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PUBLIC HEALTH ASPECTS OF *YERSINIA PSEUDOTUBERCULOSIS* IN DEER AND VENISON

A THESIS PRESENTED IN PARTIAL FULFILMENT (75%) OF THE
REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY
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EDWIN BOSI

September, 1992

DEDICATED TO

MY PARENTS (MR. RICHARD BOSI AND MRS. VICTORIA CHUAN)

MY WIFE (EVELYN DEL ROZARIO)

AND MY CHILDREN (AMELIA, DON AND JACQUELINE)

Abstract

A study was conducted to determine the possible carriage of Yersinia pseudotuberculosis and related species from faeces of farmed Red deer presented for slaughter and the contamination of deer carcase meat and venison products with these organisms. Experiments were conducted to study the growth patterns of Y.pseudotuberculosis in vacuum-packed venison stored at chilling and freezing temperatures.

The serological status of slaughtered deer in regards to Y.pseudotuberculosis serogroups 1, 2 and 3 was assessed by Microplate Agglutination Tests. Forty sera were examined comprising 19 from positive and 20 from negative intestinal carriers. Included in this study was one serum from an animal that yielded carcase meat from which Y.pseudotuberculosis was isolated.

Caecal contents were collected from 360 animals, and cold-enriched for 3 weeks before being subjected to bacteriological examination for Yersinia spp.

A total of 345 and 321 carcases surface samples for bacteriological examination for Yersiniae were collected at the Deer Slaughter Premises (DSP) and meat Packing House respectively.

A total of 70 venison sausages were purchased from local supermarkets. Direct plating and plating after 21 days cold-enrichment were carried out to examine for Yersiniae.

Venison samples were obtained from the DSP and seeded with a known approximate number of Y.pseudotuberculosis organisms. The samples were vacuum-packed and stored at temperatures of +10 ℃, +4 ℃, -1 ℃, -10 ℃, -13 ± 2 ℃, and -20 ℃; recovery and enumeration of the test organism was made at predetermined times.

The results of the Microplate Agglutination Tests showed that deer presented for slaughter at this DSP had low (1:10) or undetectable antibody titres to Y.pseudotuberculosis. The prevalence of Yersinia spp. in faeces was 5.3% (19/360) of Y.pseudotuberculosis, 2.6% (9/360) of Y.enterocolitica, 3.6% (13/360) of Y.kristensenii, 20.5% (74/360) of Y.frederiksenii, 0.6% (2/360) of Y.intermedia and 0.6% (2/360) of Y.rohdei. Five of nine strains of Y.enterocolitica isolated were found to be potentially pathogenic by means of the virulence marker tests. Two of them were identified as biotype 3 serovar O:5,27.

There was only one isolation (0.3%) of Y.pseudotuberculosis from 321 carcases sampled at the Packing House.

The prevalence of Yersinia spp. in venison sausages was 11.4% (8/70) Y.enterocolitica, 1.4% (1/70) Y.kristensenii and 5.7% (4/70) Y.intermedia.

Y.pseudotuberculosis grew very well in vacuum-packed venison stored at chilling temperature although a long lag phase was observed at -1 °C. When frozen, the organisms remained viable for a long period of time and recovered and multiplied rapidly when transferred to chill temperature.

The study showed that there was no serological evidence of yersiniosis in deer presented for slaughter during the study period despite the fact that 5.3% of the animals were carrying Y.pseudotuberculosis in their faeces. While there was a low prevalence of Y.pseudotuberculosis on carcase meat their presence could be a source of cross contamination of other carcases especially during deboning. The finding of Yersiniae in venison sausages showed that there was contamination during their preparation. The multiplication of the bacteria in vacuum-packed venison and their long survival in frozen venison are of public health concern while its presence may affect export markets.

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CHAPTER 1

GENERAL INTRODUCTION

Foodborne illness is of major public health concern worldwide (Waites & Arbuthnott, 1990). Foods of animal origin are the primary sources of many of the bacteria responsible for foodborne infections and intoxications (Roberts, 1990) and some level of unseen microbiological contamination of the carcase is inevitable in the slaughter house environment (Hathaway & Selyn, 1991).

The deer industry in New Zealand began in the early 1960's with the export of venison derived from hunted feral deer. A peak export volume of feral venison of 4000 tonnes was reached in 1971. The first licence to farm deer was issued in 1970 and there are now more than one million farmed deer. In 1981/82, Deer Slaughter Premises (DSP) were established to facilitate the growing venison export industry and by May 1992 there were 11 such facilities in the country (Wilson, pers. commun.). The venison industry registered a revenue earning of about NZ$50 million in 1990 and 1991 (Kelly, 1991). As of June 1992, the revenue earned from venison export was about NZ$81.3 million (Bryan, 1992). The local market constitutes about 350 tonnes annually (Kelly, 1991).

In the wild, Red deer (*Cervus elaphus*) are gregarious animals which tend to live in separate herds. The females are accompanied by their young of varying ages while the stags, aged over three years form their own separate herds. These two social groups go their own separate ways for the greater part of the year (Anon, 1984). The establishment of farmed herds has disrupted this lifestyle. The stressors in the new environment, including social stressors, increase the susceptibility of deer to a range of diseases. One of the most important disease of farmed deer is yersiniosis caused by infection with the Gram-negative bacterium, *Yersinia pseudotuberculosis* (Griffin, 1987). The first reported outbreak of yersiniosis in this species occurred in 1978 killing at least 60 hinds (Mackintosh et al., 1991).

The young weaners are highly susceptible to yersiniosis which occurs frequently during the cold months and is accentuated by stressors such as inadequate or poor quality feed, exposure to inclement weather conditions, transportation and yarding (Mackintosh & Henderson, 1984b). However, mature deer are also affected (Beatson, 1984 ; Jerrett et al., 1990).

Y.pseudotuberculosis has been recovered from faeces of healthy (Henderson, 1984)

and sick deer (Beatson, 1984 ; Jerrett et al., 1990) and from faeces of deer presented for slaughter (Mackintosh & Henderson, 1985).

Y.pseudotuberculosis is a facultative anaerobic pathogenic microorganism affecting a wide range of animals including man and birds (Bercovier & Mollaret, 1984). The bacterium is a psychrotroph and grows well at refrigeration temperatures (Schiemann, 1989). At the DSP, deer carcases are required to be stored at below 10°C after dressing and must be deboned in a temperature controlled room at a temperature of 10°C (Anon, 1991). The venison is vacuum-packed and exported either chilled at -1°C or frozen at temperatures of -12°C or -18°C. The shelf life of chilled vacuum-packed venison is expected to be at least 12 weeks while the frozen product can last for months without spoilage (Drew et al., 1991).

Y.pseudotuberculosis infection in man is usually self limiting (Bell et al., 1988) but severe sequelae such as reactive arthritis (Rose, 1976) and erythema nodosum (Wilkinson et al., 1969) may develop. The disease may also be associated with liver cirrhosis, haematochromatosis (Brodie et al., 1973 ; Macualay et al., 1967), intestinal haemorrhages and ileitis (Weber et al., 1970 ; Savage & Dunlop, 1976 ; Tobin et al., 1988). The pseudoappendicular syndrome in the form of an acute mesenteric lymphadenitis is the most common presentation (Winblad et al., 1966 ; Mair & Fox, 1986). A septicaemic form has been also reported but is relatively rare (Macaulay et al., 1967 ; Brodie et al., 1973 ; Bradley & Skinner, 1974 ; Paff et al., 1976).

Yersiniosis in animals is also frequently self-limiting (Bell et al., 1988). Acute yersiniosis takes the form of a fulminating septicaemia, death resulting in one to three days. The only lesion is intestinal congestion. In the subacute and chronic forms, the disease is characterized by loss of weight over a period of days or weeks, increasing listlessness, severe diarrhoea, respiratory distress, muscular weakness and incoordination (Mair, 1973). Affected deer showing signs of foetid, watery and bloody diarrhoea usually die within one to two weeks if left untreated (Mackintosh & Henderson, 1984b ; Griffin, 1987).

This study was designed to investigate the possibility of transmission of *Y.pseudotuberculosis* in faeces of deer presented for slaughter to carcase meat. Other aspects of the study include the serological status of deer presented for slaughter, the bacteriology of venison sausages and the growth of the bacterium in vacuum-packed venison stored at chill and freezing temperatures. This thesis concentrates on the public health aspects of *Y.pseudotuberculosis* in deer and venison.

and with a transparent periphery surrounding an opaque, pale lavender, raised centre. The border may be entire or undulated. The surface is often granular in the central portion with a glistening to granular transparent flat periphery (Bissett, 1981).

On Cefsulodin-Irgasan-Novobiocin (CIN) agar, the appearance of the colonies varies between different serogroups. In general, the colonies are whitish and pin-point in size, and after 24 hours incubation, they have a red centre surrounded by a translucent periphery. After 48 hours, the colonies are usually about 2.0 mm in diameter, and appear flat, dark red and dry with an entire border. More details are provided in Chapter 3.

A pH in the range from 6.0 to 8.0 is most suitable for the growth of *Y.pseudotuberculosis* (Bissett, 1981). Bercovier & Mollaret (1984) reported the pH range for growth at 5.0 to 9.6 with optimum growth occurring between 7.2 to 7.4.

b. Biochemical characteristics

Y.pseudotuberculosis strains are exceptionally constant in their biochemical characteristics with only rare individual strain variation (Bissett, 1981). They are catalase positive and oxidase negative, grow on MacConkey agar at 37°C, and are glucose fermenters (Weaver et al., 1983). They produce urease, but do not decarboxylate ornithine, lysine or arginine. A list of biochemical reactions of *Y.pseudotuberculosis* is shown in Table 2.1.

c. Serotyping

Y.pseudotuberculosis has been divided into six serogroups (I to VI), on the basis of type-specific, thermostable, somatic O-antigens. Subgroups labeled A and B have been determined for Group I, II, IV and V (Tsubokura et al., 1970 ; Bissett, 1981 ; Mair & Fox, 1986). Tsubokura et al., (1984b) have included a subgroup IIC, and two new serogroups VII and VIII into the list, but these new introductions await further study. These authors also suggested the replacement of the Roman numerals and a capital letter for each serogroup with a new number and small letter. Thus, the new system if accepted would be serogroups 1a, 1b, 2a, 2b, (2c), 3, 4a, 4b, 5a, 5b, 6, (7), and (8).

The rough antigen (R) is common to all Groups (Bissett, 1981). The antigenic structure of *Y.pseudotuberculosis* is shown in Table 2.2.

Table 2.2 Antigenic structure of *Y.pseudotuberculosis*

O-Group	O-Subgroup	Rough (R) antigen	O-antigen thermostable	H-antigen thermolabile
I [1]	A [a]	1	2,3	a,c
	B [b]	1	2,4	a,c
II [2]	A [a]	1	5,6,[16]	a,d
	B [b]	1	5,7,[16,17]	a,d
	[c]		[5,7,11,18]	ND
III[3]		1	8	a
IV[4]	A [a]	1	9,11	b,a,b
	B [b]	1	9,12	a,b,d
V [5]	A [a]	1	10,14	a,a,e(b)
	B [b]	1	10,15	a
VI[6]		1	13	a
[7]			[19]	ND
[8]			[20]	ND

ND - not determined

(Extracted from Mair & Fox, (1986); letters and numbers in [ ] are new additions or changes proposed by Tsubokura et al., (1984b).

Y.pseudotuberculosis also possesses O-1 antigen which is common to all strains, and

which could induce cross-agglutination (Tsubokura et al., 1971). There is also cross reaction between the O-antigen 5 of Group 2 and the O-factors 4 and 27 of *Salmonella* Group B, the O-antigen 9 of Group 4 with the O-factors 9 and 46 of *Salmonella* Group D and O-factor 14 of the *Salmonella* Group H (Mair & Fox, 1986). The serogroup 6 also shares common antigens with O-Group 55 of *Escherichia coli* (Mair & Fox, 1973 cited by Mair & Fox, 1986). There is a need to absorb the patient's serum with *Salmonella* of the corresponding serogroup prior to performing the agglutination test for antibodies specific for a particular serogroup of *Y.pseudotuberculosis* (Mair & Fox, 1986).

YERSINIA PSEUDOTUBERCULOSIS IN MAN

The early history of *Y.pseudotuberculosis* is described by Mair et al., (1960) and Schiemann, (1989). *Y.pseudotuberculosis* was first isolated by Malassez and Vignal in 1883 from a guinea pig inoculated with pus derived from a child with "tuberculous" meningitis. In 1889, Pfeiffer was to describe the bacterium in detail and called it *Bacillus pseudotuberculosis rodenticum.* It was in 1909 that the bacterium was first isolated, by Saisawa, from a septicaemic patient. Even with this finding, the bacterium continued to be considered only as an animal pathogen. It was not until the early 1950's, when children undergoing appendectomies were observed to have lymphadenitis, that *Y.pseudotuberculosis* was linked to this syndrome. Thereafter Knapp (1954) and Knapp & Mashoff (1954) cited by Schiemann (1989) isolated the bacterium from mesenteric lymph nodes of children undergoing appendectomies.

Yersinia is a recent addition to the family enterobacteriaceae. Previously the name *Pasteurella pseudotuberculosis* was used, but with their transfer to *Yersinia* they became *Yersinia pseudotuberculosis* (Farmer III et al., 1985). The name *Yersinia* was proposed to honour the French bacteriologist, Yersin who, in 1894, had isolated the plague bacillus, *Yersinia pestis*, during an epidemic in Hongkong (Schiemann, 1989).

Since then, a large number of cases of *Y.pseudotuberculosis* infection have been reported in the United Kingdom (Mair et al., 1960 ; Randall & Mair, 1962 ; Macaulay et al., 1967 ; Weber et al., 1970 ; Attwood et al., 1987), in Finland (Tertti et al., 1984 ; Merilahti-Palo et al., 1991), in New Zealand (Henshall, 1963 ; Rose, 1976 ; Malpass, 1981), in Japan (Sato, 1987 ; Fukushima et al., 1988 ; Fukushima et al., 1989 ; Nakano et al., 1989 ; Tsubokura et al., 1989), in Italy (Chiesa et al., 1987), in Canada (Toma, 1986), and in the United States of America (Finlayson & Fagundes, 1971 ; Paff et al., 1976).

There have been several reported outbreaks of *Y.pseudotuberculosis* in Japan (Sato,

1987 ; Nakano et al., 1989 ; Tsubokura et al., 1989) and one in Finland (Tertti et al., 1984). The outbreak of *Y.pseudotuberculosis* infection in Finland involved 19 patients between the age of 5 and 75 years. Many of the patients had been actively dealing with growing, selling and eating vegetables. The symptoms were seen in children during the early stage of the infection whereas the adults were clinically obvious in the later stage. In seven cases, the primary symptom was gastroenteritis or pseudoappendicitis. Two patients underwent appendectomies, 14 had an episode of abdominal pain,and four had diarrhoea. Ten of the 19 patients experienced postinfection complications such as erythema nodosum (6), arthritis (4), iritis (1) and nephritis (1).

In Japan, Izumi fever is now believed to be caused by Y.pseudotuberculosis (Sato, 1987 ; Tsubokura et al., 1989) sporadic outbreaks of *Y.pseudotuberculosis* infection appear to be concentrated in the 1 to 16 year age group, with a peak in children 1 to 2 years of age (Tsubokura et al., 1989). The common clinical symptoms are fever, abdominal pain, diarrhoea, nausea, and vomiting. Strawberry tongue, desquamation of the digits, and myalgia are occasionally observed. Sequelae such as erythema nodosum, arthritis, and renal insufficiency have also been reported (Tsubokura et al., 1989). In an outbreak during the summer of 1984, 39 people were ill after consuming meat products, vegetables and rice (Nakano et al., 1989). Fever was seen in all patients, exanthema in 68% and abdominal pain was experienced by 68% of the patients. The other symptoms seen were skin desquamation, dry cough, diarrhoea, vomiting, arthralgia and acute renal failure.

Y.pseudotuberculosis serogroup 1 is most frequently encountered in man in the United Kingdom (Mair, 1965) but other serogroups have been reported. Serogroup 3 was reported by Savage & Dunlop, (1976) and Tobin et al., (1988), serogroup 4 by Wilkinson et al., (1969) and Attwood et al., (1987) and serogroup 5 by Bradley & Skinner, (1973). In the United States of America serogroup 1 was reported by Finlayson & Fagundes, (1971) and Paff et al., (1976) and serogroup 3 by Paff et al., (1976). Other reports indicate the presence of serogroups 1 and 3 in Canada (Toma, 1986), serogroups 2 and 3 in Finland (Tertti et al., 1984), serogroup 1 from Poland (Borowski et al., 1971), serogroups 1b, 2a, 2c, 3, 4a, 5a, and 5b (Tsubokura et al., 1984 ; Sato, 1987) and 4b (Fukushima et al., 1988) in Japan.

Clinical Manifestations

The clinical manifestations of yersiniosis are divided into two forms namely, the primary acute infections and secondary immunological complications (Mair & Fox, 1986). In the primary acute infections, the pseudoappendicular syndrome in the form of an acute

mesenteric lymphadenitis is the most commonly observed. Eighteen per cent of a small number of cases of mesenteric adenitis in England (Mair & Fox, 1986) and 13% of a larger series from Scandinavia (Winblad et al., 1966) were due to *Y.pseudotuberculosis.*

Mesenteric lymphadenitis is not readily confirmed clinically and a mis-diagnosis of appendicitis is common, subjecting the patients to unnecessary appendectomy (Henshall, 1963 ; Malpass, 1981 ; Tertti et al., 1984 ; Nakano et al., 1989). The usual presentation of mesenteric lymphadenitis is the acute onset of pain in the middle quadrant or the right lower quadrant of the abdomen, fever and leukocytosis. At laparotomy, there is usually a normal appendix, with enlarged mesenteric lymph nodes and occasionally a terminal ileitis and colitis (Daniels, 1961 ; Paff et al., 1976). The syndrome is most common in male children (Paff et al., 1976 ; Borowski et al., 1971) and in adolescents (Paff et al., 1976 ; Mair & Fox, 1986).

Another pseudoappendicular syndrome is caused by an acute terminal ileitis. Daniels (1961) has indicated that this syndrome may be seen together with acute mesenteric lymphadenitis. His observation is shared by others (Weber et al., 1970 ; Savage & Dunlop, 1976 ; Tobin et al., 1988). The common symptoms are "colicky abdomen", nausea and fever, with enlarged mesenteric lymph nodes and inflamed terminal ileum (Weber et al., 1970 ; Savage & Dunlop, 1976). A more serious syndrome of an acute terminal ileitis associated with severe intestinal haemorrhage has been reported by Tobin et al., (1988).

Enteritis is not as commonly seen with *Y.pseudotuberculosis* infection as it is with infection with *Y.enterocolitica* (Mair & Fox, 1986). In two separate outbreaks of *Y.pseudotuberculosis* infections, diarrhoea was observed in 21% of the cases in Finland (Tertti et al., 1984) and 32% of those in Japan (Nakano et al., 1989).

Yersinia septicaemia is a rare disease and shows a predilection for elderly subjects (Paff et al., 1976 ; Mair & Fox, 1986) but has been reported in children and adolescents suffering from thalassaemia, aplastic anaemia, leukaemia and sickle-cell anaemia (Mair & Fox, 1986). Despite antibiotic therapy, the mortality rate of *Yersinia* septicaemia is about 50% (Mair & Fox, 1986).

A fatal case of *Y.pseudotuberculosis* infection associated with hepatic cirrhosis has been reported by Macaulay et al., (1967). The symptoms were anorexia, flatulence, abdominal swelling with occasional vomiting and epistaxis. Brodie et al., (1973) diagnosed a fatal case of *Y.pseudotuberculosis* infection where the patient's liver showed numerous small superficial abscesses. A case of *Y.pseudotuberculosis* infection associated with sickle-cell anaemia was seen by Bradley & Skinner (1974). In another episode of

Y.pseudotuberculosis septicaemia, the onset was insidous, with chills, fever, abdominal pain and diarrhoea. There were also symptoms of nausea, vomiting, jaundice, drowsiness, confusion, and coma. The bacterium was isolated from the blood, liver, spleen, and lymph nodes (Paff et al., 1976). Another fatal case of *Y.pseudotuberculosis* infection in an infant was reported by Borowski et al., (1971) who described petechiae on the skin, acute haemorrhagic colitis and mesenteric lymphadenitis.

A severe form of *Y.pseudotuberculosis* infection was reported in the Far-eastern territories of the former Soviet Union. It was an epidemic characterised by a scarlatiniform rash, arthralgia and acute polyarthritis (Mair & Fox, 1986). It is now referred to as the Far-eastern scarlatiniform fever. In Japan, several outbreaks were reported of Izumi fever or Izumi fever-like illness which had similar symptoms to those seen in Far Eastern fever. Studies by Sato (1987) indicated that Izumi fever is caused by *Y.pseudotuberculosis*.

The secondary immunological complications due to *Y.pseudotuberculosis* are mainly in the form of erythema nodosum and arthritis. Erythema nodosum associated with *Y.pseudotuberculosis* infections has been seen in children and young adults, particularly males, in whom mesenteric adenitis has preceded the eruption (Mair & Fox, 1986). In two cases of erythema nodosum, one patient showed numerous bluish lumps on the skin while the other showed small nodules on the extremities (Wilkinson et al., 1969). Reactive arthritis is the most frequent complication of yersiniosis. However, joint involvement is rarely reported as a complication of *Y.pseudotuberculosis* infection (Mair & Fox, 1986). Two cases of reactive arthritis caused by *Y.pseudotuberculosis* were reported in New Zealand by Rose (1976) and Malpass (1981). The host factors of age and HLA haplotype appear to determine the outcome of the infection. Affected subjects with HLA-27 have a greater risk of developing post-infection autoimmune arthritis because they eliminate the bacteria very slowly (Gransfor et al., 1989 cited by Robins-Browne & Hartland, 1991).

Transmission

Y.pseudotuberculosis is ubiquitous (Blake et al., 1991) and can be recovered from domestic animals such as cattle, sheep, goats and deer (Hodges et al., 1984a), small mammals and birds (Mair, 1973 ; Mackintosh & Henderson, 1984a ; Toma, 1986 ; Fukushima & Gomyoda, 1991), and from surface water and soil (Mackintosh, 1988 ; Fukushima, 1992). The role of animals, birds and their products as a source of infection for man has not been established. However, there is recent evidence of human infection through water contaminated by faeces of cats (Fukushima et al., 1989) and wild animals (Fukushima et al., 1990 ; Fukushima & Gomyoda, 1991). This evidence is supported by the results of

restriction endonuclease typing of isolates, from human patients and from the puddle, sand and the cat and those from the mountain water and wild animals.

Daniels (1961) isolated *Y.pseudotuberculosis* 1a from the faeces of a patient who also had a high agglutination titre (1:2560). *Y.pseudotuberculosis* 1a was also recovered from the faeces of his canary. It has also been suggested that human infection may have been acquired from a dog (Macaulay et al., 1967). While the owner was clinically ill due to infection with *Y.pseudotuberculosis* serogroup 1a, the dog had a high antibody titre to the same organism. A case in Poland also showed high antibody titres in hens to *Y.pseudotuberculosis* with a concurrent infection in the owner (Borowski et al., 1971).

Food animals, especially pigs, are known to harbour *Y.pseudotuberculosis* (Tsubokura et al., 1976 ; Merilahti-Palo et al., 1991 ; Fenwick, pers. comm.,) however, only on one occasion has the organism been recovered from retailed pork (Fukushima, 1985) and pasteurized milk (Greenwood & Hooper, 1989). It has been suggested that human infection may be acquired through the consumption of pork and milk.

Diagnostic Methods

a. Culture

Although the isolation of *Y.pseudotuberculosis* usually establishes a diagnosis this is not always the case, as patients suffering from the immunopathological forms of yersiniosis may not be excreting the organism (Heeseman et al., 1987). The organism may not always be recovered from the infected mesenteric lymph nodes (Mair & Fox, 1986).

b. Histology

The lesion starts with lymphoid tissue hyperplasia, followed by diffuse hyperplasia of histiocytes, and formation of epithelioid granulomas. Later, there is a central coagulative necrosis of the granulomas associated with polymorphonuclear cell infiltrations leading to the formation of yellowish microabscesses (El-Maraghi et al., 1979 ; Mair & Fox, 1986). These histological findings are observed in the mesenteric lymph nodes. The appendix may be mildly inflamed, with occasional microabscesses (Mair & Fox, 1986).

c. Serology

The slide agglutination test for the presence of antibodies in serum provides a rapid presumptive diagnosis but, due to the risk of spontaneous agglutination, a positive slide test

must be confirmed by the tube agglutination test (Mair & Fox, 1986). To demonstrate *Y.pseudotuberculosis* antibody using the tube agglutination test, a suspension of a live, or phenol or formalin-killed culture is used in the case of human sera. Heat-killed organisms are not agglutinated or only weakly agglutinated by human sera (Knapp, 1956) cited by Mair & Fox (1986).

The agglutination test is specific only for serogroups 1, 3 and 5; serogroups 2, 4 and 6 are less specific (Mair & Fox, 1986). Group 2 *Yersinia* share a common antigen with *Salmonella* group B, group 4 with *Salmonella* group D and H (Mair & Fox, 1986) and group 6 with *E.coli* group 55 (Mair & Fox, 1973 cited by Mair & Fox, 1986).

Stahlberg et al., (1987) conducted Enzyme Immunosorbent Assay (EIA) and Immunoblot against sera of 14 patients involved in an outbreak of *Y.pseudotuberculosis* 3 infection in Finland and found that the EIA was able to detect IgM and IgG antibodies to *Y.pseudotuberculosis* 3 in 14 and 12 patients respectively. The immunoblot analysis revealed that *Yersinia* Outer membrane Proteins (YOPs) 4 and 5 are recognised in most patients, YOPs 2 and 3 less frequently, and YOP 1 seems not to induce any antibody response detectable by immunoblotting.

It has been observed that patients with enteric infection do not produce a detectable level of agglutinins to *Y.pseudotuberculosis* until several days after the onset of illness. The antibody titre reaches its peak by the second week of illness. A four fold or greater rise or fall, and a minimum titre of 1:80 is regarded as significant (Sato, 1987). In another study high titres of agglutinins of between 1:160 - 1:10,240 were detected in patients with acute mesenteric lymphadenitis within 24 hours of the onset of symptoms (Mair & Fox, 1986). In a further study the authors regarded a titre of 1:160 as significant, and a titre of more than 1:320 as evidence of infection (Attwood et al., 1987). The agglutinins decline rapidly in titre and usually disappear within three months (Sato, 1987) although some suggest they last for five months (Attwood et al., 1987) or even up to six months (Mair & Fox, 1986). The difference in the duration of antibody titre may be dependent on the initial titre level as has been observed by workers involved in the serological study of yersiniosis in deer (Mackintosh et al., 1986).

Pathogenesis and Virulence

The virulence of *Yersiniae* depends on the presence of a 40 to 50 megadalton (Md) plasmid (Gemski et al., 1980 ; Portnoy et al., 1981). There are several properties encoded by this virulence-associated plasmid such as the calcium dependency at 37°C (Gemski et al.,

1980 ; Berche et al., 1982), autoagglutination at 37°C (Laird & Cavanaugh, 1980), hydrophobicity at 37°C (Martinez et al., 1983 ; Lachica & Zink, 1984), resistence to inactivation by normal human serum (Pai & D_EStephano, 1982), production of V and W antigens and *invasin* an outer membrane protein (OMP)(Portnoy et al., 1981 ; Bolin et al., 1982 ; Kapperud et al., 1985), and virulence for laboratory animals (Portnoy et al., 1981).

Other characteristics have been used as markers of virulence such as biotype (Schiemann & Devenish, 1982), serotype (Une et al., 1977), invasiveness in the guinea pig conjunctiva (Sereny test)(Boyce et al., 1979), production of heat-stable enterotoxin (Pai & Mors, 1978 ; Robins-Browne et al., 1979) and the development of diarrhoea in mice (Laird & Cavanaugh, 1980).

Extensive research is being carried out to understand the pathogenicity of *Yersiniae*. Robins-Browne & Hartland (1991) have updated the issue in their paper. An entero-invasive pathogen is only successful if it is able to penetrate the intestinal mucosa and colonize the gut-associated lymph nodes (Robins-Browne et al., 1985). All pathogenic and non-pathogenic strains of *Yersiniae* possess the gene, *inv* which produces *invasin*, a protein localized on the outer membrane (Pepe & Miller, 1990). When *Yersiniae* bind to cells, they presumably activate a phagocytic process, even in cells that are normally non-phagocytic, inducing them to ingest the bacteria. In this sense, pathogenic *Yersiniae* promote their own uptake (Robins-Browne & Hartland, 1991).

Miller et al., (1990) described another chromosomal gene *ail* (attachment invasion locus), which codes for a protein produced by *Y.enterocolitica*. This protein promotes bacterial adhesion to some cells and invasion of others. A chromosomal gene of *Y.enterocolitica* called *yst* is involved with the production of heat-stable enterotoxin (Delor et al., 1990), which is secreted into the culture medium by most clinical isolates (Robins-Browne et al, 1979). Carniel et al., (1989) described yet another chromosomally-encoded virulence determinant which is involved in the uptake of iron by *Yersiniae*.

Y.pseudotuberculosis, *Y.enterocolitica* and *Y.pestis* carry virulence plasmids. These species display a distinctive phenotypic characteristic called "calcium dependency at 37°C" in media with low calcium ions. In this situation, the bacteria secrete a series of plasmid-encoded proteins called YOPs (Yersinia Outer Membrane Proteins) (Heeseman et al., 1986 ; Michiels et al., 1990). Some YOPs mediate bacterial adherence to human epithelial cells and protect against non-specific killing by complement components in normal human serum (Kapperud et al., 1987). Other YOPs may protect the bacteria from phagocytosis, while others induce cytotoxic damage to host cells (Lian et al., 1987 ; Rosquist et al., 1988).

Robins-Browne & Hartland (1991) give a good account of the pathogenesis of *Y.enterocolitica* based on the studies undertaken of late. Because a majority of the cases of yersiniosis occur in the temperate regions, the bacterium would have been growing in the food at temperatures below 30°C. At these temperatures the bacteria show motility, which favours their ability to penetrate the intestinal mucus, and establish contact with the epithelium. Production of heat-stable enterotoxin is also enhanced at this temperature.

In complicated cases the clinical signs and symptoms are usually restricted to the intestinal tract, with diarrhoea and abdominal discomfort resulting from mucosal inflammation and the effects of the enterotoxins. Mesenteric adenitis occurs if the bacteria are transported, probably within phagocytes and via the draining lymphatics, to the lymph nodes. The fact that the infection is self-limiting may be explained by the inability of the bacteria to acquire sufficient iron to sustain their growth *in vivo* (Robins-Browne et al., 1987). However, *Yersiniae* may cause systemic infection in hosts suffering from iron overload, in particular those who are receiving treatment with the iron chelator, desferroxamine B (Robins-Browne & Prpic, 1983). In addition to the gastrointestinal syndrome some immunopathological diseases associated with *Yersinia* infection such as arthritis, erythema nodosum and uveitis (Heeseman et al., 1987) may develop.

Isolation using cold-enrichment

Both *Y.pseudotuberculosis* and *Y.enterocolitica* are psychrotrophic microorganisms that grow well at refrigeration temperature (Mair & Fox, 1986 ; Schiemann, 1989). *Y.enterocolitica* is able to grow rapidly at 2°C and 5°C when present in pure culture (Nielsen & Zenther, 1984), and still grows, but at a slower rate at 0°C (Hanna et al., 1977b) and -2°C (Gill & Reichel, 1989).

Cold-enrichment was introduced to enhance the growth and recovery of *Yersiniae*. The protocol was initially developed for the isolation of *Y.pseudotuberculosis* by incubating at +4°C for several days (Paterson & Cook, 1963 ; Tsubokura et al., 1973), but is now extended to *Y.enterocolitica* and related species (Van Noyen et al., 1981).

The principle of cold-enrichment has been studied using *Y.enterocolitica* as the model. *Y.enterocolitica* and related species are known to be poor competitors with the spoilage organisms commonly found in milk (Stern et al., 1980) and meat (Kleinlein & Untermann, 1990). The bacterium grows more slowly than most non-*Yersiniae* and could be overgrown and masked when streaked onto a weakly selective agar if other bacteria are present (Aulisio et al., 1980). Schiemann (1982) observed that the growth of

Y.enterocolitica ceased when a competing organism reached a stationary phase.

The beneficial effect of cold-enrichment appears to result from a temperature induced reduction of the growth rates of non-*Yersinia* species present so that *Y.enterocolitica* comes closer to attaining its potential maximum population before the other organisms reach the stationary phase. At +4°C the growth of other organisms is inhibited so that *Y.enterocolitica* has a selective advantage (Schiemann & Olson, 1984).

There are several enrichment broths used for the recovery of *Yersinia spp*. As an example, the Bile-Oxalate-Sorbose (BOS) broth was developed mainly for the isolation of *Y.enterocolitica* serovar O:8 which has been one of the most difficult to recover from food (Schiemann, 1979 ; Lee, 1977). On the other hand, *Y.enterocolitica* serovar O:5,27 is only isolated in BOS broth with great difficulty. When 2.5% sodium chloride was added to the media, improved growth of serovar 0:5,27 was observed. This however affects the recovery of *Y.enterocolitica* serovar O:3 which is sensitive to sodium chloride (Schiemann, 1982).

It is still difficult to select a single enrichment system that will be equally efficient for the recovery of all pathogenic forms of *Y.enterocolitica*. One must consider the types of *Yersiniae* being sought when selecting an enrichment system and obviously the use of several systems will provide the greatest recovery potential (Schiemann, 1982). Due to the influence of food and sample types, the level and conditions of *Yersiniae* present, and the presence of extraneous microorganisms, it is advisable to use more than one medium for both the enrichment and the plating procedures (Cox et al., 1990).

Phosphate Buffered Saline (PBS) has been recommended as the cold enrichment diluent of choice for the isolation of *Y.pseudotuberculosis* (Tsubokura et al., 1973) and *Y.enterocolitica* (Kounev, 1989a). The latter recommended the use of PBS because of its poor nutritive content which means that it does not support the accumulation of hydrogen peroxide in the presence of injured cells.

Cold-enrichment at +4°C for 21 days results in considerable delays in isolation of *Yersinia spp*. (De Boer & Seldan, 1987). However, the period can be shortened substantially by increasing the incubation temperature to about 15°C (Schiemann & Olson, 1984). Doyle & Hugdahl (1983) found that the recovery of *Yersiniae* is better when the samples are in a small volume, enriched in PBS and incubated at 25°C. There are suggestions that incubation at +4°C may cause stress to the already injured cells making recovery the more difficult (Kounev, 1989b) and that the additional recoveries obtained by cold-enrichment were types of *Y.enterocolitica* that were not clinically important (Van Noyen et al., 1981).

Treatment

Yersiniosis is a self-limiting disease usually with an uneventful recovery (Henshall, 1963 ; Paff et al., 1976 ; Mair & Fox, 1986 ; Bell et al., 1988). Gastrointestinal symptoms usually subside in two to three weeks without recourse to antibiotics (Mair & Fox, 1986). However, treatment is indicated when the infection is persistent or recurrent (Paff et al., 1976), or in patients with septicaemia, intra-abdominal sepsis, severe terminal ileitis (Mair & Fox, 1986) and prolonged elevation of serologic titres (Weber et al., 1970).

Y.pseudotuberculosis and *Y.enterocolitica*, except for a few strains with resistence plasmids, are susceptible to those antibiotics which are usually active against gram-negative rods. *Y.enterocolitica*, by virtue of its beta-lactamase activity, is resistent to penicillin, ampicillin, cephalothin and carbenicillin, all of which are active against *Y.pseudotuberculosis*. Only *Y.enterocolitica* serovar O:8 is suceptible to ampicillin. In adults, tetracycline is the drug of choice while chloramphenicol, streptomycin and gentamicin should be reserved for serious cases. Co-trimoxazole is also effective in the treatment of yersiniosis (Mair & Fox, 1986). Weber et al., (1970) and Paff et al., (1976) recommended streptomycin and tetracycline for the treatment of yersiniosis.

In vitro tests have shown that *Y.pseudotuberculosis* is sensitive to ampicillin, cephalothin, chloramphenicol, kanamycin, penicillin, streptomycin and tetracycline (Finlayson & Fagundes, 1971). In another study, Kanazawa et al., (1987) showed that *Y.pseudotuberculosis* is sensitive to penicillin, chloramphenicol, the first generation cephalosporins, the beta lactamase-stable third generation cephalosporins and the new beta-lactams, such as carbapenem.

Y.pseudotuberculosis is resistent to ampicillin (Paff et al., 1976), Colistin (Finlayson & Fagundes, 1971), lincomycin and novobiocin (Kanazawa et al., 1987), and erythromycin (Finlayson & Fagundes, 1971 ; Kanazawa et al.,1987).

The *in vitro* sensitivity test on *Y.pseudotuberculosis* isolates from red deer showed that the isolates were sensitive to ampicillin, streptomycin chloramphenicol, gentamicin, kanamycin, neomycin, trimethoprim-sulphamethoxazole, and tetracycline (Hodges et al., 1980).

The difference in response to ampicillin between the human strains (Paff et al., 1976) and with the human (Finlayson & Fagundes, 1971) and deer (Hodges et al., 1980) strains may be due to the presence of resistence plasmids carried by the former and perhaps due to the longer and greater use of ampicillin in man. All the isolates were resistent to

bacitracin, erythomycin, novobiocin and moderately resistent to furazolidone. The antibiotics, neomycin, streptomycin, tetracycline or a trimethoprim-sulphonamide combination have been recommended for the treatment of *Y.pseudotuberculosis* infection in deer (Hodges et al., 1980).

Vaccination

The only known commercial vaccine against *Y.pseudotuberculosis* "Yersinavax" has been developed for deer in New Zealand (Mackintosh et al., 1991). The vaccine, prepared from multiple strains of *Y.pseudotuberculosis* which had been formalin-killed and aluminium hydroxide adjuvanted was tested on 2216 deer calves but did not give 100% protection against the disease (Mackintosh et al., 1986). A further trial using oil adjuvanted killed multistrain *Y.pseudotuberculosis* gave a significant protection against the development of yersiniosis. The results showed that after deer were stressed and challenged orally 54% of unvaccinated and 31% of vaccinated test animals developed yersiniosis (Mackintosh et al., 1991). A trial in 1990 showed that 30% of the vaccinated and stressed group and 30% of the unvaccinated non-stressed control group developed yersiniosis. Of the unvaccinated, stressed controls, 60% developed clinical yersiniosis (Mackintosh et al., 1991). The disease was observed to be stress-induced, therefore management plays an equally important part in reducing yersiniosis in farmed deer. A field trial in 1991 involving 2463 calves on 17 farms was conducted under natural conditions to test the vaccine made from formalin-killed multistrains Y.pseudotuberculosis sergroups 1, 2 and 3 plus concentrated supernatant, and with adjuvant DEAE dextran (Mackintosh et al., 1992). Outbreaks of yersiniosis were observed in three farms. The rate of clinical cases in the unvaccinated herd ranged from 14.5 to 21% whereas it was from 4 to 8.5% in the vaccinated group. There were more deaths in the non-vaccinated than in the vaccinated group. From the above results the vaccine appeared to have reduced the number of cases of clinical infection and death.

***YERSINIA PSEUDOTUBERCULOSIS* IN DEER**

Yersiniosis due to *Y.pseudotuberculosis* has emerged as a major disease of farmed deer (Henderson, 1983) and is recognized as one of the leading causes of death in deer in New Zealand (Beatson, 1984 ; Griffin, 1987), Australia (Jerrett et al., 1990), and in the United Kingdom (Fletcher, 1982). Red deer (*Cervus elaphus*) appears to be the species most commonly infected with *Y.pseudotuberculosis* but there are also reports of Wapiti (*Cervus elaphus canadiensis*) (Henderson, 1984), Chital deer (*Axis axis*) (Jerrett et al., 1990), and Fallow deer (*Dama dama*) (Chapman et al., 1979) being affected. *Y.pseudotuberculosis* serogroups 1, 2 and 3 have been recovered from farmed red deer in

New Zealand (Henderson, 1984) and Australia (Jerrett et al., 1990). Serogroups 1 and 2 were isolated from the Chital deer (Jerrett et al., 1990) and serogroup 1 from Wapiti (Henderson, 1984). In Japan, *Y.pseudotuberculosis* serogroups 3, 4b and 5a were isolated from feral deer (Fukushima & Gomyoda, 1991) whereas serogroups 1b, 2 and 3 were recovered from deer in Canada (Toma, 1986).

Deer are highly susceptible to *Yersinia* infection (Henderson, 1983). The disease mostly affects young weaners (Henderson, 1984 ; Beatson, 1984 :Mackintosh, 1988 ; Jerrett et al., 1990) although infection in mature deer is also encountered (Beatson, 1984 ; Jerrett et al., 1990). The disease is observed to occur frequently during winter (Henderson, 1984 ; Jerrett et al., 1990) and spring (Jerrett et al., 1990) and is believed to be precipitated by stressors such as poor quality feed, inclement weather conditions (Mackintosh & Henderson, 1984b), underfeeding and transportation (Jerrett et al., 1990 ; Mackintosh and Henderson, 1984b). Infection, presumed to follow the ingestion of faecally contaminated feed, initially leads to a transient subclinical enteritis with local lymphatic invasion. Subsequent stress may trigger a fulminating septicaemia. In the chronic form of the disease the affected animal is diarrhoeic and generally loses condition over one to two weeks preceding death (Griffin, 1987). The diarrhoea is watery, foetid, and sometimes blood-tinged (Mackintosh & Henderson, 1984b). A significant proportion of sudden deaths in deer is caused by yersiniosis (Beatson, 1984 ; Jerrett et al., 1990).

On necropsy, the ileum, caecum and colon are severely affected. The intestinal wall is thickened and a fibrinonecrotic pseudomembrane covers the mucosal surface. The contents of the intestines is fluid and blood-tinged. The mesenteric lymph nodes are enlarged, oedematous and haemorrhagic while the abdominal viscera may be congested (Beatson, 1984).

While the postmortem findings in New Zealand are basically similar to those observed elsewhere, workers in Australia reported that about 50% of the cases there showed green faecal staining of the perineum, and that the ileocaecal lymph nodes were severely affected (Jerrett et al., 1990).

There are also species difference seen between Red and Chital deer. For example, multifocal erosions of the small and large intestinal mucosa appear to be more prevalent in Chital than in Red deer, and multifocal pale foci 1-2 mm in diameter were observed in the liver of most Chital but were not found in Red deer (Jerrett et al., 1990).

Microscopically, the affected intestines show multifocal dense accumulations of neutrophils surrounding bacterial colonies in the superficial lamina propria (Henderson,

1983 ; Jerrett et al., 1990). The liver is congested with small disseminated foci of necrosis and polymorphonuclear infiltration throughout the parenchyma. The mesenteric lymph nodes have scattered focal areas of necrosis with an associated marked polymorphonuclear infiltration and colonies of bacteria (Henderson, 1983).

Y.pseudotuberculosis has also been isolated from faeces of healthy (Henderson & Hemmingsen, 1983) and sick, scouring deer (Jerrett et al., 1990), from the intestinal tract and mesenteric lymph nodes of dead animals and occasionally from parenchymatous organs (Anon, 1978a). There are also reports of the isolation of *Y.pseudotuberculosis* from aborted foetuses and foetal membranes (Anon, 1979c).

YERSINIA PSEUDOTUBERCULOSIS IN CATTLE

Y.pseudotuberculosis serogroups 1, 2 and 3 have been recognized in cattle in New Zealand (Hodges & Carman, 1985), and in Australia (Slee et al., 1988). Serogroup 1a has been identified in the United Kingdom (Mair & Harbourne, 1963), serogroups 1b and 3 in Canada (Toma, 1986), and serogroup 2b in Japan (Tsubokura et al., 1989). *Y.pseudotuberculosis* has also been reported in cattle in the United States of America (Brown & Davis, 1989).

The bacterium has been incriminated in cattle showing signs of enteritis with profuse and foetid diarrhoea which sometimes contained flecks of blood (Slee et al., 1988 ; Callinan et al., 1988 ; Brown & Davis, 1989 ; Harihayan & Bryenton, 1990) but also apparently healthy cattle have been found to carry the bacteria in their faeces (Hodges & Carman, 1985 ; Bullians, 1987 ; Tsubokura et al., 1989).

Y.pseudotuberculosis has been isolated from the liver (Anon, 1979b ; Callinan et al., 1988), lymph nodes (Callinan et al., 1988 ; Harihayan & Bryenton, 1990), intestinal contents (Slee et al., 1988 ; Callinan et al., 1988), and lungs (Langford, 1969 ; Callinan et al., 1988), of aborted foetuses (Mair & Harbourne, 1963 ; Toma, 1986 ; Jerrett & Slee, 1989), and from bovine placental membranes (Langford, 1969) and cotyledons (Jerrett & Slee, 1989).

Younger animals appear to be more susceptible to the disease (Brown & Davis, 1989), but all ages can be affected (Slee et al., 1988). Calves less than three months of age were observed to be uninfected and were presumably protected by the presence of maternal antibody (Slee et al., 1988). Clinical enteritis in cattle appears mainly to be caused by *Y.pseudotuberculosis* belonging to serogroup 3 (Slee et al., 1988) whereas members of

serogroup 1a (Mair & Harbourne, 1963) and 1b (Toma, 1986) have been associated with abortion which occurred at the 4th, 5th (Langford, 1969) or 6th month (Mair & Harbourne, 1963) of gestation.

Apparently healthy cattle were found to have agglutination titres of between 1:10 to 1:80 to *Y.pseudotuberculosis* (Slee et al., 1988). A cow that aborted in the 6th month of gestation had a titre of 1:640 and two in-contact animals had titres of 1:80 and 1:160 (Mair & Harbourne, 1963).

Following experimental inoculation of cattle with *Y.pseudotuberculosis* serogroup 3, positive antibody titres were detected between day 4 and 7 after the intial challenge and peak titres of 1:160 to 1:320 were detected on the 9th and 16th day respectively (Slee et al., 1988). The cattle excreted the bacteria in their faeces from day 1 to 9 after challenge but showed no clinical or haematological changes. The latter was observed when the excretion of the organism became profuse at day 7 to 11. The clinical signs were fever and diarrhoea while a haematological change was indicated by a left shift in the blood neutrophils. At necropsy, heavy growth of *Y.pseudotuberculosis* was recovered from the small intestines, colon and faeces, and from the palatine tonsil and mediastinal lymph nodes but not from an animal killed at 72 days after the initial challenge.

YERSINIA PSEUDOTUBERCULOSIS IN GOATS

Y.pseudotuberculosis serogroups 2b and 3 have been identified in goats in New Zealand (Lanada, 1990 ; Fenwick, pers. comm.,), serogroup 1 and 3 in Australia (Slee & Button, 1990), serogroup 3 in Canada (Toma, 1986) and the United States of America (Cappucci et al., 1978), serogroup 1a in the United Kingdom (Jones et al., 1982), and serogroup 1 (Morita et al., 1973) and 1b (Tsubokura et al., 1989) in Japan.

Slee & Button (1990) found that serogroups 1 and 3 are responsible for a watery, non-bloody diarrhoea in goats in Australia. This organism has also been found to cause generalized infection (Morita et al., 1973) and abortion (Cappucci et al., 1978 ; Witte et al., 1985) in goats.

The bacterium has been isolated from the intestinal contents of recently captured feral goats (Anon, 1979a), mastitic goats milk (Cappucci et al., 1978 ; Jones et al., 1982), placenta and abomasal contents of dead newly born kids (Witte et al., 1985) and from the faeces of healthy goats (Orr et al., 1987 ; Lanada, 1990).

Goats experimentally exposed to *Y.pseudotuberculosis* serogroup 3 excreted the organism in their faeces after day 5, and also developed a mild, left shift in blood neutrophils. Antibodies were detected by day 9 (Slee & Button, 1990).

YERSINIA PSEUDOTUBERCULOSIS IN SHEEP

Y.pseudotuberculosis has been recognized as a cause of sporadic outbreaks of a fatal disease in sheep in Australia (Philbey et al., 1991). The consistent lesions in the intestinal tract of affected sheep were an acute segmental, suppurative, erosive enterocolitis with the formation of microabscesses around colonies of bacteria (Slee & Skilbeck, 1992). Two separate outbreaks of abortion caused by *Y.pseudotuberculosis* have been reported in ewes (Anon, 1982 ; Karbe & Erickson, 1984).

The bacterium has been isolated from aborted foetuses (Hartley & Kater, 1964), the liver (Watson & Hunter, 1960 ; Karbe & Erickson, 1984), and from stomach contents of aborted foetuses (Watson & Hunter, 1960 ; Dennis, 1966 ; Karbe & Erickson, 1984), from lungs, kidneys and the placenta (Karbe & Erickson, 1984), from diarrhoeic faeces (Hariharan & Bryenton, 1990 ; Slee & Button, 1990 ; Philbey et al., 1991), and from mesenteric lymph nodes of sheep (Anon, 1979b). *Y.pseudotuberculosis* has also been recovered from a ram with infectious epididymo-orchitis (Jamieson & Soltys, 1947) and from faeces of healthy lambs at an abattoir (Bullians, 1987).

Organisms belonging to serogroup 3 appear to predominate in sheep (Philbey et al., 1991 ; Slee & Skilbeck, 1992), and can affect young lambs from two to four months of age but the isolation of *Y.pseudotuberculosis* is most frequent from one to two year old sheep with no apparent clinical evidence of disease (Slee & Button, 1990 ; Slee & Skilbeck, 1992). Members of serogroup 1 (Philbey et al., 1991) and lb (Jamieson & Soltys, 1947 ; Toma, 1986) have also been recovered from sheep.

Sheep may excrete the bacteria for a period of one to 14 weeks (Philbey et al., 1991). When experimentally infected, the animals excreted the organism from day 2, and then developed mild, non-bloody diarrhoea with a left shift in neutrophils on days 4 to 6. Serum antibodies were detected on day 7 after the initial challenge (Slee & Button, 1990).

Karbe & Erickson (1984) inoculated pregnant ewes intravenously with *Y.pseudotuberculosis*. The ewes infected at 2.5 to 3.5 months of gestation showed an elevated temperature and developed a severe purulent placentitis and endometritis. These lesions were associated with foetal death, abortion, and ewe mortality. Ewes infected when

they were at about four months of gestation suffered from a more focal placentitis of varying severity resulting in stillbirth or delivery of moribund, weak or healthy lambs. Stillborn lambs infected with *Y.pseudotuberculosis* may have focal necrotizing hepatitis (Karbe & Erickson, 1984).

YERSINIA PSEUDOTUBERCULOSIS IN SWINE

Yersinia pseudotuberculosis serogroups 2b and 3 have been recognized in New Zealand (Fenwick, pers. comm.,). In Australia, serogroup 3 is a cause of enteritis in pigs (Slee & Button, 1990 ; Harper et al., 1990). Serogroups 1b (Tsubokura et al., 1970, 1976), 2b, 2c, 3, 4b, 5a, and 5b (Tsubokura et al., 1989) have been isolated in Japan whereas serogroups 1b and 3 have been recovered from pigs in Canada (Toma, 1986 ; Hariharan & Bryenton, 1990) and serogroup 1 from Norway (Kapperud, 1986). Serogroup 3 appears to be most commonly isolated from pigs in Canada (Toma, 1986), Japan (Tsubokura et al., 1989), Australia (Slee & Button, 1990 ; Harper et al., 1990), Italy (Chiesa et al., 1987), Finland (Merilahti-Palo et al., 1991) and Brazil (de Barcellos & de Castro, 1981).

Y.pseudotuberculosis has been recovered from apparently healthy swine (Zen-Yoji et al., 1974 ; Tsubokura et al., 1976 ; Blackall, 1977 ; Mair et al., 1979), and from pigs with diarrhoea (de Barcellos & de Castro, 1981 ; Harper et al., 1990 ; Hariharan & Bryenton, 1990). Young pigs aged between 10 and 14 weeks appear most susceptible to the disease (de Barcellos & de Castro, 1981 ; Harper et al., 1990).

Swine may be an important reservoir for human infection with *Y.pseudotuberculosis* (Tsubokura et al., 1976 ; Shiozawa et al., 1988). The bacterium has been recovered from retailed pork in Japan (Fukushima, 1985).

Experimentally infected pigs began excretion of the bacteria on day 8, and had developed mild, non-bloody diarrhoea and a left shift in blood neutrophils. Antibodies were detected by the agglutination test on day 10. At necropsy, there were no gross lesions except for some vascular congestion in the distal jejunum and ileum. The bacterium was recovered from the intestinal contents (Slee & Button, 1990).

YERSINIA PSEUDOTUBERCULOSIS IN HORSES

Y.pseudotuberculosis serogroup 2a has been recovered from a 10 week old foal

which died after developing pneumonia (Mair & Ziffo, 1974). The postmortem findings were numerous microabscesses throughout the liver, spleen and both lungs. Pure growth of the bacterium was cultured from these organs.

Y.pseudotuberculosis has been isolated from horses using inoculation of infected material into guinea pigs (Pfeiffer, 1889, Schlaffke, 1921, cited by Mair & Ziffo, 1974). The same authors also mentioned the works of Monteverde & Banadea (1935) who obtained a pure culture of *Y.pseudotuberculosis* from a suppurating submaxillary lymph node of a mare.

YERSINIA PSEUDOTUBERCULOSIS IN CATS

Y.pseudotuberculosis serogroups 1b and 2a have been recognized in cats in Canada (Toma, 1986) while in Japan, serogroups 1b, 2b, 2c, 3 and 4b are involved (Tsubokura et al., 1989). In New Zealand, serogroups 1, 2 and 3 have been isolated from feral cats (Mackintosh & Henderson, 1984a).

Y.pseudotuberculosis infection in cats was reported as early as 1902 in Amsterdam and in several other European countries (cited by Mair et al., 1967). In the United Kingdom, two cases were reported. One of the cats was emaciated, and passing loose, black foetid faeces, and had urinary incontinence with frequent micturition. The other cat was jaundiced and had abdominal discomfort. *Y.pseudotuberculosis* 2a was isolated from both cats (Mair et al., 1967). Evidence of yersiniosis due to *Y.pseudotuberculosis* in urban cats in the United Kingdom has also been reported (Robinson, 1972). Obwolo & Gruffydd-Jones (1977) reported the isolation of *Y.pseudotuberculosis* serogroup 2b from the kidneys and lungs of a cat which showed inappetance, emaciation, jaundice and enlarged kidneys.

A case of *Y.pseudotuberculosis* was reported in a cat from Papua New Guinea that died in quarantine in Australia. The cat was anorexic, with vomiting and diarrhoea (O`Sullivan et al., 1976). In Canada, *Y.pseudotuberculosis* was isolated from numerous yellowish-white, slightly raised, 1-2 mm subcapsular nodules on the liver of a cat presented with anorexia, lethargy and occasional vomiting (Spearman et al., 1979).

In contrast to most other species adult cats appear to be more commonly affected than kittens and young cats (Pallaske & Meya, 1932 cited by Mair et al., 1967). However, Obwolo & Gruffydd-Jones (1977) stated that *Y.pseudotuberculosis* infection is apparently rare in cats. It is difficult to reproduce the disease artificially in cats (Goret et al., 1957) and cats may be subclinical carriers which spread the organism (Mollaret, 1965, both works cited

by Obwolo & Gruffydd-Jones (1977).

In Japan, the isolation rate of *Y.pseudotuberculosis* from cats is between 0.2% (Kaneuchi et al., 1987) and 3.2% (Yanagawa et al., 1978 cited by Kaneuchi et al., 1987).

It is likely that the cat is infected through eating rodents and birds, believed to be the natural reservoirs of *Y.pseudotuberculosis* (Mair et al., 1967). Cats have been incriminated as the source of *Y.pseudotuberculosis* infection in man (Albrecht, 1910 ; Paul & Weltmann, 1934 ; Mollaret, 1965) as cited by Mair et al., (1967). Fukushima et al., (1989) reported the first evidence of *Y.pseudotuberculosis* being transmitted to man via water contaminated by infected cat faeces. The restriction endonuclease patterns of the *Y.pseudotuberculosis* isolated from the patients, soil, sand, and the cat showed identical plasmid profiles.

YERSINIA PSEUDOTUBERCULOSIS IN DOGS

Y.pseudotuberculosis was isolated from a dog which died of gastroenteritis and anaemia (Collet et al. 1955 cited by Obwolo, 1976). However, *Y.pseudotuberculosis* serogroups 1b, 2b, 2c, 3, 4a, 4b, 5a, 6, and 7 were recognised in healthy dogs in Japan (Tsubokura et al., 1989). In New Zealand, the bacterium has also been recovered from apparently healthy dogs (Fenwick & Madie, pers. comm., 1992).

In two separate cases in the United Kingdom, pet dogs were observed to have significant seroconversion to *Y.pseudotuberculosis* serogroup 1a while in one case, their owner (Macaulay et al. 1967) and in another case, a member of the owner's family (Randall & Mair, 1962) were clinically affected with the disease. There is a suggestion that this may constitutes a zoonosis as in the former, the owner was bitten by the dog while in the latter, the boy took over his father's daily chore of feeding the animals.

YERSINIA PSEUDOTUBERCULOSIS IN WILDLIFE AND BIRDS

Wild rats and birds have been incriminated as the reservoirs of *Y.pseudotuberculosis* (Obwolo, 1976). The source of repeated outbreaks among a colony of guinea pigs was traced to green feeds contaminated with excreta of wood pigeons (Paterson & Cook, 1963). In Japan, wild animals are believed to be important reservoirs of *Y.pseudotuberculosis* (Fukushima et al., 1990 ; Fukushima & Gomyoda, 1991). In New Zealand, *Y.pseudotuberculosis* has been isolated from feral cats, rats, hares and birds (Mackintosh & Henderson, 1984a). The bacterium has also been isolated from rodents in Italy (Chiesa et

al., 1987).

Outbreaks of *Y.pseudotuberculosis* infection among chinchilla and canaries have been reported in Canada (Langford, 1972) and *Y.pseudotuberculosis* has been recovered from the lion, kangaroo, shrew, hedgehog, python and tortoise (Obwolo, 1976). Mammals and birds in zoos in the United Kingdom have been shown to be infected (Parsons, 1991). Of 4776 animals necropsied at Regent's Park during the period 1981 to 1987, 38 yielded *Y.pseudotuberculosis* while at Whipsnade Park, 2871 necropsies yielded 19 isolates. The majority of the animals were found to be thin, and white necrotic foci were seen throughout the liver and spleen. The lungs, kidneys, intestines and various lymph nodes were less commonly affected. Fifteen out of 57 isolates which had been typed comprised of serogroups 1a, 1b and 2a (Parsons, 1991). Birds in Zoos in Japan have also been shown to be infected (Kanzaki et al., 1988 cited by Sanekata et al., 1991).

The presence of *Y.pseudotuberculosis* in wildlife has been studied by workers in Japan, Canada, United States of America, United Kingdom and New Zealand and their findings are shown in Table 2.3.

YERSINIA PSEUDOTUBERCULOSIS IN ARTHROPODS

Y.pseudotuberculosis has been isolated from ixodid ticks but its significance is unclear because infected ticks failed to transmit infection to susceptible hosts (Obwolo, 1976). *Y.pseudotuberculosis* has been found to be pathogenic for human lice (Krynski & Becla, 1964) while fleas remain carriers of the bacteria for up to 35 days after feeding on infected guinea pigs, but have not been shown to transmit the infection to susceptible guinea pigs (Blanc & Baltazard, 1944 cited by Obwolo, 1976).

YERSINIA PSEUDOTUBERCULOSIS IN THE ENVIRONMENT

Y.pseudotuberculosis is ubiquitous in nature (Blake et al., 1991) and has been isolated from surface water (Mackintosh, 1988) mountain streams (Fukushima et al., 1988 ; Fukushima, 1992), and soils (Fukushima et al., 1989 ; Mackintosh, 1988). The bacterium is able to withstand extremely cold climates, and may be viable for extended periods of time in the cold environment and in the carcases of birds and other animals (Blake et al., 1991). Yersiniosis has been associated with the winter and spring seasons (Mackintosh, 1988 ; Blake et al., 1991 ; Fletcher, 1982 ; Jerrett & Slee, 1989) or the cold months (Mair et al., 1960) but is less frequently seen during the summer season (Sato, 1987).

YERSINIA PSEUDOTUBERCULOSIS IN FOOD

Paterson & Cook (1963) observed that their guinea pig colonies were infected with *Y.pseudotuberculosis* through consumption of green feed contaminated by faeces of wood pigeons. The authors suggested that persons engaged in horticulture or in the handling or preparation of contaminated vegetables would be exposed to the risk of contracting *Y.pseudotuberculosis*. One outbreak of *Y.pseudotuberculosis* infection in man in Finland incriminated vegetables as the source of the infection (Tertti et al., 1984). In Japan, two outbreaks of the disease were believed to be caused by eating vegetables, meat products and rice at a local restaurant (Sato, 1987).

Y.pseudotuberculosis serogroup 4b has been isolated from retailed pork in Japan (Fukushima, 1985). This is believed to be the first isolation of this bacterium from meat. The same serogroup 4b has also been recovered humans in Japan (Fukushima et al., 1988). *Y.pseudotuberculosis* serogroup 1a has also been recovered from pasteurized milk (Greenwood & Hooper, 1989). The recovery of this bacterium in food animals suggest that infection in man may increase in the future (Zen-Yoji et al., 1974).

Table 2.3 *Y.pseudotuberculosis* in wildlife and birds in selected countries

Country	Species	Serogroup	Reference
Japan			
	Mice	4b & 5a	Fukushima et al. (1988)
	Mole	6	"
	Deer	3,4b,5	Fukushima & Gomyoda, (1991)
	Hare	1b, 2b	"
	Marten	4b	"
	Raccoon dog	1b,2b,4b,5a,6	"
	Rats	1b, 4a	Kaneko et al., (1979)
	Monkey		
	Erythrocelous patas		Hirai et al., (1974)
New Zealand			
	Hare	1, 2	Mackintosh & Henderson, (1984a)
	Mouse	1	"
	Rat	1	"
	Rabbit	1, 2	"
	Possum		Anon. (1977)
	Malayan Otter		Anon. (1978c)
	Cavie		Anon. (1980)
Canada			
	Muskoxen	1b	Blake et al., (1991)
	Raccoon	1b	Hariharan & Bryenton, (1990)
	Beaver	1b	Hackling&Sileo, (1974)
	Snowshoe hare		"
	Rodent	1b	Toma, (1986)

USA			
	Chinchillas		Leader & Baker, (1954)
	Bushbaby		Chang et al., (1980)
	Monkey		
	(*Macaca spp.)*		Bronson et al., (1972) Rosenberg et al., (1980) Strickland et al., (1982)
	(*Cercocebus spp.)*		Bronson et al., (1972)
	Blesbok (*Damaliscus dorcas)*		Baskin et al., (1977)
	Dik Dik (*Madogna kirkii)*		"
	Anteater		"
Japan			
	Duck	1b	Fukushima & Gomyoda (1991)
	Wigeon	4b	"
	Cockatoo	3	Sanekato et al., (1991)
	Seagull	1b	Kaneuchi et al., (1989)
	Bunting	4b	Hamakazi et al., (1989)
	Pied wagtail	3	"
New Zealand			
	Canary		Anon, (1975; 1977)
	Sparrow	1	Mackintosh & Henderson, (1984a)
	Seagull	2	"
	Starling	1	"
	Mallard duck	1	"
	King parrot		Anon. 1980

Canada			
	Crow	1a	Hacking & Sileo, (1974)
	Purple martin	1b	"
	Wildbird	1a,1b,2a,3	Toma, (1986)
	Canary	1	"
USA			
	Grackles		Clark & Locke, (1962)
	Turkey		Mathey et al., (1954)
UK			
	Stock-doves		Clapman, (1953)

BRIEF REVIEW OF OTHER *YERSINIA* SPECIES

YERSINIA ENTEROCOLITICA

Y.enterocolitica was first described in the United States about half a century ago by Schleifstein and Coleman (cited by Schofield, 1992). This bacterium has now emerged as a frequent human foodborne pathogen (Van Noyen et al., 1981) in Europe particularly in Belgium and the Scandinavian countries where *Y.enterocolitica* infections in humans are second only to Salmonellosis (Van Noyen et al., 1981) ; in Australia it has by-passed Shigella to become the third most common cause of gastroenteritis in man (Feeney et al., 1987).

Y.enterocolitica is a gram-negative short rod or coccobacillus. It is motile at 28°C but non-motile at 37°C. There are seven biotypes namely, 1A, 1B, 2, 3, 4, 5 and 6. The classification of biotype 6 is designated for strains formerly called biogroup 3A-3B, and is only provisional pending further studies. Biogroups 1B, 2, 3, 4 and 5 include only pathogenic strains belonging to a small number of serogroups (Wauters et al., 1987)

Serotyping is based on the O-antigen which is present in the cells in the form of lipopolysaccharides. The serotypes 0:3, 0:5, 0:8, and 0:9 are considered to be pathogenic for humans (Schofield, 1992) but only serotypes 0:3 and 0:9 are regularly associated with disease in man (Van Noyen et al., 1981 ; Bottone, 1981) while serotype 0:8 and 0:5,27 predominate in the United States (Bottone, 1981). However, serotype 0:8 was responsible for two major outbreaks in the United States (Shayegani & Parsons, 1987). It appears that serotype 0:3 is emerging as a common cause of yersiniosis in the United States (Lee et al., 1991).

Y.enterocolitica is ubiquitous and has been isolated worldwide from a variety of human specimens such as mesenteric lymph nodes, abscesses, faeces, sputa and from wounds (Bottone, 1981), from animals and birds (Shayegani & Parsons, 1987 ; Tsubokura et al., 1989), and from different foodstuffs such as meat, ice cream and oysters, and from water (Morris & Feeley., 1976).

Y.enterocolitica tolerates high concentrations of bile salts. Consequently, most of the common enteric media have been used successfully to isolate *Y.enterocolitica* with MacConkey and Salmonella-Shigella agar being the media of choice (Feeley, 1981). The Cefsulodin-Irgasan-Novobiocin (CIN) agar introduced by Schiemann in 1979 has now become the common medium for the isolation of *Yersinia spp*. It is highly selective and

supports the growth of *Y.enterocolitica* and related species while being at the same time, differential enough to distinguish colonies of *Yersinia* from most other Gram-negative bacteria (Schiemann, 1982 ; Mair & Fox, 1986).

Clinical disease due to *Y.enterocolitica* may appear in three phases. The first is the acute primary phase lasting one to two weeks and exhibiting acute inflammatory abdominal disease such as acute enteritis or mesenteric lymphadenitis. The secondary phase which follows is dominated by acute inflammatory symptoms in the connective tissues or in the skin. Lesions may be found in the joints causing arthritis and in the skin in the form erythema nodosum or erythema multiforme. Ocular syndromes (iridocyclitis) and glomerulonephritis have also been recorded (Larsen, 1987). The tertiary or chronic phase may follow and is manifest as chronic inflammatory rheumatic diseases such as classical rheumatoid arthritis or ankylosing spondylitis. This phase is often preceded by a history of secondary phase symptoms and could be considered as a progression of a "not-cured" secondary phase disease. Usually no bacteria can be isolated during the tertiary phase but agglutinins are detected in serum (Larsen, 1987).

In *Y.enterocolitica* infection, the common symptoms are abdominal pain, fever and diarrhoea (Asakawa et al., 1973 ; Black et al.,1978 ; Morse et al., 1984 ; Tacket et al., 1985). The infection is usually a self-limiting diarrhoeal illness in infants and younger children, and terminal ileitis and mesenteric adenitis in older children and adults. It is an important cause of pseudoappendicitis (Van Noyen et al., 1987). Complications such as bacteraemia, intestinal perforation, ileocolic intussusception, peritonitis, and pharyngitis have been reported and can be fatal. Postinfection complications include reactive arthritis and erythema nodosum (Mair & Fox., 1986 ; Lee et al., 1991).

Y.enterocolitica survives very well in nature, especially at low temperature (Morris & Feeley, 1976).However, it has been recovered from animals in Nigeria (Adesiyun et al., 1986 ; Okoroafor et al., 1988), and in Singapore (Ho & Koh, 1981) which are tropical countries.

Y.enterocolitica infection may spread from person to person (Toivanen et al., 1973) and via contaminated foods. Outbreaks of yersiniosis in the United States have been associated with the consumption of contaminated chocolate milk (Black et al., 1978), pasteurized milk (Lofgren et al., 1982), soybean curd (Tacket et al., 1985), milk and chow mein (Morse et al., 1984) and pork chitterlings (Lee et al., 1991). There is no reason to doubt that transmission is via the oral route (Wormser & Keusch, 1981).

There have been several major outbreaks of *Y.enterocolitica* infections of humans in

Japan but their sources have not been established (Asakawa et al., 1973).

The acquisiton of *Y.enterocolitica* through contaminated blood during transfusion has been reported (Ulyatt et al., 1991 ; Wilkinson et al., 1991 ; Grossman et al., 1991).

Animals harbouring this bacterium may appear clinically normal or they may be ill, often with a diarrhoeal illness (Wormser & keusch, 1981). Pigs have been recognised to harbour pathogenic strains of *Y.entercolitica.* Occasionally, dogs, cats and deer have been found to harbour strains which have disease-producing potential for humans. In some instances, diarrhoeal disease in the dog and cat, possibly related to *Y.enterocolitica* infection in the pet owner have been reported (Gutman et al., 1973) cited by Wormser & Keusch (1981).

YERSINIA KRISTENSENII

Yersinia kristensenii, formerly called Atypical *Y.enterocolitica* or *Y.enterocolitica*-like, is a "new" species of the *Enterobacteriaceae* comprised of Sucrose-negative strains. Serogroups O:11, O:12 and O:28 are most commonly encountered (Bercovier et al., 1980c). The name was given in honour of the Danish microbiologist, M. Kristensen.

Y.kristensenii has been isolated in several countries in Europe, the United Kingdom, Japan, the United States of America, Australia and the United Kingdom (Bercovier et al., 1980b), New Zealand (Henderson, 1984 ; Lanada, 1990) and in Brazil (Falcao, 1987).

Of 115 strains collected by the National Centre of *Yersinia* (Institut Pasteur, Paris), 18% are from humans, 61% from animals, 11% from water, 6% from soil and 4% from vegetables. Most of the human strains isolated were from stools of asymptomatic carriers and, after cold-enrichment, from patients with diarrhoea. *Y.kristensenii* is commonly recovered from soil and seems to be well adapted to the environment. The organism has been isolated from a large number of animal species including horses, frogs, sheep, wild rodents, and monkeys. It has also been recovered from earthworms, food, water, sewage and soil. In man, isolations have been from urine, blood, eye and stool (Bercovier et al., 1980c). Farmer III et al., (1985) also isolated the bacterium from human faeces, blood and urine, and from animals, water and soil.

Some strains of *Y.kristensenii* have been found to possess virulence markers (Prpic & Davey, 1985) and in the production of heat-stable enterotoxin (Kapperud & Langeland, 1981). Some strains of *Y.kristensenii* may be pathogenic to man.

YERSINIA FREDERIKSENII

Yersinia frederiksenii, formerly called Atypical *Y.enterocolitica* or *Y.enterocolitica*-like, is a "new" species of *Enterobacteriaceae* that is positive only for rhamnose (Ursing et al., 1980). The physiological and cultural characteristics are similar to those of *Y.enterocolitica sensu stricto* (Bercovier et al., 1980a). The name was given in honour of the Danish microbiologist, Wilhelm Frederiksen (Ursing et al., 1980).

Y.frederiksenii has been recovered in 15 countries in Europe and in North America, Israel, Australia (Ursing et al., 1980) New Zealand (Henderson, 1984 ; Lanada, 1990), Brazil (Falcao, 1987). and Japan (Fukushima et al., 1989).

The bacterium is commonly isolated from fresh water and seems to be well adapted to an aquatic life. Of 201 strains of *Y.frederiksenii* collected by the National Centre of *Yersinia*, Institut Pasteur, Paris 53% were derived from sewage, 23% from humans, 10% from cattle and pigs, 7% from fish, 4% from food such as milk and sandwiches, and 2% from wild rodents. Two strains were recovered from soil (Ursing et al., 1980).

Y.frederiksenii has been isolated from wounds and the respiratory tract (Farmer III et al., 1985). Other isolations were from human stools (98%), and from blood and sputum (2%). The organism was isolated from stools after cold-enrichment and was rarely associated with typical gastrointestinal disease. While *Y.frederiksenii* is considered an environmental strain in Japan (Fukushima, 1987b) it is elsewhere believed to be an opportunistic human pathogen (Ursing et al., 1980).

YERSINIA INTERMEDIA

Yersinia intermedia, formerly called Atypical *Y. enterocolitica* or *Y.enterocolitica*-like, is like *Y.kristensenii* and *Y.frederiksenii* a "new" species of *Enterobacteriaceae* comprised of rhamnose, melibiose and raffinose-positive strains (Brenner et al., 1980). Physiological and cultural characteristics are similar to those of *Y.enterocolitica sensu stricto*. The name was suggested by E. J. Bottone and B. Chester (Bercovier et al., 1980a).

There are eight biotypes (1 to 8) differentiated by their biochemical reactions to D-melibiose, L-rhamnose, alpha-methyl-D-glucoside, D-raffinose and Simmon's citrate. Serogroups O:4 and O:17 are recognized to predominate (Brenner et al., 1980)

Y.intermedia has been isolated in 15 countries in Europe and in North America,

Japan and Australia (Brenner et al., 1980). The bacterium is also found in countries such as New Zealand (Henderson, 1984 ; Lanada, 1990), Brazil (Falcao, 1987), and Nigeria (Agbonlahor et al., 1986).

Y.intermedia seems to be a normal component of the aquatic ecosystem (Brenner et al., 1980). In a study of 321 strains of *Y.intermedia*, 74% were isolated from fresh water or sewages. The others were from fish, oysters, shrimps, or snails (8%), wild rodents (4%), milk, cream or meat (2%), and from man (13%). Farmer III et al., (1985) isolated three strains from wounds, stools and urine of man.

Cold-enrichment technique is required for isolation of this bacterium from clinical and non-clinical specimens. The human isolates are not usually associated with gastrointestinal illness but as suggested by Brenner et al., (1980) *Y.intermedia* should be considered an opportunistic pathogen for man. Only six years later Agbonlahor, (1986) identified *Y.intermedia* as the cause of diarrhoea in children in Nigeria.

YERSINIA ROHDEI

Y.rohdei is a "new" species of *Enterobacteriaceae* which utilizes citrate, and ferments raffinose and melibiose. It is negative to indole and the Voges-Proskauer test. Several strains have been isolated from faeces of healthy dogs, and from surface water in Germany (Aleksic et al., 1987). The same authors also isolated the bacterium from a patient suffering from diarrhoea and abdominal cramps in the United States of America and from a healthy child in Germany.

These bacteria are negative to virulence marker tests such as the calcium dependency, congo-red uptake, and autoagglutination tests and they also fail to produce a heat-stable enterotoxin. *Y.rohdei* is therefore classified as an environmental microorganism (Aleksic et al., 1987).

YERSINIA ALDOVAE

Yersinia aldovae, formerly called *Y.enterocolitica*-like Group X_2 is a "new" species of *Enterobacteriaceae* isolated from aquatic ecosystems (drinking and river water, and fish) and from soil. The name was given in honour of Eva Aldova, the Czechoslovakian microbiologist who first isolated this organism (Bercovier et al., 1984).

It has been recovered from faeces of goats in New Zealand (Lanada, 1990) but has

not been associated with disease in man or animals.

YERSINIA MOLLARETTI

Yersinia mollaretti, formerly called *Y.enterocolitica* biogroup 3A is a "new" species of *Enterobacteriaceae* which ferments L-sorbose and inositol (Wauters et al., 1988). The name was given in honour of Henri Mollaret.

Most strains have been isolated from environmental sources, but a few have been recovered stools of both healthy persons and patients with diarrhoea. They are also frequently isolated from meat, raw vegetables, soil and water. The organism lacks virulence markers and there is no evidence that it is pathogenic for man (Wauters et al., 1988).

YERSINIA BERCOVIER

Yersinia bercovier, formerly called *Y.enterocolitica* biogroup 3B is a "new" species of *Enterobacteriaceae*. The name was given in honour of Herve `Bercovier (Wauters et al., 1988).

The bacterium has been isolated from human stools, animals, raw vegetables, and from soil and water samples. There is no evidence of pathogenicity for man (Wauters et al., 1988).

YERSINIA RUCKERI

Yersinia ruckeri is the causative agent of "red mouth disease" of Rainbow and Steelhead trout, and Sockeye and Chinook salmon. It is placed in the genus *Yersinia* because of its phenotypic similarily to *Yersinia* and its Guanine and Cytosine content, which is similar to that of other members of the genus (Farmer III et al., 1985).

Most isolations have been from fish hatcheries in the United States of America. The bacterium has been commonly isolated from kidneys of affected fish. The first case of "red mouth disease" in fish in New Zealand was reported in 1991 (Colin, 1991). One human isolation of *Y.ruckeri* in the United States of America was from bile. It is however, extremely rare for this organism to be found in human clinical specimens (Farmer III et al., 1985).

CHAPTER 3

EVALUATION OF CEFSULODIN-IRGASAN-NOVOBIOCIN (CIN) AGAR AND THE LYSINE ARGININE IRON AGAR (LAIA) SLANT FOR THE ISOLATION AND PRESUMPTIVE IDENTIFICATION OF *YERSINIA SPP.*

Introduction

MacConkey agar, Salmonella-Shigella (SS) agar, Xylose-Lysine Deoxycholate (XLD) agar, Deoxycholate- Citrate-Lactose (DCL) agar, Hektoen agar, Bismuth Sulphite agar, and Lactose-Saccharose-Urea (LSU) agar have been used for the isolation of *Yersinia spp.* MacConkey agar has remained the most popular isolation medium for *Y.enterocolitica* (Schiemann, 1989) and has been preferred for the isolation of *Y.pseudotuberculosis* (Tsubokura et al., 1973). However, this medium lacks the differential property to allows easier identification of *Yersinia spp.* on the basis of colonial morphology. Thus, in 1979, Schiemann developed a selective and differential medium for the isolation of *Y.enterocolitica*. Cefsulodin and Novobiocin are incorporated in this medium making it selective for *Yersinia*. The ability to ferment mannitol producing red colonies makes differentiation from other Gram-negative bacteria relatively simple. Cefsulodin-Irgasan-Novobiocin (CIN) agar was not designed to recover *Y.pseudotuberculosis* and there are limitations to this medium because *Y.pseudotuberculosis* may be inhibited by the antibiotics, Novobiocin and Cefsulodin (Mair & Fox, 1986 ; Fukushima & Gomyoda, 1986 ; Schiemann, 1989).

Other bacteria which also grow on CIN agar producing similar red colonies have been identified as *Citrobacter spp.*, *Serratia spp.* and *Enterobacter spp.* (Schiemann, 1979 ; Schiemann, 1982 ; Mair & Fox, 1986).

The Lysine-Arginine-Iron Agar (LAIA) slant developed by Weagant (1983) has been evaluated on 27 strains of *Y.enterocolitica* and other enterobacteriaceae. All of the strains of *Y.enterocolitica* which were tested produced the typical reactions of an alkaline slant (purple), an acid butt (yellow), no gas or hydrogen sulphite formation.

The LAIA slant consists of five tests in one tube. The principle of this test is to differentiate between bacteria which decarboxylate lysine and arginine, ferment glucose, deaminate lysine and arginine, and produce gas and hydrogen sulphite. Those that decarboxylate these amino acids produce an alkaline substrate which is indicated by a

purple colour throughout the medium. Those that do not decarboxylate lysine and/or arginine but ferment glucose produce purple colour slant and yellow coloured butt. The yellow butt is due to the fermentation of glucose. The LAIA slant differentiates bacteria that deaminate amino acids, which is shown by the red band in the agar and also detects bacteria which produce gas and hydrogen sulphite and ferment glucose.

Yersinia spp. do not decarboxylate lysine or arginine but ferment glucose (Farmer III et al., 1985). They do not deaminate amino acids nor produce gas and hydrogen sulphite. Therefore, they produce the typical reactions of an alkaline slant, an acid butt, no gas and hydrogen sulphite production in the LAIA slant.

The LAIA slant has proven to be reliable for the presumptive identification of *Y. enterocolitica* and has been used by Mollee & Tilse (1985) for the presumptive identification of *Y.enterocolitica* isolated from stools cultured on CIN agar. The authors found the medium to be very reliable, and to give no false positive results.

This study was conducted to evaluate the performance of CIN agar as an isolation medium and to determine the reliability of the LAIA slant as a presumptive identification media for *Yersinia spp.*

Materials and Methods

The CIN agar (Difco laboratories Detroit Michigan USA) was prepared according to the manufacturer's instructions. One litre of distilled water was added to 59.5 g of Yersinia Selective agar (Difco laboratories Detroit Michigan USA) and dissolved by heating. The medium was autoclaved for 15 minutes at 121°C and allowed to cool to 45°C to 50°C. The antibiotic supplements (Difco laboratories Detroit Michigan USA) were suspended in sterile distilled water and added to the medium. Amounts of 15 to 20 ml were poured into sterile petri dishes and stored at +4°C until used within two weeks (Petersen, 1985).

The LAIA slant was prepared according to Weagant (1983) by dissolving 34.5 gm of Lysine-Iron agar (LIA) (Difco laboratories Detroit Michigan USA) and 10g of l-Arginine (Sigma) in one litre of distilled water. Amounts of 6.0 ml were dispensed into screw-capped plastic tubes and autoclaved for 15 minutes at 121°C. The medium was cooled with the plastic tubes in a slanting position and stored at +4°C.

The CIN agar was evaluated with one strain each of *Y.pseudotuberculosis* belonging to serogroups 1 to 6, one strain each of the *Y.enterocolitica* biotypes 1A and 5, serotypes O:1,2,3; O:2,3; O:3; O:5,27; O:6,30; O:7,8; O:8 and O:9. The performance of the agar in

regards to one strain of each species of *Y.kristensenii, Y.intermedia, Y.frederiksenii* and *Y.rohdei* was also evaluated. The agar was also evaluated with other bacteria such as *Salmonella panama, Salmonella typhimurium, Klebseilla oxytoca, Escherichia coli, Serratia marcescens, Serratia liquefaciens, Citrobacter freundii, Enterobacter agglomerans, Proteus vulgaris, Pseudomonas stutzeri, Pseudomonas maltophilia, Alcaligenes faecalis and Listeria monocytogenes.*

The LAIA slant was evaluated with a strain of each of the *Y.pseudotuberculosis* serogroups 1 to 6, and 10 strains of *Y.pseudotuberculosis* which had been isolated from goats in the Manawatu (Lanada, 1990), one strain of each *Y.enterocolitica* serotypes 0:1,2,3; 0:2,3; 0:3; 0:5,27; 0:6,30; 0:7,8; 0:8; and 0:9, 10 strains of *Y.enterocolitica* biotypes 1A, and 17 strains of biotype 5. The other test organisms comprised 10 strains of each of *Y.kristensenii, Y.frederiksenii, Y.intermedia,* and *Y.rohdei.* Several species of *Enterobacteriaceae* comprising *Salmonella panama* (one strain), *Salmonella typhimurium* (one strain), *Klebsiella oxytoca* (two strains), *Escherichia coli* (two strains), *Serratia marcescens* (two strains), *Serratia liquefaciens* (one strain), *Citrobacter freundii* (two strains), *Enterobacter agglomerans* (one strain), and *Proteus vulgaris* (two strains) and other species such as *Pseudomonas stutzeri, Pseudomonas maltophilia, Alcaligenes faecalis* and *Listeria monocytogenes* one strain each were also used for the study.

The *Y.pseudotuberculosis* and *Y.enterocolitica* strains were obtained from the Pasteur Institute, Paris except for *Y.enterocolitica* 0:8 (ATCC 9610) which was obtained from the New Zealand Communicable Disease Centre (NZCDC). *Y.enterocolitica* biotypes 1A, and 5, *Y.kristensenii, Y.frederiksenii, Y.intermedia* and *Y.rohdei* were isolated from goats in the Manawatu region (Lanada, 1990). The NZCDC also provided the strains of *Salmonella panama, Salmonella typhimurium, Citrobacter freundii, Enterobacter agglomerans* and *Serratia liquefaciens.* The strains of *Citrobacter freundii, Serratia marcescens, Proteus vulgaris and Alcaligenes faecalis* were provided by Dr.John Brooks of the Department of Biotechnology, Massey University. The strains of *Klebsiella, Escherichia, Pseudomonas* and *Listeria* were obtained from Mr. Stan Fenwick of the Department of Veterinary Pathology and Public Health, Massey University.

The test organisms were grown on CIN agar and Blood agar, and incubated at 28°C for 24-48 hours. A colony of each culture from the Blood agar plate was inoculated into Tryptone water (Difco laboratories Detroit Michigan USA), and incubated at 28°C for 24 hours. The broth culture was inoculated into the LAIA slant using a sterile Pasteur Pipette, and incubated at 28°C for 48 hours.

Result

All of the *Yersinia spp.* tested grew on CIN agar producing colonies having a typical bullseye appearance at 24 hours post-inoculation. There were morphological changes within species when incubated for 48 hours. The *Yersinia spp.* produced the typical reactions of an alkaline slant (purple colour), an acid butt (yellow colour), and no gas and no hydrogen sulphite formation in the LAIA slant. The non-*yersinia spp.* which grew on CIN agar were *Citrobacter freundii, Serratia marcescens, Serratia liquefaciens, Pseudomonas stutzeri and Pseudomonas maltophilia.* Only *Enterobacter agglomerans* produced the same reactions as *Yersinia* in the LAIA slant. The growth of *Yersinia spp.* is summarized below:-

Y.pseudotuberculosis

Overall, all the serogroups 1 to 6 produced colourless pin-point colonies after 18 hours incubation and by 24 hours colonies showed red centres with translucent peripheries. The colonies appeared glistening, round and with entire borders. After 48 hours incubation, all serogroups produced dry colonies which were 2.0 mm or less in diameter. Only serogroup 4 produced small colonies which were 1.0 mm or less in diameter. Serogroups 1, 2, 3 and 5 produced dark red colonies while serogroups 4 and 6 had lighter red colonies. All serogroups produced flat colonies with entire borders except for serogroups 4 and 5 which had irregular borders. A thin light pink periphery was observed only with serogroup 2 after 24 hours incubation (Fig. 3.1.a., 3.1.b., 3.1.c., 3.1.d., 3.1.e., and 3.1.f.).

Y.enterocolitica

Serovar O:1,2,3

After 24 hours incubation, the colonies were 0.5 mm or less in diameter and were smooth and raised with dark red centres and translucent peripheries. After 48 hours, the colonies were up to 2.0 mm diameter, and were dry, smooth and raised with entire borders and dark red centres surrounded by translucent peripheries.

Serovar O:2,3

After 24 hours incubation, the colonies were pin-point and colourless. At 48 hours, the colonies were 1.0 mm or less in diameter and flat, mucoid with dark red centres and translucent peripheries.

Serovar O:3

After 24 hours incubation, the colonies were less than 1.0 mm in diameter and were smooth, raised and round with entire border, and dark red centres surrounded by thin translucent peripheries. After 48 hours, the colonies were about 4.0 mm in diameter and were dark red with irregular border and dry appearance.

Serovar O:5,27

After 24 hours incubation, the colonies were 1.0 mm or less in diameter and were smooth with a raised and glistening, dull red centre and a translucent periphery. After 48 hours, the colonies were 1.5 mm or less in diameter and were flat, round, mucoid, with a light pink centre which was surrounded by a translucent periphery.

Serovar O:6,30

After 24 hours incubation, the growth was sparse. The colonies were 1.5 mm or less in diameter and were smooth and raised with dark red centres and translucent peripheries. At 48 hours, the colonies were 2.5 mm or less in diameter and were flat, dry, and dark red and irregularly shaped.

Serovar O:7,8

After 24 hours incubation, the colonies were 1.5 mm or less in diameter and were smooth and raised with a glistening, dark red centre and a thick translucent periphery. At 48 hours, the colonies were dry and flat with a round, mucoid, red centre which gradually turned to pink.

Serovar O:8

After 24 hours incubation, the colonies were less than 2.0 mm diameter with a red centre and a translucent periphery. They were glistening and flat with an entire border. After 48 hours, the colonies were about 3.0 mm or less in diameter and were flat, mucoid and red with an entire border, but with no translucent periphery.

Serovar O:9

After 24 hours incubation, the colonies were 1.5 mm or less in diameter and were smooth and raised with a glistening, dark red centre and surrounded by a translucent

periphery. After 48 hours, the colonies were dry and flat and with a round and mucoid, red centre which gradually turned light pink.

Y.enterocolitica **Biotype 1A**

After 24 hours incubation, the colonies were 2.0 mm or less in diameter and were reddish and round with an entire border and a translucent periphery. After 48 hours, the colonies were 5.0 mm or less in diameter and were mucoid and flat with an entire border. There was a rough translucent periphery and a red centre which gradually turned light yellow.

Y.enterocolitica **Biotype 5**

After 24 hours incubation, the colonies were pin-point in size, red and glistening. After 48 hours, the colonies were 2.0 mm or less in diameter and were raised, dry, mucoid and round with an entire border and a translucent periphery.

Y.kristensenii

After 24 hours incubation, the colonies were 1.5 mm or less in diameter and were red and round with an entire border and a translucent periphery. After 48 hours, the colonies were 4.0 mm or less in diameter and were flat, dry, and dark red, with an irregular shape and without a transparent periphery.

Y.intermedia

After 24 hours incubation, the colonies were 2.0 mm or less in diameter and were red and mucoid with a round, entire border and a translucent periphery. After 48 hours, the colonies were 4.0 mm or less in diameter and were flat with an entire border and a mucoid translucent periphery with a diffuse red colour becoming dark yellow.

Y.frederiksenii

After 24 hours incubation, the colonies were 2.0 mm or less in diameter and were round, mucoid and red with a translucent periphery. After 48 hours, the colonies were 3.0 mm or less in diameter and were flat and mucoid with an entire border and a pale periphery turning to pink.

Y.rohdei

After 24 hour incubation, the colonies were 1.0 mm or less in diameter and were round, raised and red with a translucent periphery. After 48 hours, the colonies were 3.0 mm or less in diameter and were raised and round, mucoid, with a diffuse red centre and a pale periphery.

Non-*Yersinia spp.*

The colonies of *Citrobacter* and *Serratia spp.* were about 2.0 mm in diameter and were mucoid, round, raised and red with a translucent periphery. After 48 hours, the colonies were 4.0 mm diameter and were mucoid, raised and with an entire border and no translucent periphery. *Serratia marcescens* produced deep red colonies whereas *Serratia spp.* showed a paler red colour. The *Pseudomonas spp.* had light pink coloured colonies.

In LAIA slant non-*Yersinia spp.* produced variable reactions after 48 hours incubation at 28°C. *Serratia spp.*, *Klebiella oxytoca*, *Escherichia coli* and *Alcaligenes faecalis* produced an alkaline reaction (purple) throughout the medium. *Citrobacter freundii* and *Salmonella spp.* produced an alkaline medium and hydrogen sulphite (black precipitate). *Proteus vulgaris* produced an alkaline slant and an acid butt, and also a red band below the slant. *Pseudomonas spp.* produced a bluish slant and a yellow butt while *Listeria monocytogenes* produced an entire acid medium. Only *Enterobacter agglomerans* produced an alkaline slant and an acid butt which was similar to that produced by *Yersinia spp*. The summary of the results is shown in Table 3.1.

Table 3.1. Growth and reactions of *Yersinia spp.* and other species on CIN agar and LAIA slant respectively

Species	Growth on CIN agar	Reactions in LAIA slant*
Yersinia spp.	+	K/A
Enterobacter agglomerans	-	K/A
Serratia marcescens	+	K/K
Serratia liquefaciens	+	K/K
Citrobacter freundii	+	K/K H_2S
Proteus vulgaris	-	K/A red band
Alcaligenes faecalis	-	K/K
Klebsiella oxytoca	-	K/K
Escherichia coli	-	K/K
Pseudomonas stutzeri	+w	Blue/K
Pseudomonas maltophilia	+w	Blue/K
Listeria monocytogenes	-	A/A
Salmonella panama	-	K/K H_2S
Salmonella typhimurium	-	K/K H_2S

*slant/butt

K - alkaline (purple colour)

A - acid (yellow colour)

$+^w$ - sparse growth

Discussion

The CIN agar has been recommended as the medium of choice because it is highly selective and supports good growth of *Y.enterocolitica* and related species (Harmon et al., 1983 ; Mair & Fox, 1986 ; Fukushima & Gomyoda, 1986). It was also found to recover more *Y.pseudotuberculosis* than the Virulence-*Yersinia-enterocolitica* (VYE) agar

(Fukushima, 1987b). Several other species of bacteria have been shown to grow on CIN agar. e.g. *Enterobacter spp., Serratia spp.,Citrobacter spp., Proteus spp., Alcaligenes spp., Morganella spp., Pseudomonas spp., Klebsiella spp., Providencia spp., Escherichia spp., Aeromonas spp., Hafniae spp.,* CDC Group VE-2 and API Group 2 (Schiemann, 1979 ; Head et al., 1982 ; Petersen, 1985 ; Kachoris et al., 1988). *Citrobacter spp., Serratia spp.,* and *Enterobacter spp.,* are frequently confused with *Y.enterocolitica* on CIN agar (Schiemann, 1979 ; Schiemann, 1982 ; Mair & Fox, 1986).

The results of this study are consistent with the findings of other workers that CIN agar supports the growth of *Yersinia spp* and that it is highly selective against most of the common enteric bacteriae.

The LAIA slant has only been evaluated with 27 strains of *Y.enterocolitica* and found to be reliable (Weagant, 1983). In this study, several species of *Yersinia* were tested and gave similar results to those obtained by Weagant. Thus, the LAIA slant is recommended as a presumptive identification media for *Yersinia spp.* Nevertheless, it may be unreliable for *Y.ruckeri* as some strains of this organism decarboxylate arginine (Farmer III et al., 1985). Another potential source of confusion is *Enterobacter agglomerans* which can produce the typical *Yersinia* reactions in LAIA slants. The tested strain of *E.agglomerans* did not grow on CIN agar. If other strains are found to grow on CIN agar they can be eliminated through the biochemical tests used in the definitive identification (Mair & Fox, 1986).

Conclusion

This study has shown that CIN agar supports the growth of both pathogenic and non-pathogenic strains of *Yersinia.* A few other Gram-negative bacteria may also grow on the medium with similar colonial and phenotypic morphology to *Yersinia* but they can be eliminated by inoculation into the LAIA slant, and by biochemical tests. The use of CIN agar as a primary isolatory medium and the LAIA slant as a presumptive identification medium for *Yersinia spp.* is recommended.

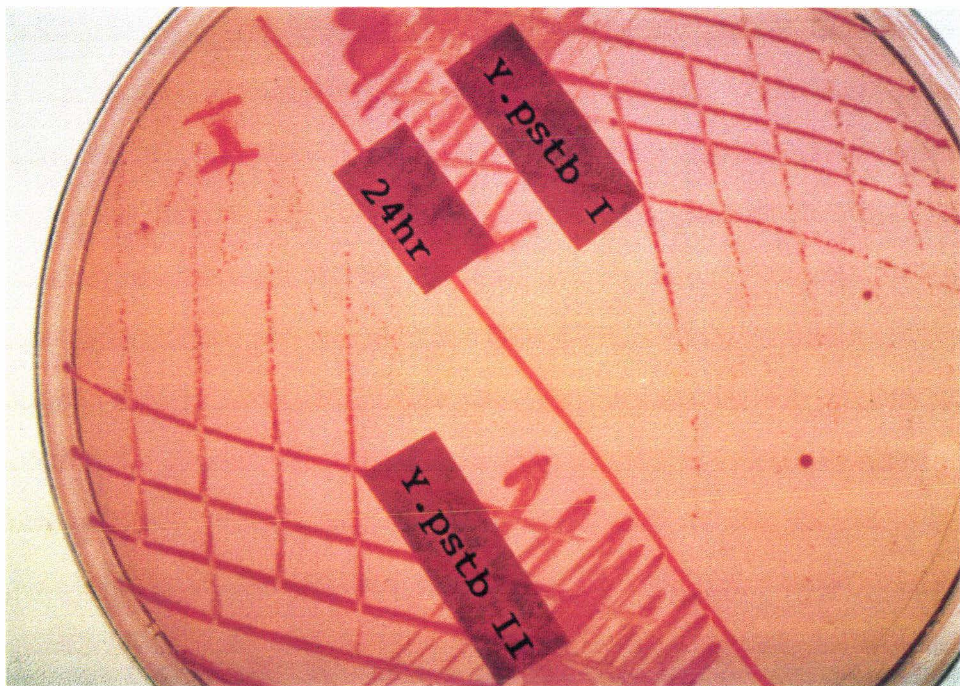


Fig. 3. 1. a Growth of *Y.pseudotuberculosis* I and II on CIN Agar After 24 Hours Incubation at 28 ° C

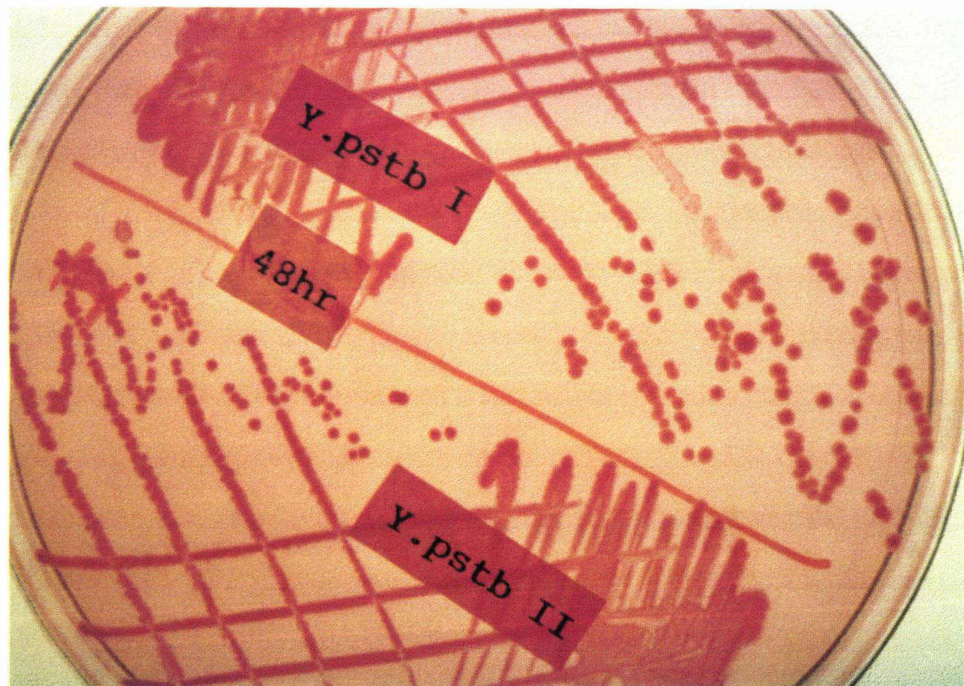


Fig. 3. 1. b Growth of *Y.pseudotuberculosis* I and II on CIN Agar After 48 Hours Incubation at 28°C

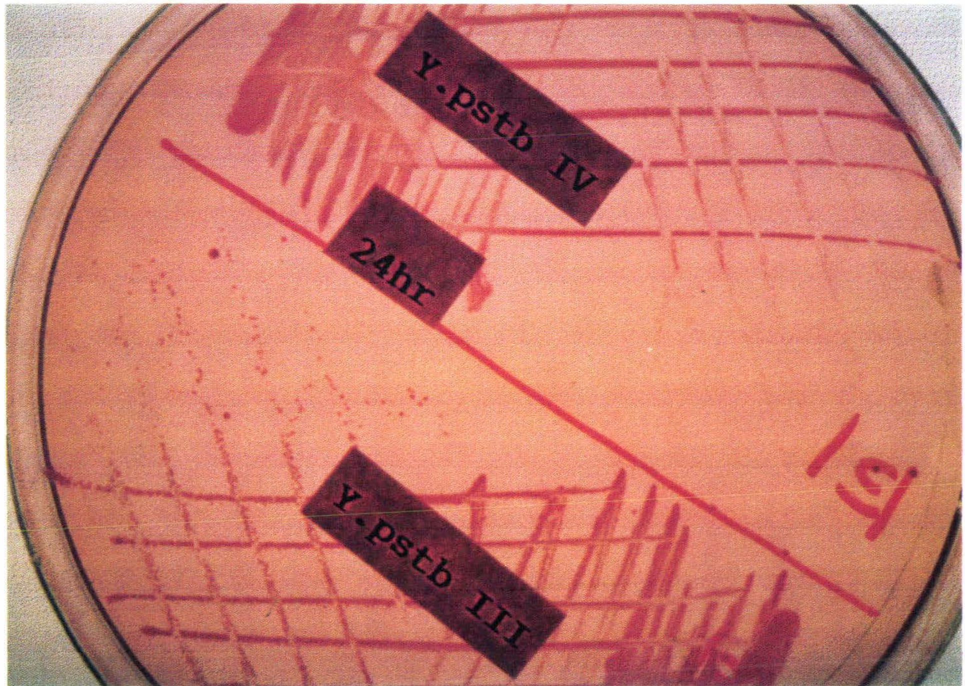


Fig. 3. 1. c Growth of *Y.pseudotuberculosis* III and IV on CIN Agar After 24 Hours Incubation at 28°C

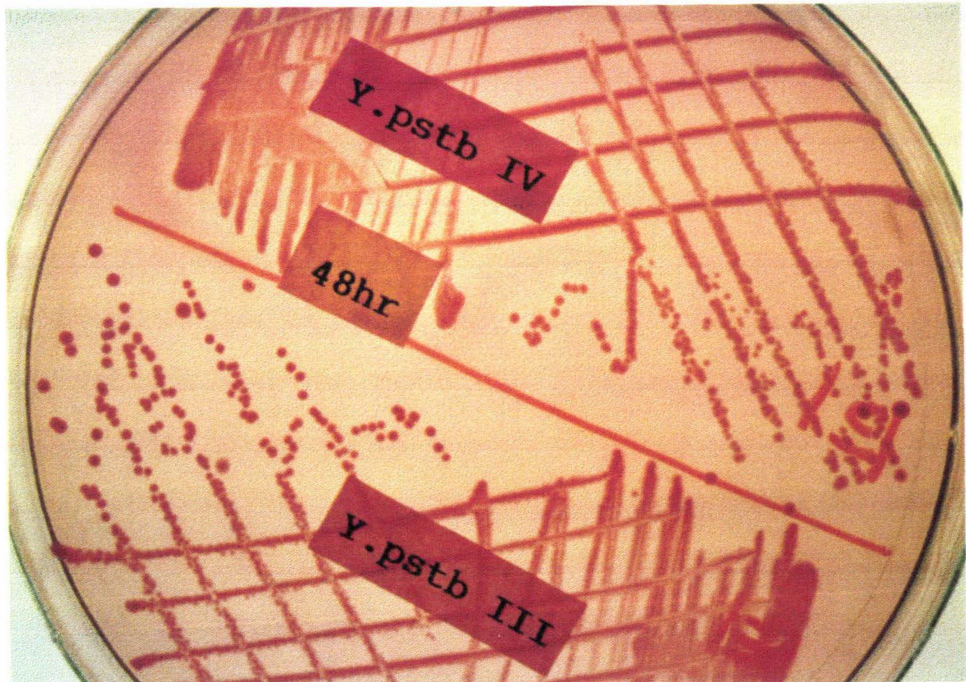


Fig. 3. 1. d Growth of *Y.pseudotuberculosis* III and IV on CIN Agar After 48 Hours Incubation at 28°C

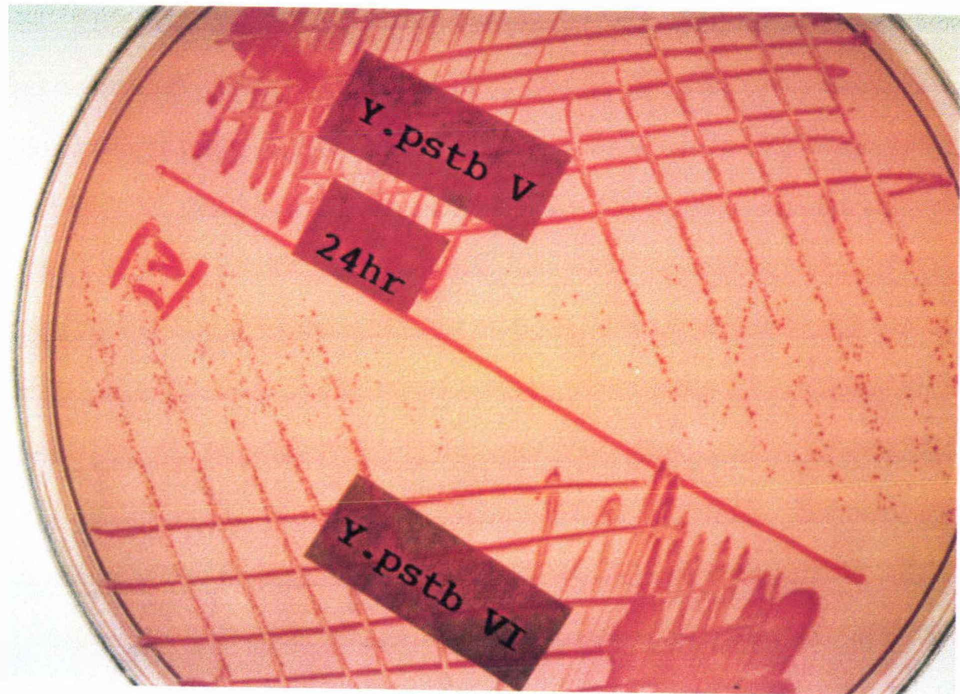


Fig. 3. 1. e Growth of *Y.pseudotuberculosis* V and VI on CIN Agar After 24 Hours Incubation at 28° C

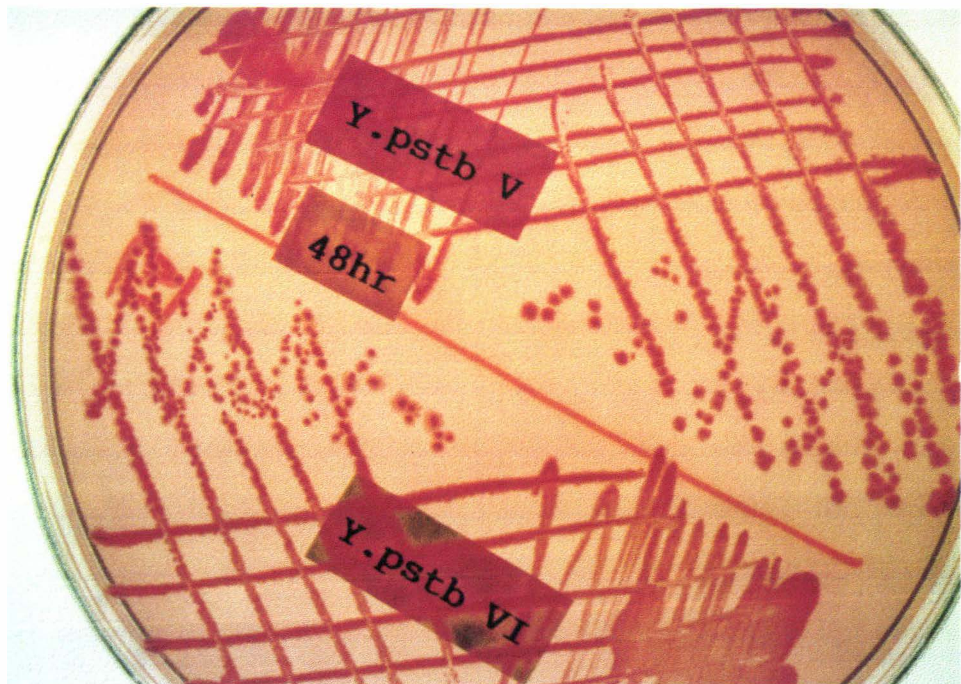


Fig. 3. 1. f Growth of *Y.pseudotuberculosis* V and VI on CIN Agar After 48 Hours Incubation at 28°C

CHAPTER 4

EVALUATION OF THE 6-WELL NUNC PLATES FOR THE ENUMERATION OF *YERSINIA PSEUDOTUBERCULOSIS*

Introduction

There are several methods of enumerating viable bacterial cells. In this study, the spread plate method is used. The standard procedure is to dispense 0.1 ml of the inoculum onto the medium in a standard petri dish which is 9.0 cm in diameter. The inoculum is then spread evenly using a sterile wire loop, and incubated at the appropriate temperature for 24 to 48 hours before counting the number of colonies. Only plates with 200 or less colonies are taken into consideration. The number of viable cells is calculated by multiplying the number of colonies on the plate with the reciprocal dilution factor of the original culture (Cruickshank, 1965).

Before use in the enumeration of *Y.pseudotuberculosis* the performance of the 6-well plates, which require less than half the volume of medium used in standard petri dishes, was evaluated.

Materials and Methods

The Cefsulodin-Irgasan-Novobiocin (CIN) agar (Difco Laboratories Detroit Michigan USA) was prepared according to the manufacturer's instructions. Amounts of about 8 ml and 20 ml of the medium were poured into the 6-well plates (Nunclon, InterMed, Denmark) and standard petri dishes respectively.

Each of the wells measures 4.0 cm in diameter, and the amount of media used is about 6 to 8 ml per dish. The standard petri dish measures about 9.0 cm in diameter and uses about 15 to 20 ml of media.

A pure culture of a field isolate of *Y.pseudotuberculosis* from fatal yersiniosis in a deer designated Yellow 6 was grown on blood agar for 24 hours at 28°C. A colony was inoculated into Tryptone water (Difco Laboratories Detroit Michigan USA) and incubated at 28°C for 24 hours. A series of ten-fold dilutions was made using sterile normal saline. The 10^{-6} dilution was chosen for the test.

Using an Oxford pipette (Pipetman 200, Gibson, France) with sterile disposable tips 0.05 ml of inoculum was dispensed into each of the six wells on the plate. The plate was rocked gently so that the inoculum covered the surface of the medium. For the standard petri dish, 0.1 ml of the same inoculum was dispensed into each of six dishes, and spread evenly with a sterile wire loop. The plates were incubated at 28°C for 48 hours and the colonies on each plate counted.

Result

The result of the count is shown in Table 4.1.

Table 4.1 Number of colonies of *Y.pseudotuberculosis* on Nunc plates and standard petri dish

Replicate No.	No. of colonies			
	Nunc Dish /0.05 ml	Petri Dish /0.1 ml	Nunc dish /1.0 ml*	Petri dish /1.0 ml*
1	5	9	100	90
2	4	9	80	90
3	5	14	100	140
4	5	9	100	90
5	5	11	100	110
6	5	5	100	50
Mean	4.8	9.5	96.7	95

* calculated figure

The result of a two sample t-test was $p= 0.8984$ meaning that there is no significant difference between the results on the Nunc plates and those on the Petri dishes. Based on the number of colonies on the Nunc plates and the Petri dishes the calculated cell density of the stock culture solution was Log_{10} 7.97 and Log_{10} 7.95 per ml respectively.

Discussion

The result of the two-sample T-test indicates that there is no significant difference between the counts in the 6-well plates and those in the Petri dishes but it was observed that the counts in the 6-well plates were more consistent than those in the Petri dishes. The only obvious technical difference between the two methods was the use of a wire loop to spread the inoculum on the agar in the Petri dishes.

Conclusion

The 6-well plate was found to be acceptable as an alternative to the standard petri dish for use in the enumeration of *Y.pseudotuberculosis* and to be preferable because of the reproducibility of results and the savings on medium.

CHAPTER 5

EFFECTS OF CEFSULODIN-NOVOBIOCIN ON THE RECOVERY OF *YERSINIA PSEUDOTUBERCULOSIS*

Introduction

When the Yersinia Selective Agar (YSA) base is incorporated with the antibiotics, Cefsulodin (4 mg) and Novobiocin (2.5 mg), the medium is commonly referred to as Cefsulodin-Irgasan-Novobiocin (CIN) agar. CIN agar was found to be selective for *Y.enterocolitica* and support the growth of related species (Mair & Fox, 1986). However, Fukushima & Gomyoda (1986) found that the growth of *Y.pseudotuberculosis* may be inhibited by the antibiotics, Cefsulodin and Novobiocin.

Bacteria which have been frozen may be injured so that recovery on selective agar is even more difficult. The ability of injured cells to recover from freezing is dependent on the availability of complex nutrients in the recovery media (Nakamura & Dawson, 1962).

This study was conducted to determine whether there was any difference in the rate of recovery of *Y.pseudotuberculosis* from a frozen venison sample cultured onto *Yersinia* Selective agar and onto CIN agar.

Materials and Methods

A known number ($\log_{10}$ 6.26/0.1 ml) of *Y.pseudotuberculosis* (strain Yellow 6) was inoculated onto a piece of venison (149 cm^2 and 58 g) which was then vacuum packed and frozen at -20°C for eight weeks. To recover the bacteria from the venison, the sample was at first thawed at +4°C for three hours. Then 50 ml of 0.1% Peptone saline was added and the sample stomached (Colworth 400, London) for two minutes. A series of ten-fold dilutions was made with the suspension using sterile normal saline. The 10^{-2} dilution was chosen for enumeration. A volume of 0.05 ml of inoculum was dispensed into each of 18 wells containing YSA base (Difco Laboratories Detroit Michigan USA) and into each of 18 wells containing CIN agar (Difco Laboratories Detroit Michigan USA). The plates were incubated at 28°C for 48 hours, and the colonies counted.

Results

The number of *Y.pseudotuberculosis* colonies were counted in each dish and the result is shown in Table 5.1.

Table 5.1Number of *Y.pseudotuberculosis* colonies in 6-well plates containing YSA base and CIN agar

No.	No. of *Y.pseudotuberculosis* colonies on YSA base	CIN Agar
1	36	19
2	49	37
3	47	25
4	49	27
5	38	32
6	49	24
7	42	44
8	50	31
9	42	36
10	49	30
11	38	44
12	52	16
13	43	26
14	47	39
15	49	21
16	53	30
17	40	35
18	48	37
Mean	45.6	27.3

The two-sample t-test result is p= 0.000. This means that there is a highly significant difference between YSA base and CIN agar in favour of YSA base for the recovery of *Y.pseudotuberculosis* from frozen venison.

Discussion

Freezing causes metabolic injury and may also result in the death of bacterial cells (Straka & Strokes, 1959 ; Nakamura & Dawson, 1962 ; Morichi & Irie, 1973). As mentioned earlier, media with complex nutrients offer a better chance of recovering injured cells (Nakamura & Dawson, 1962).

This study showed that the antibiotics, Cefsulodin and Novobiocin, inhibited the growth of *Y.pseudotuberculosis* on CIN agar a finding which is consistent with the work of (Fukushima & Gomyoda, 1986). On the other hand this agar has adequate nutrients for the recovery of freeze injured *Y.pseudotuberculosis*.

Freezing can reduce the number of normal flora in the meat samples. It is therefore possible to recover and enumerate *Y.pseudotuberculosis* from venison with low bacterial contamination using the YSA base. In chill temperatures, the number of normal flora in venison may be very high and can mask the growth of *Y.pseudotuberculosis* making enumeration difficult (Fig. 5.1). Thus, in such a situation, CIN agar is recommended for use but there will be an under-estimation of the concentration of *Y.pseudotuberculosis* in the experimental works. The CIN agar was used to enumerate *Y.pseudotuberculosis* from samples that were frozen and chilled in order to maintain consistency in the protocol and results.

Conclusion

The study showed that CIN agar can be used effectively in the isolation of *Y.pseudotuberculosis*. This medium was therefore used for the further studies on the growth behaviour of *Y.pseudotuberculosis* in vacuum-packed venison stored at chilling and freezing temperatures. Although there would be an under-estimation in the number of viable cells, the media was practical when dealing with samples with a high concentration of normal flora and for the purpose of maintaining consistency in the protocol.

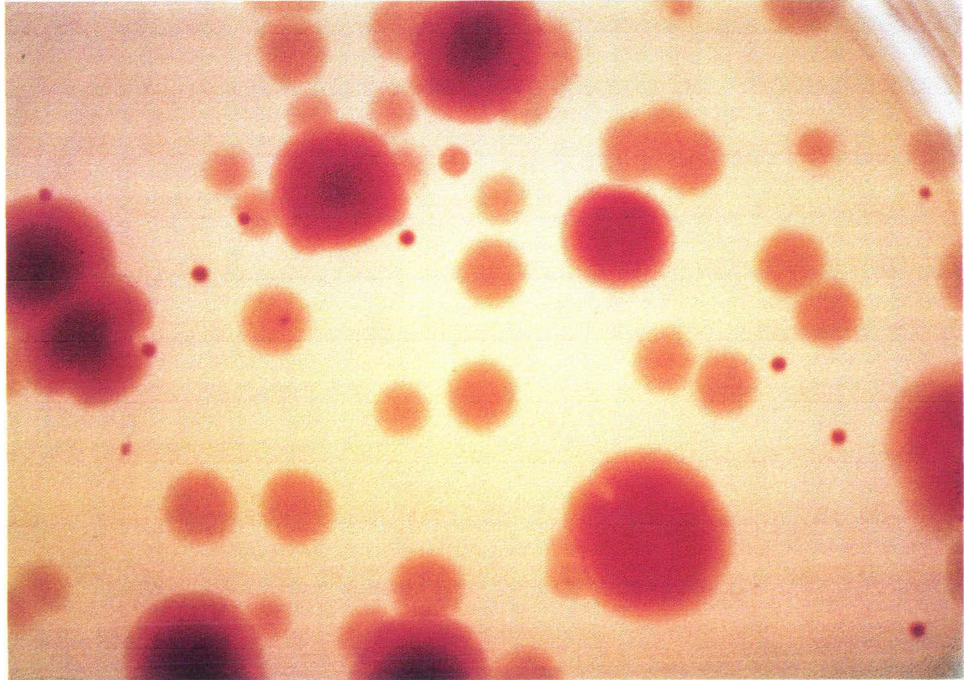


Fig. 5. 1 Growth of *Y.pseudotuberculosis* on CIN Agar (Small Red Colonies) Among Spoilage Bacteria Making Enumeration of the Bacterium Difficult

CHAPTER 6

PREVALENCE OF *YERSINIA PSEUDOTUBERCULOSIS* AND RELATED SPECIES IN FAECES AND ON CARCASES OF FARMED RED DEER PRESENTED FOR SLAUGHTER AT THE DEER SLAUGHTER PREMISES, FEILDING

Introduction

The first recorded outbreak of yersiniosis due to *Y.pseudotuberculosis* in farmed deer in New Zealand occurred in 1978, killing about 60 hinds (Mackintosh, 1988). Another 16 fatal cases were seen in deer recently transported or yarded (Anon, 1979b).

Between June 1979 and September 1981, a total of 326 cases of yersiniosis in deer were studied at the Invermay Animal Health Laboratory and 65 strains (20%) of *Y.pseudotuberculosis* and 18 strains (5.5%) of *Y.enterocolitica* were recovered. However, seven strains (0.7%) of *Y.pseudotuberculosis* and 297 strains (30.5%) of *Y.enterocolitica* were isolated from faeces of 974 clinically normal, young and aged Red deer, Wapiti, Wapiti cross, White Tail, Fallow and feral deer (Anon, 1982). The mere isolation of *Yersinia spp.* from faeces does not necessarily imply an aetiological role in the particular clinical disease suffered by the animal.

In a national survey carried out among healthy animals on 122 deer farms in New Zealand five isolates of *Y.pseudotuberculosis* were recovered from 3810 animals (0.13%) (Henderson & Hemmingsen, 1983). Of a total of 120 adult stags from 21 commercial farms surveyed for *Y.pseudotuberculosis* at a Deer Slaughter Premises in the lower South Island, only two isolates (1.2%) were made from the large intestine and ileocaecal lymph node (Mackintosh & Henderson, 1985).

Hodges et al., (1984b) tested 439 faecal samples from young weaners and recovered 47 isolates (10.7%) of *Y.pseudotuberculosis*. In another study, Henderson (1984) isolated *Y.pseudotuberculosis* from seven (10.7%) of 922 faecal samples from farmed and feral deer. The author also recovered 176 isolates (19%) of *Y.enterocolitica*, 56 isolates (6.5%) of *Y.frederiksenii*, 29 isolates (3.1%) of *Y.kristensenii*, and eight isolates (0.9%) of *Y.intermedia*. The *Y.pseudotuberculosis* isolates were made from Red deer and Wapiti.

From 1979 to 1982, a total of 234 isolates of *Y.pseudotuberculosis* were made from various species of animals and birds (Hodges et al., 1984a). Of these, 117 strains (50%)

were from deer. It was observed that *Y.pseudotuberculosis* serogroup 3 appears to predominate in deer and domestic animals in the North Island (Hodges et al., 1984a) while serogroup 1 predominates in deer (Anon, 1982) and small mammals and birds in the South Island (Mackintosh & Henderson, 1984a).

The present study was conducted to investigate the prevalence of *Y.pseudotuberculosis* and related *Yersinia* species in faeces of farmed red deer presented for slaughter, and to determine whether contamination of carcases with these organism occurred.

Materials and Methods

Slaughtering of farmed deer at the Feilding DSP

The DSP at Feilding processes an average of 120 animals per day. The majority of the deer slaughtered at this plant originate from various farms in the lower part of the North Island but occasionally animals from the South Island are slaughtered (Signal, pers. comm.,).

Deer presented for slaughter at Deer Slaughter Premises must undergo *ante mortem* and *post mortem* inspections. The principal aim of the *ante mortem* inspection is to ensure that the animals are healthy and free from diseases that may render the meat they yield unfit for human consumption. Only clean animals are considered for slaughter (Wilcockson, 1986).

Following *ante mortem* inspection the animals are driven into the stunning box where they are stunned by means of a captive bolt pistol. After stunning the animals are simultaneously immobilised and electrically stimulated by a low voltage system (80v) applied for 90 seconds (Anon, 1991). Electrical stimulation facilitates the sticking operation and increases the rate of *post mortem* pH-decline (Madie, pers. comm.,). An electrode is attached to the lip or muzzle while the hook from which the animal is suspended serves as the other electrode. The animals are stuck while being electrically stimulated. After bleeding, the oesophagus is clamped by a plastic ring which is pushed into the thoracic cavity by means of a rodding instrument in order to prevent the back-flow of ruminal content. The hide of the head and forelegs is loosened and the lower parts of the legs cut off at the carpal joint.

The carcases are automatically transferred to the next work station where further

freeing of the hide of the upper limbs is completed. The carcases are then inverted and the penis and scrotum freed from the skin and placed inside a plastic bag. The loose hide of the head, neck and shoulder is then fastened to chains on both sides in order to facilitate the automatic removal of the entire hide. The operator assists the hide removal by using his knife to detach the hide in certain areas where breaks in the skin may occur or where there is likely to be excessive removal of meat with the hide. The lower hindlegs are removed at the level of the tarsal joint.

The carcases are now moved to the next work station where the operator opens the abdomen by making a long incision in the midline from the pelvis to the sternum. The penis and scrotum in the plastic bags are removed from the carcases by cutting through the pelvic bones with a bone-saw. This is followed by the head being detached at the atlanto-occipital joint and presented for inspection. The kidneys and the abdominal organs are removed and placed in a tray on a viscera trolley. Next, the pluck is removed and likewise placed in a tray on the trolley. The viscera are then inspected by the Meat Inspector.

After evisceration and inspection excessive fat and minor blemishes are trimmed off. The carcases are then weighed and the tissue depth measured at the 12th rib. This is followed by stamping of the carcases with the official inspection legend. Finally the carcases are transferred to the chillers for cold storage overnight before being transported in a refrigerated truck to Hastings for deboning. The carcases must reach an internal temperature of less than 7°C which must be maintained throughout the storage and transportation period prior to deboning.

Blood, caecal content and meat surface samples were collected from thirty deer each month for one year at the DSP Feilding. This number is statistically significant at the 95% level of confidence, assuming the prevalence of infection to be 1% in a infinite population (Cannon & Roe, 1982).

A total of 225 (62.5%) of the 360 farmed red deer sampled were stags between one to two years of age. The rest were hinds of varying ages from yearlings to five years old (Table 6.3).

A total of 360 faecal samples were collected from 360 deer. However, only 345 carcases and 282 carcases out of 360 were sampled at Feilding and Hastings respectively. Fifteen carcases at Feilding were not sampled because swabbing in the chiller was not permitted. At Hastings, 78 carcases were lost to deboning and 39 carcases were subsequently substituted with other available carcases (Table 6.4).

Sampling Methods

A. Blood

Samples of blood were taken during exsanguination after the initial flush of blood from the sticking wound. About 8 ml of blood was collected from each test animal into a plain, sterile, 10 ml test tube (Fig. 6.2)

B. Caecal Content

These were collected after the completion of the viscera inspection by incising the caecum with a clean pair of scissors. Approximately one gram of caecal content was scooped out and placed in a container with 10 ml sterile Phosphate Buffer Solution (PBS) (Fig. 6.3.a., 6.3.b., & 6.3.c.).

C. Meat Surface

These were collected shortly after the carcases had entered the chiller. From each carcase three samples were taken from pre-determined areas, i.e. the gluteal region, the flank and the neck (Fig. 6.4.a., 6.4.b., & 6.4.c.). These areas were chosen according to the assumption that they represent sites where contamination is most likely to occur. These sampling sites have been recommended by Nottingham et al., (1974) for beef carcase.

A sterile template with a 10 cm^2 circular hole was placed on the site and a sterile cotton swab wetted with PBS was then rubbed on the delineated area. A second cotton swab was run over the area to remove any microorganism loosened from the meat surface by the moisture from the first swab. Both cotton swabs were placed in a container with 10 ml PBS. The same template and sampling technique were used for the two other sites. The sampling side of the carcase was identified by a sticker secured to the Achilles tendon. At the boning plant in Hastings, the same sampling procedure was conducted but on the other side of the carcase.

Microbiological Examination

The faecal samples and carcase swabs were cold-enriched at +4°C for 21 days. From each sample a loopful of material was streaked onto Cefsulodin-Irgasan-Novobiocin (CIN)

agar (Difco Laboratories Detroit Michigan USA).

The CIN agar plates were incubated at 28°C for 24 to 48 hours. Small red colonies showing a bullseye appearance on the plate (Fig. 6.5.a & 6.5.b) were picked up and inoculated into Tryptone water (Difco Laboratories Detroit Michigan USA) and incubated at 28°C for 24 hours. The broth culture was inoculated into Lysine-Arginine-Iron Agar (LAIA) slants (Difco Laboratories Detroit Michigan USA)(Sigma) and Triple-Sugar-Iron Agar (TSIA) slants (Difco Laboratories Detroit Michigan USA) with a sterile Pasteur pipette, and incubated at 28°C for 48 hours. Only those cultures producing an alkaline slant (purple colour) and an acid butt (yellow colour), no gas and hydrogen sulphide formation on LAIA slant, and an alkaline slant (red colour) and an acid butt (yellow colour), no or little gas formation and no hydrogen sulphide production on TSIA slant were considered for further tests. Suspect isolates were inoculated into urea agar slopes and ornithine decarboxylase broth, and incubated at 28°C for 48 hours. Only cultures that hydrolized urea were considered for further examination.

Organisms that do not decarboxylated ornithine broth may be *Y.pseudotuberculosis* or *Y.enterocolitica* biotype 5. Other *Yersinia spp.* decarboxylate ornithine. The biochemical tests for the identification of *Yersinia spp.* comprise motility, Voges-Proskauer, indole, Simmon's citrate, aesculine hydrolysis, lysine decarboxylase, arginine dihydrolase, malonate utilization, and sucrose, trehalose, rhamnose, melibiose, raffinose, alpha-methyl-D-glucoside, sorbose, sorbitol, cellobiose, xylose, maltose, adonitol, salicin, arabinose and lactose fermentations. The preparation of these media is described in Appendix I. The isolation technique is shown in Fig. 6.1 and the biochemical reactions of *Yersinia spp.* is shown in Table 6.1.

Serotyping

The isolates identified as *Y.pseudotuberculosis* and *Y.enterocolitica* biotype 3 were subcultured on blood agar. The hyperimmune serum for *Y.pseudotuberculosis* serogroup 1, 2 and 3 was raised on rabbits.

The protocol for raising hyperimmune sera from rabbits was that recommended by the National Centre of *Yersinia*, Institut Pasteur, Paris. The first step was the preparation of antigens. *Y.pseudotuberculosis* serogroup 1, 2 and 3 were grown separately on blood agar and incubated at 28°C for 48 hours. The culture of serogroup 1 was harvested using sterile cotton swabs and resuspended in 10 ml sterile normal saline. The same procedure was used for the other two serogroups. The suspensions were autoclaved for one hour at 121°C and stored at +4°C.

After the rabbits had been bled from an ear vein for pre-immune serum assessment the heat-killed cultures were inoculated into each of two rabbit per serogroup. Initially, 0.5 ml of the culture was injected intravenously using the vein from the other ear. This was followed by intravenous injection of 1.0 ml of culture on the 4th day, 2.0 ml on day 8 and finally 4.0 ml on day 12. Blood was collected from the animal via the ear veins or by cardiac puncture on the eighth to the tenth day following the last injection and kept at +4°C. The serum was harvested by gently mascerating the blood clot and centrifuging it for 10 minutes at 1500 r.p.m. before aspirating the serum and storing it at +4°C. The specificity of all sera was confirmed with reference strain of bacteria.
For serotyping of *Y.enterocolitica* serovar O:5,27 a commercial hyperimmune serum was used (Eco-bio, Belgium).

A drop of the hyperimmune serum was placed on a clean glass slide. A loopful of the culture was mixed with the serum and rocked gently for 30 to 60 seconds. A positive agglutination test is indicated by clumping within 60 seconds.

Virulence Marker Tests

The autoagglutination test (Laird & Cavanaugh, 1980) and the Congo-Red Magnesium Oxalate test (Riley & Toma, 1989) were used to determine the presence of virulence plasmids in the isolated microorganisms.

a. Autoagglutination Test

The media were prepared as described in Appendix I.18. Two ml of the medium was dispensed into a small tube and inoculated with an isolated colony grown on trypticase- soy agar (Difco Laboratories Detroit Michigan USA). The tube was then incubated at 37°C for 24 hours. Agglutination-positive strains formed a layer of irregularly-edged flocculent growth at the bottom of the tube with the medium almost clear. In negative cases, most of the bacteria were suspended in the medium, resulting in an homogenous, cloudy suspension (Fig. 6.6).

b. Congo-Red Magnesium Oxalate Agar (CRMOX)

The media were prepared as shown in Appendix I (19). The strains tested were first subcultured on blood agar at 28°C for 24 hours. An isolated colony was plated onto

CRMOX and incubated at 37°C for 24 hours. The CRMOX agar is selective for bacteria which are calcium dependent and also take up the Congo-red dye. These two characteristics are associated with the presence of virulence plasmids. CRMOX-negative strains produce only large, colourless colonies while CRMOX-positive strains produce both small, red colonies and large, colourless colonies. The small size of the colonies indicates calcium dependency while the red colour indicates the uptake of Congo-red dye (Fig. 6.7).

Results

A total of 19 isolates (5.3%) of *Y.pseudotuberculosis*, 9 (2.6%) of *Y.enterocolitica*, 13 (3.6%) of *Y.kristensenii*, 74 (20.6%) of *Y.frederiksenii*, and two (0.6%) each of *Y.intermedia* and *Y.rohdei* were made from caecal content of farmed red deer slaughtered at the Feilding Deer Slaughter Premises over a period of 12 months (Table 6.2). There was a single isolation (0.3%) of *Y.pseudotuberculosis* from a carcase at the Hastings Packing House.

Eleven strains isolated from caecal content and one carcase strain of *Y.pseudotuberculosis* belonged to serogroup 3 while the other eight strains were serogroup 2. Two of the four strains of *Y.enterocolitica* biotype 3 were serovar 0:5,27.

All strains of *Y.pseudotuberculosis*, four strains of *Y.enterocolitica* biotype 3 (including 2 of serovar O;5,27) and one strain of biotype 5 were positive to the autoagglutination and congo-red magnesium oxalate virulence marker tests.

Seventeen of the 20 (85%) *Y.pseudotuberculosis* isolates were from hinds despite the fact that only 37.5% of the animals sampled were hinds. Nine of the isolates were from yearling hinds (Table 6.5).

There were numerous isolates which appeared morphologically similar to *Y.enterocolitica* on CIN agar and although they showed consistent biochemical characteristics they remained untypeable. They hydrolysed urea and aesculin but decarboxylated ornithine after five days of incubation. They produce acetoin at 28°C but not at 37°C, and utilised sucrose, melibiose, raffinose, and salicin. Seven of these isolates (FF63/9(2), FF73B/10, FF36/11, FF62b/12, FF53/01, FF71/01 and FF69/05) are stored at -20°C for further investigation. Three isolates (F52/11, FF79s/12 and FF60/01) are also stored for further investigation. They appeared to be *Y.enterocolitica* but their biochemical characteristics did not fit into the biotyping scheme.

Discussion

The results showed that *Y.pseudotuberculosis* is carried in the intestinal tract of farmed red deer presented for slaughter, and that it appears that the organism is only very rarely transferred to the carcase during processing.

The isolate of *Y.pseudotuberculosis* from the carcase at Hastings may or may not come from the deer. It could have been derived from the workers who handled the carcases during transportation to Hastings or from the processing environment. Based on the biochemical profile (Appendix IIA) this particular isolate from the carcase which was serogroup 3 failed to utilize arabinose and was thus different from the isolates obtained from the caecal content. This would support the suggestions that this isolate may not have originated from deer.

The low prevalence of *Yersinia* in carcases of deer despite the frequent isolation of the bacterium from faeces is not surprising as the inverted pelting technique results in very low carcase contamination levels (Drew & Fennessy, 1988).

In this study, most animals were found to carry *Y.pseudotuberculosis* during the late autumn (42% of the isolates) and winter (42%), a finding which is consistent with those of other workers in New Zealand (Mackintosh, 1988) and Australia (Jerrett et al., 1990).

Twenty strains of *Y.pseudotuberculosis*, four of *Y.enterocolitica* biotype 3 and one of biotype 5 were found to be pathogenic based on the positive results obtained from the autoagglutination, congo-red uptake and calcium dependency tests. There are however reservations in regards to the pathogenic potential of the other species of *Yersiniae*. For example, *Y.intermedia*-like organism have been shown to be responsible for diarrhoea in children in Nigeria (Agbonlahor, 1986). Virulence markers are also found in many of the so-called environmental strains of *Yersiniae* (Prpic & Davey, 1985) while *Y.frederiksenii* and *Y.kristensenii* are known to produce a heat-stable enterotoxin (Kapperud & Langeland, 1981).

Other significant findings of this study were the isolation of *Y.enterocolitica* serovar O:5,27 which is one of the common serotypes in human infection in the United States of America (Bottone, 1981), and the recovery of *Y.rohdei*.

The relationship between age, sex and the isolation rate must be interpreted with care. It must be realised that slaughter animals are not a true random sample of the population. They are also not necessarily representative of the age structure and sex ratio of the population from which they are derived (Cannon & Roe, 1982).

Conclusion

This study has established that apparently healthy farmed red deer may carry *Y.pseudotuberculosis* in their faeces. However, the results indicate that, at least at this DSP, carcase contamination is a very rare event (1/666). The one isolate was made when the prevalence of faecal carriage was highest and at the Hastings Plant after the carcase had been handled during transportation and cooled to below 7°C for 18 to 24 hours. As the potential risk of carcase contamination is greater during the cold months (winter and late autumn) there is a need for those involved in the deer slaughter and processing to be aware of this and to take special precautions when handling the animals during this period.

The origin of the sole *Y.pseudotuberculosis* isolate from the carcase at Hasting is not clear. Although it was serogroup 3, this particular strain did not utilize arabinose. It is therefore recommended to undertake a more extensive study in several DSPs which use different processing techniques and concentrate on the autumn and winter months. More animals and a larger surface area should be sampled.

Fig. 6.1 Isolation and identification scheme for *Yersinia spp.*

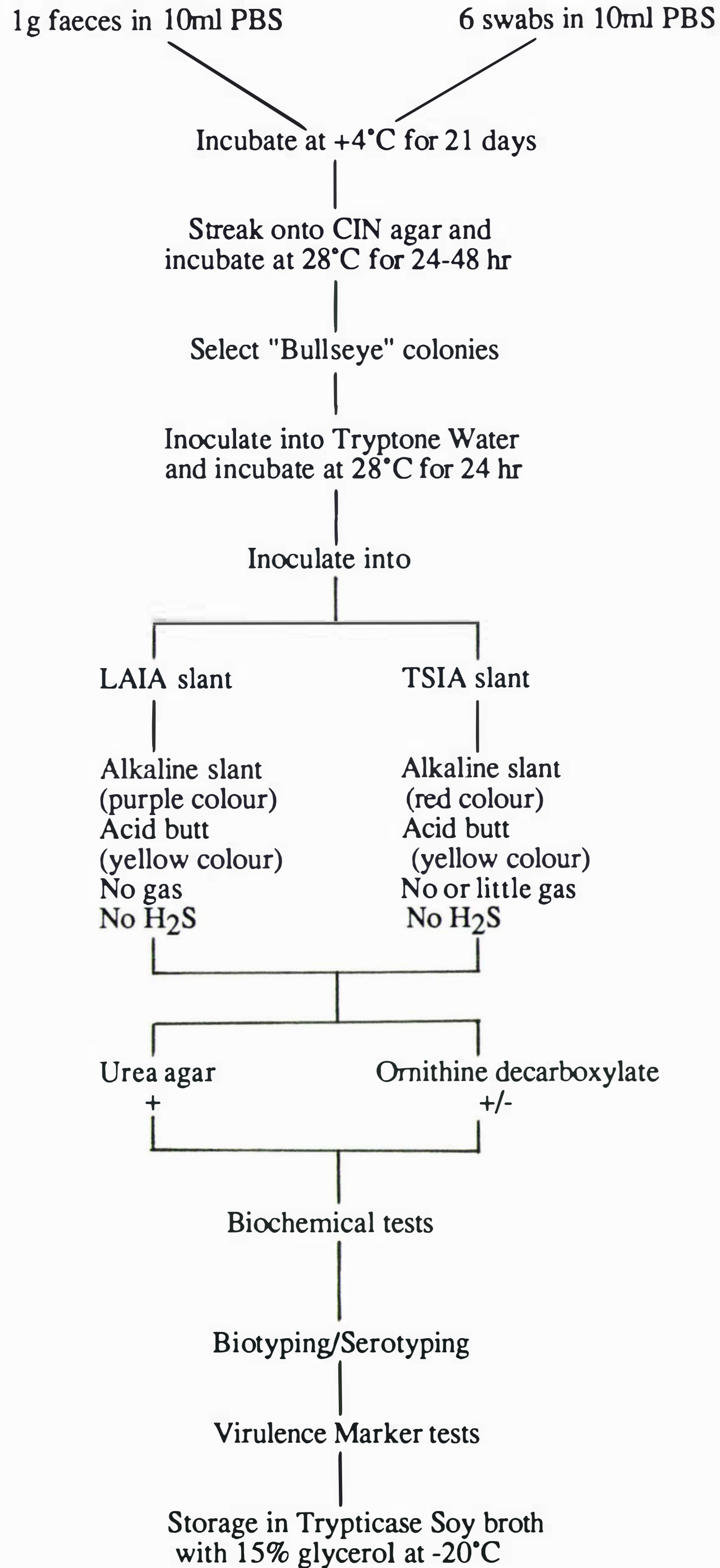


Table 6.1 Differential Biochemical reactions of *Yersinia species*

Biochemical test		Species			
		Y.pstb[a]	*Y.ent*[b]	*Y.fred*[c]	*Y.kris*[d]
Urease		+	+	+	+
Ornithine decarboxylase		-	+[1]	+	+
Voges-Proskauer	28°C	-	+[1]	+	+
	37°C	-	-	-	-
Methyl-red	28°C	+	+/(+)	+	+
Motility	28°C	+	+/(+)	+	+
	37°C	-	-	-	-
Indole		-	-	-	-
Lysine decarboxylase		-	-	-	-
Arginine dihydrolase		-	-	-	-
Simmon's citrate		-	-	V	-
Aesculine hydrolysis		+	V	+	-
Malonate utilization		V[k]	-	-	-
Sucrose		-	+[1]	+	-
D-trehalose		+	+[1]	+	+
L-rhamnose		+	-	+	-
Melibiose		+	-	-	-
Raffinose		V	-	-	-
Alpha-CH_3-D-glucoside		-	-	-	-
L-sorbose		-	+[1]	+	+
D-sorbitol		-	+[1]	+	+
Maltose		+	+	+	+
D-xylose		+	V[m]	+	+
Cellobiose		-	+	+	+
L-arabinose		+	+	+	+
Salicin		V	V	+	-
Lactose		-	-	V	V
Adonitol		-	-	-	-

Table 6.1 (Contd)

Biochemical test	Species					
	Y.inter[e]	*Y.ald*[f]	*Y.roh*[g]	*Y.mol*[h]	*Y.ber*[i]	*Y.ruc*[j]
Urea hydrolysis	+	+	+[n]	+	+	+
Ornithine decarboxylase	+	+	+[n]	+	+	+
Voges-Proskauer 28°C	+	+	-	-	-	-/(+)
37°C	-	-	-	-	-	-
Methyl-red 28°C	+	+	+	+	+	+
Motility 28°C	+	+	+	+	+	+
37°C	-	-	-	-	-	-
Indole	+	-	-	-	-	-
Lysine decarboxylase	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-
Simmon's citrate	V	+	+[n]	-	-	(+)
Aesculine hydrolysis	+	+	-	+[n]	+	(+)
Malonate utilization	-	-	-	-	-	-
Sucrose	+	-	+	+	+	-
D-Trehalose	+	+	+	+	+	+
L-Rhamnose	+	+	-	-	-	-
Melibiose	+	-	V	-	-	-
Raffinose	+	-	V	-	-	-
Alpha-CH_3-D-glucoside	+	-	-	-	-	-
L-sorbose	+	-	nt	nt	nt	nt
D-sorbitol	+	+	+	+	+	-
Maltose	+	-	(+)	+	+	+
D-xylose	+	+	+	+	+	-
Cellobiose	+	-	+	+	+	-
L-arabinose	+	+	+	+	+	-
Salicin	+	-	-	V	+[n]	-
Lactose	-	-	(+)	-	-	-/(+)
Adonitol	-	-	-	-	-	-

keys : + = positive; (+) = 90% or more positive between 4 to7 days; - = negative; V = 10.1% -89-9% positive; and nt = not tested.
note : All tests were performed at 28°C except for *Y.bercovieri, Y. mollaretti* and *Y.ruckeri* which is at 25°C.

[a] Kapperud and Bergan (1984)
[b] Bercovier et al., (1980b)
[c] Ursing et al., (1980)
[d] Bercovier et al., (1980c)
[e] Brenner et al., (1980)
[f] Bercovier et al., (1984)
[g] Aleksic et al., (1987)
[h] Wauters et al., (1988)
[i] Wauters et al., (1988)
[j] Ewing et al., (1978)
[k] Serogroup 4 strains positive
[l] Most negative strains biotype 5
[m] Reaction varies between biotype
[n] May be delayed

Table 6.2 Prevalence of *Yersinia spp.* in faeces of 360 farmed Red deer presented for slaughter at the DSP, Feilding

Sampling Mth/year	Species isolated					
	Y.pstb	*Y.ent*	*Y.krist*	*Y.fred*	*Y.inter*	*Y.roh*
06/91	4	1	-	-	-	-
07/91	5	-	-	-	-	-
08/91	-	3	1	9	-	-
09/91	-	-	5	20	-	-
10/91	1	1	4	2	-	-
11/91	-	2	-	9	-	-
12/91	-	2	1	14	1	-
01/92	1	-	-	1	-	-
02/92	-	-	-	5	1	2
03/92	-	-	-	5	-	-
04/92	-	-	1	9	-	-
05/92	8	-	1	-	-	-
Total	19	9	13	74	2	2
%	5.3	2.6	3.6	20.6	0.6	0.6

The prevalence of *Y.pseudotuberculosis* in carcase of farmed Red deer was 1/321 (0.3%). The sole isolate was recovered in the May sampling.

Table 6.3 Age and sex of 360 farmed Red deer sampled at the DSP Feilding during between June, 1991 and May, 1992

	Age (years) and Sex (M/F)					
Mth/Yr	<1	1 - 2	2 - 3	3 - 4	4 - 5	>5
06/91	-/-	1/16	-/ 3	-/-	-/-	-/-
07/91	-/-	-/ 5	-/ 7	-/ 5	-/ 2	-/11
08/91	-/-	30/-	-/-	-/-	-/-	-/-
09/91	-/-	30/-	-/-	-/-	-/-	-/-
10/91	-/-	29/ 1	-/-	-/-	-/-	-/-
11/91	-/-	20/-	-/-	-/-	-/-	-/10
12/91	-/-	30/-	-/-	-/-	-/-	-/-
01/92	-/-	30/-	-/-	-/-	-/-	-/-
02/92	-/-	30/-	-/-	-/-	-/-	-/-
03/92	-/-	-/-	-/-	-/30	-/-	-/-
04/92	-/-	25/-	-/-	-/-	-/-	-/ 5
05/92	-/30	-/-	-/-	-/-	-/-	-/-
Total	-/30	225/22	-/10	-/35	-/2	-/26
% (F)	8.3	6.1	2.8	9.7	0.6	7.2
% (M)	-	62.5	-	-	-	-

Table 6.4 Number of faecal samples and carcase swabs collected between June, 1991 and May, 1992

Mth/Yr	Number of sample		
	Feilding Faeces	Feilding Carcase	Hasting Carcase
06/91	30	30	23
07/91	30	30	23
08/91	30	30	30
09/91	30	30	0/10*
10/91	30	30	26
11/91	30	30	11/19*
12/91	30	30	29
01/92	30	30	20/10*
02/92	30	30	30
03/92	30	30	30
04/92	30	30	30
05/92	30	15	30
Total	360	345	321

* replacement of missed carcases

Table 6.5 Approximate age and sex of farmed red deer positive to *Yersinia pseudotuberculosis*

I.D.	Serogroup	Age(yr)	Sex
FF39/6	3	1.5	M
FF43/6	3	5.0	F
FF49/6	3	8.0	F
FF59/6	3	6.0	F
FF37/7	2	2.5	F
FF72/7	3	1.5	F
FF73/7	3	3.5	F
FF74/7	3	2.0	F
FF80/7	3	2.0	F
FF85/10	3	1.5	M
FF69/01	3	2.0	M
FF64/05	3	1.0	F
HC67/05	3	1.0	F
FF73a/05	2	1.0	F
FF74a/05	2	1.0	F
FF75/05	2	1.0	F
FF76/05	2	1.0	F
FF77/05	2	1.0	F
FF78a/05	2	1.0	F
FF80a/05	2	1.0	F

FF - Feilding Faeces HC - Hasting Carcase

Fig. 6.2 Blood Collection Using a Sterile Test Tube

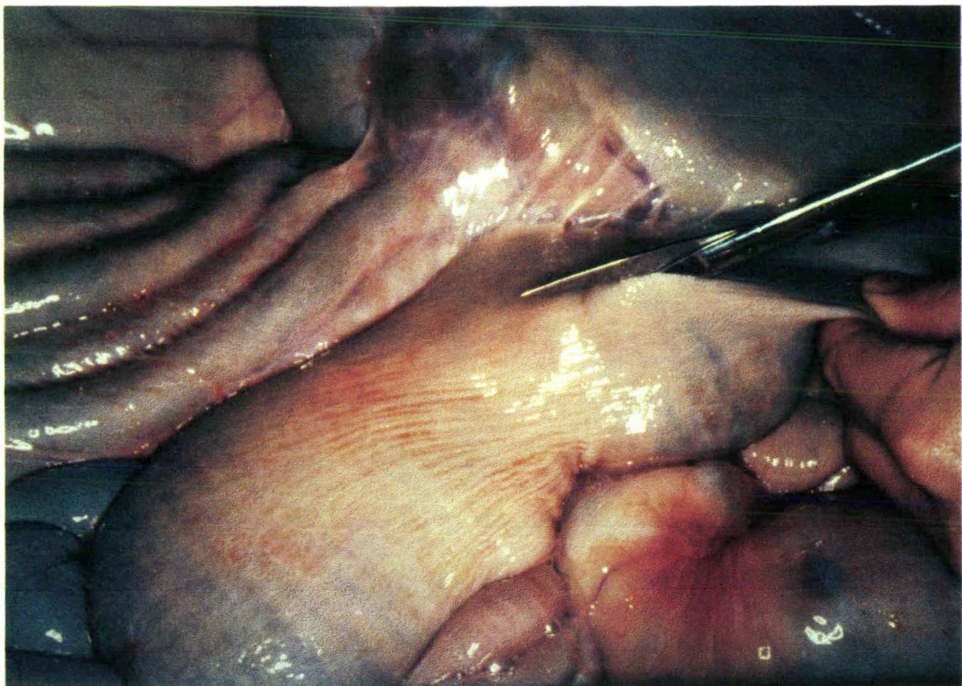


Fig. 6.3. a Opening of the Caecum Using a Pair of Clean Scissor

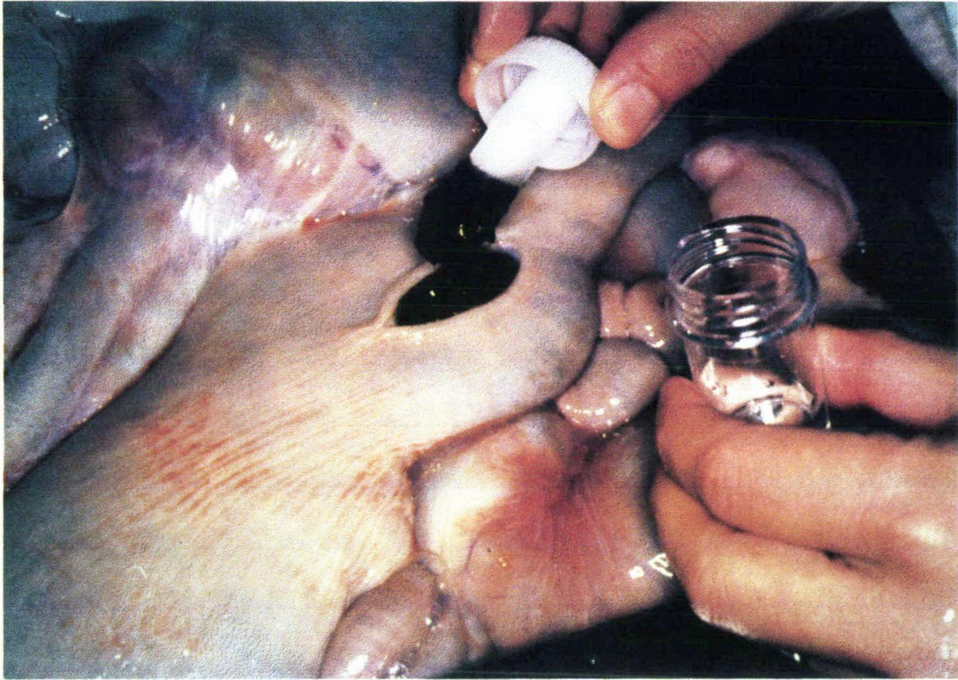


Fig. 6. 3. b Caecal Content Scooped Out and Mixed with 10 ml Phosphate Buffer Solution (PBS)

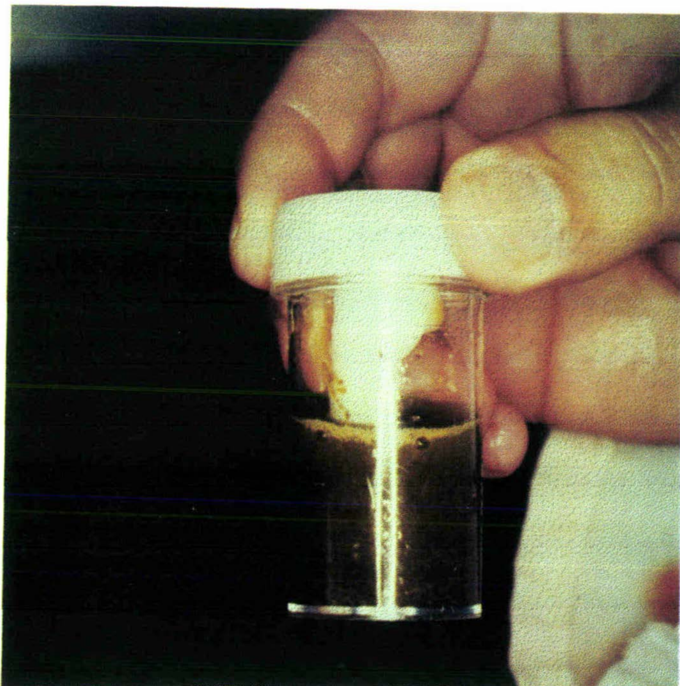


Fig. 6. 3. c Caecal Content with 10 ml PBS in a Sterile Plastic Container

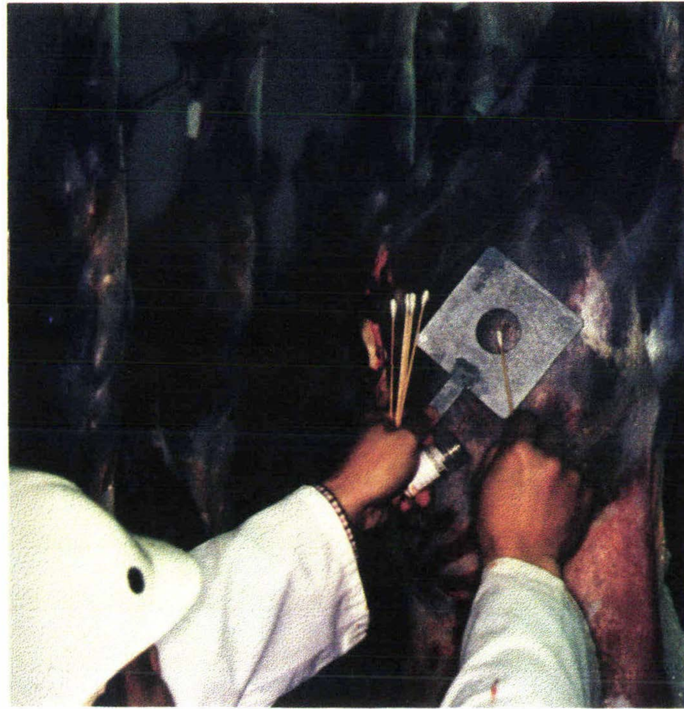


Fig. 6. 4. a Carcase Swabs Taken in the Gluteal Region

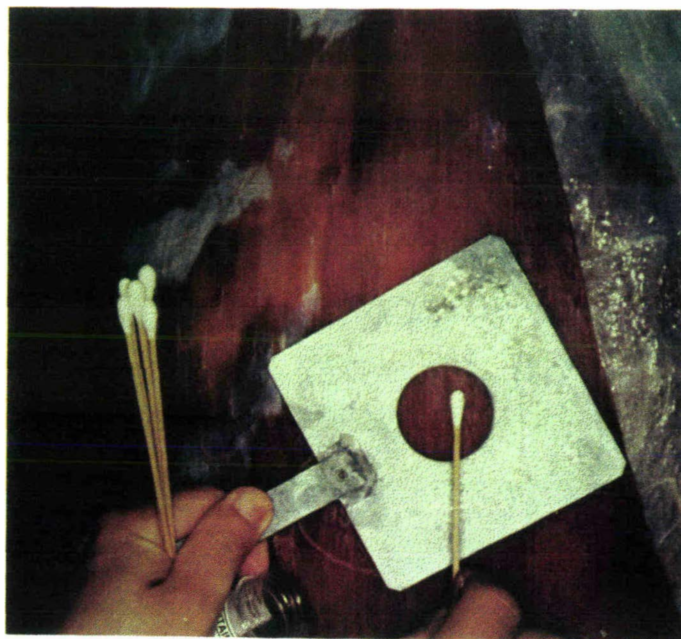


Fig. 6. 4. b Carcase Swabs Taken at the Mid-Trunk

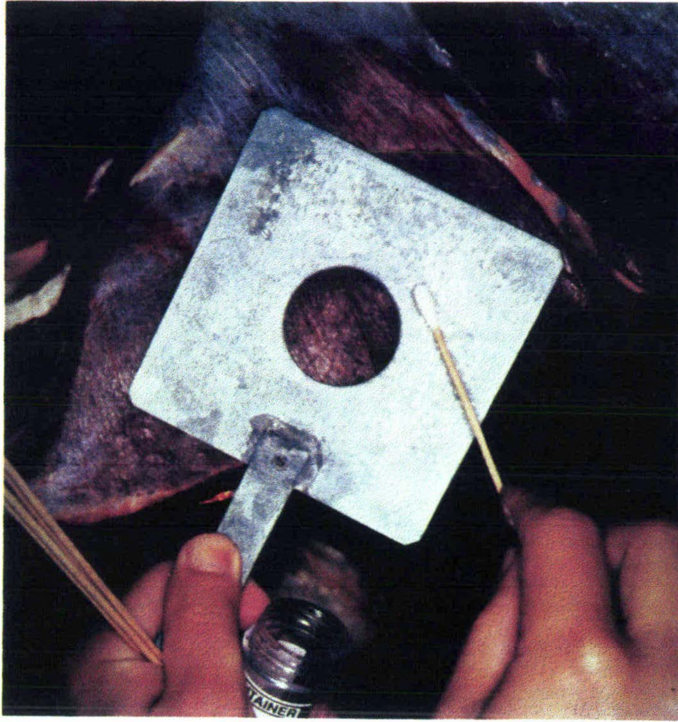


Fig. 6. 4. c Carcase Swabs Taken at the Neck

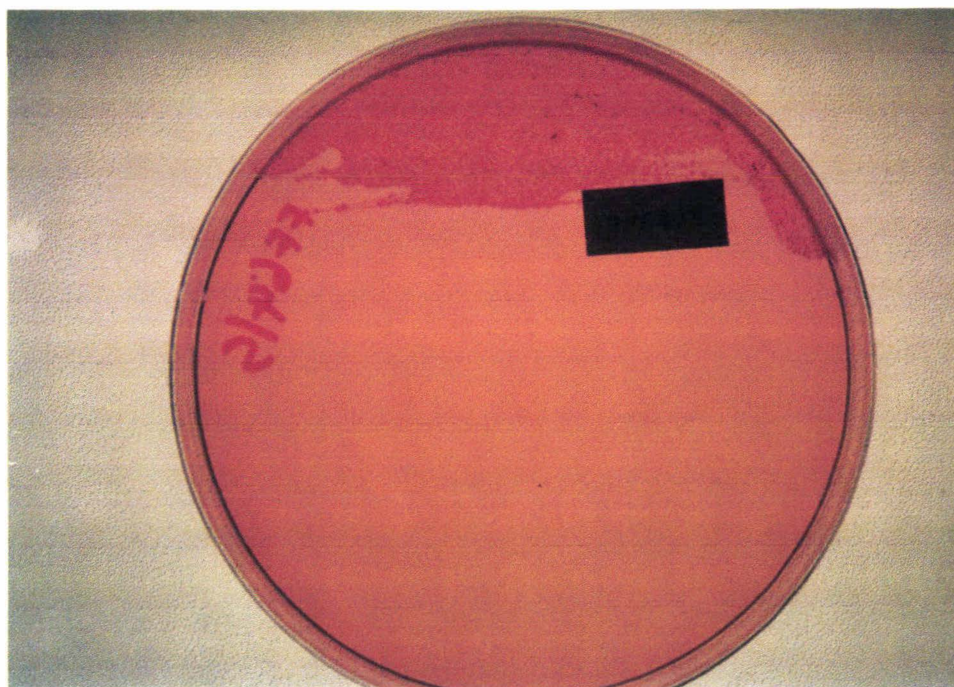


Fig. 6. 5. a Pin-Point Colonies of *Y.pseudotuberculosis* from Faecal Sample (FF64/5) on CIN Agar at 24 Hours Incubation at 28 °C

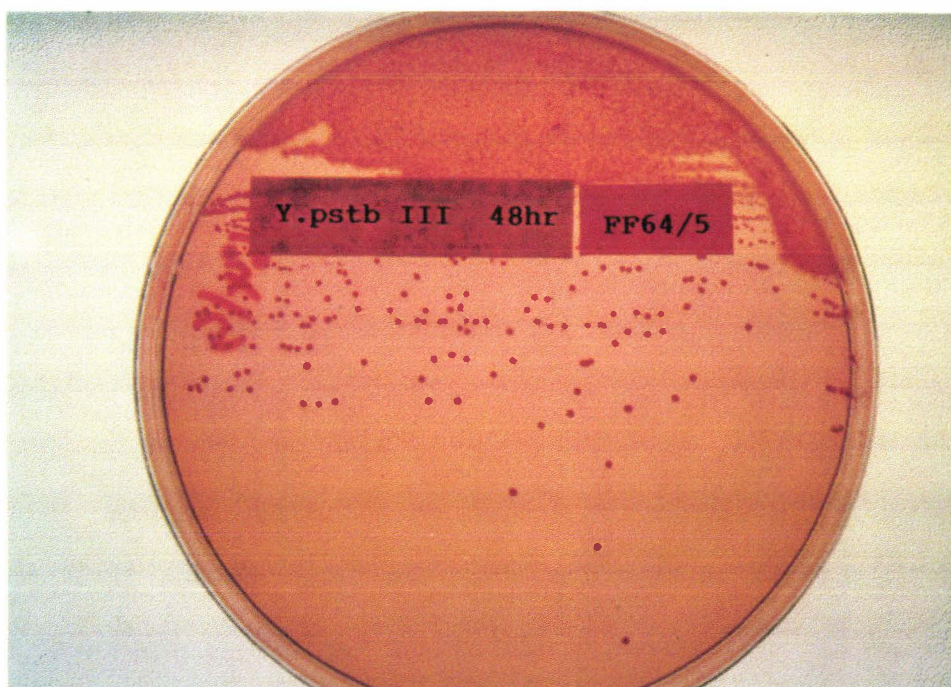


Fig. 6. 5. b Dark-Red and Dry Looking Colonies of *Y.pseudotuberculosis* from Faecal Sample (FF64/5) on CIN Agar at 48 Hours Incubation at 28°C

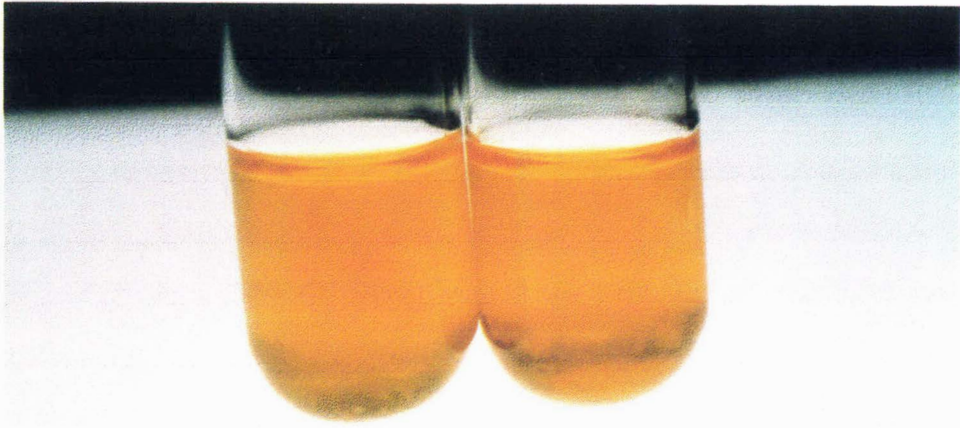


Fig. 6. 6 Positive Autoagglutination Test Showing "Coagulation" After 24 Hours Incubation at 37° C

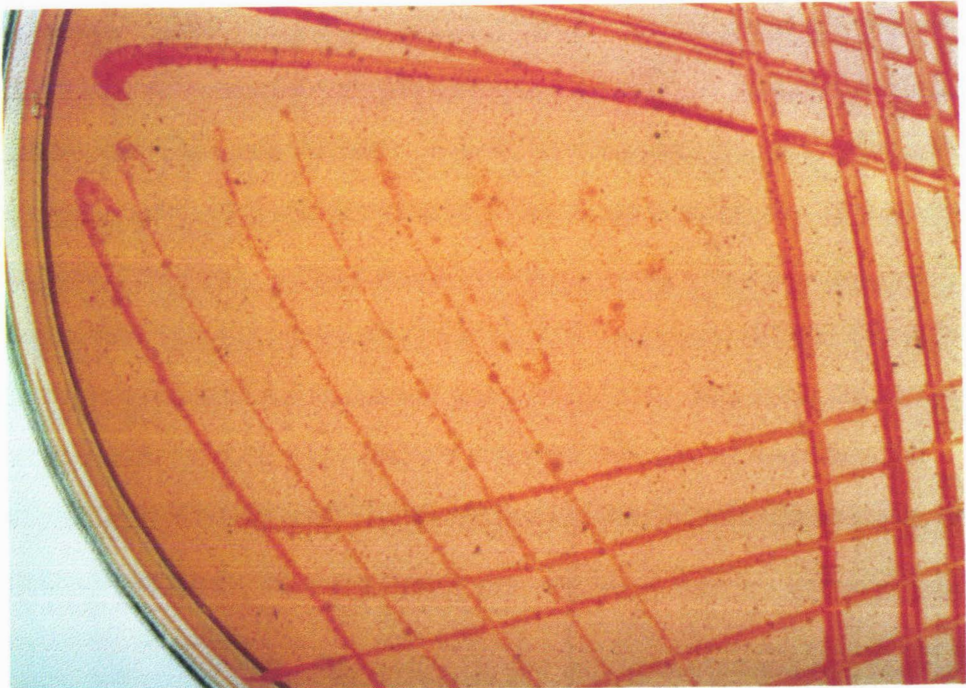


Fig. 6. 7 CRMOX-Positive Strains Showing Both Large and Small Colonies After 24 Hours Incubation at 37°C

CHAPTER 7

A STUDY OF THE PRESENCE OF AGGLUTINATING ANTIBODIES TO *YERSINIA PSEUDOTUBERCULOSIS* IN DEER PRESENTED FOR SLAUGHTER

Introduction

Yersiniosis due to *Y.pseudotuberculosis* is almost entirely a disease of young and recently captured deer (Mackintosh, 1988). The bacterium also affects other animals including man, reptiles and birds (Obwolo, 1976)(refer to Chapter 1). The majority of clinical cases occur in the winter period when the animals, particularly young weaners, are under the greatest environmental stress and may be exposed to feed shortages. The organism appears to survive in the environment under cold and wet conditions (Mackintosh, 1988 ; Blake et al., 1991).

In one investigation, Red deer calves were monitored throughout the autumn-winter-spring period. It was observed that subclinical infection spread slowly through the group between May and August, during which period faecal isolation of *Y.pseudotuberculosis* was made from 10 to 20% of the calves. By August, 97% of the calves had agglutination titres of 1:80 but no clinical yersiniosis was observed (Mackintosh et al., 1986 ; Mackintosh, 1988). The majority of the animals appear to experience a subclinical infection and develop natural immunity. Natural infection results in moderate macroscopic agglutination titres averaging 1:80 to 1:160, and lasting for one to three months (Mackintosh, 1988).

The current serological tests based on agglutination are not very satisfactory due to the variable immunological response and much cross-reactivity within the serogroups of *Y.pseudotuberculosis* and between some serogroups of *Y.pseudotuberculosis* and other species of *Enterobacteriaceae*. (Mair & Fox, 1986). Thus, the ELISA test has been recommended because of its superior sensitivity and specificity (Griffin, 1988).

This study was conducted to determine the antibody titres in the serum of deer presented for slaughter using the microplate agglutination test.

Materials and Methods

A 96-well microplate dish was used. To make a serum dilution of 1:10, 10 ul of

serum which had been heat inactivated at 56°C for 30 minutes was added to 90 ul of a mixture of sterile normal saline and 0.005% w/v Safranine (BDH Chemical, UK) in the first well. Into each of the other wells was dispensed 50 ul of saline-safranine diluent. Then, 50 ul of the diluted serum in well number one was transferred to well number two to make a serum dilution of 1:20. This process was carried through to well number five thereby reaching a dilution of 1:160. Finally, 50 ul of heat-killed antigen (Davies, 1990) was dispensed into each well. All sera were tested in duplicate against serogroups 1, 2 and 3 of *Y.pseudotuberculosis*. The plates were incubated at 37°C for two hours and stored at 4°C overnight. The plates were allowed to stand at room temperature for two hours before a reading was made using a black background (Davies, 1990). The negative controls were the pre-immune sera from rabbits and positive controls were known rabbit hyperimmune sera.

Of a total of 360 serum samples, only 40 were tested for the presence of agglutinating antibodies. Twenty sera were from animals with positive faecal culture, the remainder were from negative animals.

Results

The result of the microplate agglutination tests showed that sera had no significant antibody titres to serogroups 1, 2 or 3. Mackintosh et al., (1986) suggested that 1:40 is a significant titre. Few sera had titres of 1:10 but most did not show any agglutination. The result is shown in Table 7.1.

Table 7.1 Antibody titres to *Y.pseudotuberculosis* serogroups 1, 2 and 3 in deer presented for slaughter at the DSP, Feilding

Serum I.D.	Faecal +/-	*Y.pseudotuberculosis* serogroup 1 (titre level)	2	3
S39/6	+	-	-	-
S43/6	+	-	-	-
S49/6	+	-	-	-
S59/6	+	-	-	-
S37/7	+	-	1:10	-
S72/7	+	-	-	-
S73/7	+	-	-	-
S74/7	+	-	-	-
S80/7	+	-	-	-
S85/10	+	-	-	-
S69/01	+	-	-	-
S64/5	+	-	1:10	1:10
S67/5*	+	-	1:10	-
S73/5	+	-	1:10	-
S74/5	+	-	-	-
S75/5	+	-	-	1:10
S76/5	+	-	1:10	-
S77/5	+	-	-	-
S78/5	+	-	1:10	-
S80/5	+	-	-	-
S73/6	-	-	-	-
S61/7	-	1:10	1:10	1:10
S50/8	-	-	-	-
S55/8	-	-	-	-
S70/8	-	-	-	-
S57/9	-	-	-	-
S65/9	-	-	-	-
S96/9	-	-	-	-
S65/10	-	-	-	-
S91/10	-	-	-	-
S36/11	-	-	-	-
S48/11	-	-	-	-
S59/11	-	-	-	-
S65/12	-	-	-	-
S82/12	-	-	-	-
S41/01	-	-	-	-
S47/01	-	-	-	-
S38/02	-	-	-	-
S56/02	-	-	-	-
S70/02	-	-	-	-

* carcase positive

Discussion

The microplate agglutination test was conducted to estimate antibody titres of *Y.pseudotuberculosis* in sera of deer. Only serogroups 1, 2 and 3 of *Y.pseudotuberculosis* were studied. The result showed that the sera of 40 animals did not have a significant level of antibodies although 20 of the samples came from animals with positive faecal or carcase isolates. This finding is contrary to that of other workers using the agglutination tests with live cultures (Mackintosh et al., 1986) who found that deer which had positive faecal cultures on at least one occasion had agglutination titres ranging from 1:40 to 1:640.

This study indicates that serology using the microplate agglutination test is not a reliable method for the detection of animals excreting the organism in their faeces. The use of heat-killed organisms may have caused the low or non-detection of agglutinins in the sera. This study did not compare with the live organisms (antigen) because the positive controls showed positive agglutination. There is also the possibility that the antibody titres were on the declining phase.

Deer with natural infection have been found to have serological titres of 1:80 to 1:160 and the rate of titre decline was dependent on the peak titre recorded (Mackintosh, 1988). If the peak titres were 1:160, 1:320 and 1:640, the titre had usually declined to undetectable levels by 8, 12 and 16 weeks respectively.

The common serological technique using crude antigens obtained from total cells is not specific. There are potentials for cross-reactions between certain serogroups of *Y.pseudotuberculosis* with certain groups of *Salmonella* (Mair & Fox, 1986).

In man, immunoblotting (Stalhberg et al., 1987 ; Heesemann et al., 1987) and Enzyme Immunosorbent Assay (EIA) (Stalhberg et al., 1987) have been used for serological diagnosis of yersiniosis.

Conclusion

The microplate agglutination test used in this study did not detect antibodies that would indicate that these deer were carrying *Y.pseudotuberculosis* in their intestinal tract. The use of heat-killed organisms and the declining phase of the antibody titres were reasons which may have contributed to the low or non-detection of agglutinins. Therefore, this test or one that uses live-organisms cannot be used as a substitute for faecal culture if the aim is to assess the prevalence of carrier animals.

CHAPTER 8

PREVALENCE OF *YERSINIA SPECIES* IN RETAILED VENISON SAUSAGES

Introduction

Yersinia pseudotuberculosis has been associated with several outbreaks of foodborne illness in Japan (Nakano et al., 1989; Tsubokura et al., 1989) and in Finland (Tertti et al., 1984). The outbreak in Japan was believed to be caused by the consumption of meat products, vegetables and rice whereas in Finland, those affected were actively dealing with growing, selling and buying vegetables.

Yersinia enterocolitica has been associated with several outbreaks of foodborne illness caused by the consumption of contaminted chocolate milk (Black et al., 1978), Chow mein and milk (Morse et al., 1984), milk (Tacket et al., 1985 ; Lofgren et al., 1986), and pig chitterlings (Lee et al., 1991) in the United States of America, and milk in Japan (Asakawa et al., 1973).

Inoue & Kurose (1975) isolated pathogenic strains of *Y.enterocolitica* serovar O:5 from the intestinal content of cows, and also from beef.

Several studies conducted in swine indicate that pathogenic strains of *Y.pseudotuberculosis* and *Y.enterocolitica* are carried by this species, and have subsequently been recovered from pork (Fukushima, 1985 ; Nesbakken & Kapperud, 1985 ; Anderson et al., 1991).

Deer are highly susceptible to infection with *Y.pseudotuberculosis* and in sporadic outbreaks, stock losses of up to 10% have been recorded (Mackintosh et al, 1986). Many deer have been found to acquire subclinical infection and excrete the organisms intermittently in their faeces for prolonged periods (Mackintosh & Henderson, 1984b). Other species such as *Y.enterocolitica*, *Y.intermedia*, *Y.frederiksenii* and *Y.kristensenii* have also been isolated from deer (Henderson, 1984).

Because many deer carry *Yersinia spp.* in their faeces it seemed likely that contamination of carcases could occur during processing with the resulting risk of foodborne infection to the consumer. This study was conducted to investigate the prevalence of *Yersinia spp.* in retail venison sausages.

Materials and Methods

A total of 70 venison sausages were purchased from supermarkets in Palmerston North. This type of sausage in addition to venison also contains mutton, cereals, seasoning, spices and preservatives. The sausages were processed according to the recommendation of Mair & Fox (1986). A total of 25 g of each sausage was mixed with 225 ml of Phosphate Buffered Peptone (Difco Laboratories Detroit Michigan USA) and stomached for two minutes in a Stomacher (Colworth 400, London). Direct plating of a loopful of the suspension was made onto Cefsulodin-Irgasan-Novobiocin (CIN) agar and incubated for 24 to 48 hours at 28°C. The rest of the suspension was kept in a clean jar and incubated for 21 days at +4°C. After the cold-enrichment, the suspension was streaked onto CIN agar and incubated at 28°C. After 24 hours incubation a red colony with a typical bullseye morphology was picked up, inoculated into Tryptone water (Difco Laboratories Detroit Michigan USA), and incubated at 28°C for 24 hours. The cultures from direct plating and after cold-enrichment were screened using Lysine-Arginine-Iron Agar (LAIA) and Triple-Sugar-Iron Agar (TSIA) slants, and following the protocol described in Fig. 6.1.

Results

A total of eight strains (11.4% of the samples) of *Y.enterocolitica*, one strain (1.4%) of *Y.kristensenii* and four strains (5.7%) of *Y.intermedia* were isolated from the venison sausages (Table 8.1). All of the strain of *Y.enterocolitica* were biotype 1A.

Table 8.1 Prevalence of *Yersinia spp.* from retailed venison sausages in Palmerston North

Date of packing	No. of samples	Number of Isolation of *Yersinia spp.* *Y.ent.* B1A	*Y.krist.*	*Y.inter*
26.09.91	6	-	-	-
17.12.91	6	-	-	-
31.12.91	6	-	-	-
23.01.92	7	1	-	1
30.01.92	7	2	-	1
11.02.92	7	-	-	-
27.02.92	6	-	-	2
19.03.92	6	3	-	-
17.03.92	7	-	-	-
07.04.92	6	1	1	-
23.04.92	6	1	-	-
total	70	8	1	4
%		11.4	1.4	5.7

Y.ent. - *Y.enterocolitica* *Y.krist.* - *Y.kristensenii*

Y.inter - *Y.intermedia*

Discussion

Yersinia enterocolitica is commonly isolated from a variety of animals, foods and surface water. Foods are found to be contaminated with environmental strains of *Y.enterocolitica* and related species (Lee, 1977). The organisms survive well in nature, especially at low temperature (Morris & Feeley, 1976). Of particular public health significance is the carriage by food animals of pathogenic strains of *Yersiniae* which may be transmitted to the meat they yield and subsequently to various meat products (Nesbakken & Kapperud, 1985 ; Kapperud, 1986).

In a study conducted by Nesbakken & Kapperud, (1985) *Y.enterocolitica*, *Y.intermedia*, *Y.kristensenii* and *Y.frederiksenii* were isolated from retailed raw pork and cooked foods. Ternstron & Molin (1987) recovered *Y.enterocolitica* biotype 1A, *Y.intermedia* and *Y.frederiksenii* from beef and pork, and *Y.enterocolitica* biotype 1A and *Y.intermedia* from chicken. In Brazil, *Y.enterocolitica* and related species were isolated from milk, groundnuts, cheese and lettuce (Falcao, 1987 ; Warnken et al., 1987).

Y.enterocolitica and *Y.intermedia* were recovered from meat, dairy products, seafoods and vegetables in Italy (Chiesa et al., 1987).

This study revealed that venison sausages on sale to consumers in Palmerston North are contaminated with environmental strains of *Yersiniae*. The source of contamination was not established, and venison cannot be directly incriminated because these sausages were mixed with mutton and other substances. Bullians (1987) isolated *Y.enterocolitica* and related species from lambs at an abattoir. Therefore, the deer and/or the sheep may have been the sources of contamination. However, non-animal constitutents, processing water, the processing environment and personnel could also be responsible for the contamination.

The potential pathogenic role of the so-called environmental strains of *Yersiniae* must be viewed with caution. In Nigeria, *Y.intermedia*-like was found to be responsible for cases of acute diarrhoea in humans (Agbonlahor, 1986). Other workers (Prpic & Davey, 1985) have discovered that certain strains of *Y.intermedia, Y.frederiksenii* and *Y.kristensenii* possess some virulence markers. The production of heat-stable enterotoxin has been observed in some strains of *Y.kristensenii* and *Y.frederiksenii* (Kapperud & Langeland, 1981).

Conclusion

Although no potentially pathogenic strains of *Yersiniae* were recovered from venison sausages in this study, it is impossible to rule out their presence. It has been suggested that a multisystem isolation protocol comprising several enrichments and isolation media be used for the purpose of successfully isolating as many strains of *Yersiniae* as possible (Schiemann, 1982 ; Cox et al., 1990).

This study showed that *Yersinia* is a common contaminant of venison sausages. While the source of contamination was not established, there is a need to educate those in the food industry on the importance of food hygiene and the consumers on the importance of adequate cooking of food.

CHAPTER 9

GROWTH OF *YERSINIA PSEUDOTUBERCULOSIS* IN VACUUM-PACKED VENISON STORED AT CHILLING AND FREEZING TEMPERATURE

Introduction

Y.pseudotuberculosis and *Yersinia enterocolitica* are psychrotrophs and grow well under refrigeration temperatures (Mair & Fox, 1986 ; Schiemann, 1989). Psychrotrophic microorganisms have a temperature range for growth of between -8 and +30°C; the optimum temperature for growth is between 15 to 20°C (Petersen et al., 1991). Many studies have been carried out to determine the growth behaviour of *Y.enterocolitica* in seafood (Peixotto et al., 1977), beef and pork (Hanna et al., 1977a & 1977b), meat (Kleinlein & Untermann, 1990), and in milk (Stern et al., 1980) at various temperatures. However, there is only one report of a growth study of the growth of *Y.pseudotuberculosis* in pork (Fukushima, 1987b).

Venison is a dark red meat due to a relatively high content of iron. It is also high in protein and low in fats, energy and cholesterol (Drew & Seman, 1987). Therefore, venison has all of the best attributes of red meat with none of the perceived undesirable characteristics of other types of meat (Drew et al., 1991). The venison processed in New Zealand for the overseas markets is vacuum-packed and exported in either chilled form at -1°C (Seman et al., 1989) or frozen at -12°C or -18°C (Drew et al., 1991). According to one study chilled vacuum-packed venison at -1°C has a shelflife of at least 12 weeks without spoilage while in the frozen state it may last for up to 28 months without any deterioration in eating quality (Drew et al., 1991).

This study was conducted to investigate the growth pattern of *Y.pseudotuberculosis* on venison at chilling and freezing temperatures. The growth potential of *Y.enterocolitica* (ATCC 9610) was assessed in the -20°C experiment as a comparison because this organism, or closely related strains have been widely used in similar experiments in the literature.

Materials and Methods

Venison samples were derived from the ventral abdominal muscles. Fatty and membranous tissues were trimmed off, and the meat cut into pieces of approximately equal

size and weight (6 cm x 10 cm and 2 cm thick and weighing between 30 to 100 g). The pH of the venison samples was determined using a Solomat, MPM 2000 instrument (Solomat Corp. Stamford, USA) with a combination pH-electrode (Broadley-James Corp. Santa Ana, USA). Only meat with a pH > 5.6 was used in these experiments. Meat samples were kept in non-permeable plastic bags which were supplied by the New Zealand Venison Company Limited (Hastings).

The test organisms used were *Y.pseudotuberculosis* serogroup 3 strain Yellow 6 and FF85/10, and *Y.enterocolitica* ATCC 9610. Yellow 6 was isolated from a 6 month old hind belonging to the Deer Production Unit, Massey University which died from yersiniosis on the 31 May,1991. FF85/10 was isolated from faeces of a deer presented for slaughter in October, 1991. *Y.enterocolitica* ATCC 9610 was obtained from the New Zealand Communicable Disease Centre (NZCDC).

Each culture was grown on Blood agar and incubated at 28°C for 24 hours. A colony was picked up with a sterile straight wire and introduced into Tryptone water (Difco Laboratories Detroit Michigan USA) and incubated overnight at 28°C. Ten-fold serial dilutions of the broth culture were prepared using sterile normal saline. The enumeration of viable bacteria was by the plate spread method (Cruickshank, 1965). The isolatory media were kept at 28°C for about two hours to allow drying and help to absorb the inoculum rapidly. For each dilution 0.05 ml of inoculum was dispensed onto duplicate Cefsulodin-Irgasan-Novobiocin (CIN) agar microplates (Nunclon) and incubated at 28°C for 48 hours (Fig. 9.1). The number of colonies was counted and an mean value was calculated for each dilution, on which the number of viable cells in the original suspension was determined. Venison samples were inoculated with either a "high" dose or "low" dose of organism. The 10^{-4} dilution was used for the low-dose inoculum whereas the 10^{-2} dilution was used for the high-dose inoculum.

A 0.1 ml volume of the inoculum was added to 1.0 ml of sterile distilled water, and spread over the venison in the plastic bag using a sterile Pasteur pipette. In twelve out of the 18 experiments two samples per time were used (Appendix IV). After inoculation, the air in each plastic bag was evacuated and sealed using a vacuum-packaging machine (Lindgren, Auckland) that was set at 100% vacuum and stored at the specified temperatures. The "day 0" samples were recovered and titrated immediately after vacuum-packaging.

In order to recover the organisms at the predetermined times the plastic bag was opened, and 50 ml of sterile 0.1% Peptone (Difco Laboratories Detroit Michigan USA) saline solution was added and the contents stomached (Colworth, UK) for two minutes. If the samples were frozen, thawing at +4°C was carried out for at least three hours before

stomaching. Ten-fold serial dilutions were prepared from the resulting suspension. Counting of viable bacteria was carried out as described earlier. In one experiment, the venison samples were pre-heated at 65°C for 30 minutes to reduce the number of resident bacteria without causing excessive damage to the venison. This was done to determine the growth of *Y.pseudotuberculosis* on vacuum-packed venison in the absence of a competiting normal flora.

Results

The results of the growth experiments are shown as; total colony-forming units (CFUs) per sample, total CFU per g (gram) and total CFU per cm^2 and given in Graph I to XVIII. For the purpose of explaining the result of the experiments, the CFU per g will be used. Detailed results are tabulated in Appendix IV (1 to 18).

a. Growth at +10°C

Y.pseudotuberculosis grew rapidly in vacuum-packed venison at +10°C with a 2.73 log increase in the number of CFUs within 18 days before spoilage was detected (Graph I & App. IV.1).

b. Growth at +4°C

At 4°C, Yellow 6 showed a 3.85 log increase in number (Graph II & App. IV.2) while FF85/10 showed a 2.64 log increase in the number of CFUs within 28 days (Graph III & App. IV.3). When the venison samples were pre-heated and stored at +4°C, there was a 6.04 log increase in number of CFUs within 28 days (Graph IV & App. IV.4).

c. Growth at -1°C

Yellow 6 and FF85/10 were capable of growing at -1°C although there was a protracted lag phase. It took 56 days to attain a 3.89 log increase in the number of CFUs for Yellow 6 (Graph V & App. IV.5) whereas the FF85/10 took only 49 days to achieve a comparable increase of 3.49 log (Graph VI and App. IV. 6).

d. Growth at -10°C

There was a decrease of 0.30 log within 88 days for samples inoculated with a low-dose inoculum and stored at -10°C (Graph VII & App. IV.7). For the high-dose inoculum,

the decreased was 0.31 log within 55 days at the same storage temperature (Graph VIII & App. IV.8). An average of 1 log decrease was observed for both the low- and high-dose inoculums when stored at -10°C for 264 days. The growth was different when the samples were stored at -13°C ± 2. There was a 0.24 log increase with the high-dose inoculum after it has been stored for 88 days (Graph IX & App. IV.9) while for the low-dose inoculum there was an decreased of 0.34 log within 55 days (Graph X & Appen. IV.10).

e. Growth at -20°C

When the temperature was -20°C, Yellow 6 at low- (Graph XI & App. IV.11) and high- (Graph XII & App. IV.12) dose inoculum showed a decrease of 0.25 log within 56 days. The low-dose inoculum showed a decrease of 0.51 log as compared to 1.78 log decrease in the high-dose inoculum within 70 days. However, in two other experiments, there was a decrease of 0.41 log within 56 days when the samples were seeded with the low-dose inoculum (Graph XVII & App. IV.17) and a 0.22 log decrease when the inoculum was high (Graph XVIII & App. IV.18).

Y.enterocolitica ATCC 9610 showed a decrease of 0.4 log for samples with the low-dose inoculum (Graph XIII & App. IV.13) and 0.86 log decrease for samples with the high-dose inoculum (Graph XIV & App. IV.14) within 70 days at -20°C.

f. Growth at +4°C after storage at -1, -10 and -20°C

Y.pseudotuberculosis (Yellow 6) in vacuum-packed venison continued to maintain a consistent growth rate when transferred to +40C after 56 days at -1°C. (Graph XV & App. IV.15). There was an erratic growth of the bacteria from samples at +4°C after storage at -10°C for eight weeks (Graph XVI & App. IV.16). There was a 3.65 log increase in the number of c.f.u after 35 days following change in storage temperature. When the low dose inoculum samples were moved to 4°C after storage at -20°C for eight weeks, there was an increase of 4.76 logs within 35 days (Graph XVII & App. IV.17). The samples with a high-dose inoculum showed a 3.02 log increase in c.f.u within 21 days after the changes in storage from -20°C to +4°C (Graph XVIII & App. IV.18).

A summary of the results is given in Tables 9.1, 9.2 and 9.3.

Table 9.1 Growth of *Y.pseudotuberculosis* in vacuum-packed venison stored at chilling temperatures

Storage temp.	Log increase CFU/g	Day	Remarks
+4°C	2.73	18	>18 d spoilage was detected (Graph I)
"	3.85	28	Yellow 6 (Graph II)
"	2.46	28	FF85/10 (Graph III)
"	6.04	28	Pre-heated samples (Graph IV)
-1°C	3.89	56	Yellow (Graph V)
"	3.49	49	FF85/10 (Graph VI)

Table 9.2 Growth of *Y.pseudotuberculosis* and *Y.enterocolitica* (ATCC 9610) in vacuum-packed venison stored at freezing temperatures

Storage temp.	Log decrease CFU/g	Day	Remarks
-10°C	0.30	88	LD inoculum (Graph VII)
"	0.31	55	HD " (Graph VIII)
"	1.0	264	LD & HD
-13°C ± 2	0.24 (+)	88	HD (Graph IX)
"	0.34	55	LD (Graph X)
-20°C	0.25	56	LD (Graph XI)
"	0.25	56	HD (Graph XII)
"	0.51	70	LD (Graph XI)
"	1.78	70	HD (Graph XII)
"	0.41	56	LD (Graph XVII)
"	0.22	56	HD (Graph XVIII)
"	0.40	70	LD ATCC 9610 (Graph XIII)
"	0.86	70	HD ATCC 9610 (Graph XIV)

Table 9.3 Growth of *Y.pseudotuberculosis* in vacuum-packed venison stored at +4°C after storage at freezing temperatures for 8 weeks

Storage temp. for 8 weeks	Log increase CFU/g	Day	Remarks
-1 °C			maintain growth (Graph XV)
-10°C	3.65	35	LD (Graph XVI)
-20°C	4.76	35	LD (Graph XVII)
"	3.02	21	HD (Graph XVIII)

LD - Low Dose HD - High Dose

Discussion

The results of this study indicate that *Y.pseudotuberculosis* is capable of growth at temperatures as low as -1 °C. The minimum level of detection for the test organism is log 2.50. This means that the organism may still be present in the samples but at too low a level to be detected.

Although *Y.pseudotuberculosis* remained viable for as long as 264 days in frozen, vacuum-packed venison, the results showed that in general there was a reduction in the number of viable cells when samples were frozen. This is consistent with the works of Straka & Strokes (1959) and Morichi & Irie (1973) who stated that frozen bacteria generally comprises both dead, sublethally injured and unharmed cells. There is an increase in the number of metabolically injured (Nakamura & Dawson, 1962) and dead (Straka & Strokes, 1959; Nakamura & Dawson, 1962) cells when freezing is prolonged.

When the samples were transferred to +4°C after frozen storage for eight weeks, the bacteria were capable of rapid growth. It appeared that low storage temperatures (-10°C) had a more detrimental effect on the bacteria than higher temperatures as can be deduced from the erratic recovery. The slow freezing rate allows the formation of large ice crystals between the cells. On further formation of the ice, the ionic strength of the extracellular fluid increases with a resulting osmotic imbalance causing water to move out from the cells. This freezes onto and enlarges the existing extracellular ice crystals which may then press on the cells causing physical damage (cited in Petersen et al., 1991). These effects may be eliminated if rapid freezing is applied. In fact, El-Kest et al., (1991) have found that rapid

freezing and lower freezing temperatures for *Listeria monocytogenes* ensure less damage or destruction of cells.

The growth pattern of *Y.enterocolitica* ATCC 9610 was similar to that of *Y.pseudotuberculosis* in frozen vacuum-packed venison. The similarity was based on the rate of decline of viable cells. However, it must be noted that different strains of *Y.enterocolitica* display different growth pattern in different substrates (Hanna et al., 1977a). There was a 4.0 log decrease within 4 weeks of *Y.enterocolitica* in frozen beef at -23 °C. However, certain strains of *Y.enterocolitica* were more tolerant to freezing than others (Hanna et al., 1977b).

The survival of microorganisms in the frozen state is due to the physical protection offered by the menstrua in which the organisms are frozen (Squires & Hartsell, 1955). In other situations, protective substances are provided such as phosphate buffer (El-Kest et al., 1991) and glycerol (Hollander & Nell, 1954 ; Howard, 1955). It has also been observed that certain organisms have an inherent ability to prevent freezing by producing unsaturated fatty-acyl residues which help to maintain membrane fluidity and hence viability (Gounot, 1991). In an earlier study, Moss & Speck (1966) described the leakage of peptides from frozen cells and this substance protected them from the lethal effects of freezing. The ability of injured cells to recover from freezing is dependent on the availability of complex nutrients in the media (Nakamura & Dawson, 1962).

The growth of *Y.pseudotuberculosis* in meat and other food products has not been studied in detail. There is only one growth study of *Y.pseudotuberculosis* serogroup 4b in raw pork. In this study which was prompted by the first isolation of *Y.pseudotuberculosis* from retailed pork in Japan, it was found that recovery of the organism from samples incubated at 25°C was difficult. At 6°C, the recovery was possible up to the 4th day after which the normal flora became more prominent (Fukushima, 1987b).

The results of the present study clearly indicates that *Y.pseudotuberculosis* can survive freezing on venison and that, on return to domestic refrigeration temperatures, proliferation to high levels over a period of several days is possible.

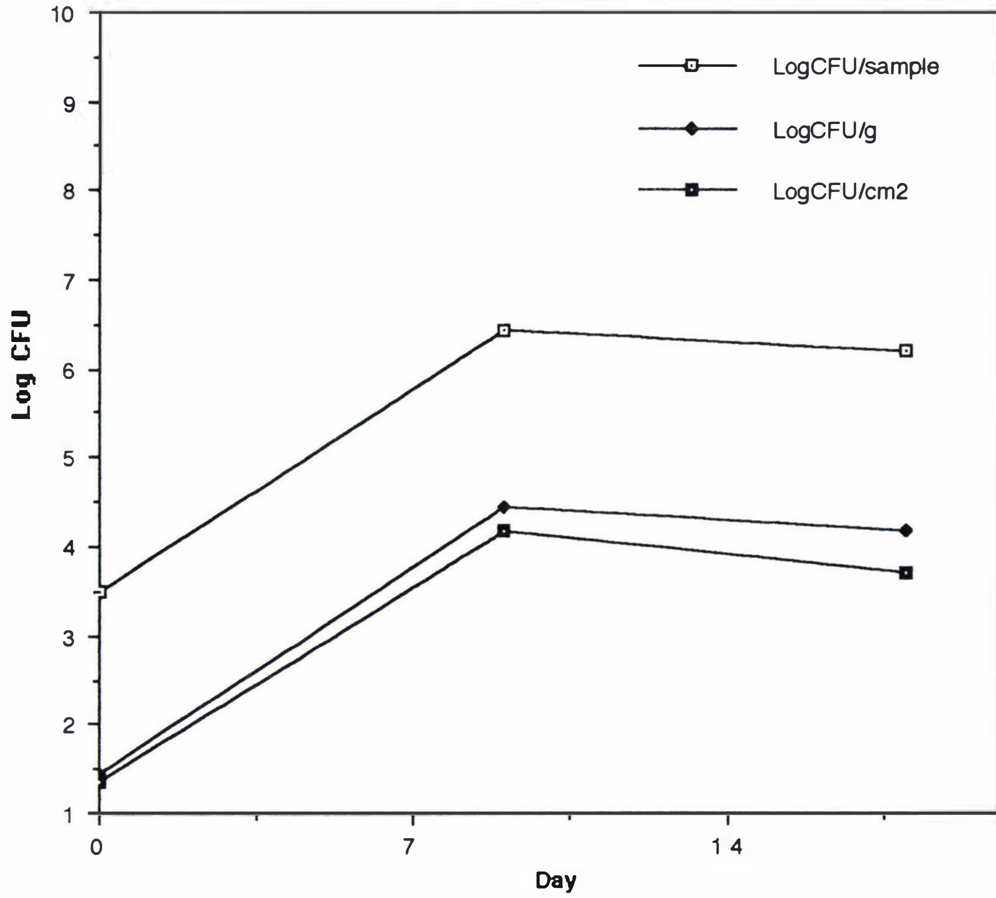
Conclusion

Y.pseudotuberculosis is able to multiply in vacuum-packed venison stored at chill temperatures, as low as -1°C. The bacterium also remains viable for a long time when the product is frozen. When such products are subsequently stored at chill temperatures, there

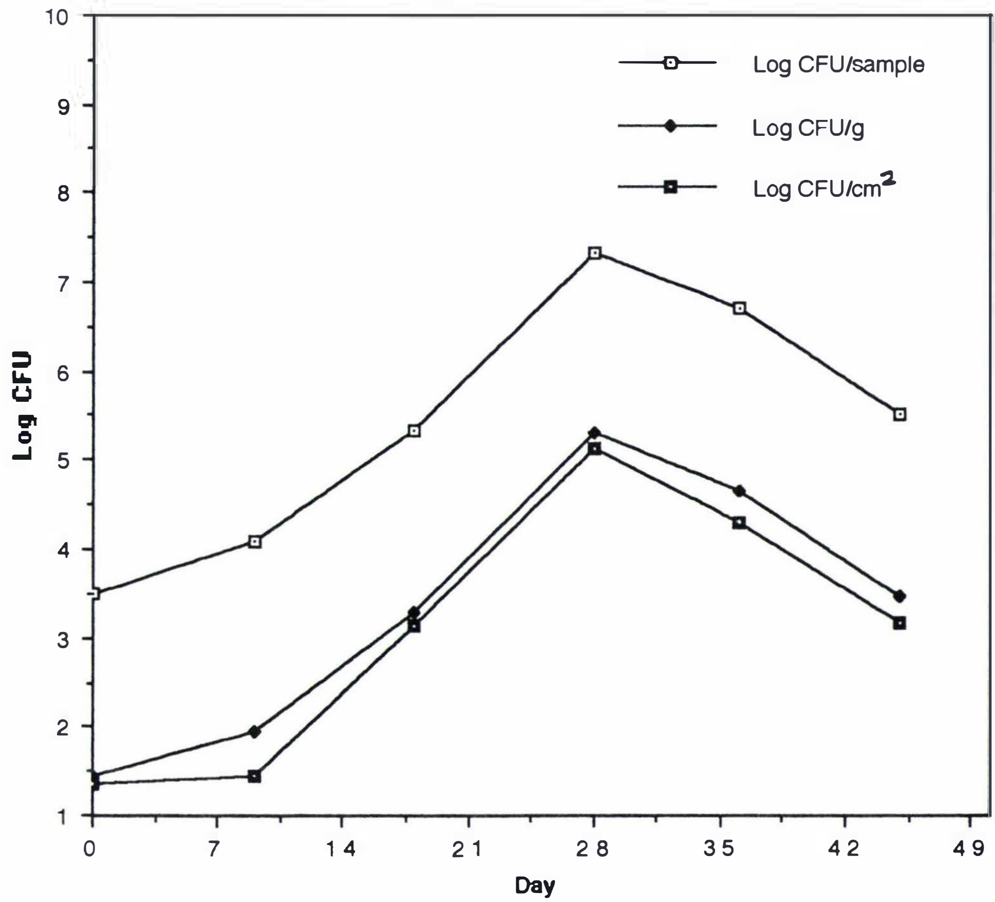
is a rapid recovery and growth of the organism.

Y.pseudotuberculosis and *Y.enterocolitica* showed a very slow decline in number of viable cells when subjected to freezing in vacuum-packed vensison. There is no indication yet whether these bacteria can produce the protective unsaturated fatty-acyl residues. It was noted that venison has a relatively high iron content (Drew & Seman, 1987) and this compound has been observed to enhance the growth of *Yersinia spp*. (Boelaert et al., 1987). Whether it is involved in protection during freezing is however unknown.

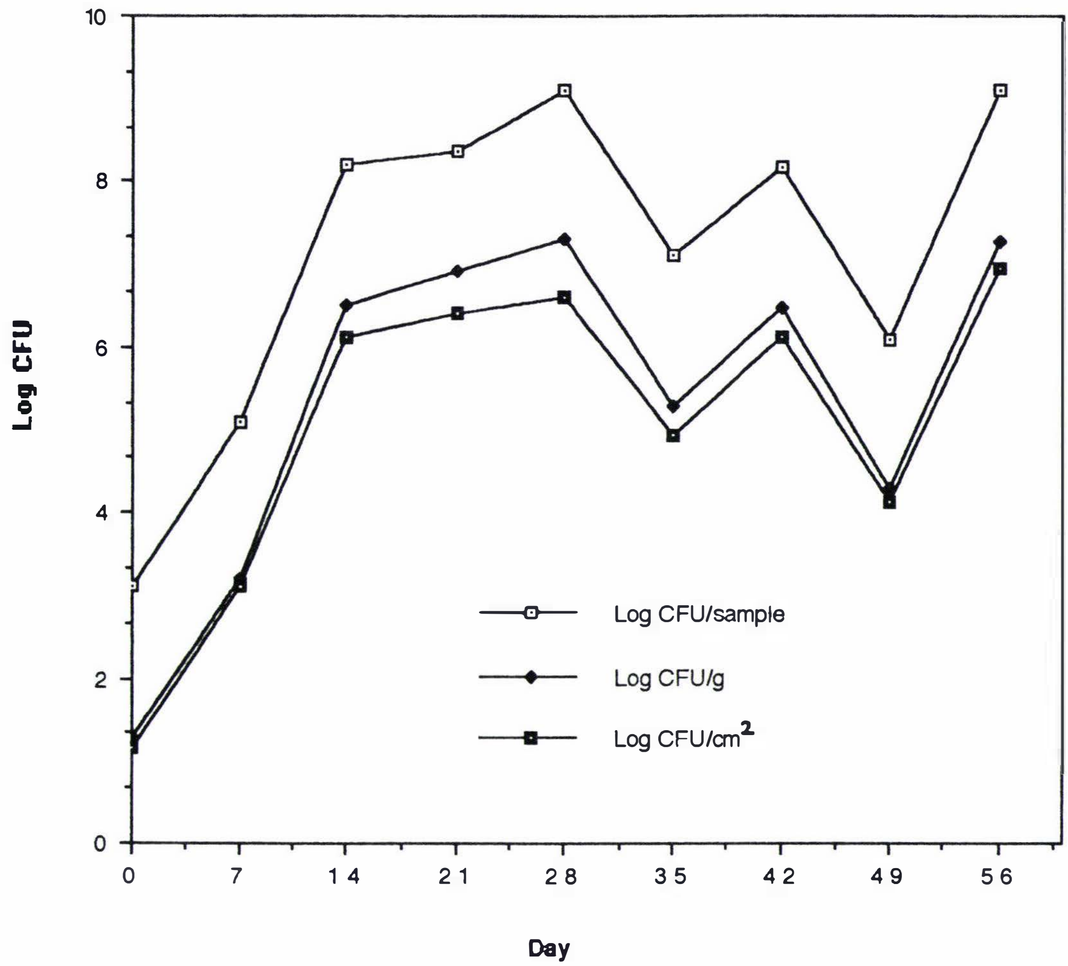
The findings are important because of their relevance to the export of chilled or frozen vacuum-packed venison. Temperature abuse in the cold chain could cause an increase in the population of the bacteria. The results of the experiments are useful to those involved in the venison industry.



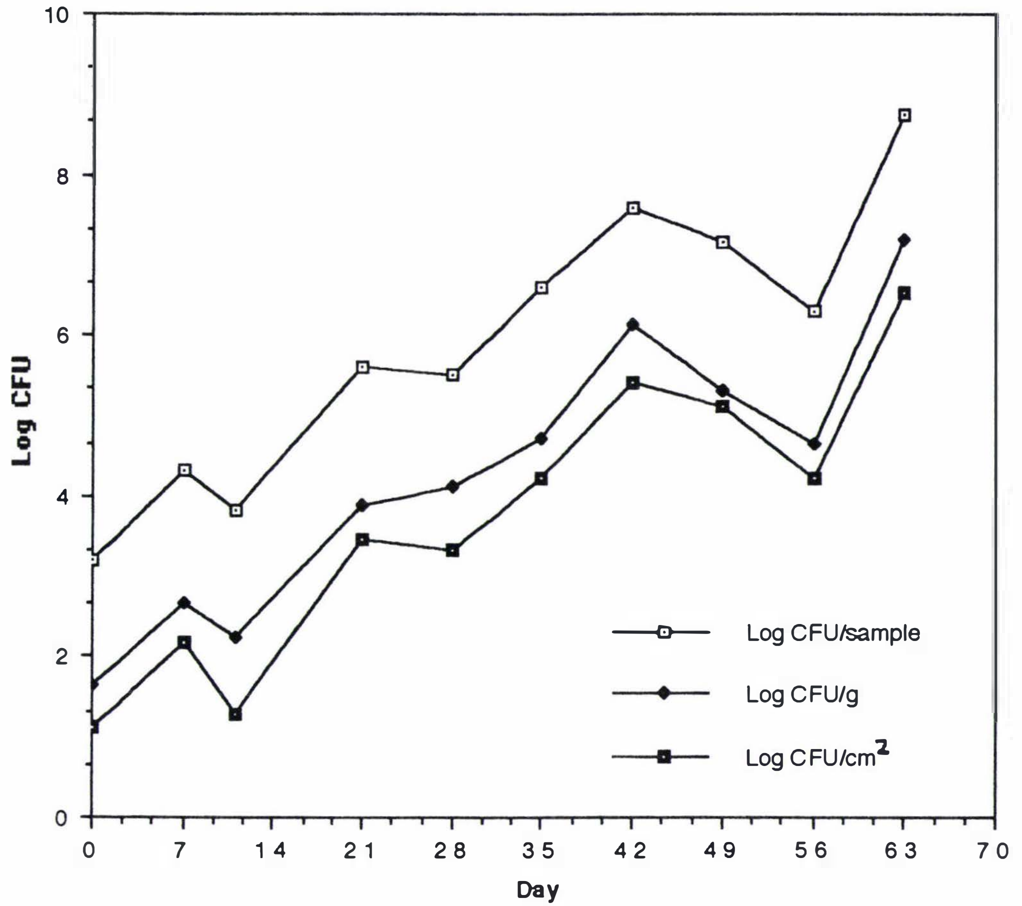
Graph I Growth of *Y.pseudotuberculosis* 3 (Yellow 6) in Vacuum-Packed Venison Stored at +10 °C



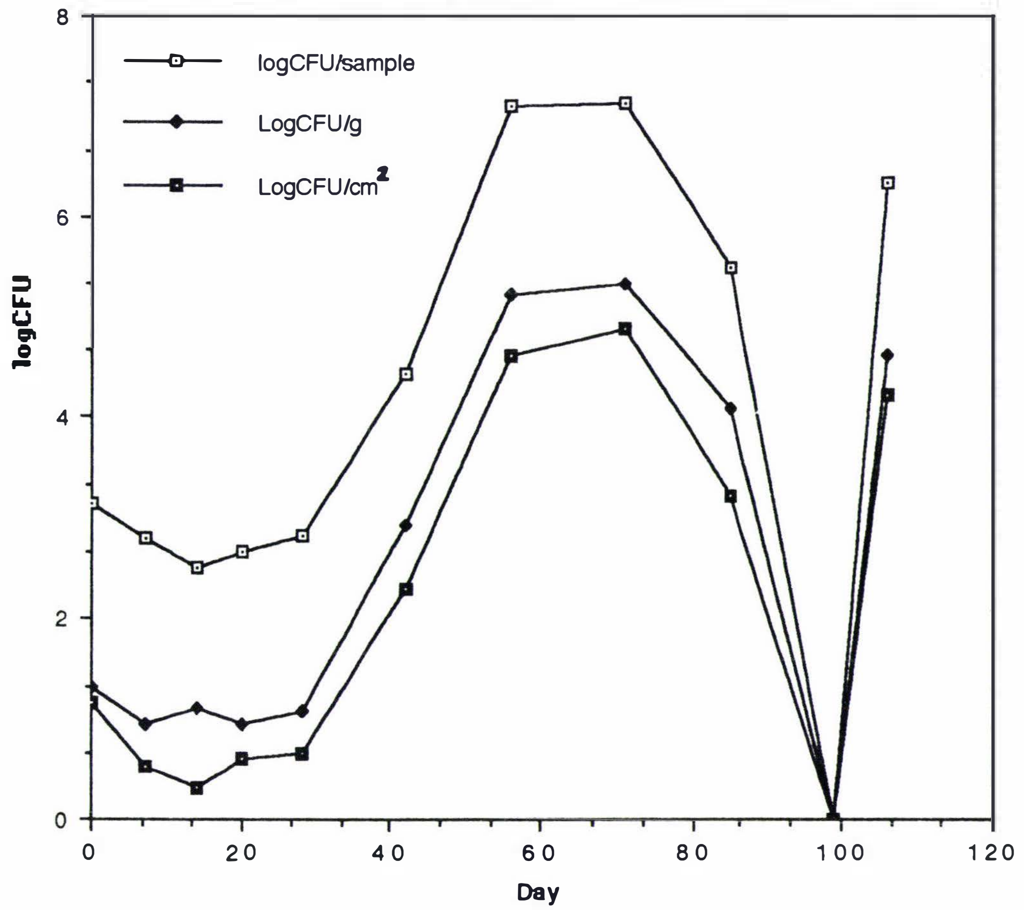
Graph II. Growth of *Y.pseudotuberculosis* 3 (Yellow 6) in Vacuum-Packed Venison Stored at +4° C



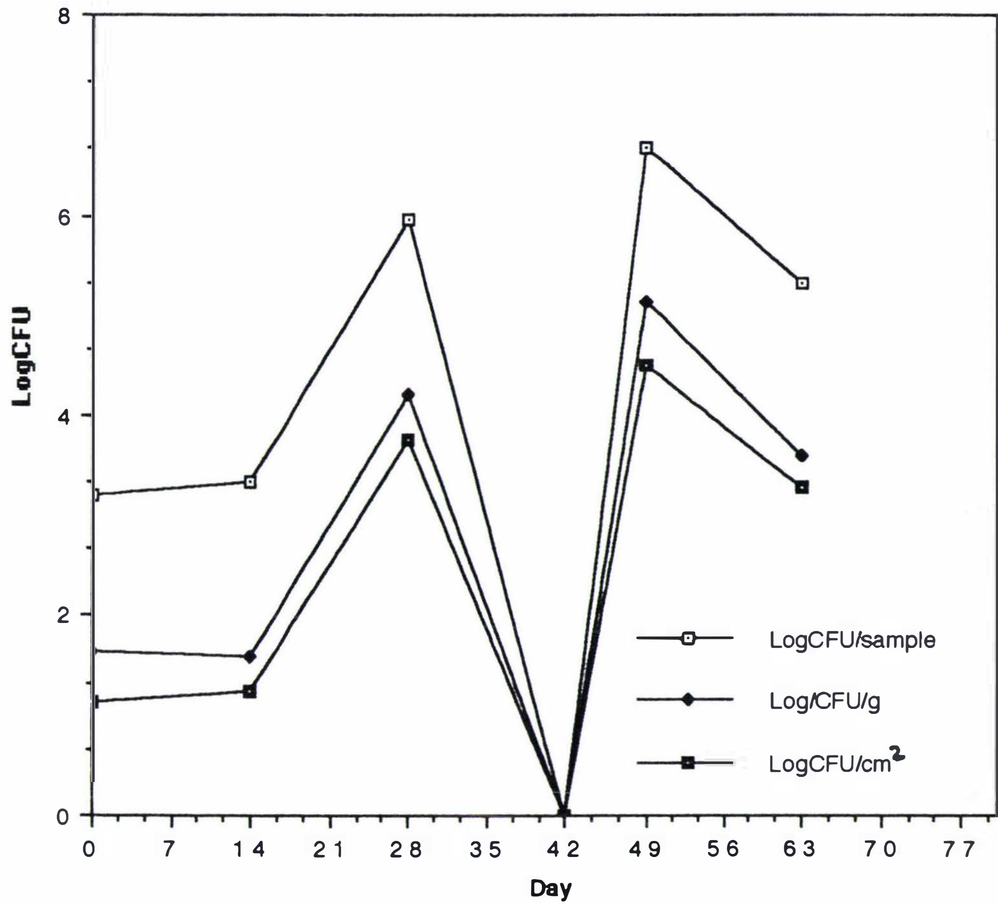
Graph III. Growth of *Y.pseudotuberculosis* 3 (FF85/10) in Vacuum-Packed Venison Stored at +4° C



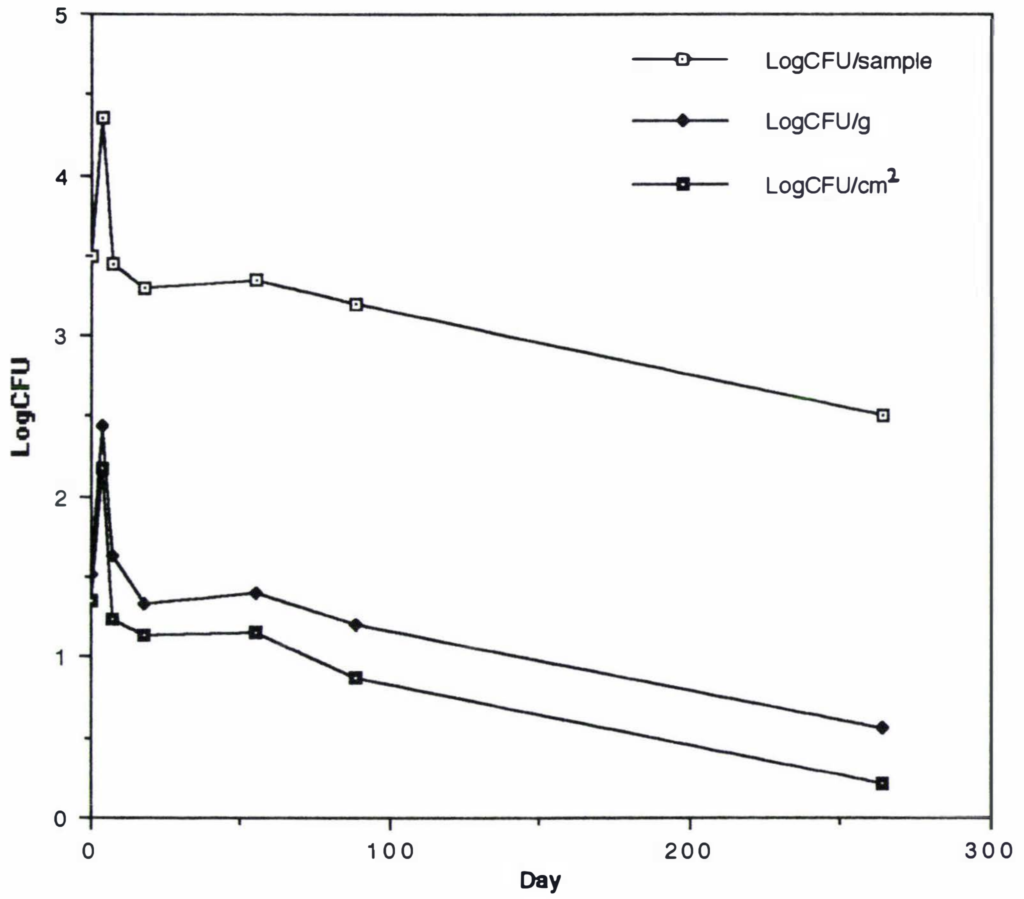
Graph IV. Growth of *Y.pseudotuberculosis* 3 (Yellow 6) in Vacuum-Packed Venison Pre-Heated Venison Stored at +4°C



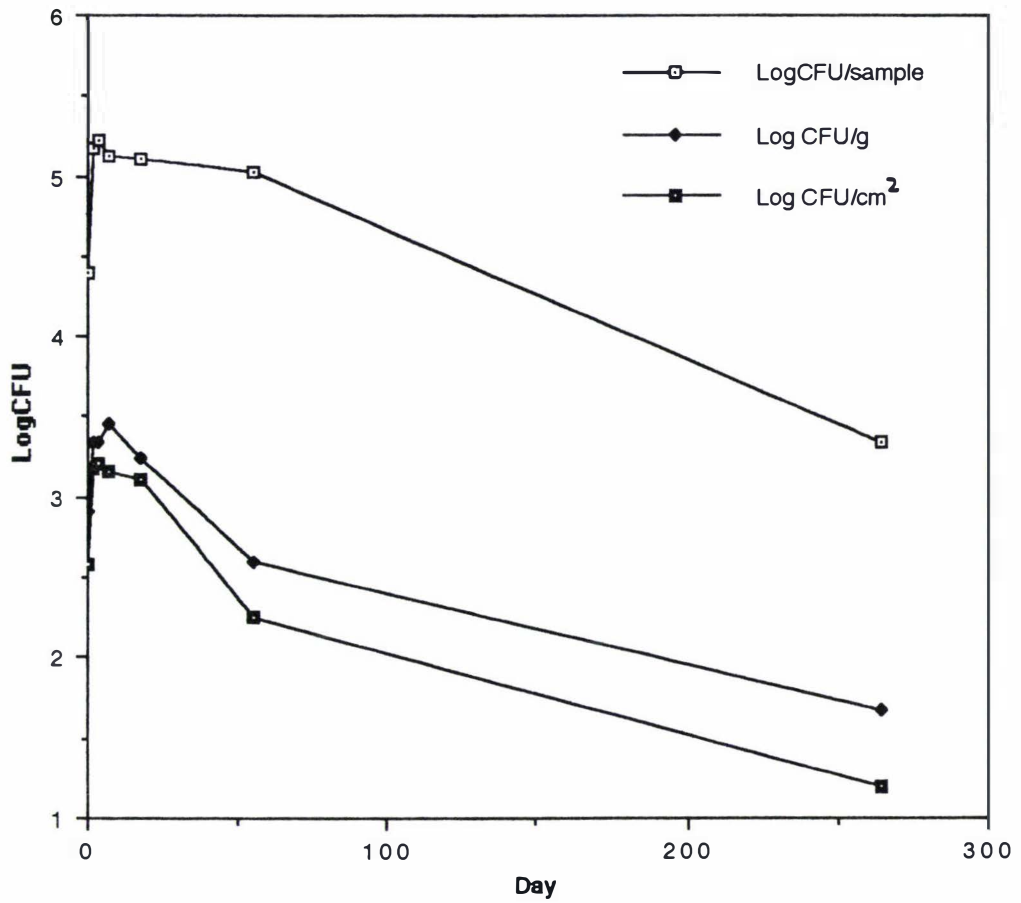
Graph V. Growth of *Y.pseudotuberculosis* 3 (Yellow 6) in Vacuum-Packed Venison Stored at -1° C



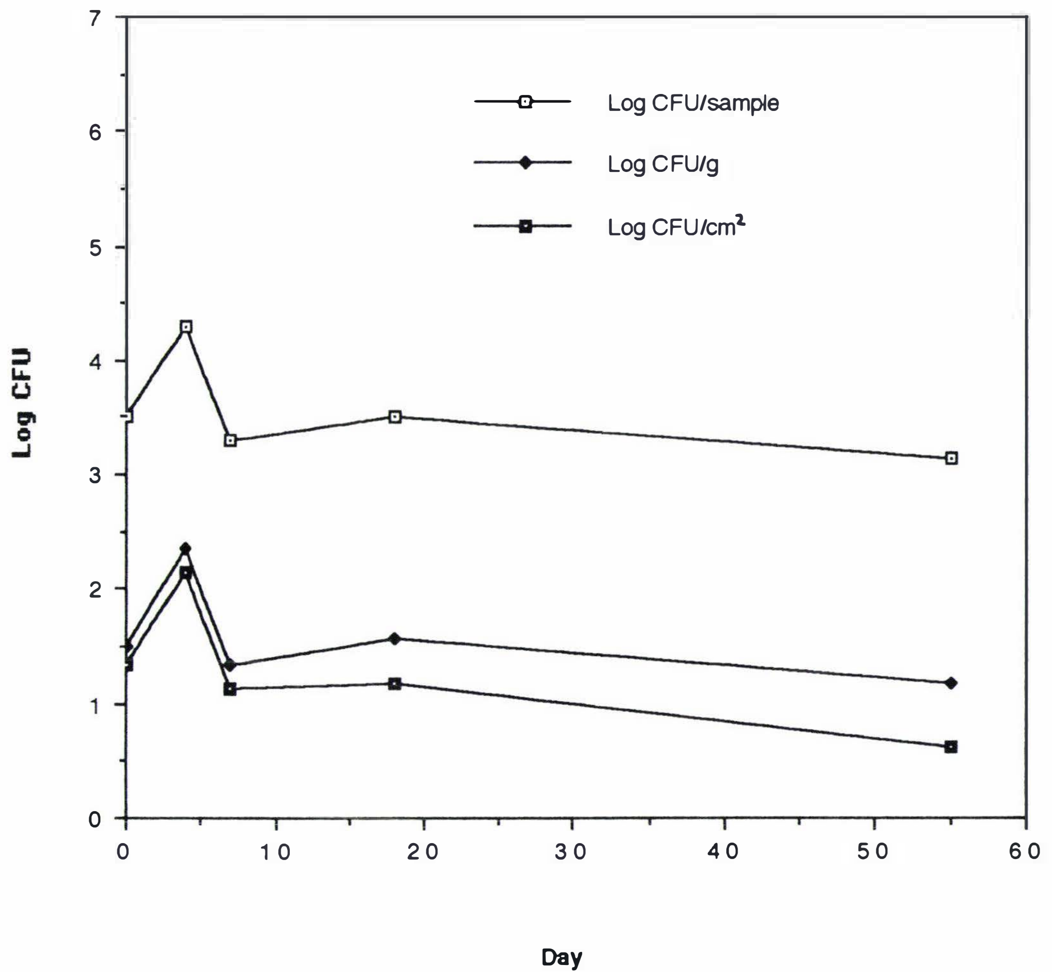
Graph VI. Growth of *Y.pseudotuberculosis* 3 (FF85/10) in Vacuum-Packed Venison Stored at -1° C



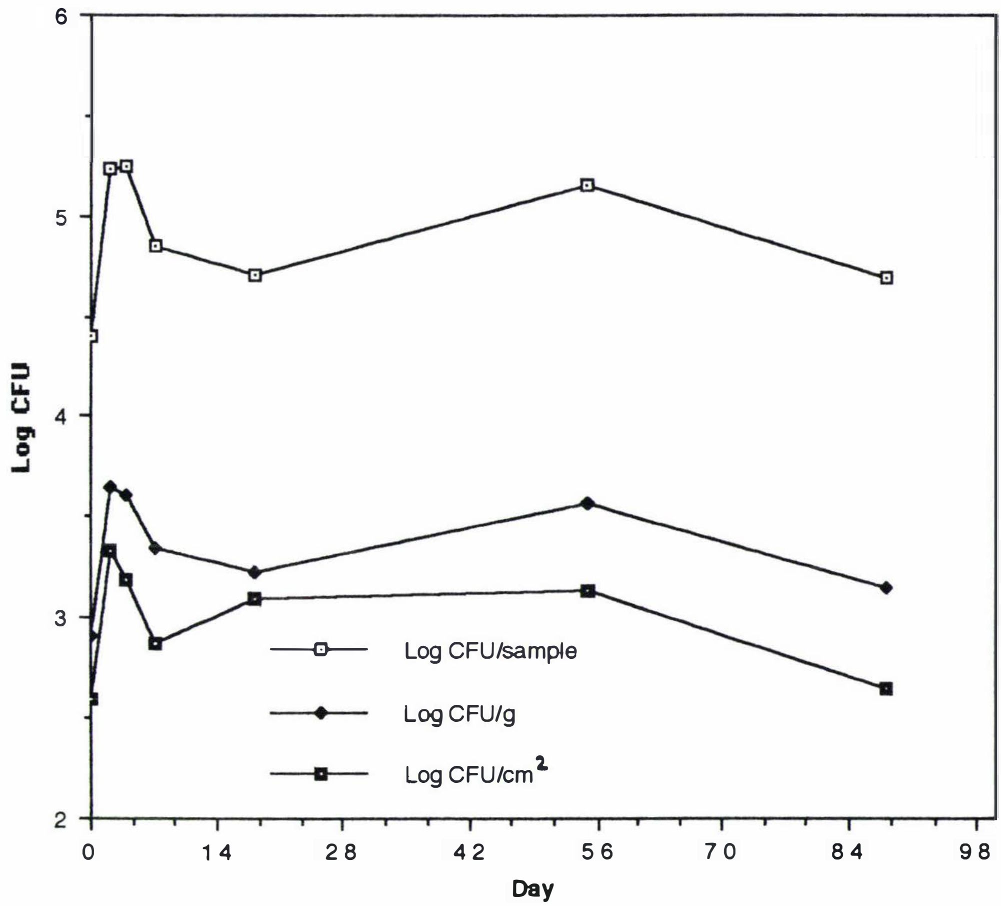
Graph VII. Growth of *Y.pseudotuberculosis* 3 (Yellow 6) in Vacuum-Packed Venison Stored at -10° C (Low-Dose Inoculum)



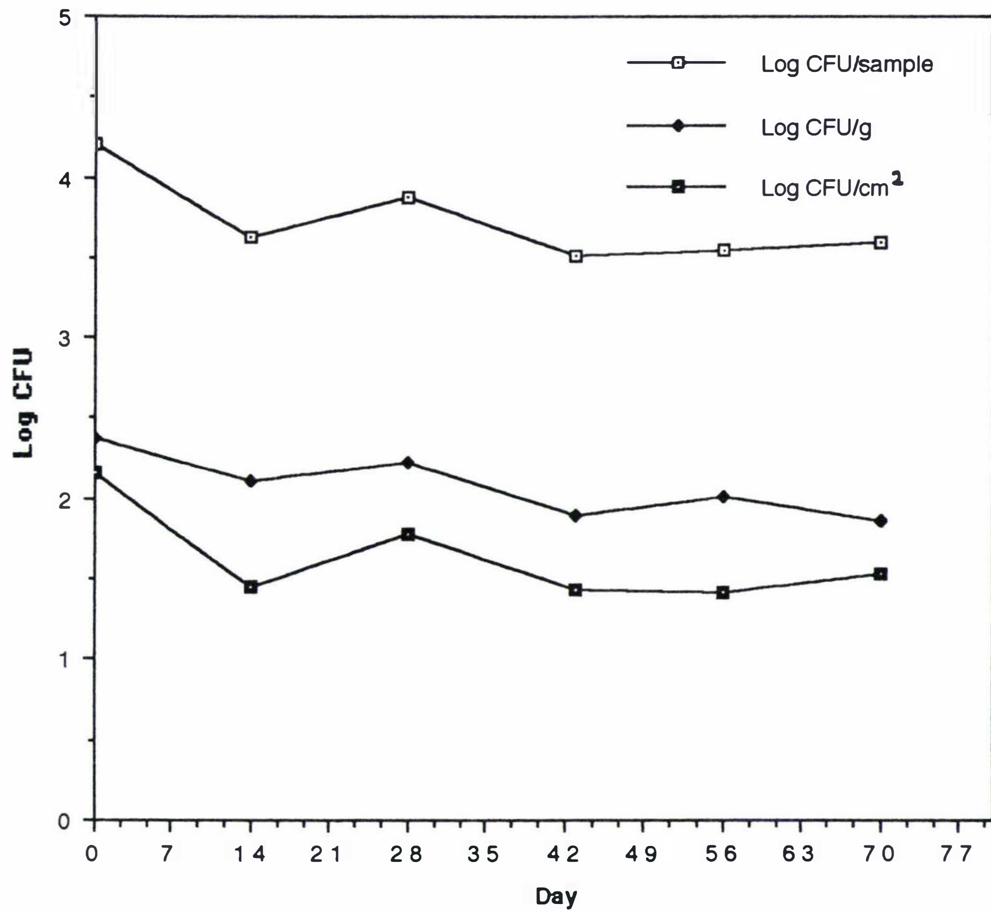
Graph VIII. Growth of *Y.pseudotuberculosis* 3 (Yellow) in Vacuum-Packed Venison Stored at -10° C (High-Dose Inoculum)



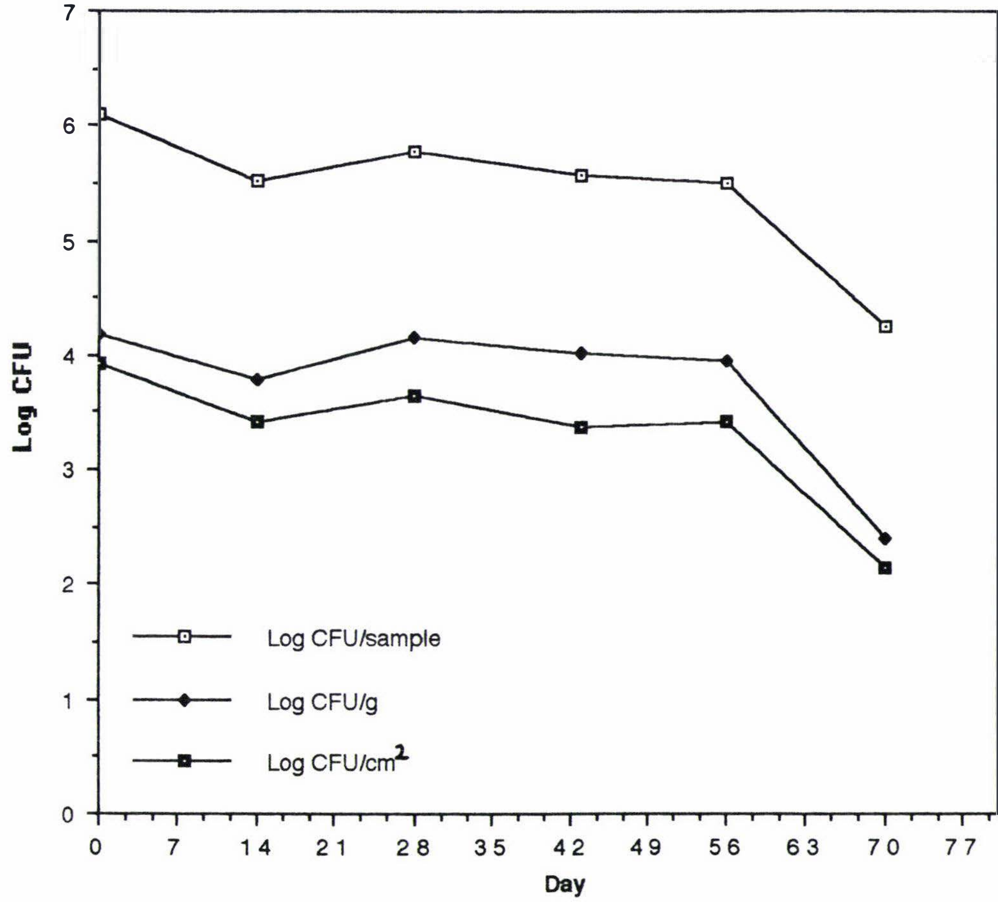
Graph IX. Growth of *Y.pseudotuberculosis* 3 (Yellow 6) in Vacuum-Packed Venison Stored at - 13° C ± 2 (High-Dose Inoculum)



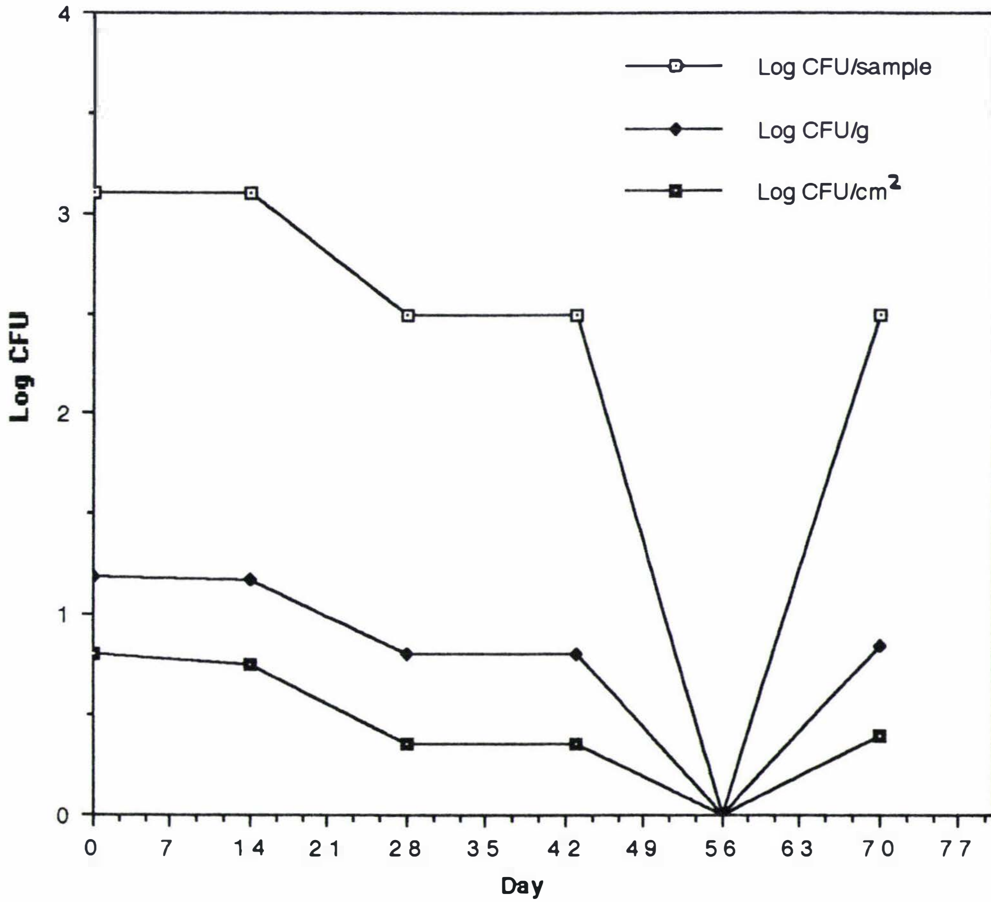
Graph X. Growth of *Y.pseudotuberculosis* 3 (Yellow 6) in Vacuum-Packed V enison Stored at - 13° C±2 (Low-Dosed Inoculum)



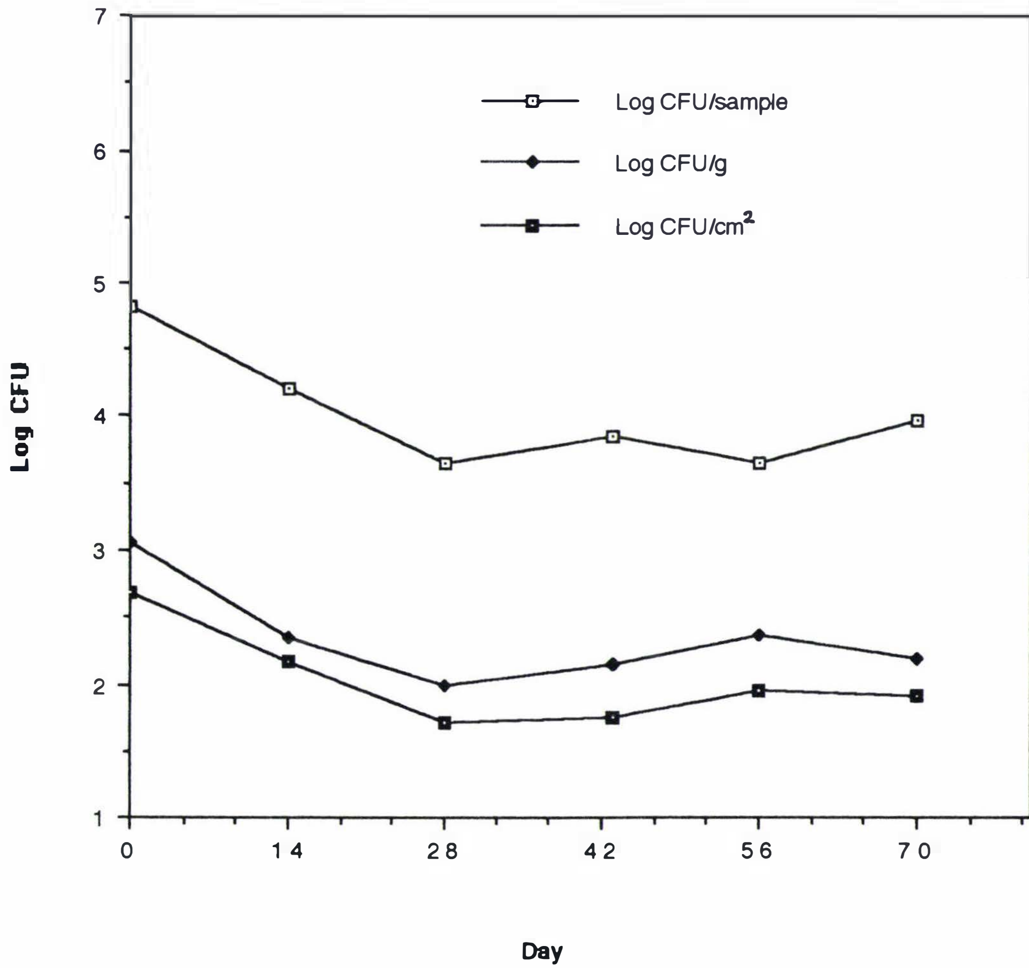
Graph XI. Growth of *Y.pseudotuberculosis* 3 (Yellow 6) in Vacuum-Packed Venison Stored at -20° C (Low-Dosed Inoculum)



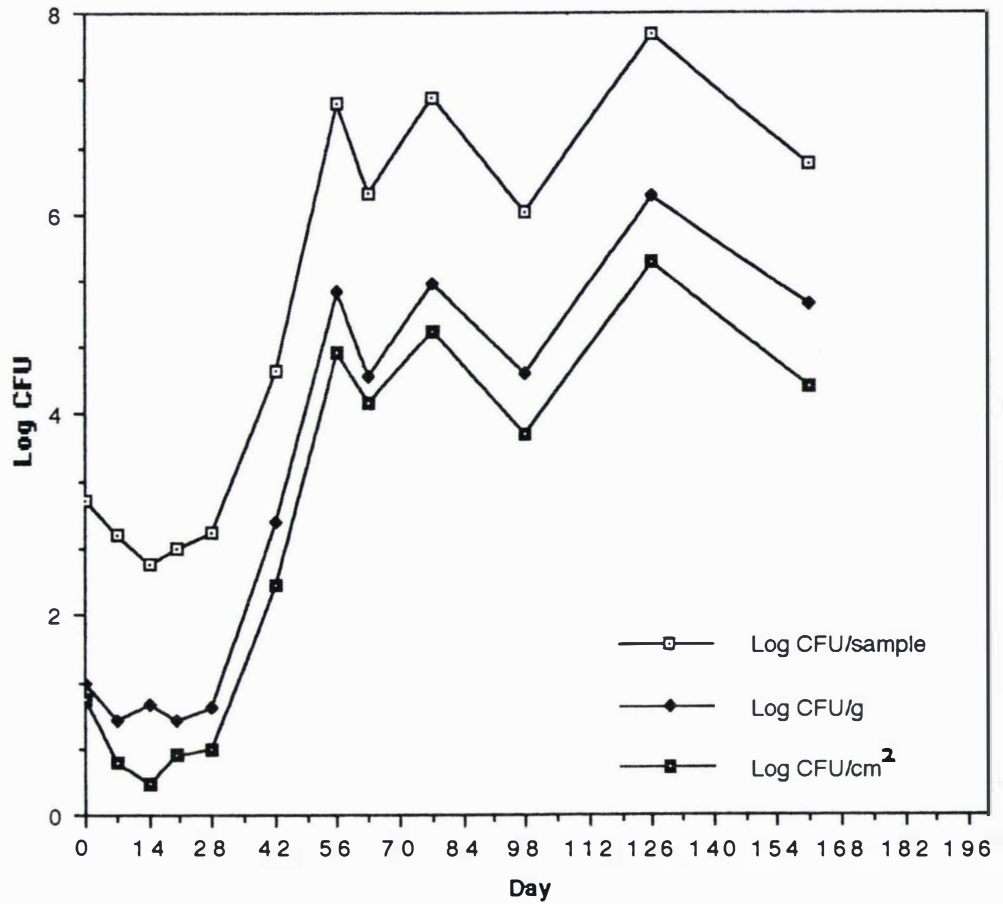
Graph XII. Growth of *Y.pseudotuberculosis* 3 (Yellow 6) in Vacuum-Packed Venison Stored at -20° C (High-Dose Inoculum)



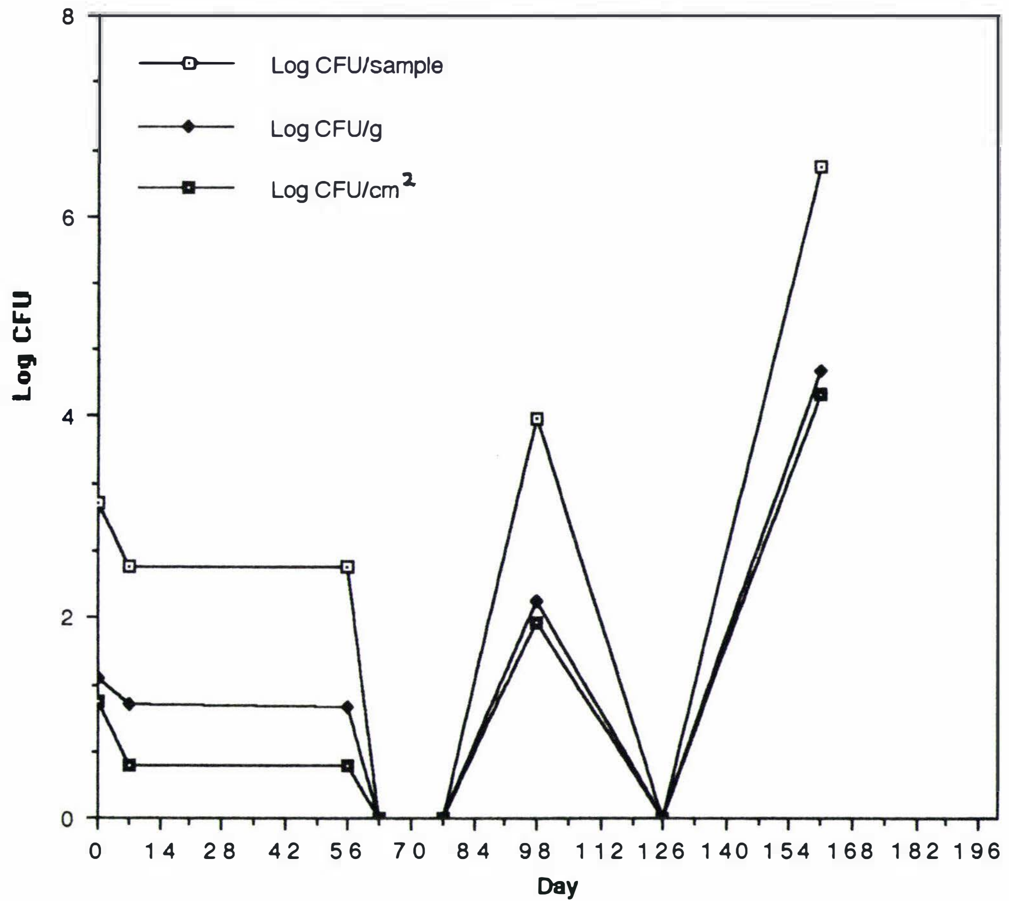
Graph XIII. Growth of *Y.enterocolitica* (ATCC 9610) in Vacuum-Packed Venison Stored at -20°C (Low-Dose Inoculum)



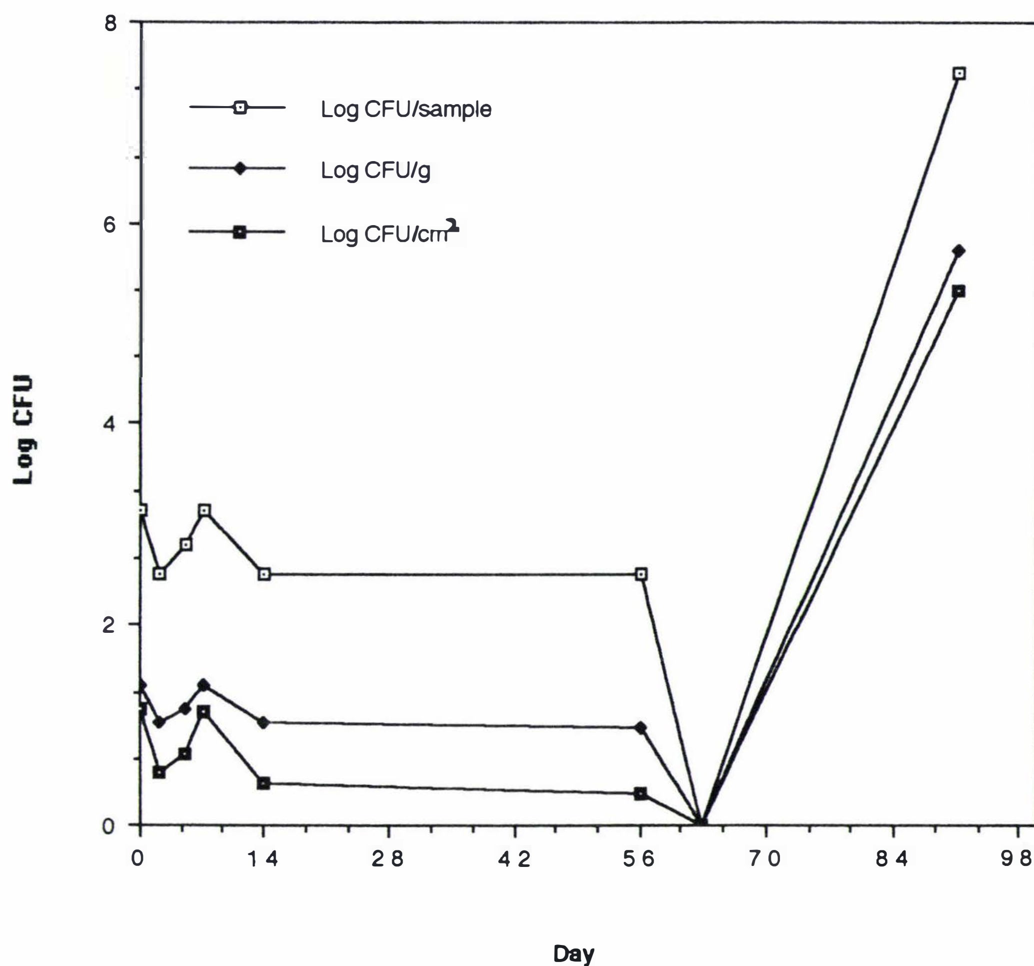
Graph XIV. Growth of *Y.enterocolitica* (ATCC 9610) in Vacuum-Packed Venison Stored at -20° C (High-Dose Inoculum)



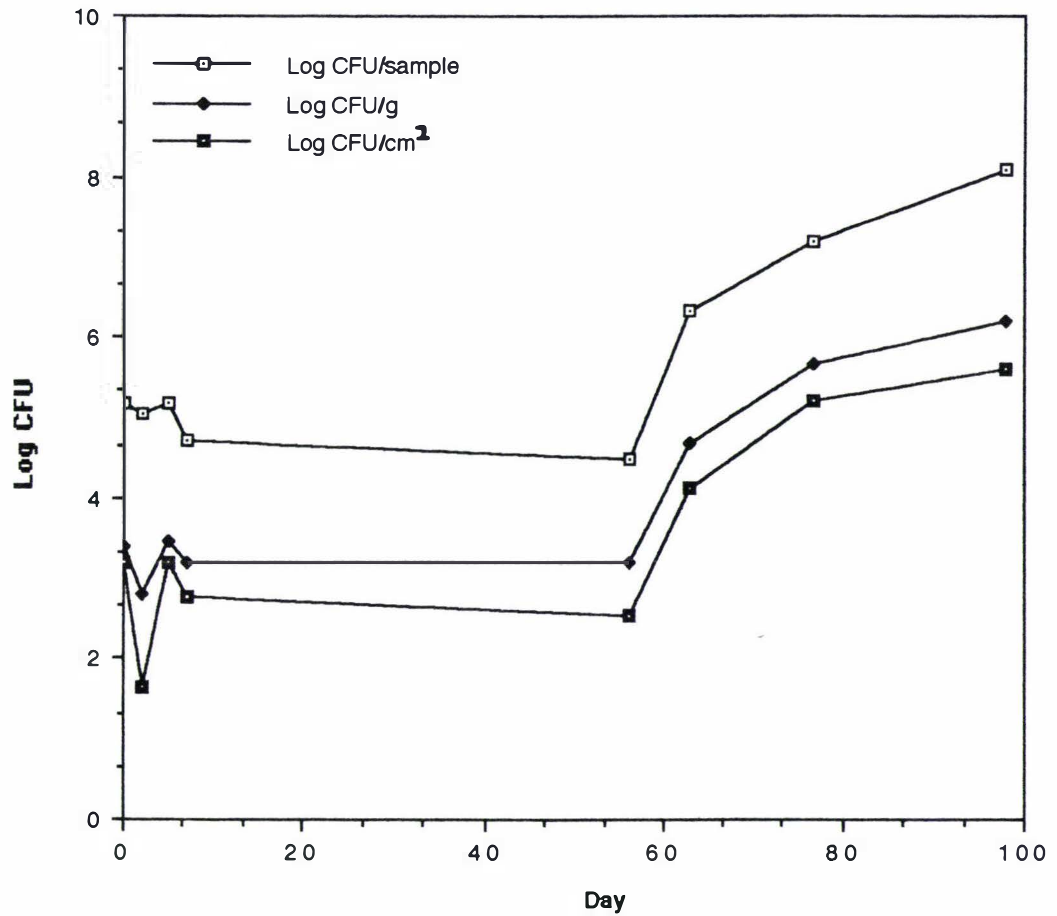
Graph XV. Growth of *Y.pseudotuberculosis* 3 (Yellow 6) in Vacuum-Packed Venison Stored at -1° C for 56 Days and then Stored at +4° C



Graph XVI. Growth of *Y.pseudotuberculosis* 3 (Yellow 6) in Vacuum-Packed Venison Stored at -10° C for 56 Days and then Stored at +4°C



Graph XVII. Growth of *Y.pseudotuberculosis* 3 (Yellow 6) in Vacuum-Packed Venison Stored at -20° C for 56 Days and then Stored at +4°C (Low-Dose Inoculum)



Graph XVIII. Growth of *Y.pseudotuberculosis* 3 (Yellow 6) in Vacuum-Packed Venison Stored at -20° C for 56 Days and then Stored at +4° C (High-Dose Inoculum)

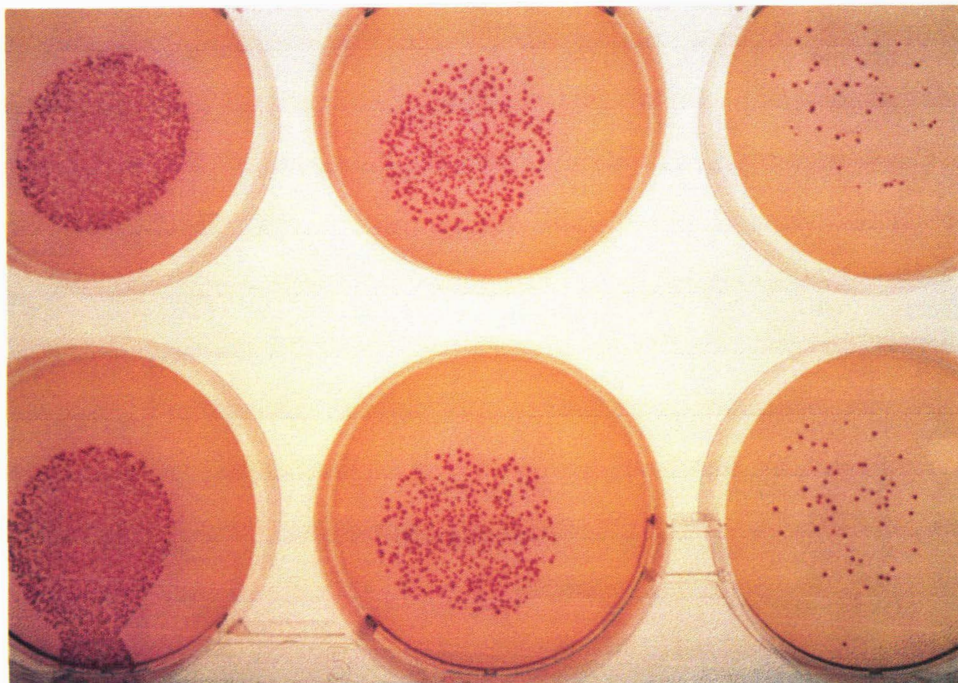


Fig. 9. 1 Enumeration of *Y.pseudotuberculosis* Using the 6-Well Nunc Plates

CHAPTER 10

GENERAL DISCUSSION

Foodborne illness is a major public health concern worldwide (Waites & Arbuthnott, 1990). Pathogenic and potentially pathogenic microorganisms are widely distributed in animals and in foods of animal origin and control of foodborne infection is a formidable undertaking. *Salmonella, Campylobacter, Listeria,* and *Yersinia* are some of the animal enteric pathogens that may be passed to man (Johnston, 1990). Microorganisms found in live animals can be carried through to raw meats after slaughter, may persist through further processing, and ultimately appear in the final retail product if insufficient attention is paid to hygiene and temperature control. Microorganisms may also be transferred to food by the foodhandler either directly or as a result of cross-contamination from hands, surfaces, utensils, and equipment which have not been adequately cleaned and disinfected between the handling of different foods (Roberts, 1990).

The cost of a foodborne disease attack may be as high as $1000 per case which considering the large number of cases means billions of dollars annually for many countries (Todd, 1987). The cost of foodborne illness includes medical expenses, loss of productivity, the "value" of deaths, losses to food suppliers (recall, destruction of the food, extra testing of food samples, reprocessing and repackaging, loss of markets) and legal actions (Todd, 1990).

Y.pseudotuberculosis is ubiquitous (Blake et al., 1991), has a worldwide distribution (Bercovier & Mollaret, 1984) and has been found in numerous animal species including birds and man (Refer to the literature review). Yersiniosis due to *Y.pseudotuberculosis* in man is rare (Bercovier & Mollaret, 1984), but a gradual increase has been suggested based on the more frequent recovery of the organism from food animals (Zen-Yoji et al., 1974). *Y.pseudotuberculosis* infection in man is usually self-limiting without recourse to antibiotics. Those affected and suffering from pseudoappendicular syndrome may undergo unnecessary appendectomies while the elderly or children with blood disorders may suffer from the more fatal septicaemic forms. Those who are immunocompromised or HLA-27 positive are more at risk of developing complications such as arthritis and erythema nodosum. Henshall (1963) believes that due to the relatively common presence of lesions of healed abscesses in the mesenteric lymph nodes detected during routine autopsies, many cases of *Y.pseudotuberculosis* infections pass unnoticed.

The venison industry is a lucrative enterprise with multi-million dollar earnings from exports. The domestic market is also substantial at 350 tonnes per year (Kelly, 1991). The major importers of New Zealand venison, Gennany, the United States of America and Japan, all have considerable yersiniosis problems, which have however, not yet been associated with the consumption of venison.

It is believed that all strains of *Y.pseudotuberculosis* are pathogenic (Mair & Fox, 1986). In addition to these deer also carry potentially pathogenic strains of *Y.enterocolitica* and other related species in their faeces. One of the major findings in this study was the isolation of *Y.enterocolitica* biotype 3 serovar O:5,27 from the faeces of deer presented for slaughter. Human cases occurred in association with the 1978 outbreak of yersiniosis in deer in New Zealand (Anon, 1983).

The roles of the so-called environmental strains comprising *Y.kristensenii, Y.intermedia* and *Y.frederiksenii* in human infection have not been elucidated. Nevertheless, *Y.intermedia*-like organisms have been associated with diarrhoea in children in Nigeria (Agbonlahor, 1986) while some strains of *Y.intermedia, Y.frederiksenii* and *Y.kristensenii* possess some virulence markers (Prpic & Davey, 1985). The production of heat-stable enterotoxin is observed in some strains of *Y.kristensenii* and *Y.frederiksenii* (Kapperud & Langeland, 1981).

The isolation of *Y.rohdei* reported in this study may be the first recorded isolation in deer.

The prevalence of *Y.pseudotuberculosis* in healthy deer has been reported as ranging from 0.13% (Henderson & Hemmingsen, 1983) to 10.7% (Hodges et al., 1984b). These figures are only useful as a guideline because there are several differences in the time of the year the studies were undertaken, age and sex of the animals, and the isolation technique. Nevertheless, out of the 20 *Y.pseudotuberculosis* isolates obtained in this study, only three were recovered from stags. This represents an isolation rate of 1.3% (3/225). Mackintosh & Henderson (1985) whose survey covered 120 stags at a DSP in the Lower South Island reported an isolation rate of 1.2%. Thus, stags 18 months and older appeared to have a relatively low prevalence of *Y.pseudotuberculosis*.

It was found that young hinds are frequently carrying the organism in their faeces. In the May sampling, there were eight isolates of *Y.pseudotuberculosis* out of 30 faecal samples from yearlings, representing about 26.7%. Overall, 17 (85%) of the 20 *Y.pseudotuberculosis* isolates were from hinds. However, it must be taken into account that deer presented for slaughter do not represent a true random sample of the deer population

as a whole (Cannon & Roe, 1982).

There is a marked increase in the number of *Y.pseudotuberculosis* isolates during the late autumn and winter. Overall, 85% of the isolates of *Y.pseudotuberculosis* were recovered during this period. Although other workers have isolated *Y.pseudotuberculosis* in deer more frequently during winter (Mackintosh, 1988 ; Jerrett et al., 1990) it has also been reported with high frequency during spring (Jerrett et al., 1990 ; Slee & Button, 1990). In this case, late autumn represents 42% of the organism isolated. It is therefore appropriate to associate the high isolation rate of *Y.pseudotuberculosis* with the cold months.

Using the autoagglutination, congo-red uptake and calcium dependency tests as virulence markers, all isolates of *Y.pseudotuberculosis* recovered from faeces and carcase meat were found to be pathogenic.

The *Y.pseudotuberculosis* recovered from New Zealand farmed deer have biochemical characteristics similar to strains studied in other countries. Serogroup 3 isolates from faeces and a carcase are non-fermenters of rhamnose and melibiose. The same characteristics were reported for human serogroup 3 isolates in Canada (Toma, 1986), from cattle in Brazil (Falcao, 1987) and Australia (Slee et al., 1988). Melibiose negative strains of serogroup 3 are also reported in swine in Japan (Tsubokura et al., 1984a). The isolate recovered from the clinical case (Yellow 6) is serogroup 3 but differs by being a non-fermenter of xylose. The isolate from the carcase is serogroup 3 but differs in being a non-fermenter of arabinose. It would be interesting to compare the biochemical characteristics of all the *Y.pseudotuberculosis* serogroups isolated from the various specimens and to identify possible associations between these and pathogenicity of the organism.

Serology is an important tool for detecting yersiniosis in man and animals. The isolation of *Y.pseudotuberculosis* from a patient may not be successful but the disease could be confirmed via serology. The macroscopic agglutination test is commonly used for titration of antibody titres. In deer, a titre of 1:40 is considered significant (Mackintosh et al., 1991) while in man, a titre of 1:160 is significant and 1:320 reflects an infection (Attwood et al., 1987). In this study, no sero-positive animals were detected although some animals were found to be shedding the organism in their faeces. There is a limitation to the microplate agglutination test and to improve the specificity and sensitivity of serological test, the ELISA test has been recommended (Griffin, 1988). Heeseman et al., (1987) and Stahlberg et al., (1987) have both used immunoblotting techniques in the detection of yersiniosis in man. The Enzyme-Linked Immunosorbent Assay (EIA) has also been found to be useful in detecting human yersiniosis (Stahlberg et al., 1987).

Retailed venison sausages were found to be contaminated with environmental strains of *Yersiniae* but as sausages were declared to also contain mutton, cereals and preservatives, the source of contamination with *Yersiniae* is obscure.

A multisystem utilizing several isolatory media and enrichment broths has been recommended for a successful recovery of pathogenic and non-pathogenic strains of *Yersiniae* especially from food. The cold enrichment technique involves incubation of samples at +4°C for several days and is supposed to enhance the isolation of *Yersiniae*. The procedure has been found useful in the isolation of *Y.pseudotuberculosis* from faeces (Paterson & Cook, 1963 ; Tsubokura et al., 1973 ; Sato, 1987). The use of Phosphate Buffer Solution (PBS) is recommended due to its non-accummulation of hydrogen peroxide in the presence of injured cells (Kounev, 1989a). This protocol, as applied in this study, has been successful in the isolation of *Y.pseudotuberculosis* from deer carcase swabs.

While the isolation of pathogenic strains of *Yersiniae* is important, the non-isolation of this organism from specimens such as food does not necessarily mean that the organism is not present. The isolation technique may not be sufficiently sensitive. In this respect, the Polymerase Chain Reaction (PCR) may be employed once a known and specific DNA sequence of the organism has been determined. The PCR should be useful in detecting *Y.pseudotuberculosis* and other pathogenic *Yersiniae* from food and meat products where the conventional recovery methods are handicapped. Such an approach is being developed for the detection of pathogenic strains of *Y.enterocolitica* (Wren & Tabaqchali, 1990 ; Fenwick & Murray, 1991 ; Nakajima et al., 1992) and *Y.pseudotuberculosis* (Nakajima et al., 1992).

Y.pseudotuberculosis is a facultative anaerobic, psychrotrophic pathogen and there are reports that this organism grows well under refrigeration temperature (Bercovier & Mollaret, 1984 ; Mair & Fox, 1986). In this study, the results of a cold storage experiment showed that *Y.pseudotuberculosis* grew rapidly under chilled temperatures. A longer lag phase was observed at -1°C. However, when the products were frozen, the organism remained viable for a long period of time with a slow reduction in number of cell. This state of slow decline in viability in the frozen state altered when the samples were shifted to +4°C. *Y.enterocolitica* ATCC 9610 appeared to behave like *Y.pseudotuberculosis* in frozen vacuum-packed venison. Venison has a high iron content (Drew & Seman, 1987) and this compound has been reported to enhance the growth of *Y.pseudotuberculosis* (Boelaert et al., 1987).

This study has provided various information useful for those involved in the deer and venison industries. *Y.pseudotuberculosis* is being carried by apparently healthy deer

and when these are presented for slaughter, faeces have the potential of being an important source of contamination of carcase meat with this organism. Such contamination may have been the source of one isolate of *Y.pseudotuberculosis* serogroup 3 which was recovered from carcase meat. A higher frequency of recovery of *Y.pseudotuberculosis* from faeces was noticed during the late autumn and winter. The risk of carcase contamination is compounded when more animals shedding the bacteria are presented for slaughter, which occurs during the colder months. A contaminated carcase may become a source of contamination to other carcases. As *Y.pseudotuberculosis* is a facultative, anaerobic psychrotroph, the conditions at the Deer Slaughter Premises, in the Packing House, in the vacuum-packs and during storage at low temperatures are conducive to its survival and potential growth.

It is therefore recommended that the deer industry looks at the implications of the findings of this study. It would be easier now to tackle the likely problem of foodborne infection in venison caused by *Y.pseudotuberculosis* or pathogenic *Y.enterocolitica*. Basically, there is a need to maintain a high standard of hygiene during the processing of deer, especially during the cold months when more deer are carrying and shedding the organism. There is also a need to determine the most appropriate method of preventing contamination of carcases prior to deboning and packing.

The introduction of a quality assurance programme for the deer industry is important so that the quality of venison from New Zealand can be guaranteed. This study provides useful information on the situation of *Yersiniae* in deer and venison which may be considered in the design of such a quality assurance programme.

List of Appendices

Appendix I. : Media and Media Preparation for the Isolation, Identification, Virulence Marker Tests and Storage of *Yersinia spp*.

Appendix IIA. : Biochemical Characteristics of *Yersinia* Isolated from Faeces and Carcase of Deer Presented for Slaughter

Appendix IIB. : Biochemical Characteristics of *Yersinia* Isolated from Retailed Venison Sausages

Appendix IIIA. : Catalogue of *Yersinia spp*. (01-66) Isolated from Faeces and Carcase of Deer Presented for Slaughter at the DSP Feilding and from Venison Sausages Purchased from Local Supermarkets, Palmerston North

Appendix IIIB. : Catalogue of *Yersinia spp*. (01-74) Isolated from Faeces of armed Red Deer Presented for Slaughter at the DSP, Feilding

Appendix IV. : Results of Growth Experiments of*Y.pseudotuberculosis* in Vacuum-Packed Venison Stored at Chilling and Freezing Temperatures, and of *Y.enterocolitica* in Vacuum-Packed Venison at Freezing Temperature

APPENDIX I

PREPARATION OF MEDIA FOR THE ISOLATION, IDENTIFICATION, VIRULENCE MARKER TESTS AND STORAGE OF *YERSINIA* ISOLATES

1. Cefsulodin-Irgasan-Novobiocin (CIN) Agar

a. *Yersinia* Selective Agar (DIFCO) 59.5 g
   Distilled water 1.0 litre
b. Dissolve by heating
c. Adjust pH to 7.4 ± 2
d. Place in two 500 ml bottles
e. Autoclave for 15 minutes at 121°C
f. Cool to 45 - 50°C
g. Resuspend *Yersinia* Antibiotic supplement Cefsulodin Novobiocin in 4 ml sterile distilled water
h. Dispense half the amount of antibiotic to each of the 500 ml agar base
i. Mix thoroughly and pour 15-20 ml amount into each petri dish
j. Allow to cool and dry before storing at +4°C
   k.Recommended to be used within two weeks (Petersen, 1985)

2. Blood Agar

i. Salt Base

a. Bacto Agar (DIFCO) 15.0 g
   Sodium Chloride 5.0 g
   Distilled water 1.0 litre
b. Dissolve by heating
c. Pour into 400 ml bottles
d. Autoclave for 15 minutes at 121°C
e. Allow to cool to 45 - 50°C
   f.Pour 10 ml amount into each petri dish and allow to cool and dry

ii. Blood Base

a. Columbia agar (DIFCO) 44.0 g
   Distilled water 1.0 litre
b. Dissolve by heating
c. Autoclave for 15 minutes at 121°C
d. Cool to 45 - 50°C
e. Add 5% v/v of Sheep blood
f. Pour 10 - 15 ml amount over the salt base
g. Allow to cool and dry, and store at +4°C

3. Lysine-Arginine-Iron Agar (LAIA) Slant Weagant, 1983)

a.	Lysine Iron Agar (DIFCO)	34.5 g
	L-Arginine (Sigma)	10.0 g
	Distilled water	1.0 litre
b.	Dissolve by heating	
c.	Dispense in 6 ml amount into screw-capped plastic tube	
d.	Autoclave for 15 minutes at 121°C	
e.	Cool in a slanting position	
f.	Store at +4°C	

4. Triple-Sugar-Iron Agar (TSIA) Slant

a.	Triple Sugar Iron Agar (DIFCO)	65.0 g
	Distilled water	1.0 litre
b.	Dissolve by heating	
c.	Dispensed in 6 ml amount into screw-capped plastic tube	
d.	Autoclave for 15 minutes at 121°C	
e.	Cool in a slanting position	
f.	Store at +4°C	

5. Urea Agar Slope

i. Agar Base

a.	Bacto Agar (DIFCO)	15.0 g
	Distilled water	1.0 litre
b.	Dissolve by heating	
c.	Dispense into 200 ml bottles	
d.	Autoclave for 15 minutes at 121°C	
e.	Cool to 45 - 50°C	

ii. Urea Base

a.	Bacto Urea agar base	29.0 g
	Distilled water	100.0 ml
b.	Mix to dissolve	
c.	Filter sterilise	
d.	Store at +4°C	

iii. Add aseptically 10% (v/v) or 20 ml of Bacto Urea Agar to 200 ml Urea agar base, mix thoroughly but gently. Dispense 3.0 ml amount into each sterile bijoux bottles and cool them in a slanting position. Store at +4 C.

6. Phosphate Buffer Solution (PBS) pH 7.6

i. Solution A

a.	Potassium dihydrogen phosphate (KH_2PO_4)	9.07 g
	Distilled water	1.0 litre

ii. Solution B

a.	Disodium hydrogen phosphate ($Na_2HPO_4.2H_20$)	11.87 g
	Distilled water	1.0 litre

iii. Mix 128 ml Solution A to 872 ml Solution B

iv. Add 9.0 g Sodium Chloride

v. Place in 500 ml bottles and autoclave for 15 minutes at 121 C

vi. When cooled, dispense aseptically in 10 ml amount into sterile plastic container

vii. or dispense in 120 ml amount in McCartney's bottles and autoclave for 15 minutes at 121 C

viii. Store at +4°C

7 Phosphate Buffer Peptone Water (pH 7.2)

a.	Peptone (DIFCO)	10.0 g
	Sodium chloride	5.0 g
	Disodium hydrogen phosphate	1.5 g
	Potassium dihydrogen phosphate	3.5 g
	Distilled water	1.0 litre

b. Adjust pH to 7.2

c. Autoclave for 15 minutes at 121°C

d. Store at +4°C

8. Recovery diluent (0.1% Peptone Saline)

a.	Peptone (DIFCO)	1.0 g
	Sodium Chloride	8.5 g
	Distilled water	1.0 litre

b. Autoclave for 15 minutes at 121°C

c. Store at +4°C

9. Tryptone Water

a.	Oxoid Tryptone (DIFCO)	20.0 g
	Distilled water	1.0 litre

b. Dissolve by gentle heating

c. Adjust pH to 7.2 - 7.4

d. Dispense in 3 ml amount in bijoux bottles

e. Autoclave for 15 minutes at 121°C

f. Store at +4°C

10. Decarboxylase Test Broth

a.	Moeller's decarboxylase broth media	10.5 g
	Distilled water	1.0 litre
b.	Dissolve by gentle heating	
c.	Add 1% L-Arginine (Sigma) or	3.0 g
	1% L-Lysine (Sigma) or	3.0 g
	1% L-Ornithine (Sigma)	3.0 g
d.	Adjust pH to 6.0	
e.	Dispense in 3 ml amount in bijoux bottles	
f.	Autoclave for 15 minutes at 121°C	
g.	Store at +4°C	

11. Aesculin Hydrolysis

a.	Aesculin (DIFCO)	1.0 g
	Ferric Iron Citrate	0.5 g
	Heart Infusion broth (DIFCO)	25.0 g
	Bacto agar (DIFCO)	15.0 g
	Distilled water	1.0 litre
b.	Dissolve by gentle heating	
c.	Dispense in 3 ml amount in bijoux bottles	
d.	Autoclave for 15 minutes at 121°C	
e.	Cool in a slanting position	

12. Methyl-Red Voges-Proskauer

a.	M.R.V.P. (Merck)	17.0 g
	Distilled water	1.0 litre
b.	Dissolve by shaking	
c.	Dispense in 3 ml amount into bijoux bottles	
d.	Autoclave for 15 minutes at 121°C	
e.	Store at +4°C	

13. Malonate broth

a.	Malonate broth (DIFCO)	8.0 g
	Distilled water	1.0 litre
b.	Dissolve by shaking	
c.	Dispense in 3 ml amount in bijoux bottles	

d.	Autoclave for 15 minutes at 121°C	
e.	Store at +4°C	

14. Simmon's Citrate Agar

a.	Simmon's citrate agar (DIFCO)	24.2 g
	Distilled water	1.0 litre
b.	Dissolve by heating	
c.	Dispense in 3 ml amount in bijou bottles	
d.	Autoclave for 15 minutes at 121°C	
e.	Store at +4°C	

15. Sugars

a.	Peptone (DIFCO)	10.0 g
	Meat Extract (GIBCO BRL SCOTLAND)	3.0 g
	Sodium Chloride	5.0 g
	Distilled water	1.0 litre
	Andrades solution	10.0 ml
b.	Dissolve and adjust pH to 7.2	
c.	Dispense in 200 ml amount	
d.	Autoclave for 15 minutes at 121°C	
e.	Add 20 ml (10%) Seitz filtered carbohydrates*	
f.	Dispense aseptically in 3 ml amount into bijoux bottles	
g.	Store at +4°C	

* comprise of sucrose, trehalose, rhamnose, melibiose, raffinose, alpha-methyl-D-glucoside, sorbose, sorbitol, cellobiose, maltose, xylose, adonitol, arabinose, salicin and lactose.

16. Tryptic Soy Broth

a.	Tryptic Soy broth (DIFCO)	30.0 g
	Distilled water	1.0 litre
b.	Dissolve by shaking	
c.	Add 15% (v/v) glycerol	150.0 ml
d.	Dispense in 3 ml amount in bijoux bottles	
e.	Autoclave for 15 minutes at 121°C	
f.	Store at +4°C	

17. Trypticase Soy Agar

a.	Trypticase Soy Agar (DIFCO)	40.0 g
	Distilled water	1.0 litre
b.	Dissolve by heating	
c.	Autoclave for 15 minutes at 121°C	
d.	Cool to 45 - 50°C	
e.	Pour 15 - 20 ml amount into petri dish	
f.	Store at +4°C	

18. Autoagglutination test

a.	Modified Minimum Essential Medium Eagle	9.72 g
	Distilled water	1.0 litre
b.	Mix for 30 minutes	
c.	Add Sodium bicarbonate	1.0 g
d.	Adjust pH to 7.0 - 7.5	
e.	Filter sterilise	
f.	Add Bovine Foetal serum (10%)	100.0 ml
g.	Store in 200 ml amount at +4°C	

19. Congo-Red Magnesium Oxalate Agar (CRMOX)

a.	Tryptic Soy agar	40.0 g
	Distilled water	1.0 litre
b.	Autoclave for 15 minutes at 121°C	
c.	Cool to 55°C	
d.	Add 0.25 M Magnesium Chloride (sterile)	80.0 ml
	0.25 M Sodium Oxalate (sterile)	80.0 ml
	20% D-galactose (filter sterile)	10.0 ml
	1% Congo-red (sterile)	5.0 ml
e.	Mix and dispense 15 - 20 ml amount into each petri dish	
f.	Allow to cool, dry and store at +4°C	

APPENDIX IIA

Biochemical characteristics of *Yersinia* isolated from faeces and carcase of deer presented for slaughter at the DSP, Feilding.

Species		*Y.pstb*	*Y.pstb*	*Y.ent.*	*Y.ent.*	*Y.ent.*	*Y.ent*
Biotype/Serotype		2	3	0:5,27	B3	B1A	B5
Number of isolates		8	12	2	2	4	1
		%+ve	%+ve	%+ve	%+ve	%+ve	%+ve
Urea hydrolysis		100	100	100	100	100	100
Motility	28°C	100	100	100	100	100	100
	37°C	0	0	0	0	0	0
Methyl Red	28°C	100	100	0	100	75	0
Voges Proskauer	28°C	0	0	100	100	100	0
	37°C	0	0	0	0	0	0
Ornithine decarboxylase		0	0	100	100	100	0
Simmon's citrate		0	0	0	0	25	0
Indole		0	0	0	0	100	0
Sucrose		0	0	100	100	100	100
Trehalose		100	100	100	100	100	0
Rhamnose		100	0	0	0	0	0
Melibiose		100	0	0	0	0	0
Raffinose		0	0	0	0	0	0
Alpha-methyl-D-glucoside		0	0	0	0	0	0
Sorbose		0	0	100	100	nc	100
Sorbitol		13	17	100	100	100	100
Cellobiose		0	0	100	100	100	100
Maltose		100	100	100	100	100	0
Xylose		100	100	100	100	100	0
Adonitol		0	0	0	0	0	0
Arabinose		100	92	100	100	100	100
Salicin		75	8	0	0	100	0
Lactose		0	0	0	0	nc	0
Malonate		0	0	0	0	0	0
Lysine decarboxylase		0	0	0	0	0	0
Arginine dihydrolase		0	0	0	0	0	0
Aesculin hydrolysis		100	100	0	0	100	0

nc - not completed

All tests done at 28°C unless specified.

APPENDIX IIA (Contd)

Species		*Y.krist*	*Y.inter*	*Y.fred*	*Y.roh*
Biotype/Serotype					
Number of isolates		13	2	74	2
Biochemical test		%+ve	%+ve	%+ve	+ve
Urea hydrolysis		100	100	100	100
Motility	28°C	100	100	100	100
	37°C	0	0	0	0
Methyl red	28°C	100	100	97	100
Voges Proskauer	28°C	0	100	27	0
	37°C	0	0	0	0
Ornithine decarboxylase		100	100	100	100
Simmon's citrate		0	0	9	0
Indole		8	100	100	100
Sucrose		0	100	100	100
Trehalose		100	100	100	100
Rhamnose		0	100	100	0
Melibiose		0	100	0	100
Raffinose		0	100	3	100
Alpha-Methyl-D-glucoside		0	50	0	0
Sorbose		100	100	100	0
Sorbitol		100	100	100	100
Cellobiose		100	100	100	100
Maltose		100	100	100	100
Xylose		92	100	100	100
Adonitol		0	0	0	0
Arabinose		100	100	100	100
Salicin		0	100	100	0
Lactose		0	0	8	0
Malonate		0	0	0	0
Lysine decasboxylase		0	0	0	0
Arginine dihydrolase		0	0	0	0
Aesculin hydrolysis		0	100	100	0

All tests done at 28°C unless specified.

APPENDIX IIB

Biochemical characteristics of *Yersinia* isolated from retailed venison sausages purchased in supermarkets, Palmerston North.

Species		*Y.ent*	*Y.krist*	*Y.inter*
Biotype/Serotype		B1A		
Number of isolates		8	1	4
Biochemical tests		%+ve	%+ve	%+ve
Urea hydrolysis		100	100	100
Motility	28°C	100	100	100
	37°C	0	0	0
Methyl Red	28°C	75	100	100
Voges Proskauer	28°C	100	0	100
	37°C	0	0	0
Ornithine decarboxylase		100	100	100
Simmon's citrate		0	0	25
Indole		100	0	0
Sucrose		100	0	100
Trehalose		100	100	100
Rhamnose		0	0	100
Melibiose		0	0	100
Raffinose		0	0	100
Alpha-methyl-D-glucoside		0	0	100
Sorbose		100	100	nc
Sorbitol		100	100	100
Cellobiose		100	100	100
Maltose		100	100	100
Xylose		100	100	100
Adonitol		0	0	0
Arabinose		100	100	100
Salicin		100	0	100
Lactose		nc	100	nc
Lysine decarboxylase		0	0	0
Arginine dihydrolase		0	0	0
Aesculin hydrolysis		100	0	100

nc - not completed

All tests done at 28°C unless specified.

APPENDIX III (A)

CATALOGUE OF *YERSINIA* SPECIES (01-66) ISOLATED FROM FAECES AND CARCASE OF DEER PRESENTED FOR SLAUGHTER AT THE DSP FEILDING AND FROM VENISON SAUSAGES PURCHASED FROM LOCAL SUPERMARKETS, PALMERSTON NORTH.

No. I.D.	Species	No. I.D.	Species
01. FF39/6	*Y.pstb* 3	36. FF52/10	*Y.krist*
02. FF43/6	"	37. FF90/10	"
03. FF49/6	"	38. FF91/10	"
04. FF59/6	"	39. FF93/10	"
05. FF37/7	*Y.pstb* 2	40. FF79b/12	"
06. FF72/7	*Y.pstb* 3	41. FF66/04	"
07. FF73/7	"	42. FF79/05	"
08. FF74/7	"	43. FF61b/12	*Y.inter*
09. FF80/7	"	44. FF41/02	"
10. FF85/10	"	45. FF39/02	*Y.rohdei*
11. FF69/01	"	46. FF40/02	"
12. FF64/05	"	47. FF63/9(2)	Untyped (UT)
13. HC67/05	"	48. FF73B/10	"
14. FF73a/05	*Y.pstb* 2	49. FF36/11	"
15. FF74a/05	"	50. FF62b/12	"
16. FF75/05	"	51. FF53/01	"
17. FF76/05	"	52. FF71/01	"
18. FF77/05	"	53. FF69/05	"
19. FF78a/05	"	54. VS8/1/3	*Y.ent*.B1A
20. FF80a/05	"	55. VS8/2/2	"
21. FF63/6	*Y.ent* B5	56. VS8/2/6	"
22. FF46/8	*Y.ent* B3	57. VS4/4	"
23. FF51/8	"	58. VS5/2	"
24. FF54/8	" 0:5,27	59. VS5/2/5	"
25. FF65/10	" "	60. VS10/1/6	"
26. FF59/11	" B1A	61. VS/11/2/4	"
27. FF63/11	" "	62. VS10/1/3	*Y.krist*
28. FF46b/12	"	63. VS4/3	*Y.inter*
29. FF81b/12	"	64. VS5/4/2	"
30. FF70/8	*Y.krist*	65. VS7/2/1	"
31. FF42/9(2)	"	66. VS7/2/4	"
32. FF57/9(1)	"		
33. FF62/9(1)	"		
34. FF64/9(2)	"		
35. FF97/9(2)	"		

Keys:

FF - Feilding Faeces : HC - Hasting Carcase : VS - Venison Sausage

APPENDIX IIIB

CATALOGUE OF *YERSINIA* (01-74) ISOLATED FROM FAECES OF FARMED RED DEER PRESENTED FOR SLAUGHTER AT THE DSP, FEILDING.

No.	I.D.	Species	No.	I.D.	Species
01.	FF50/8	*Y. fred*	48.	FF47L/01	*Y.fred*
02.	FF53/8	"	49.	FF69/02	"
03.	FF56/8	"	50.	FF70s/02	"
04.	FF61/8	"	51.	FF71/02	"
05.	FF66/8	"	52.	FF72s/02	"
06.	FF72/8	"	53.	FF90/03	"
07.	FF41/9(2)	"	54.	FF92/03	"
08.	FF43/9(2)	"	55.	FF62/8	*Y.fred**
09.	FF49/9	"	56.	FF64/8	"
10.	FF50/9(2)	"	57.	FF67/8	"
11.	FF51/9(2)	"	58.	FF40/9(1)	"
12.	FF56/9	"	59.	FF42/9(1)	"
13.	FF57/9(2)	"	60.	FF84/9(2)	"
14.	FF58/9(2)	"	61.	FF54b/12	"
15.	FF62/9(2)	"	62.	FF42s/02	"
16.	FF63/9(1)	"	63.	FF91/03	"
17.	FF64/9(1)	"	64.	FF95s/03	"
18.	FF65/9	"	65.	FF107/03	"
19.	FF89/9	"	66.	FF56/04	"
20.	FF90/9	"	67.	FF69b/04	"
21.	FF95/9	"	68.	FF70/04	"
22.	FF97/9(1)	"	69.	FF83/04	"
23.	FF98/9(1)	"	70.	FF84/04	"
24.	FF50/10	"	71.	FF85/04	"
25.	FF73A/10	"	72.	FF86/04	"
26.	FF34/11	"	73.	FF87/04	"
27.	FF38/11	"	74.	FF88/04	"
28.	FF39/11	"	75.	FF52/11	U.T
30.	FF51/11	"	76.	FF79s/12	"
31.	FF52/11	"	77.	FF60/01	"
32.	FF53/11	"			
33.	FF56/11	"			
34.	FF57/11	"			
35.	FF45s/12	"			
36.	FF46s/12	"			
37.	FF48/12	"			
38.	FF50/12	"			
39.	FF51/12	"			
40.	FF52s/12	"			
41.	FF54s/12	"			
42.	FF61s/12	"			
43.	FF62s/12	"			
44.	FF63s/12	"			
45.	FF65s/12	"			
46.	FF77s/12	"			
47.	FF80s/12	"			

Keys

Y.fred - *Y.frederiksenii* (01-54)
V.P -ve at 28°C & 37° (01-54)

*Y.fred** - *Y.frederiksenii* (55-74)
V.P +ve at 28° & -ve at 37°

U.T. - untyped

APPENDIX IV

RESULTS OF GROWTH EXPERIMENTS OF *Y.PSEUDOTUBERCULOSIS* IN VACUUM-PACKED VENISON STORED AT CHILLING AND FREEZING TEMPERATURES, AND OF *Y.ENTEROCOLITICA* IN VACUUM-PACKED VENISON AT FREEZING TEMPERATURE.

1.Growth of *Y.pstb* 3 (Yellow 6) in vacuum-packed venison stored at +10°C

	Log_{10} CFU per		
Day	sample	gram	cm^2
0	3.50	1.46	1.35
9	6.45	4.46	4.18
18	6.20	4.19	3.71
28*	ND	-	-

* spoilage detected   ND - not detected

2.Growth of *Y.pstb* 3 (Yellow 6) in vacuum-packed venison stored at +4°C

	Log_{10} CFU per		
Day	sample	gram	cm^2
0	3.50	1.46	1.35
9	4.10	1.96	1.44
18	5.33	3.30	3.13
28	7.32	5.31	5.13
36	6.70	4.64	4.29
45	5.50	3.47	3.17
53*	ND	-	-

* spoilage detected   ND - not detected

3. Growth of *Y.pstb* 3 (Yellow 6) in vacuum-packed Pre-heated venison stored at +4°C

	Log_{10} CFU per		
Day	sample	gram	cm^2
0	3.30	1.67	1.34
	3.10	1.62	0.92
	(3.20)	(1.65)	(1.13)
7	4.37	2.86	2.27
	4.27	2.47	2.09
	(4.32)	(2.67)	(2.18)
11	3.50	2.16	1.44
	4.15	2.33	2.13
	(3.83)	(2.25)	(1.29)
21	5.40	3.72	3.32
	5.81	4.03	3.63
	(5.61)	(3.88)	(3.48)

28	5.50	4.11	3.30
	5.50	4.10	3.37
	(5.50)	(4.11)	(3.34)
35	7.10	5.22	4.74
	6.10	4.19	3.73
	(6.60)	(4.71)	(4.24)
42	ND	-	-
	7.60	6.13	5.41
	(7.60)	(6.13)	(5.41)
49	ND	-	-
	7.15	5.33	5.10
	(7.15)	(5.33)	(5.10)
56	6.38	4.84	4.30
	6.20	4.48	4.15
	(6.29)	(4.66)	(4.23)
63	ND	-	-
	8.74	7.19	6.53
	(8.74)	(7.19)	(6.53)

ND - not detected ( ) - mean

4. Growth of *Y.pstb* (FF85/10) in vacuum-packed venison stored at - 1°C

	Log_{10} CFU per		
Day	sample	gram	cm^2
0	3.30	1.67	1.34
	3.10	1.62	0.92
	(3.20)	(1.65)	(1.13)
7	4.37	2.86	2.27
	4.27	2.47	2.09
	(4.32)	(2.67)	(2.18)
11	3.50	2.16	1.44
	4.15	2.33	2.13
	(3.83)	(2.25)	(1.79)
21	5.40	3.72	3.32
	5.81	4.03	3.63
	(5.61)	(3.88)	(3.48)
28	5.50	4.11	3.30
	5.50	4.10	3.37
	(5.50)	(4.11)	(3.34)
35	7.10	5.22	4.74
	6.10	4.19	3.73
	(6.60)	(4.71)	(4.24)
42	ND	-	-
	7.60	6.13	5.41
	(7.60)	(6.13)	(5.41)
49	ND	-	-
	7.15	5.33	5.10
	(7.15)	(5.33)	(5.10)
56	6.38	4.84	4.30
	6.20	4.48	4.15
	(6.29)	(4.66)	(4.23)
63	ND	-	-
	8.74	7.19	6.53
	(8.74)	(7.19)	(6.53)

ND - not detected ( ) - mean

5. Growth of *Y.pstb* 3 (Yellow 6) in vacuum-packed venison stored at -1°C

	Log$_{10}$ CFU per		
Day	sample	gram	cm^2
0	3.10	1.31	1.12
	3.15	1.35	1.17
	(3.13)	(1.33)	(1.15)
7	3.10	1.20	0.74
	2.50	0.68	0.30
	(2.80)	(0.94)	(0.52)
14	ND	-	-
	2.50	1.10	0.32
	(2.50)	(1.10)	(0.32)
20	ND	-	-
	2.67	0.94	0.60
	(2.67)	(0.94)	(0.60)
28	2.50	0.93	0.35
	3.15	1.24	0.99
	(2.83)	(1.09)	(0.67)
42	ND	-	-
	4.43	2.94	2.31
	(4.43)	(2.94)	(2.31)
56	ND	-	-
	7.11	5.22	4.63
	(7.11)	(5.22)	(4.63)
71	ND	-	-
	7.14	5.33	4.88
	(7.14)	(5.33)	(4.88)
85	ND	-	-
	5.50	4.10	3.23
	(5.50)	(4.10)	(3.23)
99	ND	-	-
	ND	-	-
106	ND	-	-
	6.33	4.63	4.22
	(6.33)	(4.63)	(4.22)

ND - not detected ( ) - mean

6. Growth of *Y.pstb* 3 (FF85/10) in vacuum-packed venison stored at -1°C

	Log$_{10}$ CFU per		
Day	sample	gram	cm^2
0	3.30	1.67	1.34
	3.10	1.62	0.92
	(3.20)	(1.65)	(1.13)
14	ND	-	-
	3.33	1.59	1.24
	(3.33)	(1.59)	(1.24)
28	ND	-	-
	5.95	4.20	3.75
	(5.95)	(4.20)	(3.75)
42	ND	-	-
	ND	-	-
49	ND	-	-

	6.68	5.14	4.51
	(6.68)	(5.14)	(4.51)
63	ND	-	-
	5.33	3.59	3.28
	(5.33)	(3.59)	(3.28)

ND - not detected ( ) - mean

7. Growth of *Y.pstb* 3 (Yellow 6) in vacuum-packed venison stored at -10°C (low-dose inoculum)

	Log$_{10}$ CFU per		
Day	sample	gram	cm^2
0	3.50	1.51	1.35
4	4.35	2.45	2.18
7	3.45	1.64	1.23
18	3.30	1.33	1.14
55	3.35	1.41	1.15
88	3.20	1.21	0.87
264	2.50	0.56	0.22

8. Growth of *Y.pstb* 3 (Yellow 6) in vacuum-packed venison stored at -1°C (high-dose inoculum)

	Log$_{10}$ CFU per		
Day	sample	gram	cm^2
0	4.40	2.91	2.59
2	5.17	3.35	3.17
4	5.23	3.52	3.21
7	5.13	3.46	3.16
18	5.11	3.25	3.12
55	5.03	2.60	2.26
264	3.35	1.67	1.19

9. Growth of *Y.pstb* 3 (Yellow 6) in vacuum-packed venison stored at -13°C ± 2 (low-dose inoculum)

	Log$_{10}$ CFU per		
Day	sample	gram	cm^2
0	3.50	1.51	1.35
4	4.30	2.35	2.14
7	3.30	1.35	1.13
18	3.50	1.56	1.17
55	3.15	1.17	0.62

10. Growth of *Y.pstb* 3 (Yellow 6) in vacuum-packed venison stored at -13°C ± 2 (high-dose inoculum)

	Log$_{10}$ CFU per		
Day	sample	gram	cm^2
0	4.40	2.91	2.59
2	5.24	3.65	3.33
4	5.25	3.61	3.19
7	4.85	3.34	2.87

18	4.70	3.23	3.10
55	5.16	3.57	3.14
88	4.69	3.15	2.60

11. Growth of *Y.pstb* 3 (Yellow 6) in vacuum-packed venison stored at -20°C (low-dose inoculum)

	Log_{10} CFU per		
Day	sample	gram	cm^2
0	4.14	2.25	2.11
	4.26	2.48	2.21
	(4.20)	(2.37)	(2.16)
14	3.63	2.11	1.44
	3.63	2.12	1.48
	(3.63)	(2.12)	(1.46)
28	3.43	1.96	1.35
	4.30	2.50	2.21
	(3.87)	(2.23)	(1.78)
43	3.55	1.86	1.47
	3.50	1.91	1.38
	(3.52)	(1.89)	(1.43)
56	3.60	2.11	1.43
	3.50	1.90	1.41
	(3.55)	(2.01)	(1.42)
70	3.30	1.57	1.23
	3.88	2.15	1.85
	(3.59)	(1.86)	(1.54)

( ) - mean

12. Growth of *Y.pstb* 3 (Yellow 6) in vacuum-packed venison stored at -20°C (high-dose inoculum)

	Log_{10} CFU per		
Day	sample	gram	cm^2
0	6.10	4.18	3.89
	6.11	4.20	3.95
	(6.11)	(4.19)	(3.92)
14	5.81	4.14	3.66
	5.25	3.45	3.18
	(5.53)	(3.80)	(3.42)
28	5.63	4.14	3.66
	5.92	4.17	3.64
	(5.78)	(4.16)	3.65)
43	5.63	4.11	3.42
	5.50	3.91	3.33
	(5.57)	(4.01)	(3.38)
56	5.46	3.77	3.42
	5.54	4.10	3.42
	(5.50)	(3.94)	(3.42)
70	4.23	2.36	2.14
	4.26	2.45	2.17
	(4.25)	(2.41)	2.16)

13. Growth of *Y.enterocolitica* (ATCC 9610) in vacuum-packed venison stored at -20°C (low-dose inoculum)

	Log_{10} CFU per		
Day	sample	gram	cm^2
0	ND	-	-
	3.10	1.19	0.80
	(3.10)	(1.19)	(0.80)
14	ND	-	-
	3.10	1.17	0.75
	(3.10)	(1.17)	(0.75)
28	ND	-	-
	2.50	0.81	0.36
	(2.50)	(0.81)	(0.36)
43	ND	-	-
	2.50	0.81	0.36
	(2.50)	(0.81)	(0.36)
56	ND	-	-
	ND	-	-
70	2.50	0.84	0.36
	2.50	0.85	0.43
	(2.50)	(0.85)	(0.40)

ND - not detected ( ) - mean

14. Growth of *Y.enterocolitica* (ATCC 9610) in vacuum-packed venison stored at -20°C (high-dose inoculum)

	Log_{10} CFU per		
Day	sample	gram	cm^2
0	5.10	3.18	2.93
	4.53	2.92	2.44
	(4.82)	(3.05)	(2.69)
14	4.15	2.28	2.13
	4.24	2.42	2.20
	(4.20)	(2.35)	(2.17)
28	3.45	1.83	1.31
	3.85	2.17	2.12
	(3.65)	(2.00)	(1.72)
43	3.85	2.13	1.83
	3.85	2.15	1.68
	(3.85)	(2.14)	(1.76)
56	4.29	2.56	2.25
	3.84	2.16	1.67
	(3.65)	(2.36)	2.12
	3.83	2.15	1.69
	(3.98)	(2.19)	(1.91)

( ) - mean

15. Growth of *Y.pstb* 3 (Yellow 6) in vacuum-packed venison stored at -1°C for 8 weeks and then stored at +4°C

	Log10 CFU per		
Day	sample	gram	cm^2
0	3.10	1.31	1.12
	3.15	1.35	1.17
	(3.13)	(1.33)	(1.15)
7	3.10	1.20	0.74
	2.50	0.68	0.30
	(2.80)	(0.94)	(0.52)
14	ND	-	-
	2.50	1.10	0.32
	(2.50)	(1.10)	(0.32)
20	ND	-	-
	2.67	0.94	0.60
	(2.67)	(0.94)	(0.60)
28	2.50	0.93	0.35
	3.15	1.24	0.99
	(2.83)	(1.09)	(0.67)
42	ND	-	-
	4.43	2.94	2.31
	(4.43)	(2.94)	(2.31)
56	ND	-	-
	7.11	5.22	4.63
	(7.11)	(5.22)	(4.63)
***			
7	ND	-	-
	6.20	4.39	4.12
	(6.20)	(4.39)	(4.12)
14	ND	-	-
	7.15	5.30	4.84
	(7.15)	(5.30)	(4.84)
21	6.56	4.94	4.31
	5.50	3.88	3.27
	(6.03)	(4.41)	(3.79)
28	8.10	6.22	5.64
	7.45	6.14	5.41
	(7.78)	(6.18)	(5.53)
35	ND	-	-
	6.50	5.10	4.28
	(6.50)	(5.10)	(4.28)

ND - not detected ( ) - mean

*********** storage at +4°C

16. Growth of *Y.pstb* 3 (Yellow 6) in vacuum-packed venison stored at -10°C for 8 weeks and then stored at +4°C

	Log$_{10}$ CFU per		
Day	sample	gram	cm^2
0	3.10	1.31	1.12
	3.15	1.47	1.17
	(3.13)	(1.39)	(1.15)
7	ND	-	-
	2.50	1.14	0.52
	(2.50)	(1.14)	(0.52)
56	ND	-	-
	2.50	1.11	0.52
	(2.50)	(1.11)	(0.52)
***			
7	ND	-	-
	ND	-	-
14	ND	-	-
	ND	-	-
21	ND	-	-
	3.98	2.17	1.95
	(3.98)	(2.17)	(1.95)
28	ND	-	-
	ND	-	-
35	ND	-	-
	6.50	(4.47)	(4.23)

ND - not detected ( ) - mean

********* storage at +4°C

17. Growth of *Y.pstb* 3 (Yellow 6) in vacuum-packed venison stored at -20°C for 8 weeks and then stored at +4°C (low-dose inoculum)

	Log$_{10}$ CFU per		
Day	sample	gram	cm^2
0	3.10	1.31	1.12
	3.15	1.35	1.17
	(3.13)	(1.39)	(1.15)
2	2.50	1.10	0.52
	2.50	0.98	0.56
	(2.50)	(1.04)	(0.54)
5	2.25	0.48	0.18
	3.33	1.83	1.25
	(2.79)	(1.16)	(0.72)
7	3.15	1.32	1.12
	3.15	1.50	1.15
	(3.15)	(1.41)	(1.14)
14	2.50	0.94	0.42
	2.50	1.12	0.44
	(2.50)	(1.03)	(0.43)
56	ND	-	-
	2.50	0.98	0.32
	(2.50)	(0.98)	(0.32)
***			

7	ND	-	-
	ND	-	-
35	7.83	6.17	5.55
	7.17	5.31	5.11
	(7.50)	(5.74)	(5.33)

ND - not detected ( ) - mean

********** storage at +4°C

18. Growth of *Y.pstb* 3 (Yellow 6) in vacuum-packed venison stored at -20°C for 8 weeks and then stored at +4°C (high-dose inoculum)

	Log_{10} CFU per		
Day	sample	gram	cm^2
0	5.25	3.53	3.23
	5.13	3.29	3.16
	(5.19)	(3.41)	(3.20)
2	5.10	3.23	1.11
	5.02	2.41	2.17
	(5.06)	(2.82)	(1.64)
5	5.22	3.56	3.23
	5.13	3.33	3.14
	(5.18)	(3.45)	(3.19)
7	4.94	3.29	3.10
	4.51	3.10	2.41
	(4.73)	(3.20)	(2.76)
56	4.60	3.27	2.69
	4.40	3.11	2.40
	(4.50)	(3.19)	(2.55)
**			
7	7.55	6.16	5.40
	5.12	3.20	2.87
	(6.34)	(4.68)	(4.14)
14	ND	-	-
	7.20	5.69	5.20
	(7.20)	(5.69)	(5.20)
21	ND	-	-
	8.10	6.21	5.60
	(8.10)	(6.21)	(5.60)

ND - not detected ( ) - mean

********** storage at +4°C

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