NOVEL SCREENING METHODS FOR THE DETECTION OF *YERSINIA ENTEROCOLITICA* IN INFECTED BLOOD USED FOR TRANSFUSION

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Abstract

Between 1991-1996, 8 patients experienced rare life-threatening reactions that followed the transfusion of blood infected with *Yersinia enterocolitica*. The first reported case occurred in 1991 and was followed by seven others that directly caused or contributed to the death of 5 of 8 patients. *Y. enterocolitica* is a food and water borne infection of the gastrointestinal tract which in adults is often asymptomatic. An unknown number of those infected experience a period of self-limiting bacteraemia. The large volume of blood collected during donation phlebotomy may contain small numbers of bacteria that can increase in number during blood bank storage, producing potentially lethal levels of bacteria and toxin. Currently there are no reliable methods available to distinguish blood donations that present the greatest risk from those that present little risk.

This thesis, reports on the evaluation of two techniques to prevent the transfusion of blood infected with *Y. enterocolitica*. The first, a molecular method, was used to amplify bacterial DNA in blood by Polymerase Chain Reaction (PCR). A 425 bp product was amplified from DNA extractions of infected blood. Results showed that the technical complexities of the methodology, together with poor sensitivity and the need for large-scale donation sampling make PCR as applied for this purpose unattractive.

An Enzyme Linked Immunosorbent Assay was developed to detect current/recent infection with *Y. enterocolitica* in healthy blood donors. Polystyrene beads were coated with bacterial proteins to detect IgA antibody to *Y. enterocolitica* in human serum. The sera from donors of confirmed unit infections, paired sera from culture-proven *Y. enterocolitica* gastrointestinal tract infection and sera from volunteer blood donors were tested. Results showed that the sera of six bacteraemic blood donors tested contained elevated levels of IgA antibody. High rates of positivity (26/27), were detected in sera from culture-confirmed GIT infection and a rate of 4.04% seropositivity was found among 495 blood donors enrolled in a clinical trial. Results showed a strong correlation between IgA seropositivity, and recipient risk associated with the transfusion of blood heavily infected with *Y. enterocolitica*. The work demonstrated how the use of a simple screening test for recent infection, could be used to exclude high risk donations and improve the safety of blood transfusion in New Zealand.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
AS-1	Additive solution - 1
BHI	Brain heart infusion
BHI-B	Brain heart infusion broth
bp	Base pair
CIN	Cefsulodin-irgasan-novobiocin
CMI	Cell mediated immunity
CPD	Citrate phosphate dextrose
CR-MOX	Congo red, magnesium oxalate
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylenebis(oxyethylenenitrilo)-tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
GIT	Gastrointestinal tract
HB_sAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCV	hepatitis C virus
HLA-B:27	Human leucocyte antigen B:27
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin-1
INF-γ	Gamma interferon
kb	Kilobase
kD	Kilodalton
lcr	Low Ca^{++} restricted
LPS	Lipopolysaccharide
MIS	Mucosal immune system
MMWR	Morbid mortality weekly report
MWM	Molecular weight marker

MQ	Millipore filtered - type 1 laboratory reagent grade water
O/N	Overnight
PCR	Polymerase chain reaction
PTH	Post transfusion hepatitis
PV	Predictive value
pYV	Yersinia virulence plasmid
RO	Reverse osmosis - type 3 laboratory reagent grade water
RP	Released protein
sIgA	Secretory immunoglobulin A
SIS	Systemic immune system
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide electrophoresis
TNF	Tumour necrosis factor
TE	Tris EDTA
Tris	Tris-(hydroxymethyl) aminomethane
WWII	World war II
Yops	Yersinia outer membrane protein

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CHAPTER 1

Transfusion transmitted endotoxaemia caused by *Yersinia enterocolitica* infected blood in New Zealand - a historical review

1.1 Introduction

The modern era of blood transfusion commenced during World War II (WWII) when blood transfusion was first used on a large scale. Since that time, the practice of blood transfusion, has provided an understanding of the importance of blood grouping and the crossmatching of donor and recipient blood groups to both improve "in vivo" survival of transfused blood and to reduce the risks of recipient death. The first Blood Banks were established in the U.S. in the years that followed WWII and resulted in the introduction of new surgical procedures reliant upon a supply of banked blood. Demand for blood in the new era, was initially met by volunteers motivated by community involvement and a desire to help others. Soon demand exceeded supply and programmes were introduced to pay blood donors for their blood. This practice remained in place in some countries for many years until the AIDS epidemic in 1982 forced a rethink of policy in the US (Blajchman, 1995).

Over the last 20-30 years the transmission of microorganisms from donor to recipient through blood transfusion has occupied an ever increasingly important role in the provision of blood services internationally. During the late 1960s it was discovered that a virus causing hepatitis could be transmitted following the transfusion of blood and blood products (Polissar, 1969). The virus became known as Hepatitis B virus (HBV) and its global spread lead to increasing cases of post transfusion hepatitis (PTH) until the introduction of laboratory testing for HBsAg in 1974 (Tung, 1971). Pre transfusion testing of donated blood rapidly decreased the number of cases but did not eliminate all PTH entirely. Another virus hepatitis "non A, non B" became linked with PTH (Tabor *et al.*, 1984; Wick *et al.*, 1985). The discovery of the agent of non A, non B hepatitis in 1989

lead to the virus being named hepatitis C virus (HCV) (Choo *et al.*, 1989). Laboratory testing for antibody to HCV in blood donors was introduced internationally shortly after the discovery of the causative agent.

In the period between the discovery of HBV and HCV, the link between Human Immunodeficiency virus (HIV) and blood transfusion became apparent. Laboratory testing for antibody to HIV commenced in blood donors in 1982 and since that date the number of HIV infections spread by blood transfusion has reduced. Today an almost unreal expectation that blood transfusion should be "entirely safe" exists among the public, concerned that a new virus similar to HIV looms to infect more victims. These perceptions have stimulated research into alternative products to human derived blood and blood products. To date successes have been few; however recombinant factor VIII used for treatment of new haemophiliacs in New Zealand provides some measure of safety for this group most at risk of acquired viral infection. Satisfactory alternatives to the use of human red cells for medium to long-term oxygen transportation remain at the research stage. Advancements in technology and an understanding of the epidemiology of the hepatitis viruses and HIV, has almost eliminated these viruses from the blood supply of developed countries. In the third world advances have been slower with HBV, HCV, HIV and infections with protozoa, in particular *Plasmodia*, *Trypanosoma* and *Leishmania* major obstacles in the provision of a safe blood supply (Gottlieb, 1993).

In 1941, the first example of a death following the transfusion of bacterially infected blood was reported in the U.S.A. (Strumia & McGraw, 1941). Around that time the estimate of the rate of unit infection with bacteria reached approximately 8.5% (Heath & Province, 1942). Since then, the use of closed system sterile collection packs, the storage of blood products at controlled temperatures, and the use of internationally standardised blood processing procedures has lead to a decline in the total number of reported cases (Sazama, 1994).

Septicaemia, endotoxic shock and disseminated intravascular coagulation (DIC) are life threatening events that occur following the transfusion of blood infected with large numbers of bacteria. Although still rare internationally the incidence of reported cases has shown a steady increase since 1985 (Morduchowicz *et al.*, 1991). Reasons are not clear, although increases have coincided with the introduction of routine plasma depletion of red

cells shortly after collection, and growing rates of GIT illness in the community (Hogman & Engstrand, 1996; Jacobs *et al.*, 1989; Jones & Hanson, 1994; MMWR, 1991; MMWR, 1997; MMWR, 1997; Morris & MacCulloch, 1996; Sazama, 1994; Theakston *et al.*, 1997). The organism most frequently implicated in post transfusion endotoxaemia is *Y. enterocolitica*, a gastric pathogen of humans and animals. During infection, small numbers of bacteria are able to enter the bloodstream and exist for short periods of time as free or intracellular bacteria (Gibb *et al.*, 1994; Gibson & Norris, 1958). If infected subjects donate blood during this stage, viable bacteria may be collected in the donation of blood. During storage in the controlled 4°C environment of the blood bank, small numbers of *Y. enterocolitica* grow in the iron-rich environment with bacterial numbers increasing the longer the blood is stored (Gibb, *et al.*, 1994; Gibson and Norris, 1958). If unit infection goes unnoticed at the time the blood is transfused, endotoxic shock, septicaemia, DIC, multi-organ failure and death can result.

Between 1982-1992, 33 reports of transfusion related endotoxaemia caused by *Y. enterocolitica* appeared in the English-language literature. These events lead to the death of 19 (59%) of the recipients (Sazama, 1994). Between 1991-1995, nine transfusion cases were reported in New Zealand alone. Eight cases were caused by *Y. enterocolitica* and 1 by *Serratia liquefaciens* infection of the transfused blood. In 7/8 cases *Y. enterocolitica* was cultured from the transfused blood. Confirmation of the eighth followed the recovery of *Y. enterocolitica* in blood cultures from the febrile hypotensive patient. In all the 9 cases contributed to the untimely death of 6 recipients (Theakston, *et al.*, 1997; Ulyatt *et al.*, 1991; Wilkinson *et al.*, 1991; Woodfield, 1991).

This thesis reviews the cases of transfusion related endotoxic shock caused by *Y. enterocolitica* in New Zealand and the risks that *Y. enterocolitica* infected blood presents to recipients of blood in New Zealand. It also investigates the potential for laboratory testing of donated blood using PCR and the application of a newly developed ELISA assay as a tool for the prevention of further cases in New Zealand.

1.2 Bacterial contamination of donated blood

In healthy individuals the blood stream is a sterile environment. Sporadic bacteria entering

the circulation are usually quickly destroyed by a functionally normal immune system. Bacteria that possess resistance to the immune system or exist in the blood as an intracellular parasite survive for longer periods. During the process of venesection bacteria may enter the blood bag, either from the skin, the environment or by an endogenous route. Usually numbers are small (<10cfu/ml) but under favourable conditions some species of bacteria are able to multiply during blood bank storage at 4°C, producing potentially lethal levels of bacterial endotoxin over the period of storage of the donated blood (Gibson and Norris, 1958).

1.2.1 Environmental bacteria as sources of infection

The introduction of disposable collection bags, improved collection techniques and closed systems for blood processing, have helped reduce levels of unit contamination reported since WWII. In spite of this, intermittent contamination traced to faulty collection equipment is occasionally reported (Khabbaz *et al.*, 1984). The most recently reported example resulted in an outbreak of *Serratia marcescens* in Scandinavia where the source of contamination was traced to the anticoagulant used in the blood bags. Faulty irradiation of the blood bag during manufacture was the cause (Heltberg *et al.*, 1993).

1.2.2 Commensal bacteria as sources of infection

During unit venesection, large bore collection needles can cut plugs of skin, which flow into the collection pack (Buchholz *et al.*, 1973; Gibson and Norris, 1958; Schifman & Pindur, 1993). Inadequate preparation or disinfection of the venepuncture site can contaminate the blood bag with the resident flora of the skin. These bacteria are normally killed by fresh plasma and under normal conditions of storage do not thrive. Commensal bacteria are common contaminants of platelet products, which are stored at room temperature. Many examples of infected platelet transfusions that produced febrile transfusion reactions in recipients have been recorded. These however are usually not life threatening (Goddard *et al.*, 1973; Katz & Tilton, 1970).

1.2.3 Endogenous sources of infection

The most serious form of unit infection follows the collection of blood from blood donors

with an infection that produces an inapparent, transient or intermittent bacteraemia, or in donors in whom there has been recent trauma to mucous membranes. Dental procedures have been implicated as the cause of low-grade infection of donated blood following the detection of flora normally resident in the oral cavity eg. *Streptococcus mitis* and *Staphylococcus epidermidis* in platelet concentrates (Morrow *et al.*, 1991). Other non-dental bacteraemias can follow irritation or trauma to mucous membranes eg. sigmoidoscopy, urinary tract infection, normal bowel movement, menstruation, and sexual intercourse (in females) (Ness & Perkins, 1980).

During infection with *Y. enterocolitica* and other organisms such as *Campylobacter enteriditis* and *Salmonella* spp., a period of low-grade bacteraemia can develop. Small numbers of pathogenic bacteria can penetrate the mucosal barrier of the large bowel, enter the lymphatics and become bloodborne (Cover & Aber, 1989; Heal *et al.*, 1987). It is not clear whether the bacteria can be found free in the plasma or whether as suggested by *Gibb et al.* that *Y. enterocolitica* is transported in the bloodstream in circulating white cells (Gibb *et al.*, 1992; Gibb, *et al.*, 1994). On retrospective questioning of donors of blood infected with *Y. enterocolitica*, a lack of symptoms of the infection at the time of donation is a common finding. This supports an endogenous bacteraemia as the mode of entry of bacteria in donated blood (Aber, 1990; Tipple *et al.*, 1990; Verhaegen *et al.*, 1998) (Bjune *et al.*, 1984; Bufill & Ritch, 1989; Gibb, *et al.*, 1994; Jacobs, *et al.*, 1989; MMWR, 1991; Theakston, *et al.*, 1997; Tipple, *et al.*, 1990).

1.2.4 Gram-negative sepsis

The development of Gram-negative sepsis involves a complicated series of effects based mainly on the composition of the bacterial cell wall. The toxic component is endotoxin, which is part of the cell envelope of Gram-negative bacteria. Endotoxin is a lipopolysaccharide (LPS) structure composed of lipid A and polysaccharide. The toxic element in the molecule is predominantly the lipid A component with the O-specific side chains less toxic (Morrison, 1983).

In most mammalian species, the injection of LPS is associated with the rapid onset of fever and hypotension, which is seen about 30 minutes after administration. Endotoxin also affects the bone marrow and circulating blood cells, inducing neutropenia, leucocytosis,

and a reduction in circulating platelet numbers. B cell and macrophage proliferation is also stimulated (Braude, 1986).

The adverse effects of endotoxin result from its capacity to stimulate the release of endogenous mediators such as tumour necrosis factor (TNF) and interleukin-1 (IL-1) from macrophages (Morrison, 1983). TNF and IL-1 are endogenous pyrogens that contribute to the febrile response in sepsis. TNF acts synergistically with IL-1 and gamma interferon (INF-γ) to trigger an inflammatory response and cause vascular damage (Young, 1990). TNF also promotes the release of prostaglandins and other lipid mediators of shock including platelet activation factor, leukotrienes C4 and D4, and thromboxane A_2 (Lefer, 1989). The adverse effects of lipid mediators is to increase vascular permeability, vaso-occlusion and contraction of pulmonary smooth muscle (Braude, 1986; Lefer, 1989). Endotoxin can also stimulate widespread coagulopathy and disseminated intravascular coagulation through activation of factor XII in the contact pathway of the coagulation cascade. Factor XIIa activates the complement pathway and the kininogen to kinin reaction, increasing polymorphonuclear chemotaxis which can lead to adult respiratory distress syndrome (Schedel, 1988).

The appropriate treatment of endotoxaemia can reduce the death rate in septic shock. In cases involving the transfusion of infected blood, the volume and the age of the blood transfused correlates with patient prognosis. Methods of treatment include the early administration of high dose antibiotic therapy eg. aminoglycosides plus an expanded spectrum cephalosporin. Management of the associated hypotension is achieved through fluid and electrolyte balance and the use of sympathomimetric amines that raise the heart rate and maintain the systolic blood pressure (Winslow *et al.*, 1973). Approx 60% of those receiving blood heavily infected with endotoxin-producing bacteria can be expected to die from multi-organ failure within 2 days.

1.2.5 Growth of *Y. enterocolitica* in blood bags

The concentration at which bacteria in a unit of blood become clinically significant is unknown, although it has been estimated to be approx. 10^5 cfu/ml (Krishnan & Brecher, 1995). Experimental models of unit infection with *Y. enterocolitica* (Buchholz *et al.*,

1992; Gibb, *et al.*, 1994; Kim *et al.*, 1992; Krishnan and Brecher, 1995; Pietersz *et al.*, 1992) demonstrate significant variation in the lag period preceding active bacterial replication. This period has been reported to vary from 7-20 days (Arduino *et al.*, 1989; Krishnan and Brecher, 1995) and precedes a rise in the viable count which plateaus at approx 10^9 organisms/ml after 12-30 days at 4°C. Visual evidence of unit infection is not obvious until 10^8 cfu/ml is reached (Kim, *et al.*, 1992; Kim *et al.*, 1992) when bacteria depleted haemoglobin bound oxygen changing the colour of the blood from red to a very dark red (almost black). The build up of CO_2 and drop in pH in the blood bag also contribute to the colour change. In contrast, sterile segments attached to the blood bag retain their colour. During the period of storage endotoxin accumulates in the blood, levels increasing with bacterial numbers (Arduino *et al.*, 1989; Kim, *et al.*, 1992).

1.3 Frequency of transfusion related endotoxic shock

The reported frequency of bacterial contamination of blood components varies. In 1987 a 2% contamination rate of blood with *Staphylococcus* spp. was reported (Mollison *et al.*, 1987). More recently rates between 0.00001 - 5% have been reported (Goldman & Blajchman, 1991; Sazama, 1994). Reasons for the variation in reported incidence is unclear may be due to the geographical incidence of the common causative bacteria, testing of different blood components, or secondary contamination during testing.

1.3.1 United States of America (USA)

Each year, over 20 million units of blood products are transfused in the USA (MMWR, 1997). No episodes of post transfusion endotoxic shock were reported to CDC or in the literature from the USA before 1985. From 1986 through 1991, 16% (29/182) of all transfusion-associated fatalities reported to the Food and Drug Administration (FDA) were associated with the bacterial contamination of blood products, mostly platelets (MMWR, 1991). The number of reports of post transfusion sepsis and death has been increasing in the USA over the last two decades. The greatest proportion follow the transfusion of red cells infected with *Y. enterocolitica.* Between 1991-1996, 10 fatalities were attributed to the transfusion of infected blood in the U.S.A. The first case of *Y. enterocolitica* infection

of platelets has been reported recently (Kuehnert & Jarvis, 1998). In a report from the U.S. General's Accounting Office, the estimated rate of bacterial-associated adverse reactions from random donor platelet pools was 0.6 per 1000 pooled units, and for Yersinia-associated RBC transfusion reactions a rate of 1 per 500,000 units of RBCs (Office, 1997).

In a review entitled "Transfusion-associated bacterial sepsis" in 1994, Wagner reported the USA incidence of post transfusion clinical sepsis caused by infected blood as similar to the rate of transmission of HCV following transfusion. These figures significantly exceed those estimated for transfusion-associated human immunodeficiency and hepatitis B virus transmission. He also found that 51% of all transfusion endotoxaemias in the USA were caused by *Y. enterocolitica* with the incidence reported for other bacteria: *Pseudomonas fluorescens* 26.5% *Pseudomonas putida* 4.1%, *Treponema pallidum* 4.1% and others 14.3% (Wagner *et al.*, 1994).

1.3.2 Germany

In a quality control data report published by the Bavarian Red Cross Blood Transfusion Service between 1985-1993 bacterial contamination was detected in 100 of 25,171 blood components tested. Single-donor platelet concentrates had a contamination incidence of 25 in 5889 (0.42%); whole blood samples 1 in 2973 (0.03%) and red cell concentrates a rate of 73 in 15,317 (0.48%); of 992 samples of fresh frozen plasma only 1 was contaminated (0.1%). Staphylococci comprised 75% and Gram-negative rods 10% of all cases. This high frequency of laboratory-detected bacterial infection contrasts with the low rate of reported transfusion-associated septicaemic events in Germany (Illert *et al.*, 1995).

1.3.3 New Zealand

Between 1990-1996 approximately 700,000 units of blood were transfused to patients in New Zealand. During this time there were a total of eleven AS-1 red cell unit infections discovered. Ten unit infections were caused by *Y. enterocolitica* and one by *Serratia liquefaciens*. Nine of the infected units were transfused to patients causing severe endotoxic shock and the death of six recipients. In 8/9 transfusion cases, the reactions were caused by large numbers of viable *Y. enterocolitica* and endotoxin, present in the blood bag. In 1997 the NZ incidence of unit infections with *Y. enterocolitica* for the period

1991-1997 was calculated to be one case per 65,000 units transfused with a fatality rate of 1:104,000 units transfused (Theakston, *et al.*, 1997). When compared to statistics for the USA, the NZ figures are approx. 80 times higher (Lee *et al.*, 1995).

Case reports for eleven unit infections that occurred between 1990 and 1996 are presented in this section. The reports cover nine actual transfusion cases (previously reported) and two examples of unpublished "non transfusion-related" unit infections. A summary of unit infections are presented in Table 1.1. Geographical distribution and the years of discovery are presented in Figure 1.1.

1) Auckland 1990: a 21 year old female was transfused with a unit of 17 day old AS-1 red cells, to correct blood loss following abdominal surgery. The transfusion was stopped after 50ml had been administered when she developed a high temperature and rigors. The patient developed severe hypotension and septicaemia and died 48 hours later despite attempts to resuscitate her. The bacteria isolated from the blood bag was *Serratia liquefaciens* and the donor history was unremarkable (Woodfield, 1991).

2) Christchurch 1991: an 84 year old male was transfused with a single unit of AS-1 red cells post operatively following hip surgery. He developed acute renal failure and hypotension and died one day after the transfusion. Blood cultures from the patient and blood bag grew *Y. enterocolitica* serotype O:3. The blood was 15 days old at the time of transfusion. Questioned after the incident the blood donor could recall a bout of diarrhoea 4 months before donating his blood. (Wilkinson, *et al.*, 1991).

3) Christchurch 1991: a 24 year old woman was transfused to correct anaemia following a course of chemotherapy to treat Hodgkin's disease. The patient developed rigors, nausea, vomiting, explosive diarrhoea, chest and back pain two hours into the transfusion of the second unit of blood. The unit of blood was 24 days old and contained *Y. enterocolitica* serotype O:3. The patient was ventilated for six days before recovering (Wilkinson, *et al.*, 1991). No donor details were available.

4) Hamilton 1991: a 61 year old male recovering from arterial bypass surgery was transfused with a unit AS-1 red cells that was 23 days old. After 30 minutes the patient complained of fever, shortness of breath and chest pain. He developed worsening

dyspnoea and hypoxia and became hypotensive. He was admitted to the intensive care ward and he responded to plasma volume expansion therapy and intravenous antibiotics to make a full recovery. *Y. enterocolitica*, serotype O:3 was isolated from the blood bag and the patient. The donor's serum contained high titred agglutinating antibody 1:5000 at the time of donation but he could not recall any gastrointestinal illness preceding the donation (Ulyatt, *et al.*, 1991).

5) Timaru 1992: a 15 year old boy died following the transfusion of a unit of blood infected with *Y. enterocolitica* serotype O:3 following a motor vehicle accident in which he broke his leg (Brett, 1993). During the transfusion the patient developed severe vomiting and temperatures and the transfusion was stopped. The donor of the unit had no previous symptoms of GIT infection. The unit of blood was 23 days old at the time it was transfused (Theakston, *et al.*, 1997).

6) Palmerston North 1993: an 18 year old male who had two days earlier undergone internal fixation of a fractured femur was transfused with a unit of AS-1 red cells that was 33 days olds. He immediately developed dyspnoea, nausea, vomiting and tachycardia after receiving approx 100ml of the unit. He recovered following intensive care treatment for hypovolaemic shock and the use of broad-spectrum antibiotic therapy. *Y. enterocolitica* serotype O:3 was recovered in blood cultures from the bag and the patient. The donor of the unit could not recall any GIT symptoms prior to donating although he did work with deer, recognised carriers of the organism (Theakston, *et al.*, 1997).

7) Palmerston North 1994: a 71 year old woman recovering from a knee joint replacement was transfused with a unit of AS-1 red cells to correct post operative anaemia. During the transfusion of the second unit the woman began to develop rigors, tachycardia, dyspnoea, chest and back pain and cyanosis. The transfusion was stopped and the patient transferred to intensive care. The 20 day old unit of red cells contained large numbers of *Y. enterocolitica* serotype O:9. The organism was also recovered in blood cultures taken from the patient. The woman died 36 hours after the transfusion. When questioned, the blood donor could not recall any symptoms of the infection at the time of donation. Three stool samples provided 20+ days after the transfusion failed to grow *Y. enterocolitica* (Theakston, *et al.*, 1997).

8) Auckland 1994: a 61 year old woman was admitted to intensive care in septic shock with nosocomial lung infection, following ventricular haemorrhage. She received 3 units of AS-1 red cells and deteriorated into shock and respiratory failure over the next few hours. Her condition continued to worsen and she died 19 hours later. A blood culture from the patient 4 hours previous to the transfusion was sterile. Blood cultures taken 6 hours after the transfusion contained *Y. enterocolitica* serotype O:3. The blood bags were not available for culture but were and the donor history was unknown (Theakston, *et al.*, 1997).

9) Auckland 1995: a 37 year old woman was admitted to hospital for an elective hysterectomy and was transfused with two units of AS-1 red cells to correct iron deficiency anaemia prior to surgery. The first unit was transfused without incidence but during the patient developed explosive diarrhoea and severe abdominal pain. The patient also became febrile and hypotensive, her condition worsening during the day. She was sedated and ventilated; however multi-organ failure developed over the next four days and she died. *Y. enterocolitica* serotype O:3 was recovered from the second unit transfused and in blood cultures taken from the patient shortly after transfusion. The infected unit used for the transfusion was 14 days old. No details concerning the recent history medical history of the donor were available (Theakston, *et al.*, 1997).

10) Masterton 1996: routine inventory check of blood stocks at Masterton Hospital revealed a unit of AS-1 red cells that was noticeably blackened at the completion of 35 days storage. Culture showed the unit to be heavily contaminated with *Y. enterocolitica* serotype O:3. When the blood donor was questioned she could not recall GIT illness at the time of donation although her husband had recently had a bout of diarrhoea (Masterton Hospital, May 1996, Fisher- Personal communication).

11) New Plymouth 1996: a unit of AS-1 resuspended red cells was returned to the Blood Bank at Taranaki Base Hospital after the laboratory technician at Hawera Hospital recognised discolouration of a unit of blood prior to crossmatch for a patient. On culture the unit contained a heavy growth of *Y. enterocolitica* serotype O:9. A review of the donor history showed the unit had been collected from a secondary school student in Taranaki. Retrospective questioning of the donor revealed recent he was passing "loose stools" at the time of donation and that a member of his family had recently recovered

from diarrhoea of unknown cause (Manawatu Regional Blood Centre, Baker - Personal communication 1996).

Case No.	Isolated from AS-1 red cells or by blood culture*	Donor GIT history	Red cells transfused Y/N	Fate of recipient
1	*S. liquefaciens*	Nil	Y	Died
2	*Y.enterocolitica* O:3	4/12	Y	Died
3	*Y.enterocolitica* O:3	unknown	Y	Survived
4	*Y.enterocolitica* O:3	Nil	Y	Survived
5	*Y.enterocolitica* O:3	Nil	Y	Died
6	*Y.enterocolitica* O:3	Nil	Y	Survived
7	*Y.enterocolitica* O:9	Nil	Y	Died
8	* *Y.enterocolitica* O:3	unknown	Y	Died
9	*Y.enterocolitica* O:3	unknown	Y	Died
10	*Y.enterocolitica* O:3	Contact	N	N/A
11	*Y.enterocolitica* O:9	Y	N	N/A

Table 1.1 Summary of NZ transfusion related endotoxaemias and unit infections.

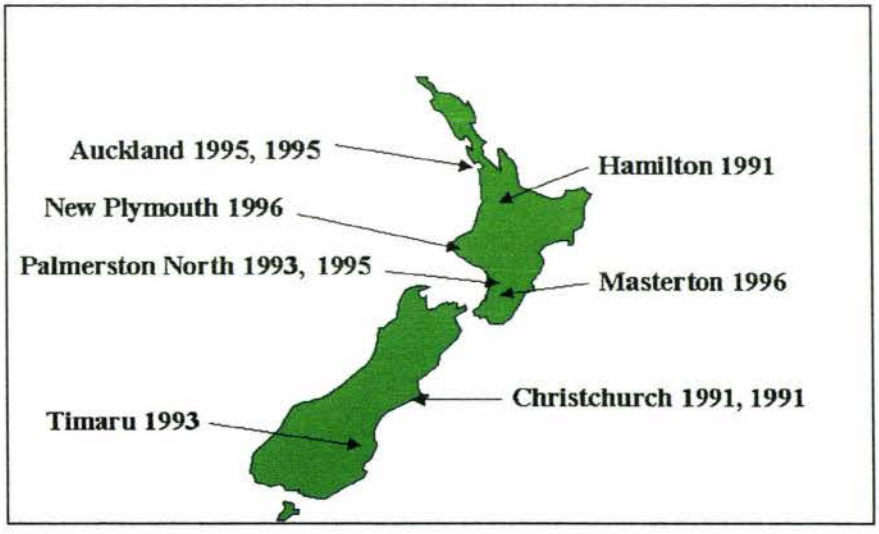


Figure 1.1 Geographical distribution of red cell unit infections with *Y. enterocolitica* in NZ.

1.4 Taxonomy of the Yersiniae

The genus Yersinia was named after the French bacteriologist Yersin following his visit to the plague epidemic in Hong Kong in 1894. During that time he isolated the causative organism *Yersinia pestis*. All members of the species *Yersinia* belong to the Enterobacteriaceae. The genus consists of *Yersinia pestis, Y. pseudotuberculosis*, and the *Y. enterocolitica* group which is comprised of *Y. enterocolitica* (biotypes I-IV), *Y. enterocolitica* (biotype V), *Y. intermedia, Y. frederiksenii, Y. kristensenii, Y. aldovae, Y. mollaretti, Y. rohdei* and *Y. bercovieri*. There is considerable overlap between the species in biochemical properties, antigenicity and ecology (Corbel, 1990).

1.5 Bacteriology of *Y. enterocolitica*

Y. enterocolitica is a small Gram-negative rod or coccobacillus (1.0-3.5 μm x 0.5-1.3 μm). They are non-acid fast, non spore forming with 1-15 peritrichous flagella which are produced during growth at temperatures below 30°C. Fimbriae are also produced. After 24 hours at 37°C on nutrient agar Y. enterocolitica produces a smooth, low, convex colony of 1-2 mm. diameter with a glistening surface and an entire or slightly crenated edge. On MacConkey agar *Y. enterocolitica* morphology appears as translucent, pale, non-lactose fermenting colonies of 1.5-2 mm. Some strains isolated from food, are lactose fermenters, producing bright pink colonies on MacConkey. On cefsulodin-irgasan-novobiocin (CIN) agar, *Y. enterocolitica* produces 1.5-2.0 mm. colonies with a deep purple/red centre, a sharp edge and is surrounded by a translucent border.

Differentiation of pathogenic from non-pathogenic strains of *Y. enterocolitica* can be performed using Congo Red-Magnesium Oxalate (CR-MOX) agar. Growth of *Y. enterocolitica* is slow so that after 24 hours at 37°C, colonies containing the Yersinia virulence plasmid (pYV^+) appear small (0.5mm) and are slightly pink in contrast to colonies lacking the virulence plasmid (pYV^-) strains that are larger (1.0-1.5mm.) and slightly pinker. After 48 hours pYV^+ colonies are small (1.0-1.5mm) and have a pink periphery with a dark red centre. pYV^- colonies are larger (2.5-3.5mm) and are entirely pink (Gemski *et al.*, 1980; Prpic *et al.*, 1987).

1.5.1 Biotypes

The *Y. enterocolitica* group is heterogeneous giving different reactions from biochemical testing performed at 29°C from those obtained at 37°C. On the basis of biochemical testing, the *Y. enterocolitica* group can be divided into 5 biotypes. Biotypes I-IV are biochemically reactive and distinct from biotype V, which is less reactive (Barton *et al.*, 1997).

1.5.2 Serotypes

At least sixty O-antigen groups are recognised in the *Y. enterocolitica* group, some of which cross-react with other bacteria. About 20 heat labile flagellar antigens are produced at temperatures below 30°C and are of little importance in serotyping. Strong association between specific biotypes and serotypes are recognised, as is a relationship between bio/serotype infections in animals and humans and geographical prevalence (Barton, *et al.*, 1997).

Biotype IA isolates are regarded as non-pathogenic but biotype IB includes serotype O:8 which is mainly found in the USA. Biotype 2 contains serotypes (O:5, 27 and O:9), biotype 3 (O:1,2,3 and O:5, 27) and biotype 4 (O:3). Biotype 5 contains serotypes O:2,3 which are only found in ruminants and small rodents.

Biotyping and serotyping are the commonest methods used to type organisms in the *Y. enterocolitica* group. The methods are useful allowing the discrimination between isolates which are potentially clinically significant and environmental contaminants (Wauters *et al.*, 1987). More recently molecular techniques such as pulse field gel electrophoresis (Iteman *et al.*, 1991) and phage typing (Gilmour & Walker, 1988) have proven useful in the epidemiologic investigation of infections.

1.6 Epidemiology of *Y. enterocolitica* infection

Y. enterocolitica is commonly isolated in countries with a cooler climate, such as those in Northern Europe, Canada, Japan (Cover and Aber, 1989; Portnoy & Martinez, 1985) and

New Zealand (Fenwick & McCarthy, 1995). In New Zealand serotype O:3 represents approximately 90% of all isolates with serotype O:9 and O5:27 less frequently encountered. Other pathogenic serotypes O:1,2 and O:8 and O:21 are not seen in New Zealand (Fenwick and McCarthy, 1995).

Yersiniosis is a notifiable disease in NZ and is the third most frequent cause of clinical gastroenteritis after *Campylobacter* and non-typhoid *Salmonella*. 1998 the cumulative rate of infection for *Y. enterocolitica* among the New Zealand population was reported by the NZ *ESR-CDC* as 15.4/100,000 (Research, 1998). The cumulative rate as at March 2000 is reported as 13.3/100,000 (Unpublished data - ESR-CDC). These contrast with reported rates of 1.6/100,000 in Australia (Australia, 1999), and 6.0/100,000 in Belgium (Verhaegen, *et al.*, 1998).

Swine are the recognised reservoir in Europe and internationally the consumption of pork has a strong association with infection. In New Zealand, the organism is more widespread and can be isolated from the stools of farm and domestic animals including cats and dogs. Fenwick and co-workers suggest that high rates of infection in NZ are related to the incidence of carriage of *Y. enterocolitica* among domestic animals and to the NZ rural/urban lifestyle that leads to frequent contact with farm animals (Fenwick and McCarthy, 1995).

1.7 Virulence factors

1.7.1 pYV plasmid

Plasmids are autonomous pieces of extrachromosomal DNA that provide bacteria with additional genetic information (Portnoy and Martinez, 1985). The Yersinia virulence plasmid (pYV) is a large 70 kilobase structure (Fig 1.3) providing capacity to encode a substantial number of products called Yersinia outer membrane proteins (Yops) (Gemski, *et al.*, 1980). The plasmid enables Yersinia spp. (*Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) to survive and multiply in the lymphoid tissues of their host. The plasmid encodes the Yop virulon, an integrated system that allows extracellular bacteria to disarm the cells involved in the immune response, to disrupt their communications, or even to induce apoptosis by the injection of bacterial effector proteins. The Yop proteins are

released through a dedicated type III secretion apparatus, called Ysc. The Ysc apparatus is composed of 25 proteins including a secretin (Cornelis & Wolf-Watz, 1997). Most of the Yops fall into two groups; some are intracellular effectors (YopE, YopH, YpkA/YopO, YopP/YopJ, YopM, and YopT), while others (YopB, YopD, and LcrV) form the translocation apparatus deployed at the bacterial surface to deliver effectors into the

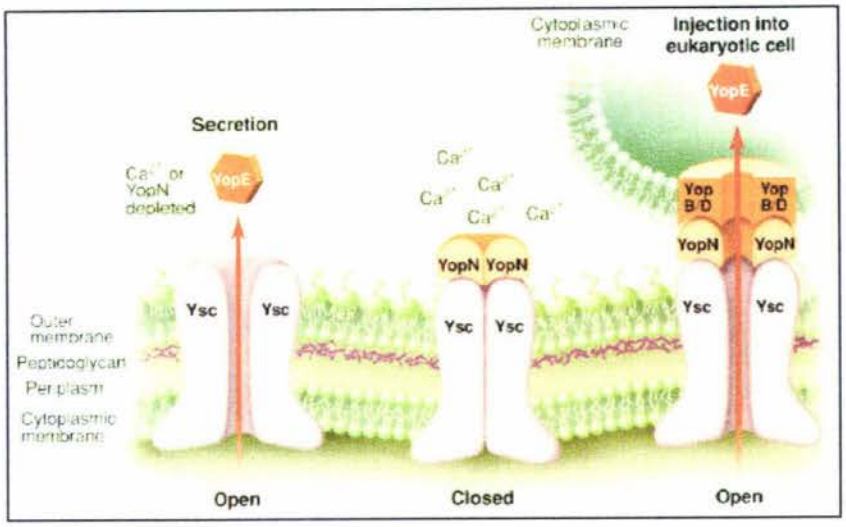


Figure 1.2 Proposed model of interaction between Yersinae and macrophages (Cornelis in *Trends in Microbiology*, 1998).

eukaryotic cells, across the plasma membrane (Cornelis *et al.*, 1998). Yop secretion is triggered by contact with eukaryotic cells and controlled by proteins of the virulon including YopN, TyeA, and LcrG, which form a plug complex closing the bacterial secretion channel (Fig 1.2).

The proper operation of the system also requires the production of small individual chaperones, called the Syc proteins, in the bacterial cytosol (Iriarte & Cornelis, 1999). Transcription of the genes is controlled both by temperature and by the activity of the secretion apparatus. The virulence plasmid of *Y. enterocolitica* and *Y. pseudotuberculosis* also encodes the adhesin YadA which forms a fibrillar matrix around pathogenic *Yersiniae* (Emody *et al.*, 1989).

1.7.2 Yop function

The full pathogenic potential of the *Yersiniae* occurs when products of both the plasmid

and the chromosome are expressed concomitantly following infection in humans and animals (Portnoy *et al.*, 1981). Strains that lack the plasmid coding capability have a reduced capacity to invade tissues and grow at 37°C in low Ca^{++} conditions. These strains are not pathogenic to humans (Portnoy and Martinez, 1985; Prpic *et al.*, 1983; Prpic *et al.*, 1985). Yop production occurs at 37°C and is partly Ca^{++} restricted, conditions that also restrict bacterial growth and motility.

Some of the Yop genes are encoded within the low calcium restricted (lcr) *lcrGVHyopBD* operon (Cornelis *et al.*, 1989) while others are located outside of this region (Hartland *et al.*, 1994). *YadA* produces the surface bacterial membrane protein YadA which prevents the binding of C1q fragments of the complement cascade (China *et al.*, 1994). YadA is produced at 37°C irrespective of Ca^{++} ion concentration and is controlled by genes outside of the *lcr* region of the plasmid. In a process described by Hartland *et al.*, the combined effects of temperature, low Ca^{++} and the production of yops N, & B/D, the cytotoxin YopE is translocated across the eukaryotic cell membrane into the cytoplasm of target cells (Hartland *et al.*, 1996).

The virulence plasmids of the pathogenic *Yersiniae* are highly conserved (Bolin *et al.*, 1988; Heesemann *et al.*, 1986). Specific antibodies to the yops and YadA are detectable in human and animal sera after infection indicating that they are expressed in vivo (Heesemann *et al.*, 1987; Heesemann *et al.*, 1988; Lahesmaa-Rantala *et al.*, 1989; Robins-Browne *et al.*, 1993; Stahlberg *et al.*, 1987; Stahlberg *et al.*, 1989). Further support for the "in vivo" expression of Yops was demonstrated when both YadA and Yops were found in the outer membrane of *Y. enterocolitica* grown intraperitoneally in guinea pigs (Skurnik, 1985). In experimental infection with rats YadA was expressed in the lamina propria following oral infection, (Skurnik & Poikonen, 1986). In humans yops and YadA have been shown to be present in the intestinal biopsies of patients with spondylarthropathies and serologic evidence of persisting *Y. enterocolitica* infection (de Koning *et al.*, 1989). *Paerregaard et al.* proved that the loss of the pYV plasmid lead to a loss of *Y. enterocolitica* virulence (Paerregaard, 1992).

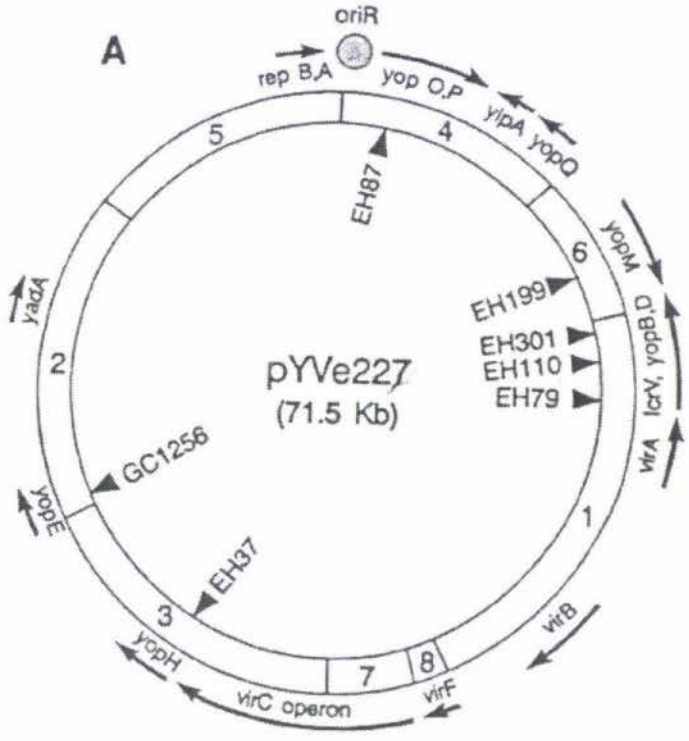


Figure 1.3 *Eco*R1 restriction map of the virulence plasmid pYVe227 of *Y. enterocolitica* showing the location and direction of transcription of genes encoding secreted and structural proteins Yops B, D, E, H, M, O, P and Q, the V antigen (*lcrV*), the lipoprotein YadA, regulatory components (*VirA, B, C, F*) and replicative functions (*repA, B, oriR*).

(From: Hartland *et al.*, Essential role of YopD in inhibition of the regulatory burst of macrophages by *Y. enterocolitica, Infection and Immunity*, Vol 62, No 10).

Initially, Yops were thought to be directly inserted into the outer membrane of the organism but have since been shown to be secreted into the extracellular space and reabsorbed back into the into the bacterial membrane eg. YadA & YlpA. YadA, YpkA, YopM, YopH, and YopE have been identified as independent virulence determinants of *Yersinia* spp. (Michiels *et al.*, 1990).

1.8 Human infection with *Y. enterocolitica*

Since its discovery *Y. enterocolitica* has become recognised as an important cause of gastrointestinal illness internationally (Cover and Aber, 1989). More recently it has become the leading cause of transfusion related endotoxic shock (Galloway & Jones, 1986; Hogman, 1996; Jacobs, *et al.*, 1989; Katz *et al.*, 1992; Kimber, 1995; Krishnan and Brecher, 1995; Mitchell & Barr, 1992; Sazama, 1994; Theakston, *et al.*, 1997; Tipple, *et al.*, 1990; Wagner, *et al.*, 1994; Wright *et al.*, 1985). In New Zealand, the number of isolations of *Y. enterocolitica* from the stools of patients with diarrhoea and abdominal pain has increased (Fenwick and McCarthy, 1995; McCarthy & Fenwick, 1991). More recently extra-intestinal disease including fulminant septicaemia, hepatic abscess and more frequently, reactive arthritis, has been identified as part of the infection (Ameratunga *et al.*, 1987; Beeching *et al.*, 1985; Lello & Lennon, 1992). Human infection with pathogenic strains of *Y. enterocolitica* occurs in all age groups with clinical illness frequently reported in the young and adolescent age groups (Bottone, 1997). Asymptomatic infection is common, especially among adults (Hoogkamp-Korstanje *et al.*, 1988). Spread of infection is via the faecal/oral route or through the ingestion of food or water contaminated by an animal or human carrier (Black *et al.*, 1978; Lee *et al.*, 1991; Shayegani *et al.*, 1983).

1.8.1 Mucosal immune system

The mucosal immune system (MIS) in animals and man provides host defense at the mucosal level (Michalek *et al.*, 1983). It acts in concert with non-immunologic protective factors inhibiting the growth of potential pathogens. The lymphoid tissues of the MIS are closely associated with the mucosa of the gastrointestinal, respiratory and uro-genital tracts. The MIS differs from the systemic immune system (SIS) in that the humoral response to stimulation via the gut associated lymphoid tissue (GALT) produces a strong

and immediate IgA response, followed by IgM and IgG antibody (McGhee *et al.*, 1987).

Following the ingestion of pathogenic strains, *Y. enterocolitica* penetrates the intestinal mucosa exhibiting a marked tropism for MIS lymphoid tissues. The organism rapidly colonises the ileal Peyer's patches of the colon (Hanski *et al.*, 1989; Hanski *et al.*, 1989). Chromosomal *ail* and *inv* genes promote attachment and penetration of the M cell layer covering the GALT. The bacteria pass through the enterocyte and emerge at the lamina propria or dome region of the lymphoid follicle (Miller *et al.*, 1989). The lamina propria is an area distal to the M cell layer which is rich in cells bearing major histocompatibility (MHC) class II antigens (B cells and macrophages) and a high concentration of T cells. IgA bearing B cells (and plasma cells) predominate with IgM, IgG and IgE B cells and plasma cells, present in lesser numbers. Bacteria that find their way into the lamina propria begin to express plasmid encoded virulence factors in the low Ca^{++}, 37°C regulated environment where secreted Yops encode resistance to phagocytosis and bacterial killing mechanisms of phagocytes. Plasmid bearing strains multiply producing micro-abscesses in the lamina propria and pYV$^-$ strains are rapidly phagocytosed and killed. (Beuscher *et al.*, 1995; China *et al.*, 1994; Green *et al.*, 1995; Hartland *et al.*, 1996; Hartland *et al.*, 1994; Kapperud *et al.*, 1985; Paerregaard *et al.*, 1991; Pai & DeStephano, 1982; Pilz *et al.*, 1992; Rosqvist *et al.*, 1988; Tabrizi & Robins-Browne, 1992). Despite resistance mechanisms some bacteria are phagocytosed by macrophages and present processed bacterial antigen to germinal B cells in the follicular lymphoid tissue. Sensitised B cells are released from the lymphoid follicles and become dispersed throughout the lymphatics and peripheral blood. Primed B cells "home" into the diffuse lymphoid areas where they secrete antibody. Antibody producing plasma cells stimulated in this way secrete predominantly monomeric forms of IgA. IgM and IgG antibody are also produced. Monomeric 7S IgA is dimerised and complexed with secretory component (SC) in its passage through the epithelial cell barrier of the mucosa. Dimeric 11S secretory IgA (sIgA) sensitises the target organism promoting phagocytosis by polymorphonuclear cells and prevents further M cell passage of bacteria by blockage of the bacteria/M cell receptor. In this way the infection is limited and bacteria are cleared from the gut. A similar mechanism acts as a defense against the invasion by normal flora in the gut. The absence of specific antibody to rarely encountered enteric pathogens such as *Y. enterocolitica* partly explains their pathogenic potential and how a poor antibody response can lead to extra-intestinal foci of infection, bacteraemia

pYV encoded factor	Function
Yad A	adhesin - mediates attachment to host cells; favours ileal colonisation; promotes interaction with polymorphs with/without opsonising conditions; essential for persistence of organisms in Peyer's patches; resistance to killing by antimicrobial polypeptides of human granulocytes
V antigen (LcrV)	protective antigen – anti-host function which can be neutralised by antibody; necessary for maximum expression of Yops
YopE	antiphagocytic; contact dependent cytotoxin; depolymerises actin microfilament
YopD	essential for the translocation of Yops E and H into target cells
YopB	essential for translocation of Yops; acts in concert with Yop D; pore former
YopH	acts as a protein tyrosine phosphatase subverting the signal transduction processes of macrophages and lymphocytes; suppresses antibacterial action of polymorphs; effect enhanced by YopE and YopA
YopM	leucine repeat protein; protein-protein interactions
YopN	controls polarised secretion; senses target cell contact?; gates the secretion channel?; negative regulation of Yop and LcrV expression
YpkA	serine/threonine kinase

Table 1.2 Yops and their function from "Foodborne Microorganisms of Public Health Significance". (Barton *et al.*, 1997)

and septicaemia in some (Stites *et al.*, 1994). The exact mechanisms of clearance of *Y. enterocolitica* remains unknown. Despite possessing resistance to phagocytosis there is evidence that a combination of both the humoral immune response and CMI are important in clearing the organism (Aleksic *et al.*, 1991; Autenrieth *et al.*, 1993; Hanski *et al.*, 1991; Kono *et al.*, 1985; Leino *et al.*, 1987; Mattila *et al.*, 1985).

1.9 Immune response to infection

Immunoblot studies of bacterial proteins show that the antibody response to infection with *Y. enterocolitica* in humans and animals produces is directed against chromosomal and plasmid encoded epitopes. (Benoit *et al.*, 1996; Cremer *et al.*, 1993; Granfors, 1979; Granfors *et al.*, 1988; Granfors *et al.*, 1980; Gronberg *et al.*, 1989; Hanski *et al.*, 1991;

Maki-Ikola *et al.*, 1992; McGhee *et al.*, 1987; Michalek *et al.*, 1983; Stuart & Woodward, 1992). Infection illicits a strong antibody response composed of at least three immunoglobulin classes with antibody detectable in the serum 7-10 days after infection. Antibody levels peak in the second or third week (IgA & IgM) or fifth week (IgG) (Bitzan *et al.*, 1987; Heesemann *et al.*, 1988; Mattila *et al.*, 1985). Controversy exists over the length of time and the antibody classes that can be detected in uncomplicated and complicated infections. Most studies using ELISA show that in uncomplicated cases IgA and IgM antibody decline rapidly to undetectable levels 2-3 months after infection, while IgG antibody persists for up to 12 months (Bitzan *et al.*, 1987; Granfors 1979). Antibodies specific for individual bacterial components decrease in a similar manner with time (Stahlberg *et al.*, 1987; Stahlberg *et al.*, 1987). Significant differences exist in the kinetics of the antibody response when complicated cases are considered. Infection that leads to rheumapathological disorders and persistent enteritis produces a prolonged IgA response that can persist for long periods, often years (Granfors *et al.*, 1989; Granfors *et al.*, 1980; Heesemann *et al.*, 1988; Lahesmaa-Rantala *et al.*, 1989; Toivanen *et al.*, 1987). A similar trend is seen in the level of IgG although this is not as pronounced as for IgA. Little is known about the status of the immune response in individuals who develop subclinical bacteraemia as a result of the infection. Studies conducted into the immune response in blood donors and presented in this thesis show that infection with *Y. enterocolitica* can produce a subclinical bacteraemia in otherwise healthy blood donors despite a good immune response to the infection.

1.10 Pathogenicity of *Y. enterocolitica*

Rabbits orogastrically infected with pathogenic strains of *Y. enterocolitica* manifest infection as a self-limiting disease in which diarrhoea and weight loss are the main features. Similar findings have been reported by others in experiments in monkeys, (Maruyama, 1973), mice, (Autenrieth *et al.*, 1994; Autenrieth & Firsching, 1996; Carter, 1975) sheep, (Hanski *et al.*, 1989; Robins-Browne *et al.*, 1993) and rats, (Gaede *et al.*, 1992). Typically colonisation of the ileum leads to histopathologic changes with inflammatory foci in the mucosal crypts of the Peyer's patches. In some animals abscess formation and spread within the lamina propria leads to mucosal ulceration and inflammation of the regional lymph nodes. (Heesemann & Gaede, 1989; Heesemann *et*

al., 1993). In most animal examples the infection is limited to the large bowel, but penetration and ulceration of the basement membrane can cause the infection to develop into fulminant septicaemia and death.

In humans *Y. enterocolitica* causes a range of clinical manifestations. Infection in adults produces sporadic stomach or abdominal pain, diarrhoea in some and occasionally other extra-intestinal sequelae such as terminal ileitis and reactive arthritis (Stolk-Engelaar & Hoogkamp-Korstanje, 1996). In the young infection causes mild, self-limiting, sometimes bloody diarrhoea, with abdominal pain closely mimicking acute appendicitis. Focal extraintestinal infection has been reported but is uncommon and information about the development of bacteraemia is scarce (Morris & Woodfield, 1998; Paerregaard, 1992). In adults abdominal pain and rheumatopathologic disorders frequently complicate the infection, causing fever and reactive arthritis which may persist for variable periods. Terminal ileitis caused by persistence of pathogenic bacteria in the bowel and more rarely fulminant septicaemia are further complications of the infection (Cover and Aber, 1989). Hepatitis, thyroiditis, pericarditis and glomerulonephritis are also reported as sequelae of chronic infection (Cremer *et al.*, 1993).

1.11 Persistence of infection

Under normal circumstances *Y. enterocolitica* is cleared from the large bowel and does not persist as part of the normal flora of the gut. In 20-30% of infections a period of chronic infection is maintained. *Toivanen et al.* showed that persistent infection occurs more commonly in those who suffer from post-infection reactive arthritis. In these cases patients are more frequently found to be HLA-B:27 positive (B:27 negative individuals may also develop severe reactive arthritis and some B:27 positive do not). The antibody response of the IgA and IgG classes is more vigorous and persists much longer. IgA1 and IgA2 subclasses are produced more vigorously, indicating local immuno-stimulation close to the intestinal epithelium. In the early phase of the infection Yersinia-IgM immune complexes are found in the circulation. In those who develop reactive arthritis the rate of lymphocyte transformation is down regulated affecting the T cell response and cell mediated immunity (Toivanen *et al.*, 1985). Those who develop reactive arthritis fail in their first line defense against the invading organism during Yersinia enteritis. Impaired CMI leads to persistence

of the microorganism in the body with bacteria remaining in the intestinal epithelium and the mesenteric lymphatics, maintaining a stimulus for prolonged antibody production (Toivanen *et al.*, 1985).

1.12 *Y. enterocolitica* bacteraemia & septicaemia

Clinical Gram-negative bacteraemia and septicaemia are reported with a number of bacteria such as *E. coli, Klebsiella spp., Enterobacter spp., Serratia spp., P. aeruginosa, and Proteus spp.* (Bone, 1993). Those most at risk include patients on immunosuppressive therapy, those recently exposed to invasive procedures, eg. insertion of bladder catheters, prostheses, drainage tubes etc. Patients with penetrating wounds, burns, trauma patients, and intestinal ulceration are also at greater risk. In the absence of transfusion idiopathic *Y. enterocolitica* septicaemia is rare.

Little is known about the low-grade bacteraemia and the stage(s) of infection leading to the bacteraemic state. In a study conducted by *Morris et al.,* 36 patients with stool culture positive *Y. enterocolitica* (serotype O:3) infection were tested for an IgG and IgA antibody response. Blood from each patient was also cultured, at about the time *Y. enterocolitica* was recovered in their stools. Of the 36 patients only one presented with clinical or recent diarrhoea. The remainder had visited their GP complaining of abdominal pain with or without joint pain. All blood cultures performed on the patients were negative. Serological confirmation of the infection using the ELISA O:3 LPS (DAKO) assay showed a poor correlation between stool culture positivity, assay positivity and bacteraemia (Morris and Woodfield, 1998). Although numbers are low findings support the low incidence of bacteraemia during *Y. enterocolitica* GIT infection (Paerregaard, 1992).

1.13 Laboratory diagnosis of *Y. enterocolitica* infection

In the acute phase of *Y. enterocolitica* infection the organism can be isolated from the stools by culture at the optimal growth temperature of 25°C. Cefsulodin-irgasan-novobiocin (CIN) agar is the selective medium of choice for the isolation of the organism from other normal intestinal flora. Pathogenic and non-pathogenic strains of *Y. enterocolitica* grow on CIN agar, while most other bacteria are inhibited. On CIN colonies

of *Y. enterocolitica* are small with a dark red centre after 24 hours incubation. After 48 hours the colonies are larger and are dark pink in colour. In suspected cases of unit infection *Y. enterocolitica* can be isolated from the blood bag using most nutrient agar eg. Brain Heart Infusion Broth/Agar incubated at 37°C. On solid media, colonies are visible after 24 hours incubation.

In the chronic stage of the infection the organism cannot usually be isolated from the stools. In these cases diagnosis of Yersiniosis can be made by intestinal biopsy or by the use of serology. (Cremer *et al.*, 1993; Granfors *et al.*, 1988; Granfors *et al.*, 1989; Granfors *et al.*, 1980; Hoogkamp-Korstanje *et al.*, 1988; Leino *et al.*, 1988; Maki-Ikola *et al.*, 1992; Nielsen 1990; Stahlberg *et al.*, 1987; Stolk-Engelaar & Hoogkamp-Korstanje, 1995). Traditional laboratory serologic testing is performed by agglutination of stained suspensions of *Y. enterocolitica* (serotypes O:3 or O:9) in serially diluted sera. The method is sensitive to IgM and IgG antibody but does not detect IgA antibody. Titres below 80 are generally considered to be normal with titres exceeding 80 (or rising titres) indicative of current infection. Today the favoured method for Yersinia serology is by ELISA which allow detection of IgA with or without IgG antibody (Benoit *et al.*, 1996; Granfors, 1979; Granfors *et al.*, 1988; Granfors *et al.*, 1989; Granfors *et al.*, 1980; Hoogkamp-Korstanje *et al.*, 1992; Larsen *et al.*, 1985; Martinez *et al.*, 1991; Michalek *et al.*, 1983; Stolk-Engelaar and Hoogkamp-Korstanje, 1995; Toivanen *et al.*, 1987; Vesikari *et al.*, 1980; Vuento *et al.*, 1984). Detection of levels of IgA and IgG antibody to *Yersinia enterocolitica* is informative about the stage of the infection. The presence of both IgA and IgG antibody correlates with acute or chronic infection; IgA alone - early stage or chronic infection; IgG alone - resolved infection (Aleksic *et al.*, 1991; Autenrieth *et al.*, 1994; Cremer *et al.*, 1993; Kihlstrom *et al.*, 1991; Lahesmaa-Rantala *et al.*, 1987; Lehtinen *et al.*, 1988; Maki-Ikola *et al.*, 1992; Soppi *et al.*, 1987; Stahlberg *et al.*, 1987; Stemerowicz *et al.*, 1990). The presence of IgA immunoglobulin in the serum of individuals is a marker of recent/current or chronic infection with *Y. enterocolitica.* If applied in the context of blood donor screening the presence of IgA antibody in the blood of donors at the time of venesection, indicates current or recent infection and an increased risk of donating blood infected with viable *Y. enterocolitica.*

CHAPTER 2

Growth of *Y. enterocolitica* in human AS-1 red blood cells during storage at 4°C.

2.1 Introduction

The concentration of clinically significant bacteria in stored blood sufficient to cause endotoxaemia and shock is unknown but has been estimated to be approx. 10^5 cfu/ml (Krishnan and Brecher, 1995). Experimental models of unit infection with *Y. enterocolitica* (Buchholz *et al.*, 1992; Gibb *et al.*, 1994; Kim *et al.*, 1992; Krishnan and Brecher, 1995; Pietersz *et al.*, 1992) report variability in the patterns of *Y. enterocolitica* growth in stored blood at 4°C, during the phases of bacterial replication. The lag phase between inoculation and detection of bacteria in blood has been reported to vary from between 7-20 days (Arduino *et al.*, 1989; Krishnan and Brecher, 1995). Stationary phase is reported to occur between 12-30 days with the viable count reaching a maximum of 10^9 organisms/ml.

This chapter reports experiments conducted to investigate the rate of growth of a strain of pYV^+ *Y. enterocolitica* serotype O:3 (PN2) in AS-1 human red cells stored at 4°C over a period of 30 days. The experiment was constructed to mimic as closely as possible the low level bacteraemia thought to exist in blood donors at the time donation and bacterial growth under usual conditions of storage. The strain used in the experiment was isolated from a unit of donated blood that caused transfusion-associated endotoxic shock and septicaemia and was an example of one of two serotypes implicated in 10 unit infections that occurred between 1991-1996 in New Zealand.

2.2 Materials

All chemicals were supplied by BDH unless otherwise stated.

1) Phosphate buffered saline

sodium chloride (Analar)	8.0g

potassium chloride (Analar)	0.6g
potassium dihydrogen phosphate (Analar)	0.2g
di-sodium hydrogen phosphate (Analar)	1.14g
Distilled water to:	1000ml

pH to 7.4 and dispensed into 50ml quantities, sterilised at 121°C /15 minutes and stored at room temperature.

2) Brain Heart Infusion Broth and Agar

BHI (Oxoid)	37.0g
Agar (Davis)	15.0g
Distilled water to:	1000ml

Powdered medium was dissolved in distilled water with stirring. pH was adjusted to 7.4 and broth dispensed into 500ml aliquots and sterilised at 121°C for 20 minutes. The broth was stored at room temperature and dispensed into smaller aliquots or following the addition of agar poured into sterile disposable Petri dishes as required. BHI agar plates were sealed in plastic bags and stored at 4°C.

3) Congo red magnesium oxalate agar (Riley & Toma, 1989)

Component # 1:	Tryptic Soy Agar (Bacto)	40 g
	Distilled water to:	825ml

Agar was suspended in water, boiled and dispensed into 825ml quantities before autoclaving at 121°C for 15 minutes.

Component # 2:	Magnesium chloride (0.25M)	
	$MgCl_2 \cdot 6H_2O$	50.8g
	Distilled water to:	1000ml

$MgCl_2$ was dissolved, autoclaved at 121°C for 15 minutes and stored at room temperature.

Component # 3:	Sodium oxalate solution (0.25M)	
	Sodium oxalate ($Na_2C_2O_4$)	33.2g
	Distilled water to:	1000ml

Sodium oxalate was dissolved, autoclaved at 121°C for 15 minutes and stored at room temperature.

Component # 4:	D-Galactose solution (20%)	
	D-Galactose	200.0g
	Distilled water to:	1000ml

Galactose was dissolved with gentle heating, filter sterilised and stored at room temperature.

Component # 5:	Congo red dye	10g
	Distilled water to:	1000ml

Congo red dye was dissolved, autoclaved at 121°C for 15 minutes. and stored at room temperature.

Final was prepared by melting TSA and cooling to 50°C. Components 2-5 (below) were aseptically added in the following amounts.

Final medium:	Component 1	825ml
	Component 2	80ml
	Component 3	80ml
	Component 4	10ml
	Component 5	5ml

Complete medium was mixed, poured into Petri dishes and stored at 4°C in sealed bags. The medium was stable for 3 months.

CR-MOX agar is used for the identification pYV^+ strains of *Y. enterocolitica* from pYV^- plasmidless strains. The medium allows the determination of congo red uptake and calcium dependent growth on the same plate (Riley and Toma, 1989). Low Ca^{++} conditions and the presence of Mg^{++} restricts the rate of growth of plasmid positive colonies so that pathogenic *Y. enterocolitica* produce tiny red colonies after 24 hours at 37°C. Non pathogenic strains grow faster are bigger and do not take up the dye.

4) Blood collection packs, (Baxter Healthcare Corporation, Fenwal Division, Deerfield, USA).

Supplied by Mid Central Health Ltd. Bags were gamma irradiated and contained 63ml of Citrate Phosphate Dextrose (CPD) anticoagulant. 100ml of additive solution 1 (AS-1)

provided with each donation pack contained; 2.2g dextrose, 900mg sodium chloride, 750mg mannitol and 27mg of adenine. Red cells reconstituted with AS-1 are referred to as AS-1 red cells in the remaining text.

5) *Y. enterocolitica* isolate

Y. enterocolitica pYV^+ (PN2) - isolated from a unit of AS-1 red cells that caused Gram-negative septicaemia and endotoxic shock in a patient following blood transfusion at Palmerston North in 1994. The strain was confirmed as *Y. enterocolitica* biotype IV serotype O:3, by Dr. Stan Fenwick, Institute of Veterinary, Animal and Biomedical Science, Massey University.

2.3 Methods

2.3.1 Donor blood collection

Two blood donors who met the requirements for blood donation from within the Mid Central Health Ltd. blood collection area were enrolled in the study. Written consent to use the donations for research purposes was obtained (see appendix 2) from each blood donor. 450ml of whole blood was collected from each volunteer into Baxter blood collection bags containing CPD. Immediately after collection each donation was centrifuged in a Heraeus Christ Cryofuge 8000 refrigerated centrifuge at 3,500 rpm for 10 minutes. Plasma was expressed into the attached plasma collection satellite bag of the Baxter blood bag set, and separated from the red cells by heat seal. Plasma was stored frozen (-30°C). 100ml of additive solution 1 (AS-1 red cell maintenance solution) was added to the red cells in each of the two donations. Red cells and additive solution were mixed and the volume divided to provide two units each containing 200ml of AS-1 red cells. The blood was transported to the research laboratory at Massey University in containers maintained at approx 4°C.

2.3.2 Unit inoculation

A frozen sample of pYV^+ *Y. enterocolitica* O:3 (strain PN2) in Glycerol/PBS was thawed, cultured onto CR-MOX agar and incubated at 37°C overnight. A single plasmid positive colony was picked from the CR-MOX agar plate and used to inoculate 20ml of Brain Heart

Infusion broth (BHI-B). The broth culture was incubated at 25°C overnight (O/N) with constant rotation and the next day serially diluted in sterile phosphate buffered saline to provide tenfold dilutions of the original. 1ml of a 10^{-8} dilution (30cfu) of the O/N culture was aseptically injected through the infusion port on each of two AS-1 red cell bags. The second unit of each pair was left uninoculated and served as a negative control. Blood bags were mixed thoroughly and maintained at 4°C ± 2°C in a temperature controlled room for the duration of the experiment.

2.3.3 Viable cell counts

Sampling of both inoculated and uninoculated units was performed on alternate days commencing at day 2 and finishing at day 30. On each day 1ml of blood was aseptically removed through the intravenous port of each blood bag in a grade 2 safety cabinet, using sterile syringes (2ml) and 21G blood collection needles (Terumo). 100µl of blood was pipetted onto Brain Heart Infusion Agar plates and aseptically spread over the entire surface. Cultures were performed in duplicate and agar plates incubated overnight (20-24 hours) at 37°C in 5% CO_2. Plates were counted the following day and the mean cfu obtained from the duplicate cultures. Confirmation of the isolation of *Y. enterocolitica* was performed by Gram stain, and colony morphology on congo red, magnesium oxalate agar (CR-MOX). From day 6 onward as bacterial numbers increased, tenfold dilutions of blood were made in sterile PBS to obtain discreet colonies within the range of 30-300 colonies per plate after 24 hours incubation.

2.3.4 PCR samples

100µl of blood from inoculated and control units were sampled and pooled on alternate days. 50µl of pooled blood was subjected to DNA extraction (see next chapter).

2.4 Results

Samples from each of the inoculated blood bags taken at day 0 and day 2 failed to grow bacteria. Viable bacteria were first detected after 4 days of incubation after which bacterial numbers rose to peak at 10^9 organisms/ml after 26 days (see Table 2.1 and Fig.2.1). After

day 26 bacterial numbers remained unchanged at days 28 and 30. Sampling stopped after 30 days. Bacterial doubling time was approximately 16 hourly. Rates of bacterial replication and the number of bacteria present in each of the two inoculated units followed a similar pattern throughout the experiment as demonstrated in the growth curve for the experiment (Fig 2.1). Both control units remained sterile throughout the period of the experiment.

Day	Unit #1 cfu/ml	Unit # 2 cfu/ml	Controls cfu/ml
0	0	0	0
2	0	0	0
4	20	100	0
6	550	400	0
8	950	800	0
10	5.1×10^3	7.2×10^3	0
12	9.6×10^4	1.1×10^4	0
14	4.6×10^5	4.0×10^5	0
16	1.4×10^6	4.1×10^6	0
18	4.5×10^7	1.0×10^8	0
20	1.9×10^7	1.1×10^7	0
22	6.8×10^8	8.5×10^8	0
24	1.4×10^9	1.7×10^9	0
26	1.5×10^9	2.1×10^9	0
28	2.0×10^9	2.1×10^9	0
30	1.8×10^9	2.0×10^9	0

Table 2.1 Growth of pYV$^+$ *Y.enterocolitica* in AS-1 red cells stored at 4°C over a period of 30 days.

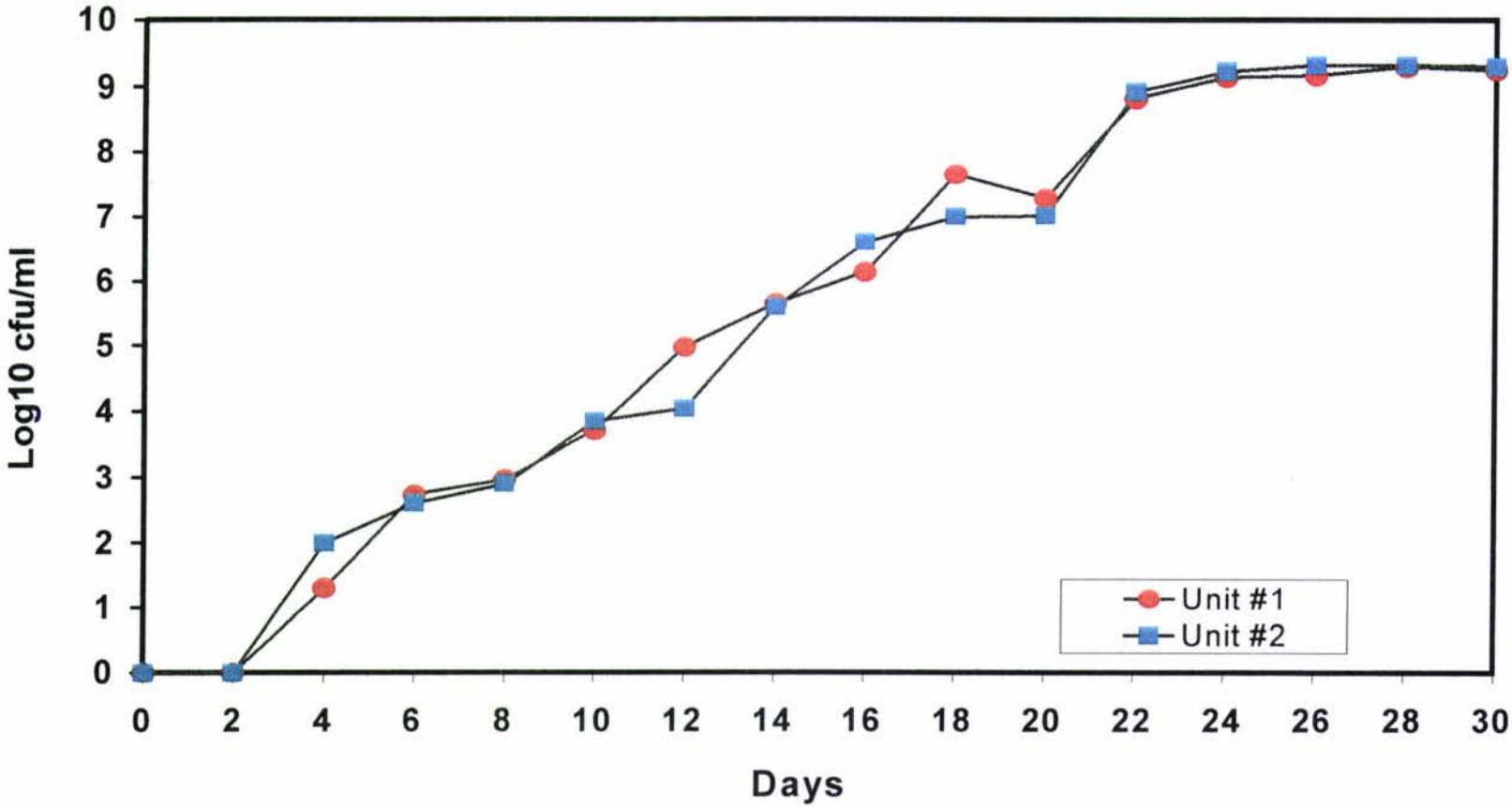


Figure 2.1 Graphical representation of the growth of pYV$^+$ *Y. enterocolitica* at 4°C following the inoculation of 30 cfu into 2 x 200ml blood bags containing human AS-1 red blood cells.

CHAPTER 3

Detection of *Y. enterocolitica* in human AS-1 red blood cells by PCR

3.1 Introduction

The theoretical sensitivity of nucleic amplification techniques such as the Polymerase Chain Reaction allows the detection of a single copy of bacterial DNA target sequence. In this chapter PCR amplification of bacterial DNA was applied to the detection of *Y. enterocolitica* in blood following the inoculation of AS-1 red cells with 30 cfu/ml of pYV^+ bacteria. Samples collected during the growth experiment described in Chapter 2 were used for PCR. This chapter presents the results of amplification of a 425 bp fragment from the attachment and invasion locus (*ail*) of *Y. enterocolitica* using primers and protocols described by Feng et al., 1992. Experimental format was guided by how the technology would apply in the blood bank laboratory. The method of sampling, DNA extraction and cycling protocols were chosen to fit envisaged workflow and time constraints.

3.2 Materials

All chemicals were supplied by BDH unless otherwise stated.

3.2.1 Agarose gel electrophoresis

1) 10X TAE buffer

Tris Base	72.6g
EDTA (Univar)	11.2g
Glacial Acetic Acid	17.1ml
Distilled water to	1000ml

The pH was adjusted to 8.5 and diluted 1/10 for electrophoresis

2) Agarose Ultrapure DNA grade (Biorad)

Agarose gels were prepared according to the method described in Sambrook et al., 1989.

3) Ethidium bromide 5µg/ml

3.2.2 PCR reagents

1) 10X PCR Buffer

As supplied by manufacturer (Gibco BRL).

2) $MgCl_2$ 50m*M*

As supplied by manufacturer (Gibco BRL).

3) dNTP's 1.25m*M* (Promega)

4) Oligonuclotide primers (Feng *et al.*, 1992).

Synthesised commercially by Oligo's etc. and supplied by Life Technologies Ltd.

5) *Taq* DNA Polymerase 5U/µl

As supplied by the manufacturer (Gibco BRL)

6) Sterile distilled water

7) λ *Hin*dIII/*Eco*RI digested size: 21.226 Kbp, 5.148 Kbp, 4.973 Kbp, 4.268 Kbp, 3.530 Kbp, 2.027 Kbp, 1.904 Kbp, 1.584 Kbp, 0.947 Kbp, 0.831 Kbp, 0.564 Kbp.

3.2.3 DNA extractions

1) TE buffer

1M Tris-HCl (pH 8.0)	10ml
0.2M EDTA (pH 8.0)	5ml
Distilled water to:	1000ml

Final pH 7.4

2) Lysis solution

Proteinase K 20 mg/ml (Boehringer Mannheim)

Dissolved in buffer containing 3% (wt/vol) Brij 35 (Sigma), 0.2*M* Tris-HCl, 0.1*M* EDTA. Final pH 7.4.

3) Tris buffered saline

NaCl	8g
KCl	0.2g
Tris base	3g
Distilled water to:	1000ml

Aliquot and sterilise at 121°C for 15 minutes. Store at RT. Final pH 7.4

3.2.4 Culture medium

1)	Brain Heart Infusion broth (Oxoid)	37.0g
	Agar (Davis)	15.0g
	Distilled water to:	1000ml

pH was adjusted to 7.4

3.2.5 Primer preparation

Primers were selected from the published *ail* sequences of *Y. enterocolitica.* Primers ail-1 and ail-2 spanned a 424 bp sequence from nucleotide 544 to 968, of the attachment and invasion locus (ail) of chromosomal DNA (Davis & Fuller, 1991; Feng *et al.*, 1992). (see appendix 6).

ail-1 5' - TTAATGTGTACGCTGCGAGTG - 3'

ail-2 5' - GGAGTATTCATATGAAGCGTC - 3'

Freeze dried primers were reconstituted in 200µl sterile distilled water according to instructions supplied by the manufacturer and dissolved with gentle agitation. 2µl was diluted in 800µl of MQ water and scanned spectrophotometrically at 260 and 280 nm using a *Shimadzu* spectrophotometer. (260nm measures nucleic acid and 280nm other molecules). A_{260} over A_{280} should be approximately 1.8-2.0.

ail-1 A_{260} = 0.215 x 400 (dilution) x 33 (conversion factor)

= 2838 µg/ml

to convert to µ*M* = $\frac{2838}{330 \times 21 \times 10^3}$

= 409 µ*M*

To obtain a 2 µ*M* concentration of primer ail 1, 2µl of stock was diluted with 409µl SDW.

ail-2 A_{260} = 0.212 x 400 x 33

= 2798.4 µg/ml

to convert to µ*M* = $\frac{2798.4}{330 \times 21 \times 10^3}$

= 403.8 µ*M*

To obtain a 2µ*M* concentration of primer ail 2, 2 µl of stock was diluted with 402 µl SDW.

T_m calculations

To establish the melting point of the primers the following formula from Sambrook was employed (Sambrook, 1989).

ail 1 T_m = 69.3 + (0.41 x GC%) – 650 / n

= 69.3 + (0.41 X 47.62) – 30.952

= 69.3 + 19.52 – 30.95

= 57.9°C

ail 2 T_m = 69.3 + (0.41 x 42.6) – 30.952

= 55.9°C

3.3 Methods

3.3.1 DNA extraction

Samples collected during the experiment described in Chapter 2 provided bacteria in the

range of 10-1.6 x 10^9 organisms/ml in human AS-1 red cells. Nucleic acid extraction was performed using a method modified from that described by Kawasaki *et al.*, 1989 (Feng et al., 1992). One hundred microlitres of blood and bacteria were lysed in Tris-EDTA buffer and pelleted. The deposit was resuspended in 50µl of Tris buffered saline and 5µl of lysis solution containing Proteinase K. Extracts were incubated at 60°C for 1 hour, boiled for 10 minutes at 98°C and stored at 4°C.

3.3.2 PCR reaction mix

Frozen stock PCR reagents were thawed and pooled in a sterile eppendorf tube. A master mix of PCR reagents and SDW was prepared to provide the final working concentrations (described below). The master mix was aliquotted into PCR reaction tubes and template DNA added prior to the thermal cycling step. Each 10µl reaction contained:

1µl	25 mM $MgCl_2$
1µl	10X PCR buffer
1µl	dNTP
1µl	ail 1 primer (0.2µ*M*)
1µl	ail 2 primer (0.2µ*M*)
0.2µl	Taq DNA polymerase (5U/ml)
3.8 µl	SDW
1µl	DNA extract

3.3.3 PCR conditions

The thermal cycler was a Hybaid Model Omn E. Samples were subjected to 40 cycles of amplification, each cycle consisting of 1 minutes of denaturation at 94°C, 2 minutes of primer annealing at 53°C and 2 minutes of strand elongation at 72°C.

3.3.4 Agarose gel electrophoresis

Five microlitres of PCR product was separated in 1.5% agarose gels in TAE buffer at 70 V for 1.5 hours. Gels were stained with ethidium bromide and visualised under UV light.

The sizes of the amplified products were compared to the λ *Hin*dIII/*Eco*RI DNA marker.

3.4 Results

DNA extracts of *Y. enterocolitica* grown in AS-1 red cells from the experiment described in chapter 2, were performed on providing bacteria in the range of 10 - $1.6x10^9$ organisms/ml. Following PCR amplification a fragment of DNA could be visualised after ethidium bromide staining of agarose gel electrophoresis. Band size was approx. 430 bp (Fig 3.1) and is comparable in size to the 425 bp product reported by Miller *et al.* and the 430 bp product reported by Feng *et al.* using the same ail primers and similar PCR protocols (Feng *et al.*, 1992; Miller *et al.*, 1990). The visual limit of sensitivity of PCR in the gel under the conditions described was 4.3 x 10^5 orgs/ml. This detection limit was averaged but not exceeded over a series of amplifications. Results are comparable to the 5.0 x 10^4 organisms/ml sensitivity reported by Feng *et al.*, for experiments using similar conditions.

Reactivity of *Y. enterocolitica* ail primers used for PCR was demonstrated by testing against a cold ethanol extraction of genomic *Y. enterocolitica* DNA. Lack of amplification of the primers was demonstrated using DNA extracts of AS-1 red cells alone. Lack of amplification by the ail primers against other bacteria sometimes isolated from blood, including *Pseudomonas* species, *Enterococcus* sp., *Klebsiella* sp., *Escherichia coli*, and isolates *of Staphylococcus aureus* has been reported elsewhere (Davis and Fuller, 1991).

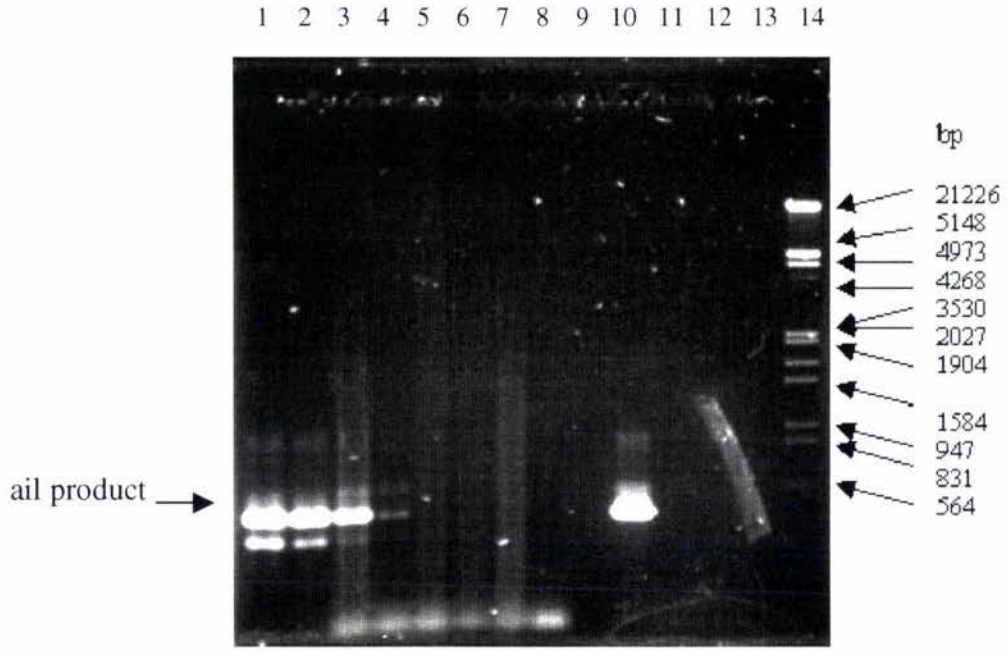


Figure 3.1 Representative gel following PCR amplification of *ail* gene from DNA extracts of citrate anticoagulated blood infected with *Y. enterocolitica*.

Lane 1, 1.5 x 10^7 organisms/ml; Lane 2, 7.3 x 10^7 organisms/ml; Lane 3, 2.8 x 10^6 organisms/ml; Lane 4, 4.3 x 10^5 organisms/ml; Lane 5, 7.3 x 10^4 organisms/ml; Lane 6, 5 x 10^3 organisms/ml; Lane 7, 850 organisms/ml; Lane 8, 450 organisms/ml; Lane 9, negative control; DNA extract of AS-1 red cells; Lane 10, positive control; genomic DNA; Lane 14, *Hin*dIII/*Eco*RI digest of λ bacteriophage DNA.

CHAPTER 4

Serologic detection of *Y. enterocolitica* infection in blood donors by IgA RP ELISA assay

4.1 Introduction

Blood donors infected with *Y. enterocolitica* at the time of donation carry the risk that their blood will contain small numbers of viable *Y. enterocolitica*. Numbers of bacteria present in blood at the time of donation are thought to be small, too small to be detected by routine bacterial culture. Routine blood donor venesection collects 450ml of blood, a volume large enough to contain many viable bacteria from donors with subclinical bacteraemia. The iron-rich environment of the blood bag provides an ideal medium for bacterial growth. During Blood Bank storage at 4°C, small numbers of bacteria are able to multiply over the period of storage (max. 35 days) producing lethal levels of bacteria and endotoxin that increase with the length of storage. Conventional techniques to detect *Y. enterocolitica* bacteraemia in the blood donor lack the required sensitivity to detect the small number of bacteria present at the time of donation.

This section investigates the use of serology to detect the antibody response associated with infection with *Y. enterocolitica* in blood donors. It also reviews the potential for the use of an ELISA assay to detect potentially dangerous donations preventing their use.

The immune response to *Y. enterocolitica* infection produces antibody to surface structures and secreted Yops (Hanski *et al.*, 1989; Hanski *et al.*, 1991; Lian *et al.*, 1987) and has been previously described. Most GIT infection with *Y. enterocolitica* in NZ occurs with serotype O:3 (90%), O:9 (8%) and O:5,27 (2%). Other global pathogenic serotypes exist eg. O:1,2,3; O:8, O:20, O:21 but these have not been isolated in NZ (personal communication S. Fenwick). Since 1991 *Y. enterocolitica* infection of donated blood has occurred with O:3 (8) and O:9 (2) serotypes in New Zealand and other serotypes eg. O:8 and O:20 elsewhere. It is therefore important that an assay to detect seroconversion should detect all common serotypes. The released proteins (RP) of *Y. enterocolitica* possess

unique amino acid sequences with little homology to proteins produced by other enterobacteriaceae. RP's are produced by pathogenic plasmid bearing strains and not by environmental strains of *Y. enterocolitica* that do not harbour the virulence plasmid. They are highly cross-reactive within the common serotypes of *Y. enterocolitica* so that the *RP's* produced by pYV^+ serotype O:3 *Y. enterocolitica*, react with antibodies produced in response to serotype O:9 or O:5,27 infections. This feature makes the RP antigens superior to the use of LPS, which is more reactive across the enterobacteriaceae and less cross-reactive with the *Yersiniae* (Nielsen *et al.*, 1996).

During the immune response to infection, immunoglobulin classes are produced at varying stages and remain for variable periods after infection (previously described). IgA antibody is produced early in the acute phase response to GIT infection with pathogenic *Yersiniae* and continues to be produced for extended periods in complicated infection (previously described). The short half-life of 6 days and a quick drop in production after the organism has been cleared makes IgA the immunoglobulin of choice for this application (Fernandez-Lago *et al.*, 1994; Granfors *et al.*, 1980; Mattila *et al.*, 1985; Nielsen *et al.*, 1996; Stites *et al.*, 1994).

This chapter describes the findings of an introductory study into seroconversion in confirmed bacteraemic blood donors using a commercially available ELISA kit (DAKO). It also reports the development of a new IgA RP bead ELISA assay and the results of performance trials against three groups of blood donor and patient sera:

Group one - samples from proven cases of unit infection (6)
Group two - culture-proven *Y. enterocolitica* infection (27)
Group three - the sera of healthy blood donors (495)

4.2 Materials

4.2.1 Released protein production

1) Ethylenebis(oxyethylenenitrilo)-tetraacetic acid (EGTA) 10X

EGTA (Boehringer Mannheim, molecular biology grade)	28.526 g
Distilled water to:	500ml

EGTA powder was dissolved in distilled water adjusted to pH 9.0-10.0 with NaOH to aid solution. Once dissolved the solution was adjusted using HCl to pH 7.4, filter sterilised (0.2µm cellulose acetate filter) and stored at RT.

2) Ammonium sulphate (Analar)

3) Dialysis tubing with 10,000 Da cut-off

4) Bio-Rad protein assay

5) MQ water

4.2.2 SDS-PAGE

1) Lower (stacking) gel buffer

Tris-HCl (Gibco BRL)	90.87g
Sodium Dodecyl Sulphate (Gibco BRL)	
Distilled water to:	500ml

pH was adjusted to 8.8 using conc. HCl. Stored at RT

2) Upper (loading) gel buffer

Tris-HCl (Gibco BRL)	30.29g
Sodium Dodecyl Sulphate (Gibco BRL)	
Distilled water to:	500ml

pH was adjusted to 6.8 using conc. HCl. Stored at RT.

3) Acrylamide/bisacrylamide

acrylamide (Gibco BRL electrophoresis grade)	75g
N,N-bis-methylene acrylamide (Gibco BRL electrophoresis grade)	2g
Distilled water to:	250ml

Stored at 4°C in the dark.

4) Ammonium persulphate (Gibco BRL)

50mg ammonium persulphate dissolved in 500µl distilled water.

5) Temed (Gibco BRL electrophoresis grade)

Undiluted stock, stored at 4°C.

6) SDS-PAGE running buffer

Tris - HCl (Gibco BRL)	15.15 g
Na Glycine (Gibco BRL)	72.05g
Sodium dodecyl sulphate	5g
Distilled water to:	5000ml

Stored at RT.

7) Sample buffer

Glycerol (analar)	2.0ml
2-mercaptoethanol (analar)	1.0ml
10% sodium dodecyl sulphate	4.0ml
Tris-HCl upper gel buffer	2.5ml
bromophenol blue 0.01%	1mg
Distilled water to:	10ml

500µl volumes were stored frozen.

8) Coomassie Blue Stock

Coomassie Brilliant Blue R-250 (Sigma)	0.125g
methanol (analar)	200ml
acetic acid (conc.)	35ml
Distilled water to:	500ml

Powdered dye was mixed in methanol and acetic acid added while mixing. Stored at RT.

9) Gel destain solution

methanol (analar)	500ml
acetic acid (analar)	100ml
Distilled water to:	400ml

Stored at RT.

10) Molecular weight marker (Gibco BRL)

Pre-stained, high range 200.0 kD, 97.4, 68, 43, 29, 18.4, 14.3 kD.

11) SDS-PAGE equipment

Biorad Mini-PROTEAN II electrophoresis cell and accessories

4.2.3 Protein electro-blotting

1) Towbin buffer

Tris-HCl	3.03g
Glycine	14.4g
Distilled water to:	1000ml

pH buffer to 8.3 and stored at RT.

1) Blotting equipment

Biorad semi-dry transblot apparatus and power pac.

4.2.4 DAKO O:3 LPS ELISA assay

1) DAKO O:3 LPS ELISA Kit

Supplied by Med-Bio NZ Ltd. and used as per manufacturer's instructions. The DAKO assay utilises microtitre trays as the solid phase and was supplied as a complete kit. This assay detected serum antibodies by their reaction with *Y. enterocolitica* O:3 lipopolysaccharide (LPS) antigen coated onto the surface of microwells. Bound antibody was detected using peroxidase-conjugated antibody to human immunoglobulins IgA and IgG. Conjugated antibody bound to fixed human antibody was detected with the addition of a chromogenic substrate with colour development proportional to levels of serum antibodies to *Y. enterocolitica* in the sample. Colour development was measured spectrophotometrically (DAKO, *Yersinia enterocolitica* O:3 ELISA, Kit Insert).

4.2.5 IgA RP bead ELISA assay

The IgA RP bead assay was developed for the screening of blood donor sera and utilised the bead technology of the Abbott Commander ELISA Processor equipment. Released

proteins (RP), produced by pathogenic *Y. enterocolitica* under controlled laboratory conditions were prepared and then used to coat polystyrene beads. Serum antibodies bound to the RP's on the bead were detected using a peroxidase labelled anti-human IgA immunoglobulin. Substrate OPD was converted from clear to a yellow/brown colour by the peroxidase and the absorbance measured spectrophotometrically.

1) 6mm polystyrene beads

Supplied by Abbott Laboratories Chicago USA, through Abbott Diagnostics Division, Auckland, New Zealand.

2) 20 & 60 well plastic reaction trays

Supplied by Abbott Laboratories Chicago USA, through Abbott Diagnostics Division, Auckland, New Zealand.

3) 6mm spectrophotometer blanking beads

Supplied by Abbott Laboratories Chicago USA, through Abbott Diagnostics Division, Auckland, New Zealand.

4) Substrate OPD (O-phenylenediamine-2HCl) and substrate diluent

1 tablet to 5ml of substrate diluent. Supplied by Abbott Laboratories Chicago USA, through Abbott Diagnostics Division, Auckland, New Zealand.

5) 1N H_2SO4 reaction stopping solution

Supplied by Abbott Laboratories Chicago USA, through Abbott Diagnostics Division, Auckland, New Zealand.

6) Goat anti-human immunoglobulin A

Peroxidase conjugated, affinity isolated, human immunoglobulin absorbed. (TAGO Immunologicals, Camarillo, California, USA). Obtained from Dainabot, Japan.

7) Phosphate Buffered Saline (pH 7.4)

Sodium chloride	8.0g
Potassium chloride	0.6g
Potassium dihydrogen phosphate	0.2g

di-Sodium hydrogen phosphate	1.14g
Distilled water up to:	1000ml

Dispensed into 50ml volumes, sterilised at 121°C for 15 minutes and stored at room temperature.

8) Stock 2.5% Casein

Casein (Sigma C-5890)	25g
0.3M sodium hydroxide	800ml

Casein was dissolved in NaOH at 37°C to aid solution. pH was readjusted 7.0 with HCl. Stored at 4°C.

9) ELISA wash solutions

PBS + 0.05% Tween 20 (microtitre tray ELISA)

MQ water (bead assay)

10) Bead ELISA equipment

Dynamic Incubator

ELISA Parallel Processing Centre (PPC)

Flexible Pipetting Centre (FPC)

Supplied by Abbott Laboratories Chicago USA, through Abbott Diagnostics Division, Auckland, New Zealand and used as per manufacturer's instruction.

11) Microtitre tray ELISA equipment

Behring ELISA II processor. Used as per manufacturer's instructions.

12) Positive control serum

Citrated plasma from a *Y. enterocolitica* bacteraemic blood donor. Plasma had been separated from the red cells by centrifugation and stored frozen immediately following donation collection. The serum was strongly reactive for IgA antibody to *Y. enterocolitica* in both the bead IgA and DAKO assays. Serum was diluted with PBS to give an OD_{492} of between 1.0-1.5. Each batch of testing included duplicate positive controls.

13) Cutoff serum

Serum from a blood donor enrolled in stage 1 of the clinical trial. Criteria used for the

selection of this serum included:

- no history of recent GIT illness for previous 3 months or contacts of
- negative stool culture for *Y. enterocolitica*
- negative blood cultures for *Y. enterocolitica*
- a serum providing an OD_{492} approximately 2 x the mean OD_{492} (0.135) of the stool culture negative sera, collected in stage 1 of the clinical trial.

On the basis of these criteria, serum from donation number 8558164 with an OD_{492} 0.129 was selected as the serum for the IgA RP bead ELISA assay.

Batches of ELISA testing included three replicates of the cutoff serum and the assay cutoff value was determined from the mean OD_{492} of the three cutoff sera. Cutoff and positive control sera were included with each batch of samples. Sera with an OD_{492} greater than the mean OD_{492} of the replicates (x3) of the cutoff serum were considered positive in the assay. Sera with an OD_{492} less than the mean cutoff value were considered negative in the assay. All positive sera were confirmed on repeat by duplicate retest and sera with OD_{492} value $\pm$ 10% of the mean of the cutoff sera were also repeated.

4.2.6 Clinical trial - blood collection

Donations from 495 blood donors enrolled in the clinical trial (described later) were collected from volunteers in two stages. All pre-donation interviews, blood collection and blood processing was conducted by staff from the Regional Blood Service of Mid-Central Health Ltd. All blood donations were collected in Baxter blood bags and processed into AS-1 red cells (as described previously).

4.2.7 Serum samples

Serum samples are identified as groups 1, 2 and 3 as previously described.

Group 1 sera

Seven serum samples from healthy blood donors asymptomatic with *Y. enterocolitica*

bacteraemia at the time of blood donation were made available for testing (see Fig 4.1). In each case the bacteraemic state at the time of donation was confirmed with the finding that the donated unit of AS-1 red cells was heavily infected with *Y. enterocolitica* after varying periods of blood bank storage at 4°C. In five cases discovery of unit infection followed the development of post transfusion endotoxic shock in the recipient. In two, discovery was made prior to transfusion after the units appeared blackened on visual inspection. In all but one case confirmation of infection with *Y. enterocolitica* was made by unit culture.

Each serum in this group was representative of the immune status of the donor, at the time the infected donation was made. Sera from each donation in this group had been held in frozen storage as outlined the NZ Standards for Blood Transfusion Services. Serum samples were provided by each of the Regional Transfusion Centres identified in (Fig. 4.1). Samples from the first three transfusion reactions identified as 1, 2 & 3 in Chapter I of this thesis were not available for testing. Insufficient volume for sample Ha1 (rf. Fig 4.1), allowed ELISA testing using the DAKO assay only.

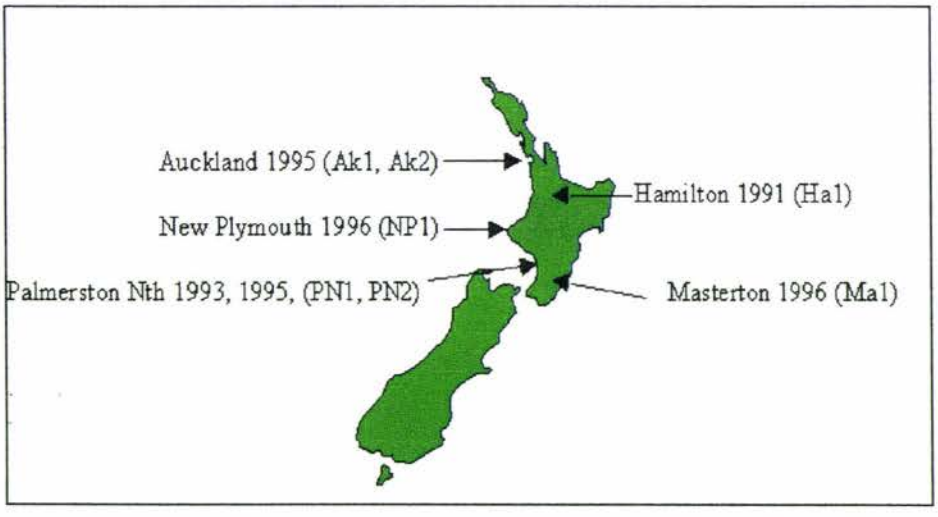


Figure 4.1 Geographical distribution and identification of group 1 serum samples.

Group 2 sera

Sera from stool culture positive *Y. enterocolitica* O:3 infection in 27 patients tested by Diagnostic Laboratory in Auckland were provided by Dr. Arthur Morris. The sera had been collected from patients as part of an investigation into the immune response and the incidence of bacteraemia in *Y. enterocolitica* GIT infection. In all of the cases, patients

had presented with GIT symptoms and returned a positive stool culture for *Y. enterocolitica.* The serum samples consisted of 26 sets of paired sera (samples A & B) and 1 set of three sera (samples A, B & C). Sample A in each set had been collected at the time of stool culture positivity for *Y. enterocolitica.* The time interval between samples A and B varied between 4 and 10 weeks. Sample B represented a convalescent serum. The single sample C had been collected approx. 12 weeks after initial stool culture positivity.

Group 3 sera (clinical trial stages 1 & 2)

Volunteer blood donors from within the MidCentral Health Ltd. blood collection region were enrolled in a trial to test the performance of the newly developed IgA RP bead assay described in this thesis. The purpose of the trial was to assess seropositivity to infection with *Y. enterocolitica* in the donor population and to check assay performance in a clinical trial setting. The trial was divided into stages 1 and 2 and collected serum from a total of 495 volunteer blood donors. All enrolled donors met the minimum requirements for blood donation as outlined on the blood donor questionnaire (see appendix) and had been free from recent gastrointestinal infection, diarrhoeal illness, for the previous month.

4.2.8 Stool culture

Donor stool samples were requested from all participants enrolled in stage 1 of the trial. The first sample was provided within 48 hours of blood donation collection and the second approximately 1 calendar month later. Blood donors seropositive in the IgA RP bead assay in stage 2 were requested to provide a single stool sample for culture *for Y. enterocolitica.* All stool samples were cultured for *Y. enterocolitica* by Medlab Central Ltd.

4.2.9 Blood culture

AS-1 red cells collected from volunteer blood donors in both stages 1 & 2 of the clinical trial were cultured using the Bactec 2000 blood culture system (described later). All cultures were performed by Medlab Central Ltd..

4.3 Method

4.3.1 Released protein preparation

In vivo, pYV^+ strains of *Yersiniae* release plasmid encoded proteins at 37°C under Ca^{++} restricted conditions (Kittelberger *et al.*, 1995; Michiels *et al.*, 1990; Robins-Browne *et al.*, 1993). Under similar laboratory conditions pathogenic *Yersiniae* can be induced to release plasmid encoded proteins into culture medium.

A single colony of pYV^+ *Yersinia enterocolitica* O:3 (PN2) was picked from an overnight culture of infected blood grown on CR-MOX agar. The colony was used to inoculate 20ml of BHI-B and the culture incubated O/N at 25°C with shaking @ 150 rpm. The next day the culture was used to aseptically inoculate 500ml of pre-warmed freshly sterilised BHI-B in a 2L Erlenmeyer flask. The baseline OD_{600} was measured (Nova Tech, Spectrophotometer) and the culture incubated at 37°C with agitation (150 rpm). The culture was sampled at 30 minute intervals until an OD_{600} of 0.4 (approx. 6.0×10^7 cells per ml) was reached. Filter-sterilised EGTA was added to a final concentration of 15mM and incubation continued for a further 120 minutes or until a final OD_{600} of 1.0 was reached. Incubation was stopped and the bacteria pelleted at 5000*g* for 20 minutes at 4°C (Sorvall RC-5B/GSA) in sterilised 300ml Nalgene centrifuge containers. The supernatant was filtered using a 0.45µm clarifying cellulose acetate filter and ammonium sulphate (47g per 100ml) added to the clarified broth, with agitation. Proteins were precipitated at 4°C overnight with constant stirring and transferred into sterile 350ml Oak-Ridge Nalgene centrifuge bottles. The proteins were pelleted at 20,000g for 1 hour at 4°C (Sorvall RC-5B/GSA), the supernatant decanted from the sedimented proteins and proteins re-dissolved in 10ml of sterile MQ. The protein samples were transferred to 1.5cm dialysis tubing (10 kDa cutoff) and dialysed against 2L of MQ water (X4) at 4°C for 48 hours. The dialysed proteins were volume reduced O/N in a vacuum chamber at 25°C and 1000 torr. negative pressure. RP's were aliquotted into 1500µl volumes, labelled and stored frozen at -80°C.

4.3.2 Biorad protein assay

Protein quantitation of the RP's was performed by Bio-Rad protein assay that was based on

the protein dye-binding of Coomassie brilliant blue (Bradford, 1976). Serial doubling dilutions of bovine serum albumin (BSA) standard (1.39mg/ml) were prepared in MQ water. 100µl of each dilution of the standard and RP's were pipetted into 4ml of working protein reagent. Tubes were mixed and absorbance was measured after 30 minutes at 495nm (Nova Tech spectrophotometer) in plastic 1cm cuvettes.

4.3.3 SDS-PAGE

Visualisation of molecular weights of the released proteins was performed by SDS-PAGE using a discontinuous buffer system. Sample and stacking gels contained Tris-Cl (pH 6.8), and upper and lower buffer reservoirs contained Tris-glycine (pH 8.3). The resolving gels contained Tris-Cl (pH 8.8). Chloride ions in the sample and stacking gel form the leading edge of the moving boundary, and the trailing edge is composed of glycine molecules. Between the trailing and leading edges of the moving boundary a zone of lower conductivity and steeper voltage gradient sweeps the polypeptides from the sample and deposits them on the surface of the resolving gel. The higher pH of the resolving gel favours ionisation of glycine and resulting glycine ions migrate through the stacked polypeptides travelling through the resolving gel immediately behind the Cl^- ions. SDS-polypeptide complexes move through the resolving gel in a zone of uniform voltage and pH and are separated according to size by acrylamide sieving (Sambrook, 1989).

SDS-PAGE gels with 4% acrylamide stacking and 12.5% acrylamide resolving gel were used for proteins within the expected range of 15-100 kDa. (Laemmli, 1970). Glass plates and spacers were assembled in the Bio-Rad aligning device, clamped into the plate holder and transferred to the casting stand. For the lower gel, buffer, acrylamide/bis and water were mixed (Table 4.1). Ammonium persulphate (50mg/500µl reverse osmosis (RO) water) and 10µl of Temed were added to the acrylamide mix and loaded into the assembled sandwich, 1cm from the top of the cutout on the front glass plate. Water-saturated isobutanol was pipetted onto the surface of the acrylamide and the gel left to polymerise for 2 hours at RT. The surface of the set lower gel was washed with Tris gel buffer and blotted dry with filter paper and the upper gel acrylamide mix prepared (as per table) and poured onto the lower gel up to the top of the glass plate cutout. A 10 well comb was inserted between the glass plates and the gel left O/N to complete polymerisation.

Next day the comb was removed, gel pockets straightened and separated as required. The sandwich assembly was removed from the casting stand and clipped into place on one side of the electrophoresis inner cooling core apparatus. This step was repeated for both sides when running two gels simultaneously. When one gel only was required a buffer dam was prepared by clamping two glass plates together and the clamp assembly fitted into the opposite side of the inner core cooling assembly.

Released proteins and MW marker (Gibco BRL) were diluted 50:50 in sample buffer, boiled for 5 minutes. then allowed to cool to RT. The inner core of the electrophoresis apparatus was filled with running buffer until a level halfway between the short and long plates was reached. Wells were rinsed with running buffer using a glass pasteur pipette and 15µl of RP sample (approx 12.5ug) loaded into each well under the electrode buffer, using a Gilson P20 pipettor and fine tip. 5µl of diluted MW marker was added to the MW lane. Positions of the samples and MW markers were recorded.

Reagents	**12.5% Lower gel**	**4.0% Upper gel**
Lower gel buffer	10ml	
MQ water	16.6ml	6.66ml
Acryl/Bis	13.4ml	1.33ml
Ammonium persulphate	0.12mg	0.03mg
Temed	10µl	10µl
Upper gel buffer		2.5ml

Table 4.1 Reagents for SDS-PAGE gels

The inner gel core was lowered into the buffer chamber of the mini-PROTEAN-II cell containing sufficient running buffer to cover 1-2 cm above the base of the gel. Bubbles were removed by swirling running buffer under the gel sandwich. The lid was fitted to match the colour coded red/black electrodes on the inner chamber and power leads connected to the power source (Bio-Rad Power Pac 300). Voltage/current settings were 200v/60mA per single gel and gels run for 45 minutes. Power was disconnected and the

inner cooling core removed from the chamber. The gel sandwich was separated from the clamp assemblies, removed from the glass plates and stained in Coomassie Blue with agitation for 60 minutes. The gel was destained until the bands were clearly visible and the background of the gel was clear (4-8 hours).

4.3.4 Protein electro-blotting

Coomassie stained released proteins of pYV^+ *Y. enterocolitica* were transblotted onto PVDF (Polyscreen) membrane using a Bio-Rad Trans-blot, semi-dry electrophoretic transfer cell. The gel was equilibrated in pre-chilled Towbin buffer for 15 minutes and presoaked filter paper (Bio-Rad) placed flat onto the surface of the platinum electrode. Prewetted PVDF membrane was cut to the size of the gel and placed on top of the filter paper. The gel was placed on the top of the membrane and air bubbles excluded. A second sheet of soaked filter paper was placed on the top of the gel, the cathode placed on the gel/membrane stack and fitted in to place on the guide posts of the apparatus. The safety cover was fitted and the power leads from the apparatus connected and plugged to a Bio-Rad power supply Model 200/2.0. Power was set to 10 volts with a transfer time of 30 minutes. The band of interest was cut from the PVDF and supplied for N-terminal protein sequencing.

4.3.5 DAKO O:3 LPS ELISA assay

The kit was supplied with all necessary reagents and consumables. Serum samples were diluted in sample diluent (1:600 for IgA and 1:6000 for IgG) and 100µl transferred to each of two wells in an LPS coated microtitre tray. Controls were diluted in the same manner as samples and included cutoff, positive controls and sample diluent control. Following the addition of serum, the trays were sealed and incubated at RT for 2 hours. Wells are washed 4 times (DAKO wash solution) and 100µl of peroxidase labelled anti-IgA and IgG added to the wells and the tray covered for 1 hour at RT. Following washing 100µl of chromogenic substrate was added to each well. After 15 minutes 100µl of dilute acid (DAKO) was added to each well to stop colour development. The OD_{490} was measured against a sample diluent blank using an ELISA reader.

4.3.6 IgA RP bead ELISA assay

A prototype assay was developed in microtitre trays because of ease of use and smaller reagent costs. 100µl of released protein diluted in fresh PBS (pH 7.8) at a concentration of 2µg/100µl was used to coat each well of a Nunc microtitre tray. Trays were left covered at RT O/N and washed before being blocked with 0.5% Casein in PBS and dried.

Sera were diluted 1:500 in 0.05% Casein PBS and 100µl transferred to each of two wells of the RP coated microtitre tray. Trays were sealed (adhesive tray seal) and incubated for 1 hour at 37°C and then washed 4 times in PBS/Tween. 100µl of peroxidase labelled anti-human IgA (1:2000 in 0.05% Casein/PBS) was added to each well, and the tray covered and incubated for 1 hour at 37°C. The tray was washed (as before) and 100µl of chromogenic substrate added to the wells. After 30 minutes 100µl of 1N H_2SO_4 was added to each well to stop colour development. OD_{492} was measured using an ELISA reader.

6mm polystyrene beads were used as the solid phase in the bead assay. Beads were pre-ground to provide consistent size and surface area for protein coating and were supplied by Abbott. Protocols established in the protein coating and blocking experiments in microtitre

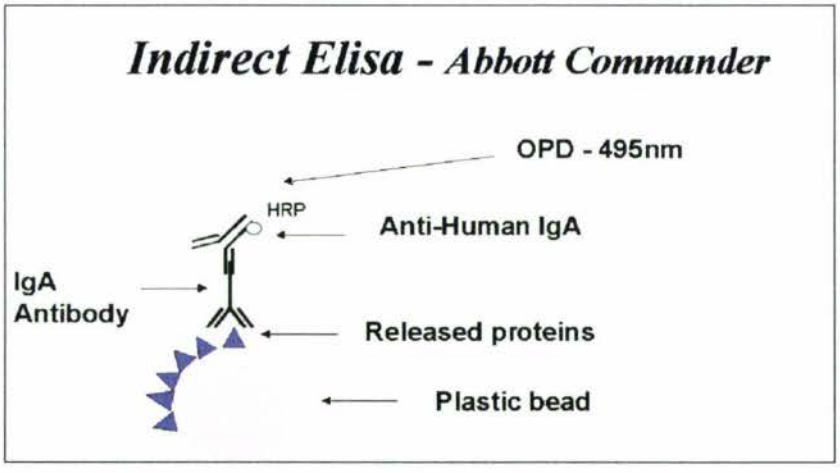


Figure 4.2 Diagrammatic representation of the Indirect ELISA bead assay developed for the Abbott Commander ELISA system.

trays, were transferred to the bead assay with minor modifications to suit the differing matrix and to accommodate suggestions made by Dainabot (Japanese division of Abbott

USA). 6mm polystyrene beads were used as the solid phase in the assay.

69.2g of plastic beads (500) were transferred to a fresh acid washed glass jar containing 250ml of 50% absolute alcohol in MQ water. The jar was sealed and rotated at 10rpm for 30 minutes. at RT. Alcohol was drained from the jar and the beads washed in fresh PBS x6 with agitation between washes. Released protein was diluted in 110ml of fresh coating buffer (0.1M phosphate buffer pH 7.8) to provide 2µg/bead (recommended volume of coating buffer 0.223ml/bead). The coating mix and beads were mixed in a clean acid washed glass jar and rotated at 7-10 rpm for 20 hours at RT on a drum roller. Coating buffer was decanted and the beads washed x4 with fresh PBS (rotated 10 minutes between each wash). 110ml of fresh blocking buffer (0.5M Glycine buffer, 0.5% Casein, pH 8.0) was mixed with the beads and rotated at 7-10 rpm for 60 minutes at RT. Beads were drained and washed x3 with fresh PBS before the addition of 110ml of 2.0% sucrose. After gentle mixing for 10 minutes the beads were transferred to a cotton mesh bag and packed into a large centrifuge bottle (Nalgene), lined with absorbent material. The beads were centrifuged (Sorvall/GSA) for 10 minutes at 2000g, transferred to a large filter paper lined glass Petri dish and dried overnight at 37°C. Beads were stored at 4°C with desiccant.

Performance of the assay

All ELISA testing was performed using the Abbott Commander ELISA Processor system. Where possible testing was performed in batches using the same cutoff and control sera, same batch of coated beads and same reagents to minimise batch to batch variation.

Test and control sera were diluted 1:400 in fresh 0.5% Casein in PBS. 200µl of diluted sample, cutoff serum and positive control were pipetted into the wells of an Abbott ELISA reaction tray. An antigen-coated bead was added to each well containing diluted serum, the tray sealed (Abbott adhesive) and incubated with shaking (Abbott Dynamic incubator) at 40°C for 30 minutes. After 30 minutes, the trays were washed with fresh MQ water in the Abbott Commander ELISA Processor and 200µl of conjugated secondary antibody (goat anti-human IgA peroxidase labelled) was added to each reaction well. Tray wells were resealed and incubated at 40°C for 30 minutes (with shaking). The wells were again washed (as before) and 300ml of OPD substrate added to each well. The trays were left

covered at RT (in the dark) for 30 minutes to allow colour development, before the reaction was stopped with 300µl of 1N H_2SO_4. The OD_{492} was measured against OPD blanking beads in blanking trays (Abbott).

4.3.7 Stool culture

All stool samples were cultured using routine procedures for the isolation of gastric pathogens. This included a direct plate culture at 37°C and Selenite F enrichment followed by subculture onto CIN selective agar.

4.3.8 Blood culture

Stage 1 Following plasma depletion and reconstitution with additive solution, 20ml of AS-1 red cells was sampled aseptically from each of the 30 units collected. A pair of BACTEC (Becton Dickinson) blood culture bottles (aerobic and anaerobic) were inoculated and incubated in a BACTEC 9000 series blood culture chamber for 7 days at 37°C. All cultures were checked automatically at 10 minute intervals for the release of radiolabelled Carbon, the indicator of bacterial growth used in the BACTEC system.

After 35 days storage at 4°C, the same AS-1 red cell units were aseptically sampled and 100ul of blood used to innoculate Brain Heart Infusion Agar. Plates were incubated for 48 hours at 37°C in 5% CO_2 for sterility.

Stage 2 AS-1 red cells from volunteer blood donors positive in the IgA RP bead assay trial were cultured onto Brain Heart Infusion Agar at the completion of 35 days storage at 4°C

4.3.9 Blood collection

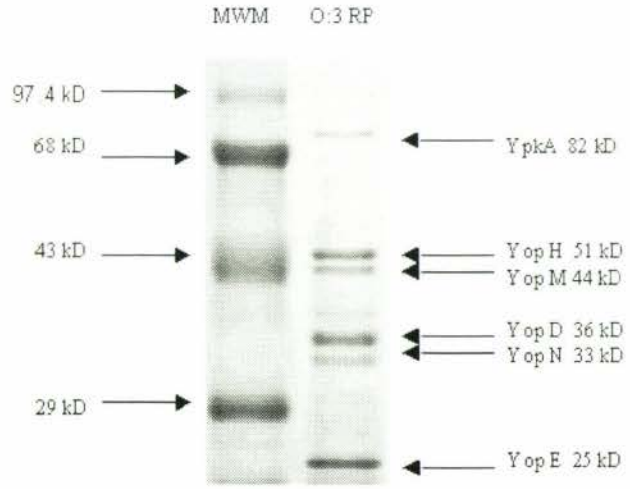
450ml of blood was collected into Baxter blood bags from 495 volunteer blood donors enrolled in the clinical trial. Anticoagulated blood was separated by centrifugation (3,500 rpm 15 minutes) into red cells and plasma using a Heraeus Christ (model 2000) Cryofuge at 4°C. Plasma was stored frozen at -30°C and red cell additive solution (Adsol) added to

the concentrated red cells. Serum collected for stage 1 of the trial was stored frozen until all 30 donations had been collected. Sera collected during stage 2 was stored overnight at 4°C prior to ELISA testing.

4.4 Results

4.4.1 SDS-PAGE

The identity of the RP's (Fig. 4.3) was inferred from their characteristic electrostatic profile as published (Hakansson *et al.*, 1993; Iriarte & Cornelis, 1999). Predominant bands in the gel are Yops H, D and E. Confirmation of position of the Yops in the gel was performed by chromatographic N-terminal, amino acid sequencing of the 36 kDa protein YopD (Amino Acid Analysis and Protein Sequencing Unit, Institute of Molecular Biosciences, Massey University). The sequence of the first five amino acids TINIK, closely resembled the published sequence of MTINIK (ENTREZ, GENBANK accession no. L06216). Methionine is traditionally a low yield amino acid in chromatographic N-terminal sequencing making determination of its presence at start of a sequence difficult. The sequence of TINIK was taken as confirmation of the position of Yop D and the accuracy of the assigned molecular weights of the Yops presented (Fig. 4.3).



MWM = molecular weight marker

Figure 4.3 Predominant bands of the released proteins of pYV$^+$ *Y. enterocolitica* strain PN2 separated by SDS-PAGE.

4.4.2 Biorad protein assay

Optical density values of the diluted standard were plotted on graph paper and a curve drawn. Protein concentration of batches of bacterial RP (Table 4.2) was calculated from OD_{492} values extrapolated against the standard curve.

RP (batch No.)	Protein mg/ml
7	0.5
8	1.0
9	1.0
10	0.4
11	0.82
12	0.83
13	0.9
14	0.7

Table 4.2 Protein quantitations of batches of released protein.

4.4.3 ELISA blocking agents

The high sensitivity of ELISA is a balance between the limitation of background signal caused by non-specifically bound reactants in test sera and reagents and retention of adequate reactivity with positive sera. Background reactivity is reduced through blocking of the solid matrix with inert or irrelevant proteins. To achieve this bovine serum albumin (BSA Sigma) and Casein (Sigma) were tested, with optimal blocking obtained using 0.5% Casein for both the tray and bead ELISA's. Blocking efficiency was established using known positive sera and pooled normal sera. BSA proved to be less effective as a blocking agent than Casein, with high OD values for the positive serum but unsatisfactory blocking effect in the blanks and pooled sera wells. Low background and strong positive control reactions were obtained for both pooled and positive control sera using a sample dilution of serum of 1:400 in 0.5% Casein/PBS. The dilution of conjugated (secondary) antibody of 1:2000 in 0.05% in Casein/PBS provided optimal reactions.

4.4.4 ELISA wash solutions

The inclusion of a weak non-ionic detergent is indicated in some ELISA applications. Wash solutions containing Tween 20 improved performance in the tray assay but its inclusion in the IgA RP bead assay disrupted the solid phase protein coat causing an increased non-specific background signal for replicates of the cutoff serum. The problem was solved with the omission of detergent from the MQ water used for the bead assay washes.

4.4.5 ELISA sample testing

Group 1 sera - DAKO LPS ELISA assay

Testing was performed according to the instructions in the DAKO Kit package insert (as described earlier). A positive result in this assay is one in which the sample OD_{490} is greater than the mean of the OD_{490} of the cutoff serum. A negative result is one in which the sample OD_{490} is less than the mean of the cutoff serum supplied with the kit. Samples ± 10% of the cutoff serum are considered to be doubtful (DAKO, *Yersinia enterocolitica* O:3 ELISA, Kit Insert). Results of ELISA testing showed that 5/7 sera from bacteraemic blood donors were positive in the DAKO assay (Table 4.3).

Sample ID	anti-IgA OD_{490}	Interpreted result	anti-IgG OD_{490}	Interpreted result
Ak1	0.62	pos	0.10	neg
Ak2	1.8	pos	0.14	neg
Ha1	0.07	neg	0.09	neg
NP1	0.42	pos	0.58	pos
PN1	0.14	neg	0.09	neg
PN2	1.92	pos	0.13	neg
Ma1	> 2.2	pos	1.68	pos

Table 4.3 Results of ELISA testing for serum IgA (cutoff 0.18) and IgG (cutoff 0.16) antibody to *Y. enterocolitica* O:3 LPS (DAKO) in bacteraemic blood donors.

Group 1 sera - IgA RP bead ELISA assay

In the anti-IgA RP bead assay all group 1 sera (insufficient Ha1) showed reactivity. Both O:9 infections reacted in the assay demonstrating the cross-reactive properties of the Yersinia released proteins (Table 4.4).

Sample ID	OD_{492}	Interpreted result
Ak1	1.7	pos
Ak2	0.6	pos
NP1	0.4	pos
PN1	1.8	pos
PN2	2.0	pos
Ma1	2.2	pos

Table 4.4 Results of ELISA testing for serum IgA antibody (cutoff OD_{492} 0.261) *to Y. enterocolitica* (released protein) in bacteraemic blood donors.

Sera with an OD_{492} exceeding the mean of the three replicates of the cutoff serum were classified as positive in the assay. A positive result indicates either current/recent or chronic infection with *Y. enterocolitica*. Sera with an OD_{492} less than the mean of the cutoff serum were classified as negative with the donor less likely to have a current or chronic infection with *Y. enterocolitica*. All sera from bacteraemic blood donations tested for IgA antibody to the RP's of *Y. enterocolitica* showed seropositivity to the infection at the time of donation (Table 4.4). Sensitivity for the IgA RP bead assay calculated using Bayesian analysis demonstrates showed that with an 80% confidence interval, 80-100% of all bacteraemic blood donors could be expected to be seropositive in the assay. Bayesian analysis allowed adjusted for low sample numbers, and reduced confidence interval from the usual 95% to 80%.

Group 2 sera

The IgA antibody response showed variability with most patients (25/27) seropositive in sample A (Table 4.5) at the time *Y. enterocolitica* was cultured in their stools (sample A). 19/27 sample A sera showed a strong IgA immune response (OD range >1.0) to the

infection with 6/27 showing a weaker antibody response (OD range 0.40-1.0). IgA antibody present in the group B samples showed that levels remained equally as strong (2/27), decreased (19/27) or increased (5/27) in comparison to group A samples. Sample No. 21 failed to react. Sample 11 showed weak seropositivity in samples A and B with

Sample ID	OD_{492} sample A	OD_{492} sample B	OD_{492} sample C	Result
1	>2.2	>2.2		Pos
2	2.103	0.318		Pos
3	<2.2	2.026		Pos
4	>2.2	1.019		Pos
5	>2.2	0.822		Pos
6	1.512	2.065		Pos
7	2.014	0.585		Pos
8	>2.2	0.64		Pos
9	0.694	0.57		Pos
10	0.495	0.918		Pos
11	0.186	0.364	0.433	Pos
12	0.335	0.492		Pos
13	0.624	0.524		Pos
14	>2.2	1.165		Pos
15	>2.2	0.513		Pos
16	0.793	0.335		Pos
17	1.948	0.996		Pos
18	0.4	0.652		Pos
19	>2.2	1.698		Pos
20	>2.2	1.809		Pos
21	**0.021**	**0.023**		**Neg**
22	>2.2	0.837		Pos
23	1.837	0.423		Pos
24	1.054	0.373		Pos
25	>2.2	0.704		Pos
26	>2.2	>2.2		Pos
27	>2.2	>2.2		Pos

Table 4.5 Serum IgA antibody (cutoff OD_{492} 0.248) to *Y. enterocolitica* RP's from stool culture positive GIT infection.

levels continuing to increase 12 weeks after initial culture positivity (Table 4.5). Findings of a poor antibody response to *Y. enterocolitica* infection have been reported to correlate

with chronicity and rheumatopathologic sequelae. The sensitivity of the bead assay at detecting antibody presence during GIT infection with *Y. enterocolitica* provided a range of 95% confidence for between 81.65 to 99.12% of cases. Overall predictive value PV_{pos} for the assay for samples from patients infected with *Y. enterocolitica* was high (96.3%) with seropositivity demonstrable in the serum of 26/27 samples.

A comparison of performance of the IgA RP bead assay and the DAKO LPS assay, against the group 2 sera (Table 4.5) showed higher rates of seropositivity in the bead assay (26/27) as compared to the DAKO LPS assay (19/27) (Personal communication, A. Morris 1998). The explanation for this is unknown. Major differences between the two assays are the target antigens used to coat the plastics and the solid phase (previously described). The IgA bead assay utilises a larger number of target proteins that were prepared from a strain of *Y. enterocolitica* recently isolated from a unit of infected blood. The DAKO assay utilised a single antigen derived from the LPS of serotype O:3. Both types of antigens are reported to illicit a strong antibody response following infection (previously described). It is probable that a combination of these factors affected the poor performance of the DAKO assay in this study.

Group 3 - Stage 1

Stool culture on the donor of unit No. 8558180 was positive for *Y. enterocolitica* at the time of donation. A repeat sample from this donor cultured 1 month later was negative for *Y. enterocolitica*. Serum from the donor of unit No. 8558180 contained elevated levels of antibody ($OD_{492} = 0.332$) in the bead IgA RP bead assay (Table 4.6) in comparison to stool culture negative donors from this group (mean $OD_{492} = 0.072$). The results of blood culture on donation No. 8558180 at day 1 and day 35 were negative for *Y. enterocolitica.*

Serum from the donor of unit number No. 8558164 gave an OD_{492} of 0.129 in the assay. This donor was stool culture negative for *Y. enterocolitica*, and returned negative blood cultures at days 1 and 35. This sample was selected as the cutoff serum for stage two using the criteria outlined previously. Replicate retesting of sera 8558180 and 8558164 in the anti-IgA RP assay showed reproducible values for both sera (CV < 5%). (data not shown).

Group 3 - Stage 2

Results of serum ELISA, stool and blood culture testing on donors/donations positive for IgA antibody to the RP's of *Y. enterocolitica* in stage 2 are presented (Table 4.7). The complete set of results of testing of sera from 465 donors enrolled in stage two of the clinical trial are presented in the appendix. All sera initially positive in the anti-IgA RP assay were confirmed reactive on duplicate retest. The assay showed good reproducibility

Sample ID	OD_{495}	Stool culture #1	Stool culture # 2	AS-1 unit culture #1	AS-1 unit culture #2
8558213	0.077	neg	neg	neg	neg
8558167	0.071	neg	neg	neg	neg
8558227	0.106	neg	neg	neg	neg
8558224	0.066	neg	neg	neg	neg
8558183	0.06	neg	neg	neg	neg
8558225	0.066	neg	neg	neg	neg
8558166	0.059	neg	neg	neg	neg
8558223	0.028	neg	neg	neg	neg
8558209	0.099	neg	neg	neg	neg
8558180	**0.332**	**pos**	**neg**	**neg**	**neg**
8558211	0.055	neg	neg	neg	neg
8558203	0.042	neg	neg	neg	neg
8558174	0.05	neg	neg	neg	neg
8558208	0.062	neg	neg	neg	neg
8558164	**0.129**	neg	neg	neg	neg
8558210	0.043	neg	neg	neg	neg
8558239	0.067	neg	neg	neg	neg
8558155	0.056	neg	neg	neg	neg
8558286	0.082	neg	neg	neg	neg
8558255	0.057	neg	neg	neg	neg
8558158	0.07	neg	neg	neg	neg
8558163	0.079	neg	neg	neg	neg
8558130	0.08	neg	neg	neg	neg
8558375	0.074	neg	neg	neg	neg
8558135	0.086	neg	neg	neg	neg
8558204	0.063	neg	neg	neg	neg
8558397	0.056	neg	neg	neg	neg
8558349	0.029	neg	neg	neg	neg
8558125	0.071	neg	neg	neg	neg

Table 4.6 Summary of results of IgA RP ELISA assay, stool and blood culture testing on volunteer blood donors enrolled in stage 1 of the clinical trial.

on duplicate sample retest (CV <5% - data not shown) for initial positive sera. Blood donors serologically positive in the assay were requested to provide a stool sample for culture. The AS-1 red cells from the serology positive donors were quarantined from blood donations serologically negative in the assay. Assay positive blood donations were stored at 4°C for 35 days and then cultured (BHI agar plate) for sterility as previously described.

20 of 495 (4.04%) blood donors tested positive for current/recent or chronic infection with *Y. enterocolitica*. 1 of the 20 (5.3%) blood donors seropositive in the assay shed viable *Y. enterocolitica* in their stools. The incidence of culture positivity in the study was of

Donor ID	OD_{492}	Cutoff	ELISA Result	Stool culture	AS-1 unit culture
8558180	0.332	0.245	**pos**	**pos**	neg
8561280	0.536	0.214	**pos**	neg	neg
8561284	0.683	0.214	**pos**	neg	neg
8561314	0.482	0.214	**pos**	neg	neg
8559372	0.609	0.214	**pos**	neg	neg
8561347	0.956	0.212	**pos**	neg	neg
8559379	2.163	0.212	**pos**	neg	neg
8559416	0.413	0.203	**pos**	neg	neg
8559419	1.124	0.203	**pos**	neg	neg
8559432	0.525	0.243	**pos**	neg	neg
8554511	0.510	0.219	**pos**	neg	neg
8561463	0.492	0.219	**pos**	not done	neg
8554534	0.642	0.209	**pos**	neg	neg
8561566	0.667	0.303	**pos**	neg	neg
8554593	1.138	0.303	**pos**	neg	neg
8554599	0.761	0.303	**pos**	neg	neg
8554601	>2.2	0.303	**pos**	neg	neg
8562266	1.261	0.347	**pos**	neg	neg
8562268	0.662	0.347	**pos**	neg	neg
8561712	0.635	0.316	**pos**	neg	neg

Table 4.7 Summary of results from IgA RP assay, stool, blood cultures.

0.202% (1/495) or approximately 20/100,000. All blood from seropositive blood donors (20) remained sterile after 35 days storage at 4°C. The overall seropositivity rate of 4.04% for IgA antibody to *Y. enterocolitica* in the trial was slightly higher than expected. This

number is likely to include a number of chronic carriers of the infection, those who have had a recent exposure to the organism and an unknown number of cross-reactions to the crude antigen(s) used in the assay. Chronic carriers of infection fail to shed bacteria in their faeces and can only be identified as such examination of Gut lymphoid tissue following colonic biopsy. This was a step considered unwarranted in this trial.

From the results presented in (Table 4.7) it can be seen that over the duration of the two months of the clinical trial, OD values for the cutoff serum increased slightly. Replacement of the blocking agent with fresh Casein failed to return the cutoff OD to the levels obtained earlier in the trial. Possible explanations for the upward drift in the results include protein coat deterioration, other reagent changes or electronic drift in the equipment used for the assays.

CHAPTER 5

Discussion

The rate of gastrointestinal infection caused by *Yersinia enterocolitica* in New Zealand is high by international standards, reflecting the rural/urban lifestyle that exposes humans to animal carriers more frequently than in other countries (Fenwick, 1992; Fenwick *et al.*, 1994; Fenwick *et al.*, 1996). Between 1991 and 1996 ten units of donated blood that were heavily infected with *Y. enterocolitica* were discovered in New Zealand. Eight units caused endotoxic shock and septicaemia and lead to the untimely death of five patients following transfusion. Two other units were identified as infected on discolouration of the blood prior to use. Incidence figures in NZ in 1997 were reported as 1 case per 65,000 units transfused with a fatality rate of one in 104,000 units transfused (Theakston *et al.*, 1997). The fatality rate at that time was estimated to be approximately 80 times higher than the reported incidence for the U.S.A of 1 case per 5 x 10^6 units transfused (MMWR, 1991; MMWR, 1997).

Internationally the incidence of transfusion related endotoxic shock following the transfusion of infected blood is low, and of secondary importance to the transmission of viruses and parasites by transfusion. The high incidence of post transfusion endotoxic shock and septicaemia caused by blood infected with *Y. enterocolitica* in NZ makes this transfusion problem arguably more significant locally than the risks presented by prions, viruses and parasites. Since 1995 the incidence of post transfusion endotoxaemia and septicaemia caused by *Y. enterocolitica* has slowed, although two unit infections were discovered in 1996 prior to use of the blood. Reasons for the decrease in incidence since are not clear, with the rate of infection with *Y. enterocolitica* (currently 14/100,000) in NZ high by comparison to other Western countries. Incidence figures for Yersiniosis between 1991-1996 are not available to allow comparison based on community prevalence.

In 1995 the NZ transfusion services first step toward prevention was introduced. Blood donors with a history of GIT illness in the preceding month (Aber, 1990; Grossman *et al.*, 1991; Theakston *et al.*, 1997) were deferred from donating blood. While it is difficult to estimate the overall impact upon reduction in incidence, the use of the questionnaire failed

to exclude four blood donors (Ak2, PN2, NP1 and Ma1) with asymptomatic bacteraemia, from donating infected blood since its introduction. This is not surprising as infection with *Y. enterocolitica* in adults is frequently asymptomatic and so donor exclusion on recent GIT symptoms is unlikely to be effective in all cases (Theakston, et., al. 1997). In 1999 the NZ Blood Service extended deferral of blood donors with recent GIT illness, (or contacts of), to three months.

In 1998 a 2 hour, room temperature delay in blood processing prior to plasma removal was introduced. Support for this step was based on reports of experimental inoculation of donated blood in which *Y. enterocolitica* failed to grow after 35 days at 4°C. In these experiments, blood from donors was inoculated with viable bacteria immediately after collection and the blood left at 20°C for 2 hours (Pietersz *et al.*, 1992). At 20°C the temperature regulated bacterial resistance to complement and phagocytosis is repressed, facilitating bacterial killing by plasma complement and phagocytes. The results of the study showed that the viable bacteria used to innoculate the bag failed to grow in the blood bag following bag storage for 35 days at 4°C. A similar study provided partial support with the finding that some, but not all similarly innoculated units remained sterile after 35 days (Sazama, 1994). Doubts about the validity of the application of these laboratory seeding experiments to actual cases of donor bacteraemia exist. The rarity of bacteraemia and technical difficulties in the identification of bacteraemic blood donors, mean confirmation of these "ex vivo" experiments will probably never be possible. Verification of these experiments under NZ conditions has never been undertaken.

Routine storage of blood for 35 days and the selection of blood closest to its expiry (some exceptions exist for medical conditions) for usage, increases the risk of receipt of a unit of blood infected with large numbers of endotoxin producing *Y. enterocolitica*. In growth experiments similar to that described in this thesis, bacterial numbers have been shown to increase with the length of storage, so that old blood (> 21 days) presents the greatest risks. Between 1995-1998 some providers of blood for transfusion in NZ modified blood storage policies, reducing the length of time of blood storage from 35 to 21 days. This policy change was implemented partly because of the fear of transfusing an infected unit and a desire to demonstrate recognition of the danger. The two most recent cases of post transfusion endotoxaemia in NZ occurred following the transfusion of blood that had been stored for 20 and 14 days respectively. This highlighted the inadequacy of short dating as

a preventative step and in 1999 the newly formed New Zealand Blood Service reestablished national uniformity for blood storage at 35 days.

Currently no method exists for the detection of donor bacteraemia at the time of donation. Microbiologic detection of unit infection during storage has been trialed but has to date failed to recover viable *Y. enterocolitica* and is not favoured by providers. Large-scale culture for *Y. enterocolitica* at the time of donation or during storage presents logistical problems when dealing with many thousands of units of blood. The variable lag period of growth (between 4-20 days) and the requirement to culture blood at least twice during the 35 day shelf life are additional complications. Other methods of bacterial detection such as staining of blood using Giemsa or Gram's stain at the time blood is released for use, have likewise been trialed by some researchers but are not favoured. Low sensitivity, interpretation difficulties and delays in the supply of blood in emergency situations further limit these options (Tipple *et al.*, 1990).

This thesis has evaluated two approaches and their application for the prevention of transfusions of blood infected with *Y. enterocolitica*. The first technique was based on the nucleic acid amplification of bacterial DNA, a procedure designed to detect unit infection by PCR. The second an ELISA assay, was applied to the detection of an antibody response to infection with *Y. enterocolitica* in blood donors and was used to identify those blood donors most at risk of donating a unit of blood while bacteraemic with *Y. enterocolitica*.

As a preliminary experiment to PCR a growth analysis of *Y. enterocolitica* in AS-1 red cells stored at 4°C was conducted. Red cell units were infected with 30 cfu of pYV^+ *Y. enterocolitica* serotype O:3 (strain PN2) and sampled to monitor the pattern of bacterial growth during storage at 4°C. Total viable cell counts performed over the next 30 days demonstrated an initial post inoculation period (lag phase) of 4 days during which bacteria were not detected. This was followed by bacterial doubling (approx. 16 hourly) from day 4 onward, that peaked at 1.6×10^9 bacteria/ml after 26 days. In the experiment the lag phase delay was shown to be shorter than the 7-20 days reported by others for similar experiments. Possible differences could be related to sample size variation, the smaller volume of the AS-1 units used in our experiments (Franzin & Gioannini, 1992) or to *Y. enterocolitica* strain differences. The results showed that maximal bacterial growth occurred between days 4 and 22. Similar patterns have been reported by others (Gibb *et*

al., 1994). Gram-negative bacterial growth in donated blood is potentially lethal with toxin levels correlating with endotoxic shock and patient prognosis (Schwalbe & Spath-Schwalbe, 1998). The number of *Y. enterocolitica* reported to produce a clinically significant level of endotoxin in stored blood has been estimated to be 10^5 bacteria/ml (Krishnan and Brecher, 1995). In this experiment 10^5 bacteria/ml was reached after 14 days, less than half the routine shelf life of the blood. This experiment demonstrated how serious transfusion reactions can occur with blood fresher than 21 days old (Stubbs *et al.*, 1991) and how reduction in the length of storage of blood to 21 days (as previously described) cannot be expected to stop serious reactions. Throughout the growth experiment all units remained unchanged in visual appearance until day 22 when the inoculated units containing 6.8×10^8 organisms/ml became blackened in comparison to the controls. This has been reported by others, a result of pO_2 desaturation of haemoglobin and a build up of CO_2 in the bag (Kim *et al.*, 1992; Kim *et al.*, 1992).

DNA extractions of bacteria in AS-1 red cells produced during the growth experiment, were prepared by Brij/Tris/EDTA/Proteinase K digestion. DNA was amplified using 21 mer primers from the *ail* gene of *Y. enterocolitica*. The PCR was standardised by varying the concentrations of $MgCl_2$ and template DNA extracted from serial dilutions of O/N BHI broth cultures of *Y. enterocolitica*. 40 cycles of amplification of the target bacterial DNA (previously described) produced a visible 430 bp fragment comparable to the amplified product reported by *Feng et al.* Best sensitivity for PCR detection was 4.3×10^5 organisms/ml, 1 log less sensitive than that reported by other workers (Feng *et al.*, 1992). The experiment demonstrated the difference between the theoretical capabilities of PCR amplification and performance in this application. Possible factors contributing to lower than expected sensitivity included the presence of red cell stroma and human DNA and the presence of haemoglobin (polymerase antagonist). These have been reported to affect sensitivity in similar experiments conducted by others (Feng *et al.*, 1992). Further experimentation with the PCR conditions, reagents, DNA extractions, and the separation of bacterial DNA from red cell stroma and human DNA may help to improve sensitivity of the PCR test system for this application.

In the opinion of the author, technical and logistical complexities mitigate against PCR and its suitability for this application. While 10^5 organisms/ml sensitivity is approximately the level of bacteria reported as clinically significant, it is unlikely that PCR could be applied

without adaptation to process large numbers of samples. In addition, factors such as the number of sample collections, variability in bacterial growth rates in blood, blood stock inventory management and the compromisation of unit sterility during sampling further compound the argument against the use of nucleic acid amplification as trialed in this experiment.

Serological investigation of three groups of sera was conducted using an assay developed to detect increased IgA antibody to *Y. enterocolitica* infection. Initial studies of the sera from bacteraemic blood donors used a commercial ELISA assay (DAKO, Denmark) to detect IgA and IgG levels of antibody to the LPS structure of serotype O:3 *Y. enterocolitica*. Results obtained in the pilot study lead to the development of a new ELISA assay for the Abbott range of Commander ELISA Processors that was representative of the equipment being used infectious disease screening in the blood transfusion industry in NZ at that time. The assay used pYV encoded protein antigens extracted from pYV^{+} *Y. enterocolitica* under controlled laboratory conditions. Recovered released proteins were used to coat 6mm plastic beads the solid matrix employed in the assay. Protocols for bead blocking, reagents, sample diluents etc. were developed using experiments against known positive and pooled blood donor sera (as previously described)

The sera from donors of blood implicated in 5 transfusion transmitted endotoxaemias and two non-transfusion related unit infections was tested using the DAKO and IgA RP bead assay. Results showed that an immune response to the infection with *Y. enterocolitica*, could be demonstrated in bacteraemic blood donor at the time of donation. The results obtained using the DAKO allowed inferences to be drawn about stages of the infections in these donors. Using this assay donor sera Ak1, Ak2 and PN2, contained levels of IgA antibody to the O:3 LPS above the cutoff supplied in the kit. Following infection with *Y. enterocolitica* IgA antibody is produced and its presence in the blood is indicative of an early response (pre IgG) to the infection or the existence of chronic infection. From the pattern of immunoglobulins against these three sera, the stage of infection during which bacteraemia most likely occurred was interpreted as being shortly after infection, before a measurable IgG response had been mounted or alternatively during chronic infection. The second of these two possibilities is perhaps less likely as IgG antibody is usually also seen in the serum of patients with chronic infection. In each of the sera from this group no IgG antibody was detected. Sera Ma1 and NP1 were positive for both IgA and IgG antibody, a

pattern consistent with an established infection. Of interest was the finding that a low-level bacteraemia existed in these donors despite the presence of opsonising antibody in the blood. Serum sample NP1 from an O:9 serotype infection showed cross reactivity with the O:3 LPS antigen in the DAKO assay yet serum sample PN1 from an O:9 infection failed to react. Sample Ha1 failed to react despite having a reported titre of agglutinating antibody of 1:5000 in testing performed immediately after the transfusion reaction caused by this blood in 1991. The validity of the argument that bacteraemia occurred in this donor in the window phase may be incorrect. Although sample numbers in this group (group 1) were small the results support the likelihood that bacteraemia occurs during or prior to the early immunoglobulin response to infection. Bacteraemia occurring during the chronic stage of the infection could not be excluded. The results showed that viable bacteria are able to survive in the bloodstream in the presence of IgG opsonising antibody. This lends support to the theory that *Y. enterocolitica* may exist as an intracellular occupant of lymphoid and/or phagocytic cells. If this is not so and bacteria exist roam free in the bloodstream, then chromosomal and plasmid encoded virulence factors must provide some protection from the effects of antibody.

A newly developed IgA RP bead ELISA assay was used to further study IgA antibody levels in 6 bacteraemic blood donors (6/7 sera tested previously), 27 paired sera from culture confirmed *Y. enterocolitica* GIT infection and sera from 495 healthy blood donors. Results obtained using the IgA RP bead assay, demonstrated the presence of IgA antibody to infection with *Y. enterocolitica* in all sera from bacteraemia blood donors at the time of donation. Performance of the bead assay was superior to that of the DAKO assay at detecting the antibody response associated with both O;3 and O:9 serotype infections the samples. Sample PN1 (O:9 infection), seronegative in the DAKO assay, was reactive in the bead assay. Although sample numbers in this group were small the results demonstrated that the sensitivity of the assay was high for the detection of antibody in the sera of blood donors bacteraemic with *Y. enterocolitica* at the time of donation.

Sensitivity of the IgA RP bead assay for detecting antibody in the sera of culture proven *Y. enterocolitica* GIT infected patients was also high 26/27 (96.3%). Reactivity of the samples varied as previously described with a strong IgA response in some patients and a weaker response in others. Comparisons between the performance of the IgA RP bead assay and the DAKO LPS O:3 assay again showed the bead assay to be superior at

detecting IgA seropositivity in response to *Y.enterocolitica* GIT infection (Morris *et al.*, 1998).

Performance of the assay against the sera of 495 normal blood donors (GIT illness free) enrolled in a clinical trial, showed IgA seropositivity in 20/495 (4.04%) donor sera tested. The 20 seropositive donors identified in the study, were representative of donors in various stages of infection and contained one blood donor actively shedding *Y. enterocolitica* in their faeces. All other seropositive donors were stool culture negative and all seropositive red cells units, were blood culture negative after 35 days storage at 4°C. The results demonstrated that low levels of IgA antibody can be found in the sera of healthy blood donors and that an unknown number of donations from seropositive blood donors can be expected to contain viable *Y. enterocolitica*. Seropositivity in the IgA RP bead assay correlates with current/recent or longer standing chronic infection with *Y. enterocolitica*. Within the seropositive group can be expected to be a number of false positive reactions, the result of cross reactivity with the RPs used as target antigen in the assay.

Overall the specificity of the assay for detection of bacteraemia in blood donors in the clinical trial was low (0/20), a figure consistent with the unknown but expectedly low incidence of bacteraemia that accompanies infection with *Y. enterocolitica*. The assay showed a low correlation between elevated IgA antibody and the presence of viable *Y. enterocolitica* in the faeces of blood donors (1/20). From the results of the clinical trial an approximation of the number of units collected annually from donors actively shedding viable bacteria using the 0.202% incidence derived from the study puts the number between 30-40 donations (165,000 donations/yr.). Depending upon the stage of the infection an unknown number of these donors may also be bacteraemic, their blood presenting significant recipient-risk following blood storage and transfusion.

The most significant finding of the study was the demonstration of seropositivity to infection with *Y. enterocolitica* in the sera of six blood donors all of whom provided blood containing viable *Y. enterocolitica* at the time of donation. In all cases, the blood donated by these donors showed to be heavily infected with *Y. enterocolitica* after variable periods of refrigerated blood bank storage. Four of the six donations caused post transfusion septicaemia and endotoxic shock resulting in the death of 3 patients. Two of the units, were discovered to be infected after colour change to the blood alerted technical staff to the

possible dangers before they were transfused.

The transfusion of stored blood infected with large numbers of *Y. enterocolitica* carries a high rate of morbidity and represents one of the greatest threats for recipients of blood transfusions in NZ. To prevent further cases, transfusion laboratories in New Zealand have over the last decade adopted varying strategies as previously discussed. This thesis has demonstrated how the identification of blood donors with current or recent infection with *Y. enterocolitica* would help to reduce risks associated with endotoxic shock caused following the transfusion of *Y. enterocolitica* infected blood. In a review of the recent medical history of seven donors of infected blood in NZ, Dr. Bart Baker from the Central Regional Blood Service in Palmerston North reported that only two donors recalled GIT illness in the months preceding donation. One other donor was a contact of a family member with recent GIT illness. Four donors had an unremarkable pre-donation medical history. Of the seven cases one donor still donated blood despite being questioned about GIT symptoms later claiming that he did not consider a recent bout of "loose stools" constituted diarrhoea or GIT disease. From the findings of this alone it is unlikely that donor deferral on recent GIT history will stop transfusion related endotoxic shock caused by *Y. enterocolitica*. Recent extension to the length of time of deferral for donors with GIT illness, is unlikely to provide additional safeguards and serve only to further increase current rates of donor deferral previously estimated to be 3-5%.

The dangers posed by the transfusion of blood infected with *Y. enterocolitica*, represent a serious health risk to recipients of blood, more so in NZ than elsewhere in the world. A decrease in incidence of cases of transfusion endotoxaemia and septicaemia caused by *Y. enterocolitica* infected blood since 1995 cannot be taken as meaning that this danger no longer exists. In 2000 statistics for Yersiniosis in the population remain similar to those reported in 1997 and figures during the peak of the unit infections are not available. If the cause of the unit infections between 1991-1996 was related to community prevalence then it is perhaps likely that there exists an undefined level of infection in the population above which *Y. enterocolitica* may again re-enter the blood supply.

The results of research presented in this thesis demonstrates the potential of a simple screening test to identify the donors most at risk of donating blood containing viable *Y. enterocolitica*. The IgA RP bead assay could be rapidly applied to large scale donation

screening in New Zealand and reduce risks of blood transfusion associated with the transfusion of *Y. enterocolitica* infected blood. The bead assay should have appeal for its simplicity, low cost and ease of application and could be introduced to eliminate donor deferral on recent GIT illness allowing a return to the collection of blood from donors currently excluded (Theakston *et al.*, 1997). Further consideration of the costs, the potential loss of 3-5% of red cell units, (although it may be possible to transfuse seropositive red cells for up to 10 days) and the expected impact upon leukodepletion that is planned for introduction in NZ in 2001 is required.

The introduction of infectious disease screening internationally followed the careful consideration of incidence of post transfusion disease, costs, and the discovery of the infectious agent causing the diseases. Today the somewhat "unreal" public expectation that providers of blood and blood products should reduce risk further has lead to the introduction of policies not based upon the rationale of the past, but on global precedent, and public demand. The findings of the work presented in this thesis demonstrate how risks associated with the transfusion of blood infected with *Y. enterocolitica* in NZ can be reduced to an acceptable level. The screening test described in this work has the potential to eliminate entirely the risk of post transfusion endotoxic shock and septicaemia caused by *Y. enterocolitica* in NZ. It is the view of this author that its introduction into routine use in areas of high endemicity such as NZ would lead to improvement in the safety of blood transfusion and improve public confidence in the safety of New Zealand's blood supply.

Appendices:

Appendix 1

Application for Ethical approval to Manawatu-Whanganui Ethics Committee

26th February 1998

Manawatu-Whanganui Ethics Committee
[redacted]
[redacted]
Palmerston North Hospital
Private Bag
PALMERSTON NORTH

RE: Manawatu-Whanganui Ethics Committee [redacted]

Dear Sir Madam

Since the beginning of 1996 I have been conducting research into the development of a diagnostic assay that might be useful in screening donated blood to eliminate serious transfusion reactions. The title of the research is "Development of a Diagnostic Test to be Used for the Detection of Bacterial Contamination of Blood with the Organism *Y. enterocolitica*.". The research has progressed to the stage that we have an assay that is ready to trial on blood donor sera.

I believe from conversations with Vicky Graham that a further formal application for ethical approval to conduct the assay trial may not be warranted. Accordingly please find enclosed the relevant details of the trial and drafts of the documentation that we hope to use in recruiting blood donors to the trial.

Yours faithfully

Chris Kendrick
Principal Investigator
Institute of Veterinary, Animal and Biomedical Science
Massey University
PO Box 11222
PALMERSTON NORTH

Appendix 2

Yersinia IgA antibody assay development

- proposed schedule for assay trial

Research into the contamination of donated blood with *Yersinia enterocolitica* is being conducted by Chris Kendrick from the Institute of Veterinary, Animal and Biomedical Sciences at Massey University. A new ELISA based assay that will detect current infection with pathogenic strains of *Yersinia enterocolitica* that may be useful in screening blood donor sera has been developed at the University. The next stage is to trial the performance of the assay in the blood transfusion setting. The purpose of the trial is to establish the background reactivity of antibodies to the released proteins of disease causing *Yersinia enterocolitica* and to assess the impact upon the processes of donation accreditation that introduction of such a screening test may have on inventory control.

It is proposed that the trial be conducted in two stages.

Stage 1: **collection of cutoff serum for the assay.**

Donor requirements

In addition to the requirements for normal donations, donors willing to participate in the trial:

- must have been free of GIT illness or symptoms for at least three months prior to unit collection
- should read and understand the information and consent forms
- should read and understand the requirements of participation
- should sign the consent form
- should be provided with containers for stool collection and a timetable outlining the dates on which samples should be returned for culture and points of drop off.

Protocol

Commencing the 30th March, enrol 25-30 healthy donors and collect a single unit of CP2D anticoagulated blood from each. Routine pre-processing protocols should apply. Plasma should be separated from the red cells in the normal manner, and stored frozen at -30^0 C separated from other plasma units. Storage and testing of the red cell units should adhere to the following protocol:

- RBC units should be stored at 4°C for 35 days, and kept separated from "non trial" RBC units
- a 1ml serum sample should be frozen and kept separate until all samples have been collected
- duplicate Yersinia IgA antibody testing to be carried out on stored sera
- RBC units to be cultured at day zero (blood culture bottles) and at day 35 (plates).

- donor stools to be cultured for *Y. enterocolitica* - within 24 hours of collection
  - approx 1 month later

Stage 2 - serology

Commencing on the 1st May 1998 enrol approx 1000 volunteers into the programme over a period of 2 months.

Donor requirements

Questioning of donors on recent GIT history should divide blood donors into:

a) those ***without*** recent GIT illness over the last 1 month
b) those ***with*** GIT illness over the previous 1 month

In addition to the requirements for normal donation, blood donors should:

- read and understand the information sheet
- read and understand the requirements of participation
- read and sign the consent form
- be informed of the arrangements for stool sample collection and culture should it be required

Lab testing

IgA antibody testing to *Y. enterocolitica* will be performed on the Abbott Commander ELISA Processing equipment currently used for donation accreditation. The equipment is located in the Department of Transfusion Medicine, Mid Central Health Ltd. All consumables for the assay will be provided by the principal investigator.

Additional testing to be performed on:

- RBC donations found to be IgA antibody positive in the screen are to be retrieved and stored at 4°C for 35 days.
- unit culture (plate culture) to be performed at the end of the normal 35 days storage.
- arrangement for stool sample collection and culture to be made on antibody positive donors

The bulk of the blood and stool culture procedures will be performed by Medlab Central Ltd. The results of testing to be held and released to Chris Kendrick and or Bart Baker. Results to be collated by Chris Kendrick.

Funding

Funding for the trial has been provided through a grant from the Palmerston North, Medical Research Fund. Settlement of costs associated with the research will be made by arrangement with the principal investigator Mr. Chris Kendrick.

NB: Dates and timeframes have been provided as guides and may not reflect actual dates of commencement or duration of the trial

Appendix 3

Blood donor information sheet & consent form

Yersinia research - stage 1

The contamination of donated blood with bacteria is a rare complication of blood transfusion worldwide. Since the first recorded example in 1991 there have been 10 reported cases of unit contamination with the organism *Yersinia enterocolitica* in New Zealand. *Y. enterocolitica* is a bacterium that causes diarrhoea mostly in the young but also in adults in whom infection often goes without notice. Bacteria present in very small numbers in healthy blood donors can be collected at the time blood donors donate blood. The bacteria are able to grow in donated blood so that during the period of refrigerated storage large numbers of bacteria may be present in the donated blood. If these units are transfused they cause life-threatening reactions in those receiving the blood.

Since 1996 this problem has been the subject of research by Mr. Chris Kendrick, a lecturer from the Institute of Veterinary, Animal and Biomedical Science at Massey University. His research entitled "Development of a Diagnostic Test to be Used for the Detection of Bacterial Contamination of Blood with the Organism *Y. enterocolitica*", has now reached the point whereby a trial is required to establish its usefulness in the testing of blood. The test has been designed to detect current and recent infection with *Yersinia enterocolitica* in blood donors. It is hoped that by being able to quickly identify those units of blood that are most at risk of causing this problem, the safety of blood transfusion can be improved in New Zealand.

To assess the usefulness of the assay the investigators require volunteers who are prepared to participate in a trial. The number required for stage 1 is 20-30 donors. Volunteers should be donors who meet all the usual criteria required of a full blood donation.

1. If you agree to participate in the trial, the donation that you make today **will not be used for transfusion purposes**. In addition a small serum sample collected with the donation will be tested for antibodies to *Yersinia enterocolitica*. The plasma from your donation will be frozen and used as a control serum for the trial. The red cells will be cultured for *Yersinia enterocolitica*. In addition if you agree to participate in this stage of the trial we require two (2) stool samples from you. The first sample we require within 24 hours of unit collection and the second 1 month later. All antibody testing will be performed in the Department of Transfusion Medicine, Mid-Central Health Ltd. and red cell unit and stool culture will be performed by Medlab Central Ltd.

2. Information collected in the trial will be collated by Mr. Chris Kendrick and/or Dr. Bart Baker, Mid Central Health Ltd. Details of the testing will remain confidential and at no time will the participant's identity be revealed in any written or verbal research reports.

3. Participation in this trial is voluntary. If you do not wish to take part you can still donate in the normal manner. If you decide at the time of donation to take part but change your mind later you can withdraw at anytime by contacting the blood service [redacted]

4. If you would like more time to consider and discuss participation please feel free to talk with other blood donors or the staff of the blood donor unit.

5. Other than the slight discomfort of donating your blood and the need to supply stools for culture there are no other associated risks or discomforts.

6. If you give your consent to participate in the trial, consent applies to laboratory testing on this occasion and not to future requests.

7. If you have any ethical concerns about the study please contact the Manawatu-Whanganui Ethics Committee on (06) 356 7773 or write to the committee at PO Box 5203, Palmerston North.

CONSENT FOR PARTICIPATION IN YERSINIA RESEARCH

I .....(NAME) consent to give a serum sample collected on the .....(DATE) to be used for testing for antibodies to *Y. enterocolitica* in the Department of Transfusion Medicine, MidCentral Health Ltd. In addition I agree to provide one (1) stool sample within 24 hours of donating this unit and a second stool sample in 1 months time. I understand that the testing for antibodies and stool culture will have no effect on my health and the unit provided at this time will not be used for transfusion purposes. I understand that my consent applies to this donation only.

SIGNATURE	NAME of DONOR (PRINT)	DATE
SIGNATURE of WITNESS	NAME of WITNESS (PRINT)	DATE

If you have any questions or concerns about the study please contact Dr. Bart Baker, Director of Transfusion, Mid-Central Health Ltd. [redacted]

Appendix 4

Blood donor information sheet & consent form

Yersinia research - stage 2

The contamination of donated blood with bacteria is a rare complication of blood transfusion worldwide. Since the first recorded example in 1991 there have been 10 reported cases of unit contamination with the organism *Yersinia enterocolitica* in New Zealand. *Y. enterocolitica* is a bacterium that causes diarrhoea mostly in the young but also in adults in whom infection often goes without notice. Bacteria present in very small numbers in healthy blood donors can be collected at the time blood donors donate blood. The bacteria are able to grow in donated blood so that during the period of refrigerated storage large numbers of bacteria may be present in the donated blood. If these units are transfused they cause life-threatening reactions in those receiving the blood.

Since 1996 this problem has been the subject of research by Mr. Chris Kendrick, a lecturer from the Institute of Veterinary, Animal and Biomedical Science at Massey University. His research entitled "Development of a Diagnostic Test to be Used for the Detection of Bacterial Contamination of Blood with the Organism *Y. enterocolitica*", has now reached the point whereby a trial is required to establish its usefulness in the testing of blood. The test has been designed to detect current and recent infection with *Yersinia enterocolitica* in blood donors. It is hoped that by being able to quickly identify those units of blood that are most at risk of causing this problem, the safety of blood transfusion can be improved in New Zealand.

To do this investigators require volunteers who are prepared to participate in the trial. The number required is approximately 1000. Suitable volunteers should be donors who:

a) meet the usual criteria required of normal blood donation
b) those donors with a recent history of gastrointestinal illness (these donations will not be used for transfusion). In addition:

1. If you agree to participate in the trial a small sample of your serum will be tested for the presence of antibodies to *Yersinia enterocolitica* in the Department of Transfusion Medicine, Mid Central Health Ltd. If your antibody test is ***negative*** your donation will be used for normal transfusion. If you test ***positive*** your donation **will not be used for transfusion** and you will be contacted and asked to provide a stool sample. Stool culture will be performed by Medlab Central Ltd. It is expected that this will involve only a few donors.

2. Information collected in the trial will be collated by Mr. Chris Kendrick and/or Dr. Bart Baker, Mid Central Health Ltd. Details of the testing will remain confidential and at no time will the participant's identity be revealed in any written or verbal research reports.

3. Participation in this trial is voluntary. If you do not wish to take part you can still donate in the normal manner. If you decide at the time of donation to take part but change your mind later you can withdraw at anytime by contacting the blood service [redacted]

4. If you would like more time to consider and discuss participation please feel free to talk with other blood donors or the staff of the blood donor unit.

5. Other than the slight discomfort of donating your blood and the possible need to supply stools for culture there are no other associated risks or discomforts.

6. If you give your consent to participate in the trial, consent applies to laboratory testing on this occasion and not to future requests.

7. If you have any ethical concerns about the study please contact the Manawatu-Whanganui Ethics Committee on (06) 356 7773 or write to PO Box 5203, Palmerston North.

CONSENT FOR PARTICIPATION IN YERSINIA RESEARCH

I .....(NAME) consent to provide a serum sample collected on the .....(DATE) to be used for testing for antibodies to *Y. enterocolitica* in the Department of Transfusion Medicine, MidCentral Health Ltd. In addition I agree to provide one (1) or (2) stool samples should I be contacted to do so.

I understand that laboratory testing for antibodies and stool culture (should it be required) will have no effect on my health and that the unit provided at this time **<u>may not</u>** be used for transfusion purposes. I understand that my consent applies to this donation only.

SIGNATURE	NAME of DONOR (PRINT)	DATE
SIGNATURE of WITNESS	NAME of WITNESS (PRINT)	DATE

If you have any questions or concerns about the study please contact Dr. Bart Baker, Director of Transfusion, Mid-Central Health Ltd. [redacted]

Appendix 5

Y. enterocolitica IgA antibody assay trial

Results of serum, stool and unit culture on blood donors from stage 2

Date	Sample ID	OD_{492}	Cutoff	Result	Stool culture	Unit culture
21/07/98	Positive	**1.321**	**0.187**			
	Positive	**1.539**				
	8559331	0.049		neg		
	8559332	0.034		neg		
	8559333	0.026		neg		
	8559334	0.041		neg		
	8559335	0.058		neg		
	8559336	0.031		neg		
	8559338	0.034		neg		
	8559339	0.027		neg		
	8559340	0.053		neg		
	8559342	0.083		neg		
	8559344	0.042		neg		
	8559345	0.041		neg		
	8559346	0.038		neg		
	8559348	0.052		neg		
	8559349	0.04		neg		
22/07/98	Positive	1.586	**0.294**			
	Positive	1.662				
	8561225	0.077		neg		
	8561227	0.201		neg		
	8561229	0.043		neg		
	8561230	0.063		neg		
	8561232	0.021		neg		
	8561235	0.051		neg		
	8561237	0.1		neg		
	8561238	0.176		neg		
	8561239	0.056		neg		
	8561240	0.073		neg		
	8561241	0.037		neg		
	8561242	0.111		neg		
	8561243	0.041		neg		
	8561246	0.235		neg		
	8561247	0.289		neg		
	8561250	0.034		neg		
	8561252	0.032		neg		
	8561253	0.045		neg		

Date	Sample ID	OD_{492}	Cutoff	Result	Stool culture	Unit culture
	8561254	0.085		neg		
	8561255	0.053		neg		
	8561256	0.204		neg		
	8561257	0.049		neg		
	8561258	0.044		neg		
	8561260	0.107		neg		
	8561261	0.042		neg		
	8561262	0.052		neg		
	8561264	0.089		neg		
	8561266	0.073		neg		
	8561267	0.021		neg		
	8561268	0.040		neg		
	8561269	0.092		neg		
	8561271	0.070		neg		
	8561272	0.038		neg		
	8561273	0.068		neg		
	8561274	0.059		neg		
	8561276	0.098		neg		
	8561277	0.259		neg		
	8561279	0.064		neg		
23/07/98	Positive	**1.658**	**0.214**			
	Positive	**1.620**				
	8561280	**0.535**		**pos**	**neg**	**neg**
	8561282	0.063		neg		
	8561283	0.043		neg		
	8561284	**0.683**		**pos**	**neg**	**neg**
	8561289	0.044		neg		
	8561290	0.052		neg		
	8561291	0.048		neg		
	8561292	0.045		neg		
	8561296	0.070		neg		
	8561297	0.108		neg		
	8561299	0.035		neg		
	8561300	0.063		neg		
	8561301	0.056		neg		
	8561302	0.037		neg		
	8561303	0.120		neg		
	8561305	0.096		neg		
	8561306	0.089		neg		
	8561307	0.152		neg		
	8561308	0.051		neg		
	8561310	0.135		neg		

Date	Sample ID	OD_{492}	Cutoff	Result	Stool culture	Unit culture
	8561311	0.067		neg		
	8561312	0.031		neg		
	8561313	0.042		neg		
	8561314	**0.482**		**pos**	**neg**	**neg**
	8561316	0.035		neg		
	8561320	0.072		neg		
	8561322	0.053		neg		
	8561323	0.056		neg		
	8561324	0.052		neg		
	8561325	0.072		neg		
	8561327	0.063		neg		
	8559364	0.058		neg		
	8559365	0.051		neg		
	8559367	0.111		neg		
	8559368	0.204		neg		
	8559370	0.069		neg		
	8559372	**0.609**		**pos**	**neg**	neg
24/07/98	Positive	1.459	**0.212**			
	Positive	1.669				
	8561330	0.073		neg		
	8561334	0.024		neg		
	8561335	0.020		neg		
	8561341	0.012		neg		
	8561342	0.048		neg		
	8561345	0.032		neg		
	8561347	**0.956**		**pos**	**neg**	neg
	8561349	0.018		neg		
	8561353	0.020		neg		
	8561356	0.032		neg		
	8561357	0.045		neg		
	8561359	0.151		neg		
	8561360	0.080		neg		
	8561362	0.043		neg		
	8561363	0.022		neg		
	8561365	0.058		neg		
	8561366	0.046		neg		
	8561367	0.076		neg		
	8561368	0.174		neg		
	8561369	0.046		neg		
	8559373	0.193		neg		
	8559374	0.049		neg		
	8559375	0.052		neg		

Date	Sample ID	OD_{492}	Cutoff	Result	Stool culture	Unit culture
	8559376	0.033		neg		
	8559377	0.026		neg		
	8559378	0.046		neg		
	8559379	**2.163**		**pos**	**neg**	**neg**
	8559381	0.047		neg		
	8559382	0.150		neg		
28/07/98	Positive	1.446	**0.203**			
	Positive	1.646				
	8559385	0.044		neg		
	8559392	0.054		neg		
	8559393	0.049		neg		
	8559394	0.068		neg		
	8559395	0.070		neg		
	8559397	0.039		neg		
	8559400	0.046		neg		
	8559401	0.171		neg		
	8559402	0.039		neg		
	8559404	0.058		neg		
	8559405	0.125		neg		
	8559406	0.042		neg		
	8559407	0.066		neg		
	8559409	0.044		neg		
	8559410	0.062		neg		
	8559411	0.040		neg		
	8559412	0.075		neg		
	8559413	0.160		neg		
	8559414	0.038		neg		
	8559415	0.034		neg		
	8559416	**0.413**		**pos**	**neg**	**neg**
	8559417	0.034		neg		
	8559418	0.069		neg		
	8559419	**1.124**		**pos**	**neg**	**neg**
	8559420	0.046		neg		
	8559421	0.033		neg		
	8559422	0.048		neg		
	8561370	0.081		neg		
	8561371	0.035		neg		
	8561374	0.050		neg		
	8561375	0.035		neg		
	8561377	0.061		neg		
	8561378	0.043		neg		
	8561379	0.041		neg		

Date	Sample ID	OD_{492}	Cutoff	Result	Stool culture	Unit culture
	8561380	0.033		neg		
	8561381	0.086		neg		
	8561382	0.048		neg		
29/07/98	Positive	1.385	**0.243**			
	Positive	1.381				
	8561383	0.051		neg		
	8561384	0.039		neg		
	8561386	0.091		neg		
	8559424	0.041		neg		
	8559426	0.056		neg		
	8559427	0.079		neg		
	8559431	0.087		neg		
	8559432	**0.525**		**pos**	**neg**	**neg**
	8559433	0.040		neg		
	8559434	0.050		neg		
	8559436	0.052		neg		
	8559438	0.070		neg		
	8559439	0.038		neg		
	8559441	0.019		neg		
	8559442	0.096		neg		
31/07/98	Positive	1.404	**0.224**			
	Positive	1.553				
	8559444	0.017		neg		
	8559445	0.176		neg		
	8559446	0.031		neg		
	8559448	0.065		neg		
	8559449	0.104		neg		
	8559450	0.039		neg		
	8559451	0.044		neg		
	8559452	0.078		neg		
	8559453	0.020		neg		
	8559459	0.022		neg		
	8559461	0.061		neg		
	8559463	0.077		neg		
	8561407	0.049		neg		
	8561408	0.079		neg		
	8561409	0.065		neg		
	8561419	0.029		neg		
	8561421	0.029		neg		
04/08/98	Positive	1.475	**0.244**			

Date	Sample ID	OD_{492}	Cutoff	Result	Stool culture	Unit culture
	Positive	1.528				
	8559464	0.096		neg		
	8559465	0.111		neg		
	8559466	0.048		neg		
	8559467	0.065		neg		
	8559468	0.079		neg		
	8559469	0.038		neg		
	8559470	0.103		neg		
	8559474	0.085		neg		
	8559476	0.043		neg		
	8559477	0.040		neg		
	8559478	0.102		neg		
	8559479	0.062		neg		
	8559480	0.035		neg		
	8559481	0.059		neg		
	8559485	0.047		neg		
	8559489	0.049		neg		
	8559493	0.035		neg		
	8559495	0.044		neg		
	8559496	0.052		neg		
	8559497	0.053		neg		
	8559498	0.042		neg		
	8559499	0.068		neg		
04/08/98	Positive	1.660	**0.219**			
	Positive	1.663				
	8554501	0.053		neg		
	8554507	0.19		neg		
	8554508	0.030		neg		
	8554509	0.036		neg		
	8554510	0.054		neg		
	8554511	0.510		**pos**	**neg**	**neg**
	8561435	0.044		neg		
	8561436	0.078		neg		
	8561438	0.027		neg		
	8561442	0.040		neg		
	8561463	0.492		**pos**	**not done**	**neg**
6/08/98	Positive	1.643	**0.309**			
	Positive	1.498				
	8554514	0.053		neg		
	8554516	0.091		neg		
	8554519	0.063		neg		

Date	Sample ID	OD_{492}	Cutoff	Result	Stool culture	Unit culture
	8554520	0.052		neg		
	8554523	0.048		neg		
	8554524	0.085		neg		
	8554525	0.086		neg		
	8554526	0.055		neg		
	8554529	0.053		neg		
	8554530	0.038		neg		
	8554534	0.642		**pos**	**neg**	**neg**
	8554536	0.043		neg		
	8554539	0.043		neg		
	8554541	0.052		neg		
	8554542	0.235		neg		
	8554545	0.146		neg		
	8554547	0.252		neg		
	8554548	0.096		neg		
	8554549	0.039		neg		
	8554550	0.038		neg		
	8554551	0.051		neg		
7/08/98	Positive	1.420	**0.263**			
	Positive	1.525				
	8561468	0.074		neg		
	8561469	0.030		neg		
	8561479	0.035		neg		
	8561490	0.094		neg		
11/08/98	Positive	1.205	**0.194**			
	Positive	1.444				
	8554564	0.134		neg		
	8554566	0.186		neg		
	8554567	0.038		neg		
	8554572	0.038		neg		
	8554573	0.060		neg		
	8554576	0.137		neg		
	8554577	0.045		neg		
	8554579	0.044		neg		
	8554580	0.026		neg		
12/08/98	Positive	1.675	**0.305**			
	Positive	1.561				
	8554585	0.051		neg		
	8561505	0.042		neg		
	8561509	0.046		neg		

Date	Sample ID	OD_{492}	Cutoff	Result	Stool culture	Unit culture
	8561511	0.291		neg		
	8561512	0.114		neg		
	8561517	0.068		neg		
	8561522	0.033		neg		
	8561524	0.072		neg		
	8561526	0.089		neg		
	8561527	0.050		neg		
	8561528	0.074		neg		
	8561531	0.120		neg		
13/08/98	Positive	1.512	**0.303**			
	Positive	1.602				
	8561538	0.053		neg		
	8561539	0.092		neg		
	8561545	0.138		neg		
	8561551	0.092		neg		
	8561552	0.098		neg		
	8561553	0.065		neg		
	8561556	0.094		neg		
	8561558	0.076		neg		
	8561559	0.269		neg		
	8561562	0.183		neg		
	8561566	0.667		**pos**	**not done**	**neg**
	8561567	0.260		neg		
	8561572	0.042		neg		
	8561574	0.050		neg		
	8561578	0.057		neg		
	8554589	0.062		neg		
	8554590	0.044		neg		
	8554591	0.046		neg		
	8554592	0.076		neg		
	8554593	1.138		**pos**	**neg**	**neg**
	8554597	0.062		neg		
	8554598	0.119		neg		
	8554599	0.761		**pos**	**neg**	**neg**
	8554600	0.072		neg		
	8554601	> 2.200		**pos**	**neg**	**neg**
	8554602	0.084		neg		
	8554603	0.110		neg		
	8554604	0.103		neg		
	8554605	0.094		neg		
17/08/98	Positive	1.388	**0.313**			

Date	Sample ID	OD_{492}	Cutoff	Result	Stool culture	Unit culture
	Positive	1.484				
	8561585	0.059		neg		
	8561588	0.066		neg		
	8561589	0.117		neg		
	8561590	0.082		neg		
	8561591	0.051		neg		
	8561592	0.051		neg		
	8561593	0.049		neg		
	8561594	0.050		neg		
	8561595	0.093		neg		
	8561596	0.083		neg		
	8561597	0.070		neg		
	8561599	0.093		neg		
	8561601	0.073		neg		
	8561607	0.131		neg		
	8561608	0.110		neg		
	8561609	0.094		neg		
	8561610	0.070		neg		
	8561611	0.042		neg		
	8561614	0.121		neg		
	8561615	0.092		neg		
	8561618	0.046		neg		
	8561622	0.043		neg		
	8561624	0.194		neg		
	8561626	0.244		neg		
	8561631	0.043		neg		
	8561635	0.090		neg		
	8561636	0.100		neg		
	8561640	0.067		neg		
	8561644	0.091		neg		
	8554607	0.058		neg		
	8554608	0.099		neg		
	"8554609	0.052		neg		
	8554611	0.088		neg		
	8554612	0.133		neg		
	8554613	0.231		neg		
	8554621	0.066		neg		
	8554623	0.044		neg		
	8554624	0.053		neg		
	8562256	0.049		neg		
19/08/98	Positive	1.436	**0.347**			
	Positive	1.679				

Date	Sample ID	OD_{492}	Cutoff	Result	Stool culture	Unit culture
	8562262	0.066		neg		
	8562263	0.072		neg		
	8562266	1.261		**pos**	**neg**	**neg**
	8562268	0.662		**pos**	**neg**	**neg**
	8562269	0.062		neg		
	8562270	0.100		neg		
	8562271	0.075		neg		
	8562272	0.074		neg		
	8562274	0.185		neg		
	8562276	0.116		neg		
	8562278	0.109		neg		
	8562279	0.061		neg		
	8562282	0.254		neg		
	8562283	0.083		neg		
	8562285	0.121		neg		
	8562286	0.166		neg		
	8562287	0.033		neg		
	8562288	0.120		neg		
	8562289	0.208		neg		
	8562292	0.045		neg		
	8562295	0.101		neg		
	8562296	0.064		neg		
	8562297	0.056		neg		
20/08/98	Positive	1.386	**0.295**			
	Positive	1.482				
	8562269	0.049		neg		
	8562300	0.067		neg		
	8562303	0.059		neg		
	8562304	0.189		neg		
	8562306	0.055		neg		
	8562308	0.049		neg		
	8562311	0.099		neg		
	8562312	0.129		neg		
	8562313	0.098		neg		
	8562314	0.126		neg		
	8562315	0.092		neg		
	8562316	0.090		neg		
	8562323	0.097		neg		
	8562326	0.028		neg		
21/08/98	Positive	1.636	**0.316**			
	Positive	1.653				

Date	Sample ID	OD_{492}	Cutoff	Result	Stool culture	Unit culture
	8561710	0.157		neg		
	8561711	0.252		neg		
	8561712	0.635		**pos**	**neg**	**neg**
	8561717	0.133		neg		
	8561719	0.062		neg		
	8561721	0.029		neg		
	8562330	0.114		neg		
	8562332	0.283		neg		
	8562335	0.073		neg		
	8562336	0.073		neg		
	8562337	0.066		neg		
25/08/98	Positive	1.646	**0.324**			
	Positive	1.573				
	8562347	0.114		neg		
	8562349	0.076		neg		
	8562350	0.048		neg		
	8562352	0.054		neg		
	8561725	0.307		neg		
	8561726	0.052		neg		
	8561727	0.083		neg		
	8561728	0.124		neg		
	8561733	0.058		neg		
	8561734	0.070		neg		
26/08/98	Positive	1.584	**0.333**			
	Positive	1.628				
	8562376	0.093		neg		
	8562377	0.042		neg		
	8562381	0.044		neg		
	8562382	0.050		neg		
	8562383	0.049		neg		
	8562385	0.080		neg		
	8562386	0.097		neg		
	8562387	0.087		neg		
	8562388	0.048		neg		
	8562390	0.061		neg		
	8562392	0.048		neg		
	8562393	0.054		neg		
	8562395	0.305		neg		
	8562396	0.056		neg		
	8562397	0.070		neg		
	8562398	0.285		neg		

Date	Sample ID	OD_{492}	Cutoff	Result	Stool culture	Unit culture
	8562399	0.065		neg		
	8562400	0.154		neg		
	8562402	0.038		neg		
	8562403	0.084		neg		
	8562407	0.071		neg		
	8562408	0.044		neg		
	8562410	0.055		neg		
	8562411	0.016		neg		
	8562412	0.062		neg		
	8562414	0.139		neg		
	8562418	0.059		neg		
	8562422	0.039		neg		
	8562424	0.045		neg		
	8562425	0.035		neg		
27/08/98	Positive	1.588	**0.334**			
	Positive	1.635				
	8562432	0.065		neg		
	8562433	0.052		neg		
	8562435	0.114		neg		
	8562436	0.072		neg		
	8562437	0.261		neg		
	8562440	0.034		neg		
	8562443	0.070		neg		
	8562445	0.044		neg		
	8562446	0.142		neg		
	8562447	0.045		neg		
	8562449	0.033		neg		
	8562450	0.215		neg		
	8562451	0.078		neg		
	8562452	0.131		neg		
	8562453	0.103		neg		
	8562454	0.053		neg		
9/09/98	Positive	1.609	**0.376**			
	Positive	1.820				
	8573530	0.061		neg		
	8573528	0.111		neg		
	8573527	0.051		neg		
	8573535	0.109		neg		
	8573537	0.045		neg		
	8573542	0.136		neg		
	8573544	0.118		neg		

Date	Sample ID	OD_{492}	Cutoff	Result	Stool culture	Unit culture
	8573545	0.085		neg		
	8573546	0.097		neg		
	8573547	0.052		neg		
	8573548	0.060		neg		
	8573549	0.058		neg		
	8573550	0.141		neg		
	8573552	0.094		neg		
	8562483	0.032		neg		
	8562484	0.040		neg		
	8562485	0.064		neg		
	8573531	0.112		neg		
	8573536	0.067		neg		
	8573541	0.322		neg		
10/09/98	Positive	1.368	**0.341**			
	Positive	1.299				
	8573557	0.054		neg		
	8573559	0.176		neg		
	8573560	0.052		neg		
	8573561	0.059		neg		

Appendix 6

BLOOD DONOR SERVICE

QUESTIONNAIRE FOR ALL BLOOD DONORS - PLEASE COMPLETE BOTH SIDES

HEALTH QUESTIONS: Please tick appropriate box	YES	NO	NURSE COMMENTS
1. Have you ever donated blood?			
2. If Yes, was it within the last 3 months?			
3. Do you have any health concerns at the present time?			
4. Have you visited your doctor or medical clinic in the last 6 months?			
5. Are you awaiting surgery?			
6. Have you had dental treatment in the last 24 hours?			
7. Have you or any of your household been ill with diarrhoea vomiting, stomach pain or upset stomach in the last 4 weeks?			
8. Apart from the oral contraceptive pill, have you taken any pills or medicines (including Aspirin) in the last 2 weeks?			
9. Have you had pills for acne treatment in the last 2 years?			
10. Have you had a cold, cough or sore throat in the last week?			
11. Have you had a cold sore or other virus infection in the last week?			
12. Have you had jaundice or hepatitis or contact with hepatitis in the last year?			
13. Have you ever lived in or visited any of the countries on the "Overseas List" provided?			
14. Have you visited a malarial zone or taken antimalarial drugs in the last 3 years? Have you ever had malaria?			
15. Have you had any vaccinations, injections, acupuncture, electrolysis, ear or body piercing, tattoos, or needlestick injury in the last year?			
16. Do you have blood pressure problems, any heart disorder, chest pains, shortness of breath or have you ever had a stroke?			
17. Have you **ever** had tuberculosis, diabetes, asthma, hayfever, epilepsy (seizures/ fits), fainting, or multiple sclerosis?			
18. Do you have a medic alert device or suffer from a severe allergy?			
19. Have you **ever** had cancer (malignancy) including melanoma?			
20. Have you had a serious illness or severe accident in the last 5 years?			
21. Have you had an operation or blood transfusion in the last year?			
22. Have you **ever** received a tissue or organ transplant (cornea, kidneys, bone, bone marrow etc)?			
23. Have you or your close relatives an unexplained neurological condition or been diagnosed with Creutzfeldt-Jakob Disease (CJD)?			
24. Have you ever had injections of human pituitary growth hormone or pituitary Gonadotrophins (fertility medicines) or have you ever been to a neurosurgeon or neurologist, or had an operation on your brain or spinal cord?			
25. WOMEN: Are you breast feeding, or have you had any pregnancies, abortions or miscarriages in the last year?			

Appendix 6

SPECIAL HEALTH QUESTIONS	YES	NO	NURSE COMMENTS
26. Have you read and understood the current pamphlet on AIDS? (All donors must have read the pamphlet on AIDS)			
27. Are you giving blood to get an AIDS test?			
28. Do you have AIDS or have you **ever** had a positive HIV test?			
29. Have you had sex with a person with AIDS or HIV infection?			
30. Have you **ever** taken recreational/bodybuilding/illegal drugs with a needle? (even once, no matter how long ago).			
31. Have you had sex with a person described in question 30 in the last 2 years?			
32. Have you been a sex worker (prostitute) during the last 10 years?			
33. Have you had sex with a sex worker (prostitute) in the last 2 years?			
34. Have you **ever** had sex with a person who has visited or lived in a country on the list/map provided?			
35. Have you or your sexual partner(s) **ever** received blood products for an inherited bleeding disorder (eg. for haemophilia, von Willibrand's disease)?			
36. Are you or your sexual partner(s) carriers of Hepatitis B or Hepatitis C?			
37. MEN: Have you had sex with another male in the last 10 years, even with safe-sex methods?			
38. WOMEN: Have you had sex with a Bi-sexual or Homosexual male in the last 2 years?			
39. Do you have any questions or queries you would like to discuss with the Medical Officer?			

ALL DONORS	SURNAME _____	AGE ____ DATE OF BIRTH ___ / ___ / ___
	FIRST NAMES _____	COUNTRY OF BIRTH _____

NEW DONORS & DONORS FROM OTHER REGIONS OR CHANGE OF ADDRESS	PRIVATE ADDRESS _____	PHONE (HOME) _____
	_____	PHONE (WORK) _____
	EMPLOYER'S NAME AND ADDRESS _____	EXTN _____
	_____	CELL PHONE _____
	WHO IS YOUR DOCTOR? (Optional) _____	DONOR'S WEIGHT _____

I have read the pamphlet called the "Blood Donors Guide to the Questionnaire" and I consent to my blood being taken, tests being performed and information being collected as outlined in this pamphlet. I have answered these questions honestly.

Your signature: _____ Date: _____

Checked by: _____

FOR OFFICE USE ONLY

Date:	Clerical:	Donation Number
Hb:	Initial:	HTLV 1
Needle in:	Needle out:	
Stripped:	Sealed:	Time:
Group:	Rh Phenotype:	
Number of Donations:	Place of last donation:	
Comments		

Appendix 7

Published *ail* nucleotide sequence for Y. enterocolitica

Yersinia enterocolitica [gi:155437] PubMed, Protein, Related Sequences

```
LOCUS       YEPAIL    2220 bp    DNA          BCT       15-FEB-1996

DEFINITION  Yersinia enterocolitica attachment invasion locus (ail) gene
            and 2 ORFs, complete cds.
ACCESSION   M29945
VERSION     M29945.1  GI:155437
KEYWORDS    attachment invasion locus protein.
SOURCE      Yersinia enterocolitica (strain 8081c) DNA.
ORGANISM    Yersinia enterocolitica
            Bacteria; Proteobacteria; gamma subdivision;
            enterobacteriaceae; Yersinia.
REFERENCE   1  (bases 1 to 2220)
AUTHORS     Miller,V.L., Bliska,J.B. and Falkow,S.
TITLE       Nucleotide sequence of the Yersinia enterocolitica ail gene
            and characterisation of the Ail protein product
JOURNAL     J. Bacteriol. 172 (2), 1062-1069 (1990)
MEDLINE     90130261

BASE COUNT      601 a    460 c    500 g    659 t

ORIGIN
1    ggatcacatc atcaataccg aagcccaaga gattagccag tgtgccagaa aaatggctcg
61   atgggacgtt ggtggaagga agcaaatatt gcttacggca cgaaaacgca tgatagatga
121  gcttcagatg tatttgccag gactgggaag tcacgtgggt aattactgtg acatccagta
181  ataaaacaga gcctctatta aaggagcttc ccaatttgaa atcagaaaaa ttacatcata
241  aacatgggtg tccagaagtc agtcggcgat atatccattt aaagagcatt gagctatgac
301  cagtattcat caactacaga acaaaaatac aggaataagt gactgatggg ataaagctga
361  ggtaagctca cagtactgta tcaatatcca tatttacata tatatcatgg atttggcatt
421  atatcatcag ccatgtcagt gatatggtta ttgtattagt attgttataa caatctggat
481  tatttttatg aaaaagacat tactagctag ttctctaata gcctgtttat caattgcgtc
541  tgttaatgtg tacgctgcga gtgaaagtag tatttctatt ggttatgcgc aaagccatgt
601  aaaagaaaat gggtatacat tggataatga ccctaaaggt tttaacctga agtaccgtta
661  tgaactcgat gataactggg gagtaatagg ttcgtttgct tatactcatc agggatatga
721  tttcttctat ggcagtaata agtttggtca tggtgatgtt gattactatt cagtaacaat
781  ggggccatct ttccgcatca acgaatatgt tagcctttat ggattactgg gggccgctca
841  tggaaaggtt aaggcatctg tatttgatga atcaatcagt gcaagtaaga cgtcaatggc
901  atacggggca ggggtgcaat tcaacccact tccaaatttt gtcattgacg cttcatatga
961  atactccaaa ctcgatagca taaaagttgg cacctggatg cttggtgcag ggtatcgatt
1021 ctaatcatct cagatagtga aaacccacct gagtgaagtg aaccccattt attggacact
1081 tttcctggcg gttgacatgg cctgatttcg gtactgcacc ggactcaggc cgtttaattt
1141 tactttgatc ctttcgttgt tgtagtaatg gatatactca tccaccgctt ttttcagttg
1201 ttctacatct tcgtattttt cattgtgcca gcattcagtc ttcagcagac caaaaaagtt
1261 ttctatcaca gcattatcca ggcagttgcc cttgcgcgac atactttgct ttacttcgcc
1321 agaccccagc cttttcttat agcttgccat ctgatattgc cagccctgat ccgagtgaag
1381 tacaggttca tcgcctgagt tcaacttctg tagcgcatca tcaagcattt tatcaatcag
1441 gttcattccg ggatgcgtat ccatctgcca ggcaacgact tcgctgttat acagatccag
1501 cacgggtgac agatacagct ttttacccct gacgttgaac tcggtcacat cgttacccac
1561 ttctggttag gggcttcggc agtaaatttt cgagcaagta tattagggac cactttaccg
1621 taggcaccct gatatgactg atatttttta cgacgcaagt tagatgcaag ctgctgttgc
1681 cgcatgagtt ttcgtacggt tttatggtta agactcccgc cctcattgcg tagggccagc
1741 gttattctgc ggtaaccata gcgaccttta tgatggtgaa acagggtttt tattctttgt
1801 ttctcatccg cataagtctc ttcacgacca ctggatttta cctgccagta gaaggtgctg
1861 cgcggaagac cggccacgta aagcaaggtc gccagtttat acagatgcct taattcagtg
1921 attattcgcg ttttttccgc tgcttctctt acaggtggta ttcactgagt gccaccgata
1981 atgcgcaggc aaagtcatta acgacccccg ccgctcaccc tgagcatggt cgttgatggc
```

```
2041 ttttatattt tccatagagc agaggatgat tctttatgtc ccgagtgaac tggggtgaac
2101 ggttatcccg gtttgccgct gaatggcaac ggacgggaat atcccctaaa gagtggtgtg
2161 agagagaagg ttattcgtgg ggaacagcga aagcgtatat ttcgataaaa gcagcgaaag
//
```

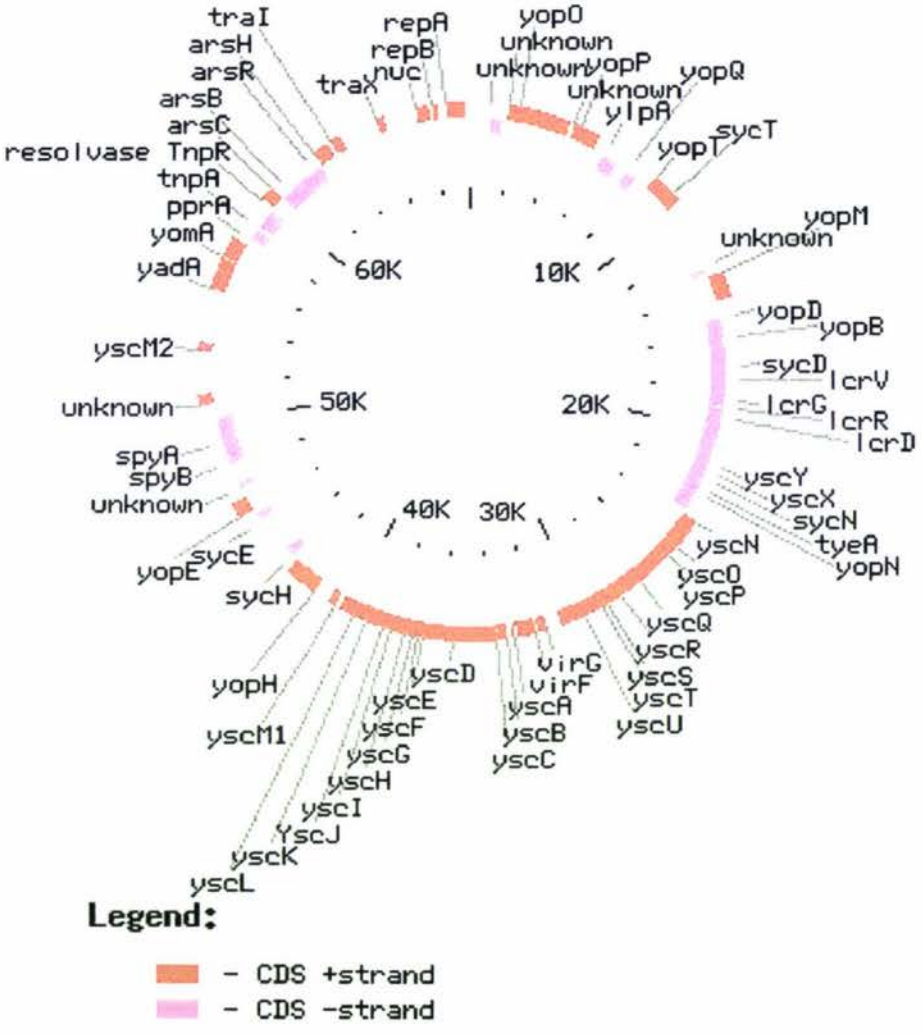
Appendix 8

Coding sequence of *Y. enterocolitica* plasmid pYVe227

Accession No: NC 002120

Total bases sequenced: 69673

Completed: Feb 28, 1999



From: http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/framik?db=Genome&gi=15220

References

Aber, R. C. (1990). Transfusion-associated *Yersinia enterocolitica*. *Transfusion* **30**, 193-5.

Aleksic, S., Burwitz, K., Bockemuhl, J. & Aleksic, V. (1991). Immune response to flagellar antigens in human infections with *Yersinia enterocolitica*. *Contributions to Microbiology & Immunology* **12**, 110-6.

Ameratunga, R., Lambert, T., Daly, D. M. & Caughey, D. E. (1987). Reactive arthritis caused by *Yersinia enterocolitica* [letter]. *New Zealand Medical Journal* **100**, 725.

Arduino, M. J., Bland, L. A., Tipple, M. A., Aguero, S. M., Favero, M. S. & Jarvis, W. R. (1989). Growth and endotoxin production of *Yersinia enterocolitica* and *Enterobacter agglomerans* in packed erythrocytes. *Journal of Clinical Microbiology* **27**, 1483-5.

Australia, N. C. D. C. C. D. N. (1999). National Notifiable Diseases Surveillance System. *[online] http://www.health.gov.au/pubhlth/cdi/cdihtml.htm.*

Autenrieth, I. B., Beer, M., Bohn, E., Kaufmann, S. H. & Heesemann, J. (1994). Immune responses to *Yersinia enterocolitica* in susceptible BALB/c and resistant C57BL/6 mice: an essential role for gamma interferon. *Infect. Immun.* **62**, 2590-9.

Autenrieth, I. B., Beer, M., Hantschmann, P., Preger, S., Vogel, U., Heymer, B. & Heesemann, J. (1993). The cellular immune response against *Yersinia enterocolitica* in different inbred strains of mice: evidence for an important role of T lymphocytes. *International Journal of Medical Microbiology, Virology, Parasitology & Infectious Diseases* **278**, 383-95.

Autenrieth, I. B. & Firsching, R. (1996). Penetration of M cells and destruction of Peyer's patches by *Yersinia enterocolitica*: an ultrastructural and histological study. *Journal of Medical Microbiology* **44**, 285-94.

Barton, M., Kolega, V. & Fenwick, S. (1997). *Yersinia enterocolitica*. Foodborne Microorganisms of Public Health Significance, Fifth edn., 493 - 513.

Beeching, N. J., Hart, H. H., Synek, B. J. & Bremner, D. A. (1985). A patient with hemosiderosis and multiple liver abscesses due to *Yersinia enterocolitica*. *Pathology* **17**, 530-2.

Benoit, C., Guiyoule, A. & Carniel, E. (1996). [Serodiagnosis of human pathogenic Yersinia infections]. [French]. *Presse Medicale* **25**, 1627-30.

Beuscher, H. U., Rodel, F., Forsberg, A. & Rollinghoff, M. (1995). Bacterial evasion of host immune defense: *Yersinia enterocolitica* encodes a suppressor for tumor necrosis factor alpha expression. *Infection & Immunity* **63**, 1270-7.

Bitzan, M., Hack, H. J. & Mauff, G. (1987). *Yersinia enterocolitica* serodiagnosis: a dual role of specific IgA. Evaluation of microagglutination and ELISA. *Zentralblatt Fur Bakteriologie, Mikrobiologie, Und Hygiene - Series A, Medical Microbiology, Infectious Diseases, Virology, Parasitology* **267**, 194-205.

Bjune, G., Ruud, T. E. & Eng, J. (1984). Bacterial shock due to transfusion with *Yersinia enterocolitica* infected blood. *Scandinavian Journal of Infectious Diseases* **16**, 411-2.

Black, R. E., Jackson, R. J., Tsai, T., Medvesky, M., Shayegani, M., Feeley, J. C., MacLeod, K. I. & Wakelee, A. M. (1978). Epidemic *Yersinia enterocolitica* infection due to contaminated chocolate milk. *New England Journal of Medicine* **298**, 76-9.

Blajchman, M. A. (1995). Bacterial contamination of blood products and the value of pre-transfusion testing. *Immunological Investigations* **24**, 163-70.

Bolin, I., Forsberg, A., Norlander, L., Skurnik, M. & Wolf-Watz, H. (1988). Identification and mapping of the temperature-inducible, plasmid-encoded proteins of Yersinia spp. *Infection & Immunity* **56**, 343-8.

Bone, R. C. (1993). Gram-negative sepsis: a dilemma of modern medicine. *Clinical Microbiology Reviews* **6**, 57-68.

Bottone, E. J. (1997). *Yersinia enterocolitica*: the charisma continues. *Clinical*

Microbiology Reviews **10**, 257-76.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-54.

Braude, A. I. (1986). Bacterial endotoxins, second edn. Philadelphia: W. B. Saunders Co.

Brett, G. (1993). Why David Burns can't rest in peace. *North and South* , 86-97.

Buchholz, D. H., AuBuchon, J. P., Snyder, E. L., Kandler, R., Edberg, S., Piscitelli, V., Pickard, C. & Napychank, P. (1992). Removal of *Yersinia enterocolitica* from AS-1 red cells. *Transfusion* **32**, 667-72.

Buchholz, D. H., Young, V. M., Friedman, N. R., Reilly, J. A. & Mardiney, M. R., Jr. (1973). Detection and quantitation of bacteria in platelet products stored at ambient temperature. *Transfusion* **13**, 268-75.

Bufill, J. A. & Ritch, P. S. (1989). *Yersinia enterocolitica* serotype 0:3 sepsis after blood transfusion. *New England Journal of Medicine* **320**, 810.

Carter, P. B. (1975). Pathogenicity of *Yersinia enterocolitica* for mice. *Infection & Immunity* **11**, 164-70.

China, B., BT, N. G., de Bruyere, M. & Cornelis, G. R. (1994). Role of YadA in resistance of *Yersinia enterocolitica* to phagocytosis by human polymorphonuclear leukocytes. *Infection & Immunity* **62**, 1275-81.

China, B., N'Guyen, B. T., de Bruyere, M. & Cornelis, G. R. (1994). Role of YadA in resistance of *Yersinia enterocolitica* to phagocytosis by human polymorphonuclear leukocytes. *Infection & Immunity* **62**, 1275-81.

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. & Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis

genome. *Science* **244**, 359-62.

Corbel, M. J. (1990). *Topley and Wilson's, Principles of Bacteriology, Virology and Immunity.*, 8th edition edn. London: Edward Arnold.

Cornelis, G. R., Biot, T., Lambert de Rouvroit, C., Michiels, T., Mulder, B., Sluiters, C., Sory, M. P., Van Bouchaute, M. & Vanooteghem, J. C. (1989). The Yersinia yop regulon. *Molecular Microbiology* **3**, 1455-9.

Cornelis, G. R., Boland, A., Boyd, A. P., Geuijen, C., Iriarte, M., Neyt, C., Sory, M. P. & Stainier, I. (1998). The virulence plasmid of Yersinia, an antihost genome. *Microbiology & Molecular Biology Review* **62**, 1315-52.

Cornelis, G. R. & Wolf-Watz, H. (1997). The Yersinia Yop virulon: a bacterial system for subverting eukaryotic cells. *Molecular Microbiology* **23**, 861-7.

Cover, T. L. & Aber, R. C. (1989). *Yersinia enterocolitica. New England Journal of Medicine* **321**, 16-24.

Cremer, J., Putzker, M., Faulde, M. & Zoller, L. (1993). Immunoblotting of Yersinia plasmid-encoded released proteins: a tool for serodiagnosis. *Electrophoresis* **14**, 952-9.

Davis, T. E. & Fuller, D. D. (1991). Direct identification of bacterial isolates in blood cultures by using a DNA probe. *Journal of Clinical Microbiology* **29**, 2193-6.

de Koning, J., Heesemann, J., Hoogkamp-Korstanje, J. A., Festen, J. J., Houtman, P. M. & van Oijen, P. L. (1989). Yersinia in intestinal biopsy specimens from patients with seronegative spondylarthropathy: correlation with specific serum IgA antibodies. *Journal of Infectious Diseases* **159**, 109-12.

Emody, L., Heesemann, J., Wolf-Watz, H., Skurnik, M., Kapperud, G., O'Toole, P. & Wadstrom, T. (1989). Binding to collagen by *Yersinia enterocolitica and Yersinia pseudotuberculosis*: evidence for yopA-mediated and chromosomally encoded mechanisms. *Journal of Bacteriology* **171**, 6674-9.

Feng, P., Keasler, S. P. & Hill, W. E. (1992). Direct identification of *Yersinia enterocolitica* in blood by polymerase chain reaction amplification. *Transfusion* **32**, 850-4.

Fenwick, S. G. (1992). Pharyngitis and infections with *Yersinia enterocolitica*. *New Zealand Medical Journal* **105**, 112.

Fenwick, S. G., Madie, P. & Wilks, C. R. (1994). Duration of carriage and transmission of *Yersinia enterocolitica* biotype 4, serotype 0:3 in dogs. *Epidemiology & Infection* **113**, 471-7.

Fenwick, S. G. & McCarthy, M. D. (1995). *Yersinia enterocolitica* is a common cause of gastroenteritis in Auckland. *New Zealand Medical Journal* **108**, 269-71.

Fenwick, S. G., Wauters, G., Ursing, J. & Gwozdz, M. (1996). Unusual *Yersinia enterocolitica* strains recovered from domestic animals and people in New Zealand. *FEMS Immunology & Medical Microbiology* **16**, 241-5.

Fernandez-Lago, L., Gomez, M., Vizcaino, N. & Chordi, A. (1994). Analysis of the immune response to *Yersinia enterocolitica* serotype-O:9-released proteins by immunoblot and ELISA. *Research in Microbiology* **145**, 553-61.

Franzin, L. & Gioannini, P. (1992). Growth of Yersinia species in artificially contaminated blood bags. *Transfusion* **32**, 673-6.

Gaede, K., Mack, D. & Heesemann, J. (1992). Experimental *Yersinia enterocolitica* infection in rats: analysis of the immune response to plasmid-encoded antigens of arthritis-susceptible Lewis rats and arthritis-resistant Fischer rats. *Medical Microbiology & Immunology* **181**, 165-72.

Galloway, S. J. & Jones, P. D. (1986). Transfusion acquired *Yersinia enterocolitica*. *Australian & New Zealand Journal of Medicine* **16**, 248.

Gemski, P., Lazere, J. R. & Casey, T. (1980). Plasmid associated with pathogenicity and calcium dependency of *Yersinia enterocolitica*. *Infection & Immunity* **27**, 682-5.

Gibb, A. P., Martin, K. M., Davidson, G. A., Walker, B. & Murphy, W. G. (1992). Bacterial growth in blood for transfusion [letter; comment]. *Lancet* **340**, 1222-3.

Gibb, A. P., Martin, K. M., Davidson, G. A., Walker, B. & Murphy, W. G. (1994). Modeling the growth of *Yersinia enterocolitica* in donated blood. *Transfusion* **34**, 304-10.

Gibson, T. & Norris, W. (1958). Skin fragments removed by injection needles. *Lancet*, 983-985.

Gilmour, A. & Walker, S. J. (1988). Isolation and identification of *Yersinia enterocolitica* and the *Yersinia enterocolitica*-like bacteria. *Society for Applied Bacteriology Symposium Series* **17**, 213S-236S.

Goddard, D., Jacobs, S. I. & Manohitharajah, S. M. (1973). The bacteriological screening of platelet concentrates stored at 22°C. *Transfusion* **13**, 103-6.

Goldman, M. & Blajchman, M. A. (1991). Blood product-associated bacterial sepsis. *Transfusion Medicine Reviews* **5**, 73-83.

Gottlieb, T. (1993). Hazards of bacterial contamination of blood products. *Anaesthesia & Intensive Care* **21**, 20-3.

Granfors, K. (1979). Measurement of immunoglobulin M (IgM), IgG, and IgA antibodies against *Yersinia enterocolitica* by enzyme-linked immunosorbent assay: persistence of serum antibodies during disease. *Journal of Clinical Microbiology* **9**, 336-41.

Granfors, K., Lahesmaa-Rantala, K. & Toivanen, A. (1988). IgM, IgG, and IgA antibodies in Yersinia infection [letter]. *Journal of Infectious Diseases* **157**, 601-2.

Granfors, K., Ogasawara, M., Hill, J. L., Lahesmaa-Rantala, R., Toivanen, A. & Yu, D. T. (1989). Analysis of IgA antibodies to lipopolysaccharide in Yersinia-triggered reactive arthritis. *Journal of Infectious Diseases* **159**, 1142-7.

Granfors, K., Viljanen, M., Tiilikainen, A. & Toivanen, A. (1980). Persistence of IgM,

IgG, and IgA antibodies to Yersinia in Yersinia arthritis. *Journal of Infectious Diseases* **141**, 424-9.

Green, S. P., Hartland, E. L., Robins-Browne, R. M. & Phillips, W. A. (1995). Role of YopH in the suppression of tyrosine phosphorylation and respiratory burst activity in murine macrophages infected with *Yersinia enterocolitica*. *Journal of Leukocyte Biology* **57**, 972-7.

Gronberg, A., Fryden, A. & Kihlstrom, E. (1989). Humoral immune response to individual *Yersinia enterocolitica* antigens in patients with and without reactive arthritis. *Clinical & Experimental Immunology* **76**, 361-5.

Grossman, B. J., Kollins, P., Lau, P. M., Perreten, J. L., Bowman, R. J., Malcolm, S. & Palko, W. M. (1991). Screening blood donors for gastrointestinal illness: a strategy to eliminate carriers of *Yersinia enterocolitica*. *Transfusion* **31**, 500-1.

Hakansson, S., Bergman, T., Vanooteghem, J. C., Cornelis, G. & Wolf-Watz, H. (1993). YopB and YopD constitute a novel class of Yersinia Yop proteins. *Infection & Immunity* **61**, 71-80.

Hanski, C., Kutschka, U., Schmoranzer, H. P., Naumann, M., Stallmach, A., Hahn, H., Menge, H. & Riecken, E. O. (1989). Immunohistochemical and electron microscopic study of interaction of *Yersinia enterocolitica* serotype O8 with intestinal mucosa during experimental enteritis. *Infection & Immunity* **57**, 673-8.

Hanski, C., Naumann, M., Grutzkau, A., Pluschke, G., Friedrich, B., Hahn, H. & Riecken, E. O. (1991). Humoral and cellular defense against intestinal murine infection with *Yersinia enterocolitica*. *Infection & Immunity* **59**, 1106-11.

Hanski, C., Naumann, M., Hahn, H. & Riecken, E. O. (1989). Determinants of invasion and survival of *Yersinia enterocolitica* in intestinal tissue. An in vivo study. *Medical Microbiology & Immunology* **178**, 289-96.

Hartland, E. L., Bordun, A. M. & Robins-Browne, R. M. (1996). Contribution of YopB

to virulence of *Yersinia enterocolitica*. *Infection & Immunity* **64**, 2308-14.

Hartland, E. L., Green, S. P., Phillips, W. A. & Robins-Browne, R. M. (1994). Essential role of YopD in inhibition of the respiratory burst of macrophages by *Yersinia enterocolitica*. *Infection & Immunity* **62**, 4445-53.

Heal, J. M., Jones, M. E., Forey, J., Chaudhry, A. & Stricof, R. L. (1987). Fatal *Salmonella* septicemia after platelet transfusion. *Transfusion* **27**, 2-5.

Heath, F. & Province, W. (1942). Preservation of human plasma: report of studies with sulphonamide compounds. *Jama* **1915**, 781-788.

Heesemann, J., Eggers, C. & Schroder, J. (1987). Serological diagnosis of Yersiniosis by immunoblot technique using virulence-associated antigen of enteropathogenic *Yersiniae*. *Contributions to Microbiology & Immunology* **9**, 285-9.

Heesemann, J. & Gaede, K. (1989). Mechanisms involved in the pathogenesis of Yersinia infections. [Review]. *Rheumatology International* **9**, 213-7.

Heesemann, J., Gaede, K. & Autenrieth, I. B. (1993). Experimental *Yersinia enterocolitica* infection in rodents: a model for human Yersiniosis. [Review]. *Apmis* **101**, 417-29.

Heesemann, J., Gross, U., Schmidt, N. & Laufs, R. (1986). Immunochemical analysis of plasmid-encoded proteins released by enteropathogenic Yersinia sp. grown in calcium-deficient media. *Infection & Immunity* **54**, 561-7.

Heesemann, J., Schroder, J. & Ulrich, M. (1988). Analysis of the class-specific immune response to *Yersinia enterocolitica* virulence-associated antigens in orogastrically infected rabbits. *Microbial Pathogenesis* **5**, 437-47.

Heltberg, O., Skov, F., Gerner-Smidt, P., Kolmos, H. J., Dybkjaer, E., Gutschik, E., Jerne, D., Jepsen, O. B., Weischer, M. & Frederiksen, W. (1993). Nosocomial epidemic of *Serratia marcescens* septicemia ascribed to contaminated blood transfusion bags.

Transfusion **33**, 221-7.

Hogman (1996). Adverse effects: bacterial contamination. *Vox Sanguinis* **70**, 78-82.

Hogman, C. F. & Engstrand, L. (1996). Factors affecting growth of *Yersinia enterocolitica* in cellular blood products. *Transfusion Medicine Reviews* **10**, 259-75.

Hoogkamp-Korstanje, J. A., de Koning, J. & Heesemann, J. (1988). Persistence of *Yersinia enterocolitica* in man. *Infection* **16**, 81-5.

Hoogkamp-Korstanje, J. A., de Koning, J., Heesemann, J., Festen, J. J., Houtman, P. M. & van Oyen, P. L. (1992). Influence of antibiotics on IgA and IgG response and persistence of *Yersinia enterocolitica* in patients with Yersinia-associated spondylarthropathy. *Infection* **20**, 53-7.

Illert, W. E., Sanger, W. & Weise, W. (1995). Bacterial contamination of single-donor blood components. *Transfusion Medicine* **5**, 57-61.

Iriarte, M. & Cornelis, G. R. (1999). Identification of SycN, YscX, and YscY, three new elements of the Yersinia yop virulon. *Journal of Bacteriology* **181**, 675-80.

Iteman, I., Baril, C., Saint Girons, I. & Carniel, E. (1991). Pulse field electrophoresis of the chromosome of the pathogenic *Yersiniae*. *Contributions to Microbiology & Immunology* **12**, 198-202.

Jacobs, J., Jamaer, D., Vandeven, J., Wouters, M., Vermylen, C. & Vandepitte, J. (1989). *Yersinia enterocolitica* in donor blood: a case report and review. *Journal of Clinical Microbiology* **27**, 1119-21.

Jones, B. L. & Hanson, M. F. (1994). Prevention of transfusion of *Yersinia enterocolitica*. *Journal of Hospital Infection* **28**, 236-8.

Kapperud, G., Namork, E. & Skarpeid, H. J. (1985). Temperature-inducible surface fibrillae associated with the virulence plasmid of *Yersinia enterocolitica* and *Yersinia*

pseudotuberculosis. *Infection & Immunity* **47**, 561-6.

Katz, A. J. & Tilton, R. C. (1970). Sterility of platelet concentrates stored at 25 C. *Transfusion* **10**, 329-30.

Katz, L., MacPherson, J. L. & Zuck, T. F. (1992). Yersinia and blood donation. *Transfusion* **32**, 191.

Khabbaz, R. F., Arnow, P. M., Highsmith, A. K., Herwaldt, L. A., Chou, T., Jarvis, W. R., Lerche, N. W. & Allen, J. R. (1984). *Pseudomonas fluorescens* bacteremia from blood transfusion. *American Journal of Medicine* **76**, 62-8.

Kihlstrom, E., Fryden, A. & Gronberg, A. (1991). Immunoblot analysis of antibody response to *Yersinia enterocolitica* in patients with reactive arthritis and enterocolitis. *Contributions to Microbiology & Immunology* **12**, 93-7.

Kim, D. M., Brecher, M. E., Bland, L. A., Estes, T. J., Carmen, R. A. & Nelson, E. J. (1992). Visual identification of bacterially contaminated red cells. *Transfusion* **32**, 221-5.

Kim, D. M., Brecher, M. E., Bland, L. A., Estes, T. J., McAllister, S. K., Aguero, S. M., Carmen, R. A. & Nelson, E. J. (1992). Prestorage removal of *Yersinia enterocolitica* from red cells with white cell-reduction filters. *Transfusion* **32**, 658-62.

Kimber, R. J. (1995). *Yersinia enterocolitica* and blood transfusion. *Medical Journal of Australia* **162**, 277.

Kittelberger, R., Hilbink, F., Hansen, M. F., Ross, G. P., Joyce, M. A., Fenwick, S., Heesemann, J., Wolf-Watz, H. & Nielsen, K. (1995). Serological crossreactivity between *Brucella abortus* and *Yersinia enterocolitica* 0:9 II the use of Yersinia outer proteins for the specific detection of *Yersinia enterocolitica* infections in ruminants. *Veterinary Microbiology* **47**, 271-80.

Kono, D. H., Ogasawara, M. & Yu, D. T. (1985). An animal model to study serum immunoglobulin response to infection by *Yersinia enterocolitica*. *Clinical & Experimental*

Rheumatology **3**, 117-21.

Krishnan, L. A. & Brecher, M. E. (1995). Transfusion-transmitted bacterial infection. *Hematology - Oncology Clinics of North America* **9**, 167-85.

Kuehnert, M. J. & Jarvis, W. R. (1998). Bacteria and safety of the blood supply. *Annals of Internal Medicine* **129**, 164-5.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-5.

Lahesmaa-Rantala, R., Heesemann, J., Lehtonen, O. P., Granfors, K. & Toivanen, A. (1989). Avidity of antibodies against released proteins of *Yersinia* spp: comparison of patients with or without reactive arthritis. *Annals of the Rheumatic Diseases* **48**, 1003-6.

Lahesmaa-Rantala, R., Lehtonen, O. P., Granfors, K. & Toivanen, A. (1987). Avidity of anti-Yersinia antibodies in yersiniosis patients with and without Yersinia-triggered reactive arthritis. *Arthritis & Rheumatism* **30**, 1176-81.

Lange, S. & Larsson, P. (1984). What do serum antibodies to *enterocolitica* indicate? *Reviews of Infectious Diseases* **6**, 880-1.

Larsen, J. H., Hartzen, S. H. & Parm, M. (1985). The determination of specific IgA-antibodies to *Yersinia enterocolitica* and their role in enteric infections and their complications. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica - Section B, Microbiology* **93**, 331-9.

Lee, A. G., Krishnan, M. D. & Brecher, M. E. (1995). Transfusion transmitted bacterial infection. *Hematology/Oncology Clinics of North America* **9**, 167-85.

Lee, L. A., Taylor, J., Carter, G. P., Quinn, B., Farmer, J. J. d. & Tauxe, R. V. (1991). *Yersinia enterocolitica* O:3: an emerging cause of pediatric gastroenteritis in the United States. The *Yersinia enterocolitica* Collaborative Study Group. *Journal of Infectious Diseases* **163**, 660-3.

Lefer, A. M. (1989). Significance of lipid mediators in shock states. *Circulatory Shock* **27**, 3-12.

Lehtinen, K., Granfors, K., Toivanen, A. & Isomaki, H. (1988). IgA anti-Yersinia antibodies and the outcome of Yersinia-triggered reactive arthritis. *Clinical & Experimental Rheumatology* **6**, 424-5.

Leino, R., Granfors, K., Havia, T., Heinonen, R., Lampinen, M. & Toivanen, A. (1987). Yersiniosis as a gastrointestinal disease. *Scandinavian Journal of Infectious Diseases* **19**, 63-8.

Leino, R., Lahesmaa-Rantala, R., Granfors, K. & Toivanen, A. (1988). IgA class antibodies against *Yersinia enterocolitica* O:3 in patients with thyroid disease. *Acta Endocrinologica* **119**, 81-4.

Lello, J. M. & Lennon, D. (1992). Yersinia arthritis versus acute rheumatic fever in a boy. *New Zealand Medical Journal* **105**, 12-3.

Lian, C. J., Hwang, W. S., Kelly, J. K. & Pai, C. H. (1987). Invasiveness of *Yersinia enterocolitica* lacking the virulence plasmid: an in-vivo study. *Journal of Medical Microbiology* **24**, 219-26.

Maki-Ikola, O., Hill, J. L., Lahesmaa, R., Toivanen, A. & Granfors, K. (1992). IgG and IgA antibody responses against porins in Yersinia-triggered reactive arthritis. *British Journal of Rheumatology* **31**, 315-8.

Martinez, M. B., Hoshino-Shimizu, S. & Falcao, D. P. (1991). *Yersinia enterocolitica*: antigenic components with diagnostic potential. *Contributions to Microbiology & Immunology* **12**, 123-6.

Maruyama, T. (1973). Studies on biological characteristics and pathogenicity of *Yersinia enterocolitica*. Experimental infection in Monkeys. *Japanese Journal of Bacteriology* **28**, 413-421.

Mattila, P. S., Valtonen, V., Tuori, M. R. & Makela, O. (1985). Antibody responses in arthritic and uncomplicated *Yersinia enterocolitica* infections. *Journal of Clinical Immunology* **5**, 404-11.

McCarthy, M. & Fenwick, S. (1991). Experiences with the diagnosis of *Yersinia enterocolitica* - an emerging gastrointestinal pathogen in the Auckland area. 1987-1989. *New Zealand Journal of Medical Laboratory Science* **45**, 19-22.

McGhee, J. R., Kiyono, H., Michalek, S. M. & Mestecky, J. (1987). *Mucosal Immunity.* Enteric immunization reveals a T cell network for IgA responses and suggests that humans possess a common mucosal immune system. New York. New York Academy of Sciences, **53**, 537-43.

Michalek, S. M., McGhee, J. R., Kiyono, H., Colwell, D. E., Eldridge, J. H., Wannemuehler, M. J. & Koopman, W. J. (1983). The IgA response: inductive aspects, regulatory cells, and effector functions. *Annals of the New York Academy of Sciences* **409**, 48-71.

Michiels, T., Wattiau, P., Brasseur, R., Ruysschaert, J. M. & Cornelis, G. (1990). Secretion of Yop proteins by Yersiniae. *Infection & Immunity* **58**, 2840-9.

Miller, V. L., Bliska, J. B. & Falkow, S. (1990). Nucleotide sequence of the *Yersinia enterocolitica ail* gene and characterization of the Ail protein product. *Journal of Bacteriology* **172**, 1062-9.

Miller, V. L., Farmer, J. J. d., Hill, W. E. & Falkow, S. (1989). The *ail* locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. *Infection & Immunity* **57**, 121-31.

Mitchell, R. & Barr, A. (1992). Transfusing *Yersinia enterocolitica. British Medical Journal* **305**, 1095-6.

MMWR (1991). From the Centers for Disease Control and Prevention. *Yersinia enterocolitica* bacteremia and endotoxin shock associated with red blood cell transfusions-

United States, 1991. *MMWR - Morbidity & Mortality Weekly Report* **40**, 176-8.

MMWR (1997). From the Centers for Disease Control and Prevention. Red blood cell transfusions contaminated with *Yersinia enterocolitica*-United States, 1991-1996, and initiation of a national study to detect bacteria-associated transfusion reactions. *MMWR - Morbidity & Mortality Weekly Report* **46**, 553-5.

Mollison, P. L., Engelfriet, C. P. & Contreras, M. (1987). *Blood Transfusion in Clinical Medicine*, 8th edn. Oxford: Blackwell Scientific Publications.

Morduchowicz, G., Pitlik, S. D., Huminer, D., Alkan, M., Drucker, M., Rosenfeld, J. B. & Block, C. S. (1991). Transfusion reactions due to bacterial contamination of blood and blood products. *Reviews of Infectious Diseases* **13**, 307-14.

Morris, A. & MacCulloch, D. (1996). Education may help reduce Yersinia sepsis from blood transfusion. *New Zealand Medical Journal* **109**, 19.

Morris, A. J. & Woodfield, D. G. (1998). Antibody screening for recent *Yersinia enterocolitica* infection in blood donors. *Transfusion* **38**, 511-2.

Morrison, D. C. (1983). Bacterial endotoxins and pathogenesis. *Reviews of Infectious Diseases* **5 Suppl 4**, S733-47.

Morrow, J. F., Braine, H. G., Kickler, T. S., Ness, P. M., Dick, J. D. & Fuller, A. K. (1991). Septic reactions to platelet transfusions. A persistent problem. *Jama* **266**, 555-8.

Ness, P. M. & Perkins, H. A. (1980). Transient bacteremia after dental procedures and other minor manipulations. *Transfusion* **20**, 82-5.

Nielsen, B., Heisel, C. & Wingstrand, A. (1996). Time course of the serological response to *Yersinia enterocolitica* O:3 in experimentally infected pigs. *Veterinary Microbiology* **48**, 293-303.

Nielsen, K. (1990). The serological response of cattle immunized with *Yersinia*

enterocolitica O:9 or O:16 to Yersinia and *Brucella abortus* antigens in enzyme immunoassays. *Veterinary Immunology & Immunopathology* **24**, 373-82.

Office, U. G. A. (1997). Blood supply: transfusion-associated risks - report to the ranking minority member, Committee on Commerce, House of Representatives. Washington, DC:. *Document no. GAO/PEMD* **97-2.**

Paerregaard, A. (1992). Interactions between *Yersinia enterocolitica* and the host with special reference to virulence plasmid encoded adhesion and humoral immunity. *Danish Medical Bulletin* **39**, 155-72.

Paerregaard, A., Espersen, F. & Skurnik, M. (1991). Role of the Yersinia outer membrane protein YadA in adhesion to rabbit intestinal tissue and rabbit intestinal brush border membrane vesicles. *Apmis* **99**, 226-32.

Pai, C. H. & DeStephano, L. (1982). Serum resistance associated with virulence in *Yersinia enterocolitica*. *Infection & Immunity* **35**, 605-11.

Pietersz, R. N., Reesink, H. W., Pauw, W., Dekker, W. J. & Buisman, L. (1992). Prevention of *Yersinia enterocolitica* growth in red-blood-cell concentrates. *Lancet* **340**, 755-6.

Pilz, D., Vocke, T., Heesemann, J. & Brade, V. (1992). Mechanism of YadA-mediated serum resistance of *Yersinia enterocolitica* serotype O3. *Infection & Immunity* **60**, 189-95.

Polissar, J. (1969). Transfusion hepatitis. Use of statistical decision theory. *Transfusion* **9**, 15-22.

Portnoy, D. A. & Martinez, R. J. (1985). Role of a plasmid in the pathogenicity of Yersinia species. *Current Topics in Microbiology & Immunology* **118**, 29-51.

Portnoy, D. A., Moseley, S. L. & Falkow, S. (1981). Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. *Infection & Immunity* **31**, 775-82.

Prpic, J. K., Robins-Browne, R. M. & Davey, R. B. (1983). Differentiation between virulent and avirulent *Yersinia enterocolitica* isolates by using Congo red agar. *Journal of Clinical Microbiology* **18**, 486-90.

Prpic, J. K., Robins-Browne, R. M. & Davey, R. B. (1985). In vitro assessment of virulence in *Yersinia enterocolitica* and related species. *Journal of Clinical Microbiology* **22**, 105-10.

Prpic, J. K., Robins-Browne, R. M. & Davey, R. B. (1987). Congo red pigmentation in *Yersinia enterocolitica* and related species. *Contributions to Microbiology & Immunology* **9**, 190-4.

Research, (1998). Surveillance data. *The New Zealand Public Health Report* **December 1998**, 86-87.

Riley, G. & Toma, S. (1989). Detection of pathogenic *Yersinia enterocolitica* by using congo red-magnesium oxalate agar medium. *Journal of Clinical Microbiology* **27**, 213-4.

Robins-Browne, R. M., Bordun, A. M. & Slee, K. J. (1993). Serological response of sheep to plasmid-encoded proteins of Yersinia species following natural infection *with Y. enterocolitica* and *Y. pseudotuberculosis*. *Journal of Medical Microbiology* **39**, 268-72.

Rosqvist, R., Bolin, I. & Wolf-Watz, H. (1988). Inhibition of phagocytosis in *Yersinia pseudotuberculosis*: a virulence plasmid-encoded ability involving the Yop2b protein. *Infection & Immunity* **56**, 2139-43.

Sambrook, J., E. F. Fritsch, and Maniatis. (1989). *Molecular cloning*: a laboratory manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.

Sazama, K. (1994). Bacteria in blood for transfusion. A review. *Archives of Pathology & Laboratory Medicine* **118**, 350-65.

Schedel, I. (1988). New aspects in the treatment of Gram-negative bacteraemia and septic shock. *Infection* **16**, 4-7.

Schifman, R. B. & Pindur, A. (1993). The effect of skin disinfection materials on reducing blood culture contamination. *American Journal of Clinical Pathology* **99**, 536-8.

Schwalbe, B. & Spath-Schwalbe, E. (1998). Endotoxin concentrations and cytokine responses in a patient with fatal transfusion-associated sepsis. *Transfusion* **38**, 703-5.

Shayegani, M., Morse, D., DeForge, I., Root, T., Parsons, L. M. & Maupin, P. S. (1983). Microbiology of a major foodborne outbreak of gastroenteritis caused by *Yersinia enterocolitica* serogroup O:8. *Journal of Clinical Microbiology* **17**, 35-40.

Skurnik, M. (1985). Expression of antigens encoded by the virulence plasmid of *Yersinia enterocolitica* under different growth conditions. *Infection & Immunity* **47**, 183-90.

Skurnik, M. & Poikonen, K. (1986). Experimental intestinal infection of rats by *Yersinia enterocolitica* 0:3. A follow-up study with specific antibodies to the virulence plasmid specified antigens. *Scandinavian Journal of Infectious Diseases* **18**, 355-64.

Soppi, E., Granfors, K. & Leino, R. (1987). Serum secretory IgA, IgA1 and IgA2 subclasses in inflammatory bowel and chronic liver diseases. *Journal of Clinical & Laboratory Immunology* **23**, 15-7.

Stahlberg, T. H., Granfors, K., Pekkola-Heino, K., Soppi, E. & Toivanen, A. (1987). Immunoblotting analysis of human IgM, IgG and IgA response to chromosomally coded antigens of *Yersinia enterocolitica* 0:3. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica - Section C, Immunology* **95**, 71-9.

Stahlberg, T. H., Granfors, K. & Toivanen, A. (1987). Immunoblot analysis of human IgM, IgG and IgA responses to plasmid-encoded antigens of *Yersinia enterocolitica* serovar O3. *Journal of Medical Microbiology* **24**, 157-63.

Stahlberg, T. H., Heesemann, J., Granfors, K. & Toivanen, A. (1989). Immunoblot analysis of IgM, IgG, and IgA responses to plasmid encoded released proteins of *Yersinia enterocolitica* in patients with or without Yersinia triggered reactive arthritis. *Annals of Rheumatic Diseases* **48**, 577-81.

Stemerowicz, R., Moller, B., Martin, P., Heesemann, J., Wenzel, B. E., Galanos, C., Freudenberg, M. & Hopf, U. (1990). Antibody activity against lipopolysaccharides, lipid A and proteins from Enterobacteriaceae in patients with chronic inflammatory liver diseases. *Autoimmunity* **7**, 305-15.

Stites, D. P., Terr, A. I. & Parslow, T. G. (1994). The Mucosal Immune System. In *Basic & Clinical Immunology*, pp. 541 - 551. Connecticut: Appleton & Lange.

Stolk-Engelaar, V. & Hoogkamp-Korstanje, J. (1995). Diagnosis in *Yersinia enterocolitica* infection. *Contributions to Microbiology & Immunology* **13**, 137-9.

Stolk-Engelaar, V. M. & Hoogkamp-Korstanje, J. A. (1996). Clinical presentation and diagnosis of gastrointestinal infections by *Yersinia enterocolitica* in 261 Dutch patients. *Scandinavian Journal of Infectious Diseases* **28**, 571-5.

Strumia, M. & McGraw, J. (1941). Frozen and dried plasma for civil and military use. *JAMA* **1925**, 1193-1201.

Stuart, P. M. & Woodward, J. G. (1992). *Yersinia enterocolitica* produces superantigenic activity. *Journal of Immunology* **148**, 225-33.

Stubbs, J. R., Reddy, R. L., Elg, S. A., Perry, E. H., Adcock, L. L. & McCullough, J. (1991). Fatal *Yersinia enterocolitica* (serotype 0:5,27) sepsis after blood transfusion. *Vox Sanguinis* **61**, 18-23.

Tabor, E., Snoy, P., Jackson, D. R., Schaff, Z., Blatt, P. M. & Gerety, R. J. (1984). Additional evidence for more than one agent of human non-A, non-B hepatitis. transmission and passage studies in chimpanzees. *Transfusion* **24**, 224-30.

Tabrizi, S. N. & Robins-Browne, R. M. (1992). Influence of a 70 kilobase virulence plasmid on the ability of *Yersinia enterocolitica* to survive phagocytosis in vitro. *Microbial Pathogenesis* **13**, 171-9.

Theakston, E. P., Morris, A. J., Streat, S. J., Baker, B. W. & Woodfield, D. G. (1997).

Transfusion transmitted *Yersinia enterocolitica* infection in New Zealand. *Australian & New Zealand Journal of Medicine* **27**, 62-7.

Tipple, M. A., Bland, L. A., Murphy, J. J., Arduino, M. J., Panlilio, A. L., Farmer, J. J. d., Tourault, M. A., Macpherson, C. R., Menitove, J. E. & Grindon, A. J. (1990). Sepsis associated with transfusion of red cells contaminated with *Yersinia enterocolitica*. *Transfusion* **30**, 207-13.

Toivanen, A., Granfors, K., Lahesmaa-Rantala, R., Leino, R., Stahlberg, T. & Vuento, R. (1985). Pathogenesis of Yersinia-triggered reactive arthritis: immunological, microbiological and clinical aspects. *Immunological Reviews* **86**, 47-70.

Toivanen, A., Lahesmaa-Rantala, R., Vuento, R. & Granfors, K. (1987). Association of persisting IgA response with yersinia triggered reactive arthritis: a study on 104 patients. *Annals of the Rheumatic Diseases* **46**, 898-901.

Tung, B. G. (1971). Testing for Australian antigen. *Transfusion* **11**, 390.

Ulyatt, D. B., May, S. J. & Leng, R. (1991). Yersinia infected blood. *New Zealand Medical Journal* **104**, 431.

Verhaegen, J., Charlier, J., Lemmens, P., Delmee, M., Van Noyen, R., Verbist, L. & Wauters, G. (1998). Surveillance of human *Yersinia enterocolitica* infections in Belgium: 1967-1996. *Clinical Infectious Diseases* **27**, 59-64.

Vesikari, T., Granfors, K., Maki, M. & Gronroos, P. (1980). Evaluation of ELISA in the diagnosis of *Yersinia enterocolitica* diarrhoea in children. *Acta Pathologica et Microbiologica Scandinavica - Section B, Microbiology* **88**, 139-42.

Vuento, R., Eskola, J., Leino, R., Koskimies, S. & Viander, M. (1984). IgM, IgG, and IgA synthesis in vitro in persons suffering from *Yersinia* arthritis and in patients with ankylosing spondylitis. *Annals of the Rheumatic Diseases* **43**, 186-91.

Wagner, S. J., Friedman, L. I. & Dodd, R. Y. (1994). Transfusion-associated bacterial

sepsis. *Clinical Microbiology Reviews* **7**, 290-302.

Wauters, G., Kandolo, K. & Janssens, M. (1987). Revised biogrouping scheme of *Yersinia enterocolitica*. *Contributions to Microbiology & Immunology* **9**, 14-21.

Wick, M. R., Moore, S. & Taswell, H. F. (1985). Non-A, non-B hepatitis associated with blood transfusion. *Transfusion* **25**, 93-101.

Wilkinson, T. J., Colls, B. M., Chambers, T. & Ikram, R. B. (1991). Blood transfusion acquired *Yersinia enterocolitica* sepsis: two cases. *New Zealand Medical Journal* **104**, 120.

Winslow, E. J., Loeb, H. S., Rahimtoola, S. H., Kamath, S. & Gunnar, R. M. (1973). Hemodynamic studies and results of therapy in 50 patients with bacteremic shock. *American Journal of Medicine* **54**, 421-32.

Woodfield, D. G. (1991). Transfusion acquired *Serratia liquefaciens* septicaemia. *New Zealand Medical Journal* **104**, 141.

Wright, D. C., Selss, I. F., Vinton, K. J. & Pierce, R. N. (1985). Fatal *Yersinia enterocolitica* sepsis after blood transfusion. *Archives of Pathology & Laboratory Medicine* **109**, 1040-2.

Young, L. S. (1990). *Gram-negative sepsis*, 3rd edn. New York: Churchill Livingstone.