation rates ($\chi^2_1 = 5.0824$, $P < 0.05$). The effectiveness of KOH post-enrichment treatment was inconsistent. MAC agar plates after KOH treatment gave significantly higher recovery rate on day 2 than MAC agar plates without the treatment ($\chi^2_1 = 5.0824$, $P < 0.05$). However, KOH did not improve recovery on CIN plates for day 2 and 3 ($\chi^2_1 = 3.7723$, $P > 0.05$).

Table 8. Recovery of *Y. enterocolitica* O:3, 1000 CFU/g feed from swine feeds using 25 g sample and 225 ml PSB at 21°C and KOH post-enrichment treatment

Sample	Recovery (%)								
	Day 2			Day 4			Day 6		
	CIN		MCIN[a]	CIN		MCIN[a]	CIN		MCIN[a]
	No KOH	KOH[b]	No KOH	No KOH	KOH[b]	No KOH	No KOH	KOH[b]	No KOH
# 1	0	0	0	0	0	0	0	0	0
# 2	0	0	0	0	0	0	0	0	0
# 3	0	0	0	67	0	0	0	0	0
+ control	0	N/A	N/A	0	N/A	N/A	0	N/A	N/A
- control	0	N/A	N/A	0	N/A	N/A	0	N/A	N/A

[a] Modified CIN : CIN with Polymyxin B Sulfate (5 IU/ml).

[b] KOH post-enrichment treatment, 0.5 ml enrichment was gently mixed in 0.13% KOH of 4.5 ml for 30 s.

Table 9. Recovery of *Y. enterocolitica* O:8, 1000 CFU/g feed from swine feeds using 25 g sample and 225 ml PSB at 21°C and KOH post-enrichment treatment followed by plating on CIN agar

Sample	Recovery (%)							
	Day 1		Day 3		Day 5		Day 7	
	No KOH	KOH[a]	No KOH	KOH[a]	No KOH	KOH[a]	No KOH	KOH[a]
# 1	0	0	0	0	0	0	0	0
# 2	0	0	0	0	0	0	0	0
# 3	0	0	0	0	0	0	0	0

[a] KOH post-enrichment treatment, 0.5 ml enrichment was gently mixed in 0.25% KOH of 4.5 ml for 20 s.

Table 10. Recovery of *Y. enterocolitica* O:3, 1000 CFU/g feed from swine feeds using 25 g sample and 225 ml PSB at 4°C followed by plating on CIN agar

Sample	Recovery (%)			
	Day 14	Day 21	Day 28	Day 35
# 1	0	0	0	0
# 2	0	0	0	0
# 3	0	0	0	0
+ control	0	0	0	0
- control	0	0	0	0

Table 11. Recovery of *Y. enterocolitica* O:8, 1000 CFU/g feed from swine feeds using 25 g sample and 225 ml PSB at 4°C with KOH[a] and without KOH post-enrichment treatment

Sample	Recovery (%)[b]											
	Day 6		Day 13		Day 20		Day 27		Day 34		Day 41	
	CIN	MAC	CIN	MAC	CIN	MAC	CIN	MAC	CIN	MAC	CIN	MAC
# 1	0	0	0	0	0	0	0	0	0	0	0	0
# 2	0	0	0	0	0	0	0	0	0	0	0	0
# 3	0	0	0	0	0	0	0	0	0	0	0	0

[a] No *Y. enterocolitica* O:8 was detected from day 6 to 41 from 0.5 ml enrichment gently mixed in 0.25% KOH of 4.5 ml for 20 to 30 s.

[b] No KOH treatment applied.

Table 12. Recovery of *Y. enterocolitica* O:3 and O:8, 1000 CFU/g feed from swine feeds using 10 g sample and 90 ml TSB with Polymyxin B (5 IU/ml) and Novobiocin (10 μg/ml) (TSBPN) at 18°C with and without KOH post-enrichment treatment

Sample		Recovery (%)													
		Day 1				Day 2			Day 3				Day 4		
		O:3[a]		O:8[a]		O:3	O:8		O:3		O:8		O:3	O:8	
		C[b]	M[c]	C[b]	M[c]	N/A	C[b]	M[c]	C[b]	M[c]	C[b]	M[c]	N/A	C[b]	M[c]
No KOH treatm -ent	# 1	100	100	100	N/A	N/A	100	33	0	0	33	0	N/A	67	0
	# 2	100	100	100	N/A	N/A	67	0	0	0	33	0	N/A	100	67
	# 3	100	100	100	N/A	N/A	0	33	0	0	0	0	N/A	67	67
	+ control	100	100	33	N/A	N/A	N/A	N/A	0	0	N/A	N/A	N/A	N/A	N/A
	- control	0	0	0	N/A	N/A	N/A	N/A	0	0	N/A	N/A	N/A	N/A	N/A
KOH treatm -ent[d]	#1	N/A	N/A	N/A	N/A	N/A	100	100	N/A		100	0	N/A	0	0
	#2	N/A	N/A	N/A	N/A	N/A	100	100	N/A		100	0	N/A	50	50
	#3	N/A	N/A	N/A	N/A	N/A	0	100	N/A		50	0	N/A	50	50

[a] Recoveries of O:3 and O:8 are separate experiments.

[b] CIN agar.

[c] MAC agar.

[d] KOH post-enrichment treatment, 0.5 ml enrichment was gently mixed in 0.25% KOH of 4.5 ml for 20 to 30 s.

TSBPN, day 1 for O:3 and O:8 and ITC, day 1 for O:3 were much better enrichment methods than PBS at 4°C and PSB at 4 and 21°C ($P < 0.05$).

Inoculum level of 100 CFU/g feed

After the experiment of 1000 CFU/g feed, both ITC enrichment and TSBPN were inoculated with 100 CFU/g feed because both were effective to recover O:3, and O:3 and O:8, respectively.

Serotype O:3 was effectively recovered (100%) in ITC enrichment after 1 and 2 day incubation (Table 13). However, serotype O:8 was poorly recovered (Table 13). The difference in recovery of serotypes O:3 and O:8 in ITC enrichment was significant ($\chi^2_1 = 32.2105$, $P < 0.05$). A two-step enrichment procedure was unsuccessful in recovery of both serotypes.

TSBPN also showed good recovery (Table 14). Both O:3 and O:8 were recovered in all three feed samples after 24 h incubation. A two-step enrichment procedure (15 h pre-enrichment followed by 24 h selective enrichment) showed good recovery for O:3, but O:8 could not be recovered from one sample (Table 14). No difference was found in recovery between normal TSBPN procedure and two-step procedure for O:3 and O:8 ($\chi^2_1 = 0.9000$, $P > 0.30$).

In comparison of ITC enrichment and TSBPN, no difference was found in recovery of O:3 after 24 h. No difference was also found in recovery of O:8 ($P > 0.05$).

TSBPN with calcium chloride (0.01%) was used to recover *Y. enterocolitica* O:8, 100 CFU/g feed. But, this method was also unsuccessful. Even positive control could not recover *Y. enterocolitica*. KOH treatment of 0.5% with 0.5% NaCl for 30 s was tried, but did not improve recovery.

Table 13. Recovery of *Y. enterocolitica* O:3 and O:8, 100 CFU/g feed from swine feeds using 10 g sample and 90 ml ITC enrichment at 22°C with one-step and two-step followed by plating on CIN agar

Sample	Recovery (%)					
	Normal procedure				Two-step[a]	
	24 h		48 h		15 h, then 24 h	
	O:3[b]	O:8[b]	O:3	O:8	O:3	O:8
# 1	100	0	100	0	0	0
# 2	100	0	100	0	0	0
# 3	100	33	100	0	0	0
+ control	67	0	100	0	0	0
- control	0	0	0	0	0	0

[a] 10 g feed and 90 ml pre-ITC enrichment omitting irgasan, ticarcillin and potassium chlorate from ITC enrichment was incubated for 15 h. Subsequently, 1 ml was transferred to ITC enrichment of 100 ml, then another 24 h incubation was taken.

[b] Recoveries of O:3 and O:8 are separate experiments.

Table 14. Recovery of *Y. enterocolitica* O:3 and O:8, 100 CFU/g feed from swine feeds using 10 g sample and TSB with Polymyxin B (5 IU/ml) and Novobiocin (10 μg/ml) (TSBPN) at 18°C with one-step and two-step followed by plating on CIN agar

Sample	Recovery (%)			
	Normal procedure[a]		Two-step[b]	
	O:3[c]	O:8[c]	O:3	O:8
# 1	100	100	100	33
# 2	100	33	100	67
# 3	100	33	100	0
+ control	100	0	67	0
- control	0	0	0	0

[a] 24 h incubation.

[b] 10 g feed and 90 ml TSB was incubated for 15 h. Subsequently, 1 ml was transferred to TSBPN of 100 ml, then another 24 h incubation was taken.

[c] Recoveries of O:3 and O:8 are separate experiments.

Inoculum level of 10 CFU/g feed

As inoculum level decreased, the difficulty of isolation of *Y. enterocolitica* from feed increased.

ITC enrichment and TSBPN were inoculated with O:3, and both O:3 and O:8, respectively at 10 CFU/g feed because they showed good recovery at 100 CFU/g feed.

ITC enrichment satisfactorily recovered O:3 after 24 h incubation (56%) and 48 h incubation (67%), but the recovery failed from one sample after 48 h incubation (Table 15).

For TSBPN, two different two-step enrichment procedures (Grain-out and grain-in) were compared to the normal procedure of 24 h incubation (Table 16). TSBPN showed satisfactory recovery of O:3 (89%) after 24 h incubation and also for grain-out (100%) and grain-in (100%) two-step procedures. However, TSBPN failed to recover O:8. Recovery in TSBPN was dependent on serotypes (O:3 and O:8) ($\chi^2_1 = 14.4000$, $P < 0.05$). No difference between ITC enrichment, 24 h and TSBPN, 24 h in recovery of O:3 was found ($\chi^2_1 = 2.4923$, $P > 0.05$).

TSB with irgasan (4μg/ml) and calcium chloride (0.01%) was devised and inoculated with O:8. The normal procedure of 24 h incubation and a two-step procedure (grain-out) were both tested, but they were not successful.

The optimum recovery rates for different enrichment procedures and inoculum levels are summarized (Table 17).

Table 15. Recovery of *Y. enterocolitica* O:3, 10 CFU/g feed from swine feeds using 10 g sample and 90 ml ITC enrichment at 22°C followed by plating on CIN agar

Sample	Recovery (%)	
	24 h	48 h
# 1	67	100
# 2	33	0
# 3	67	100
+ control	33	100
- control	0	0

Table 16. Recovery of *Y. enterocolitica* O:3 and O:8, 10 CFU/g feed from swine feeds using 10 g sample and 90 ml TSB with Polymyxin B (5 IU/ml) and Novobiocin (10 μg/ml) (TSBPN) at 18°C with one-step and two-step followed by plating on CIN agar

Sample	Recovery (%)					
	Normal procedure[a]		Two-step (Grain-out)[b]		Two-step (Grain-in)[c]	
	O:3[d]	O:8[d]	O:3	O:8	O:3	O:8
# 1	67	0	100	0	100	0
# 2	100	0	100	0	100	0
# 3	100	0	100	0	100	0
+ control	100	67	100	0	100	100
- control	0	0	0	0	0	0

[a] 24 h incubation.

[b] 10 g feed and 90 ml TSB was incubated for 15 h. Subsequently, 1 ml was transferred to TSBPN of 100 ml, then another 24 h incubation was taken.

[c] 10 g feed and 90 ml TSB was incubated for 15 h. Subsequently, another 24 h incubation was taken after the addition of Polymyxin B (5 IU/ml) and Novobiocin (10 μg/ml).

[d] Recoveries of O:3 and O:8 are separate experiments.

Table 17. Summary of methods to recover *Y. enterocolitica* O:3 and O:8 from swine feeds

Method	Plating agar	Serotype	Level of inoculum (CFU/g feed)	Day(s) of enrichment	Temperature (°C) of incubation	Recovery (%)
PBS	CIN	O:3	1000	14 to 35	4	0
PBS	CIN	O:8	1000	35	4	11
ITC	CIN	O:3	1000	1	22	100
ITC	SSDC	O:8	1000	2	22	17
PSB	CIN	O:3	1000	4	21	22
PSB	CIN	O:8	1000	1 to 7	21	0
PSB	CIN	O:3	1000	14 to 35	4	0
PSB	CIN	O:8	1000	6 to 41	4	0
TSBPN	CIN	O:3	1000	1	18	100
TSBPN	CIN	O:8	1000	1	18	100
TSBPN-KOH	MAC	O:8	1000	2	18	100
ITC	CIN	O:3	100	1 or 2	22	100
ITC	CIN	O:8	100	1	22	11
TSBPN	CIN	O:3	100	1	18	100
TSBPN	CIN	O:8	100	1	18	56
ITC	CIN	O:3	10	1	22	56
TSBPN	CIN	O:3	10	1	18	89

Discussion

The recovery rates of both PBS and PSB at 4°C were poor on both O:3 and O:8 in addition to being time-consuming procedures, although PSB is known to be superior to PBS in cold enrichment (De Boer et al., 1982; Mehlman, 1978). The inferiority of PSB to ITC enrichment in this study agrees with other researches with pork samples (Nesbakken et al., 1991). There is some disagreement on the efficiency of cold enrichments of PBS or PSB for pathogenic strains like O:3 and O:8. Some researchers (De Boer et al., 1982; De Boer and Nouws, 1991; Van Noyen et al., 1980; Wauters et al., 1988) found weak recovery capacity of these methods for pathogenic strains. However, Doyle et al. (1981) successfully isolated O:8 from porcine tongues by PBS and Cox and Bailey (1990) recovered O:3 and O:8 appropriately by PBS. Sometimes, these were superior to ITC enrichment (Hariharan et al., 1995; Hoorfar and Holmvig, 1999), which is in contrast to the results in this study using animal feeds. Anyway, this study indicates that the prevalence of *Y. enterocolitica* in swine or pork samples (De Boer and Seldam, 1987; Doyle et al., 1981; Jiang et al., 2000; Letellier et al., 1999; Nesbakken and Kapperud, 1985) and poultry (De Boer et al., 1982) may have been greatly underestimated when PBS or PSB was used for enrichment.

PSB at 21°C was also ineffective and the increase of incubation temperature of PSB did not result in better recovery.

The effectiveness of ITC enrichment for serotype O:3 was confirmed in this study. The recovery rate for O:8 was relatively poor. Wauters et al. (1988) mentioned the efficacy of ITC enrichment may be limited to O:3. MRB from which ITC enrichment

derived is inhibitory to O:8 (Schiemann, 1983). Irgasan, magnesium chloride or potassium chlorate in ITC enrichment does not have an effect on O:8 (Schiemann, 1980; Toora et al., 1994a).

Normally two to five days of incubation time for ITC enrichment were used (De Boer and Nouws, 1991; De Giusti et al., 1995; De Zutter et al., 1995; Hariharan et al., 1995; Hoorfar and Holmvig, 1999; Wauters et al., 1988), but our study showed that 1 or 2 day incubation is most desirable for isolation from feed samples.

TSBPN was the most preferred recovery method in this study because it was more sensitive than other enrichment methods and recovered both O:3 and O:8. There was no statistical difference for recovery capacity, compared to ITC enrichment at 10 CFU/g feed for O:3, but the difference was found at 1000 CFU/g feed for O:8. Different use of agar plates, CIN agar for TSBPN and SSDC agar for ITC enrichment may be another reason for the difference. In addition, a TSBPN is a simple procedure, compared to ITC enrichment, which requires many ingredients. TSBPN favored O:3 more than O:8 (Table 16), similar to ITC enrichment. Nonpathogenic *Y. enterocolitica* strains were not tested in this study, but TSBPN is more likely to be favorable for nonpathogenic strains because virulent Congo Red+ strains were found to be more susceptible to polymyxin B and novobiocin than were nonvirulent strains (Robins-Browne et al., 1986). Thus, nonpathogenic strains should be more resistant to these antibiotics.

The 1 day incubation gave much higher recovery rate than 3 day incubation (Table 12) for recovery of O:3 and O:8 from swine feed samples. Accordingly, TSBPN for 24 h incubation was most efficient to recover both O:3 and O:8 from swine feeds on basis of

recovery rate and incubation time in this study. Longer incubation times have been used for milk samples (Landgraf et al., 1993).

CIN agar plates were preferred in this study because of very noticeable colony morphology with dark red center and colorless border and its high selectivity. MAC agar was used without any major problems at 1000 CFU/g inoculum level in this study. However, colorless and small colonies of presumptive *Y. enterocolitica* made it hard to pick colonies. Also, the absence of selective supplements made it hard to isolate colonies in plates with heavy contamination. Wauters et al. (1988) and De Boer and Nouws (1991) recommended SSDC agar rather than CIN agar to recover O:3 from ITC enrichment, but no benefit of the use of SSDC was found in this study. Relatively less differentiated colony morphology and impossibility of storage due to lack of autoclaving made the use of SSDC agar undesirable. Another disadvantage is that SSDC agar is less suitable to recover pathogenic strains than O:3 (Wauters et al., 1988). In this study, O:8 was recovered from one CIN agar plate at 100 CFU/g feed on day 1 from ITC enrichment (Table 13), but no O:8 was recovered from SSDC plates even at 1000 CFU/g feed (Table 7).

Some researchers (Aulisio et al., 1980; De Boer et al., 1982; Doyle et al., 1981) reported the usefulness of KOH post-enrichment treatment for better recovery of *Y. enterocolitica*, but no difference was found for KOH treatment in this study except for MAC agar plates on day 2, TSBPN (Table 12). Since KOH treatment is used to eliminate background flora in highly contaminated foods, highly selective media may make this step unnecessary. When CIN plates are employed for isolation, KOH treatment may not be useful because CIN plates are already highly selective for *Y. enterocolitica*.

Nevertheless, the value of KOH treatments still remains because injured *Y. enterocolitica* is hard to recover on CIN plates and some strains like *Y. enterocolitica* biotype 3B serotype O3 (Fukushima and Gomyoda, 1986), and also plasmid-bearing virulent strains (Bhaduri et al., 1997) are possibly inhibited on CIN plates. Accordingly, the combination of MAC agar and KOH treatment can be valuable. In this study, the KOH treatment was only effective for MAC plates, day 2 in TSBPN on which high number of background microflora are expected (Table 12). The value of MAC agar – KOH treatment was shown compared to CIN agar – KOH treatment by Doyle and Hugdahl (1983), and it indicates possible CIN agar inhibition against some *Y. enterocolitica*.

Two-step enrichment procedure was performed for ITC enrichment and TSBPN, but no improved recovery was found. The two-step procedure was used by a few researchers (Bhaduri et al., 1997; Schiemann, 1982; Toora et al., 1994b). An advantage of the two-step procedure was believed to prevent selective agents from suppressing the growth of virulent *Y. enterocolitica* at the onset of growth (Bhaduri et al., 1997; Schiemann, 1982), but the addition of irgasan or novobiocin to CIN broth base was actually found to increase the growth rate of *Y. enterocolitica* O:3 at 25 or 37°C (Logue et al., 2000). Thus, the addition of antibiotics such as irgasan or novobiocin in the later stage can adversely affect the recovery because unlimited growth of other background microflora can easily suppress the growth of *Y. enterocolitica*.

The feeds used in this study were free from antibiotics, but currently 70 to 80% of growing feeds, and 50 to 60% of finishing feeds contain antimicrobial agents in US (Cromwell, 2001). If the feeds containing antimicrobial agents are used for recovery of *Y. enterocolitica*, the inhibitory effects of the antimicrobial agents to *Y. enterocolitica* may

be still minimal because the antimicrobials used in feeds are used normally at subtherapeutic levels and also diluted in enrichments.

Research to develop enrichment media should be based on recovery of both O:3 and O:8 (especially low levels) equally. Considering the poor recovery rates of ITC enrichment and TSBPN for O:8 compared with O:3 in this study, efficient enrichment to recover O:8 is needed because O:8 is the most prevalent serotype in US. Serotype O:8 is normally more sensitive to enrichment chemicals (Doyle et al., 1981; Lee et al., 1980). YER-BOS by Schiemann (1982) recovered relatively low number of inoculum of *Y. enterocolitica* O:8 (in food), as low as 30 CFU/g, but this procedure is complicated. The difficulty in making an enrichment which can recover various *Y. enterocolitica* without bias may be related to the heterogeneity of biochemical traits of serotypes of *Y. enterocolitica*.

Also, the development of recovery enrichment media for *Y. enterocolitica* should be focused on highly nutritious media, as suggested by Schiemann (1989). TSBPN and ITC enrichment are more nutritious media than others evaluated in our study. Incubation temperatures of 12 to 22°C are more practical to shorten incubation time compared to cold enrichment (weeks). In addition, not only the effects of individual selective supplements, but also the combination or mutual effects of selective agents (Toora, 1994a) should be studied because they can have nullifying effects on each other (Schiemann, 1980). The combination of selective supplements is not always additive. In this study, for example the addition of calcium chloride to TSBPN gave negative effects to recover serotype O:8 while calcium chloride by itself enhanced the growth of *Y. enterocolitica* in other studies (Toora, 1994a).

In conclusion, the media found optimal in recovery of O:3 and O:8 from swine feeds were TSBPN, 24 h.

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Part III:

Transformation of *Yersinia enterocolitica* with pGFPuv plasmid vector

Abstract

Green fluorescent proteins (GFPs) producing bacteria have been increasingly used in food microbiology area because of easy identification of it. GFP producing *Y. enterocolitica* has never been used in this area. *Y. enterocolitica* was transformed with the pGFPuv plasmid vector which encodes for GFP by using electroporation. Fluorescence of *Y. enterocolitica* colonies on Luria-Bertani (LB) and also CIN agars was visualized under UV light. The presence of pGFPuv vectors inside the cells was confirmed on agarose gel by electrophoresis at 3.3 kb. Because the presence of green fluorescent *Y. enterocolitica* can be easily identified, its use is promising for many applications such as evaluation of culture media for isolation of *Y. enterocolitica* from naturally contaminated materials. This technique is also very useful for evaluation of spatial location or gene expression of cells.

Introduction

Green fluorescent proteins (GFPs) are from a Pacific Northwest jellyfish, *Aequorea victoria* (Cubitt et al., 1995). Transformed microorganisms with GFP gene such as *E. coli* O157:H7 and *Salmonella* have been increasingly used in the study of food microbiology (Ajjarapu and Shelef, 1999; Burnett et al., 2000; Gandhi et al., 2001; Pao et al., 2001; Wachtel et al., 2002) because their presence or spatial location can be easily identified based on visualization. Another advantage of the use of GFP gene is that no factors or substrates for the generation of green light are required and no considerable toxicity of GFP was observed (Misteli and Spector, 1997).

In this study, the transformation of *Y. enterocolitica* with pGFPuv plasmid vector (Clontech, Palo Alto, CA) encoding for GFP was tested for its potential use.

Materials and methods

Y. enterocolitica was transformed with the pGFPuv plasmid vector (Clontech, Palo Alto, CA) with the assistance of Paul Ebner, a graduate student in the animal science department. The pGFPuv vector encodes for *Aequorea Victoria* green fluorescent protein (GFP) that gives bright fluorescence when excited by standard UV light, and contains ampicillin resistance selection marker (Anonymous).

Competent cells

Cells were made electro-competent using the technique outlined in the electroporator II instruction manual (Anonymous, 1997). Briefly, *Y. enterocolitica* culture was grown in 25 ml of Luria-Bertani (LB) broth (10 g Bacto tryptone [Difco, Detroit, MI], 5.0 g yeast extract [Difco], 10.0 g NaCl [Difco] per 1 liter) at 37°C overnight. The resulting culture was used to inoculate 500 ml of LB broth and grown at 37°C to mid-log growth phase ($O.D._{600} = 0.55$). LB broth of 125 ml containing mid-log phase *Y. enterocolitica* was transferred to a chilled 250 ml centrifuge tube. The culture was pelleted by centrifugation at 3500 rpm and 0°C for 15 min. The culture was resuspended in 125 ml ice-cold water, pelleted and resuspended in 62.5 ml ice-cold water. The culture was again pelleted, resuspended in 5 ml ice-cold, 10% glycerol (Fisher Scientific) and transferred to 25 ml centrifuge tube. The glycerol containing culture was pelleted and resuspended in 240 µl 10% ice-cold glycerol. Individual aliquots of 40 µl were stored at -70°C until further use.

Electroporation

Electro-competent cells (40 µl) were briefly mixed with 1 µl of pGFPuv (500 ng/µl) and transferred to an 0.1 cm electroporation cuvettes (Invitrogen, Carlsbad, CA). Cells

were transformed by administering a rapid 1500 volt shock (Labconco, Kansas City, MO) using the Electroporator II (Invitrogen, San Diego, CA). Transformed cells were then transferred to 1 ml SOC broth medium (2.0 g bacto tryptone [Difco], 0.5 g yeast extract [Difco], 1 ml 1 M NaCl [FisherBiotech], 0.25 ml 1 M KCl [Mallinckrodt, Paris, KY], 1 ml 2 M Mg^{2+} [Mallinckrodt], and 1 ml 2 M glucose [Mallinckrodt] per 100 ml) and incubated for 1.25 h at 37°C to increase their viability and transformation efficiency (Drury, 1994).

Isolation of fluorescent *Y. enterocolitica*

Individual colonies containing pGFPuv were isolated by spreading 100 μl of transformed cell culture on LB agar plates containing 100 μg/ml ampicillin (Sigma, St. Louis, MO) and a 100 μl overlay of 100 mmol isopropyl-beta-D-thiogalactoside (IPTG) to increase gene expression (Chalfie et al., 1994). Plates with no ampicillin were also spread as controls. Cultures were incubated for 16 to 24 h at 37°C, then observed under UV light (350-450 nm) (Transilluminator, San Gabriel, CA) for the presence of bright green colonies.

Verification of transformation

Individual green fluorescent colonies were inoculated into 5 ml of 2YT broth (16 g tryptone peptone, 10 g yeast extract, and 5 g NaCl per 1 liter) and grown overnight at 37°C. Plasmid DNA was isolated using Quantum Prep Plasmid Miniprep kit (BIO-RAD, Hercules, CA) according to the manufacturer's direction.

Plasmid DNA was separated by traditional electrophoresis in 0.9% agarose and 0.5X TBE (TBE, 10X: 108 g tris-base [Mallinckrodt], 55 g boric acid [FisherBiotech], and 20 ml 0.5 M EDTA (pH 8.0) [FisherBiotech] per 1 liter).

DNA samples (14 μl) were combined with 3 μl sample buffer (4 g sucrose [FisherBiotech] and 2.5 mg bromophenol blue [ICN, Cleveland, OH] in 10 ml tris-base EDTA (TE)) and loaded into the individual wells of the agarose gel. A super-coiled DNA ladder (Promega, Madison, WI) was included as a molecular weight reference. Samples were electrophoresed for 1 h at 110 V. Gels were visualized over a UV transilluminator (Fisherbiotech) and photographed for further analysis.

Streaking on CIN agar plates with fluorescent *Y. enterocolitica*

Three isolated fluorescent colonies were grown in each BHI broth for overnight, then streaked on two CIN plates for use of CIN plates with fluorescent *Y. enterocolitica*.

Results

Many green fluorescent colonies of *Y. enterocolitica* were observed under UV lights on the LB agar plates. Non-ampicillin treated plates were found to have no fluorescent colonies. Also, original SOC medium gave larger growth of fluorescent colonies than the medium with 1:1 or 1:10 dilution with saline.

The presence of pGFPuv vectors inside *Y. enterocolitica* cells was verified on agarose gel by electrophoresis. The band at 3.3 kb, corresponding with one of pGFPuv vector, was apparent (Fig 1).

Three transformed isolates were grown in each BHI broth overnight, then streaked on CIN plates. After incubation, the plates were observed under an UV box. Two isolates fluoresced on CIN plates, but one did not.

Discussion

Y. enterocolitica was successfully transformed with green fluorescent protein (GFP) plasmid. The colonies fluoresced on CIN agar plates as well as LB agar plates. An advantage of the use of fluorescent *Y. enterocolitica* lies in that the identification of the microorganism is much easier. The reason why one isolate among three fluorescent isolates did not fluoresce on CIN plates may be due to the absence of ampicillin in BHI broth and CIN agar plates. The possibility of loss of pGFPuv vectors should be a concern when transformed cells are used in a scientific study. In addition, low brightness on plates without IPTG was somewhat problematic to identify fluorescent colonies.

Although this transformation was successful, fluorescent *Y. enterocolitica* was not used in this study to evaluate enrichment methods. Ampicillin must be maintained in culture media to keep pGFPuv plasmids inside the cells. We were concerned that addition of ampicillin could disrupt the consistency of this study because ampicillin was not used in all media.

Integration of GFP gene into the chromosome of *Y. enterocolitica* should be more desirable than insertion of the GFP plasmid into the cells to prevent cells from losing the GFP gene during a scientific study (Gandhi et al., 2001).

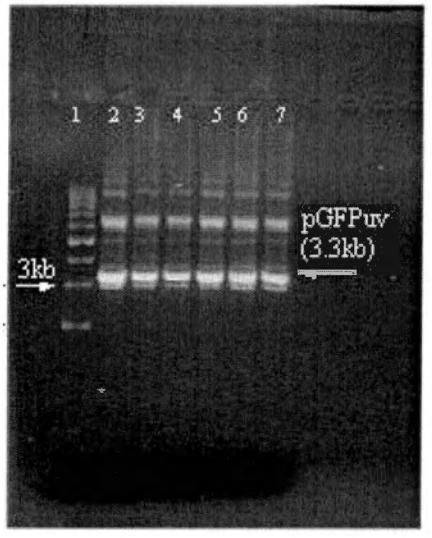

Figure 1. Plasmid DNA of transformed *Y. enterocolitica* separated by agarose gel electrophoresis. Arrows indicate a 3kb size standard and presence of pGFPuv in each transformed sample. Lane 1, super-coiled DNA ladder ; Lane 2 through 7, transformed *Y. enterocolitica* with pGFPuv vector.

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TSB with Polymyxin B (5 IU/ml) and Novobiocin (10 μg/ml) (TSBPN) at 18°C for 1 day incubation followed by plating on CIN agar plates was most the efficient and desirable enrichment method to recover *Y. enterocolitica* O:3 and O:8 from swine feeds in this study.

Tremendous efforts of many scientists over the last 20 years have made it possible to develop efficient enrichment methods for *Y. enterocolitica*. Nevertheless, the absence of enrichment broth which is equally efficient for various serotypes of *Y. enterocolitica* and the relative insensitivity of cultural methods leading to underestimation of prevalence of pathogenic *Y. enterocolitica* (Johannessen et al., 2000) still remain a problem. Compared to cultural methods, PCR and colony hybridization methods are known to be rapid, highly specific and more sensitive (Durisin et al., 1997; Johannessen et al., 2000; Lambertz et al., 1996; Nesbakken et al., 1991). However, drawbacks of PCR and colony hybridization methods still exist in that PCR method can have a high incidence of false positive (Lambertz et al., 1996) and colony hybridization method is costly (Sheridan et al., 1998). Thus, research is needed to develop efficient culture methods which can recover various serotypes/biotypes of *Y. enterocolitica* and to eliminate drawbacks of rapid methods. Optimal methods should be selected after consideration to each different sample type.

GFP producing *Y. enterocolitica* can be a versatile tool in the study of food microbiology. It can be used to track the contamination by *Y. enterocolitica* in a food matrix, or to assess storage conditions of foods or preservation effects. Insertion of the

GFP gene to the chromosome of *Y. enterocolitica* prevents the cells from losing the GFP gene and is recommended for GFP transformed cells which might be used routinely.

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Vita

Joo-Sung (Paul) Kim was born on May 26, 1974, and is the first son of Kil-Yong Kim and Bok-Nyon Kim. He received his elementary, middle, and high school education in Seoul, South Korea, graduating from On-Soo high school in February of 1993. He then entered Korea University in Seoul majoring in food technology. He graduated from Korea University with a B. S. in food technology in division of life science in February, 2000. In August of 2000, he started his graduate study toward a Master of Science in Food Science department under the direction of Dr. F. Ann Draughon at the University of Tennessee, Knoxville. Upon completion of his graduate research in July, 2002, he was awarded his Master of Science degree.

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