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ASSESSMENT OF THE SENSITIVITY OF CURRENT STANDARD PROCEDURES
FOR THE ISOLATION OF *YERSINIA ENTEROCOLITICA* FROM PORK MINCE

A DISSERTATION PRESENTED IN PARTIAL FULFILMENT (25%) OF THE
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ABSTRACT

Y. enterocolitica and related species have been isolated from many types of food. The majority of isolates differ in biochemical and serological characteristics from typical pathogenic strains and are termed non-pathogenic or environmental strains. Usually the number of *Y. enterocolitica* organisms present in food products is low compared with the dominant background flora. The ability of current enrichment procedures to recover pathogenic strains of *Y. enterocolitica* from different foods is often inadequate probably because different strains require different conditions for optimum growth (De Boer 1992). An efficient enrichment procedure should confer some selective advantage to the desired type of microorganism by promoting its growth relative to the competing microflora. At present, there is no single ideal isolation procedure available for the recovery of pathogenic strains of *Y. enterocolitica* from foods.

The aim of this study was to determine the recovery rate of *Y. enterocolitica* biotype 4/serotype O:3 from samples of pork mince inoculated with known numbers of the microorganism using different enrichment parameters (Time, temperature and pH) and Cefsulodin-Irgasan-Novobiocin (CIN) agar as the selective medium. The experiment was conducted in two trials using different bacterial dilutions. Three pork mince samples in duplicate were inoculated with known quantities of *Y. enterocolitica* biotype 4/serotype O:3 organisms and subjected to cold enrichment in phosphate buffered saline (PBS) with a pH of 7.6, 6.6 and 5.5 at 25°C for 2 days, 10°C for 7 days and 4°C for 21 days. CIN agar was used as the selective medium. Pre-inoculation control samples were selected and plated in CIN on day 0 and on day 21 after PBS enrichment at 4°C.

In Trial one *Y. enterocolitica* organisms were recovered from all 3 samples incubated at 25°C for 2 days and from 1 out of 3 inoculated samples incubated at 4°C for 21 days. There were no organisms recovered from other inoculated samples. The control sample did not show any environmental contamination with *Yersinia* species. In Trial two, *Y. enterocolitica* was recovered from 1 out of 3 duplicate samples enriched in PBS with pH 6.6 and incubated at 25°C for two days. *Y. enterocolitica* was not recovered from other inoculated samples. *Y. intermedia* was isolated from all pH, temperature and time combinations and also from control samples.

The following conclusions can be drawn from this experiment. Incubation at high temperature (25°C) and short duration (48 hours) can be used as an efficient method for isolating *Y. enterocolitica* from pork samples. The standard incubation period of 21 days required for cold enrichment at 4°C is too long for the isolation of pathogenic strains, because of possible growth of environmental microorganisms. A pH of 6.6 is less efficient than 7.6 for enrichment although occasional isolation can be made using this pH. Enrichment in PBS with a pH of 5.5 with any time as well as temperature combinations and incubation at 10°C for 7 days are not ideal for isolation of pathogenic *Yersinia enterocolitica* strains. Of the three enrichments (PBS 7.6, 6.6, 5.5) used in this experiment, PBS with pH 7.6 was found to be most efficient to others.

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CHAPTER ONE
REVIEW OF LITERATURE
SECTION-A
THE GENUS *YERSINIA*

INTRODUCTION

The genus *Yersinia* that belongs to the family *Enterobacteriaceae* was named after the French bacteriologist Yersin who first isolated *Yersinia pestis* in 1894 (Bercovier and Mollaret 1984). There are at present at least 11 species recognised within the genus *Yersinia*. Of these, *Y. pestis* is the cause of plague, a serious human illness. *Y. pseudotuberculosis* is the cause of epizootic disease in several animal species (Mair 1975). In more recent years another member of the genus, *Yersinia enterocolitica* has been reported with increasing frequency as the cause of foodborne disease in humans throughout the world, particularly in temperate countries (Swaminathan *et al* 1982). *Y. pseudotuberculosis* and *Y. enterocolitica* produce similar clinical signs in humans and animals including acute and chronic enteritis, mesenteric lymphadenitis, and septicaemia (Schiemann 1989). Erythema nodosum and reactive polyarthritis are found in susceptible humans with the HLA-B27 tissue factor (Robins-Browne 1992).

Unlike *Y. pestis* and *Y. pseudotuberculosis*, *Y. enterocolitica* is a biochemically heterogenous group including both pathogenic and non-pathogenic strains, which have been isolated from different sources such as water, milk, animals, meat, tofu, etc.; and are widespread in terrestrial and aquatic environments. (Kapperud 1991).

Yersinia ruckeri is the cause of septicaemic disease (red mouth) in rainbow trout and certain related fishes and can be isolated from kidney tissue of the diseased fish (Rucker 1966). As an epizootic in hatcheries, the disease is an important economic problem in fish farming industries. *Yersinia ruckeri* has not been isolated from humans (Ewing *et al* 1978).

The other species of the genus *Yersinia* are *Y. frederiksenii*, *Y. kristensenii*, *Y. intermedia*, *Y. aldovae*, *Y. rhodei*, *Y. mollaretii* and *Y. bercovieri* (Bercovier *et al* 1984, Wauters *et al* 1987).

YERSINIA PSEUDOTUBERCULOSIS

Malassez and Vignal in 1883 made the first report of *Yersinia pseudotuberculosis* (Bisset 1981). The organism has been isolated from many outbreaks of tuberculosis like disease in guinea pigs and was named *Pasteurella pseudotuberculosis* for many years (Schiemann 1989). Since Dickinson and Mocqout (1961) isolated *Y. pseudotuberculosis* from the alimentary contents of healthy pigs, investigators have studied the properties of this organism and sought methods to distinguish it from closely related members of other genera.

Y. pseudotuberculosis is a well-recognised pathogen with a worldwide distribution (Parsons 1991). Infection has been described in several animal species (Mair 1975, Paff *et al* 1976) including birds, rodents and primates in zoological gardens (Keymer 1976, Jones 1980). Many reports from Europe, Canada and Japan describe pigs as a source of infection (Narucka and Westendoorp 1977). *Y. pseudotuberculosis* has consistently been recovered from intestinal contents and tonsils from apparently healthy slaughtered pigs, in different parts of the world (Toma and Deidrick 1975) and has also been isolated from raw pork (Fukushima 1985). Masshoff and Dolle (1953) isolated the organism from lymph-nodes of children undergoing surgery for appendicitis and Daniels (1962) suggested that the organism was responsible for mesenteric lymphadenitis frequently observed at laporatomy on children showing clinical signs of appendicitis. *Y. pseudotuberculosis* has been associated with a variety of other human diseases including terminal ileitis, arthritis and septicaemia. Several large outbreaks have been reported in Japan since 1981 (Inoue *et al* 1984, Sanbe *et al* 1987).

YERSINIA ENTEROCOLITICA

Gilbert in 1932 made the first description of human illness caused by *Y. enterocolitica* (cited by Winblad *et al* 1966). Ten years later, Schleifstein and Coleman proposed the name "*Bacterium enterocoliticum*" to describe bacteria isolated from samples submitted to the New York State Department of Health. Little information was gathered during the following 20 years until Winblad *et al* (1966) described eight serogroups of *Yersinia enterocolitica* and related species. Frederiksen in 1964 officially named *Bacterium enterocoliticum* as *Yersinia enterocolitica* (cited by Ostroff 1995).

The decade of the 1970's heralded a new interest in *Yersinia* as reports from throughout the world emphasised its importance as a human pathogen and improved cultural methods facilitated an increase in isolation of the organism. In 1972, Mollaret described 642 cases of yersiniosis diagnosed at the Pasteur Institute in Paris over a four-year period. Later, Mollaret *et al* (1979) described isolates from 35 countries on six continents. *Yersinia enterocolitica* infection has now become recognised as a major cause of gastro-enteritis in the developed world particularly in Europe, North America and Japan. Belgium serves as a valuable source of information regarding the epidemiology and ecology of *Y. enterocolitica* (Vandepitte *et al* 1973, Van Noyen *et al* 1981, Tauxe *et al* 1987). In Denmark, *Y. enterocolitica* O:3 infection ranks second only to campylobacteriosis with approximately 800 registered cases per year (Christensen 1987). In the other Scandinavian countries, the isolation rates of *Yersinia enterocolitica* from patients with gastro-enteritis compare with those of *Salmonella* and other enteric pathogens (Kapperud 1986). Laboratory reports indicate that infections due to *Y. enterocolitica* increased in England and Scotland in the last decade. (Cover and Aber 1989). In the United States, the ingestion of contaminated food has been implicated as a mode of transmission of many sporadic outbreaks (Black *et al* 1978, Morse *et al* 1984). *Yersinia* infections in the US have a tendency to occur in rural communities and the increase in rural rates of infection may reflect an increased exposure to environmental or animal reservoirs of organisms (Snyder *et al* 1982). Reports of sporadic cases and outbreaks of human yersiniosis are also well established by investigators in Japan (Zen-

Yoji and Maruyama 1972, Chiesa *et al* 1987). In Australia, South Australia leads in reported cases of human yersinosis followed by Queensland and New South Wales (Robins-Browne 1992). The disease is uncommon in tropical and developing countries (Robins-Browne 1992) although there are reports of the isolation of *Y. enterocolitica* from pigs in Nigeria (Adesiyun *et al* 1986) and of outbreaks of water associated *Y. enterocolitica* in humans in Bangladesh (Samadi *et al* 1982). Isolation of *Y. enterocolitica* has also been reported from the West Indies (Adesiyun *et al* 1992), Latin America (Falcao 1981) and Singapore (Ho and Koh 1981).

There is a definite pattern in the geographical distribution of serotypes and biotypes of *Y. enterocolitica* with O:3 and O:9 being the most prevalent pathogenic strains for humans in Europe, Canada and Japan and USA. An increase in the number of O:3 isolation has been reported from around the world making this the most frequently isolated strain (Ostroff 1995). However, in the last decade an increase in *Y. enterocolitica* O:9 infections has been reported from Great Britain, and serogroup O:8 from Western Europe and Japan (Chiesa *et al* 1991b).

GENERAL CHARACTERISTICS OF *YERSINIA* SPECIES

THE BACTERIUM

Yersiniae are gram negative, asporogenic bacteria appearing as straight rods or coccobacilli, 0.5-0.8 μm in diameter and 1.3 μm in length. Pleomorphism occurs depending on the type of medium, incubation time and temperature used. A capsule is not present but an envelope occurs in *Y. pestis* grown at 37°C and in cells from in vivo samples. Yersiniae are facultatively anaerobic, having both a respiratory and fermentative type of metabolism. They ferment glucose and other carbohydrates with acid production but little or no gas. They are oxidase negative and catalase positive. With few exceptions nitrate is reduced to nitrite. Phenotypic characteristics are often temperature dependant, and usually most characteristics are expressed by cultures incubated at 25-29°C rather than at 35-37°C. Yersiniae occur in a broad spectrum of both live and inanimate habitats

with some species adapted to specific hosts. They are non-motile when cultured at 37°C but motile when cultured at 22-29°C except *Y. pestis* which is always non-motile. (Bercovier and Mollaret 1984) Fresh isolates may require a few subcultures to express their motility. Motile organisms have 2-15 peritrichous flagellae characterised by a long wave motion (Nilehn 1969). Other properties such as biochemical characteristics and virulence factors are also temperature dependent.

Unlike the biochemical homogeneity found among strains of *Y. pestis* and *Y. pseudotuberculosis*, strains of *Y. enterocolitica* differ substantially in their biochemical reactions. Two biotyping systems have been developed. The five biotypes as defined by the Wauters system are based on eight biochemical reactions; most human strains are biotype 4, although biotype 1 and 2 are occasionally pathogenic for humans (Wauters 1981, Stern and Pierson 1979) Using a similar system with several additional biochemical reactions, five biotypes were described by Nilehn in 1969. Typical strains of *Y. enterocolitica* ferment sucrose and utilise rhamnose, citrate and melibiose (Bottone 1977). Atypical strains usually ferment rhamnose or are unable to ferment glucose. *Y. enterocolitica* can be divided into four major groups by DNA-techniques (Brenner *et al* 1980). The enteropathogenic strains all belong to one subgroup and were identified as the typical *Y. enterocolitica* like-bacteria. More than 60 O-antigens have been identified which have resulted in more than 50 different serotypes within the *Y. enterocolitica* group of organisms.

GROWTH REQUIREMENTS

Enteropathogenic yersiniae grow fairly well on simple media such as MacConkey and deoxycholate agar showing reasonable growth within 24-48 hours. They multiply at temperatures between 4°C and 42°C with an optimum range of 27-30°C. (Barton 1992). Growth of *Y. enterocolitica* at 0-2°C in milk after 20 days has been observed (Leistner *et al* 1975) and Hanna *et al* (1977) reported growth of *Y. enterocolitica* strains in raw beef at 0-1°C after prolonged storage. *Y. enterocolitica* grows well at 25°C but requires additional factors for growth at 37°C (Swaminathan *et al* 1982). The organism withstands

freezing and survived in chicken stored at -18°C for more than 90 days (Leistener *et al* 1975). The success of low temperature enrichment is not because all other bacteria stop growing or die, but because the growth rates of microbial antagonists are slowed relative to that of *Y. enterocolitica* (Schiemann 1989).

Y. enterocolitica is destroyed by standard pasteurisation methods (Francis *et al* 1980) and stressed after mild heat treatment (Hanna *et al* 1988). However, heat treatment for 3 minutes at 60°C can destroy the organism (Hanna *et al* 1977). *Y. enterocolitica* has been reported to grow and survive for long periods in water and milk cartons (Stanfield *et al* 1985). One study reported proliferation of *Y. enterocolitica* in stored blood at 4°C and subsequent sepsis in the recipient patient (Jones *et al* 1993).

The optimum range for the growth of *Y. enterocolitica* is pH 7.6-7.9 (Hanna *et al* 1979, Falcao *et al* 1979). Growth of *Y. enterocolitica* in brain heart infusion was better at pH 7 and 8 than 6 or 9 (Hanna *et al* 1977) and little or no growth occurred at pH 5 (Hanna *et al* 1977).

Y. pseudotuberculosis can survive under alkaline condition better than any other known Gram-negative bacterium (Aulisio *et al* 1980). *Y. pseudotuberculosis* serotype 4b survived but did not grow in experimentally inoculated raw pork at 6°C and at 25°C (Fukushima 1987).

Basic nutritional requirements such as glutamic acid, thiamine, cystine and pantothenate are essential for the growth of *Y. pseudotuberculosis* (Burrows and Gillett 1966). Schiemann (1989) reported that at 28°C *Y. pseudotuberculosis* required no supplements such as thiamine or pantothenate but that these were needed for growth at 37°C .

ISOLATION

Yersiniae can grow well on enteric media and the colonies strongly resemble several other common *Enterobacteriaceae*. Yersiniae may be isolated from clinical and food samples by direct plating on a variety of differential and selective agars used for enteric bacteria. Most strains of *Y. enterocolitica* will grow on selective enteric media, and will appear as small, lactose-negative colonies on MacConkey and Salmonella-Shigella agars in 48 hours (Schiemann 1989, Aulisio *et al* 1980). Schiemann (1979) developed a differential medium called cefsulodin-irgasan-novobiocin (CIN) which is highly selective for *Y. enterocolitica*. This medium is reported to provide a high rate of recovery of strains of *Y. enterocolitica* representing various serotypes. The medium requires 24 hours incubation at 25°C for the development of a unique colonial morphology for presumptive identification. *Y. enterocolitica* appear as colonies 0.5-1.0 mm in diameter with dark red "bull's eye" centres and transparent borders. Other media like Bismuth sulphite agar and MacConkey agar with Tween 80 have also been used. Incubation at 37°C is not recommended since *Yersinia* grows slowly and may be overgrown by other enteric microorganisms. Furthermore, the virulence plasmid is unstable and may be lost spontaneously during overnight growth at 37°C.

Samples for isolation of *Yersinia* can come from a wide range of sources like faecal material and throat and rectal swabs from pigs (Greenwood and Hooper 1985), palatine tonsils from pigs (De Boer *et al* 1986), wild animals (Kapperud 1975 and 1981), drinking water and environmental surface swabs (Langeland 1983) and abscesses, blood, lymph-nodes and stools from humans (Carniel and Mollaret 1990). Using standard bacteriological procedures the isolation and identification of *Yersinia* is not difficult from normally sterile sites. Recovery of the organism from stools, on the other hand, is hampered by the lack of development of the characteristic colonial morphology as well as the slow growth of the organism in competition with the normal faecal flora. Laboratory diagnosis can be facilitated by an awareness of the unique temperature-related growth characteristics of the species, as well as employment of selective media. Cold enrichment

at 4°C can be useful in detecting small numbers of organisms and organisms from highly contaminated specimens (Greenwood *et al* 1975).

Nesbakken and Kapperud (1985) used three different isolation procedures for the recovery of naturally occurring yersiniae from porcine tonsils and concluded that phosphate buffered saline (PBS) and cold enrichment should be included in any isolation procedure regardless of the type of yersiniae sought. The procedure was the most effective single method for the recovery of *Y. enterocolitica* O:3/biotype 4, biotype 1 and *Y. kristensenii*. Several authors (Greenwood and Hooper 1985, Davey *et al* 1983, and Ratnam *et al* 1983) have recommended the combination of cold enrichment in M/15 PBS at pH 7.6 with subsequent plating onto CIN agar. This combination was extremely efficient not only for the recovery of *Y. enterocolitica* serotypes O:3 and O:9 but also for other types and species of the genus from a variety of samples. Methods for isolation of *Yersinia* from foods are problematic due to competition and overgrowth by the food microflora.

For efficient recovery of yersiniae from food samples, the use of low temperature enrichment similar to that used for faecal specimens is necessary (Schiemann 1989). Incorporation of bile salts and sorbitol (Mehlman *et al* 1978) or peptone (Weagnant *et al* 1983) in the enrichment medium improves the rate of isolation. Modified Rappaport broth is effective for isolation of *Y. enterocolitica* O:3 and O:9, but is less useful for other biotypes or serotypes (Wauters 1973).

Aulisio *et al* (1980) observed that *Y. enterocolitica* tolerated short exposures to weak alkali better than other *Enterobacteriaceae* and taking advantage of this property, mixed aliquots of cold enrichment cultures from naturally and experimentally contaminated food samples with 0.5% potassium hydroxide before streaking on MacConkey agar or CIN agar. The alkali treatment was reported to increase the yield of both *Y. enterocolitica* and *Y. pseudotuberculosis* four fold, and the sensitivity one hundred fold.

Y. pseudotuberculosis will grow on most clinical isolation media, such as blood agar and MacConkey agar. However CIN agar is the preferred medium, since it is more differential for *Yersinia* species, although slightly inhibitory to some strains of *Y. pseudotuberculosis* (Fukushima 1985). Mair and Fox (1986) recommend the use of lactose-sucrose-urea agar as a selective medium for faecal samples.

IDENTIFICATION

Suspect *Yersinia* colonies from CIN agar or other plates should be picked for further screening. Many biochemical tests have been developed for the presumptive diagnosis of *Yersinia* organisms. *Yersinia* are lactose negative, do not produce hydrogen sulphide in triple sugar iron (TSI) medium, but are urease positive. Presumptive identification is achieved by inoculating a colony on to TSI and urea agar (Wauters 1973). Urease positive strains (pink colour) that show TSI acid butt (yellow) plus alkaline (red) or acid (yellow) slant (A/A or K/A), and no gas or blackening (H₂S production) after 24 hours at 28-29°C are verified by Gram staining and testing for motility at 28°C and absence of motility at 37°C (Lassen 1975, Christensen 1980). Weagant (1983) developed lysine-arginine-iron agar (LAI) medium for presumptive identification of *Y. enterocolitica* isolates. This medium is a modification of lysine iron agar (LIA) to lysine-arginine-iron agar used for *Yersinia*. Positive reaction on LAI are alkaline slant (purple), acid butt (yellow), no H₂S, no gas formation after 24 hours at 28-29°C. The reaction of *Y. enterocolitica* on this medium is more reliable and distinctive than LIA. Biochemical tests including sugars, urea, aesculin, methyl red (MR), Voges-Proskauer (VP), Simmons citrate, arginine, ornithine and lysine are used to differentiate the various *Yersinia* species.

The main biochemical tests that differentiate *Y. pseudotuberculosis* from *Y. enterocolitica* are ornithine decarboxylase, sucrose and sorbitol; *Y. pseudotuberculosis* is negative whereas *Y. enterocolitica* is positive for these three tests. *Y. pseudotuberculosis* and most strains of *Y. enterocolitica* are urease positive (Quinn *et al* 1994).

BIOTYPES AND SEROTYPES

Yersinia species are differentiated from one another by distinguishing biochemical and serological reactions. Unlike the biochemical homogeneity found among strains of *Y. pestis* and *Y. pseudotuberculosis*, strains of *Y. enterocolitica* differ substantially in their biochemical reactions (Bercovier and Mollaret 1984, Kapperud and Bergan 1984). Determination of its antigenicity enables the identification of the serogroup to which a certain strain of *Y. enterocolitica* belongs. It is this variability that resulted in the establishment of several new species and subgroups of *Y. enterocolitica*.

Two biotyping systems have been developed. Initially Nilehn (1969) grouped *Y. enterocolitica* into five biotypes. Using a similar system with several additional biochemical reactions, the five biotypes as defined by Wauters system are identified by eight biochemical tests (Wauters *et al* 1987) which include the pyrazinamide, 5-D glucosidase and proline peptidase reactions. Most human strains are biotype 4, although biotypes 1 and 2 are occasionally pathogenic for humans. Wauters *et al* (1988b) divided biotype 1 to differentiate the non-pathogenic environmental strains (1A) from the North American pathogenic strains (1B) and also proposed the creation of biotype 6 to accommodate the non-pathogenic *Y. enterocolitica* strains biotype 3A and 3B within biotype 3. Biotype 3A and biotype 3B are now called *Y. mollaretii* and *Y. bercovieri* respectively (Wauters *et al* 1988b). Table-1 shows the biochemical differentiation of 6 *Y. enterocolitica* biogroups (Wauters *et al* 1987) and Table-2 presents the differentiation of species within the genus *Yersinia* (Bercovier and Mollaret 1984, Wauters *et al* 1987).

Serotyping is determined by bacterial agglutination using rabbit hyperimmunised serum with anti-O antisera in conjunction with biotyping to define those strains of *Y. enterocolitica* that are potentially pathogenic for humans. The serotypes that have the most clearly defined potential for human disease are O:3, O:9, O:5,27 and O:8. (Mair and Fox 1986). The significance of other strains is often uncertain and cross-reactions with strains of other biogroups or species may occur. For example, non-pathogenic strains cross-reacting with antisera to O:3 and O:8 are found in biogroup 1A, and some *Y.*

frederiksenii share a common antigen with O:3 or O:9. The accurate identification of pathogenic strains necessitates consideration of both biochemical and antigenic characteristics.

Y. pseudotuberculosis has been separated into six serogroups. Type I to VI (Thal 1978). Subtypes A and B have been identified for types I, II, IV and V (Tsubokura *et al* 1970).

Table-1: Biochemical differentiation of *Y. enterocolitica*.

Biogroups	1A	1B	2	3	4	5	6
Lipase	+	+	-	-	-	-	-
Aesculin/Salicin	+/-	-	-	-	-	-	-
Indole	+	+	Delayed positive	-	-	-	-
Trehalose/Nitrate	+	+	+	+	+	-	+
5-D-Glucosidase	+	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+	+	-
Proline peptidase	V	-	-	-	-	-	+

Table-2: Biochemical differentiation of species within the genus *Yersinia*.

Biochemical Test	<i>Y. pestis</i>	<i>Y. pseudotuberculosis</i>	<i>Y. enterocolitica</i>	<i>Y. frederiksenii</i>	<i>Y. intermedia</i>	<i>Y. kristensenii</i>	<i>Y. aldovae</i>	<i>Y. bercovieri</i>	<i>Y. mollaretii</i>	<i>Y. rohdei</i>
Indole	-	-	V	+	+	V	-	-	-	-
Ornithine	-	-	+	+	+	+	+	+	+	+
Motility 25 °C to 28 °C	-	+	+	+	+	+	+	+	+	+
Fermentation of sucrose	-	-	+	+	+	-	-	+	+	+
Rhamnose	-	+	-	+	+	-	+	-	-	-
Cellobiose	-	-	+	+	+	+	-	+	+	+
Sorbitol	-	-	+	+	+	+	+	+	+	+
Melibiose	V	+	-	-	+	-	-	-	-	V

+ = 90% or more strains are positive; - = 90% or more strains are negative; V = 11-89% of strains are positive. Adapted from Bercovier *et.al* 1984; Wauters *et.al* 1987. All tests were performed at 25 °C to 28°C.

EPIDEMIOLOGY

ENVIRONMENT

Y. enterocolitica and related species are widely distributed in the terrestrial and aquatic environments, which are the sources of the organism for warm-blooded animals. Although Mehlman *et al* (1978) reported that *Y. enterocolitica* does not survive or multiply in water at low temperatures in the absence of organic matter, the organism has been isolated from water by a number of investigators (Toma and Deidrick 1975, Harvey *et al* 1976, Schiemann 1978 and Kapperud 1981). In general, isolates of *Y. enterocolitica* from water differ from those implicated in human disease. (Toma 1973, Schiemann 1978). The transmission of *Y. enterocolitica* to humans via contaminated water is possible, but there are few published reports of outbreaks except with serotype O:8. Serotype O:8, the predominant human pathogenic strain of *Y. enterocolitica* in USA, appears to be rare in pigs (Schiemann 1989), and is most likely transmitted to humans by ingestion of water from streams, lakes or ponds and by consumption of food washed with contaminated water (bean sprouts, tofu) and milk products (Black *et al* 1978). *Y. intermedia* and *Y. frederiksenii* are found mainly in fresh water, fish and food and are occasionally isolated from humans. *Y. kristensenii* is mainly found in soils and other environmental samples as well as foods but is rarely isolated from humans (Kreig 1984).

Yersinia pseudotuberculosis is widespread in the environment probably through contamination by faeces of infected rodents and birds (Kaneko and Hashimoto 1981, Carniel and Mollaret 1990).

HUMANS

Although Winblad (1973) indicates that the organism may be transmitted from person to person by contact, there is still lack of evidence as to whether humans serve as a reservoir of *Y. enterocolitica* and if the organism is transmitted via the faecal-oral route. There are reports of the isolation of *Y. enterocolitica* from butchers, food handlers, and

kindergarten and primary school children. The rate of isolation from butchers and kindergarten children was greater than from food handlers and primary school children (Shayegani *et al* 1981). A hospital outbreak of *Y. enterocolitica* infection in Finland was due to contact with an infected patient who apparently transmitted the organism to hospital personnel (Toivannen *et al* 1973). Yersiniosis in young children who had no contact with infected animals has been reported (Albert and Lafleur 1971, Lafleur *et al* 1972).

ANIMALS

For a long time animals have been regarded as the main source of human *Y. enterocolitica* infections and a large number of surveys have been carried out to identify animal reservoirs (Wauters and Janssens 1976, Ahvonen 1973, Tsubokura *et al* 1973, Leistener *et al* 1975, Toma and Deidrick 1975, Christensen 1980, Hurvell *et al* 1981). Pigs appear to be the principal carriers of serovars of *Yersinia enterocolitica* that are pathogenic to humans, for example serotypes O:3 and O:9 (Schiemann 1989, Carniel and Mollaret 1990). Although a definite connection between isolates from pigs and human illness is still to be established, it has been suggested that pigs play an important role in human infection (Pedersen 1979, Wauters 1979). Pigs are healthy carriers and detection of infection at routine, organoleptic meat inspection is consequently not possible (Christensen 1987). *Y. enterocolitica* has been frequently isolated from the tonsils and intestines of pigs and these animals are considered to be major reservoirs of the O:3 serotype. The incidence of *Y. enterocolitica* in animals varies, not only between countries, but also within a country. Conflicting isolation rates may depend not only on the kind of specimens examined but also on the distribution within the herds (Christensen 1980). Christensen (1980) studied the transmission of *Y. enterocolitica* O:3 within and between herds of pigs in Denmark by examination of faecal specimens and concluded that *Y. enterocolitica* O:3 had a herd or farmwide distribution and that open management type farming in which 6-8 weeks old pigs are purchased by the farmers from various pig markets or pig producers was an important factor in the spread of *Y. enterocolitica* between the pig herds.

Y. enterocolitica infections in cattle, sheep, goat, chinchillas and hares are also on record (Swaminathan *et al* 1982) and dogs and cats have been incriminated as potential reservoirs of this micro organism (Ahvonen *et al* 1973, Toma *et al* 1973). Dogs, cats and occasionally rats may be faecal carriers of O:3 and O:9, and the intimate contact between man and pets suggests a potential reciprocal transmission, although such an epidemiological link has not been confirmed (Carniel and Mollaret 1990). Wild animals, including rodents, birds and insects have also been shown to harbour *Y. enterocolitica* (Botzler *et al* 1976, Kaneko *et al* 1978, Kaneko and Hashimoto 1981). The bio-serotypes most often isolated from these species are not those commonly involved in human infections.

Y. enterocolitica was recovered from crabs (Faghri *et al* 1984), and *Y. enterocolitica* biotype 2, serotype O:5, 27, demonstrating virulence characteristics was recovered from a common garter snake (Kwaga *et al* 1993).

Like *Y. enterocolitica*, *Y. pseudotuberculosis* is widely distributed among domestic pets (Yanagawa *et al* 1978, Fukushima *et al* 1984 and 1989, Tsubokura *et al* 1984), farm animals (Tsubokura *et al* 1984), wild animals (Bercovier *et al* 1978), birds, and occasionally reptiles (Obwolo 1980) and most animal species are potential carriers of *Y. pseudotuberculosis* with deer, goats and rodents being the animals most commonly involved (Kappeurd 1975, Fukushima *et al* 1987). The organism has a worldwide distribution with many reports from zoo animals and aviaries in the Northern Hemisphere (Barton 1992).

FOOD

Although the epidemiology of *Y. enterocolitica* is incompletely understood, this organism is recognised as a significant foodborne pathogen. *Y. enterocolitica* and related species have been isolated from many types of foods in many countries and several studies indicate that consumption of contaminated food (Lee *et al* 1981, Tacket *et al* 1984, Kapperud 1991) and water (Toma *et al* 1973, Harvey *et al* 1976) are important

factors in the epidemiology of *Y. enterocolitica* infections. In outbreaks of yersiniosis in the USA, the food items incriminated were chocolate milk, powdered milk, pasteurised milk, chow-mein, tofu, bean sprouts and chitterlings (Kapperud 1991). The majority of food isolates differ in biochemical and serological characteristics from typical clinical strains and are usually termed non-pathogenic or environmental strains. These include the related ubiquitous species *Y. frederiksenii*, *Y. kristensenii*, *Y. intermedia*, *Y. aldovae*, *Y. rhodei*, *Y. mollaretii* and *Y. bercovieri*. In a survey from Palmerston North, Hudson (1992) isolated *Y. enterocolitica* from ready to eat cooked meat products and concluded that contamination occurred after cooking.

SEASONAL PREVALENCE

Y. enterocolitica differs from other enteric pathogens in that human infections appear to peak in autumn and winter months (Robins-Browne 1991). In Belgium and Sweden, infection due to *Y. enterocolitica* occurs most frequently from September to December peaking in October (Vandepitte *et al* 1973, Wauters 1979). In South Africa, the incidence of *Y. enterocolitica* infection is highest during the period between December and May (Rabson and Koornhof 1973). The increased incidence of clinical infections due to *Y. enterocolitica* and *Y. pseudotuberculosis* during the cold season has at least two reasons: first, the bacteria survive and multiply better in the environment at low temperatures; and secondly, animals which are healthy carriers of the organism excrete large numbers in their faeces after a stress like cold weather or starvation (Carniel and Mollaret 1990).

TRANSMISSION

The transmission of *Y. enterocolitica* is to a large extent unknown. The most likely portal of entry of the organism in both animals and in man is the digestive tract. Since *Y. enterocolitica* has been isolated from the intestinal contents of a great number of animals, it has been presumed that this organism would follow the same epidemiological pattern as other *Enterobacteriaceae* like *Salmonella* and *Escherichia* species. The

proposed mode of infection with *Y. enterocolitica* in humans is by the intake of contaminated food, less likely is contact with infected animals or person-to-person infection (Morris and Feeley 1976, Kapperud 1991). Several investigators (Toma *et al* 1973, Harvey *et al* 1976, Schiemann 1978, Kapperud 1981) have reported that water may be the main reservoir for *Y. enterocolitica* and that transmission may result from consumption of contaminated water.

Many countries report transmission *Y. enterocolitica* through pork products, since pigs are known healthy carriers of *Y. enterocolitica* and contamination can occur during meat processing. Cross contamination can also occur in the kitchen from raw pork products to other foods, which are not subsequently heat-treated (Lee *et al* 1990).

Transmission by blood transfusion has been reported (Cover and Aber 1989). The yersiniae present during transient bacteraemia in the donor may multiply in blood products stored at 4°C, without altering their appearance. Accordingly, large numbers of bacteria may be infused directly into patients receiving blood transfusion. Administered in this manner, even environmental strains of *Y. enterocolitica* could be as lethal as enteropathogenic strains (Robins-Browne 1992). A septic shock syndrome produced upon transmission is fatal in about 70% of cases (Jacobs *et al* 1989).

Transmission of *Y. pseudotuberculosis* occurs by consumption of contaminated food but contact with infected animals including pets is considered to be an alternative mode of transmission to humans (Carniel and Mollaret 1990).

YERSINIOSIS IN HUMANS

YERSINIA PSEUDOTUBERCULOSIS

Sporadic human infection with *Yersinia pseudotuberculosis* has been reported from all continents but only a few outbreaks are on record (Van Noyen *et al* 1995, Teritti *et al* 1984, Inoue *et al* 1984, Sato 1987). Before 1954, only a systemic form of *Y.*

pseudotuberculosis infection was recognised (Schiemann 1989). The disease resembles typhoid, and causes hepatic and myoproliferative disease in adults. Most reports suggest that the incidence of *Y. pseudotuberculosis* is less than that of *Y. enterocolitica* and that manifestation of the disease is usually less severe in man than in animals (Carniel and Mollaret 1990).

Y. pseudotuberculosis infection is now known to be associated with a variety of clinical signs like mild fever, diarrhoea, mesenteric lymphadenitis, terminal ileitis, arthritis and septicaemia (Mollaret 1965, Mair and Fox 1986). In most cases the organism migrates to the ileum and mesenteric lymph nodes, but does not colonise the organs or invade the blood stream (Carniel and Mollaret 1990). Abdominal pain in the right lower abdomen, which mimics appendicitis, often leads to surgery. The disease is usually self-limiting and the clinical symptoms disappear even without treatment, although intestinal necrosis has occasionally been reported (Ahvonen 1972). Reactive polyarthritis and erythema nodosum which occur a few weeks after the acute phase are frequently associated with the presence of the HLA-B 27 antigen (Aho *et al* 1973), however polyarthritis, arthralgia and other joint involvements are rarely reported as a complication of infection (Mair and Fox 1986). A disease called Far-eastern scarlatiniform fever or epidemic pseudotuberculosis occurs in the Far East of former USSR (Somov 1973). The disease is characterised by fever, scarlatiniform rash, arthralgia and acute polyarthritis. Other symptoms include fever and gastro-intestinal lesions (Mair and Fox 1986). Infection involving renal failure in children reported in Japan differs from European reports in which this has not been described (Bottone 1977). A few years ago infection due to *Y. pseudotuberculosis* was the most common cause of human yersiniosis in France, however this has gradually changed with the prominence of *Y. enterocolitica* infection (Carniel and Mollaret 1990).

YERSINIA ENTEROCOLITICA

Y. enterocolitica is an invasive enteric pathogen. The serotypes that have the most clearly defined potential for human disease are O:3, O:9, O:5,27 and O:8. (Mair and Fox

1986). The bacteria enter through the epithelium of the terminal ileum, penetrate the lamina propria and multiply in Peyer's patches and lymph nodes leading to lymphadenitis (Carniel and Mollaret 1990, Robins-Browne 1992). Usually the infection is limited to this area of the intestine and its lymph nodes, but occasionally the microorganisms can cause abscess formation in organs such as the liver and spleen (Robins-Browne 1989). The clinical presentation of acute yersiniosis in humans varies according to age (Robins-Browne 1992). The majority of clinical infections occur in children under five years and are associated with serotype O:3. Watery diarrhoea with occasional blood in stools, fever, headache and abdominal pain are common clinical signs. Diarrhoea is the most common symptom caused by serotype O:3, O:9 and O:5,27 (Carniel and Mollaret 1990). In children over five years of age the infection is often presented with abdominal pain (right iliac fossa syndrome), fever and little or no diarrhoea. Pseudoappendicular symptoms similar to those caused by *Y. pseudotuberculosis* are produced by serotype O:8 infection (Carniel and Mollaret 1990). Exudative pharyngitis and tonsillitis are symptoms commonly seen in adult patients (Robins-Browne 1992). Meningitis, endocarditis, sinusitis, septic arthritis, osteomyelitis and cellulitis of the lower limbs are other clinical signs reported in *Yersinia enterocolitica* infection. Although septicaemia is a rare complication, several cases have been reported in South Africa (Rabson and Koornhof 1972). Reactive arthritis, the most important immunological sequelae of yersiniosis in adult patients is quite common in Scandinavian countries where serogroup O:3 strains and the HLA-B27 histocompatibility antigen are highly prevalent (Robins and Browne 1992). Arthritis follows the onset of diarrhoea or the pseudoappendicular symptoms after 2-4 weeks. Kerato-conjunctivitis, urethritis, erythema nodosum, acute proliferative glomerulonephritis and rheumatic fever-like endocarditis have been reported from Scandinavian countries (Ahvonen 1972). Laboratory confirmation of infection is difficult and takes longer than for other gastrointestinal pathogens and often the reported number of cases do not reflect the true incidence of the disease (De Boer 1992).

YERSINIOSIS IN ANIMALS

YERSINIA PSEUDOTUBERCULOSIS

Yersinia pseudotuberculosis (serotype I, II and III) has been documented as a cause of severe infection in many species of mammals, including humans, primates, domestic ungulates, dogs, cats, rodents and birds (Fukushima *et al* 1984, Mackintosh and Henderson 1984a and 1984b, Wilson 1984, Slee and Button *et al* 1990). Outbreaks accompanied by high mortality may reach epidemic proportions in susceptible hosts. After oral entry the bacteria pass through the gastrointestinal tract and invade the mesenteric lymph nodes of the terminal ileum followed by spread of the organism to distal lymph nodes and subsequent invasion of the spleen and liver where micro-abscesses are formed. Rapid invasion can lead to septicaemia and death of the animal (Carniel and Mollaret 1990). Other clinical signs such as diarrhoea for 48 hours before death, advanced depletion of fat, varying degrees of enteritis, abomasitis, marked oedema of the mesentery and congestion of the lungs have also been reported (Macintosh and Henderson 1984a; Slee and Button 1990). *Yersinia pseudotuberculosis* is considered to be the possible cause of abortion in sheep in Germany (Mirle *et al* 1991). *Y. pseudotuberculosis* is commonly found in rodents, which probably represent the main source of infection for pigs. Diarrhoea in pigs has been reported in Canada (Schiemann 1989), Brazil (De Barcellos *et al* 1981) and New Zealand (Hodges *et al* 1984).

YERSINIA ENTEROCOLITICA

Domestic animals, including pigs, cattle, sheep, dogs and cats have been suggested as potential reservoirs of infection. (Robins-Browne 1989). *Y. enterocolitica* has been isolated from farmed deer (Henderson 1984), sheep (Slee and Button 1990) goats (Buddle *et al* 1988) dogs and cats (Fantasia *et al* 1985) and pigs (Carniel and Mollaret 1990).

Most human pathogenic *Y. enterocolitica* are able to colonise the lymphoid tissue of pigs without causing any clinical signs (Fukushima *et al* 1984). A study from China reporting the isolation of *Y. enterocolitica* from pigs with diarrhoea suggests that the bacteria may also be capable of producing overt clinical infections in pigs (Zheng 1987). Neonatal pigs are colonised and become intestinal and pharyngeal carriers but do not usually develop disease (Fukushima 1984, Schiemann 1989). Catarrhal enteritis in the small and large intestine and micro-abscesses in the intestine and liver have also been reported (Erwerth and Natterman 1987). *Y. enterocolitica* enteritis in colostrum deprived newborn piglets supplemented with human milk formula caused reduced milk uptake, decreased body weight gain, reduced liver weight and micro-abscesses in the liver and small intestines (Shu *et al* 1995).

In Australia sheep infected with *Y. enterocolitica* had watery diarrhoea and thickening of the mucosa with fibrinous inflammation of the spiral colon (Slee and Button *et al* 1990). Experimental *Y. enterocolitica* infection in ewes produced placentitis (Corbel *et al* 1992) and some strains of *Y. enterocolitica* (biotype I and II or *Y. intermedia*) produced abortion in sheep (Corbel *et al* 1992).

Y. enterocolitica strains has been isolated frequently from bovine faeces in the UK (Davey *et al* 1983) and in Australia (Hughes 1979). Many other animals, including wild animals, birds and insects have been shown to harbour *Y. enterocolitica*. However, symptoms associated with the presence of this organism are rarely recognised and the majority of strains isolated was found to be non-pathogenic (Kapperud 1981).

YERSINIOSIS IN NEW ZEALAND

HUMAN INFECTION

Human infection has been reported in this country due to both *Y. enterocolitica* and *Y. pseudotuberculosis* (Henshall 1963). The infection of *Y. pseudotuberculosis* was the first reported isolation from humans outside Europe. Reactive arthritis has been a

sequel to *Y. pseudotuberculosis* infection in some cases in New Zealand (Rose 1976). Malpass (1981) reported a case of mesenteric lymphadenitis involving *Y. pseudotuberculosis* in a soldier.

Although an early study conducted in Palmerston North failed to isolate *Y. enterocolitica*, leading to the conclusion that the organisms is only an occasional cause of enteritis in this country (Watson *et al* 1979). However, infection with this organism in humans is now considered to be the second most common foodborne infection after campylobacteriosis (McCarthy and Fenwick 1990). *Y. enterocolitica* serotypes O:3, O:5,27, O:8 and O:9 have been reported in New Zealand (Beeching *et al* 1985, Wright *et al* 1995), however about 90% of human yersiniosis in this country is due to biotype 4, serotype O:3. Fenwick and McCarthy (1995) who analysed data from the Auckland area observed a bimodal age distribution of infections. Infection was found to be most prevalent in young children of 0-4 years and in young adults of 20-23 years of age and more common in males than in females. Diarrhoea, reactive arthritis, and enteritis are the most commonly observed clinical signs (Ameratunga *et al* 1987, Jones and Burns 1987). Pharyngitis and rheumatic fever-like symptoms have been reported in a young boy (Lello and Lennon 1992). Fatal transfusion-related sepsis has been recorded in New Zealand. (Wilkinson *et al* 1991, Ulyatt *et al* 1991). It has been suggested that the psychrotrophic properties of *Y. enterocolitica* are the main reason for the multiplication and survival of the organism in stored blood products. Since *Y. enterocolitica* relies on free iron for growth, it was also suggested that prolonged storage of blood resulting in progressive haemolysis and increase in the iron concentration which is favourable for its growth (Wilkinson *et al* 1991).

ANIMAL INFECTION

Yersiniosis due to *Y. pseudotuberculosis* serotypes I, II, III, has become one of the most common causes of death in deer in New Zealand (Beatson 1984, Macintosh 1992). The growing incidence of yersiniosis in young deer due to food shortage and after stresses especially in the winter months is a great economic problem in deer farming. An

outbreak of yersiniosis caused by *Y. pseudotuberculosis* has been recorded in pigs. (Hodges *et al* 1984).

Sporadic infection and disease due to *Y. enterocolitica* has been reported in stressed deer (Henderson 1984). *Y. enterocolitica* biotype 4, serotype O:3 is known to occur at a high prevalence in New Zealand pigs which is similar to other parts of the world (De Allie 1994). Yersiniosis has been reported in other domestic animals such as cattle (Hodges *et al* 1984) and in sheep, where the organism was associated with abortion (Hartley and Kater 1964). In this country, there is an increased rate of isolation of *Y. enterocolitica* biotype 5 from domestic animals such as deer (Henderson 1984), sheep (Bullians 1987), and goats (Buddle *et al* 1988, Lanada 1990). In Europe this biotype has been infrequently isolated from rabbits, hares (Bercovier *et al* 1978, Nilehn 1969) and goats (Krostad *et al* 1972). An outbreak of diarrhoea in a goat-herd having a history of mixing with pigs has been reported (Orr *et al* 1987). Several strains of *Y. pseudotuberculosis* and *Y. enterocolitica* were isolated from tonsils and faecal matter from slaughtered pigs (De Allie 1994). Dogs may carry biotype 4, serotype O:3 asymptotically and hence might act as a potential source of infection for people and other animals (Fenwick *et al* 1994). Bosi (1992) reported the isolation of *Y. enterocolitica* serotype O:5,27 from a slaughtered deer, but no history or clinical signs were recorded. A wide range of clinical symptoms has been recorded in domestic animals in New Zealand, including diarrhoea in hoggets (McSporran *et al* 1984) and suppurative enteritis in cattle and sheep (Belton and McSporran 1988).

DIAGNOSIS

Definitive diagnosis is based on the isolation and identification of the organism from appropriate clinical specimens taken when symptoms are present. The diagnosis from food and milk samples also depends on many factors including the number of organisms present in the samples, the competing microflora, etc. The routine procedures used for the identification of *Yersinia* species including cold enrichment, selective enrichment and biochemical confirmation usually take 2-4 weeks to complete. Special

tests are performed to differentiate pathogenic from non-pathogenic strains. In most human enteric infections, in the absence of positive culture results, yersiniosis has been included in the differential diagnosis (Bottone 1981). In many instances *Y. enterocolitica* infection resembles inflammation of the terminal ileum and proximal large intestine caused by other bacteria like *Salmonella typhi*.

Serological testing has been used extensively in many countries for the diagnosis of *Yersinia* infections, following presumptive screening and biochemical identification. Enzyme linked immunosorbent assay (ELISA), radio immuno assay (RIA) and the complement fixation test (CFT) are also widely used (Robin-Browne 1992). They are practical and reliable methods because of their high sensitivity, however sera from some patients show prozone phenomenon (antigen-antibody reaction is incomplete in the presence of a high titre of antibodies) (Robin-Browne 1992). False positive reactions have occurred with *Y. enterocolitica* serotype O:9, due to antigenic similarities with organisms like *Brucella abortus*, *Escherichia coli*, *Morganella* species and *Salmonella* species. A four-fold increase in titre between acute and convalescent sera indicates a reliable and positive diagnosis (Robins-Browne 1992). Enzyme immunoassay (EIA) using Yops (*Yersinia* outer membrane proteins) as antigen has been used in the diagnosis of *Y. pseudotuberculosis* and *Y. enterocolitica* infections in sheep (Robins-Browne 1992). The indirect immunofluorescence (IF) test has been used to detect *Y. enterocolitica* O:3 isolated from the tonsils of pigs (Shiozawa *et al* 1991). Polymerase chain reaction (PCR) based on DNA testing is a new method for the genotypic characterisation of microorganisms (Nakajima *et al* 1992). A two-step PCR for the detection of pathogenic *Y. enterocolitica*, based on the use of two pairs of oligonucleotides in a nested configuration (Kapperud *et al* 1990a and 1990b) has the potential for providing a rapid, specific as well as sensitive method for detection of pathogenic bacteria. The use of immunomagnetic separation to concentrate the bacteria followed by PCR for identification has been suggested (Rasmussen *et al* 1995). An immunoblotting method using monoclonal antibodies was developed to detect *Y. enterocolitica* serotype O:3 from the faeces of pigs (Li *et al* 1992).

TREATMENT

Enteritis caused by infection with yersiniae is usually self-limiting, and specific treatment is rarely required. In the early stages of infection, antibiotic therapy is not advised, but systemic infection (septicaemia, intra-abdominal abscessation and mesenteric lymphadenitis) should be treated without delay, although patients with *Yersinia* infections generally show a poor response to antibiotics due to the intracellular nature of the organism (Robins-Browne, 1992). Like other *Enterobacteriaceae* most strains of pathogenic *Yersinia* produce beta-lactamase which makes them resistant to narrow-spectrum and short-acting antibiotics (Robin-Browne 1992). Therefore antibiotic sensitivity tests are often carried out prior to chemotherapy. *In vitro* susceptibility of *Y. enterocolitica* to aminoglycosides, tetracycline, chloramphenicol and sulpha combinations is well established. Combinations of antibiotics are useful in acute and septicaemic conditions (Horstein *et al* 1985). Trimethoprim-sulphamethoxazole is a useful treatment with any enteric infections. At present there is no general indication for the treatment of pigs with *Yersinia* infection, since clinical signs are so rare. *In vitro* studies indicate that isolates from pigs are sensitive to oxytetracycline, furazolidone, neomycin and sulphonamides. Adding tetracycline as a feed additive reduces the faecal excretion of the organism (Taylor 1992).

PREVENTION AND CONTROL

YERSINIA PSEUDOTUBERCULOSIS

Yersiniosis caused by *Yersinia pseudotuberculosis* is a zoonotic disease, which most often occurs after contact with sick animals (Ahvonen *et al* 1973). Avoiding contact with clinically ill animals including rodents and birds is the best way to prevent human infection particularly in children (Morita *et al* 1968, Schieman *et al* 1989). Improved hygienic practices, decreased stress e.g. sheltering during extreme weather conditions and the use of a formalinised vaccine containing *Y. pseudotuberculosis* serotype I, II, III have substantially reduced the incidence of disease in domestic deer. A sustained immunity is

conferred in young deer after administration of two consecutive doses of the vaccine a month apart (Macintosh 1993).

YERSINIA ENTEROCOLITICA

Asymptomatic intestinal carriage and faecal excretion of *Y. enterocolitica* by pigs and occasionally pet animals may serve as a source of infection for humans (Schieman 1989). Pig-shed hygiene is likely to play a major role in transmission of *Y. enterocolitica* between pigs since the organism is spread via the faecal oral route. Improved shed hygiene, reduced stock density, reduced stress and improved drainage may help to control the infection. Since flies play a role in the spread of *Y. enterocolitica* infection (Fukushima *et al* 1979), control of insects and rodent reservoirs helps to decrease the incidence of *Yersinia* infection (Taylor 1992).

In the meat-works bacterial contamination of meat usually occurs from oral and intestinal contents and from the slaughter house environment. In many countries meat inspection protocols insist on incision of mandibular lymph nodes, which may contribute to the contamination of carcasses. Special attention should be paid during excision of tongue, pharynx and particularly the tonsils and lymph nodes (Nesbakken 1988). Extreme care must be taken while removing the intestines and the circumanal incision (Anderson 1988, Nesbakken *et al* 1994). Properly constructed and maintained facilities and equipment are critical for the control of *Yersinia*.

The principal sources for outbreaks of yersiniosis have been processed and prepared food. Therefore, key factors in the prevention and control of *Yersinia* must focus on the use of clean and wholesome raw ingredients and proper sanitation of the processing environment. *Yersinia* species are more susceptible to disinfectants than other enteric bacteria and are readily killed by chlorine and other commercial sanitisers (Harakeh *et al* 1985). Because *Y. enterocolitica* is able to propagate at low temperatures, refrigerated storage will not guarantee control of this organism (Kapperud 1991).

Human infection due to *Yersinia* can also occur as a result of water contamination (Toma *et al* 1975, Harvey *et al* 1976, Schiemann 1978) and use of untreated water may play a role in contamination of meat during processing. Cross contamination of food can also occur prior to cooking, so care should be taken when preparing raw pork products together with other foods.

SECTION-B

YERSINIA AND FOOD HYGIENE

INTRODUCTION

Y. enterocolitica can cause disease in humans and animals, but often the source of infection is not known. Several large outbreaks of *Y. enterocolitica* infection worldwide have indicated food as the main source of infection (Lee 1977, Black *et al* 1978, Kapperud 1991). *Yersinia* species are ubiquitous and may be found in water, soil, vegetables, milk and a wide variety of meats, but the only known natural reservoir for *Y. enterocolitica* is pigs (Cover and Aber 1989). The isolation of *Y. enterocolitica* from foods, including pork, beef and chicken is well-established (Lee 1977, Hanna *et al* 1977, Mollaret 1979, Kapperud 1991). The ability of this organism to grow at low temperatures assists in its survival and multiplication during chilled storage of foods such as raw and cooked meat and in milk (Hanna *et al* 1977, Lee *et al* 1981). The organism can also survive freezing temperatures for long periods of time.

The presence of *Yersinia* species in food is not always associated with disease in consumers because most yersiniae are not pathogenic (Doyle *et al* 1981). Therefore, all isolates from foods should be tested for pathogenicity (enterotoxin production, cellular invasion and plasmid-encoded virulence). The ability of yersiniae to compete with other meat microbes is usually poor (Stern *et al* 1980), however elevated pH in meat favours the growth of *Y. enterocolitica* due to decreased growth potential of the competing microflora (Anderson *et al* 1991).

MEAT

PORK AND PORK PRODUCTS

The high prevalence of *Y. enterocolitica* in pigs throughout the world indicates that pigs may act as a reservoir for *Yersinia enterocolitica* infections in humans. The

predominant human serotype O:3 has consistently been recovered from caecal and mesenteric lymph nodes of apparently healthy pigs (Toma *et al* 1975). Moreover, it has been documented by many investigators that pork and pork products made from raw or under-cooked pork serve as a potential source of *Y. enterocolitica* infection for humans (Wauters and Janssens 1976, Asakawa *et al* 1979, Hanna *et al* 1977, Lee *et al* 1981, Fukushima 1985, Christensen 1987). Wauters and Janssens (1976) isolated *Y. enterocolitica* from 75 out of 142 pork tongues obtained from 34 different butcher shops in 19 Belgian communities. In the Netherlands, Narucka and Westendoorp (1977) recovered 22 *Y. enterocolitica* isolates of diverse serotypes as well as 7 isolates of *Y. pseudotuberculosis* from throat swabs of 163 healthy pigs. In a survey conducted in Norway, *Yersinia* species were isolated from the tonsils of 200 (43.4%) of 461 freshly slaughtered pigs. *Y. enterocolitica* comprised (92.7%) of the total number of isolates, while *Y. kristensenii*, *Y. intermedia* and *Y. pseudotuberculosis* comprised 0.3%, 0.5%, and 0.5% respectively (Nesbakken and Kapperud 1985). In Denmark, a tonsil swab survey of 2218 slaughtered pigs for the prevalence of *Y. enterocolitica* serotype O:3 revealed that 25% of the pigs and 82% of herds tested were positive (Pedersen 1976 and 1979). Japanese authors believe their results show that colonisation of tonsils by *Y. enterocolitica* is an important factor in contamination of pig carcasses and pork products (Inoue *et al* 1984). They suggested that alteration in slaughter techniques could prevent much of this contamination. *Y. enterocolitica* has also been isolated from pork products in Germany (Leistner 1975), the USA (Lee *et al* 1981) and Canada (Schiemann and Fleming 1981).

The incidence of *Y. enterocolitica* O:3 on carcass surfaces of freshly slaughtered pigs varies depending on a number of factors including the evisceration technique used. Manual evisceration caused the greatest incidence of *Y. enterocolitica* on the carcass surface, whereas the use of an automated bung cutter reduced the incidence markedly, particularly if supplemented with the use of plastic bags enclosing the anus and rectum (Anderson *et al* 1991, Nesbakken *et al* 1994).

BEEF AND CHICKEN

Cattle and sheep are generally not considered to be important carriers of pathogenic biotypes of *Y. enterocolitica*. However, beef sometimes may be consumed without adequate heating and there has been concern about *Y. enterocolitica* contamination of beef. A survey in Germany revealed the presence of non-pathogenic strains of *Y. enterocolitica* from faecal and meat samples of cattle (Leistner *et al* 1975) and environmental biotypes of *Y. enterocolitica* have been recovered from beef in Japan and USA (Inoue and Kurose 1975, Hanna *et al* 1976). Several strains of atypical *Y. enterocolitica* were isolated from vacuum packaged beef and lamb cuts (Hanna *et al* 1976). Leistner *et al* (1975) reported isolation of *Yersinia* species including *Y. enterocolitica* from samples of chicken from retail markets in Germany. The organism has also been isolated from chicken in Japan (Inoue *et al* 1984) and from turkey meat (Guthertz *et al* 1976). In New Zealand, the presence of *Y. enterocolitica* in ready to eat meat products including beef and chicken bought from retail supermarkets is very low and the majority of isolated strains have been found to be non-pathogenic serotypes (Hudson *et al* 1992).

SEAFOODS

Seafood has been implicated as the cause of foodborne *Y. enterocolitica* infection especially when consumed raw or undercooked. Strains of *Y. enterocolitica* have been isolated from oysters (Toma 1973, Lee 1977), mussels, shrimps, crabs (Peixotto *et al* 1980) and fish (Kapperud 1981).

OTHER FOODS

MILK AND MILK PRODUCTS

Y. enterocolitica has been isolated from raw milk in Australia (Hughes 1979), Canada (Schiemann 1978) and USA (Shayegani *et al* 1982). Schiemann (1978) reported

the presence of *Y. enterocolitica* in 18.2% samples of raw milk in the southern region of Canada and Christensen (1987) reported the isolation of the organism from 10% of 251 raw milk samples examined in Denmark. *Y. enterocolitica* has also been isolated from milking machines, raw milk and cream in Czechoslovakia (Aldova *et al* 1979). Although the serotypes of the isolates from milk were not in all cases the same as those recovered from people, consumption of raw milk or dairy products made from raw milk could pose a potential public health hazard. There are a few reports of the isolation of *Y. enterocolitica* from pasteurised milk and ice-cream (Schiemann and Toma, 1978, Black *et al* 1978). Isolation of *Y. enterocolitica* from pasteurised milk may indicate either a defect in the pasteurisation process or post-process contamination (Shayegani *et al* 1982). In the latter case *Y. enterocolitica* may proliferate well because of reduced competition from the limited surviving flora (Stern *et al* 1980) The presence of *Y. enterocolitica* in pasteurised milk should therefore be a cause for concern to dairy microbiologists.

VEGETABLES

Contamination by *Y. enterocolitica* of vegetables stored under refrigeration has been reported. In France, strains of *Y. enterocolitica* were recovered from carrots, tomatoes and green salads (Delmas and Vidon 1985), from bananas (O:3) (Mollaret 1972), from vegetables (Mehlman *et al* 1978) and from contaminated tofu (Soybean curd) in USA (Aulisio *et al* 1983). *Y. pseudotuberculosis* was recovered from 3.16% of stored fresh onions, carrots, beets and potatoes in the Soviet Union (Kuznetsov 1975, cited by Lee *et al* 1981).

FOODBORNE OUTBREAKS

Confirmed outbreaks of foodborne yersiniosis caused by *Y. enterocolitica* are comparatively rare (WHO 1987), however, extensive outbreaks have occurred in some countries, most notably in USA and Japan, and sporadic cases have been reported from many other countries (Cover and Aber 1989, Schiemann 1989). In USA, six major foodborne outbreaks have been documented and different food vehicles were identified

as sources of infection. One outbreak of intestinal illness due to *Y. enterocolitica* infection resulting in an unusual large number of appendectomies in Oneida county, New York, was caused by consumption of contaminated chocolate milk (Black *et al* 1978). Two large outbreaks of *Y. enterocolitica* infection involving 137 children and one adult who had gone for a camping trip were reported in Quebec, Canada (Kasatiya 1976). Also, in Japan, several large outbreaks have been reported involving infection with *Y. enterocolitica* serotype O:3 in school children, but the source of infection was not identified (Asakawa *et al* 1973). Although large outbreaks have only been traced to the consumption of contaminated milk products and water, sporadic outbreaks have been associated with eating of contaminated meat especially pork products (Lee *et al* 1990).

Infection in humans with *Y. pseudotuberculosis* is less frequent than with *Y. enterocolitica* (Carniel and Mollaret 1990, Van Noyen *et al*, 1995). Except for a few outbreaks reported from Finland and Japan, most cases have been sporadic (Terti *et al* 1984, Inoue *et al* 1988).

PUBLIC HEALTH SIGNIFICANCE OF FOODBORNE *YERSINA* SPECIES

The recovery of typical pathogenic biotypes from foods is infrequent with the exception of raw pork tongues and occasionally liver and meat products. Human pathogenic biotypes have been recovered from only a few types of food and from water samples, but there is substantial evidence that environmental biotypes of *Y. enterocolitica* are fairly common in drinking water, and foods such as raw milk, meats, oysters, fish and vegetables (Lee *et al* 1981, Kapperud 1991, De Boer *et al* 1992). The consumption of these foods and water would expose people to a regular intake of environmental biotypes of *Y. enterocolitica* of which the rhamnose positive biotype may be associated with human illness (Bottone 1977). Illness due to environmental strains is rare, however, confirmed cases of human infection caused by environmental biotypes of *Y. enterocolitica* suggest that a few strains may be virulent and can cause human disease under specific conditions (Bottone 1977).

ISOLATION AND IDENTIFICATION OF *Y. ENTEROCOLITICA* FROM FOOD

Y. enterocolitica and related species have been isolated from many types of food. The majority of isolates differ in biochemical and serological characteristics from typical pathogenic strains and are usually termed 'non-pathogenic' or environmental strains which include *Y. frederiksenii*, *Y. kristensenii*, *Y. intermedia*, *Y. aldovae*, *Y. rohdei*, *Y. mollaretii* and *Y. bercovieri*. The environmental strains, except for rare cases, are of little clinical significance (Kapperud 1991). The strains associated with human disease mainly belong to the serogroups O:3, O:5,27, O:8 and O:9. The increasing interest in *Y. enterocolitica* infection and the role of foods in some outbreaks of yersiniosis has led to the development of improved procedures for isolation of this organism from foods during the last 10-15 years. As the number of *Y. enterocolitica* organisms in foods usually is low and as there is often a quantitatively dominating background flora, isolation methods commonly involve enrichment of the sample, plating onto selective agar media followed by confirmation of typical colonies.

There are many problems related to the recovery of low numbers of *Y. enterocolitica*. The ability of current enrichment procedures to recover pathogenic strains of *Y. enterocolitica* from different foods is often inadequate probably because different strains require different conditions for optimum growth. Whereas serotypes O:3 and O:9 can grow in enrichment media used for *Salmonella* (Wauters *et al* 1973) serotype O:8 strains are sensitive to chemical compounds used in enrichment media and are very difficult to recover from foods. The environmental biotypes of *Y. enterocolitica* have intermediate to low resistance to selective enrichment broths and are easily recovered from foods. Their presence in foods makes it more difficult to recover pathogenic strains, probably due to competition for nutrients (De Boer *et al* 1992). Recovery procedures developed for environmental biotypes from uncontaminated foods are probably not applicable for the isolation of typical human pathogenic biotypes. In addition, recovery of *Y. enterocolitica* from many types of foods has not been evaluated by studies involving inoculation and subsequent recovery (Mehlman *et al* 1978).

ENRICHMENT

An efficient enrichment procedure should confer some selective advantage to the desired microorganisms, thereby allowing them to multiply more rapidly than the competing microflora. Being a psychrotrophic organism *Y. enterocolitica* is able to multiply at 4° C and enrichment at this temperature for 2-4 weeks is widely used as the first step in isolation procedures. At this low temperature, the growth rate of competitive bacteria is slowed sufficiently to enable *Y. enterocolitica* to multiply to numbers necessary for isolation on plating media. A large number of enrichment procedures, involving incubation at temperatures between 4° C and 35° C for time periods from 24 hours to 21 days have been proposed for the isolation of *Y. enterocolitica*. The procedures may be divided into prolonged enrichment periods, selective enrichment at higher temperatures for shorter periods, and two step enrichment procedures. Cold enrichment media include simple buffers like phosphate buffered saline (PBS), PBS modified by addition of 1% sorbitol and 0.15% bile salts (PBSSB) (Mehlman *et al* 1978), PBS with 0.5% peptone (Weagant 1983), trypticase soy broth (Schiemann 1983a) and tris-buffered peptone water, pH 8.0 (Greenwood and Hooper 1985). Cold enrichment using PBS has been widely used for the isolation of yersiniae from food and environmental samples (Fukushima *et al* 1984b). The addition of sorbitol (1%) and bile salts (0.15%) to reduce the incubation time and increase the selectivity of the medium has been used frequently in recovery methods (Mehlman *et al* 1978, Nesbakken *et al* 1985). Wauters (1973) formulated a modified Rappaport broth (MRB) containing magnesium chloride, malachite green and carbenicillin, in which the sample was enriched at 25°C for 2 days. The MRB has been used for many years as the first choice of media for the isolation of the major pathogenic serotypes of *Y. enterocolitica* in Europe although it has been shown to be the least satisfactory medium for the isolation of *Yersinia* species from food and environmental samples (Davey *et al* 1983, Delmas and Vidon 1985). Carbenicillin in MRB has been shown to inhibit strains of the common North American serotype O:8. (Schiemann, 1983, Gilmour and Walker 1988) and also the growth of certain strains of serotype O:3 (Schiemann 1983). In contrast, Wauters *et al* (1988) found that serotype O:3 strains are not inhibited by carbenicillin and that exclusion of this antibiotic results in a

decrease in selectivity of the enrichment medium. Another study of two modified selenite media effective for the recovery of certain strains of *Y. enterocolitica* from meats found that it is critical to limit the sample size of blended meat suspension to 0.2 g per 100 ml enrichment medium to restrict the growth of competitive bacteria, otherwise the slower growing *Y. enterocolitica* would be overgrown by the faster growing normal bacterial flora (Lee *et al* 1981). Several two step enrichment procedures have also been proposed for the isolation of *Y. enterocolitica* from foods. These involve pre-enrichment of the sample in a medium of low selectivity (Primary enrichment) followed by secondary enrichment in a medium of high selectivity. Schiemann developed a two step enrichment procedure in 1982 for the isolation of *Y. enterocolitica* from food. In this procedure, pre-enrichment for 9 days at 4 °C in yeast extract rose bengal broth is followed by selective enrichment in bile oxalate sorbose (BOS) broth at 22°C for 5 days. As the pre-enrichment medium is less selective, it will allow multiplication of small inoculates and repair of injured cells. BOS broth was found especially useful for the isolation of serotype O:8 strains, whereas strains of serotype O:5,27 were more difficult to recover (Schiemann 1983). In 1988, Wauters *et al* developed a new enrichment broth named ITC, derived from modified Rappaport broth and based on the selective agents irgasan, ticarcillin and potassium chlorate. In comparative studies, ITC broth was especially effective for the recovery of *Y. enterocolitica* from pork and porcine tonsils, while cold and two-step enrichments yielded better results for non-pathogenic strains (Wauters *et al* 1988, Kwaga *et al* 1990, De Boer and Nouws 1991). At the present time, no single enrichment procedure can be recommended for the isolation of *Yersinia* species from foods, and the use of multiple enrichment media is often advisable (De Boer 1992).

TEMPERATURE AND TIME

The incubation period of 21 days typically required for cold enrichment is often unacceptably long for use in the quality assurance of foods. Also, food and other environmental samples may contain large numbers of other psychrotrophic microorganisms, which may multiply and compete with *Yersinia* species during the cold enrichment period (Schiemann 1983). The types of other microorganisms present, the

ratio between these and *Y. enterocolitica* and the method of enrichment affect the time required for the isolation of *Y. enterocolitica* from mixed cultures (Swaminathan *et al* 1982). Consequently, it might be anticipated that enrichment procedures that are selective purely as a result of low incubation temperatures would be less effective when applied to environmental samples than when used for pathogenic strains. Schiemann *et al* (1983a) showed that an incubation of 15°C for two days was as efficient as incubation for 3 weeks at 4°C. Doyle *et al* (1981) were able to isolate *Yersinia* species by incubation in PBS for 1-3 days at 25°C. Greenwood and Hooper (1985) compared incubation in tris-buffered 1% peptone water at 21°C and 9°C and found that enrichment at 21°C was a suitable alternative to 9°C for *Yersinia* species from food samples but for maximum recovery, prolonged incubation was necessary. Several other enrichment procedures involving incubation at higher temperature for shorter periods and using selective media have been proposed (Hanna *et al* 1977 and 1980, Schiemann 1983a, Fukushima 1985, Ibrahim and Macrae 1991).

EFFECT OF pH

The effects of pH on the growth of *Y. enterocolitica* were determined in brain heart infusion at pH levels ranging from 5-9. Growth was rapid between pH 7 and 8, with the most ideal pH range being 7.6-7.9. Little or no growth was found over 24 hours incubation at pH 5 (Hanna *et al* 1977 and 1979). Falcao *et al* (1979) suggested a pH range of 7.6-7.9 as the ideal for optimum growth. The recovery of *Y. enterocolitica* from a sauce and its presence in pickled products indicate survival of *Yersinia* at much lower pH levels (Kutznetsov *et al* 1975).

ALKALI TREATMENT

Alkali treatment of samples was found to improve the isolation rate of strains of *Y. enterocolitica*, since these bacteria are more tolerant of high pH than most other Gram negative bacteria. By treating samples with 0.5% potassium hydroxide (KOH) solution before plating, the background flora is strongly reduced, making it easier to differentiate

Yersinia colonies from similar colonies on the isolation medium (Aulisio *et al* 1980). Following alkali treatment *Y. enterocolitica* may be recovered after only 1-3 days enrichment in PBS incubated at 25° C (Fukushima 1985). The length of exposure of *Y. enterocolitica* to the alkali solution is critical as times longer than 15 seconds may result in the loss of some strains (Doyle *et al* 1981). Alkali treatment has also been used for the isolation of *Yersinia* species from environmental samples (Doyle *et al* 1981). However, Schiemann (1983a) found that various factors, including medium, temperature and growth phase can influence the alkali tolerance and reduce the effectiveness of the treatment. Weagant (1983) concluded that a specific length of time for alkali treatment cannot be recommended and that streaking three to four successive plates from a KOH treated sample at 10 seconds intervals enhances the probability of isolating colonies of *Y. enterocolitica*, even in the presence of numerous other micro-organisms. Fukushima (1985) found that KOH treatment of meat samples is a valuable, rapid method for direct isolation of yersiniae from meat contaminated with more than 10² cells per gram. Wauters *et al* (1988) found that direct plating after KOH treatment is only suitable for strongly positive materials. Despite these limitations, a post enrichment alkali treatment could profitably be used for the isolation of yersiniae with relatively little extra cost or effort.

PLATING MEDIA

In general, the two most important factors to consider when choosing a selective plating medium are a high recovery rate of the desired microorganism and inhibition of the growth of competing microorganisms. Increasing the selective properties of a medium however, may result in reduced recovery of the desired microorganism or in failure to recover atypical strains. Therefore it is often necessary to reach a compromise (De Boer 1992). The production of a distinctive colony type by the desired microorganism is an additional advantage, in that it reduces the number of strains requiring identification by biochemical or other means.

Different agar plating media have been used for the isolation of *Y. enterocolitica* from clinical specimens and food. Initially, agar media like MacConkey agar, Salmonella-Shigella agar, deoxycholate-citrate agar and bismuth sulphite agar, designed for isolation of enteric pathogens, were used for *Y. enterocolitica*. Lee (1977) modified MacConkey agar with Tween 80 to improve differentiation of *Yersinia* colonies from lactose negative colonies. However, lipolytic *Yersinia* strains, which are easily recognised on this medium as white wrinkled colonies surrounded by sheen, are usually non-pathogenic (De Boer *et al* 1986). Salmonella-Shigella agar was found to be more selective for *Y. enterocolitica* after addition of sodium deoxycholate and calcium chloride (SSDC) (Wauters 1973 and Wauters *et al* 1988a). Colonies of *Y. enterocolitica* on this medium are small, round and colourless. However, some species of *Morganella*, *Proteus*, *Serratia* and *Aeromonas* can grow on SSDC and differentiation of *Yersinia* from colonies of these organisms can be difficult. Schiemann (1979) developed the now commonly used Cefsulodin-irgasan-novobiocin (CIN) agar as a selective and differential medium for *Yersinia enterocolitica*, on which organisms capable of fermenting mannitol, like yersiniae, produce red coloured "bulls eye" colonies. CIN medium was found to be inhibitory to *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*, but some *Enterobacter*, *Aeromonas* and *Proteus* strains show a colony appearance similar to *Yersinia* (De Boer *et al* 1986). The addition of 1 mg/ml of streptomycin improves the selectivity of CIN agar, but colonies of *Y. enterocolitica* become smaller (Schiemann 1989). The commercial availability of this medium makes it convenient to use. An additional advantage is that prepared agar plates may be stored for up to one month at 2°C without any reduction in the recovery rate of *Yersinia* species (Pedersen 1985). However, the same author recommended the use of CIN medium within 14 days of preparation since the recovery rate and colony size of *Y. enterocolitica* reduced during prolonged storage. On CIN agar, colonies of pathogenic and environmental *Yersinia* strains appear similar. Fukushima (1985) developed a selective medium called virulent *Yersinia enterocolitica* agar (VYE) for the isolation of pathogenic strains. These form red colonies on the VYE medium, while most environmental *Yersinia* strains form dark colonies with a dark peripheral zone as a result of aesculin hydrolysis. Of the media developed for the isolation of *Y. enterocolitica*, (CIN) agar (Schiemann

1979) is the most commonly used agar medium. Like BOS enrichment medium, CIN agar was developed only after basic studies on factors affecting the growth and inhibition of a range of serotypes (Schiemann 1979). This agar is highly selective for *Yersinia* species, allows a high recovery rate and produces a distinctive colony type (Gilmour and Walker 1988). Consequently, the confirmation rate for presumptive strains on CIN agar is high and only *Aeromonas hydrophila*, *Citrobacter freundii*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* show similar colony morphology (Harmon *et al* 1984). Although CIN agar is the most efficient medium for isolation of *Yersinia* species from food, at present no single plating medium can effectively isolate all strains.

IDENTIFICATION

Strains that produce the characteristic colony morphology on the selective agar should be identified by screening procedures involving two or more of the following media: triple sugar iron (TSI) or Klingler's iron agar (KIA) slopes, urea agar slopes, lysine decarboxylase broth, and a motility medium. In general, strains of *Y. enterocolitica* will produce an acid butt and slope on TSI or acid butt and alkaline slope on KIA with no gas or hydrogen sulphide produced in either; they are urease positive and motile at 22-30°C (Swaminathan *et al* 1982). The use of more than two primary screening tests is recommended for *Y. enterocolitica*, in particular for strains isolated from food and environmental samples as these may occasionally be biochemically atypical and may not produce the characteristic reactions (Bercovier *et al* 1978). Devenish and Schiemann (1981) have suggested that only KIA and urea agar slopes are required for confirmation of suspect colonies on CIN agar.

Some biochemical activities of *Yersinia* strains e.g. cellobiose, raffinose, indole, ornithine decarboxylase and Voges-Proskauer are temperature dependent (Bercovier and Mollaret 1984). Therefore, these tests are preferably incubated at 25°C or 30°C, rather than at 37°C. Many rapid tests are used for the differentiation of *Yersinia* from other genera. Miniaturised identification kits like API 20E (Bio Merieux) and the Minitex system (BBL) are valuable for rapid identification of *Y. enterocolitica*.

COMPARISON OF DIFFERENT ISOLATION TECHNIQUES FOR *YERSINIA* SPECIES FROM FOOD SAMPLES

Schiemann (1983a) proposed tryptone soy broth as the medium of choice for enrichment. Pre-enrichment in tryptone soy broth followed by enrichment in BOS showed improved recovery of human pathogenic strains of *Y. enterocolitica* from inoculated foods. In one study, the occurrence of *Y. enterocolitica* in foods was evaluated using different enrichment procedures. Most positive samples obtained within the shortest period of time were found using enrichment in PBSSB and BOS, with alkali treatment before plating on to CIN agar. However, only environmental serogroups of *Y. enterocolitica* were isolated (Delmas and Vidon 1985). The enrichment in BOS isolated more non-pathogenic strains than pathogenic strains of *Y. enterocolitica* from pork products (Wauters *et al* 1988a, De Boer and Nouws 1991, Kwaga *et al* 1990). In a different study, cold enrichment in PBSSB gave high isolation rates, mainly of non-pathogenic environmental strains of *Yersinia* species (De Boer 1992, De Boer *et al* 1986). In these studies MRB was found to be a suitable medium for the isolation of serogroup O:3 and O:9 strains, but much less efficient for the isolation of environmental strains. Enrichment in ITC was found to be the most efficient method for the recovery of strains of serotypes O:3 (Wauters *et al* 1988a). The usefulness of this enrichment broth for the isolation of other pathogenic serotypes remains to be determined. Alkali treatment often resulted in a marked increase in the number of *Yersinia* isolations. This occurred especially when high numbers of competing organisms were present, and when a plating medium with low selectivity, such as MacConkey agar was used (De Boer *et al* 1986).

Although *Yersinia* colonies can be difficult to recognise on MacConkey agar, this has been shown to be a useful medium for the isolation of *Y. enterocolitica* (Mehlman *et al* 1978, Schiemann 1979b, De Boer *et al* 1986). In several comparative studies CIN agar was found to be the most selective plating medium for *Yersinia* species (Aldova *et al* 1980, Harmon *et al* 1983, Gilmour and Walker 1988). However in one study *Y. enterocolitica* biotype 3b, serogroup O:3 strains were inhibited on CIN agar (Fukushima 1985). Differentiation of *Yersinia* colonies from other colonies is not always

easy on CIN agar (Fukushima 1985, De Boer *et al* 1986). Plating ITC enrichments on to SSDC isolated more O:3 strains than plating on to CIN agar (Wauters *et al* 1988a, De Boer 1992), but SSDC proved to be unsuitable after alkali treatment (Wauters *et al* 1988a). Addition of selective agents such as magnesium chloride, malachite green, bile salts, irgasan and the antibiotics carbenicillin, ticarcillin and cefsulodin to *Yersinia* isolation media results in growth inhibition of the Gram positive and part of the Gram negative flora.

Because the isolation media presently in use are not highly selective with respect to identification of pathogenic strains of *Yersinia* species, confirmation of the identity of presumptive colonies is always necessary. At present, no single isolation procedure is available for the recovery of all pathogenic strains of *Y. enterocolitica* from foods. Cold enrichment in PBS, PBSB, two step enrichment with TSB and BOS and enrichment in ITC are the procedures most commonly used. The commercially available CIN and SSDC are the most commonly used agar media for plating.

These enrichment and plating media are not particularly selective for *Y. enterocolitica* as they also support the growth of several other members of the *Enterobacteriaceae*. This makes the isolation of low numbers of yersiniae in samples containing contaminants rather difficult (De Boer 1992). Moreover, non-pathogenic environmental *Y. enterocolitica* strains are common in raw foods and hinder the isolation of pathogenic strains (Mehlman *et al* 1978). Cold enrichment and two step enrichment are efficient methods for the recovery of a wide spectrum of serotypes of *Y. enterocolitica*. However, usually laboratories are interested in the isolation of pathogenic serotypes and as the colony appearance of pathogenic and environmental strains on CIN and SSDC agar is similar, much work is required to select pathogenic strains from these media (De Boer 1992). This highlights the need for the development and evaluation of agar media selecting for pathogenic serotypes like VYE agar (Fukushima 1985). This also holds for SSMC agar, which is probably less suitable for pathogenic strains other than serotype O:3. For the recovery of strains of serotype O:8, enrichment in BOS and plating on CIN seems to be the most efficient procedure. Selection of the proper

enrichment procedure will depend on the bio/serotypes of *Yersinia* species sought and the type of food to be examined. The use of more than one medium for both enrichment and plating will obviously result in higher recovery rates of *Yersinia* species from foods (De Boer 1992).

IDENTIFICATION OF PATHOGENIC *YERSINIA* SPECIES FROM FOOD SAMPLES

Since *Y. enterocolitica* is an ubiquitous microorganism its presence may be expected in many foods. The majority of strains isolated are non-pathogenic (Delmas and Vidon 1985, De Boer 1986), so the mere presence of this organism may not be sufficient to render a food product unfit for human consumption. Consequently there is a need to determine the pathogenicity of isolates. Virulent strains of *Y. enterocolitica* contain a 70-kilobase-virulence plasmid (45 Mdas) and loss of this plasmid is accompanied by a loss in the ability to invade cells (Farmer *et al* 1992). The virulence activity of *Y. enterocolitica* is associated with a few bio/serogroups. Therefore identification of pathogenic strains depends upon serogrouping and biotyping of the isolates (Bottone 1981). In Europe, strains of serotypes O:3 (biogroup 4), O:9 (biogroup 2) and O:5,27 (biogroup 2) are the most frequently isolated human pathogenic strains. In North America, strains causing yersiniosis in humans usually belong to biogroup 1b (serotypes O:4, O:8, O:13a, 13b, O:18, O:20).

YERSINIA OUTER MEMBRANE PROTEINS (YOPS)

All virulent strains of *Y. enterocolitica* possess a plasmid approximately 70 Kilo base-pairs termed pVYC (Plasmid involved in *Yersinia* virulence) (Farmer *et al* 1992). The yops are a set of virulence and virulence accessory proteins synthesised and secreted by the three species of *Yersinia* pathogenic for humans. A few of these proteins are secreted into the growth medium and adsorbed back onto the cell surface, largely because of their extreme hydrophobicity and tendency to form aggregate. At least one yop is a true outer membrane protein (yop-1) comprised of identical 50 kd sub-units which combine to form intertwined chains that appear as delicate fibrillae on the cell surface

(Cornelis *et al* 1987). These structures mediate bacterial adherence to human epithelial cells and protect the bacteria from non-specific killing by complement in normal human serum. The role of other yops in virulence is not well defined. Some may protect the bacteria from phagocytosis while others induces cytotoxic damage to host cells.

Yersinia carrying this plasmid show calcium dependency, which is characterised by restricted growth at 37°C in media containing low concentration of calcium ions (Gemski *et al* 1980). Plasmid-bearing *Yersinia*, cultivated under those growth-restricting conditions, devote most of their energy towards the production and secretion of a series of plasmid encoded proteins, including several designated yops (Laird and Cavanaugh 1980). Many *Yersinia* virulence factors are thermally regulated in their expression and/or activity. Magnesium oxalate (MOX) was used to demonstrate calcium dependency at 37°C for some strains of *Y. enterocolitica* (Mair and Fox 1986). Gemski *et al* 1980, identified a profound difference in growth inhibition at 37°C on magnesium oxalate agar for invasive strains but not for non-invasive strains of *Y. enterocolitica*. Kapperud (1991) described that prolonged storage or subculturing might cause a loss of plasmids in strains of virulent *Y. enterocolitica*.

IN VITRO TESTS

There are many distinct features, which characterise strains of pathogenic *Y. enterocolitica* e.g. the ability to invade cells (virulence) and the ability to produce a heat-stable enterotoxin (Bercovier *et al* 1978, Pai and Mors 1978). Several *in vitro* tests have been used to determine the potential virulence of *Yersinia* isolates including tests for pyrazinamidase (Kandolo 1985), uptake of Congo red (Prpic *et al* 1985), calcium dependency at 37°C on magnesium oxalate agar (Gemski *et al* 1980), auto-agglutination at 37°C (Laird and Cavanaugh 1980), salicilin fermentation and aesculin hydrolysis (Shayegani *et al* 1981)).

Pyrazinamidase test

The pyrazinamidase test, which measures the activity of the enzyme pyrazin-carboxylamidase was used to define 381 isolates of yersiniae with respect to pathogenicity (Kandolo and Wauters 1985). A good correlation between virulence and a negative test for pyrazinamidase was found. The absence of pyrazinamidase activity is positive for pathogenic strains although it does not differentiate between plasmid positive and negative strains (Kandolo and Wauters 1985).

Congo red dye uptake

The ability of virulent strains of *Y. enterocolitica* to take up Congo red dye from media has been proposed as a simple method to detect pathogenicity (Prpic *et al* 1983). Although all virulent strains incorporate the dye, some avirulent strains also do, so that this method can only be used as a simple screening procedure (Prpic *et al* 1985). Congo red is now added to magnesium oxalate agar and this combination is referred to as Congo red-magnesium oxalate agar (CR-MOX). Subculturing of freshly picked pathogenic strains of *Y. enterocolitica* on CR-MOX produce small red colonies indicating presence of plasmids. Non-pathogenic strains lacking plasmids produce large colourless colonies on the same medium(Prpic *et al* 1983).

Calcium dependency at 37°C

Calcium dependency has been observed for *Y. enterocolitica*, which is correlated with the presence of the virulence plasmid and so provides a simple, inexpensive test (Gemski *et al* 1980). Virulent strains of *Y. enterocolitica* incubated on calcium free media do not grow or produce only small colonies at 37°C, but grow well at 25°C (Mair and Fox 1986).

Auto-agglutination

Virulent strains of *Y. enterocolitica* tend to agglutinate spontaneously and form a pellet at the bottom of test tubes when grown in tissue culture media incubated at 37°C. Auto-agglutination does not occur at 26°C or at either temperature with avirulent strains (Laird and Cavanagh 1980). Like calcium dependency, auto-agglutination provides a simple, inexpensive test to assess the pathogenicity of *Y. enterocolitica* strains. Une (1977), demonstrated that pathogenic strains of *Y. enterocolitica* were able to penetrate cultured He-La cells, while non-pathogenic strains of *Y. enterocolitica* did not. Screening *Y. enterocolitica* strains for the presence of a specific plasmid should provide an effective and rapid *in vitro* procedure to test for virulence. Environmental *Yersinia* species however may also possess plasmids of similar size, which presently have no known recognised function. Therefore plasmid detection should not be used as a sole indicator of virulence (Gilmour and Walker 1988). Salicin and aesculin are both β -glucosides that vary in the chemical structure of the compound attached to D-glucose by a beta-linkage. Both these compounds are hydrolysed by the enzyme β -glucosidase. These two tests are used regularly for differentiation of pathogenic from non-pathogenic strains. Pathogenic strains are negative and non-pathogenic are positive.

IN VIVO TESTS

Animal models

Initially, animal models were used to determine the invasive ability of *Y. enterocolitica*. The Sereny test or guinea pig conjunctivitis model (Sereny 1955) was used to assess the pathogenic potential of *Y. enterocolitica* (Pai and Mors 1978). Other tests like the suckling mouse assay for the production of the heat stable enterotoxin (Pai and Mors 1978) and the mouse intra-peritoneal challenge (Aulisio *et al* 1983) were used to test for virulence. The disease in mice mimics the major pathological features of human disease (Carter 1975). However animal testing tends to be costly and is subject to increasing public opposition and now has been largely replaced by *in vitro* tests.

CHAPTER TWO
EXPERIMENT
SECTION-A
MATERIALS AND METHODS

INTRODUCTION

Y. enterocolitica and related species have been isolated from many types of food. The majorities of food isolates differ in biochemical and serological characteristics from typical clinical strains and are usually called non-pathogenic or environmental strains. The growing interest in *Y. enterocolitica* infection and the role of foods in some outbreaks of yersiniosis in humans has led to the development of improved procedures for the isolation of this organism from foods. As the number of *Y. enterocolitica* organisms in food are usually low and as there is often a significant background flora, direct isolation on selective plating media is seldom successful (De Boer and Nouws 1991). The isolation of low numbers of *Y. enterocolitica* from food can be achieved only by a combination of appropriate methods of enrichment coupled with plating on selective and differential media. The long incubation period required by standard methods of cold enrichment is often unacceptable in connection with quality assurance of foods. Incubation at higher temperatures for shorter periods with the use of selective and differential media has been frequently documented in the literature (Doyle *et al* 1981, Greenwood and Hooper 1985). Many laboratories now use cold enrichment in PBS, modified with 1% sorbitol and 0.15% bile salts as PBSSB (Mehlman *et al* 1978), and plating on CIN agar (Wauters 1977), as the preferred procedure for the isolation of *Y. enterocolitica*, but at present no single isolation procedure is entirely satisfactory for the recovery of all pathogenic strains of *Y. enterocolitica* from foods.

ARTIFICIAL INOCULATION OF *Y. ENTEROCOLITICA* IN PORK PRODUCTS AND SUBSEQUENT RECOVERY

Little work has been done on artificial inoculation and subsequent isolation of *Yersinia* organisms from pork products. Recovery of *Y. pseudotuberculosis* (Fukushima 1985) and *Y. enterocolitica* (Fukushima and Gomyoda 1986) from inoculated raw ground pork incubated at 25° and 6°C was made only after inoculation of high number of these organisms. These results were supported by evidence of inhibition of growth of *Y. pseudotuberculosis* and *Y. enterocolitica* by *Enterobacteriaceae* and environmental *Yersinia* organisms.

In USA, Hanna *et al* (1977) studied the growth of *Y. enterocolitica* by artificial inoculation of raw and cooked beef and pork products. With three strains of *Y. enterocolitica*, increases in counts occurred on raw beef held over a 10 day period at 0-1°C. When inoculated raw or cooked beef and pork were stored at 7°C for 10 days or at 25°C for up to 24 hours, large increases in *Y. enterocolitica* counts occurred. *Y. enterocolitica* counts were found to be higher in cooked than raw products. In another study in USA, Harmon *et al* (1984) inoculated pork homogenate with different strains of *Y. enterocolitica* for the purpose of studying the effectiveness of different plating media for subsequent isolation. Of the seven plating media (CIN agar, bismuth sulphite agar, MacConkey agar, MacConkey-Tween 80 agar, cellobiose-arginine-lysine agar, desoxycholate citrate agar and *Salmonella-Shigella* agar) used, CIN agar was found to be the best for isolation of *Y. enterocolitica* from artificially inoculated pork homogenates.

The aim of this study was to determine the recovery rate of *Y. enterocolitica* from fresh pork mince inoculated with known numbers of a reference strain of *Y. enterocolitica* using different enrichment parameters (time, temperature and pH), cold enrichment and CIN as a selective plating medium.

OUTLINE OF METHODS USED

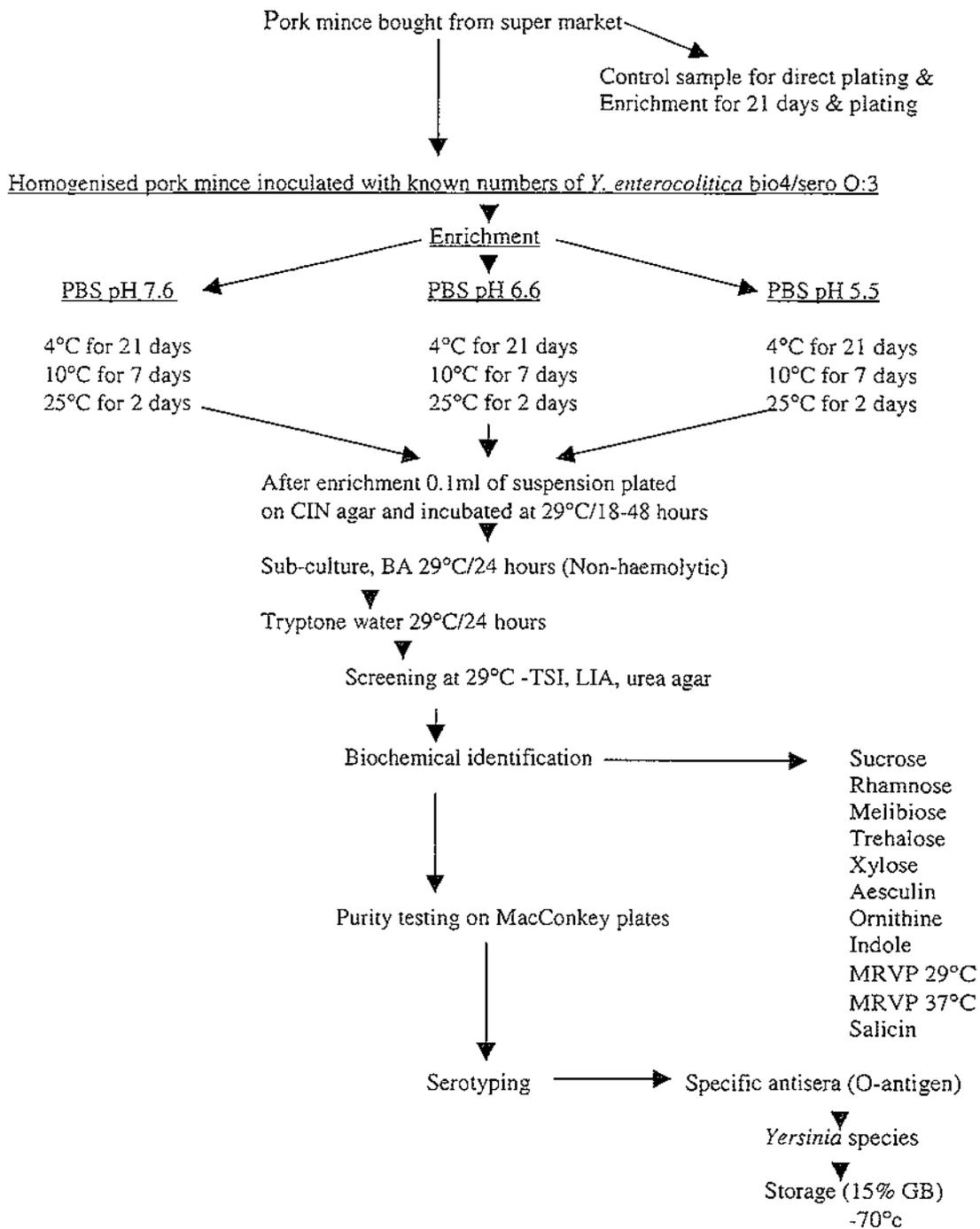
Pork mince was purchased from local supermarkets and 15g aliquots of samples were prepared by careful weighing. Selected samples were inoculated with known quantities of *Y. enterocolitica* biotype 4/serotype O:3 organisms and cold enriched in PBS with a pH of 7.6, 6.6 or 5.5 at 25°C for 2 days, 10°C for 7 days and 4°C for 21 days. CIN agar (DIFCO, USA) was used as a selective plating medium after enrichment (Table 3&4).

See the appendix for preparation of media used for the isolation, identification, virulence testing and storage.

PREPARATION OF BACTERIAL CULTURE

A reference culture of *Y. enterocolitica* biotype 4/serotype O:3 was obtained from Massey University Veterinary Microbiology Laboratory. This strain was used for the experimental contamination of the pork mince samples. Ten-fold dilutions of bacterial culture were made to arrive at a known number of organisms in each dose of inoculum. A drop of thawed isolate previously frozen was streaked on blood agar (BA). This was incubated overnight (18 hours) at 29°C. One loop of bacterial growth was picked from the BA plates and inoculated into 3 ml of brain heart infusion broth (BHI) the next morning and incubated for 8 hours at 29°C. The number of viable bacteria present in the inoculum was calculated by counting colonies grown from ten-fold dilutions by using procedures for the viable bacterial count (Quinn 1989). 1 ml of incubated BHI broth was added to 9 ml of normal saline to make a 1 in 10 suspension. In the same way 10^2 , 10^3 , 10^4 , 10^5 , 10^6 and 10^7 dilutions were made. From each dilution, 20 microlitres of bacterial inoculum was transferred onto a blood agar plate in form of a drop. Four drops from each dilution were placed separately on one BA plate using a disposable measuring loop. After the drops were dried well, the plates were incubated for 24-48 hours at 29°C. Average colony counts were made from 4 drops on a blood agar plate starting from the

Table 3: Flow diagram used for the isolation of *Yersinia* species



CIN - (Cefsulodin-Irgasan-Novobiocin agar)
 BA - (Blood agar)
 TSI - (Triple-sugar-iron agar)
 LIA - (Lysine-argine-iron-agar)
 GB - (Glycerol broth)

highest dilution (10^7). Then calculations were made to determine the number of colonies present in each lower dilution ($10^2, 10^3, 10^4, 10^5$ and 10^6).

PROCESSING THE SAMPLES FOR ENRICHMENT

Each sample of pork mince was handled using separate disposable, sterile, wooden spatulas and weighed in separate sterile containers using a Mettler PJ 360 digital weighing balance (Watson and Victor LTD). The samples were placed in separate sterile stomacher bags with the appropriate PBS solution and homogenised in a stomacher apparatus (Colworth 400, London), for 10 minutes. Before the homogenates were transferred to sterile bottles the fat debris floating on the homogenate was removed using a sterile tea filter. Later, samples were divided as described for each trial and bacterial inoculations were made. The bacterial inoculum was mixed thoroughly before and after inoculation using a vortex mixer (Chiltern Auto MT 19).

TRIAL ONE

A half kg minced pork was purchased from a supermarket in Palmerston North. A 5g control sample was collected from the pork mince and homogenised in 45 ml of PBS using the stomacher. A loop of dilute from 10ml sample of the homogenate was used for direct plating on CIN on day 0. Another 10ml sample was cold enriched at 4°C for 21 days before plating.

Three 15g samples of pork mince were separately homogenised in the stomacher each with 135 ml of PBS at pH 7.6, 6.6 and 5.5 (See table 3). From each preparation 10ml were transferred to sterile bottles. For each pH group triplicate samples were processed (A, B, C). Each sample contained 10 ml of pork homogenate. The remainder of the homogenate was discarded. Five hundred microlitres of the 10^5 dilute contained 10,000 organisms, was added to each 10ml sample of pork homogenate and vortexed for a minute. These samples were labelled as A, B and C. Later each sample was divided into three equal parts and kept separately in 3 individual bottles for different enrichment

procedures (25°C for 2 days, 10°C for 7 days and 4°C for 21 days). Thus triplicate samples (A, B, C) were prepared for each pH value (7.6, 6.6, 5.5) and for each enrichment procedure (25°C for 2 days, 10°C for 7 days and 4°C for 21 days). The number of organisms was determined as 2.3×10^3 per 3.5 ml after dividing 10.5 ml mixture of pork mince homogenate and bacterial suspension into 3 equal parts of 3.5 ml each (Table 3).

The samples were classified as follows:

TABLE 4:

Classification samples for Trial one:

SAMPLES	25 °C for 2 days			10 °C for 7 days			4 °C for 21 days		
	A	B	C	A	B	C	A	B	C
5.5									
6.6									
7.6									

Each sample contained 2.3×10^3 organisms after inoculation.

TRIAL TWO

A half kg of minced pork was purchased from a different retail market in Palmerston North. A control sample (5g) was homogenised in 45 ml of PBS using the stomacher. A loop of dilute from 10ml sample of homogenate was used for direct plating on CIN agar on day 0, and a 10ml sample was cold enriched at 4°C for 21 days before plating on to CIN.

Three 15g samples of pork mince were homogenised individually in the stomacher with 135 ml of PBS at pH 7.6, pH 6.6 and 5.5. For all three pH groups three sets of duplicate bottles each containing 5mls of homogenate were made. Each of the three duplicate samples was inoculated with 500-microlitres of bacterial suspension containing either 5×10^2 , 5×10^3 or 5×10^4 organisms. The inoculated samples were vortexed

for 1 minute and incubated separately at different enrichment temperatures and times i.e. 25°C for 2 days, 10°C for 7 days and 4°C for 21 days (Table 4).

The samples were classified as follows:

TABLE 4:

Classification samples for Trial two:

	25 °C for 2 days	10 °C for 7 days	4 °C for 21 days
SAMPLES	A1 B1 A2 B2 A3 B3	A1 B1 A2 B2 A3 B3	A1 B1 A2 B2 A3 B3
5.5			
6.6			
7.6			

Number of organisms after inoculation

A1 and B1 = 5×10^2 , A2 and B2 = 5×10^3 , A3 and B3 = 5×10^4

PLATING OF SAMPLES

After the respective incubation periods, a loopful of homogenate was plated onto CIN agar (DIFCO, USA). The plates were incubated at 29°C and examined after 24-48 hours for growth and cultural characteristics of the bacteria. Colonies typical of yersiniae (pink 'bulls-eye' colonies surrounded by a transparent border, varying in size from pin-point after one day to 2.5 mm in diameter after two days incubation) were picked off and sub-cultured onto BA plates at 29°C for 24 hours. Haemolytic cultures were rejected and oxidase tests were performed to select oxidase negative colonies. From BA plates, selected colonies were transferred to bijoux bottles containing tryptone water. These samples were incubated for 24 hours and used as inoculum for further identification.

PRESUMPTIVE SCREENING FOR *YERSINIA* SPECIES

Triple-sugar-iron agar (TSI), lysine-iron agar (LIA) and urea slopes were inoculated. Cultures which showed one or more of the following reactions, were rejected: H₂S production (blackening on TSI), excessive gas production & urease negative. Cultures which gave either an acid slant/acid butt (A/A) or an alkaline slant/acid butt (K/A) reaction on TSI, and an alkaline slant/ acid butt (K/A) on LIA, plus urease positive (pink on urea agar), with motility at 29°C were considered as presumptive yersiniae. At this point a further tryptone water culture was inoculated and incubated for a further 24 hours. This was used as the inoculum for biochemical characterisation and subsequent identification of the isolate. All *Yersinia* species recovered during this study were stored at -70°C in 15% glycerol broth.

BIOTYPING AND SEROTYPING OF *YERSINIA* ISOLATES

Organisms confirmed as *Yersinia* by morphology and cultural characteristics were further identified on the basis of biochemical characteristics as described by Bercovier and Mollaret (1984), Wauters *et al* (1987) (Table 2). Purity plating was made from the samples used for biochemical testing on MacConkey agar to confirm that no cross-contamination had occurred during processing before biochemical testing. Each isolate was subjected to the following biochemical tests at 29°C; Ornithine decarboxylase, methyl red, Voges-Proskauer (also at 37°C), indole production, aesculin hydrolysis and acid production from the following carbohydrates; sucrose, L-rhamnose, D-melibiose, D-trehalose, D-xylose and salicin. All tests were read daily for three days.

Biotyping of all *Y. enterocolitica* isolates were done based on Wauters classification system (Table 1). *Y. enterocolitica* biogroup 4 showed positive reaction to Trehalase and Voges-Proskauer at 29°C not at 37°C and negative reaction to lipase, aesculin, salicin, indole, 5-D-glucoside and proline peptidase. Serotyping by slide agglutination was carried out using commercially available typing sera (Eco-Bio, Belgium) representing O-antigen factors available for *Y. enterocolitica* serotype O:3.

SECTION B

RESULTS

The experiment was conducted in two separate trials. The results are presented in Tables 7, 8 and 9 (Trial one) and 10, 11 and 12 (Trial two). The aim was to recover *Yersinia enterocolitica* from artificially inoculated samples of minced pork.

Trial one

The number of inoculated organism in each sample of 5ml homogenate was 2.3×10^3 .

Table 6 shows the results of trial one in which samples were inoculated with *Y. enterocolitica*, enriched in PBS at pH 7.6 and incubated at 25°C for two days, 10°C for 7 days and 4°C for 21 days. *Y. enterocolitica* was recovered from all three inoculated samples at 25°C for 2 days and from one out of 3 inoculated samples incubated at 4°C for 21 days. There were no organisms recovered from other inoculated samples.

Table 7 shows the results of trial one in which samples were inoculated with *Y. enterocolitica* enriched in PBS at pH 6.6 and incubated at 25°C for two days, 10°C for 7 days and 4°C for 21 days. *Y. enterocolitica* was not isolated from any sample.

Table 8 shows the results of trial one in which samples were inoculated with *Y. enterocolitica*, enriched in PBS at pH 5.5 and incubated at 25°C for two days, 10°C for 7 days and 4°C for 21 days. *Y. enterocolitica* was not recovered from any sample. In trial one, the control sample (Table 13) did not show any contamination with *Yersinia* species.

Trial two

The number of organisms used was as follows: A1 and B1 = 5×10^2 , A2 and B2 = 5×10^3 , A3 and B3 = 5×10^4 per 5ml of homogenate

Table 9 shows the results of trial two in which samples were inoculated with *Y. enterocolitica* enriched in PBS at pH 7.6 and incubated at 25°C for two days, 10°C for 7 days and 4°C for 21 days. *Y. enterocolitica* was not recovered from any inoculated sample. *Y. intermedia* was isolated from two samples incubated at 25°C for two days, from two samples incubated at 10°C for 7 days and from one sample incubated at 4°C for 21 days.

Table 10 shows the results of trial two in which samples were inoculated with *Y. enterocolitica* enriched in PBS at pH 6.6 and incubated at 25°C for two days, 10°C for 7 days and 4°C for 21 days. *Y. enterocolitica* was recovered from one sample (in duplicate) inoculated with 10^4 organisms and incubated at 25°C for two days. *Y. intermedia* was isolated from 1 sample incubated at 25°C for two days and one sample incubated at 10°C for 7 days. All other samples were negative.

Table 11 shows the results of trial two in which samples were inoculated with *Y. enterocolitica*, enriched in PBS at pH 5.5 and incubated at 25°C for two days, 10°C for 7 days and 4°C for 21 days. *Y. enterocolitica* was not recovered from any inoculated sample. *Y. intermedia* was isolated from one sample incubated at 4°C for 21 days.

In trial two, *Yersinia intermedia* was isolated from the control sample enriched in PBS at pH 7.6 and incubated at 4°C for 21 days (Table 12).

Samples positive in the screening tests were selected for biochemical identification and biotyping. Identification of *Yersinia enterocolitica* serotype O:3 was made by slide agglutination tests using specific antiserum.

KEY TO THE TABLES:

A/A = ACID SLANT AND ACID BUTT

K/A = ALKALINE SLANT AND ACID BUTT

YI = *YERSINIA INTERMEDIA*

YE BIO 4 = *YERSINIA ENTEROCOLITICA* BIOTYPE 4

MR = METHYL RED

VP = VOGES-PROSKAUR

nl = NON-LACTOSE FERMENTERS

nh = NON-HAEMOLYTIC

x = NOT PERFORMED

+ = POSITIVE

- = NEGATIVE

TABLE 6:
TRIAL ONE

pH 7.6	25 °C for 2 days			10 °C for 7 days			4 °C for 21 days		
SAMPLES	A	B	C	A	B	C	A	B	C
TSI	A/A	A/A	A/A	A/A	A/A	A/A	K/A, GAS	A/A	A/A
LIA	K/A	K/A	K/A	K/A	K/A	K/A	K/A, GAS	K/A	K/A
UREA	+	+	+	-	-	-	-	-	+
MOTILITY 29°C	+	+	+	-	+	+	+	+	+
MOTILITY 37°C	-	-	-	-	+	+	+	+	-
MacCONKEY	nl	nl	nl	x	x	x	x	x	nl
BLOOD AGAR	nh	nh	nh	x	x	x	x	x	nh
MR 29°C & 37°C	++	++	++	x	x	x	x	x	++
VP 29°C & 37°C	+-	+-	+-	x	x	x	x	x	+-
ORNITHINE	+	+	+	x	x	x	x	x	+
NITRATE	+	+	+	x	x	x	x	x	+
SIMON'S CITRATE	-	-	-	x	x	x	x	x	-
INDOLE	-	-	-	x	x	x	x	x	-
SUCROSE	+	+	+	x	x	x	x	x	+
TREHALOSE	+	+	+	x	x	x	x	x	+
RHAMNOSE	-	-	-	x	x	x	x	x	-
MELIBIOSE	-	-	-	x	x	x	x	x	-
α-METHYL GLUCOSIDE	-	-	-	x	x	x	x	x	-
SORBITOL	+	+	+	x	x	x	x	x	+
CELLOBIOSE	+	+	+	x	x	x	x	x	+
RESULT	Y.E bio.4	Y.E bio.4	Y.E bio.4	-	-	-	-	-	Y.E bio.4

Samples inoculated with *Y. enterocolitica*, enriched in PBS at pH 7.6 and incubated at 25°C for 2 days, 10°C for 7 days and 4°C for 21 days.

TABLE 7:
TRIAL ONE

pH 6.6	25 °C for 2 days			10 °C for 7 days			4 °C for 21 days		
SAMPLES	A	B	C	A	B	C	A	B	C
TSI	H ₂ S	H ₂ S	H ₂ S	K/A	A/A	-	A/A	K/A, GAS	K/A, GAS
LIA	H ₂ S	H ₂ S	H ₂ S	K/A	K/A	-	K/A	K/A, GAS	K/A, GAS
UREA	+	+	+	-	-	-	-	-	-
MOTILITY 29°C	-	+	+	-	-	-	-	-	-
MOTILITY 37°C	-	+	+	-	-	-	-	-	-
MacCONKEY	x	x	x	x	x	x	x	x	x
BLOOD AGAR	x	x	x	x	x	x	x	x	x
MR 29 C & 37°C	x	x	x	x	x	x	x	x	x
VP 29 C & 37 °C	x	x	x	x	x	x	x	x	x
ORNITHINE	x	x	x	x	x	x	x	x	x
NITRATE	x	x	x	x	x	x	x	x	x
SIMON'S CITRATE	x	x	x	x	x	x	x	x	x
INDOLE	x	x	x	x	x	x	x	x	x
SUCROSE	x	x	x	x	x	x	x	x	x
TREHALOSE	x	x	x	x	x	x	x	x	x
RHAMNOSE	x	x	x	x	x	x	x	x	x
MELIBIOSE	x	x	x	x	x	x	x	x	x
α-METHYL GLUCOSIDE	x	x	x	x	x	x	x	x	x
SORBITOL	x	x	x	x	x	x	x	x	x
CELLOBIOSE	x	x	x	x	x	x	x	x	x
RESULT	-	-	-	-	-	-	-	-	-

Samples inoculated with *Y. enterocolitica*, enriched in PBS at pH 6.6 and incubated at 25° C for two days, 10° C for 7 days and 4° C for 21 days.

TABLE 8:
TRIAL ONE

pH 5.5	25 °C for 2 days			10 °C for 7 days			4 °C for 21 days		
	A	B	C	A	B	C	A	B	C
TSI	K/A, GAS	H ₂ S	H ₂ S	A/A	K/A, GAS	K/A, GAS	K/A	K/A, GAS	K/A, GAS
LIA	K/A, GAS	H ₂ S	H ₂ S	K/A	K/A, GAS	K/A, GAS	K/A	K/A, GAS	K/A, GAS
UREA	-	-	-	-	-	-	+	+	+
MOTILITY 29°C	+	+	+	-	-	-	+	+	+
MOTILITY 37°C	+	+	+	-	-	-	+	+	+
MacCONKEY	x	x	x	x	x	x	x	x	x
BLOOD AGAR	x	x	x	x	x	x	x	x	x
MR 29°C & 37°C	x	x	x	x	x	x	x	x	x
VP 29°C & 37°C	x	x	x	x	x	x	x	x	x
ORNITHINE	x	x	x	x	x	x	x	x	x
NITRATE	x	x	x	x	x	x	x	x	x
SIMON'S CITRATE	x	x	x	x	x	x	x	x	x
INDOLE	x	x	x	x	x	x	x	x	x
SUCROSE	x	x	x	x	x	x	x	x	x
TREHALOSE	x	x	x	x	x	x	x	x	x
RHAMNOSE	x	x	x	x	x	x	x	x	x
MELIBIOSE	x	x	x	x	x	x	x	x	x
α-METHYL GLUCOSIDE	x	x	x	x	x	x	x	x	x
SORBITOL	x	x	x	x	x	x	x	x	x
CELLOBIOSE	x	x	x	x	x	x	x	x	x
RESULT	-	-	-	-	-	-	-	-	-

Samples inoculated with *Y. enterocolitica*, enriched in PBS at pH 5.5 and incubated at 25°C for two days, 10°C for 7 days and 4°C for 21 days.

TABLE 9:
TRIAL TWO

pH 7.6	25 °C for 2 days						10 °C for 7 days						4 °C for 21 days					
	A 1	B 1	A 2	B 2	A 3	B 3	A 1	B 1	A 2	B 2	A 3	B 3	A 1	B 1	A 2	B 2	A 3	B 3
TSI	H 2 S	K A	A A	H 2 S	A A	A A	K A	A A	K A	A A	A A	A A	A A	A A	H 2 S	A A	-	A A
LIA	H 2 S	K A	K A	H 2 S	K A	K A	K A	K A	K A	K A	K A	K A	K A	K A	H 2 S	K A	-	K A
UREA	+	+	+	-	+	-	+	+	+	+	+	-	+	-	-	-	-	-
MOTILITY 29°C	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
MOTILITY 37°C	+	-	-	+	+	+	-	+	-	+	-	+	-	+	+	+	+	+
MacCONKEY	x	nl	nl	x	x	x	nl	x	nl	x	x	x	nl	x	x	x	x	x
BLOOD AGAR	x	n h	n h	x	x	x	n h	x	n h	x	x	x	n h	x	x	x	x	x
MR 29°C & 37°C	x	+	+	x	x	x	+	x	+	x	x	x	+	x	x	x	x	x
VP 29°C & 37°C	x	+	+	x	x	x	+	x	+	x	x	x	+	x	x	x	x	x
ORNITHINE	x	+	+	x	x	x	+	x	+	x	x	x	+	x	x	x	x	x
NITRATE	x	+	+	x	x	x	+	x	+	x	x	x	+	x	x	x	x	x
SIMON'S CITRATE	x	-	-	x	x	x	-	x	-	x	x	x	-	x	x	x	x	x
INDOLE	x	-	-	x	x	x	-	x	-	x	x	x	-	x	x	x	x	x
SUCROSE	x	+	+	x	x	x	+	x	+	x	x	x	+	x	x	x	x	x
TREHALOSE	x	+	+	x	x	x	+	x	+	x	x	x	+	x	x	x	x	x
RHAMNOSE	x	+	+	x	x	x	+	x	+	x	x	x	+	x	x	x	x	x
MELIBIOSE	x	+	+	x	x	x	+	x	+	x	x	x	+	x	x	x	x	x
α-METHYL GLUCOSIDE	x	-	-	x	x	x	-	x	-	x	x	x	-	x	x	x	x	x
SORBITOL	x	+	+	x	x	x	+	x	+	x	x	x	+	x	x	x	x	x
CELLOBIOSE	x	+	+	x	x	x	+	x	+	x	x	x	+	x	x	x	x	x
RESULT	-	Y I	Y I	-	-	-	Y I	-	Y I	-	-	-	Y I	-	-	-	-	-

Samples inoculated with *Y. enterocolitica*, enriched in PBS at pH 7.6 and incubated at 25°C for two days, 10°C for 7 days and 4°C for 21 days.

TABLE 10:
TRIAL TWO

pH 6.6	25 °C for 2 days						10 °C for 7 days						4 °C for 21 days					
	A 1	B 1	A 2	B 2	A 3	B 3	A 1	B 1	A 2	B 2	A 3	B 3	A 1	B 1	A 2	B 2	A 3	B 3
TSI	A A	A A	A A	H 2 S	K A	K A	A A	A A	A A	A A	A A	A A	A A	A A	A A	A A	H 2 S	A A
LIA	K A	K A	K A	H 2 S	K A	K A	K A	K A	K A	K A	K A	K A	K A	K A	K A	K A	H 2 S	K A
UREA	+	-	+	-	+	+	-	+	+	-	+	-	-	-	-	-	-	-
MOTILITY 29°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MOTILITY 37°C	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
MacCONKEY	x	x	x	x	nl	nl	x	nl	x	x	x	x	x	x	x	x	x	x
BLOOD AGAR	x	x	x	x	n h	n h	x	n h	x	x	x	x	x	x	x	x	x	x
MR 29°C & 37°C	x	x	x	x	+	+	x	+	x	x	x	x	x	x	x	x	x	x
VP 29°C & 37°C	x	x	x	x	+	+	x	+	x	x	x	x	x	x	x	x	x	x
ORNITHINE	x	x	x	x	+	+	x	+	x	x	x	x	x	x	x	x	x	x
NITRATE	x	x	x	x	+	+	x	+	x	x	x	x	x	x	x	x	x	x
SIMON'S CITRATE	x	x	x	x	-	-	x	-	x	x	x	x	x	x	x	x	x	x
INDOLE	x	x	x	x	-	-	x	-	x	x	x	x	x	x	x	x	x	x
SUCROSE	x	x	x	x	+	+	x	+	x	x	x	x	x	x	x	x	x	x
TREHALOSE	x	x	x	x	+	+	x	+	x	x	x	x	x	x	x	x	x	x
RHAMNOSE	x	x	x	x	+	-	x	+	x	x	x	x	x	x	x	x	x	x
MELIBIOSE	x	x	x	x	+	-	x	+	x	x	x	x	x	x	x	x	x	x
α-METHYL GLUCOSIDE	x	x	x	x	-	-	x	-	x	x	x	x	x	x	x	x	x	x
SORBITOL	x	x	x	x	+	+	x	+	x	x	x	x	x	x	x	x	x	x
CELLOBIOSE	x	x	x	x	+	+	x	+	x	x	x	x	x	x	x	x	x	x
RESULT			-	-	Y I	Y E b 4	-	Y I		-	-	-	-	-	-	-	-	-

Samples inoculated with *Y. enterocolitica*, enriched in PBS at pH 6.6 and incubated at 25°C for two days, 10°C for 7 days and 4°C for 21 days.

TABLE 11:
TRIAL TWO

pH 5.5	25 °C for 2 days						10 °C for 7 days						4 °C for 21 days					
	A 1	B 1	A 2	B 2	A 3	B 3	A 1	B 1	A 2	B 2	A 3	B 3	A 1	B 1	A 2	B 2	A 3	B 3
TSI	K A	A A	A A	-	H 2 S	A A	A A	A A	A A	H 2 S	A A	A A	K A	A A	A A	A A	A A	A A
LIA	K A	K A	K A	-	H 2 S	K A	K A	K A	K A	H 2 S	K A	K A	K A	K A	K A	K A	K A	K A
UREA	+	+	+	-	-	-	-	+	-	+	-	+	-	-	-	-	+	-
MOTILITY 29°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MOTILITY 37°C	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
MacCONKEY	x	x	x	x	x	x	x	x	x	x	x	x	ni	x	x	x	x	x
BLOOD AGAR	x	x	x	x	x	x	x	x	x	x	x	x	h	x	x	x	x	x
MR 29°C & 37°C	x	x	x	x	x	x	x	x	x	x	x	x	+	x	x	x	x	x
VP 29°C & 37°C	x	x	x	x	x	x	x	x	x	x	x	x	+	x	x	x	x	x
ORNITHINE	x	x	x	x	x	x	x	x	x	x	x	x	+	x	x	x	x	x
NITRATE	x	x	x	x	x	x	x	x	x	x	x	x	+	x	x	x	x	x
SIMON'S CITRATE	x	x	x	x	x	x	x	x	x	x	x	x	-	x	x	x	x	x
INDOLE	x	x	x	x	x	x	x	x	x	x	x	x	-	x	x	x	x	x
SUCROSE	x	x	x	x	x	x	x	x	x	x	x	x	+	x	x	x	x	x
TREHALOSE	x	x	x	x	x	x	x	x	x	x	x	x	+	x	x	x	x	x
RHAMNOSE	x	x	x	x	x	x	x	x	x	x	x	x	+	x	x	x	x	x
MELIBIOSE	x	x	x	x	x	x	x	x	x	x	x	x	+	x	x	x	x	x
α-METHYL GLUCOSIDE	x	x	x	x	x	x	x	x	x	x	x	x	-	x	x	x	x	x
SORBITOL	x	x	x	x	x	x	x	x	x	x	x	x	+	x	x	x	x	x
CELLOBIOSE	x	x	x	x	x	x	x	x	x	x	x	x	+	x	x	x	x	x
RESULT		-	-	-	-	-	-	-	-	-	-	-	Y I	-	-	-	-	-

Samples inoculated with *Y. enterocolitica*, enriched in PBS at pH 5.5 and incubated at 25°C for two days, 10°C for 7 days and 4°C for 21 days.

TABLE 12:
CONTROL SAMPLES

pH 7.6.	TRIAL ONE		TRIAL TWO	
	DAY 0	21 DAYS & 4° C	DAY 0	21 DAYS & 4° C
TSI	H ₂ S	A/A	A/A	A/A
LIA	H ₂ S	K/A	K/A	K/A
UREA	+	+	+	+
MOTILITY 29°C	x	+	+	+
MOTILITY 37°C	x	+	+	-
MacCONKEY	x	x	x	NL
BLOOD AGAR	x	x	x	NH
MR 29°C & 37°C	x	x	x	++
VP 29°C & 37°C	x	x	x	+-
ORNITHINE	x	x	x	+
NITRATE	x	x	x	+
SIMON'S CITRATE	x	x	x	-
INDOLE	x	x	x	-
SUCROSE	x	x	x	+
TREHALOSE	x	x	x	+
RHAMNOSE	x	x	x	+
MELIBIOSE	x	x	x	+
α-METHYL GLUCOSIDE	x	x	x	-
SORBITOL	x	x	x	+
CELLOBIOSE	x	x	x	+
RESULT	-	-	-	Y.I

This table shows the results of control (uninoculated pork mince samples) for trial one and trial two.

SUMMARY OF RESULTS:

In trial one, each sample contained 2.3×10^3 *Y. enterocolitica* after inoculation. *Y. enterocolitica* was isolated from all (100%) samples incubated in PBS pH 7.6 at 25°C for 2 days, 1 out of 3 (33%) samples incubated at 4°C for 21 days and no yersiniae were recovered after incubation at 10°C for 7 days.

In trial two, the number of *Y. enterocolitica* in each sample after inoculation was: A1 and B1 = 5×10^2 , A2 and B2 = 5×10^3 , A3 and B3 = 5×10^4 . *Y. enterocolitica* was only isolated from 1 out of 2 (50%) samples incubated in PBS pH 6.6 at 25°C for 2 days (B3).

DISCUSSION

The increasing interest in *Y. enterocolitica* as a cause of foodborne infections has resulted in the development of improved procedures for the isolation of this organism from foods (De Boer 1992). An efficient enrichment procedure should confer some advantage to the desired microorganisms, thereby allowing them to multiply more rapidly than the competing microflora. Being a psychrotrophic organism, *Y. enterocolitica* is able to multiply at 4°C and several enrichments involving incubation at this and higher temperatures up to 35°C, for time periods from 24 hours to 21 days, have been proposed. Cold enrichment media include simple buffers like phosphate buffered saline (PBS). Recovery of *Y. enterocolitica* from many types of foods has been evaluated by studies involving inoculation and subsequent recovery of the test microorganism (Hanna *et al* 1977, Mehlman *et al* 1978, Fukushima and Gomyoda 1986).

Investigation of the efficacy of different enrichment procedures is important in particular in terms of the time involved in isolating pathogenic strains of *Y. enterocolitica* from pork samples. Obviously a rapid, sensitive and specific method would be an advantage to the food industry.

During this study two trials were conducted. In these trials, *Y. enterocolitica* was isolated from experimentally inoculated samples enriched in PBS at pH 7.6 and incubated at 25°C for only 2 days (Table 1). This result clearly indicates that pathogenic strains of *Y. enterocolitica* can be isolated from pork samples following incubation at high temperature for a short time. Schiemann (1989) showed that incubation at 15°C for 2 days was as efficient as incubation at 4°C for 3 weeks. Doyle *et al* (1981) were able to isolate *Yersinia* species by incubating in PBS for 1-3 days at 25°C. The isolation of inoculated organisms after incubation at high temperature for a short duration in this experiment is consistent with the earlier reports (Ibrahim and Macrae 1991).

In Trial one, 1 positive isolate was made out of 3 samples enriched at 4°C for 21 days in PBS pH 7.6. The 21 day-incubation period typically required for cold enrichment

is often unacceptably long for use in the quality assurance of foods (De Boer 1992). Other environmental samples such as water may contain large numbers of other psychrotrophic microorganisms from *Enterobacteriaceae*, which can multiply and compete with *Yersinia* species thus reducing the recovery rate of yersiniae (De Boer 1992).

In Trial two, 1 positive isolation was made from 3 duplicate samples enriched at 25°C for 2 days in PBS pH 6.6. This isolation was made only from a sample inoculated with 10^4 *Yersinia enterocolitica*. One reason that positive isolations were not made from other samples even with inoculation of 10^4 organisms may have been due to the presence of relatively high numbers of *Y. intermedia*.

No isolations of *Yersinia enterocolitica* were made from samples enriched in PBS pH 5.5. This clearly showed the significant effect of low pH (5.5) on the growth and isolation of *Yersinia enterocolitica* from both trials. Hanna *et al* (1979) showed similar results.

The ability of current enrichment procedures to recover pathogenic strains of *Y. enterocolitica* from different foods is often inadequate, probably because different strains require different conditions for optimum growth (Wauters *et al* 1973, Falcao *et al* 1979, De Boer 1992). In the present study, the growth of *Y. enterocolitica* was variable even under what is usually considered optimum conditions of enrichment at 4°C for 21 days (Table 1 and Table 10).

The study used two different pork mince samples bought from two different local supermarkets. *Yersinia intermedia* was isolated from one and not from the other. Interestingly, in the second trial, *Yersinia intermedia* was isolated from all pH groups and from almost all temperature and time combinations. This organism was also isolated from control samples in the second trial indicating the possibility of *Y. intermedia* contamination during meat processing, storage or distribution. The environmental *Yersinia* species or environmental biotypes of *Y. enterocolitica* have intermediate to low resistance to selective enrichment broths and are easily recovered from foods. Their

presence in foods makes it much more difficult to recover pathogenic strains, possibly due to competition for nutrients (De Boer 1992). Most environmental isolates are different biochemically and serologically from typical pathogenic strains.

Some enrichment and plating media used for the isolation of *Yersinia* species are not particularly selective for *Yersinia enterocolitica* as they also support the growth of several other *Enterobacteriaceae*. This makes the isolation of low numbers of *Yersinia enterocolitica* more difficult from contaminated samples. In this study there appeared to be a marked effect caused by the presence of environmental yersinae on the growth and isolation of *Y. enterocolitica*.

CONCLUSIONS

The following conclusions can be drawn from this experiment.

Time and Temperature:

- Pathogenic strains of *Y. enterocolitica* can be isolated from pork samples after incubation at high temperature (25°) for a short duration (48 hours), especially if the number of environmental contaminants is low.
- Although growth of *Y. enterocolitica* was variable under different enrichment procedures, this experiment showed that the isolation of *Y. enterocolitica* was difficult even following ideal enrichment and isolation conditions (4°C for 21 days in PBS at pH 7.6).
- The standard incubation period of 21 days used for cold enrichment at 4°C is too long for the isolation of pathogenic strains.

- Incubation at 10°C for 7 days period is not ideal for isolation of pathogenic *Yersinia enterocolitica* strains.

Enrichment pH:

- A pH of 6.6 is not as ideal as 7.6 for enrichment although occasional isolations can be made using this pH for enrichment. Enrichment in PBS at pH 5.5 with any time temperature combinations is not ideal for isolation of pathogenic *Yersinia enterocolitica* strains.

Effect of environmental contaminants:

- *Y. intermedia* grew readily in all three enrichment combinations irrespective of the presence of pathogenic strains of *Y. enterocolitica*.
- The presence of high numbers of *Y. intermedia* appeared to have suppressing effect on the growth of the inoculated organisms.
- Correlation between isolation of pathogenic strains of *Y. enterocolitica* and time, temperature and pH was noted in many cases.
- Of the three enrichments (PBS 7.6, 6.6, 5.5) used in this experiment, PBS pH 7.6 was been found to be superior.

This study only used biochemical and serological identification of pathogenic *Yersinia enterocolitica* and biochemical differentiation of environmental *Yersinia* species. Future studies should aim at molecular characterisation of *Yersinia* species and important pathogenic strains of *Yersinia enterocolitica* in order to improve the correct identification of these organisms. This will help to improve sensitivity of the test used and reduce the time taken to isolate the pathogenic strains of *Yersinia enterocolitica* found in pork products.

APPENDIX

Preparation of media used for the isolation, identification, virulence testing and storage of *Yersinia* isolates

Aesculin Hydrolysis

1. To 1 litre of distilled water, add the following materials:

1 gm Aesculin (DIFCO)

0.5 gm Ferric Iron Citrate

25 gm Heart Infusion Broth

15 gm Bacto Agar (DIFCO)

2. Dissolve by gentle heating.

3. Mix thoroughly and dispense 3 ml amounts in bijoux bottles.

4. Sterilise by autoclaving for 15 minutes at 121°C.

5. Cool in a slanting position and store at 4°C.

Blood agar

This medium comprises a salt base and a blood base.

Salt base

1. To 1 litre of distilled water add 15.0 gm Bacto Agar (DIFCO) and 5.0 gm Sodium Chloride and dissolve by heating.

2. Autoclave for 15 minutes at 121°C.

3. Cool to 45-50°C.

4. Dispense 10 ml amounts into each petri dish and allow to cool and dry.

Blood base

1. To 1 litre of distilled water, add 44.0 gm of Columbia Blood Agar Base (DIFCO) and dissolve by heating.

2. Autoclave for 15 minutes at 121°C.

3. Cool to 44-45°C.

4. Add 5% (W/V) of sheep blood.
5. Dispense 10-15 ml amounts over the already prepared salt base.
6. Allow to cool and dry, and store at 4°C.

Carbohydrate test media

1. To 1 litre of distilled water, add the following:
 - 10 gm peptone (DIFCO)
 - 3 gm Meat Extract (GIBCO, BRL)
 - 5 gm Sodium Chloride
 - 10 ml Andrades Solution
2. Dissolve by stirring and adjust pH to 7.2.
3. Dispense in 200 ml bottles.
4. Autoclave for 15 minutes at 121°C.
5. Add 20 ml (10%) Seitz filtered carbohydrates*.
6. Dispense aseptically in 3 ml amounts into bijoux bottles. Store at 4°C.

* The following were used: Sucrose, trehalose, rhamnose, melibiose, raffinose, α methyl-glucoside, sorbose, cellobiose, maltose, xylose, arabinose, salicin and lactose.

Cefsulodin-Irgasan-Novobiocin Agar (CIN) (Schiemann 1979)

The preparation of this medium is achieved by combination of *Yersinia* selective agar and *Yersinia* antimicrobial supplement cefsulodin novobiocin (CN).

1. To 1 litre of distilled water, add 59.5 gm of *Yersinia* Selective Agar Base (DIFCO) and dissolve completely by boiling.
2. Sterilise by autoclaving for 15 minutes at 121°C.
3. Cool to 45-50°C.
4. Aseptically add 10 ml rehydrated *Yersinia* antimicrobial supplement CN (DIFCO,)
5. Mix thoroughly, avoiding the formation of air bubbles, and dispense 15-20 ml amounts into sterile petri dishes.
6. Allow to cool and dry, store at 4°C.

Congo Red-Magnesium Oxalate Agar (CR-MOX) (Riley and Toma 1989)

1. To 825 ml of distilled water, add 40 gm of Tryptic Soy Agar (DIFCO) and dissolve by boiling.
2. Autoclave for 15 minutes at 121°C
3. Cool the molten medium to 55°C, and add the following solutions:
 - 80 ml of 0.25 M Magnesium Chloride (2.38 gm/100 ml)
 - 80 ml of 0.25 M Sodium Oxalate (3.55 gm/100 ml)
 - 10 ml of 20% D-galactose
 - 5 ml of 1 % Congo red.
4. Sterilise all solutions by autoclaving for 15 minutes at 121°C, except for D-galactose solution, which is filter-sterilised.
5. After thorough mixing, dispense 15-20 ml amounts into sterilised petri dishes.
6. Allow to cool and dry. Store at 4°C.

Decarboxylase test broth

1. To 1 litre of distilled water add 10.5 gm Moeller's decarboxylase base medium and dissolve by gentle heating.
2. Dispense into 200 ml bottles.
3. To each 200 ml amount, add either 2 gm L-Arginine (SIGMA), 2 gm L-lysine (SIGMA), or 2 gm L-ornithine (SIGMA).
4. Adjust pH to 6.0.
5. Dispense 3-ml amounts into bijoux bottles.
6. Autoclave for 15 minutes at 121°C. Store at 4°C.

Glycerol Broth (15%)

1. To 300 ml of distilled water add the following:
 - 2.4 gm Nutrient Broth.
 - 45 ml Glycerol.

2. Mix together (don't need to heat).
3. Dispense 3 ml amounts into bijoux bottles.
4. Autoclave for 15 minutes at 121°C. Store at 4°C.

Lysine Iron Agar (LIA) (Weagant 1983)

1. Add 34.5 gm Lysine Iron agar (DIFCO) to 1 litre of distilled water.
2. Dissolve by heating.
3. Dispense in 6 ml amounts into screw-capped polycarbonate tubes.
4. Autoclave for 15 minutes at 121°C.
5. Cool in slanting position. Store at 4°C.

MR-VP Medium

1. Dissolve 17 gm Bacto MR-VP Medium (DIFCO) in 1 litre of distilled water.
2. Dispense into test tubes in 10 ml amounts.
3. Sterilise by autoclaving for 15 minutes at 121°C.

Methyl Red Test

To 5 ml of culture in trypton water add 5 drops of methyl red solution. The indicator solution is prepared by dissolving 0.1 gm Bacto Methyl red in 300 ml of 95% alcohol and diluting to 500 ml in distilled water. A positive reaction is indicated by a distinct red colour, showing the presence of acid. A negative reaction is indicated by a yellow colour.

MacConkey Agar

1. To 1 litre of distilled water, suspend the required amount of medium and heat to boiling with gentle swirling to dissolve completely.
2. Add 10 gm of lactose to Bacto MacConkey Agar Base and swirl the flask to dissolve completely.

3. Sterilise by autoclaving for 15 minutes at 121°C.
4. Cool to 45-50°C.
5. Dispense in approximately 20-ml amounts into sterile petri dishes.
6. Allow to cool and dry, store at 4°C.

Phosphate buffered saline (pH Range of 5.3-8)

1. Solutions required:

M/15 Sodium phosphate dibasic (Na_2HPO_4) and M/15 Potassium acid phosphate (KH_2PO_4)

2. M/15 Sodium phosphate dibasic (Na_2HPO_4)

Dissolve 9.465g of the salt in distilled water making into 1 litre.

3. M/15 Potassium acid phosphate (KH_2PO_4)

Dissolve 9.07g of the salt in distilled water making into 1 litre.

4. M/15 buffer solution (PBS)

The following volumes of each solution in millilitres are used:

pH	M/15 Sodium phosphate dibasic (ml)	M/15 Potassium acid phosphate (ml)
5.5	40	960
6.6	400	600
7.6	880	120

5. Required pH

Obtained by mixing up the stock solutions in a jar and simultaneously reading with a pH meter (Orion research digital ionalyser/501).

Pyrazinamidase Slopes (Kandolo and Wauters 1985)

1. Prepare buffer solution by dissolving 2.37 gm of Tris-Maleate in 500 ml of distilled water.
2. Add the following :

- 15 gm Tryptic Soy Agar (DIFCO)
- 1.5 gm Yeast Extract (DIFCO)
- 0.5 gm Pyrazine carboxamide (MERCK)

3. Dissolve by boiling.
4. Dispense 5ml amounts into screw-capped polycarbonate tubes.
5. Sterilise by autoclaving for 15 minutes at 121°C.
6. Cool in a slanting position, and store at 4°C.

Simmons Citrate Agar

1. To 1 litre distilled water add 24.2 gm Bacto Simmons Citrate Agar.
2. Heat to boiling to dissolve completely.
3. Mix thoroughly and dispense 3 ml amounts in bijoux bottles.
4. Sterilise by autoclaving for 15 minutes at 121°C.
5. Cool in slanting position and store at 4°C.

Triple-Sugar-Iron Agar (TSIA)

- 1 To 1 litre of distilled water add 65.0 gm TSIA (DIFCO) and dissolve by heating.
2. Dispense 6 ml amounts into screw-capped polycarbonate tubes.
3. Autoclave for 15 minutes at 121°C.
4. Cool in a slanting position, and store at 4°C.

Tryptone Water

1. To 1 litre of distilled water add 20 gm Tryptone (DIFCO) and dissolve by gentle heating.
2. Dispense in 3 ml amounts into bijoux bottles.
3. Autoclave for 15 minutes at 121°C, and store at 4°C

Urea Agar

This medium comprises an agar base and a urea base.

Agar Base

1. To 1 litre of distilled water add 15.0 gm Bacto Agar (DIFCO), and dissolve by heating.
2. Dispense into 200 ml bottles.
3. Autoclave for 15 minutes at 121°C.
4. Cool at 45-50°C.

Urea Base

1. To 100 ml distilled water add 29.0 gm Bacto Urea Agar (DIFCO) and mix to dissolve.
2. Filter sterilise and store at 4°C.

To prepare the medium, aseptically add 20 ml of Bacto Urea Agar to 200ml Agar Base. Mix thoroughly and dispense 3 ml amount into each sterile Bijoux bottle. Cool in a slanting position. Store at 4°C.

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