

**STUDIES ON YERSINIOSIS IN HUMAN AND SELECTED
ANIMAL POPULATIONS IN JOS AND ITS ENVIRONS**

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DECLARATION

I hereby declare that this work is the product of my own research efforts undertaken under the supervision of Professor S.E.Agina and has not been presented elsewhere for the award of degree or certificate. All sources have been duly distinguished and appropriately acknowledged

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CERTIFICATION

This is to certify that the research work for this thesis and the subsequent preparation of the thesis by Joseph A.E.Okwori (Mat. No. PGNS/UJ/0172/04) were carried out under my supervision.

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DEDICATION

This thesis is dedicated to THE HOLY TRINITY and the loving memory of my Late Dad, Pa Okwori Emmanuel Ameh (1936-2001).

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ABSTRACT

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are important food and water borne pathogens that has recently emerged world wide. This study was aimed at characterizing the prevalence of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in human and selected animal populations in Jos and its environment. Three thousand stool samples from human and selected animal species from 5 different locations in Jos and its environs were surveyed over a period of three years. Human samples were made up of equal number of 750 apparently healthy individuals and 750 diarrhoeic subjects. The samples were analysed bacteriologically for the presence of *Yersinia* species using appropriate culture methods and selected molecular techniques. Presumptive identification was made based on characteristic morphological appearances of the colonies. No pathogen was recorded among the healthy subjects indicating that there are no asymptomatic patients of *Yersinia* in Jos. Of the seven hundred and fifty (750) diarrhoeic patients screened, (46)6.1% were positive for *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. Among the positive subjects were 13 who had undergone appendectomy previously. The total percentage of occurrence of the *Yersinia* organisms among males and females were (21) 2.8% and (25) 3.3% respectively. Of the 1500 animal samples screened, (749) 49.9% were found infected with *Y. enterocolitica* (532) 35.4% and *Y. pseudotuberculosis* (217) 14.5%). High occurrence rate was recorded in pigs (271) 18.1%, followed by Sheep (189) 12.6%, poultry (163) 10.9%, and Dogs (126) 8.4%. High prevalence rate was recorded in Vom (231) 15.4%, Bukuru (182) 12.1%, and Bassa (128) 8.5%. The women in Bassa had the highest occurrence of yersiniosis, (9) 1.2%. Experimentally infected laboratory animals for pathogenicity test, at post mortem showed black spotted, necrotic lesion on the liver with evidence of gastroenteritis in rabbits. Unlike the European countries where *Y. enterocolitica* serotype 0:3 predominates, biotypes 1A, 1B and serotype 0:9 were found prevalent among strains of *Y. enterocolitica* isolated in this part of the world. Two principal DNA based methods polymerase chain reaction (PCR) and elongated pulsed field gradient electrophoresis (PFGE), were used to characterize *Y. enterocolitica* and *Y. pseudotuberculosis* in this study. All strains of *Y. enterocolitica* isolated in Jos, Plateau State, Nigeria, were differentiated by PCR which exhibited a characteristic differential genomic feature from the French isolates used as control, whereby arginine is replaced with guanine at position 320 on the sequence nucleotide bond. The PFGE based on *NOI* enzymes profiles differentiated *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* strains into 4 and 7 variants respectively. The overall incidence of *Y. enterocolitica* and *Y. pseudotuberculosis* infections was low in the population studied but a striking geographic and demographic variation was noted. Statistically, the distribution of *Yersinia species* in humans ranked highest amongst the age groups 11-20 and in pigs amongst animals ($P < 0.05$). There is no significant difference using the t- test in the occurrence of *Yersinia* species during the wet and dry seasons ($P > 0.05$). This work represents the first attempt to map out novel potential virulent markers of *Y. enterocolitica* and *Y. pseudotuberculosis* in Plateau State, Nigeria, using multispacer-typing (MST) and PFGE technology. The prevalence of yersiniosis in Jos suggests that animals and animal products can serve as reservoir host for *Yersinia* species.

Therefore constant screening to select infected animals for treatment and hygienic handling of animal products is hereby recommended.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

The term yersiniosis refers to infections caused by either *Yersinia enterocolitica* or *Yersinia pseudotuberculosis* (Aleksic and Bockemuhl, 1990; Iwata *et al*, 2005), which appear as enteritis and sometimes septicemia in humans and animals (Mair, 1973; Hurvell, 1981).

The genus *Yersinia* is classified into the family *Enterobacteriaceae* which is a group of gram negative coccobacilli, oxidase negative and facultatively non sporulating anaerobes with optimal growth at 27°C (Iwobi, 2003), and includes species with different degrees of pathogenicity (Boyd and Cornelis, 2001; Thoerner *et al*, 2003). Based on differentiating biochemical traits, 11 species, have been identified to date, which are further divided into different biotypes. Typically, the serotypes are linked to geographical distribution, severity of human disease and animal reservoir (Salyers and Whitt, 1994; Boyd and Cornelis, 2001).

The three species of *Yersinia* that cause disease in humans and animals include *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Yersinia pestis* (Bercovier *et al*, 1984b; Aleksic *et al*, 1987; Wauters *et al*, 1988b; Bronfin, 2002). These three pathogenic species differ considerably in invasiveness; while *Yersinia enterocolitica* and *Yersinia psuedotuberculosis* can cross the gastro-intestinal mucosa to infect underlying tissues, *Y. pestis* is injected into the body by an insect bite and thus, does not have to penetrate any body surface on its own (Cornelis *et al*,

1998).

Yersinia pseudotuberculosis and *Y. pestis* are closely related species that share nearly 97% gene homology (Trebesius *et al*, 1998; Achtman *et al*, 1999; Garcia, 2002). Recent studies strengthened by whole scale genome sequencing proposed that *Y. pestis* is a recently emerged clone of *Y. pseudotuberculosis* (Achtman *et al*, 1999; Radnedge *et al*, 2002). In recent years, these bacteria have been isolated from a variety of environmental sources (Botzler *et al*, 1968; Kapperud, 1977; Bercovier *et al*, 1978; Alonso *et al*, 1979, Agbalika *et al*, 1985); but their enzootiology, including the identity of their natural reservoirs, is not yet fully understood. Some of these bacteria commonly occur in aquatic ecosystems while others are associated more frequently with terrestrial ecosystem (Kapperud, 1991).

Yersiniosis is frequently characterized by a severe form of gastroenteritis with diarrhea and/or vomiting in young children, with the most common symptoms being fever and abdominal pain (Hocking *et al*, 1997; CDC/MMWR, 1991). In older children, *Yersinia* infection mimics appendicitis (El-Sherbini *et al*, 1999) and mesenteric lymphadenitis (Winblad, 1973; Mollaret *et al*, 1979). Gastroenteritis has been reported among young adults (Ray *et al*, 2004). The bacteria may also cause infection of other sites (such as wounds, joints, and urinary tract), reactive arthritis (Lindsay, 1997) and occasionally bacteraemia (CDC/MMWR, 1991).

More rarely *Y. enterocolitica* infections have been found to be the cause of enteric fever, septicaemia and a broad spectrum of other extra intestinal diseases, particularly in hosts that are very elderly and debilitated or immunologically

compromised (Winblad, 1973). *Yersinia* is thought to be a significant foodborne pathogen, even though pathogenic isolates have seldom been recovered from foods (De Boer, 1995). While pigs have been shown to be a major reservoir for human pathogenic strains of bioserotypes 4/0:3 (Andersen *et al*, 1991; Kapperud, 1991; De Boer, 1995), the transmission route from pigs to humans remains unproven.

Y. enterocolitica frequently colonizes pigs without apparent clinical symptom in these animals. Outbreaks of the disease have been associated with a variety of contaminated food products, and both outbreaks and sporadic disease have been linked to the consumption of pork by-products (Tauxe *et al*, 1987; Lee *et al*, 1991; Ostroff *et al*, 1994). It has not been possible to demonstrate any phenotypic or genotypic differences between human and porcine isolates (Hurvell, 1981; Kapperud and Bergan, 1984; Fredriksson-Ahomaa, 2001). Pigs are known source for human *Y. enterocolitica* infection. Other food producing animals, such as cattle harbour mostly non pathogenic *Y. enterocolitica* strains (Siriken, 2004). Difficulties associated with the isolation of *Y. enterocolitica* from foods and clinical samples stem from the high number of background flora which over-shadows the organisms. Direct isolation even on selective media is seldom successful and time-consuming enrichment steps are needed (Fredriksson-Ahomaa and Korkeala, 2003). No single procedure is currently available which will recover all pathogenic serotypes (De Boer, 1992).

With the advent of whole genome sequencing, DNA-based methods, including PCR and DNA colony hybridization, food borne pathogens can be detected more rapidly and with greater sensitivity and specificity (Jagow and Hill, 1986; Hill,

1996).

In 1966 only 23 cases of yersiniosis were known or published throughout the world (Toma, 1973) and more than 4000 cases were reported in 1974. Centre for Diseases Control (CDC) estimated recently that about 17,000 cases of yersiniosis occur annually in the United State of America (USA) and it is a far more common disease in North Europe, Scandinavia, Japan (CDC/MMWR, 1991) and Canada (Toma, 1973;Toma and Lafleur, 1974).

Annual incidence rates of reported *Y. enterocolitica* infections in Finland have varied from 564 to 873 cases per 5 million persons during 1995-1999 (Anon, 2000). The infectious rate may, however be much higher since only the most serious cases are registered. Yersinia-triggered reactive arthritis often occurs in Nordic countries, where bioserotype 4/0:3 are especially prevalent (Sievers *et al*, 1972; Bottone, 1999). The epidemiology of *Yersinia* infection is complex and poorly understood. Most cases of yersiniosis occur sporadically without an apparent source (Kapperud, 1991; Bottone, 1999; Smego *et al*, 1999).

The disease has been reported in every country in which the organism was searched for (Bercovier *et al*, 1980). In many countries, *Y. enterocolitica* is a common cause of acute bacterial gastroenteritis, rivaling *Campylobacter* species and *Shigella* species in frequency (Cover and Aber, 1989). In the United States, *Y. enterocolitica* has been an uncommonly reported pathogen, although in recent years it has emerged as an occasional cause of sporadic illness and foodborne disease outbreaks associated with water, contaminated milk, bean sprouts, tofu and

chitterlings (Cover and Aber, 1989; Lee *et al*, 1990). In France an average *Y. enterocolitica* contamination rate of 33.5% was observed from food and pork products (Delmas and Vidon, 1985).

Most of the reported cases of yersiniosis in Africa have been in Zaire, Rwanda (Makula *et al*, 1969) and South Africa (Rabson and Koornhofi, 1972). Most of the reported cases in Nigeria were by Agbonlahor *et al*, (1981) Lombin *et al*, (1985) Kwaga *et al*, (1986) Okoroafor *et al*, (1988) Onyemelukwe, (1993) Igumor *et al*, (1994) Omoigberale and Abiodun, (2002) and Okwori *et al*, (2005). Yersiniosis has attracted much attention since the last decade and since it was first described by Schleifstein and Coleman in 1939.

1.2 STATEMENT OF THE PROBLEM

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are important food-borne pathogens that can cause yersiniosis in humans and animals. The epidemiology of *Y. enterocolitica* and *Y. pseudotuberculosis* is complex and remains poorly understood. Most cases of yersiniosis occur sporadically without an apparent source. The main sources of human infection are assumed to be pork and pork products, as pigs are known to be major reservoir of pathogenic *Y. enterocolitica*. However, no clear evidence shows that such a transmission route exists. *Y. enterocolitica* and *Y. pseudotuberculosis* are the species of *Yersinia* most frequently isolated from human and animal infections in the industrialized world.

1.3 JUSTIFICATION OF THE STUDY

The justification of this study is to discourage the quick services of unnecessary performances of appendectomies in humans with related or similar clinical symptoms (as the organisms micmics the clinical symptoms of appendicitis) and to discourage unprotected contact and breeding of animals in residential houses.

1.4 AIMS AND OBJECTIVES OF THE STUDY

- To determine the prevalence of the organism in human and selected animal populations in Jos and its environs and to assess their involvement in related clinical symptoms.
- To establish pathogenicity features using laboratory animals and to alert the public on the severity of the infection.
- To carry out molecular characterization of the various biotypes and serogroups for rapid identification and diagnosis.
- To identify the public health implication of the organism and to establish control strategies

CHAPTER TWO

LITERATURE REVIEW

2.1 HISTORY AND TAXONOMY

No chapter in the exposition of bacterial pathogen as evasive as *Y. enterocolitica* is ever fully written said Edward Bottone in his splendid review article ‘*Yersinia enterocolitica*: the charisma continues’ (Bottone, 1997). This is quite true of the organisms *Y. enterocolitica* and *Y. pseudotuberculosis*.

In 1939, Schlieffstein and Coleman, working at the New York State Department of Health, described five bacterial isolates, which they thought resembled *Actinobacillus lignieri* and *Pasteruella pseudotuberculosis*. Two of the bacterial strains were isolated from facial lesions and three were obtained from the intestines of humans exhibiting symptoms of enteritis. The bacteria were Gram-negative, motile when grown at 20°C non-motile when grown at 37°C, antigenically related, and highly virulent for mice. They also found that the bacteria were biochemically similar to a bacterium isolated in 1934 from facial abscesses by McIver and Pike who tentatively identified it as *Flavobacterium pseudomallei whitemore*. Schlieffstein and Coleman concluded that the bacterium was a new, unidentified species, and because three strains were from enteric contents, they proposed the name *Bacterium enterocoliticum* (Schlieffstein and Coleman, 1939). Ten years later, Van Longhem (1944) proposed the genus name *Yersinia* (in the family *Enterobacteriaceae*), in honour of a French bacteriologist Alexander Yersin, who first isolated the plague bacillus *Yersinia (Pasteurella) pestis* during an epidemic in Hong kong in 1894.

Twenty years later, a Danish Microbiologist (Whilhel, 1964) re-examined the isolates (*Bacterium enterocoliticum*) of Schleifstein and Coleman and found similar biochemical reactions in all these strains. The characteristics of this group of bacteria were sufficiently distinct to separate them from *Yersinia pseudotuberculosis*; yet they resemble *Yersinia pseudotuberculosis* sufficiently to justify their classification in the genus *Yersinia* as a species. Hence Frederiksen proposed the species name *Yersinia enterocolitica*.

The following year, Smith and Thal (1965), substantiated Frederiksen's assignment of this species to *Yersinia*; however, because some of the strains examined by Frederiksen were indole-positive they proposed the less inclusive name *Yersinia X* until the status of the indole positive strains could be established.

The taxonomy of *Yersinia* was revolutionized by Brenner *et al*, (1976), who applied the DNA- DNA hybridization technique, in addition to classic biochemical tests, for the classification of *Y. enterocolitica* and *Y. pseudotuberculosis*. Four years later, as a result of these studies, three groups of *Y. enterocolitica*-like bacteria were separated from *Y. enterocolitica* and given their own species designation (*Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*) (Brenner *et al*, 1976; Ursing *et al*, 1980; Bercovier *et al*, 1980). These studies also resolved the above-mentioned dilemma of Smith and Thal, concerning the *Yersinia X* classification and uncertainty about the taxonomy of the indole positive and indole-negative strains. The DNA hybridization studies revealed that both indole positive and indole negative strains of *Y. enterocolitica* were closely related (DNA hybridization relatedness of 79 to 100%)

to be considered one species rather than two different species. Thus, four yersinia species (*Y. enterocolitica*, *Y. intermedia*, *Y. frederiksenii*, and *Y. kristensenii*) were established in 1980. The recent, rapid advances in understanding the biology of *Y. enterocolitica* also have resulted in an appreciation of the fact that many *Yersinia* stains originally identified as *Y. enterocolitica* actually are different *Yersinia* species. Within *Y. enterocolitica* sensu strictu, there was sufficient biochemical heterogeneity to have initially warranted the establishment of several biogroups or biotypes. For example, Nilehn (1969) proposed placing the strains into ‘biogroups’, according to their biochemical properties and she identified five biogroups in same year. The biogrouping was later modified by Wauters (1970) who, omitted some of Nilehn’s substrates and used lecithinase activity as an additional criterion for biotyping *Y. enterocolitica*. This scheme was further modified by Wauters *et al*, (1987) and the five biogroups of *Y. enterocolitica* were subsequently expanded to six. Two of these biogroups (3A and 3B) were subsequently speciated. Bercovier *et al*, (1984) proposed the name *Y. aldovae* for *Y. enterocolitica* isolates that had been recovered from aquatic ecosystems and were formally known as *Y. enterocolitica* like group X2. Three years later, Aleksic *et al*, (1987) proposed the species designation *Y. rohdei* for a group of strains isolated from human and dog faeces. *Yersinia ruckeri*, the red mouth disease – causing bacterium, is the oldest *Y. enterocolitica*-like species, and was given the species designation by Ewing *et al*, (1978). However, the validity of this classification has been questioned by several investigators who believe that *Y. ruckeri* actually may be a distinction, perhaps not even yersinia species. Jensen *et al*, (1969) proposed the name *Y. philomiragia* for a bacterium first

isolated from a dead muskrat in Northern Utah and from samples in the same area in 1959 (Type strain ATCC 25015).

Following intensive taxonomic studies in the 1980s, the genus *Yersinia* at present includes 10 established species, *Yersinia pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica*, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. bercovier*, *Y. mollaretti*, *Y. rohdei* and *Y. aldovae*. The taxonomic status of *Y. ruckeri*, a fish pathogen is still uncertain (Murray *et al*, 2003).

Y. pestis, *Y. pseudotuberculosis* and certain strains of *Y. enterocolitica* are of pathogenic importance for humans and certain warm blooded animals, whereas the other species are of environmental origin. According to the present knowledge may at best act as opportunistic organisms (Murray *et al*, 2003). Members of the genus *Yersinia* exhibit 10 to 32% relatedness to other members of the family *Enterobacteriaceae* by DNA –DNA Hybridization. The DNA G + C content of the genus is in the range of 46.0 to 48.0 mol %. Based on the DNA - DNA hybridization, *Y. enterocolitica* exhibits 43 to 64% of relatedness to *Y. pestis* and *Y. pseudotuberculosis* (Ewing, 1986). The genetic difference between *Y. pestis* and *Y. pseudotuberculosis*, on the other hand, is on the subspecies level and would taxonomically justify the inclusion of both into the species (Achtmann *et al*, 1999; Bercovier *et al*, 1980).

On the basis of antibody reactions to differentiate lipopolysaccharide structures *Yersinia* species, can also be divided into different serological groups. Typically the serotype, are linked to geographical distribution, severity of human

disease and animal reservoir (Salyers and Whitt, 1994; Boyd and Cornelis, 2001).

Yersinia pestis is an agent of bubonic plague, the famous black death that swept through the middle ages in 1347 – 1350 with estimated 25million deaths in Europe (about ¼ of population). Between 1972 – 1990 an average of 13 cases per year was reported in the South West of USA with fatality rate of 10%. To day, isolated cases of *Y. pestis* infection are reported sporadically in the USA, India, and Madagascar (Perry and Fetherston, 1997). The focus of this thesis will be on *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, the two species of animal pathogens which can cause a diversity of human diseases (Laird and Cavanaugh, 1980; Lee *et al*, 1981; Tauxe, 1987) and are distributed worldwide but occur mainly in the moderate and subtropical climatic areas of the USA, Europe, North central, and East Asia, South Africa, and Australia. On the other hand, they are rare or lacking in the tropical regions of Africa and South East Asia (Murray *et al*, 2003).

2.2 YERSINIA PSEUDOTUBERCULOSIS

The designation *pseudotuberculosis* derives from the characteristic histopathological aspect found in mesenteric lymph nodes of lymphoid hyperplasia cases which closely resemble those observed during tuberculosis infection (caseating lesions containing giant cells). *Y. pseudotuberculosis* has a wide distribution in most countries with cold climates and is recognised as an important agent of sporadic and epidemic human disease (Murray *et al*, 2003). *Y. pseudotuberculosis* is the least common of the three main agents of yersinia infections in human with variable hosts, including domestic and sylvatic animals (Asim, 2003). The bacterium is a primary

pathogen of wild and domestic animals in all continents. Nearly all-animal species are potential carriers of *Y. pseudotuberculosis* and asymptomatic carriage can evolve into a fulminating and fatal infection when the animals are subjected to stress (famine, cold temperatures, etc). In humans, *Y. pseudotuberculosis* infections are not frequent although outbreaks associated with consumption of water or food supplies contaminated with animals faeces have been reported; but has only rarely been isolated from soil, water and foods. Humans develop varying degrees of illness from abdominal pain and fever to septicemia, but a mesenteric adenitis that mimics an acute appendicular syndrome (pseudoappendicitis) is the most common clinical presentation (Schiemann, 1989). In most instances, the infection is self-limiting and can be effectively treated with antibiotic therapy.

A striking character of *Y. pseudotuberculosis* is the high degree of sequence identity of *Y. pestis* (the causative agent of plague), which is intriguing given the markedly different epidemiological and clinical features of the two species. In this regard, it should be noted that *Y. pestis* has been proposed to be a recently emerged clone of *Y. pseudotuberculosis* (Achtman *et al*, 1999). Among *Y. pseudotuberculosis* strains there is little or no variation in biochemical reactions except with the sugars melibiose, raffinose and salicin. Serologically (based on a heat-stable somatic antigen), the *Y. pseudotuberculosis* strains are classified into six groups with each serogroup containing pathogenic strains. Gemski *et al*, (1980) reported that serogroup III strains harbour a 42- mdal plasmid as do serogroup II strains that are lethal to adult mice. The association of yersiniosis in human with the presence of a 42- mdal plasmid in *Y. pseudotuberculosis* has been established. Virulence genes

present on the chromosome of *Y. pseudotuberculosis* have been identified (Isberg and Falkow, 1985; Isberg *et al*, 1987). The *inv* gene of *Y. pseudotuberculosis* is homologous with that of *Y. enterocolitica* and encodes for an invasion factor for mammalian cells. Transfer of *inv* gene into *E. coli* K-12 resulted in the expression of the invasive phenotype in *E. coli* (Iserberg and Falkow, 1985). The *Inv* gene is thermoregulated (Iserbeg *et al*, 1988), it encodes for a 103 Kdal protein, invasins, which binds to specific receptors on mammalian cells and facilitates the entry of *Y. pseudotuberculosis* into tissue (Iserbeg and leong, 1988). Tests for *Y. pseudotuberculosis* virulence are not as abundant as those for *Y. enterocolitica*. However, tissue cell invasive and plasmid carrying isolates of *Y. pseudotuberculosis* may be identified by colony hybridization. At present there are six serogroups represented by Roman numeral I-VI. Serogroups I, II, III and IV have subtypes, but antiserum to one serogroup type will cross-react with the subtype strain and vice versa. Strains belonging to serogroups II and III are lethal when fed to adult mice even though these strains do not elaborate lipase. Hela cell invasive strains are esculin-positive, which is contrary to findings with *Y. enterocolitica* (FDA/CFSAN, 2001). *Y. pseudotuberculosis* strains harbour a 41-48 mdal plasmid and will autoagglutinate at 37°C.

Y. pseudotuberculosis is now known as the causative agent of Izumi fever whose aetiology remains long unknown and some cases of *Y. pseudotuberculosis* infection satisfy the criteria of Kawasaki syndrome an important illness of children in Japan (Isubokura *et al*, 1989; Sunahara *et al*, 2000). The first case to our knowledge of chronic prostatitis due to *Y.*

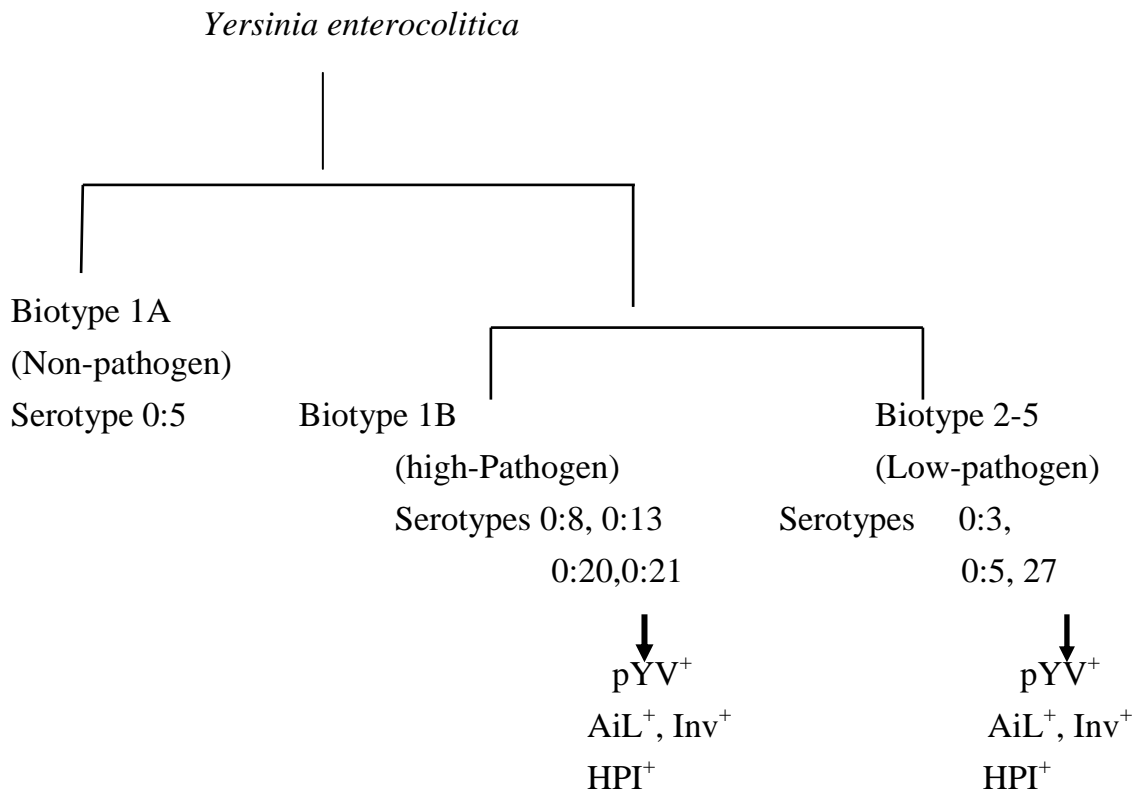
pseudotuberculosis was reported by Naiel and Raul (1988).

2.3 YERSINIA ENTEROCOLITICA

Y. enterocolitica is an important food and water-borne pathogen that has recently emerged worldwide (Najdenski *et al*, 1994., Ostroff, 1995; Ray *et al*, 2004). Infection due to *Y. enterocolitica* was first observed in 1933 in the United States of America (Karachalios *et al*, 2002). It is widely distributed in nature in aquatic and animal reservoirs, with swine serving as a major reservoir for human pathogenic strains (Bottone, 1997). Inanimate reservoirs include lakes, streams, well-water, soil, vegetables (Cover and Aber, 1989). Because *Y. enterocolitica* can also survive in cold environments, disease outbreaks have also been traced to infected blood samples stored at 4°C (Tipple *et al*, 1990). Various biotyping schemes have been defined over the years for differentiation of the *Y. enterocolitica* strains. In the revised biotype scheme proposed by Wauters *et al*, (1987), *Y. enterocolitica* strains are divided in to 5 biotypes (BT) based on their differing biochemical and ecological characteristics. In terms of pathogenicity, two broad groups are identified: the non-pathogenic isolates represented by biotype 1A strains, and the pathogenic group. The latter is further subdivided into two groups, namely the high-pathogenicity, mouse lethal group comprising biotype 1B strains (typified by serotypes 0:8, 0:13, 0:20 and 0:21) and the low-pathogenicity, non-mouse lethal groups (represented by biotypes 2-5) (Carniel, 2002).

As a mouse virulence determinant, a high pathogenicity island (HPI) has been identified, which codes for synthesis and uptake of the siderophore yersiniabactin, an

iron sequestering low molecular weight compound invaluable in the iron-limiting environment of the host (Heesemann, 1987; Carniel *et al*, 1996; Pelludat *et al*, 1998; Carniel, 2002). To date, the HPI defines essentially the differences in pathogenicity existing between the low and high-pathogenicity *Y. enterocolitica* strains, with the low-pathogenicity strains lacking this chromosomal locus. All other known virulence markers are however shared between the two groups. The presence of the HPI has also been demonstrated in *Y. pseudotuberculosis* and *Y. pestis* (Buchrieser *et al*, 1998a; Buchrieser *et al*, 1998b., De Almedia, *et al*, 1993). In terms of geographical distribution, the low and high pathogenicity *Y. enterocolitica* species exhibit some preferences: the high-pathogenicity organisms are more frequently isolated in the USA (so-called New World Strains), while the low-pathogenicity isolates (so-called Old World Strains) are predominantly isolated in Europe and Japan (Aleksic and Bockemuhl, 1990).



PYV – Plasmid for *Yersinia* virulence

Ail – attachment invasion locus

Inv – invasin

HPI – high pathogenicity island

(Fredriksson-Ahomaa Maria and Korkeala Hannu, 2003)

Figure 1: An overview of the species diversity/variability existing among the *Y. enterocolitica* Strains.

2.3.1 Representational difference analysis as a tool in the elucidation of the genetic variability among *Y. enterocolitica* strains.

The presence of chromosomal “pathogenicity islands” in several bacterial pathogens suggests that bacterial diversity may commonly involve horizontal acquisition of substantial blocks of chromosomal DNA encoding a series of related gene products which convey a new set of virulence properties on the recipient (Hacker *et al*, 1997). A variety of techniques have been developed to detect regions of DNA that differ between two closely related genomes that may be involved in genomic diversity. One such method is representational differences analysis (RDA), otherwise referred to as suppression subtractive hybridization (Lisitsyn *et al*, 1993; Lisitsyn and Wigler, 1995; Diatchenko *et al*, 1996). Originally developed as a tool in eukaryotic organisms to identify genetic polymorphisms in human neoplasia, this tool has since then been expanded to screen for genetic variability among different bacterial species, and even between closely related strains of the same species. In the work by Tinsley and colleagues for example, the method of RDA was successfully applied to map out genetic differences between *Neisseria meningitidis* and *Neisseria gonorrhoeae*, two closely related bacteria which have developed two different pathogenicity strategies (Perrin *et al*, 1999; Tinsley and Nassif, 1996; Choi *et al*, 2002).

In another study by Morrow *et al*, (1999) and Emmerth *et al*, (1999) RDA was successfully applied to detect genomic differences responsible for the different host propensities of two closely related pathogenic *Salmonella enterica* serovars, *S. typhimurium* and *S. typhi*. *S. typhimurium* is a frequent cause of gastroenteritis in

humans yet typically causes a lethal systemic infection in genetically susceptible mice. *S. typhi* on the other hand is the aetiologic agent of human typhoid fever, capable of causing potentially fatal systemic infection in humans, but is completely avirulent in nonprimate hosts such as mice. Their study revealed a novel fimbrial operon and transcriptional regulator that were unique to the tested *S. typhimurium* strain but absent in the *S. typhi* genome.

The *Y. enterocolitica* species represents a highly heterogeneous group of bacteria ranging from the non-pathogenic 1A strains to the high-pathogenicity 1B and low pathogenicity isolates. Although, closely related, the 1A and 1B organisms differ significantly with respect to pathogenicity. The biotype 1A isolates are generally considered avirulent while the high-pathogenicity 1B strains are known to cause a broad range of gastrointestinal diseases.

2.4 OVERVIEW OF THE PATHOGENESIS OF *YERSINIA* SPECIES

Infections due to *Y. enterocolitica* and *Y. pseudotuberculosis* usually ensue after ingestion of the microbes in contaminated food (Black *et al*, 1978) or water (Keet, 1974) or rarely through direct person to person transmission e.g. in kindergartens, schools or by direct inoculation through blood transfusion in hospitals (Stenhouse and Milner, 1982). *Y. enterocolitica* is a common cause of human infection whereas *Y. pseudotuberculosis* is primarily an animal pathogen. The incubation period varies between 4 and 7 days in *Y. enterocolitica* and is unknown for *Y. pseudotuberculosis* (Murray *et al*, 2003).

Both species have affinity for the lymphoid tissue and penetrate into the ileal mucosa via the M - cells of Peyer's Patches. From the basolateral site they invade the

intestinal epithelial cells-resulting in gastroenteritis. However, more important is the successful elimination of the phagocytes which they actualize with the aid of secreted outer proteins (Yops) in a three-step interaction. The first step is the paralysis of phagocytic machinery by injecting a set of effector proteins by use of a type III secretion system. The second step includes the suppression of tumour necrosis factor alpha production in macrophages, impeding the recognition by bacterial lipopolysaccharide and suppressing the immune response. The third step finally leads to induction of apoptosis and cell death in macrophages (Murray *et al*, 2003). For effective colonization of the host, a medley of virulence factors comes into play (Bottone, 1997). Some of these factors are chromosomally encoded like the invasin InVA, the adhesive factor myf (Mucoid Yersinia factor), the enterotoxin Yst, and proteins involved in iron acquisition. In addition all yersinia species harbour a 70-kb virulence plasmid (designated PYv for yersinia virulence plasmid by Portnoy and Falkow, 1981; Portnoy *et al*, 1984) which codes for an array of tightly regulated and sophisticated antihost factors that guide the invading yersinia pathogen past numerous host defence mechanisms. The PYv plasmid carries a number of important virulence genes whose product fall under four broad categories, namely, the adhesin protein (YadA), translocated antiphagocytic protein called Yops (for *Yersinia* outer proteins), proteins dedicated to the processing and extracellular secretion of Yops designated Ysc (for “Yop Secretion”), and a complex regulatory network (LCR proteins for low calcium response) (Cornelis *et al*, 1998; Boyd and Cornelis, 2001). Thus pathogenic yersiniae may spread and penetrate into lymph nodes, where they multiply. The inflammatory response causes pain in the lower abdominal region,

which is a typical symptom and which may be mistaken for appendicitis (Murray *et al*, 2003).

Intestinal yersiniosis may present in three clinical forms viz: enteritis, terminal ileitis or mesenteric lymphadenitis causing “pseudoappendicitis,” and septicemia (Bottone, 1997). Watery, sometimes bloody stool are characteristic of *Y. enterocolitica* infection but are rarely caused by *Y. pseudotuberculosis*. The association of human illness with consumption of *Y. enterocolitica* contaminated-food, animal wastes and unchlorinated water is well documented (Aulisio *et al*, 1982; Aulisio *et al*, 1983). Refrigerated foods are potential vehicles because contamination is possible at the manufacturing site (Aulisio *et al*, 1982) or in the home (Aulisio *et al*, 1983) and the organism could survive and grow during refrigerated storage. Since it was discovered, *Y. enterocolitica* has become an important cause of diarrhoea in much of the industrialized world (Cover and Aber, 1989; Lee *et al*, 1990). Bloody diarrhoea is observed mainly in adults and less frequently in children and it is often accompanied by fever, vomiting and abdominal pain. Terminal ileitis mesenteric lymphadenitis and pseudoappendicitis may also be produced by *Y. enterocolitica* but these are the characteristic symptoms in *Y. pseudotuberculosis* infections that are especially common in children older than 5 years and adolescents. Whereas adults usually overcome intestinal yersiniosis within 1 or 2 weeks, disease in children may last for up to 4 weeks (Bottone, 1997).

The careful delineation of *Y. enterocolitica* virulence traits, which required several years of dedicated work and many elegant experiments, also led to the

assumption that only *Yersinia* strains possessing the above virulence markers are capable of causing disease in humans. However, even in the case of *Y. enterocolitica*, there is not always a strong association between the presence of virulence markers and the production of disease. A good example of this observation is the classical study of Noble *et al*, (1987) in Vancouver, Canada. The authors examined more than 200 *Yersinia* strains (isolated from the stools of 171 patients) for the PYv, calcium dependence, autoagglutination, Congo red dye uptake, pyrazinamidase activity, fermentation of salicin and hydrolysis of esculin, and analyzed the data to determine whether they correlated with the symptoms of the patients. The results obtained with the individual tests or with group tests did not consistently correlate with the clinical symptoms. Thus, strains of *Y. enterocolitica* and *Y. enterocolitica* - like species lacking many virulence factors were significantly associated with the occurrence of diarrhoea in patients in whom no other infectious or noninfectious causes of enteric disease could be identified. The recovery of an unusually large number of *Yersinia* strains not belonging to recognized pathogenic groups/species also has been reported (Bissett *et al*, 1990) in the United States of America. According to a surveillance study conducted by the California Department of Health, a large proportion (ca. 40%) of *Yersinia* strains isolated from the faeces of patients with diarrhoea belonged to nonpathogenic *Yersinia* biogroups and/or species. No other cause of diarrhoea was identified, which suggests that nonpathogenic *Yersinia* were responsible for gastroenteritis in at least some of the patients from whom the bacteria were isolated. Another example is a study conducted on the epidemiology and pathogenicity of *Y. enterocolitica* among children

in two prospectively monitored cohorts in Santiago, Chile (Morris *et al*, 1991). In this study, a group of biogroup 1A strains which should have been pathogenic on the basis of traditional virulence marker criteria were significantly associated with diarrhoea (P=0.02, McNemar matched-pair analysis). Several other reports (Cafferkey *et al*, 1993; Ratnam *et al*, 1982; Sholey and Freeman, 1984) implicate nonpathogenic *Y. enterocolitica* and *Y. enterocolitica*-like species as agents of human disease, and suggest that their ability to cause disease is related to their having virulence factors/markers different from those described for classical pathogenic *Y. enterocolitica* strains. However, precisely what those virulence markers might be is not yet well understood. Among several possible candidates, production of enterotoxins and novel invasion mechanisms are the most frequently proposed.

A successful hallmark of bacterial pathogenesis lies not only in the synthesis of a plethora of virulence factors, but also in the efficient translocation or diversity of these antihost effector molecules into the target. Among pathogenic bacteria, six transport systems have been clearly elucidated to be involved in the transport of proteins to the extracellular milieu. These include the signal sequence independent pathway (type I), the main terminal branch of the general secretion pathway (type II or secretion), the contact dependent pathway (type II), the type IV pathway, the *Bordetella pertussis* filamentous haemagglutinin secretion pathway (TPS, two-partner secretion) and the autotransporter pathway (He *et al*, 1991; Binet *et al*, 1997; Collazo and Galan, 1997; Henderson *et al*, 1998; Burns, 1999)

2.5 PREVALENCE OF *YERSINIA ENTEROCOLITICA* AND *YERSINIA PSEUDOTUBERCULOSIS* IN ANIMALS AND MAN

2.5.1 Prevalence in animals

Yersiniosis is an emergent zoonotic disease, which is cosmopolitan in nature (Inchinohe *et al*, 1991; Miller, 1992). Animals have long been suspected of being a reservoir for *Y. enterocolitica* and *Y. pseudotuberculosis* and hence, sources of human infections. Swine are the most common reservoir of these pathogens. Outbreaks of disease have been associated with a variety of contaminated food products, and both outbreaks and sporadic disease have been linked to the consumption of pork byproducts (Tauxe *et al*, 1987; Lee *et al*, 1991; Ostroff *et al*, 1994). Numerous studies have been carried out to isolate *Y. enterocolitica* from a variety of animals (Hurvell, 1981), including wild animals (Ahvonen *et al*, 1973; Kaneko *et al*, 1978; Kaneko and Hashimoto, 1981; Shayegani *et al*, 1983; Kaneuchi *et al*, 1989; Suzuki *et al*, 1995; Wuthe *et al*, 1995; Sulakvelidze *et al*, 1996) and farm animals (Ahvonen *et al*, 1973; Szita *et al*, 1980). However, most of the strains isolated from animal sources differ both biochemically and serologically from strains isolated from man with yersiniosis. Human pathogenic strains of *Y. enterocolitica* typically have only been isolated from slaughtered pigs. The highest prevalence of *Y. enterocolitica* belonging to bioserotypes associated with human yersiniosis has been obtained in pigs tonsils, with bioserotype 4/0:3 being the most common. Experimental infection of pigs has shown that *Y. enterocolitica* remains longer (Nilsen *et al*, 1996; Thibodeau *et al*, 1999), and the number of isolates is higher (Shiozawa *et al*, 1991) in tonsils than in faeces.

Y. enterocolitica of bioserotype 4/0:3 has a world-wide distribution in the pig

population, but the prevalence does vary between herds in many countries. This herd-wise distribution has been demonstrated by culture methods in Denmark, Norway, Finland and Canada (Christensen, 1980; Asplund *et al*, 1990; Andersen *et al*, 1991); Letellier *et al*, 1999) and the serological tests in Denmark and Norway (Nielsen and Wegener, 1997; Skjerve *et al*, 1998). By culture method, 18% (Andersen *et al*, 1991) to 64% (Asplund *et al*, 1990) of the herds have been negative for *Y. enterocolitica* 4/0:3. By contrast, serological investigations have been shown that 70% - 90% of the slaughter herds in Denmark and 63% in Norway are infected with serotype 0:3, and that nearly all finishing pigs infected herds are seropositive (Nielsen and Wegener, 1997; Skjerve *et al*, 1998).

The source and mode of *Y. enterocolitica* infection in pigs is still unclear. This organism has not been isolated from small breeding pigs, but rather from pigs that have been moved to the first or second fattening pens. These findings suggest that infected faeces and pen floors are likely the most important sources of infection (Fukushima *et al*, 1983a). More intensive farming and production system have probably contributed to the high prevalence of pathogenic *Y. enterocolitica* in pigs. The prevalence of bioserotype 4/0:3 has been shown to be highest in large pig farms with open management, where small pigs have been purchased from various pigs markets or pig producers (Christensen, 1987b; Skjerve *et al*, 1998).

The presence of symptomless carriers together with the widespread occurrence of *Y. enterocolitica* 4/0:3 in herds render control of this bacterium at farm level difficult. Strict slaughter hygiene remains important in reducing contamination

in slaughterhouses (Skjerve *et al*, 1998).

Pet animals, such as cats and dogs, have been suspected of being reservoirs for human infections with *Y. enterocolitica* because of their close contact with humans (Schiemann, 1989). However, strains of *Y. enterocolitica* 4/0:3 have only occasionally been isolated from dogs and cats (Ahvonen *et al*, 1973; Yanagawa *et al*, 1978; Pedersen and Winblad, 1979). These strains have mostly been isolated from apparently healthy dogs (Fukushima *et al*, 1984b; Fantasia *et al*, 1985; Fredriksson-Ahmoa *et al*, 1999). Dogs can asymptotically carry *Y. enterocolitica* in the pharynx and excrete the organism in faeces for several weeks after infection (Fenwick *et al*, 1994).

Monkey species especially new world monkeys such as the squirrel monkey (*Saimiri sciureus*) seem to be sensitive to *Y. pseudotuberculosis* (Iwata *et al*, 2005). Many cases of yersiniosis in breeding monkeys have been reported and *Y. pseudotuberculosis* in particular frequently causes fatal infection (Hirai *et al*, 1974 ; Rosenberg *et al*, 1980; McArthur and Wood, 1983; Maruyama *et al*, 1983; Murata and Hama, 1992; Sasaki, 1996; Kageyama *et al*, 2002). Similarly, there have been some reports of monkey infection with *Y. enterocolitica* (Baggs *et al*, 1976; Skavlen *et al*, 1985; Damme *et al*, 1987).

2.5.2 Prevalence in Humans

Y. enterocolitica and *Y. pseudotuberculosis* have been isolated from humans on all continents (Bottone, 1999; Sunahara *et al*, 2000). Bioserotype 4/0:3 is the most common type of *Y. enterocolitica* recovered from humans with diarrhoea (Bissett *et*

al, 1990; Gonzalez *et al*, 1990; Bucci *et al*, 1991; Kontiainen *et al*, 1994; Munk Petersen *et al*, 1996; Stolk-engelaar and Hoogkamp-Korstanje, 1996). The highest incidence of enteritits caused by this type has been found in young children (Stolk-Engelaar and Hoogkapm-Korstanje, 1996). However, Morris *et al*, (1991) have also isolated strains of bioserotype 4/0:3 at a high rate from asymptomatic children. Annual incidence rates of reported *Y. enterocolotica* infections in Finland have varied between 564 and 873 cases per 5 million persons during 1995-1999 (Anonymous, 2000). The infection rate is probably much higher since only the most serious cases are registered. The prevelance of *Y. enterocolotica* 0:3/0:9 specific antibodies was relatively high in Finland (19% and 31% by enzyme immunoassay and immunoblotting, respectively) and in Germany (33%, 43%) when healthy blood donors were studied (Maki-Ikola *et al*, 1997). This may indicate a high amount of subclinical *Yersinia* infections in the healthy population.

Y. enterocolitica can cause gastro-intestinal symptoms ranging from mild self-limited diarrhoea to acute mesenteric lymphadenitis evoking appendicitis (Ahvonen, 1972b). Sometimes focal disease, such as pharyngitis, cellulitis, subcutaneous abscess, pneumonia and meningitis, may occur without gastro-intestinal illness (Tacket *et al*, 1983; Rose *et al*, 1987; Cover and Aber, 1989; Bin-Sagheer *et al*, 1997). The incubation period of *Y. enterocolitica* enterocolitis ranges from 1 to 11 days. The minimal infective dose for humans has not been determined. Symptoms of enterocolitis typically persist for 5 to 14 days, but they occasionally last for several months. The duration of the excretion of the organism in stool has been reported to range from 14 to 97 days (Cover and Aber, 1989). The clinical manifestations of

infection depend on factors such as the age and physiological state of host and pathogenic properties of the particular strain (Cover and Aber, 1989).

Most commonly, *Y. enterocolitica* infections occur in young children (Stolk-Engelaar and Hoogkamp-Korstanje, 1996). In patients under 5 years of age, yersiniosis presents as diarrhoea, often with low-grade fever and sometimes with abdominal pain (Hoogkamp-Korstanje and Stolk-Engelaar, 1995). The symptoms can even be so faint and short-lived that yersiniosis is not diagnosed, despite faecal carriage (Olsovksy *et al*, 1975; Van Ossel and Wauters, 1990). In older children and young adults, acute yersiniosis can be present as a pseudo-appendicular syndrome, which is frequently confused with appendicitis (Stoddard *et al*, 1994). Sepsis is a rare complication of *Y. enterocolitica* infection, except in patients who have a predisposing underlying disease (Kellogg *et al*, 1995) or are in an iron-overloaded state (Cover and Aber, 1989; Hopfner *et al*, 2001). Sepsis can also occur during blood transfusion (Mitchell and Brecher, 1999). One source of *Y. enterocolitica* contaminated red blood cell concentrate has been reported to be a blood donor with asymptomatic bacteremia (Strobel *et al*, 2000).

Normally, yersiniosis is a self-limited disease, but sometimes long-term sequelae, including reactive arthritis, erythema nodosum, uveitis, glomerulonephritis and myocarditis, will occur. Post-infection complications usually develop within one week to one month of initial infection, and these may be the only obvious clinical manifestation of *Yersinia* infection (Sievers *et al*, 1972; Ahvonen 1972b; Toievanen *et al*, 1985). Reactive arthritis and erythema nodosum are the most common

complications (Ahvonen, 1972b; Sievers *et al*, 1972; Leirisalo-Repo, 1987). Reactive arthritis associated with urethritis and/or conjunctivitis is often termed Reiter's disease. *Yersinia*- triggered reactive arthritis (Aho *et al*, 1974; Leirisalo-repo and Suoranta, 1988), reiter's disease (Aho *et al*, 1974) and uveitis (Careless *et al*, 1997) are strongly associated with the human leukocyte antigen. HLA-B27 *Yersinia* triggered reactive arthritis often occurs in Nordic countries, where HLA-B27 and Bioserotype 4/0:3 are especially prevalent (Sievers *et al*, 1972; Bottone, 1999).

2.6 EPIDEMIOLOGY OF *YERSINIA ENTEROCOLITICA* AND *YERSINIA PSEUDOTUBERCULOSIS*

Y. enterocolitica and *Y. pseudotuberculosis* are distributed world wide but occur mainly in the moderate and subtropical climatic areas of the Americas, Europe, East Asia, South Africa and Australia. On the other hand they are rare or lacking in the tropical regions of Africa and Southeast Asia (Murray *et al*, 2003). Nearly 66% of *Y. enterocolitica* cases occur among infant and small children, while 75% of *Y. pseudotuberculosis* cases involve 5-20 year olds (FDA /CFSAN, 2001).

Predisposing factors to these infections include cold temperatures, iron overload, immunosuppression especially in cases of administration of antacids and H₂ blockers and pH of 5.0 – 9.0 (Bronfin, 2002). Also, *Y. enterocolitica* bacteriamiae and endotoxin shock associated with red blood cell transfusion has been reported in the USA (Arduino *et al*, 1991).

In 1998, confirmed cases of *Y. pseudotuberculosis* through contaminated homogenized milk were reported to British Columbia Centre for Disease Control

Society. In 1991 children consuming untreated drinking water in Okayama, Japan, were exposed to *Y. pseudotuberculosis* infections. Similar reports have been documented in Czechoslovakia in the 1980s, and also in the Finland and Japan where outbreaks constituted sporadic cases (Olovsky *et al*, 1975; Anno, 2000).

Foodborne outbreaks of *Y. enterocolitica* bioserotype 4/0:3 are uncommon, although it is theoretically possible for this bacterium to contaminate and then grow in many types of refrigerated foods (Kapperud, 1991). In Japan and Czechoslovakia, some large outbreaks have been documented (Olovsky *et al*, 1975; Kapperud, 1991). In all cases, *Y. enterocolitica* serotype 0:3 were the causative agent, but the source of infections went undetected. In the USA, one outbreak of serotype 0:3, which were implicated with chitterlings (a dish made from big intestine), have been reported (Lee *et al*, 1990). In addition, six major outbreaks of other serotypes have occurred in the USA; five of these were caused by serotype 0:8. The outbreaks were associated with chocolate milk (Black *et al*, 1978), powdered milk and chow mein (Shayegani *et al*, 1983), tofu (Tacket *et al*, 1985), bean sprouts (Cover and Aber, 1989) and pasteurized milk (Ackers *et al*, 2000). A rarer serotype, 0:13, has caused an outbreak where pasteurized milk was the common source (Tacket *et al*, 1984). However, most *Y. enterocolitica* 4/0:3 infections are sporadic (Robins-Browne, 1997).

2.6.1 Possible transmission routes

Pigs are considered to be the main source of human *Y. enterocolitica* 4/0:3 infections, even though a definite connection between isolates from pigs and human

infections have still to be established. Elevated serum antibody concentrations have been found among people involved in swine breeding or pork production, suggesting a direct transmission of this bacterium from pigs to humans. In Finland, slaughterhouse workers and pig farmers were observed to have elevated antibody levels to *Y. enterocolitica* 0:3 twice as frequently as grain-or berry farmers (Seuri and Granfors, 1992) or randomly selected blood donors (Merilahti-Palo *et al*, 1991). Similar differences have also been discovered between people involved in swine slaughtering practices and office personnel in Norway (Nesbakken *et al*, 1991b). A close genetic relationship between pig isolates and human isolates has been shown by REAP (Nesbakken *et al*, 1987), REAC (Kapperud *et al*, 1990b), ribotyping (Anderson and Saunders, 1990) and PFGE (Asplund *et al*, 1998).

Pet animals have also been suspected of being sources for human infections because of their close contact with humans (Schiemann, 1989). However, direct transmission from pets to humans has yet to be proven. The most common transmission route of pathogenic *Y. enterocolitica* is thought to be faecal-oral via contaminated food (Schiemann, 1989; Smego *et al*, 1999), although pathogenic isolates have seldom been received from food samples (De Boer, 1995; Ostroff, 1995). *Y. enterocolitica* 4/0:3 infection has been associated with consumption of raw or undercooked pork and untreated water in case control studies (Tauxe *et al*, 1987; Ostroff *et al*, 1994; Satterthwaite *et al*, 1999).

Direct person-to-person contact has not been demonstrated, but Lee *et al*, (1990) reported *Y. enterocolitica* 0:3 infections in infants who were probably

exposed to infection by their caretakers. Indirect person-to-person transmission has apparently occurred in several instances by transfusion of blood product (Mitchell and Brecher, 1999). In these cases, the most likely source of *Yersinia* has been blood donors with subclinical bacteremia (Feng *et al*, 1992).

2.7 GROWTH CONDITIONS FOR *Y. PSEUDOTUBERCULOSIS* AND *Y. ENTEROCOLITICA*

Y. pseudotuberculosis and *Y. enterocolitica* are psychrotrophic bacteria that have the ability to replicate at temperatures between 0 - 4°C. The doubling time at the optimum growth temperature (approximately 28 to 30°C) is around 34 min (Schiemann, 1989). Although they can grow at temperatures as low as 0°C, the organisms grow much more slowly as temperatures drop below 50°C (Goverde *et al*, 1994; Harrison *et al*, 2000). It has been shown that the number of *Y. enterocolitica* on pork can reach log 9cfu per cm² after 5 days at 10°C (Nissen *et al*, 2001). Goverde *et al*, (1994) demonstrated that PYv-positive strains grew slower than PYv-negative ones at 30-35°C and 1-10°C. *Yersinia* withstands freezing and can survive in frozen foods for extended periods even after repeated freezing and thawing. However, it is susceptible to heat and can be destroyed by pasteurization at 71.8°C for 18 seconds (Toora *et al*, 1992).

Yersinia is able to grow over a pH range from approximately 4 to 10, with an optimum pH of around 7.6 (Robins-Browne, 1997) and can survive alkaline conditions better than other gram-negative bacteria (Aulisio *et al*, 1980). However, since few foods have high alkaline pH tolerance it is relatively unimportant. The bacterium's tolerance of acidic conditions, on the other hand is of great significance.

The ability to survive the high acidity of some foods and to pass through the stomach suggests that *Y. enterocolitica* is relatively acid-resistant. Although the mechanism of acid tolerance is unknown, it may be due to the activity of urease, which catabolises urea to release ammonia, which in turn elevates the cytoplasmic pH (De Koning-Ward and Robins-Browne, 1995). Tolerance of *Y. enterocolitica* to acid depends on the acidulant used, the environmental temperature, the composition of the medium, and the growth phase of the bacteria (Brocklehurst and Lund, 1990). Acetic acid has been shown to be a more effective inhibitor than either lactic or citric acid (Brocklehurst and Lund, 1990).

Y. enterocolitica grows most efficiently in an environment with pH 5.0-9.0 hence the increased incidence of the disease in patients who are on antacids and H₂ blockers. *Y. enterocolitica* and *Y. pseudotuberculosis* are facultatively anaerobic bacteria that can grow in anaerobic conditions. These organisms can also grow well in modified atmosphere at 8°C (Harrison *et al*, 2000), but with higher levels of CO₂, the length of lag phase will increase and growth will be slower (Pin *et al*, 2000). *Yersinia enterocolitica* have been shown to grow well on meat packaged in vacuum or in modified atmosphere and stored at 5°C (Doherty *et al*, 1995, Bodnaruk and Draughon, 1998), even in the presence of high background flora (Barakat and Harris, 1999; Bredholt *et al*, 1999). Nissen *et al*, (2001) demonstrated that *Y. enterocolitica* can grow well on both decontaminated and untreated pork when packaged in vacuum and stored at 10°C. However, the growth of serotype 0:3 in raw minced meat has been found to be inhibited by natural microflora of the meat in some studies (Fukushima and Gomyoda, 1986; Kleinlien and Untermann, 1990).

Y. enterocolitica can tolerate salt (NaCl) at concentrations of up to 5% (Stern *et al*, 1980, Robins-Browne, 1997). The inhibition caused by NaCl is strongly dependent on storage temperature. Brine concentration of 4.5% inhibits growth of *Y. enterocolitica* completely at 2°C and only partly at 5°C (Nielsen and Zeuthen, 1985). *Y. enterocolitica* can tolerate both sodium nitrate and nitrite of up to 20mg/ml for 48 hours *in vitro* (De Giusti and de Vito, 1992). However, a nitrite concentration of only 80mg/kg has been reported to inhibit the growth of *Y. enterocolitica* in fermented sausages (Asplund *et al*, 1993).

2.8 LABORATORY DIAGNOSIS

Certain chromosomal and plasmid-located virulence genes such as *invA*, *ail*, *yst*, *yadA*, and *virF*, have been used for the detection of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* by polymerase chain reaction (PCR), and DNA colony blot hybridization. These methods however are not required in the routine laboratory diagnosis because isolation of *Y. enterocolitica* and *Y. pseudotuberculosis* usually are not problematic. For patients with chronic infections however, it may be difficult to isolate from the specimens obtained during surgery such as organ tissues and lymph nodes, and for such situations in site indirect immunofluorescence, an RNA - targeted PCR and a fluorescent in situ hybridization method have been recommended (Hoogkamp-korstanje *et al*, 1988; Trebesius *et al*, 1998), and such tests are available only in specialized laboratories.

2.8.1 Isolation of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*

Faeces are the most common clinical specimens examined for the presence of

Y. enterocolitica and *Y. pseudotuberculosis*. Therefore, rapid isolation of the organisms requires selective media, which provide good nourishment for *Yersinia* and inhibit growth of normal microflora. Enrichment of stool specimen is usually not necessary for patients with diarrhoea when the number of excreted organisms is low (Murray *et al*, 2003). During acute gastroenteritis or with organ abscesses, pathogenic *Y. enterocolitica* is often the dominant bacteria and can easily be isolated by direct plating on conventional enteric media (Ahvonen, 1972a). Because of high number of background flora and the low number of pathogenic strains of *Yersinia* in food and environmental samples, direct isolation even on selective media is seldom successful. To increase the chances of *Yersinia* strains in samples, enrichment media prior to isolation on solid media is required (De Boer, 1992).

2.8.2 Cold enrichment culture

The psychrotrophic nature of *Y. enterocolitica* and *Y. pseudotuberculosis* is unusual among enteric bacteria, and consequently, enrichment in different solutions at 4°C for prolonged periods has been used for isolation of *Yersinia spp.* (Eiss, 1975). Cold enrichment in phosphate-buffered solution (PBS) has been widely used for clinical, food and environmental samples (Oosterom, 1979; Pai *et al*, 1979; Kontiainen *et al*, 1994; Funk *et al*, 1998; Letellier *et al*, 1999). To increase sensitivity, sorbitol (1%) and bile salts (0.15%) have been added to PBS. This phosphate buffer saline broth with sorbitol and bile salts (PBSSB) has frequently been used in isolation methods, especially for foods (Mehlman *et al*, 1978; Schiemann, 1982; Harmon *et al*, 1983; Logue *et al*, 1996; NCFA, 1996). In addition, nutritional media, such as tryptic soya broth (TSB), have been reported to yield

better results, particularly when food and environmental samples are studied (Van Pee and Stragier, 1979).

One major disadvantage encountered with cold enrichment is the long incubation period, typically 21 days, which is unacceptable for quality assurance of foods. Doyle and Hugdahl (1983) have shown that incubation in PBS for 1-3 days at 25°C is as efficient as enrichment at 4°C for some weeks. Another problem with cold enrichment is the presence of other psychrotrophic bacteria in foods. These bacteria also multiply during the enrichment. By treating cold enrichments with potassium hydroxide (KOH), the background flora can be reduced, making selection of *Yersinia* colonies easier (Schiemann, 1983b). This alkali treatment was developed by Aulisio *et al*, (1980) after they observed that *Yersinia* spp. are more tolerant of alkali solutions than many other gram-negative bacteria.

2.8.3 Selective enrichment culture

Several selective media for isolation of *Y. enterocolitica* and *Y. pseudotuberculosis* at higher temperatures have been developed (Wauters, 1973; Lee *et al*, 1980; Schiemann, 1982; Wauters *et al*, 1988a; Toora *et al*, 1994), with different antimicrobial agents being used as selective supplements in these media. Wauters (1973) formulated a Modified Rappaport Broth (MRB) containing magnesium chloride, malachite green and carbenicilin, in which the sample was incubated at 25°C for 2-4 days. Later, Wauters *et al*, (1988a) developed an enrichment broth derived from the modified Rappaport base, supplemented with irgasan, ticarcilin and potassium chlorate (ITC). Both media have been shown to be

efficient for recovery of strains of bioserotype 4/0:3, but inhibitory for strains of bioserotype 2/0:5, 27 and 1B/0:8 (Oosterom 1979; Wauters *et al*, 1988a; Kwaga *et al*, 1990; De Boer and Nouws, 1991). Schiemann (1982) developed a bile-oxalate-sorbose (BOS) medium for the isolation of *Y. enterocolitica*, particularly for strains belonging to bioserotype 1B/0:8. Pre-enrichment in low-selectivity medium prior to selective enrichment in MRB (Harmon *et al*, 1983; NCFA, 1996) or BOS (Schiemann, 1983a; Walker and Gilmour, 1986; Wauters *et al*, 1988a; Cox and Bailey, 1990) has also been used for isolation of *Y. enterocolitica* from foods.

2.8.4 Selective Agar Plates

Several different selective agar plating media have been used for isolation of *Y. enterocolitica*. Initially, plating media, such as MacConkey agar (MCA), Deoxycholate citrate agar (DCA) and Salmonella-Shigella agar (SSA), developed for other enteropathogens were used (Nilehn, 1969a). On these media, *Y. enterocolitica* strains grow well but slowly and are easily overgrown by other enteric bacteria because of the low selectivity. Of the traditional enteric media, the most widely used is MCA (Doyle and Hugdahl, 1983; Fukushima, 1985; Sierra *et al*, 1995). Both modifying existing enteric media and development of entirely new media have achieved improvements in selectivity. SS-agar was made more selective for *Y. enterocolitica* by addition of sodium deoxycholate and CaCl₂ (Wauters, 1973; Wauters *et al*, 1988a). Used in combination with ITC enrichment, recovery of strains of bioserotype 4/0:3 is good (Wauters *et al*, 1988a). This agar is widely used because of its high selectivity and commercial availability (IOS, 1994).

However, differentiation of *Yersinia* from competing organisms, such as *Morganella*, *Proteus*, *Serratia* and *Aeromonas*, can be difficult. In several comparative studies, Cefsulodin igrasan Novobiocin (CIN) agar have been found to be the most selective plating medium for *Yersinia* spp. (Head *et al*, 1982; Harmon *et al*, 1983; Schiemann, 1983a; Walker and Gilmour, 1986; Cox and Bailey, 1990). Organisms capable of fermenting mannitol, like *Yersinia*, produce red “bull’s eye” colonies on CIN agar. Only *Citrobacter freundii*, *Enterobacter agglomerans* and species of *Aeromonas* and *Klebsiella* produce similar colony morphology (Devenish and Schiemann, 1981; Harmon *et al*, 1983). Other selective media have been developed for the isolation of *Y. enterocolitica* strains (Bercovier *et al*, 1984a., Fukushima, 1987) but CIN agar is the most generally accepted because of its high selectivity and the high confirmation rate of presumptive isolates. Moreover, the commercial availability of this medium makes it convenient to use.

2.9 IDENTIFICATION OF *YERSINIA ENTEROCOLITICA* AND *YERSINIA PSEUDOTUBERCULOSIS*

Devenish and Schiemann (1981) determined the minimum number of biochemical tests required for identifying *Yersinia* amongst bacteria growing and presenting similar colony morphology on CIN agar; two tests, Kligler’s iron and Christenesen’s urea tests, were sufficient. *Y. enterocolitica* can be identified with biochemical tests such as fermentation of sucrose, rhamnose and melibiose (Schiemann, 1989). Commercial rapid identification tests provide suitable alternatives to the conventional tube tests (Cox and Mercuri, 1978; Manafi and Holzhammer, 1994; Varetas *et al*, 1995; Neubauer *et al*, 1998; Linde *et al*, 1999).

The API 20E system, widely used for identification of presumptive *Yersinia* isolates, has been shown to be accurate in identifying of *Y. enterocolitica* (Archer *et al*, 1987; Sharma *et al*, 1990; Neubauer *et al*, 1998). This kit system has a positive identification rate of 93% for *Y. enterocolitica* incubated at 28°C instead of 37°C (Archer *et al*, 1987). In the study by Sharma *et al*, (1990), identification of *Y. enterocolitica* biotypes 3, 4 and 5 was excellent, with a positive predictive value of 99%, when the strips were incubated at 28°C for 18-24h. All pathogenic *Y. enterocolitica* strains were correctly identified with API 20E by Neubauer *et al*, (1998). *Y. enterocolitica* isolates have also been identified with PCR targeting the 16S rRNA gene combined with sequencing (Neubauer *et al*, 2000a). Although the majority of isolates recovered from non-human sources are non-pathogenic, thus having no clinical significance, it is important to assess the pathogenicity of isolates (Kapperud, 1991).

2.10 PATHOGENICITY TESTS FOR *YERSINIA ENTEROCOLITICA*

The pathogenicity of *Y. enterocolitica* can be studied by animal tests such as the guinea pig conjunctivitis model (Sereny test) (Sereny, 1955), suckling mouse assay, mouse intraperitoneal challenge, and mouse diarrhoea and splenic infection following oral challenge (Aulisio *et al*, 1983; Bakour *et al*, 1985). However, because animal testing tends to be costly and is subject to increasing public opposition, it has largely been replaced by in vitro tests.

2.11 PHENOTYPIC TESTS FOR *YERSINIA ENTEROCOLITICA*

Classification and nomenclature systems of all organisms are historically

based on observable, morphological characteristics. A number of phenotypic characteristics associated with the virulence plasmid of *Yersinia* have been described (Gemski *et al*, 1980; Heesemann *et al*, 1983; Lachica and Zink, 1984; Skurnik *et al*, 1984; Skurnik, 1985). Calcium dependence, measured by growth restriction on magnesium oxalate agar (Gemski *et al*, 1980; Bhaduri *et al*, 1990), autoagglutination at 35-37°C (Skurnik *et al*, 1984), uptake of Congo red (Prpic *et al*, 1983; Riley and Toma, 1989) and crystal violet (Bhaduri *et al*, 1987) are the most popular indirect markers for identifying pathogenic strains of *Y. enterocolitica*. The pyrazinamidase (PYZ) test (Kandolo and Wauters, 1985) and the tissue culture invasiveness assay (Lee *et al*, 1977) are proven indicators of potentially pathogenic isolates (Noble *et al*, 1987; Miller *et al*, 1989; Farmer III *et al*, 1982). However, both of these tests measure functions that are chromosomally mediated, and thus, cannot replace pathogenicity tests, since they are only correlated with the ability of the strain to harbour the plasmid, and not to the presence of the plasmid itself. No single phenotypic virulence-associated characteristic has been shown to be a reliable indicator of pathogenicity (Noble *et al*, 1987).

2.12 GENOTYPIC TESTS FOR CLASSIFICATION AND IDENTIFICATION OF *YERSINIA SPECIES*

Genotypic tests are more recent approaches to the classification and identification of microorganisms, which involves the comparison of genotypic or genetic characteristics. Because animal tests are less desirable and phenotypic tests are time-consuming and not reliable, a number of rapid and specific DNA hybridization tests for identifying pathogenic bacteria have been developed (Hill and

Keasler, 1991). These methods are based on specific segments of DNA that have known virulence functions. Several DNA colony hybridization assays have been used to verify the pathogenicity of *Y. enterocolitica* isolates (Miller *et al*, 1989; Robins-Browne *et al*, 1989; Delor *et al*, 1990; Kapperud *et al*, 1990a; Ibrahim *et al*, 1992a).

Pathogenicity of bacteria can be determined rapidly with polymerase chain reaction (PCR). In this method, DNA sequences are specifically amplified with oligonucleotide primers to give over 10^6 -fold amplification of the selected region within a few hours (Saiki *et al*, 1988). In addition to speed, amplification of the target DNA with PCR offers maximum sensitivity and specificity (Kwaga *et al*, 1992). Numerous PCR methods (Wren and Tabaqchali, 1990; Fenwick and Murray, 1991; Nakajima *et al*, 1992; Rasmussen *et al*, 1994b; Ibrahim *et al*, 1997) have been developed to confirm pathogenicity of *Y. enterocolitica* isolates.

Genotypic markers for pathogenicity of *Y. enterocolitica* include both plasmid and chromosomal loci. When the full pathogenicity of *Yersinia* is being determined, the plasmid encoded virulence determinant, the target for PCR or colony hybridization, must be selected (Kapperud *et al*, 1990a; Wren and Tabaqchali, 1990; Bhaduri *et al*, 1997). The diagnostic value of the primers or probes that target plasmid-encoded sequences has been questioned because accidental loss of the plasmid during isolation yields false-negative result (Fenwick and Murray, 1991; Blais and Phillippe, 1995).

2.13 DETECTION OF PATHOGENIC *YERSINIA* SPECIES WITH DNA-BASED METHODS

2.13.1 DNA extraction:

DNA can be extracted from the cell either by lysing the cell wall to release the DNA or by using more laborious DNA purification procedures. Heat is routinely used, prior to PCR, to break down the cell wall of microbes and inactivate heat-labile PCR inhibitors (Lantz *et al*, 1994). However, when natural samples are studied, heat treatment alone is insufficient for *Y. enterocolitica* (Kaneko *et al*, 1995). Proteinase K treatment is most commonly used before heat treatment in the PCR methods designed for direct detection of *Y. enterocolitica* in natural samples. Proteinase K degrades cell wall proteins and PCR-inhibitory proteins and polypeptides in the sample and prevents heat-stable DNase contamination (Maas and Dahloff, 1994). *Y. enterocolitica* possess nuclease activity to break down the PCR product., this can be prevented by proteinase K treatment prior to PCR (Bhaduri and Cottrell, 1997; Nakajima *et al*, 1994). Dickinson *et al*, (1995) have shown that by increasing the amount of proteinase K from 0.2 to 1mg per ml and by using isopropanol precipitation of DNA, *Y. enterocolitica* can effectively be detected directly in raw chickens and cheese samples. DNA purification has been carried out by traditional pheno-chloroform extraction and ethanol precipitation in some PCR assays developed to detect *Y. enterocolitica* directly in natural samples (Harnett *et al*, 1996., Nilsson *et al*, 1998; Ozbas *et al*, 2000; Weynants *et al*, 1996). This method is however, laborious, time-consuming, and unsuitable for large numbers of samples. Numerous commercial DNA purification kits are available to make DNA isolation faster and easier. Some of these kits have also been used in PCR assays designed for

Y. enterocolitica (Arnold *et al*, 2001; Bhaduri and Pickard, 1995; Boyapuelle *et al*, 2001; Jourdan *et al*, 2000; Sandery *et al*, 1996).

Several DNA colony hybridisation assays with gene probes targeting the virulence-related DNA sequences of *Y. enterocolitica* have also been developed for biological samples (Jagow and Hill, 1986; Miliotis *et al*, 1989; Nesbakken *et al*, 1991a; Goverde *et al*, 1993; Durisin *et al*, 1997; Weagant *et al*, 1999). Colony hybridization does not require isolation of pure cultures, and it enables rapid detection and enumeration of all pathogenic bioserotypes. High background flora does, however, reduce the efficiency of hybridization because target cells grow insufficiently in the presence of competing microflora (Durisin *et al*, 1997). Despite this, Nesbakken *et al*, (1991a) found that the prevalence of pathogenic *Y. enterocolitica* in Norwegian pork products was substantially higher with the colony hybridization method than with the culture method.

2.13.2 Polymerase Chain Reaction (PCR) for the detection of pathogenic *Yersinia enterocolitica*

Pathogenic bacteria can be detected from natural samples rapidly and with high specificity and sensitivity using PCR (Candrian, 1995; Olsen *et al*, 1995; Hill, 1996; Scheu *et al*, 1998). Several methods have been developed to detect *Y. enterocolitica* in clinical, food and environmental samples. PCR have some disadvantages (Harris and Griffiths, 1992), one of the most serious being the high sensitivity of the technique. Small concentrations of contaminating DNA may result from cross-examination, reagents or accumulation of PCR products in the laboratory by repeated amplification of the same target sequences. To minimise contamination, laboratories must take specific precautions such as the use of disposable material,

separate sets of pipettes only for PCR and analysis of amplification products in an area separate from that where reagents and samples are prepared. Another drawback of PCR is its inability to distinguish between viable and non-viable cells. However, this problem can be overcome with a short pre-enrichment step before PCR is carried out. A further disadvantage is that many materials, such as food, faeces and blood, contain substances inhibitory to PCR (Rossen *et al*, 1992; Lantz *et al*, 1994). Removal of such inhibitors is important.

However, sample preparation must remain fairly simple. The short enrichment culture procedure without DNA Isolation is one of the best approaches because it is easy to perform and gives a high sensitivity. In addition, enrichment culture procedures are helpful in distinguishing live cells from dead cells (Lantz *et al*, 1994). However, Lantz *et al*, (1999) have demonstrated that high concentrations of target bacteria will inhibit PCR when an enrichment step has been used. The presence of large amounts of other bacteria has also been shown to inhibit the PCR reaction (Rossen *et al*, 1992). Inhibition caused by high bacterial concentration can be avoided by a 100-fold dilution of the enrichment culture (Lantz *et al*, 1999).

In epidemiological studies, differentiation of species into types is necessary to ascertain the prevalence of pathogenic types in particular regions as well as to identify reservoirs of infection, transmission vehicles and routes. To differentiate *Y. enterocolitica* strains, both phenotyping and genotyping has been used.

2.14 BIOTYPING OF *YERSINIA ENTEROCOLITICA* STRAINS

Biotyping has been extensively used because *Y. enterocolitica* comprises a biochemically heterogeneous group of bacteria (Bercovier *et al*, 1980) The biotyping

scheme proposed by Wauters *et al*, (1987) has been widely adopted and is based on the following reactions: tween-esterase activity, indole production, acid from salicin, trehalose and xylose, nitrate reduction, P-galactosidase (ONPG) activity, Voges-Proskauer reaction, proline peptidase activity, esculin hydrolysis and pyrazinamidase activity. With these reactions *Y. enterocolitica* is divided into six different biotypes: 1A, 1B, 2-5. Biotype 1A consists of non-pathogenic strains, and biotype 1B and 2-5 include strains that are associated with disease in man and animals. The most widespread strains of *Y. enterocolitica* belong to biotype 4.

2.15 SEROLOGICAL TYPING OF *YERSINIA ENTEROCOLITICA* STRAINS

Strains of *Y. enterocolitica* can also be subdivided on the basis of serotypes; with this being the most commonly used typing method for *Yersinia*. Serotyping is mostly based on LPS surface O antigen, and more seldom on H (flagellar) or K(fimbriae) antigens. Since the initial description of Windblad (1967) of eight O antigens, the list has been extended to 76 (Wauters *et al*, 1991). Aleksic and Bockemuhl, (1990) have proposed a revised and simplified typing scheme, which includes 20 antigenic factors for *Y. enterocolitica* alone. Serotype 0:3 is most frequently isolated from humans globally. Other serotypes obtained from humans include serotype 0:9 and 0:5, 27, particularly in Europe, and serotype 0:8 in the USA. However, several O antigens, including 0:3; 0:8 and 0:9 have been found in both pathogenic and non-pathogenic strains (Aleksic, 1995). An accurate biochemical characterisation is needed for or after serological typing to allow the correct assessment of the relevance of strains especially from foods and the

environment. This is because related species and biotype 1A strains are widely distributed in these samples (Wauters *et al*, 1991; Hoorfar and Holmwig, 1999).

2.16 PHAGE TYPING OF *YERSINIA ENTEROCOLITICA* STRAINS

Two schemes (Swedish and French) are used for phage typing of *Y. enterocolitica* (Schiemann, 1989). Of these, the French scheme has been used more often and recognises 12 phage types 1-X (including IX a-c). The Swedish scheme recognises seven phages (A1, A2, B1, B2, C32, C61, E1) and is used less frequently. Neither of these schemes has produced a large number of distinct epidemiological types because many strains fall into the same phage types. Strains of bioserotype 4/0:3 and phage type VIE predominate in Europe and Japan (Kapperud, 1991), whereas phage type 1Xb has been isolated in Canada (Toma and Deidrick, 1975) and in the USA (Dolye *et al*, 1981). Baker and Farmer III (1982) have developed a set of 24 phages, which offers a marked improvement for differentiation. Because of the need to maintain stocks of biologically active phages and control strains, phage typing is available at only a few laboratories.

2.17 RESTRICTION ENDONUCLEASE ANALYSIS OF THE PLASMID (REAP)

Plasmid analysis, the first bacterial typing tool, has been used for differentiating bacterial strains (Farber, 1996). Plasmids are isolated from each isolate and then separated electrophoretically in an agarose gel to determine their number and size. Pathogenic strains of *Y. enterocolitica* contain only one virulence plasmid of about 70 kb (Vesikari *et al*, 1981; Heesemann *et al*, 1983; Skurnik, *et al*, 1983). To increase discriminatory power, the isolated plasmid is cut with different frequent-cutting restriction enzymes (Heesemann *et al*, 1983; Nesbakken *et al*,

1987). Restriction endonuclease analysis of the plasmid (REAP) yields specific patterns for each bioserotype however, within bioserotype 4/0:3, the diversity of the REAP patterns is limited.

2.17.1 Restriction endonuclease analysis of the chromosome (REAC)

Chromosomal DNA restriction analysis was the first of the chromosomal DNA-based typing schemes. In this method, endonucleases with relatively frequent restriction sites are used to cut the DNA, thereby generating hundreds of fragments ranging from 0.5 to 50kb in size (Maslow *et al*, 1993). A major limitation of this technique is the difficulty in interpreting complex profiles, which consist of hundreds of bands that may be unresolved and overlapping. Kapperud *et al*, (1990b) have used REAC to study polymorphism in restriction fragment patterns among *Y. enterocolitica* isolates belonging to different bioserotypes. A total of 22 distinct REAC patterns were distinguished among the 72 *Yersinia* strains examined, and the patterns varied clearly between bioserotypes. Some variation occurred among strains within the same bioserotype, but strains of bioserotype 4/0:3 were homogenous.

2.18 RIBOTYPING OF *YERSINIA ENTEROCOLITICA* STRAINS

To avoid problems associated with complex REAC patterns, probes, which hybridise to specific DNA sequences, are used. Ribotyping refers to the use of nucleic acid probes to recognise ribosomal genes, which are present in all bacteria (Farber, 1996). In practice, chromosomal DNA is isolated and a frequent-cutting enzyme is used to cut the DNA into small fragments. Fragments are separated by electrophoresis through an agarose gel. The separated DNA fragments are transferred from the agarose to either a nitrocellulose or nylon membrane by

Southern blotting (Southern, 1975). Probing is usually done with labeled probes containing *E. coli* 23S, 16S and 5S, rRNA sequences. After probing, fragments containing a ribosomal gene will be highlighted, creating a fingerprint pattern containing approximately 1 to 15 bands that can be compared easily among isolates. Ribotyping has been used to characterise *Y. enterocolitica* isolates in several studies (Andersen and Saunders, 1990; Blumberg *et al*, 1991; Iteman *et al*, 1996; Mendoza *et al*, 1996; Lobato *et al*, 1998; Fukushima *et al*, 1998). A close relationship has been found between the ribotypes and bioserotypes of *Y. enterocolitica* isolates. Although variation exists among isolates belonging to the same bioserotype, genetic diversity is limited among isolates of bioserotype 4/0:3.

2.19 MULTISPACER TYPING (MST)

This is an ideal technique (epidemiological marker) for tracing isolates obtained from a wide variety of sources (e.g. community or Hospital bacterial outbreaks) and may even be applied in clinical specimens for bacterial identification at the strain level. The MST approach allows easy comparison and exchange of results obtained in different laboratories based on intergenic region sequencing as these regions are considered potentially variable due to lack of selection pressure.

2.20 PULSED-FIELD GEL ELECTROPHORESIS (PFGE) TYPING

Pulsed-field gel electrophoresis is a variation of agarose gel electrophoresis that permits analysis of the large fragments of bacterial DNA. For PFGE, bacterial isolates grown both in broth and on solid media are combined with molten agarose and poured into small moulds. The embedded bacteria are then subjected to *in situ*

detergent-enzyme lysis and digestion with an infrequently cutting restriction enzyme. The digested bacterial plugs, containing the whole genome, are inserted into an agarose gel and subjected to electrophoresis in an apparatus in which the polarity of the current is changed at predetermined intervals. The pulsed field allows clear separation of large molecular length DNA fragments, ranging from 10kb to 800 kb. PFGE provides a highly reproducible restriction profile, which typically shows distinct, well-resolved fragments representing the entire bacterial genome in a single gel (Logonne, 1993). Because of the high discriminatory power, and good intra and interlaboratory reproducibility, PFGE is still one of the best methods available when compared with the newer typing methods (Olive and Bean, 1999).

A number of studies have been conducted to characterise *Y. enterocolitica* with PFGE (Iteman *et al*, 1991; Buchrieser *et al*, 1994; Najdenski *et al*, 1994; Saken *et al*, 1994; Hosaka *et al*, 1997). Iteman *et al*, (1996) compared PFGE with ribotyping and REAP, and found PFGE to be the most suitable technique for epidemiological tracing of *Y. enterocolitica*. PFGE allows subtyping of strains belonging to same bioserotype (Buchrieser *et al*, 1994; Najdenski *et al*, 1994; Saken *et al*, 1994). Najdenski *et al*, (1994) showed that the pulsotype resembles the biotype more closely than the serotype and that the genome of *Y. enterocolitica* is stable *in vitro*. The global homogeneity of the pulsotypes among strains of bioserotype 40:3 has been shown to be high (Najdenski *et al*, 1994; Saken *et al*, 1994; Asplund *et al*, 1998). Although strains of bioserotype 4/0:3 can be subdivided into several pulsotypes, most strains fall into one or two dominating pulsotypes, decreasing the discriminatory power of PFGE.

2.21 APPLICATIONS OF PFGE IN MICROBIOLOGY

Originally, Schwartz, Cantor and colleagues (1983) developed PFGE for separation of eukaryotic DNA, specifically chromosomes of the yeast *Saccharomyces cerevisiae*. This useful technique was rapidly applied to the analyses of chromosomal and extrachromosomal DNAs of many other eukaryotic organisms.

The first application of PFGE with prokaryotic DNA was in microbial genetics for mapping the chromosome of *Escherichia coli* (Smith *et al*, 1987). PFGE has subsequently been useful in mapping and chromosomal size estimation of numerous prokaryotes (Cole and Girons, 1994). Indeed, the application of PFGE to the study of prokaryotic genomes has led to the discovery that some bacteria possess a linear rather than circular chromosome, and some possess multiple chromosomes (Cole and Girons, 1994).

Mapping and sizing of genomes using PFGE requires the cleavage of DNA by restriction endonucleases into a reasonable number of fragments, then size-dependent electrophoretic separation of the fragments (Voss *et al*, 1995., Willinger *et al*, 1994). The choice of an appropriate restriction enzyme requires consideration of the organism's genome composition. For example, a bacterial genome that is >45%G + C typically has rare occurrences of the tetranucleotide CTAG (McClelland *et al*, 1987). Therefore using a restriction endonuclease which had this tetranucleotide as part of its recognition sequence may be an appropriate choice for genomes with a high G + C content. Likewise an organism with a genome that has a high A + T content may be suitably cut with a restriction endonuclease which recognizes predominant G and C. The sizes of these macrorestriction fragments are determined by comparison to known size markers and, when summed up give a reasonable

estimate of the genome size. By combining fragment size data from several single restriction endonuclease digests and double digests, a physical map of the genome can be generated. The application of this method has contributed a wealth of information to the field of bacterial genomics (Cole and Girons, 1994).

2.22 RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD)

Randomly amplified DNA (RAPD) assay, also referred to as arbitrary primed PCR, is a variation of the PCR technique employing a single short (typically 10 base pairs) primer that is not targeted to amplify any specific bacterial sequence. The primer hybridises at multiple random chromosomal locations and initiates DNA synthesis at low annealing temperatures. The resulting PCR products present a variety of different sized DNA fragments that are visualized by agarose gel electrophoresis (Farber, 1996). RAPD is a very simple and quick method, but its reproducibility is low and standardization of the technique is difficult (Olive and Bean, 1999). Some studies have characterized *Y. enterocolitica* isolates with RAPD (Rasmussen *et al*, 1994a; Odinet *et al*, 1995; Leal *et al*, 1999). This method allows discrimination between *Y. enterocolitica* isolates belonging to different bioserotypes and, also in some cases, between isolates belonging to the same bioserotype (Odinot *et al*, 1995; Leal *et al*, 1999).

2.23 CHEMOTHERAPY

Intestinal infections with *Y. enterocolitica* and *Y. pseudotuberculosis* are mostly self-limited and need no specific antibiotic treatment. In immunocompromised patients, enteritis may be treated prophylactically. Oral

deoxycycline or trimethoprim-sulfamethoxazole has been recommended for such patients as well as for patients with complicated gastrointestinal or focal extraintestinal infections in general (Cover and Aber, 1989).

In contrast to *Y. pseudotuberculosis*, *Y. enterocolitica* serogroups 0:3 and 0:9 produce two chromosomally determined Beta-lactamases (types A and B, respectively), which are variably expressed (Stock *et al*, 2000). These account for resistance to ampicillin, cephalothin and carbencillin. *Y. enterocolitica* serotype 0:8 is susceptible to ampicillin but is variably resistant to carbenicillin and cephalothin and, like most biotype 1B strains, produces a type A beta-lactamase (Stock *et al*, 2000). In vitro antibacterial activity does not necessarily reflect in vivo efficacy.

Clinically, administration of broad-spectrum antibiotics often in combination with aminoglycosides has resulted in successful outcomes for most patients with extra intestinal infections including septicaemia (Bottone, 1997). Flouroquinolones (ciprofloxacin) and expanded spectrum cephalosporins such as cefotaxime and ceftriaxone may be regarded as the most active antimicrobial agents for the treatment of *Y. enterocolitica* 0:3 infections (Rastawicki *et al*, 2000). *Y. enterocolitica* strains are usually susceptible in vitro to chloramphenicol, colistin, cotrimoxazole, gentamicin, kanamycin, nitrofurazone, streptomycin and tetracyclines but resistant to penicillin and first generation cephalosporins. Antibiotic treatment is not usually necessary in uncomplicated diarrhoea, enteritis or terminal ileitis, and there is little evidence that it alters the course of the disease (Topley and Wilson, 1990).

Treatment of *Y. pseudotuberculosis* with suitable antibiotic may be effective in the early phases of the disease. The sensitivity of *Y. pseudotuberculosis* to antibiotics varies between isolates and should be determined before treatment is begun. Many strains are sensitive to ampicillin, chloramphenicol, cotrimoxazole, spectinomycin and tetracyclines. It is highly sensitive invitro to some of the newer Beta lactam antibiotics, the minimum inhibitory concentrations of aztreonam, cefixime, cefotaxime, ceftizoxime and ceftriaxone were reported to be 0.01mg/1 for most strains (Kanazawa *et al*, 1987). Generally, *Y. pseudotuberculosis* and *Y. enterocolitica* are susceptible to many antibiotics but may be resistant to penciillin and it's semi-synthetic derivatives.

The usefulness of antibiotic treatment in patients with reactive arthritis and other immunopathological disorders is still under discussion (Murray *et al*, 2003). Although persistence of the organisms in the intestines or mesenteric lymph nodes may be assumed, prospective and retrospective studies so far have not yielded solid evidence in favour of antibiotics therapy (Murray *et al*, 2003).

2.24 CONTROL OF YERSINIA SPECIES

Protective immunity to plaque can be conferred on mice or rats by immunization with killed virulent or living virulent strains of *Y. pestis* that produce the F1 antigen at 37°C. Killed avirulent strains are much less protective than killed virulent strains. Protective immunity to *Y. pestis* has been produced in guinea pigs by immunization with a living attenuated strains of *Y. pseudotuberculosis* (Thal, 1956). However, reciprocal immunity against *Y. pseudotuberculosis* was not

produced by vaccination with *Y. pestis* (Thal *et al*, 1967).

Killed whole-cell vaccines are protective against bubonic plague in man but they are thought to be less effective against pneumonic infection (WHO, 1970). Living attenuated vaccines based on strains such as EV76 are also protective in man but produce an unacceptable incidence of adverse reactions (Meyer *et al*, 1974a). Attempts have been made to produce cell-free *Y. pestis* vaccines but none has proved more effective than whole-cell preparations. According to Girard (1953) vaccines protective for guinea-pigs and mice should also protect man. The purified F1 antigen is protective in mice but requires an adjuvant if used in guinea-pigs. The V and W antigens are also immunogenic in mice but not in guinea-pigs. Murine toxin appears to contribute little to protective immunity against plague. A non-toxic cell-free alkaline extract of *Y. pestis* was reported by Smith and Packman (1966) to protect both mice and guinea-pigs and was suggested for trials in man. Ribosome preparations of *Y. pestis* have also been reported to stimulate immunity (Johnson, 1972).

Cellular vaccines for the prevention of *Y. pseudotuberculosis* or *Y. enterocolitica* infections have not been developed, but immunity to both organisms has been produced by cell-free extracts containing V antigen (Une and Brubaker, 1984) and to the former by chromosomally encoded antigens (Simonet *et al*, 1985). Vaccines for the protection of fish against *Y. ruckeri* usually consist of killed whole-cell preparations. These can be given by parenteral injection, by the oral route or by repeated immersion of the fish in vaccine suspensions. All methods seem to

stimulate protective immunity but the antigens required for this have not been characterized

CHAPTER THREE

MATERIALS AND METHODS

The prevalence of *Y. enterocolitica* and *Y. pseudotuberculosis* was studied in the faecal samples from animals and humans with both cultural, biochemical and serological methods and by polymerase chain reaction and DNA sequencing.

3.1 STUDY POPULATIONS

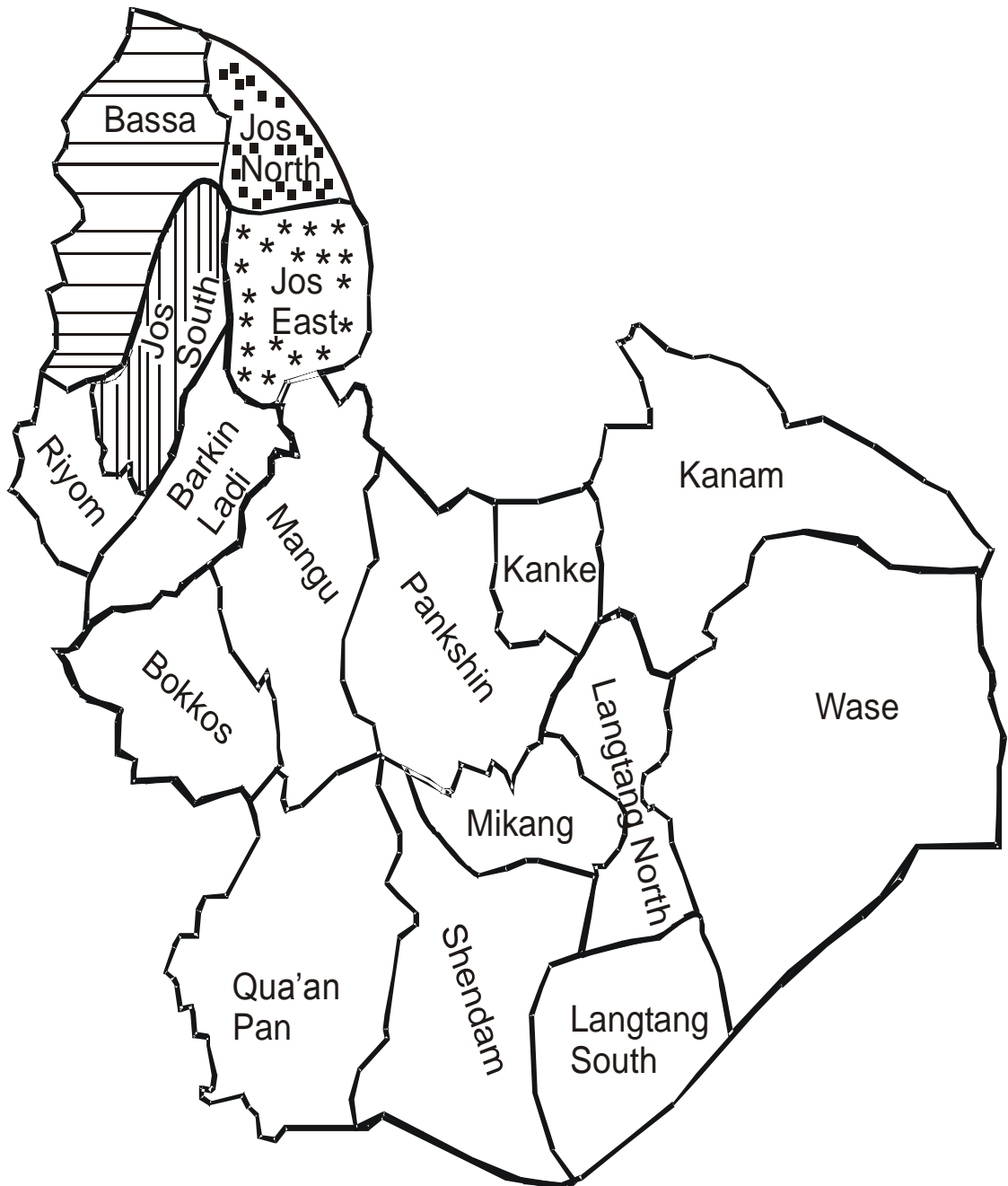
The study populations were made up of apparently healthy individuals, diarrhoeic patients and some randomly selected animal species (pigs, sheep, poultry and dogs).

3.2 STUDY AREA





The principal areas of investigation were: –

- Vom (premises of the National Veterinary Research Institute and residential quarters in Jos-South Local Government Area).
- Bukuru (main market and residents - Jos-South Local Government Area)
- Jos (Residents and abattoirs - Jos-North Local Government Area)
- Jos East (markets and residents - Jos-East Local Government Area).
- Bassa (markets and residents - Bassa Local Government Area).

MAP OF PLATEAU STATE INDICATING STUDY AREAS COVERED



KEY:

-  Jos South and Vom
-  Jos North
-  Jos East
-  Bassa

(Department of Geography, University of Jos, Nigeria, 2006)

3.3 COLLECTION OF FAECAL SAMPLES

Stool samples and rectal swabs from both humans and animals were collected from five (5) different locations in Jos and its environs and assessed for their *Yersinia* species contents. A total of 3000 faecal samples and rectal swabs were collected between June, 2001 and August, 2004 by convenient random sampling. Human faecal samples (1500) collected were made up of 750 each from apparently healthy individuals and diarrhoeic patients respectively.

Animal faecal samples and rectal swabs (1500) collected were made up 75 each from the selected species of animals investigated. Three hundred (300) samples were collected per study area. All samples were taken to the laboratory within three hours of defaecation. All animals screened appeared healthy with no history or signs of illness.

3.4 CULTURING OF THE SAMPLES

➤ Cold enrichment culture

The psychrotrophic *Y. enterocolitica* and *Y. pseudotuberculosis* is unusual among other *Enterobacteriaceae*. They can only cause infection in the presence of sufficient calcium ie they are calcium dependent at temperature above 30°C. Consequently, cold enrichment in phosphate buffered saline (PBS) or phosphate buffered saline with sorbitol and bile salts (PSB) have been widely used for clinical, food and environmental samples (Shiozawa *et al*, 1987; De Boer and Nouws, 1991; Logue *et al*, 1996; Funk *et al*, 1998; Letellier *et al*, 1999).

1g of faecal samples were aseptically inoculated into 10 ml of phosphate

buffered saline (PBS), homogenized for about 30 seconds, treated with sodium hydroxide to reduce the load of normal flora (Schiemann, 1983a) and incubated at 4°C for 21 days (FDA/CFSAN, 2001; Fredriksson -Ahomaa and Korkeala, 2003).

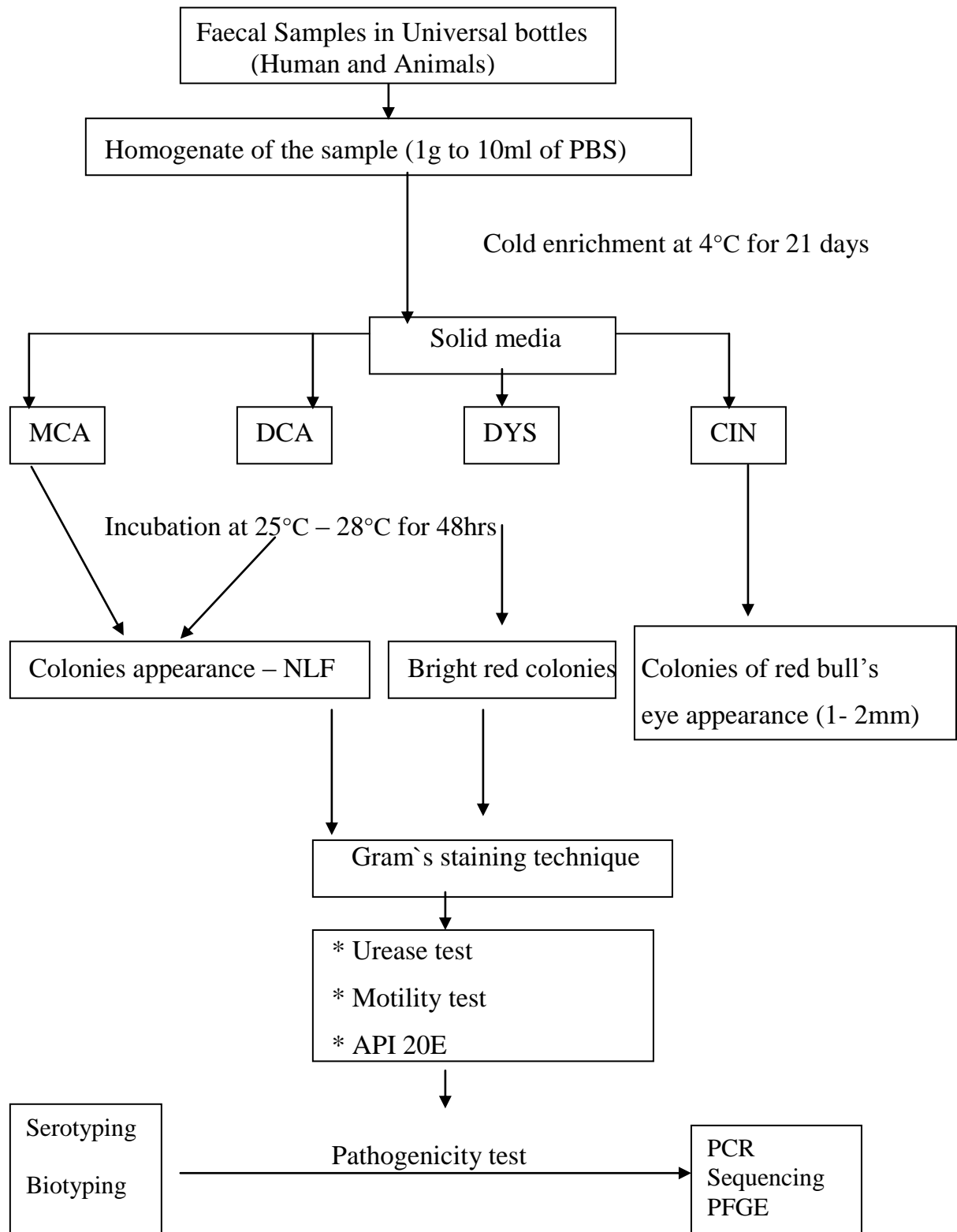


Figure 2: Flow chart for the isolation, identification and characterization of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from faecal samples

➤ **Bacterial isolation**

The methods used for the culture and isolation of *Yersinia* species was a modification of the methods of Van-Noyen *et al*, (1981). Faecal samples in PBS were further subcultured onto selected solid media (MCA (without salt) DCA, DYS) by streaking and incubated at 25°C for 18-48 hours. Culture plates were examined after incubation. Red mucoid colonies were rejected while small colonies of about 1-2 mm in diameter, flat colourless or pale pink were selected. The selected colonies were further re-inoculated onto Cefsulodin Irgasan Novobiocin (CIN) agar plates Christensen's urea agar and bile aesculin agar slopes by stabbing with a straight wire and was incubated at 25°C or room temperature for 18 – 24 hours.

3.5 DATA MANAGEMENT AND ANALYSIS

Laboratory results were entered and managed using the Microsoft package for social sciences version 14 program, (2006). Descriptive statistical analysis was done using ANOVA and the student T- test for the comparison of results between individual groups of patients and the animals. The prevalence of yersiniosis were compared among age groups (Okwori *et al*, 2007).

3.6 PRESUMPTIVE IDENTIFICATION

Preferences were given to typical isolates resembling *Yersinia* species with the following results (FDA/CFSAN, 2001):

- | | |
|---------------------------------------|-----------------------|
| ▪ Grams stain reaction | Negative coccobacilli |
| ▪ Urease reaction | Positive |
| ▪ Motility test @25-30 ⁰ C | Positive |
| ▪ Motility test @ 37 ⁰ C | Negative |

On CIN agar, presumptive identification was made based on characteristic appearance of colonies with dark red bull's eye surrounded by a transparent border (Barrow *et al*, 1983; FDA/CFSAN, 2001). Isolates were further re- identified, confirmed by phenotypic characterization using API-20E strips (Bio-merieux, la Balme- les –Grottes, France) (Figs 7, 8) (Archer *et al*, 1987; Sharma *et al*, 1990; Neubauer *et al*, 1998) and compared with control reference strains obtained from the Bacterial Research Department National Veterinary Research Institute, Vom. Confirmed isolates of *Y. enterocolitica* and *Y. pseudotuberculosis* were lyophilized at the Viral Vaccine Department, National Veterinary Research Institute, Vom, into ampoules and stored at 4°C until required for further investigation.

Y. enterocolitica and *Y. pseudotuberculosis* were differentiated from each other as shown on figure 8 by API – 20E strip test.

3.7 BIO AND SEROTYPING OF *YERSINIA ENTEROCOLITICA* ISOLATES

All confirmed *Y. enterocolitica* isolates were biotyped according to the revised scheme of Wauters *et al*, (1987). Subdivision into six biotypes (1A, 1B, 2, 3, 4, and 5) was based on the following reactions: Pyrazinamidase activity, esculin hydrolysis, salicin acidification, tween-esterase activity, indole production, xylose acidification and nitrate reduction (Table 1).

Table 1: Biochemical tests used for biotyping of *Yersinia enterocolitica* strains

Test	1A	1B	2	3	4	5
Pyrazinamidase	+	-	-	-	-	-
Esculin hydrolysis	+	-	-	-	-	-
Salicin (acid production)	+	-	-	-	-	-
Tween-esterase	+	+	-	-	-	-
Indole production	+	+	+	-	-	-
Xylose (acid production)	+	+	+	+	+	-
Nitrate reduction	+	+	+	+	+	+

(Kandolo and Wauters, 1985)

➤ **Biotyping of *Yersinia enterocolitica* strains**

All *Yersinia* strains were biochemically characterized and the *Y. enterocolitica* strains were assigned to one of the six biotypes defined by Bercovier *et al*, (1980). The API 20E enteric identification system (Biomereux, France) was used for biochemical identification. Cultures were incubated at 25°C for 18 to 24 hours. API 20E biochemical tests were done according to the manufacturers` instructions.

➤ **Serotyping of *Yersinia enterocolitica* strains**

In all studies, serotyping of *Y. enterocolitica* was carried out with slide agglutination using commercially prepared serum agglutinant anti- *Y. enterocolitica* 0:3, and anti- *Y. enterocolitica* 0:9 (containing the corresponding antibodies) (Bio-Rad, Marnes-La-Coquette, France) (Thoerner *et al*, 2003).

3.8 STORAGE OF ISOLATES BY LYOPHILIZATION

All isolates of *Y. enterocolitica* and *Y. pseudotuberculosis* were lyophilized or freeze-dried at the Viral Vaccine laboratories, (National Veterinary Research Institute, Vom, Nigeria) into ampoules and stored in the deep freezer at -4°C until required for further studies.

3.9 PATHOGENICITY TEST

3.9.1 Sources of rabbits and guinea pigs

Ten New Zealand breeds of rabbits weighing between 50-750 grams were obtained from the small animal laboratory Department, National Veterinary Research Institute, Vom, Nigeria. Also ten Abyssinian breeds of guinea pigs

weighing 200 - 400g were obtained from the same source as above.

3.9.2 Sources of reference *Yersinia* strains

The standard strains of *Yersiniae* used were provided by Bacterial Research Laboratories, National Veterinary Research Institute, Vom, Nigeria. Other reference strains of *Y. enterocolitica* and *Y. pseudotuberculosis* were obtained at the later stage of work from the Faculty of Veterinary Medicine, University of Helsinki, Finland.

3.9.3 Sereny test

Sereny test is the demonstration of keratoconjunctivitis in experimental animals such as rabbit (Sereny, 1955).

The animals were divided into five groups of four with each containing two rabbits and two guinea pigs. The groups were named A, B, C, D and E. Group A was infected with local strains of *Y. enterocolitica*, Group B with local strains of *Y. pseudotuberculosis*. Group C was infected with standard strains of *Y. enterocolitica* (serotype 0:3), Group D was infected with standard strain of *Y. pseudotuberculosis* (serotype III), while Group E served as negative control. The route of inoculation was oral. Growers mash ("Guinea Feed" Edo state, Nigeria) was used for feeding the animals for the period of the experiment.

The animals were housed in separate cages (45 by 46 by 32cm). They were first observed and acclimatized to laboratory conditions for a period of 9 days. Prior to the experiment the animals were monitored for 4 weeks for any noticeable clinical symptoms. Faecal samples were screened bacteriologically to ensure the absence of the *Yersinia* species. The animals were also screened serologically for *Y.*

enterocolitica and *Y. pseudotuberculosis* antibodies prior to experiment to ensure that they were free. Each cage was identified by a letter and each animal (rabbits and g/pigs) was identified by a number. The body temperature was recorded daily to ensure that the animals remained afebrile. Drinking water was available ad libitum, and diet distributed every morning. Each group of the animals were housed separately in a roofed block building with cross ventilation, in an environmental temperature of $25 \pm 1^{\circ}\text{C}$ and relative humidity of 50%. Animal care and manipulations were conducted in accordance with the Consortium Guide (1988).

3.9.4 Preparation of inoculum

The test organisms were cultured on trytone soy agar supplemented with 0.6% Yeast extract (TSYE). The TSYE plates were incubated at room temperature for 48hrs. A pure colony was picked with a wireloop and this was used to inoculate 10ml of trytone soy yeast extract broth (OXOID). The broth was incubated at 25°C for 48hrs and then centrifuged at 3,000 rpm for 30 minutes. The deposit was washed twice in sterile phosphate buffered saline (pH 7.3) and finally resuspended in the same medium for oral inoculation. Concentration of bacteria was adjusted to 10^8 cfu per ml (Miles and Misra, 1938; Agbonlahor *et al*, 1983).

3.9.5 Peroral challenge of animals

Animals in groups A-D were deprived of drinking water for 24hrs and then allowed to drink from aqueous bacterial suspension containing about 1×10^8 cfu/ml for 24hours of the respective bacteria as described above. The inocula were then withdrawn and the animals served with clean water after 24hrs period. Those in-group E were also served with clean drinking water. They were then observed for

weight loss, sign of diarrhoea and death for 3 weeks. The oral route of infection more accurately reflects the natural route of infection. During this period, faecal specimens were collected from the rabbits at weekly intervals for culture. After three weeks, the animals were sacrificed, dissected and the viscera examined. The heart blood was also cultured to reclaim the organisms. The sera from the infected animals were tested against the respective organisms.

3.10 HISTOPATHOLOGY

Tissues from the small intestine and liver were removed at postmortem of the infected animals (carcasses). The lumen of the small intestine was flushed with PBS, prior to been embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The sections were scored for increased numbers of macrophages and neutrophils, granuloma formation, inflammatory infiltrates, necrosis, and bacterial colonization. A minimum of five animals per time point was examined.

3.11 MOLECULAR STUDIES

3.11.1 Selection of Target Sequences

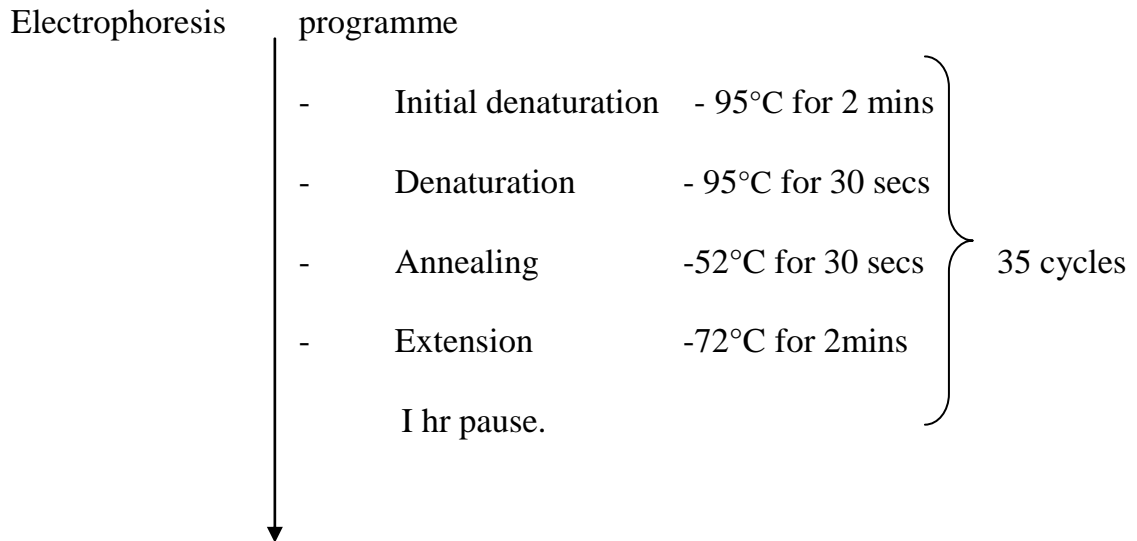
The variability of *Y. enterocolitica* isolates was examined based on non-coding sequences from the complete renamed sequences of *Yersinia pestis* and *Yersinia pseudotuberculosis* as obtained in the Gene bank.

3.11.2 Multispacer (MST) Primer Design

The Genome sequence of *Yersinia pestis* and *Yersinia pseudotuberculosis* with Genbank accession numbers NC – 005810 and NC - 00408 were aligned using the blast method on a computer soft ware. *Yersinia pestis* and *Yersinia*

pseudotuberculosis show 90% DNA sequence homology (Portony *et al*, 1984; Parkhill *et al*, 2001) whereas both share about 50% DNA sequence homology with *Y. enterocolitica* (Bercovier *et al*, 1980; Brenner *et al*, 1976) and to test this hypothesis a comparison of the genome sequence of two closely related organisms (*Y. pestis* and *Y. pseudotuberculosis*) were attempted to develop a genome-based method for selecting sequences suitable for strain typing or characterization.

PCR Amplification



Automated DNA sequencing

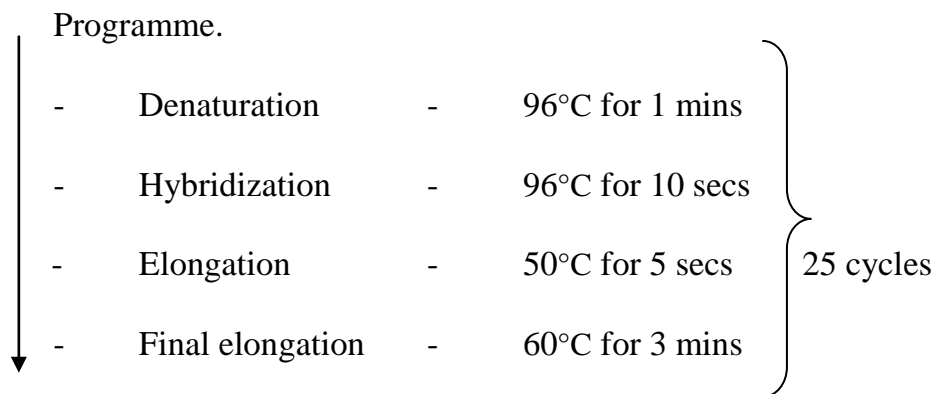


Figure 3: Programmes for PCR amplification and sequencing reactions

Table 2: List of primers and alignment spacers used for PCR amplification and sequences of *Yersinia* species

Primers	Nucleic acid sequence (5`-3`)	Product length (bp)
YPO2576_YPO2577F: TCAGATTGCTTGCTGTAGTC 40	(20 bases)	346bp
YPO2576_YPO2577R: TAAAATTCGCTACCGTCTCC 40	(20 bases)	
YPO2043_YPO2044F: TGCCCGTAGAACGGGCTG 40	(18 bases)	404bp
YPO2043_YPO2044R: GATTAACAACACACTAGATGTG 40	(22 bases)	
YPO1831_YPO1832F: GTCATGAATATGGGTGTGTG 40	(20 bases)	360bp
YPO1831_YPO1832R: CAAGCTATATCTAATCAACTGC 40	(21 bases)	
YPO1651_YPO1652F: CAATTGTTTCGTC AATTTTGTC 40	(21 bases)	539bp
YPO1651_YPO1652R: GTCAGCAATACCATTATTAGTC 40	(22 bases)	
YPO1285_YPO1286F: TTGGGATAAGGCCATCGAC 40	(19 bases)	275bp
YPO1285_YPO1286R: CAATCTGAGCGACAGTCC 40	(18 bases)	
YPO1143_YPO1144F: GTAAGAAGAATTTTCGGCCTG 40	(20 bases)	328bp
YPO1143_YPO1144R: CAAAGACCAGACTTTTTTAATAG 40	(22 bases)	
YPO0909_YPO0910F: CACCAACAATTATCACACTC 40	(20 bases)	92bp
YPO0909_YPO0910R: AGCATTGATGGCATTGAATC 40	(20 bases)	
YPO0859_YPO0860F: ATGCTTGACCCATCCCGTAA 40	(20 bases)	451bp
YPO0859_YPO0860R: CAAGCAGAGTTTGTAGCATTG 40	(20 bases)	
YPO0409_YPO0410F: CAAATCCAAGAGCGCAAAC 40	(19 bases)	105bp
YPO0409_YPO0410R: TCGCAGTCGAAAGGCCTC 40	(18 bases)	
YPO0092_YPO0093F: AGGGGTGTATCGCATCAAAG 40	(20 bases)	434bp
YPO0092_YPO0093R: TCGCCTGCTGAACTTTAAC 40	(19 bases)	

Key: YP - *YP* - *Yersinia pestis* - *Yersinia pseudotuberculosis*

F – Forward

R – Reverse

Bp - Base pair

A- Arginine, G- Guanine, T- Thymidine, C- Cytosine

(Altschul *et al*, 1990)

3.11.3 DNA extraction

Genomic DNA was extracted from fresh isolates of *Y. enterocolitica* grown into late log phase by using the QIAamp tissue kit (QIAGEN, Hilden, Germany) according to the manufacturers instructions.

A high quality undegraded chromosomal DNA was isolated according to the method described in current protocols (Ausubel *et al*, 1999) as follows. Bacterial cells were lysed with sodium dodecyl sulphate (SDS) and proteinase K. Cell wall debris, polysaccharides and remaining proteins were then removed by selective precipitation with hexadecyltrimethyl ammonium bromide (CTAB) and high molecular weight DNA was recovered from the resulting supernant by isopropanol precipitation.

3.11.4 Procedure for DNA extraction using Qiagen test kit

Sterile plastic tubes were arranged serially and 180 µl of ATL buffer and 20 µl of proteinase k added making a total volume of 200ul. Colonies were harvested, mixed and vortexed properly in these tubes, followed by incubation at 56°C for 1-2hours or 65°C for 30 minutes. Repeated centrifugations were made, 200 µl of buffer ATL was then added, and then vortexed to mix properly, before the mixture was re-incubated at 70°C for 10 minutes. Ethanol (200µl) was then added to the suspension and the filterate housed in another plastic tube with a central filter pad.

This was followed by centrifugation for 1 minute at 8000rpm and the supernatant discarded and replaced with another tube containing 500ml of AW1

buffer and 500ml of buffer AW2. 200ml buffer AE or disfilled water was added and left at room temperature for 2-5 minutes for ellution.

The preparation was re-centrifuged and the supernatant stored until required at 4°C.

3.11.5 PCR amplification and sequencing:

The primers used for MST PCR were obtained from Eurogentic (Seraing, Toidi, Belgium). Their specificity was predicted by using blast software (Altschul *et al*, 1990; Fournier *et al*, 2004; Drancourt *et al*, 2004). Polymerase chain reactions (PCR) were carried out in a PTC-200 automated thermal cycler (MJ Research, Waltham, Mass). Two microlitres (2.0µl) of the DNA preparation were amplified in a 50µl reaction mixture containing 50ml each primer, 200µl each dATP, dCTP, dGTP, and dTTP (Invitrogen, Gaithersburg Maryland, USA), 1µl of eloNgase polymers (Invitrogen), 2ml of EloNgase buffer A, and 8µl of eloNgase Buffer B.

The following conditions were used for Amplification:

- an initial 2 minutes of denaturation at 95°C was followed by 35 cycles.
- denaturation for 30 seconds at 95°C.
- annealing for 30 seconds at various temperatures and extension for 1 minute at 72°C.

Amplification was completed by holding the reaction mixture for 3 minutes at 72°C to allow complete extension of the PCR products. PCR products were purified by using a Q1Aquick spin PCR purification kit (Q1AGEN, Germany) as described by the manufacturer. Sequencing reactions were carried out by using the

dRhodamine Terminator cycle sequencing ready reaction kit with Amplitaq polymerase FS (Perkin-Elmer-coignieers, France) as described by the manufacturer. For all PCR products, sequences for both DNA strands were determined twice. Sequencing products were resolved by using an ABI 3100 automated sequencer (Perkin-Elmer, France). Sequence analysis was performed by using the ABI prism DNA sequencing analysis software package (Version 3.0; Perkin-Elmer, France) sterile water was used as a negative control in each assay.

For a typical 50µl reaction volume, the following components were pipetted into a PCR test-tube:

3.11.6 Reaction components PCR

DNA	5 µl
Primer forward (F)	1 µl
Primer Reverse (R)	1 µl
DNTP (dATP, dCTP, dGTP, dTTP)	5 µl
10xTaq-Reaction buffer (mgCl ₂)	5 µl
Nitrogen Tag-polymerase (5U/ul)	0.3 µl
H ₂ O	33 µl
MgCl ₂	2.5 µl

A negative control with water as template DNA was always included in the reactions and 5.0µl of the finished PCR product was checked on an agarose gel before purification with the QiaGen PCR purification kit.

3.11.7 Agarose gel electrophoresis

The agarose gel was prepared by mixing an appropriate proportion of agarose (to a final concentration of 0.7-2% depending on the MW of the sample DNA) with 1x Tris-acetate-EDTA (TAE) buffer, the mixture cooked and after cooling poured into precast agarose chambers. The DNA was then mixed with loading buffer, loaded onto spurs on the gel and electrophoretically separated by voltage application, utilizing the 1 x TAE solution as the running buffer. Following the electrophoretic run, gels were stained in ethidium bromide solution and the DNA visualized under ultraviolet radiation.

3.11.8 Macrorestriction analysis with pulsed field gel electrophoresis (PFGE)

Plugs were washed once with TE buffer (10mM Tris-HCl, 1 mM EDTA) before proteinase K was inactivated with phenylmethylsulfonyl fluoride. Plugs were further washed two times with Tris-chloride and EDTA (TE) buffer before restriction endonuclease digestion was performed according to the manufacturer's instructions.

All samples were electrophoresed using a Gene Navigator system (Pharmacia, Uppsala, Sweden) with a hexagonal electrode through a 1% (w/v) agarose gel (SeaKem Gold; FMC Bioproducts) in a 0.5 c TBE buffer (Amresco, Solon Oh, USA) at 12°C and 200 V. switching times from 1 to 15 s over 18 h for xbaI, xhoI and AclI, from 1 to 18 s over 20 h for NotI and SpeI, and from 1 to 20 s over 20 h for ApaI were used. Low-Range, Mid-Range I and Lambda Ladder PFG markers (New England Biolabs) were used for fragment size determination. The gels were stained

for 30 min in 1 liter of running buffer containing 50µl of ethidium bromide (10mg/ml and destained in running buffer until appropriate contrast was obtained for standard photography and/or digital imaging with an Alpha imager 2000 documentation system (Alpha Innotech, San Leandro, CA, USA). The banding patterns were interpreted visually. Isolates were considered to be different when a one-band difference between fragments over 70 kb was observed. (See plate 9).

3.11.9 DNA sequencing

DNA Sequencing was done by the dideoxy-chain terminating method on an automated ABI prism DNA Sequencer. The ensuing chromatograms were processed with Chromas software and BLASTN and BLASTX programs provided by National Center for Biotechnology Information (NCBI) and the Institute for genomic research (TIGR), and also the *Y. pestis* and *Y. enterocolitica* gene banks from Sanger Center were employed for in-depth homology searches.

3.11.10 Bioinformatics (Westend *et al*, 2002)

Bioinformatic tools were utilized for sequence analysis, alignments and similarity searches. The two primary databanks that were extensively utilized were Genbank and European Molecular Biology Laboratory (EMBL).

3.11.11 Primary databanks

1. The Genbank in the USA is under the auspices of National Center for Biotechnology (NCBI) and is an official sequence data bank, which contains protein, and nucleotide sequences from more than 55,000

different organisms. All sequences are identified or tagged with a unique accession number. A Genbank sequence is usually divided into two parts:

- The Annotation which contains a precise and detailed information about the sequence and
 - The sequence itself. The ENTREZ (France) search machine is coupled with the Genbank and allows a specific search based on an accession number, organism, gene, protein or author.
2. The EMBL nucleotide sequence database is the European equivalent of the Genbank and utilizes the sequence retrieval system (SRS), a search machine similar to the ENTREZ for specialized searches of the database and many other databanks over the web interface.

3.11.12 Basic local alignment search tool (BLAST)

Variations of the BLAST algorithm (Altschul *et al*, 1990) have been incorporated into several popular programs for searching protein and DNA databases for sequence similarities. BLAST programs have been written to compare protein or DNA queries with protein or DNA databases in any combination, with DNA sequences often undergoing conceptual translation before any comparison is performed. The blast program compares protein queries to protein databases, as a prototype for BLAST, although the ideas presented extend immediately to other versions that involve the translation of a DNA query or database. Some of the refinements described are applicable as well to DNA-DNA comparison, but have yet to be implemented.

BLAST is a heuristic that attempts to optimize a specific similarity measure. It permits a tradeoff between speed and sensitivity, with the setting of a 'threshold' parameter, T. A higher value of T yields greater speed, but also an increased probability of missing weak similarities. The BLAST program requires time proportional to the product of the lengths of the query sequence and the database searched. Since the rate of change in database sizes currently exceeds that of processor speeds, computers running BLAST are subjected to increasing load. However, the conjunction of several new algorithmic ideas allow a new version of BLAST to achieve improved sensitivity at substantially augmented speed.

In addition to the text-based SRS and ENTREZ search engines described above, the BLAST search was also extensively utilized. The BLAST search enables comparison of a particular sequence of interest with available databanks, leading to identification of similar sequences or relationship with previously described gene families.

The following BLAST programs were employed in this study:

- BLASTN: compares a nucleic acid query sequence with nucleic acid databanks directly.
- BLASTX: compares a translated nucleotide sequence with protein sequence databanks.
- TBLASTX: compares a translated nucleotide sequence with a database of translated nucleotide sequences.
- BLASTP: compares a protein query with a protein database.

The BLAST program provided by NCBI and BLAST 2 maintained by the Swiss Institute of Bioinformatics were extensively used for sequence analysis (Appendix – A).

3.11.13 Elongated pulsed field gradient electrophoresis

DNA extraction was performed according to the methods of Fredriksson-Ahomaa *et al.* (1999) and Niskanen *et al.* (2002). Briefly, a single colony grown on blood agar was inoculated into 5 ml of tryptic soy broth and incubated for 18 h at 30°C. The cells from 2ml of tryptic soy broth were washed once in 5 ml of cold Tris-sodium chloride buffer (PIV) (10mM Tris (pH 7.5), 1 M NaCl) and then resuspended in 750 µl of cold PIV. Next, 0.5 ml of cell suspension was mixed with 0.5 ml of 2% (wt/vol) low-melting-temperature agarose (InCert agarose; BioWhittaker Molecular Applications, Rockland, Maine) and cast in GelSyringe dispensers (New England Biolabs, Beverly, Mass.). The plugs were lysed at 37°C with shaking for 3 h in 2.5ml of lysis solution (6 mM Tris (pH 7.5), 1 M NaCl, 100 mM EDTA (pH 7.5), 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% sodium lauroyl sarcosine, 20µg of RNase/ml, 1 mg of lysozyme/ml) and completed with a 1-h EDTA-sodium lauroyl sarcosine buffer with proteinase K(ESP) (0.5MEDTA (pH 8.0), 10% sodium lauroyl sarcosine, 100 µg of pronase/ml) wash at 50°C. The plugs were stored at 4°C in fresh ESP solution. Before digestion, pronase was inactivated with Protease inhibitor (Pefablock SC - AEBSF; Roche, Mannheim, Germany). Restriction endonuclease digestion was performed according to the manufacturer's instructions. DNA of *Y. enterocolitica* was digested with NotI enzyme (New England Biolabs). *Y. pseudotuberculosis* isolates were digested with both *NotI* and *SpeI* enzymes

separately. The samples were electrophoresed at 12°C through a 1% (wt/vol) agarose gel (SeaKem Gold; FMC Bioproducts) in a 0.5x Tris-borate-EDTA buffer (Amresco, Solon, Ohio) at 200 V by using a Gene Navigator system (Pharmacia, Uppsala, Sweden) with a hexagonal electrode. Interpolation protocols with ranges from 1 to 18 s for 20 h for *NotI* and from 1 to 15 s for 18 h for *SpeI* were used. A mid-range PFGE marker (New England Biolabs) was used for fragment size determination. The gels were stained for 30 min in 1 liter of running buffer containing 50µl of ethidium bromide (10 mg/ml) and photographed under UV light with an Alpha Imager 2000 documentation system (Alpha Innotech, San Leandro, Calif.). By following standard procedures the banding patterns were interpreted visually for the determination of *NotI* and *SpeI* profiles. Profiles were considered to be different when a one-band difference was observed.

CHAPTER FOUR

RESULTS

4.1 PREVALENCE AND DISTRIBUTION OF *Y. ENTEROCOLITICA* AND *Y. PSEUDOTUBERCULOSIS* AMONG HUMAN AND ANIMALS STUDIED IN DIFFERENT STUDY AREAS

Of the three thousand (3000) faecal samples screened from both humans and animals, 750 samples from apparently healthy individuals (humans) yielded no isolate. This means that there was no human carrier of *Yersinia* species detected in these studies. Some of the 750 faecal samples 46/750 (6.2%) collected from diarrhoeic patients yielded pathogens. A high prevalence of yersiniosis was recorded among age groups of 11 and 20 years 17 (2.3%) ($P < 0.05$) while 14 (1.9%) was documented in Bassa Local Government Area (Table 4). An isolate rate of 25 (3.3%) of *Y. pseudotuberculosis* was recorded as compared to 21 (2.8%) of *Y. enterocolitica*. Most of the patients found with *Y. pseudotuberculosis* had previous clinical history of appendectomies. (Tables 5 and 6 show a total percentage of 3.3% prevalence of yersiniosis among women ($P > 0.05$).

Men in Vom recorded high percentage of 1.1% compared to the other study areas such as Bukuru Market, Jos market, Jos East residences, which had 0.5%, 0.1%, and 0.4% respectively. This may be attributed to the fact that most patients screened were livestock attendant working directly with animals. Table 7, shows a total of 50% positive cases of yersiniosis among all the different animal species studied with pigs 271 (18.1%) and sheep 189 (12.6%) ranking highest in Vom 231 (15.4%) Bukuru (12.1%) and Bassa 128 (8.5%).

Of the 4 selected animal species screened in the different study areas, the faecal samples collected from dogs in NVRI Vom residents recorded the highest prevalence of *Yersinia* species (4.9%). *Y. enterocolitica* ranked highest (35.5%) compared to *Y. pseudotuberculosis* (14.5%) in the overall isolation rate specifically with high prevalence in pigs 271 (18.1%) ($P < 0.05$) sheep 189 (12.6%) and poultry 163 (10.9%) (Table 8).

Table 9 show the prevalence of *Y. enterocolitica* and *Y. pseudotuberculosis* in the study areas as 231(15.4%) in Vom, 182 (12.1%) in Bukuru, and 128 (8.5%) in Bassa. The occurrence of these 2 organisms as recorded in this study shows that the organisms are truly principal agents of yersiniosis.

Table 10 show the seropositive, biotypes and genotypes of *Y. enterocolitica* isolates studied. All isolates gave positive reaction to typing sera 0:9. The different biotypes and genotype observed are as shown on the Table.

Tables 11 and 12 show the seasonal distribution of *Y. enterocolitica* and *Y. pseudotuberculosis* in human and animals during the wet and dry periods ($P > 0.05$).

Table 3: Differential biochemical reactions for identification of *Y. enterocolitica* and *Y. pseudotuberculosis* by API 20E

BIOCHEMICAL TEST	YE (99.3 % *)	YP (99.7% *)
Betagalactosidase (ONPG)	-	-
Arginine dihydrolase (ADH)	-	-
Lysine decarboxylase (LDC)	-	-
Ornithine decarboxylase (ODC)	+	-
Citrate utilization (CIT)	-	-
Hydrogen sulphate (H ₂ S)	-	-
Urease (URE)	+	+
Tryptophane deaminase (TDA)	-	-
Indole (IND) production	+	-
Voges proskauer (VP)	-	-
Gelatine Hydrolysis (GEL)	-	-
Glucose (GLU)	+	+
Mannitol (MAN)	+	+
Inositol (INO)	+	-
Sorbitol (SOR)	+	+
Rhamnose (RHA)	-	+
Sucrose (SAC)	+	-
Melibiose (MEL)	-	+
Amydaline (AMY)	+	-
Arabinose (ARA)	+	+
Oxidase (OXD)	-	-

YE - *Y. enterocolitica*

YP - *Y. pseudotuberculosis*

API - Appareils et procede d` identification

* - Percentge confirmation using API software

Table 4: Distribution of yersiniosis among different age groups of diarrhoeic subjects.

Study Area	Age group in years						Total (%)
	0-10	11-20	21-30	31-40	41-50	51-Above	
Vom	3/26 (0.4)	4/70 (0.5)	1/11 (0.1)	2/25 (0.3)	2/15 (0.3)	0/3 (0)	12/150 (1.6)
Bukuru	0/20 (0)	3/20 (0.4)	2/25 (0.3)	1/13 (0.1)	0/15 (0)	1/47 (0.1)	7/150 (0.9)
Jos	0/15 (0)	2/50 (0.3)	1/15 (0.1)	0/5 (0)	1/20 (0.1)	0/45 (0)	4/150 (0.5)
Jos East	2/8 (0.3)	3/40 (0.4)	2/50 (0.3)	1/30 (0.1)	1/20 (0.1)	0/2 (0)	9/150 (1.2)
Bassa	2/10 (0.3)	5/60 (0.7)	6/40 (0.8)	1/27 (0.1)	0/9 (0)	0/4 (0)	14/150 (1.9)
Total	7/79 (0.9)	17/240 (2.3)	12/151 (1.3)	5/100 (0.7)	4/79 (0.5)	1/101 (0.1)	46/750 (6.2 %)

Since $P < 0.05$, there is a statistically significant difference amongst the medians at the 95% confidence level.

Statistical package : Microsoft package for Social Sciences version 14 program, 2006.

Statistical procedure: One way ANOVA

Table 5: Frequency of occurrence of *Y. enterocolitica* (YE) and *Y. pseudotuberculosis* (YP) among diarrhoeic patients studied

Study Area	YE (%)	YP (%)	Total (%)
Vom	5 (0.7)	7 (0.9)	12/150 (1.6)
Bukuru	3 (0.4)	4 (0.5)	7/150 (0.9)
Jos	3 (0.4)	1 (0.1)	4/150 (0.5)
Jos East	2 (0.3)	7 (0.9)	9/150 (1.2)
Bassa	8 (1.1)	6 (0.8)	14/150 (1.9)
Total	21 (2.8)	25 (3.3)	46/750 (6.1)

Since $P > 0.05$, there is not a statistically significant difference amongst the medians at the 95% confidence level.

Statistical package: Microsoft package for Social Sciences version 14 program, 2006.

Statistical procedure: Student T- test

Table 6: Distribution of yersiniosis among sex groups of diarrhoeic patients

Study Area	Sex		Total (%)
	Male (%)	Female (%)	
Vom	8/70 (1.1)	4/80 (0.5)	12/150 (1.6)
Bukuru	4/62 (0.5)	3/88 (0.4)	7/150 (0.9)
Jos	1/65 (0.1)	3/85 (0.4)	4/150 (0.5)
Jos East	3/68 (0.4)	6/82 (0.8)	9/150 (1.2)
Bassa	5/67 (0.7)	9/83 (1.2)	14/150 (1.9)
Total	21/332 (2.8)	25/418 (3.3)	46/750 (6.1)

Since $P > 0.05$, there is not a statistically significant difference amongst the medians at the 95% confidence level.

Statistical package: Microsoft package for Social Sciences version 14 program, 2006.

Statistical procedure: Student T- test

Table 7: Prevalence of yersiniosis among different animals studied

Study Area	Number positive in selected animal species				Total
	Pigs (%)	Sheep (%)	Poultry (%)	Dogs (%)	
Vom	28/75 (1.9)	70/75 (4.7)	60/75 (4.0)	73/75 (4.9)	231/300 (15.4)
Bukuru	52/75 (3.5)	60/75 (4.0)	55/75 (3.7)	15/75 (1.0)	182/300 (12.1)
Jos	58/75 (3.9)	35/75 (2.3)	10/75 (0.7)	12/75 (0.8)	115/300 (7.7)
Jos East	65/75 (4.5)	11/75 (0.7)	16/75 (1)	1/75 (0.07)	93/300 (6.2)
Bassa	68/75 (4.5)	13/75 (0.9)	22/75 (1.5)	25/75 (1.7)	128/300 (8.5)
Total	271/375 (18.1)	189/375 (12.6)	163/375 (10.9)	126/375 (8.4)	749/1500 (49.9)

(Equal number of samples collected from each animal)

Since $P < 0.05$, there is a statistically significant difference amongst the medians at the 95% confidence level.

Statistical package : Microsoft package for Social Sciences version 14 program, (2006).

Statistical procedure: Student T- test

Table 8: Distribution of *Y. enterocolitica* and *Y. pseudotuberculosis* among different animals studied.

Animal	Number sampled	Positive YE isolate	Positive YP isolate	Total (%)
Pigs	375	251 (16.7)	20 (1.3)	271 (18.1)
Sheep	375	100 (6.7)	89 (5.9)	189 (12.6)
Poultry	375	115 (7.7)	11 (0.7)	163 (10.9)
Dogs	375	115 (7.7)	11 (0.7)	126 (8.4)
Total	1500	532 (35.4)	217 (14.5)	749 (49.9 %)

(YE:*Yersinia enterocolitica* YP:*Yersinia pseudotuberculosis*)

Since $P < 0.05$, there is a statistically significant difference amongst the medians at the 95% confidence level.

Statistical package : Microsoft package for Social Sciences version 14 program, 2006.

Statistical procedure: Student T- test

Table 9: Prevalence of *Y. enterocolitica* and *Y. pseudotuberculosis* among animals in the study areas

Study area	Number of samples examined	Positive YE (%)	Positive Yp (%)	Total (%)
Vom	300	175 (11.7)	56 (3.7)	231/300 (15.4)
Bukuru	300	64 (4.3)	118 (7.9)	182/300 (12.1)
Jos	300	42 (2.8)	73 (4.9)	115/300 (7.7)
Jos East	300	60 (4.0)	33 (2.2)	93/300 (6.2)
Bassa	300	90 (6.0)	38 (2.5)	128/300 (8.5)
Total	1500	431 (28.7)	318 (21.2)	749/1500 (49.9).

Since $P < 0.05$, there is a statistically significant difference amongst the medians at the 95% confidence level.

Statistical package : Microsoft package for Social Sciences version 14 program, 2006.

Statistical procedure: Student T- test

Table 10: Bio/serotypes and genotypes of *Y. enterocolitica* isolates

No of Isolate	Origin	Serogroup	Biotype	Genotype	
				P/1285	P/1143
1 – 39	Human Faeces	0:9	1A	1	1
40 – 48	Human Faeces	0:9	1B	1	1
1 – 221	Pigs	0:9	1A	1	1
222 – 271	Pigs	0:9	1A	1	1
1 – 189	Sheep	0:9	1A	1	1
1 – 163	Poultry	0:9	1A	1	1
1 – 126	Dogs	0:9	1A	1	1
French Control	A Human Faeces	0:9	1B	2	2
“	B Human Blood	0:9	1B	2	2
“	C Human Faeces	0:9	1A	3	3
“	D Human Faeces	0:9	1A	4	4
“	E Human Blood	0:9	1A	5	5

Table 11: Seasonal distribution of *Y. enterocolitica* and *Y. pseudotuberculosis* in humans

Season	Sample size	Positive (%)		Total (%)
		YE	YP	
Dry	350	8 (1.1)	16 (2.1)	3.2
Wet	350	17 (2.3)	5 (0.7)	2.9
Total	750	25 (3.3)	21 (2.8)	6.1

(YE: *Yersinia enterocolitica* YP: *Yersinia pseudotuberculosis*)

Since $P > 0.05$, there is not a statistically significant difference amongst the medians at the 95% confidence level.

Statistical package : Microsoft package for Social Sciences version 14 program, 2006.

Statistical procedure: Student T- test

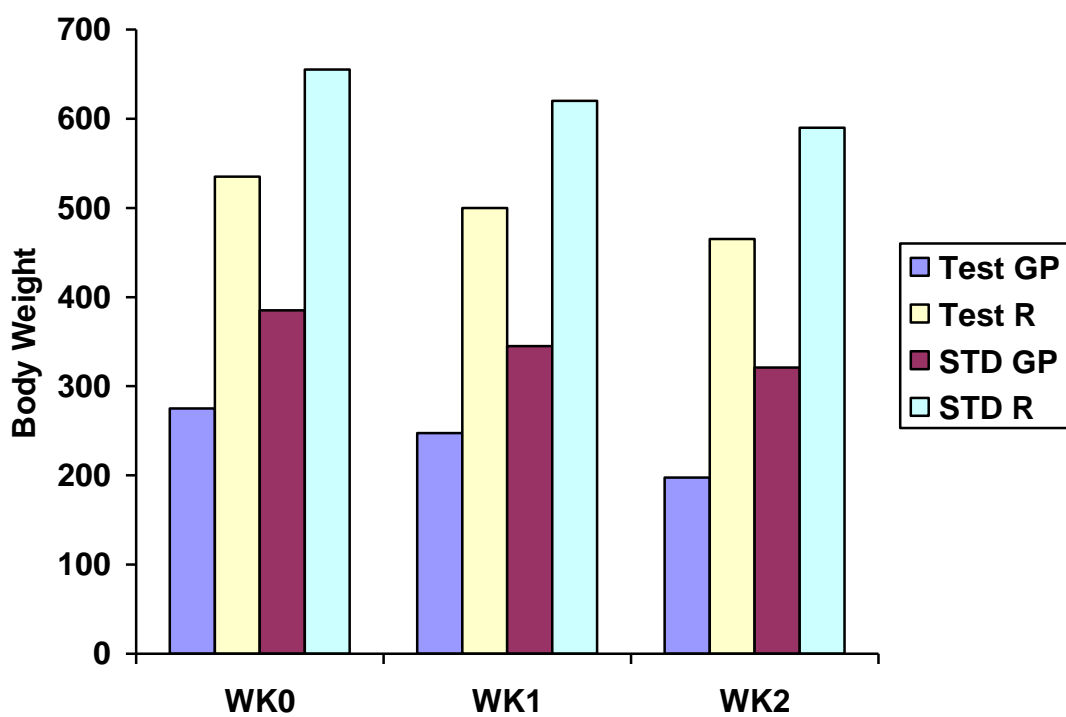
Table12: Seasonal Distribution of *Y. enterocolitica* and *Y. pseudotuberculosis* in animals

Season	Sample Size	Positive (%)		Total (%)
		YE	YP	
Dry	750	202(13.5)	100(6.7)	20.1
Wet	750	330(20.2)	117(7.8)	29.8
Total	1500	532(35.5)	217(14.5)	49.9

Since $P > 0.05$, there is not a statistically significant difference amongst the medians at the 95% confidence level.

Statistical package : Microsoft package for Social Sciences version 14 program, 2006.

Statistical procedure: Student T- test



Test GP - *Test Guinea pig*
Test R - *Test Rabbit*
STD GP - *Standard/Control Guinea pig*
STD R - *Standard/Control Rabbit*

Figure 4: Variations in body weight of animals inoculated with *Yersinia pseudotuberculosis*

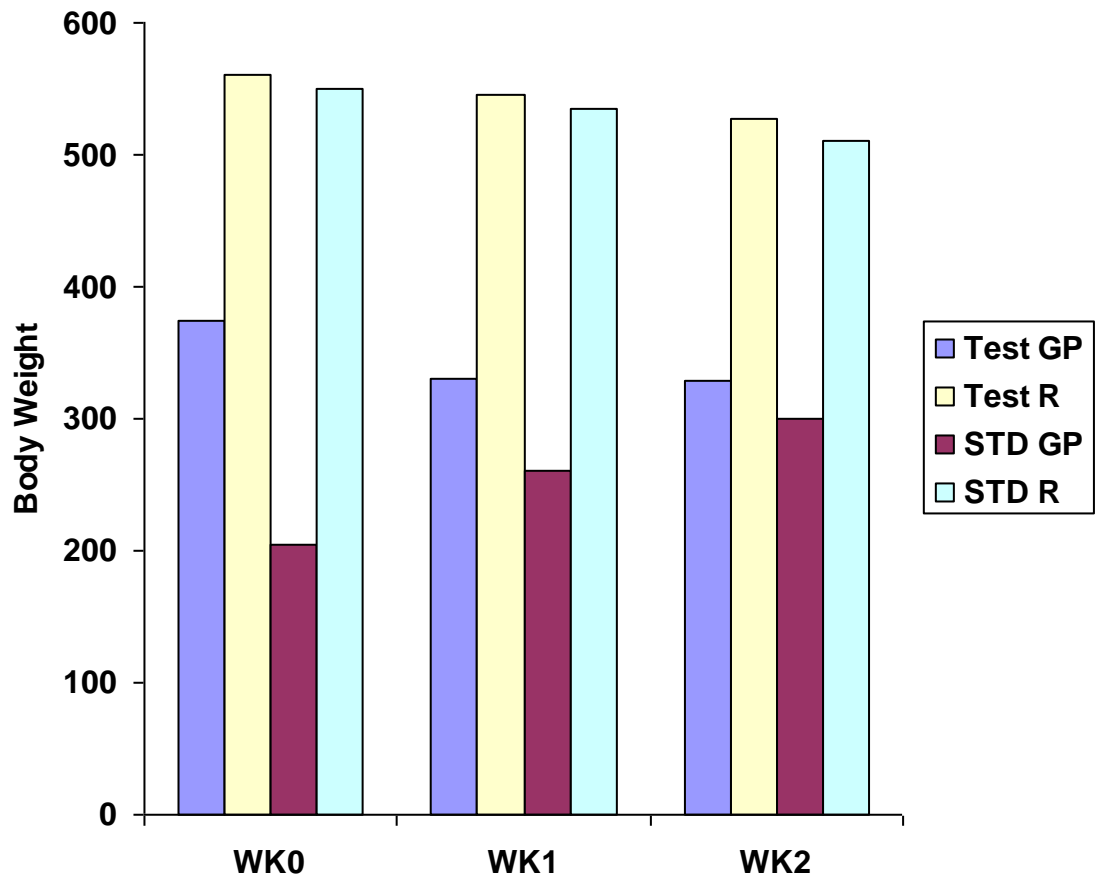


Figure 5: Variations in body weight of animals inoculated with *Yersinia enterocolitica*

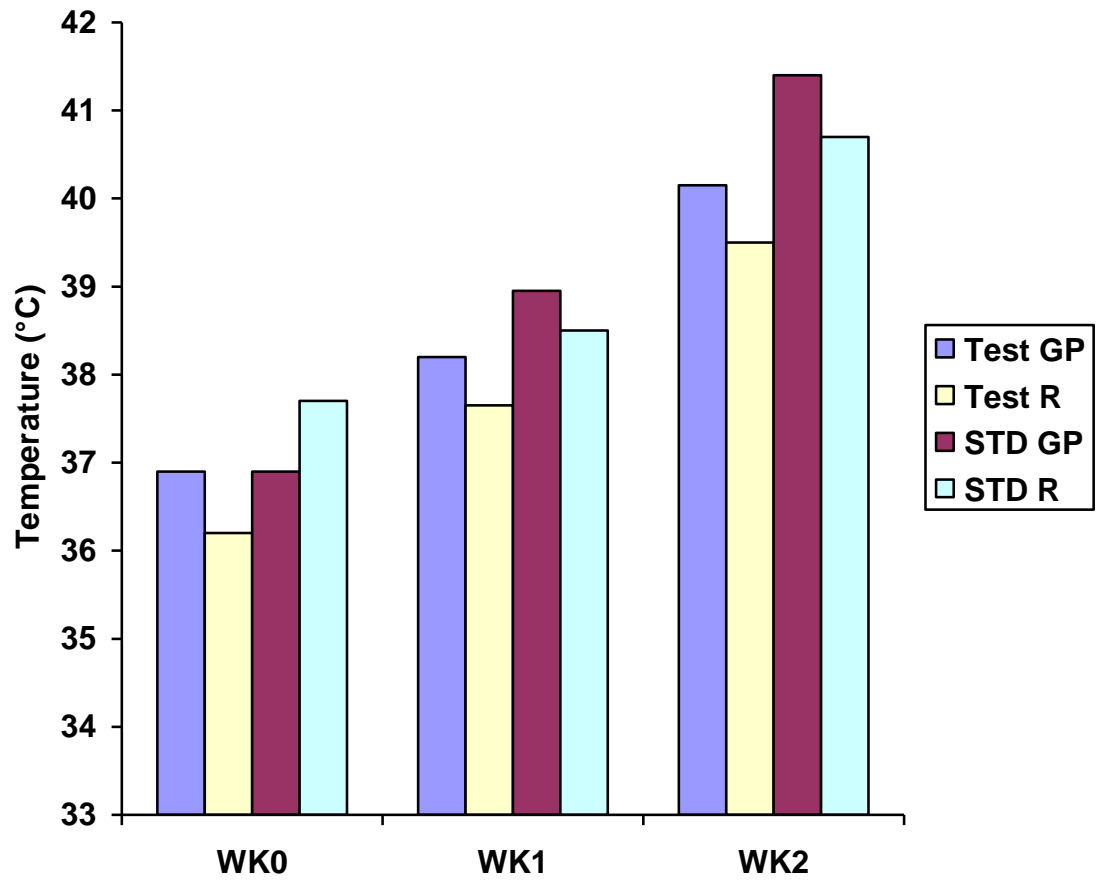


Figure 6: Body temperature variations in animals inoculated with *Y. pseudotuberculosis*

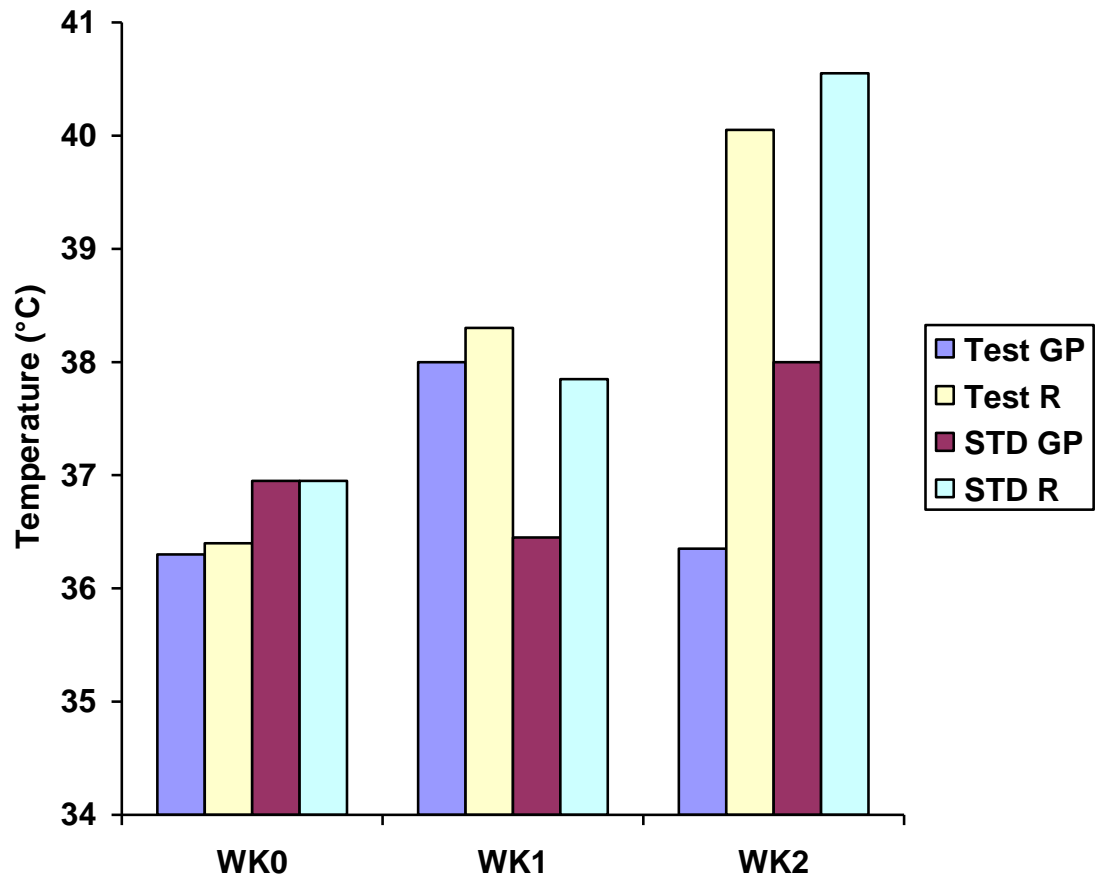


Figure 7: Body temperature variations in animals inoculated with *Y. enterocolitica* isolated from Vom (animals).

4.2 PATHOGENCITY TEST

4.2.1 Host response to *Yersinia pseudotuberculosis* infection

Six days after inoculation of animals with the bacterial broth culture, signs of depression and isolation, progressive ruffling of the fur as well as steady loss of weight leading to gross emaciation were observed. Body temperatures of animals were also observed to increase. The faecal samples showed the presence of large quantities of the organism. The experimental rabbits died on the eleventh day of post inoculation. When post mortem of the inoculated animals was conducted, severe necrotic lesions were seen on the whole matrix of the liver with some coalescing with consequent hepatic hypertrophy. On close examination, black spotted necrotic lesions were seen on heart tissues of the dead animals. Enlarged lymph node especially the mesenteric, pigmented lungs and renal hypertrophy were also observed. There were no inflammatory symptoms observed on the gastrointestinal tract.

The organisms were also recovered from culture of the heart blood made on CIN agar. Sera samples tested for the presence of corresponding antibodies gave visible agglutination.

4.2.2 Host response to *Yersinia enterocolitica* infection

At the sixth day of post inoculation, the rabbits showed signs of depression and isolation. Ruffling of the fur was observed. Profuse diarrhoea was noticed at between 9th and 11th days. Acute lateral paralysis was noted from the 15th day. Body temperatures were also seen to have increased. The organisms were recovered in large numbers from cultures of faecal samples. On the 22nd day of post inoculation,

the animals were sacrificed and prominent haemorrhagic and distended blood vessels draining the gut wall were significantly noted suggesting gastroenteritis. The faecal content of the gut wall had a watery consistency. The heart, lungs, liver, kidney and lymph nodes were neither enlarged nor pigmented.

Guinea pigs showed much weight loss than rabbits infected with *Y. pseudotuberculosis* (Fig. 4) while those infected with *Y. enterocolitica* did not show any significant weight loss (Fig. 5).

The serum of the infected animals reacted with the organisms. However, the guinea pigs did not show any significant clinical condition. The Rabbits however showed a rapid rise in temperature relative to low temperature in guinea pigs thereby constituting a level of immunity to the organism (Fig. 6 & 7). Studies in the rabbit model appeared to have further elucidated the clinical roles played by the isolates in the illnesses of the patients from whom they were isolated.

4.3 HISTOPATHOLOGY

All tissues analysed from the infected animals demonstrated polarized profile and inflammatory cell influx throughout the course of the infection. These were detected by the application of H & E staining technique. Details are as shown on plates 2 - 5 below.



Plate 1: Necrotic ↑ and black spotted lesions ↓ on the visceral organs of an infected rabbit with *Yersinia enterocolitica*.

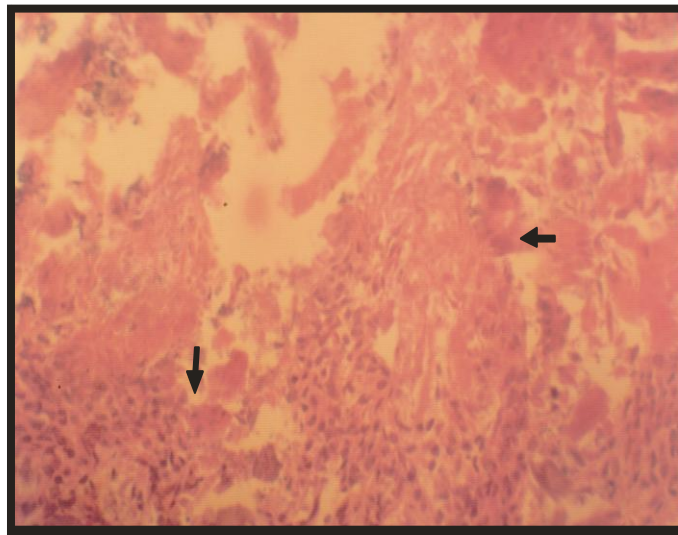


Plate 2: Histological tissue of rabbit (liver)infected with *Y. enterocolitica* showing pyloric gland dislodged (↓) with hypnotic nodules (←) (H&E stained).

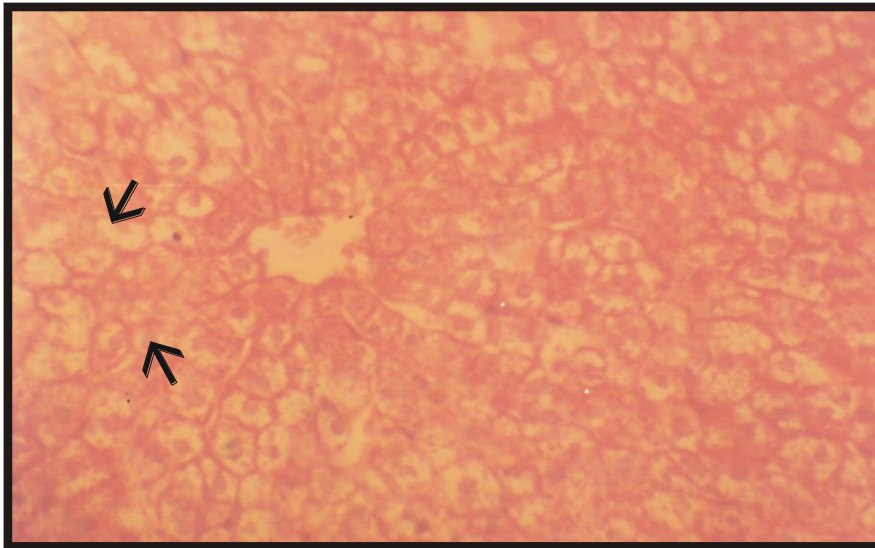


Plate 3: Histological tissue of rabbit (Liver) infected with *Y. enterocolitica* showing liver plate staining (pink disease ↙) with clear cytoplasmic appearance (↗). (H & E Stained)

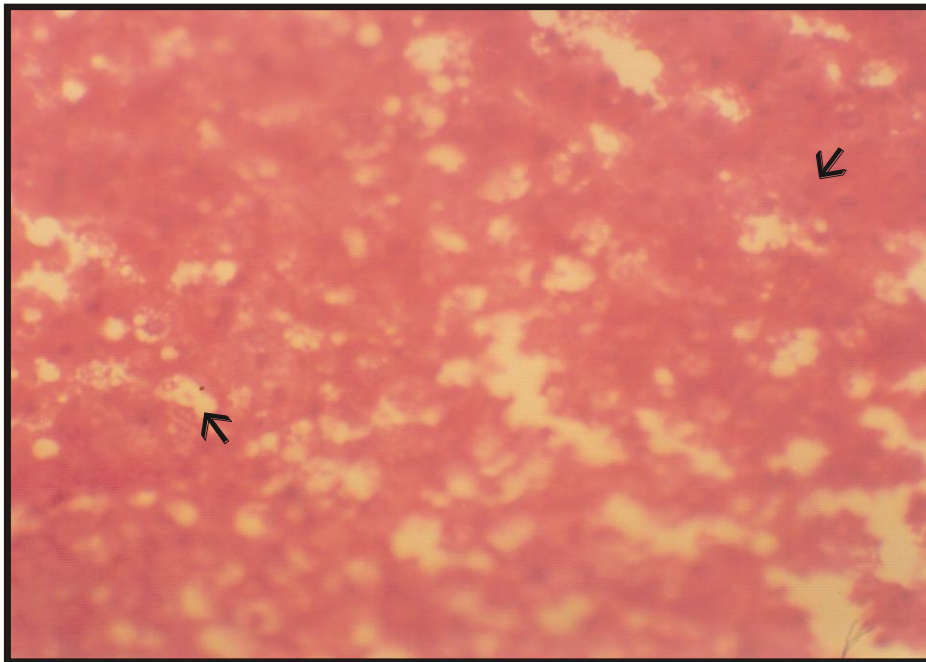


Plate 4: Histological tissue (Liver) of guinea pig infected with *Y. enterocolitica* showing highly vacuolated with less defined cell wall. Some cells annucleated while others perinucleated with central vein filled with colloids (H & E Stained)

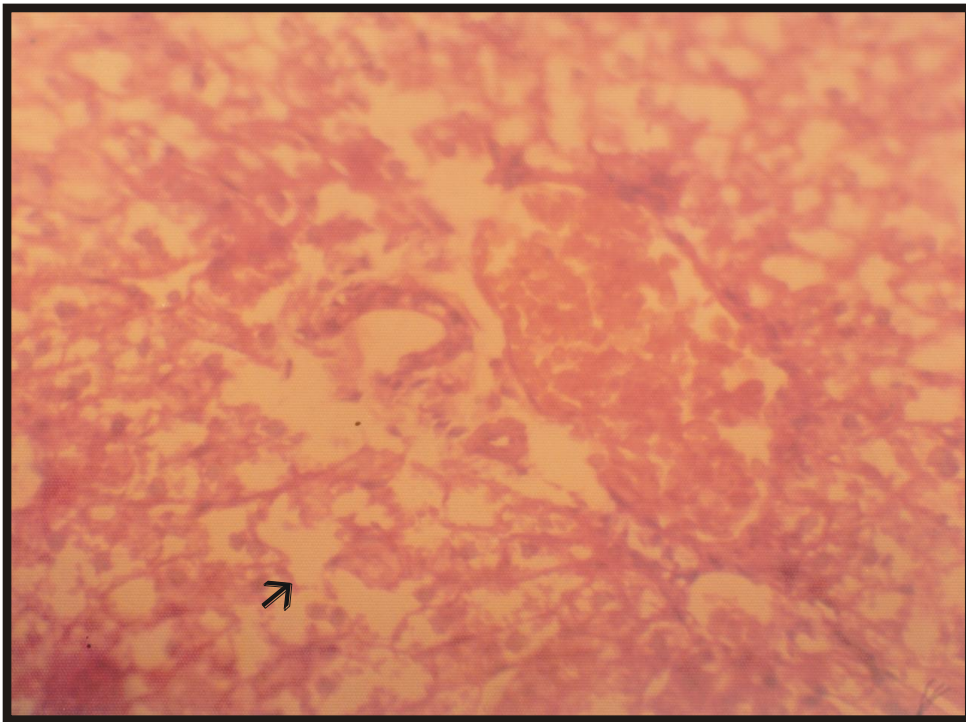


Plate 5: Histological tissue rabbit (liver) infected with *Y. enterocolitica* showing warty cytoplasm with lobular demarcation. (↗)

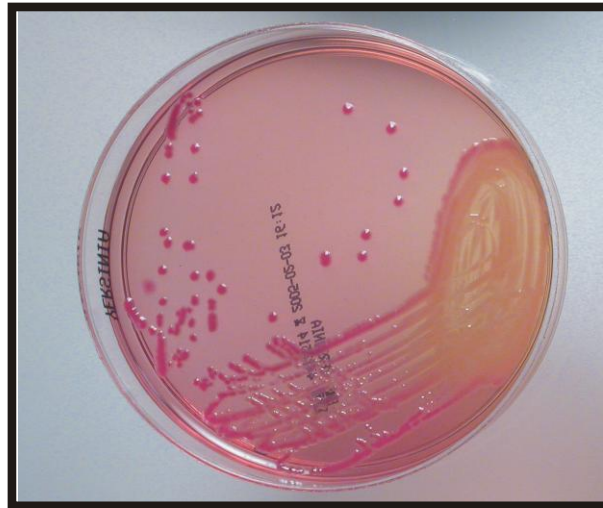


Plate 6: Pure culture of *Y. enterocolitica* on CIN Agar
(Characteristic: Bull's eye appearance)

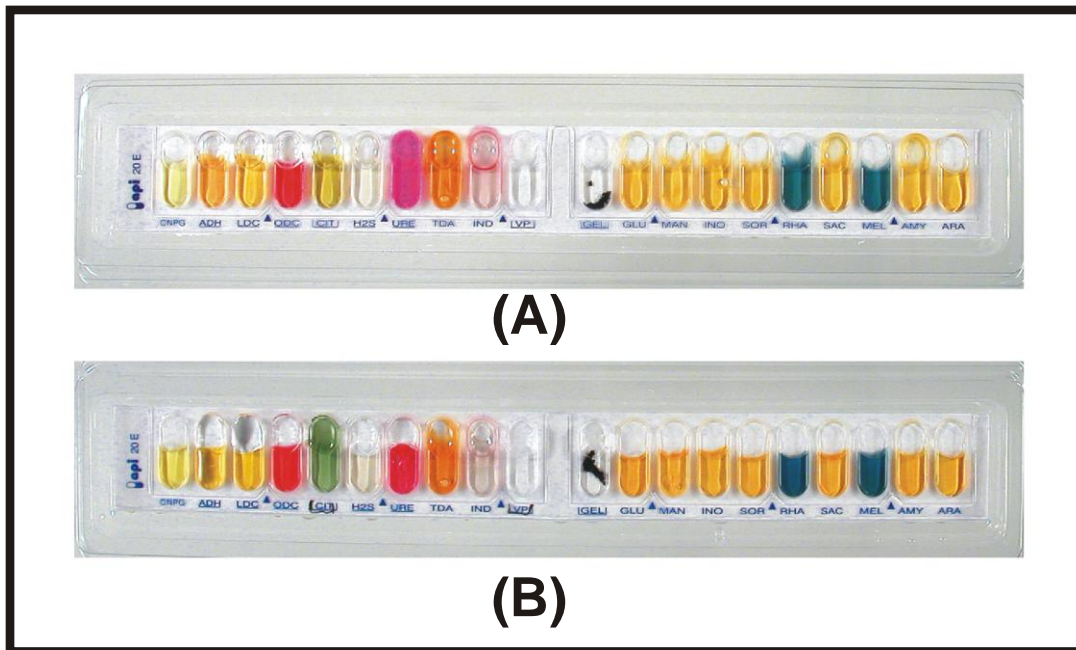


Plate 7: Phenotypic characterization of control organism (A) and test (B) strains of *Yersinia enterocolitica* by API - 20E

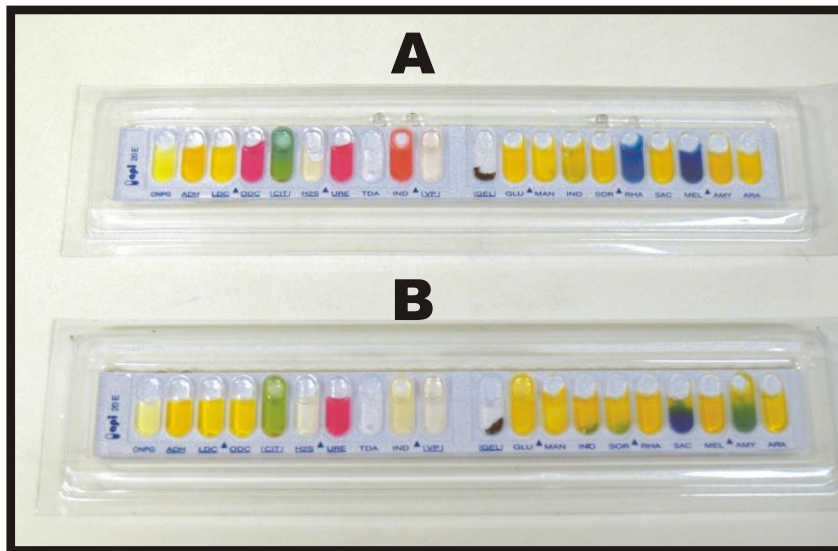
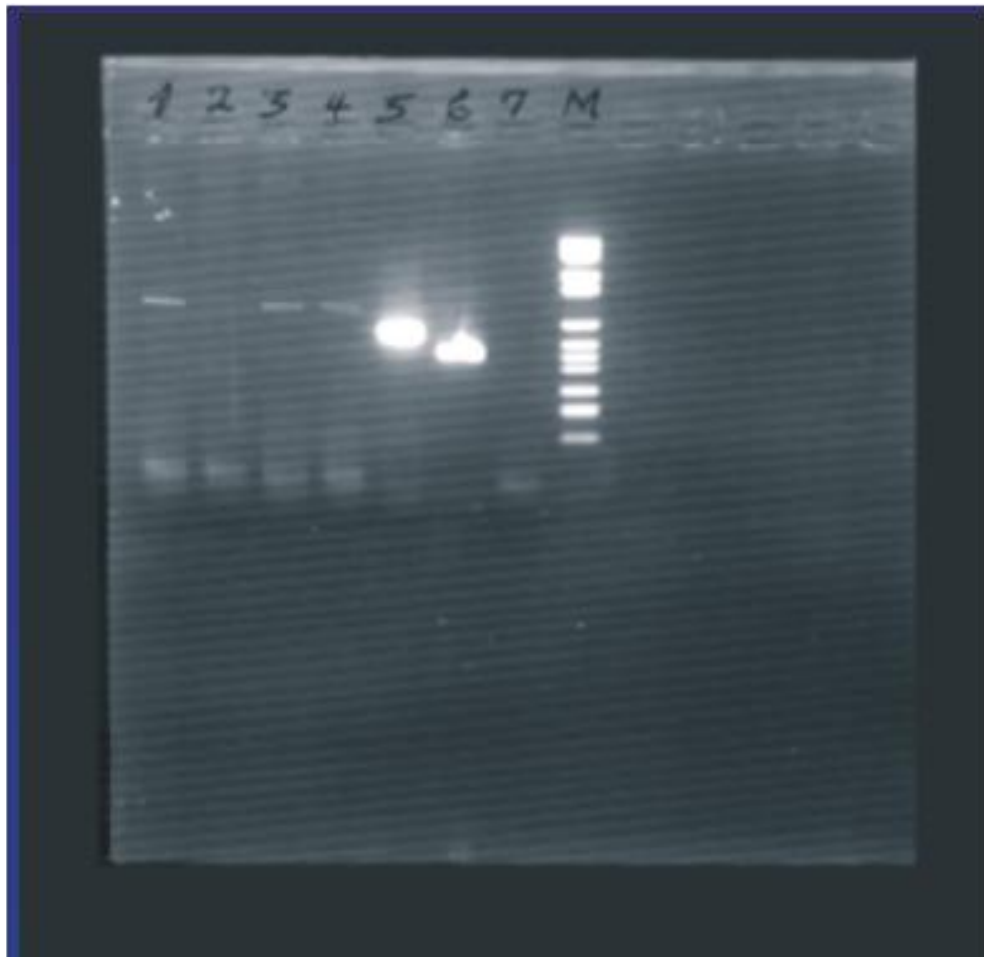


Plate 8: Phenotypic characterization of *Y. enterocolitica* (A) & *Y. pseudotuberculosis* (B) by API 20E



Labels:	1-4	-	<i>Yersinia enterocolitica</i>
	5	-	<i>Yersinia pseudotuberculosis</i>
	6	-	<i>Yersinia pestis</i>
	7	-	Negative control
	M	-	Marker

Plate 9: PCR for the genus *Yersinia* indicating genetic variability.

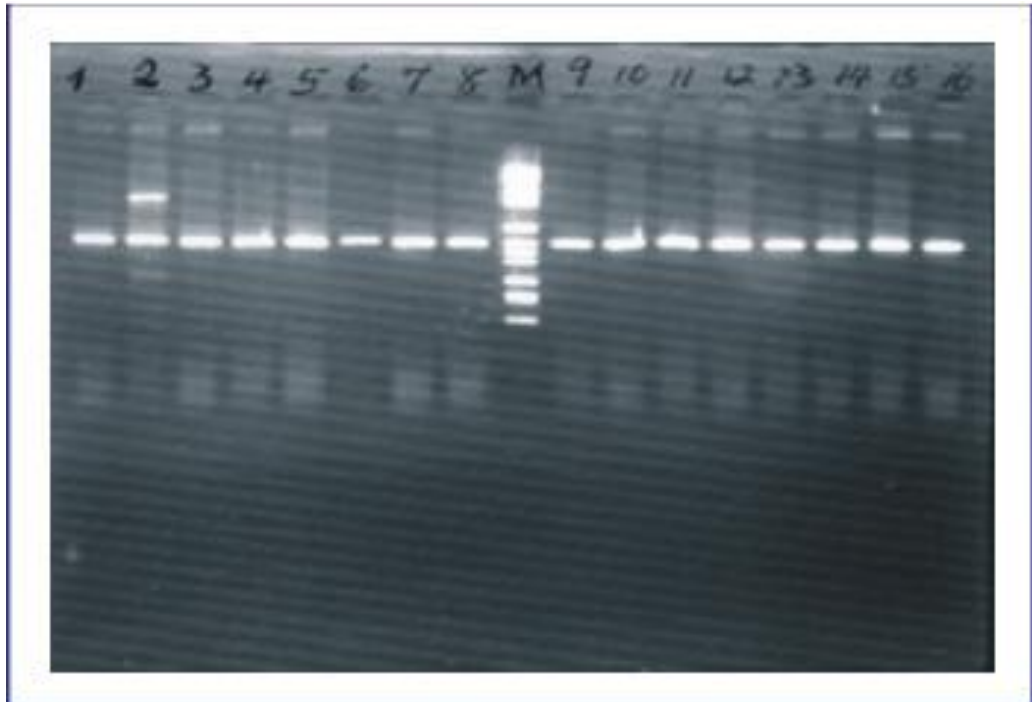


Plate 10: PCR for *Y. enterocolitica* strains (1-16) indicating no genetic variability

4.4 MOLECULAR TYPING BY POLYMERASE CHAIN REACTION (PCR)

Of the 10 pairs of primers used for this study, only five pairs were found reactive. The most reactive of the primers (YP 1285) was used for PCR/Sequence analysis.

The genotypes for all isolates collected from the different study area in Jos, Plateau State of Nigeria were found to be same. Some French isolates were used as controls and in comparison were found to be different from all isolates from Nigeria by an indication of markers of mutation. Differences in the genetic composition of the organisms were observed in the 2 countries by this mutation at position 320 where **A** was replaced by **G** as below (Table 13).

Surprisingly, three of the French isolates (control organisms) originally isolated and identified as *Y. enterocolitica*, used in this study with same primers and sequence analysis showed only 51% similarity with standard reference *Y. enterocolitica* at the Genbank using MULTALIN multiple alignment.

A second control check using 16srRNA (Universal Primers) indicated 98% similarity.

Primer 1285

	310	320	330	340	350	360
1yp1285	CTGAACCAGCATT	TTTTAGATTAAAAAGTAAACTGAAAGAGATCGGGTTAGGTGGTGAAG				
2yp1285	CTGAACCAGCATT	TTTTAGATTAAAAAGTAAACTGAAAGAGATCGGGTTAGGTGGTGAAG				
3YP1285	CTGAACCAGCATT	TTTTAGATTAAAAAGTAAACTGAAAGAGATCGGGTTAGGTGGTGAAG				
5YP1285	CTGAACCAGCATT	TTTTAGATTAAAAAGTAAACTGAAAGAGATCGGGTTAGGTGGTGAAG				
4YP1285	CTGAACCAGCATT	TTTTAGATTAAAAAGTAAACTGAAAGAGATCGGGTTAGGTGGTGAAG				
55yp1285	CTGAACCAGCATT	TTTTAGGTTAAAAAGTAAACTGAAAGAGATCGGGTTAGGTGGTGAAG				
57yp1285	CTGAACCAGCATT	TTTTAGGTTAAAAAGTAAACTGAAAGAGATCGGGTTAGGTGGTGAAG				
60YP1285	CTGAACCAGCATT	TTTTAGGTTAAAAAGTAAACTGAAAGAGATCGGGTTAGGTGGTGAAG				
61YP1285	CTGAACCAGCATT	TTTTAGGTTAAAAAGTAAACTGAAAGAGATCGGGTTAGGTGGTGAAG				

Plate 11: Differences in genetic composition of Nigerian and French strains of *Y. enterocolitica*

4.5 GENOTYPIC VARIABILITY OF *YERSINIA ENTEROCOLITICA* AND *YERSINIA PSEUDOTUBERCULOSIS* STRAINS BY ENLONGATED PULSED FIELD GRADIENT ELECTROPHORESIS (PFGE)

The genotypic profile of *Y. enterocolitica* (n = 58) and *Y. pseudotuberculosis* (n = 22) isolated from human, animal, food and environmental samples in Jos metropolis were assessed by the enlongated PFGE (Table 13). The isolates were typed with *NotI* and *SPEI* enzymes. The *NotI* enzymes were more sensitive than the *SPEI* enzymes as the discriminatory index increased from about 30 to 90%. Hence the isolates of *Y. enterocolitica* yielded 4 profiles with *NotI* enzymes (Plate 12 and Table 14), indicating 4 new variants of *Yersinia enterocolitica* strains. Strains **i/iv** were found in human, pig, lettuce and fermented cow milk samples. Strain **i** was found widely distributed among human and animal populations and their products in Jos metropolis. Strain **iii** was uniquely found only among sheep. *Yersinia pseudotuberculosis* gave 7 profiles with the *NotI* enzymes meaning 5 different strains (Plate 13 and Table 15). Strain **Na** was found widely distributed in human, sheep, fermented cow milk and fish, while **Nb** strains were only found in pigs.

Table 13: Distribution of pathogenic *Y. enterocolitica* (YE) and *Y. pseudotuberculosis* (YP) strains screened by elongated PFGE.

Origin	Number of strains	Number of YE(%)	Number of YP(%)
Human	38	(84.2)32^a	(15.8)6^a
Pig	20	(75)15	(25)5
Sheep	3	(66.7)2	(33.3)1
Fermented milk	11	(45.5)5	(54.5)6
Lettuce	2	(100)2	-
Fish	6	(33.3)2	(66.7)4
Total	80	(72.5)58	(27.5)22

(a = Number of strains)

Since $P < 0.05$, there is a statistically significant difference amongst the medians at the 95% confidence level.

Statistical package : Microsoft package for Social Sciences version 14 program, 2006.

Statistical procedure: One way ANOVA

Table 14: Phenotypic and genotypic characterization of pathogenic *Y. enterocolitica* strains

Origin	Number of strain	Biotype	Serotype	PFGE (<i>Not 1 Profile</i>)
Human	32	2(31) 4(1)^a	O:2(3) O:9(29)^a	Ni
Pigs	15	2(14) 4(1)	O:2(6) O:3(1)	Ni/ii
Sheep	2	2(2)	O:3(1) O:9(1)	Niii
Fermented milk	5	4(5)	O:1/O:2(2) O:9(3)	Ni/ii
Lettuce	2	2(1) 4(1)	O:2(1) O:9(1)	Niv
Fish	2	2(2)	O:9(2)	Ni/iv

(a = Number of strains)

Since $P > 0.05$, there is not a statistically significant difference amongst the medians at the 95% confidence level.

Statistical package : Microsoft package for Social Sciences version 14 program, 2006.

Statistical procedure: Student T- test

Table 15: Serotypes and genotypic characterization of pathogenic *Y. pseudotuberculosis* strains

Origin	Number positive	Serotype	PFGE (<i>NOt 1 Profile</i>)
Human	6	O:1	Na
Pigs	5	O:1	Nb
Sheeps	1	O:1	Na
Fermented milk	6	O:1	Na
Lettuce	0	-	-
Fish	4	O:1	Na

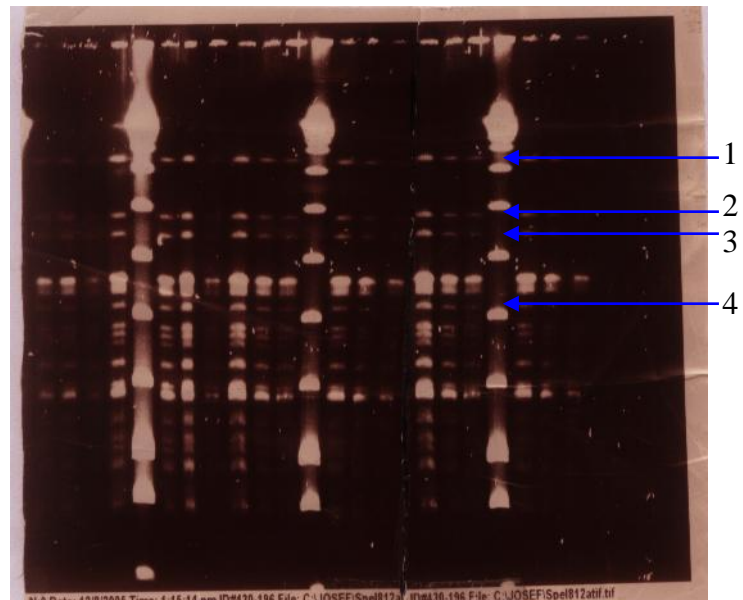


Plate12: Four variable profiles of *Y. enterocolitica* with PFGE (*Not I* enzymes)
(Note: 1 - 4 above are the separate bands representing the variations in the profiles)

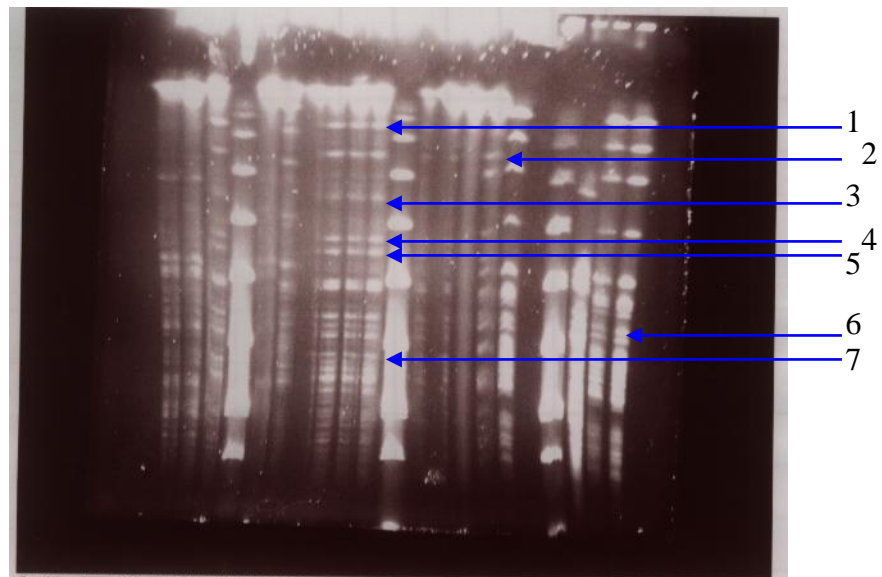


Plate 13: Seven variable profiles of *Y. pseudotuberculosis* with PFGE (*Not I* enzymes)
(Note:1 - 7 above are the separate bands representing the variations in the profiles)

CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

5.1 DISCUSSION

Yersinia enterocolitica and *Y. pseudotuberculosis* were considered rare micro-organisms for a long time, but during the last two decades it has been isolated all over the world from animals, raw food materials, environments, water and human beings (Slee and Skilback, 1992; Shepel *et al*, 2001; Fredriksson-Ahomaa and Korkeala, 2003; Ray *et al*, 2004; Okwori *et al*, 2007). This is similar to the present study. Complications (due to yersiniosis) such as bacteraemia, needless or mistaken surgery and even death have been reported world wide but description of observed clinical manifestation or patho-anatomical changes are sparse (Tachet *et al*, 1985; Schiemann, 1989).

The results obtained in the current studies supports the findings of Fredriksson-Ahommaa and Korkeala (2003) who documentd the low occurrence of pathogenic *Y. enterocolitica* in clinical, foods and environmental samples as could have been due to methodological problems. Hence the use of improved methods, selective culture media (CIN) and careful handling of clinical samples in this investigation has revealed the occurrence of *Y. enterocolitica* in this part of the world. This is in disagreement with the claims of Murray *et al*, (2003) who reported the rarity of *Y. enterocolitica* and *Y. pseudotuberculosis* in African countries.

5.2 APPARENTLY HEALTHY INDIVIDUALS

This investigation did not incriminate *Y. enterocolitica* and *Y.*

pseudotuberculosis amongst apparently healthy subjects, as there were no isolates made from the screened samples. This goes to explain that there were no carriers of the pathogen among the subjects investigated. This finding is in support of claims by Lal *et al*, (2003) who in their studies in India recorded no isolates from apparently healthy 170 control subjects. Similarly, these findings does not support the claims by some scholars in Brazil (Nunes and Ricciardi, 1986; Warnken *et al*, 1987, Ceccarelli *et al*, 1990; Tassinari *et al*, 1994) who have frequently isolated *Y. enterocolitica* from both healthy and sick humans and animals in the state of Sao Paulo, Brazil.

5.3 DIARRHOEIC PATIENTS

The results of this study on human diarrhoeic patients indicates that not all diarrhoea cases are due to *Y. enterocolitica* as less than 25% of the faecal samples screened gave positive results. This finding is similar to the work of Lal *et al*, (2003), who in India made a 2.05% isolation of *Y. enterocolitica* from 2000 diarrhoeic samples investigated. The rate of isolation of this organism from diarrhoeae stools from several other countries has been reported to be 2-5% (Lal *et al*, 2003). Similar findings have been reported in Nigeria (Agbonlahor *et al*, 1983; Anyawu, 1995). Onyemelukwe (1993) in Enugu, Nigeria, isolated *Yersinia enterocolitica* from a number of children between the ages of 0-12 years. Infants may acquire these pathogens directly by eating contaminated food or indirectly via the contamination of objects (e.g. bottles, toys and care provider's hands) that infants subsequently place in their mouths.

In some parts of the world, raw ground pork is commonly given to babies

without teeth, but this practice is not known to be common in the USA (Tauxe *et al*, 1987). The distribution *Y. enterocolitica* among age groups and its isolation rate recorded among diarrhoeic patients in this study is quite comparable to that reported from other part of the world (Singh *et al*, 1983; Varghese *et al*, 1984; Ram *et al*, 1987) where *Y. enterocolitica* is considered a major gastro-intestinal pathogen (Ostroff, 1995). This correlates with a study by Hoogkamp-Konstainje and Stolk-Engelaar (1995) who found 109 (42.1%) patients (above 25 years of age) out of 350 infected with *Y. enterocolitica*.

Y. enterocolitica usually causes a syndrome of gastroenteritis with diarrhoea as the predominantly presenting symptom (Cover and Albert, 1989). Although the clinical information obtained in our surveillance system is limited, this study is in agreement with the report of Brofin, (2002) that *Y. enterocolitica* and *Y. pseudotuberculosis* are causes of pseudoappendicitis and sometimes monoarthritis after a diarrhoeal illness (Ray *et al*, 2004) as most of the diarrhoea cases studied had history of appendicectomy in the past. The low isolation rate (2.8%) of *Y. pseudotuberculosis* as compared with *Y. enterocolitica* (3.3%) is in agreement with the work of Bottone, (1999) who reported that *Y. enterocolitica* is the most common cause of human yersiniosis globally. The high incidence of yersiniosis recorded among female diarrhoeic patients in BASSA (1.9%) and Vom (1.6%) as against all other study areas buttressed the fact that the organism thrives well in cold climatic region as these 2 places (BASSA and Vom) seem to be the coldest part of Jos. The high prevalence of *Y. enterocolitica* among female patients in Bassa, Vom, and Jos East may be due to unkept residential habit at the outskirts of Jos city, as they often

engaged in small poultry and piggery farms within their premises. Although they do not keep these animals in larger numbers than other study areas, yet they often practice the semi-intensive and extensive system of farming. In these practices the animals are left to roam about in the farmland, human dwellings (premises) and village joints. This exposes inhabitants to the animal droppings, urine and saliva. The women in these areas seem to have recorded highest number of isolates simply because of their enterprising nature compared to their male counterparts. They are also involved in the preparation of pepper-flavoured meat dish popularly called “Kayanchiki” (intestines of animals) and could get infected easily while preparing this meat.

Another likely way is the indiscriminate use of animal droppings as source of energy for cooking in place of firewood and as manure for farming. Jos and Bukuru are central busy cities of Plateau state where such practices do not take place. They probably may have recorded low prevalence of the pathogen due to these facts.

The high isolation rate of *Y. enterocolitica* (3.3%) in all the 5 study areas as compared with *Y. pseudotuberculosis* (2.8%) among the diarrhoeic patients is in agreement with reports of Lal *et al*, (2003). Researchers have isolated *Y. enterocolitica* from patients in many countries worldwide but the disease appears to favour cooler climates. The serotypes 0:3/0:8/0:9 are most often associated with human infections. While serotype 0:3 predominates in countries where the disease is endemic (Belgium, Canada, Japan, Norway, and Denmark) Cover and Albert, 1989; Ostroff, 1995). Isolation of the bacterium in developing countries is uncommon.

5.4 INCIDENCE OF *YERSINIA ENTEROCOLITICA* AND *YERSINIA PSEUDOTUBERCULOSIS* IN ANIMALS

The overall incidences (50%) of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in the selected animal population investigated are rather high. This aspect of the study confirms the zoonotic nature of the organism as reported by Schiemann *et al*, 1981; Tauxe *et al*, 1987; Wauters *et al*, 1988 b). These findings have revealed that *Y. enterocolitica* and *Y. pseudotuberculosis* are common pathogens in the selected species of animals as reported in a similar study by Ray *et al*, (2004).

In this study, 50% of the animals screened were found with the organism. This may be considered high compared with the report of Brewer and Corbel (1983) who reported 5.2% frequency of occurrence from similar studies. Again, the overall frequency of occurrence obtained in this study may be attributed to the selective culture media used. It is not surprising that pigs ranked highest (18.1%) among the four different animals screened followed by sheep (12.5%). Pigs are known to be the most important reservoir of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* (Okwori *et al*, 2005). These findings are similar to reports in other parts of the world particularly in India where *Y. enterocolitica* was frequently isolated from intestinal contents of slaughtered pigs and rectal swabs of apparently healthy pigs (Verma and Mishra, 1984). In Japan yersiniosis due to *Y. pseudotuberculosis* has been isolated from many animals including monkey (Iwata *et al*, 2005) dogs, pigs, rodent, rabbits, deer and birds (Fukushima and Gomyoda, 1986). Isolation of *Y. enterocolitica* and *Y. pseudotuberculosis* is yet another evidence of these organisms in Jos, Plateau State, Nigeria

as documented in similar studies by Okoroafor *et al*, (1988) and Okwori *et al*, (2005).

Pigs are known to carry the same serotypes found in man (Tereza *et al*, 1999). In a similar study conducted in Finland, (Fredriksson-Ahomaa, 2001) the main source of sporadic human *Y. enterocolitica* 4/0:3 infections was traced to pigs. A total of 80% of the 212 human strains obtained in Helsinki city, Finland were indistinguishable from strains of pig origin. The discovery of identical genotypic ribotype patterns (1/0:9) in both pigs and humans in this study lends further support to the epidemiologic evidence that swine might be the source of infection among the diarrhoeic patients investigated. These claims correlate with the findings of Hurvell, (1981) Schiemann, (1989) and Kapperud, (1991) who reported that healthy pigs are potential carriers of *Y. enterocolitica* 0:3/biovar 4 and 0:9 biovar 2 which are pathogenic to humans. The association between yersiniosis in man and the consumption of pork in Belgium (Tauxe *et al*, 1987) and Norway (Ostroff *et al*, 1994) identified raw or undercooked pork as the main source of infection.

The apparently low incidence of the infections in Islamic countries where consumption of pork is restricted, as obtained in the Northern part of Nigeria further buttressed the fact that pork is the source of infection with *Y. enterocolitica* and *Y. pseudotuberculosis* (Anon, 2006). Samadi *et al*, (1982) has documented similar findings in Bangladesh.

The prevalence of yersiniosis in sheep (12.6%) in this study indicates that most flocks in the study area were infected. Both *Y. enterocolitica* and *Y. pseudotuberculosis* are known enteropathogens of sheep and are excreted in large

numbers during the acute stage of infection (Slee and Skilbeck, 1992).

This finding proves that the organisms are prevalent in the study areas. It also further suggests that sheep are the maintenance host as shown by Slee and Skilbeck (1992).

Several authors have reported seasonality of infection by *Yersinia* species in a range of domestic and wild mammals, human beings and birds (Fukushima *et al*, 1990). In these studies, incidence of yersiniosis in both human and animals was highest between October to December of each year.

In the work conducted by Fukushima *et al*, (1997) a high prevalence of *Y. pseudotuberculosis* was recorded in chicken in Japan. This is very relevant to the present study where the frequency of occurrence of *Y. pseudotuberculosis* in poultry ranked 10.9% compared with *Y. enterocolitica*. Of all the animal species screened dogs in Vom recorded the highest number of *Yersinia* species (4.9%). This could be due to the high exposure of dogs (as cannivorous animals) to infected carcasses improperly discarded after post mortem examination by Veterinarians at the National Veterinary Research Institute, Vom. This finding is in line with that of Fenwick *et al*, (1994) who stated that dogs could carry *Y. enterocolitica* bioserotype 4/0:3 asymptotically and excrete this organism in their faeces for weeks. These findings were not in agreement with those of Aleksic *et al*, (1987) who in their studies on human and dog faeces isolated pure cultures of *Yersinia rohdei*.

5.5 SEROLOGICAL TYPING OF *YERSINIA ENTEROCOLITICA* AND *YERSINIA PSEUDOTUBERCULOSIS*

Serotype 0:9 were found prevalent in all the samples investigated (both human and animals). This is in disagreement with the findings of Agbonlahor *et al*, (1983) who reported serotype 0:3 in gastroenteritis in Lagos, Nigeria and Adesiyun *et al*, (1986) who also in Zaria, Nigeria reported serotype 0:8. This is an indication that other serotypes are not prevalent in this part of the world. This finding supports the reports of Cover and Alber (1989) and Ostroff *et al*, (1994) that serotypes 0:3/0:8/0:9 were most often associated with human disease, while serotype 0:3 predominated in countries such as Belgium, Canada, Japan, Norway, and Denmark where the disease is endemic. Before the 1980s *Y. enterocolitica* 0:8 was the most frequently identified serotype in human diseases in the USA. Serotype 0:3 emerged during the 1990s and was recognized as a substantial cause of gastroenteritis in Black children living in urban areas of USA. It appears to be largely acquired from pork in the USA (Lee *et al*, 1991; Lee *et al*, 1990; Metchock *et al*, 1991). The present studies do not support the claims by Toma and Lafeur (1981) that *Y. enterocolitica* serotype 0:3 accounts for about 75% of all human isolates in Canada. Although, both outbreak and sporadic disease have been linked to the consumption of pork by-products (Tauxe *et al*, 1987). The global distribution of *Y. enterocolitica* serotypes remains to be established (Schiemann *et al*, 1981).

5.6 BIOTYPING OF *YERSINIA ENTEROCOLITICA*

All *Y. enterocolitica* isolates were biotyped and belonged to either biotype 1A or 1B incidentally this is similar to the findings in several parts of the world where a significant proportion of *Y. enterocolitica* isolated from clinical cases of

gastroenteritis has been found to be biotype 1A (Morris *et al*, 1991). Worldwide isolation of biotype 1A *Y. enterocolitica* from Human, animals and diverse environment as well as interest in their pathogenicity warrants further studies. *Y. enterocolitica* strains embrace a heterogenous group of bacteria with different biochemical and antigenic properties, as well as geographical predisposition. MST was applied to identify novel genetic markers that could account for the variability observed within this bacterial group. Two main groups are identifiable among *Y. enterocolitica* serotypes, namely non-pathogenic biotype 1A organisms and the high pathogenicity, mouse lethal serotype 1B. To date, the high pathogenicity island appears to define essentially the difference between the high-pathogenicity and low-pathogenicity strains, with the low-pathogenicity organism lacking this locus (Iwobi, 2003).

Recently, Grant *et al*, (1998) showed that contrary to previously held conceptions, some non-pathogenic *Y. enterocolitica* 1A strains do harbor some potential virulence markers that confer an invasive phenotype to them. Their conclusion was that some clinical isolates of *Y. enterocolitica* which lack classical virulence markers may be able to cause disease via virulence mechanisms that differ from those previously characterized. Robins-Browne *et al*, (1989) for example reported on a 1A strain of serogroup 0:6 that produced a novel heat-stable enterotoxin termed YST-II. This toxin differs from YST-I in a number of ways including its mechanism of action, which does not appear to involve activation of guanylate cyclase.

In the light of the foregoing, it becomes apparent that a better understanding of the genetic content and variability within *Y. enterocolitica* species will shed interesting light on the diversity that exists within this bacterial group.

The seasonal distribution of yersiniosis in human differs significantly from that of animals, while that of human have been found to be between October to January (Winter period) as reported by Lee *et al*, (1991) Lal *et al*, (2003) that of animals have been found to be within the wet season (i.e May to August). Globally the winter period is a festive period all over the world and so *Y. enterocolitica* being a food borne pathogen could easily be consumed along with food materials or in drinks. Statistically, of particular interest is the approximately P-value for the test. Since the P-value is greater than or equal to 0.05, there is not a significant difference in the distribution of the 2 years for *Y. enterocolitica* and *Y. pseudotuberculosis* and the wet and dry seasons at the 95.0% confidence level (Student T- test).

On the other hand a high prevalence rate of yersiniosis among the selected animals was recorded in the wet season. This could be attributed to increased grazing area of fresh pasture favoured by the rains. This increases the chances of the animals becoming infected by feeding on pasture contaminated by their faecal wastes, especially among the sheep as reported in similar studies in Australia by Slee and Skilbeck (1992).

5.7 PATHOGENICITY TEST

Yersinia enterocolitica infections in animals are often accompanied with mastitis, enteritis, septicaemia, sporadic cases of abortion and placentitis. Others are

ruffling of the fur, paralysis and emaciation. *Yersinia pseudotuberculosis* infections on the other hand are characterized by emaciation, ruffling of the fur, septicaemia, severe abscesses on the viscera as well as mesenteric adenitis (Topley and Wilson, 1990).

It can be seen from this work that the two species of *Yersinia* studied have the same incubation period in the laboratory animals studied (9 days). This incubation period was seen to be similar to that in man (6-10 days). This could be as a result of their near similar physiologic disposition as obtained in man. The signs of diarrhoea shown by rabbits infected with *Yersinia enterocolitica* could be attributed to the heat stable enterotoxin produced by the organism which resembles the ST toxin of *Escherichia coli*. This toxin is however not produced by *Y. pseudotuberculosis* hence its inability to cause diarrhoea. However, guinea pigs inoculated with *Yersinia enterocolitica* failed to show any clinical signs of disease. The chronic and rounded nodules on the viscera of animals are strongly suggestive of the invasive nature of the *pseudotuberculosis* toxin (lipopolysaccharide and endotoxin) as well as *Yersinia* outer protein (Portnoy *et al*, 1984 and Jani, 2002). It also reveals the potency of a chromosomally encoded protein called invasin (*inv*) in the outer membrane that facilitates the intracellular penetration of the above mentioned toxins (Isberg and Falkow, 1985). It may also be right to note that unlike *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* has the viscera as their predilection site. Because these virulent factors are absent in *Yersinia enterocolitica* it is unable to produce the lesions typical with *Yersinia pseudotuberculosis*.

It is also likely that fibrillar haemagglutinin or adhesin (adh) possessed by the *Yersinia species* for attachment to epithelial cells is responsible for the agglutination yielded by the sera of the infected animals against the respective organisms as haemagglutinins on the surfaces of some viruses (e.g. myxoviruses) are homologous to antigenic sites on the surfaces of red blood cells. It can therefore be seen that the two species under study have the same incubation period and could both produce septicaemia, emaciation as well as ruffling of fur.

Yersinia pseudotuberculosis was also seen to produce lesions on the viscera of both rabbits and guinea pigs under study. The organism could be said to be highly invasive. *Yersinia enterocolitica* on the other hand produce gastroenteritis as well as paralysis only in rabbits and no symptoms in guinea pigs. Thus the guinea pig model could be said to be unsuitable for the pathogenicity studies of the organism.

The LD50 of the isolates of *Yersinia pseudotuberculosis* for rabbits could be said to be 1×10^8 organisms since half the number of rabbits inoculated with the organism died.

5.8 GENOTYPING OF *YERSINIA ENTEROCOLITICA* STRAINS

It has been observed that the bio/sera/phage typing of *Y. enterocolitica* is not sufficiently discriminating (Tereza *et al*, 1999). Hence the Multispacer technique (MST) has been successfully applied in recent times to map out colonial genomic differences between closely related bacterial genera and even between isolates of the same species. It has been applied to the genotyping and diagnosis of *Y. pestis* (Drancourt *et al*, 2004) *Rickettsia conorii* (Fournier *et al*, 2004) and *Bartonella*

quintana (Foucault *et al*, 2005) all in France.

In these studies, the comparative differences in spacers and mutation at position 320, where A was replaced by G, between the Nigerian isolates of *Y. enterocolitica* and French isolates used as controls were not unusual. This may be due to environmental pollution or geographical heterogeneity in accordance with the findings of Siriken (2004). This work represents the first attempt to map out novel, potential virulent markers of *Y. enterocolitica* strains in Plateau state, Nigeria, using multispacer typing technique. These data provide the first human and animal population based estimates of laboratory confirmed *Y. enterocolitica* infection in Jos, Plateau State, Nigeria. The overall incidence of *Y. enterocolitica* and *Y. pseudotuberculosis* infection was low in the population studied but striking geographic and demographic variation was noted. These findings will require further investigations.

5.9 GENOTYPIC VARIABILITY OF *YERSINIA ENTEROCOLITICA* AND *YERSINIA PSEUDOTUBERCULOSIS* STRAINS BY ELONGATED PULSED FIELD GRADIENT ELECTROPHORESIS (PFGE)

The DNA based molecular methods such as PFGE provide sensitive assays for subtyping of *Y. enterocolitica* and *Y. pseudotuberculosis* strains of the same boiserotypes (Fredriksson – Ahomaa *et al*, 1999). Interpretation of PFGE patterns was at times challenging because of the large amount of fragments very closely spaced together. The PFGE results based on the *NotI* enzymes differentiated *Y. enterocolitica* and *Y. pseudotuberculosis* strains. These findings are consistent with previous reports by Niskanen *et al*, (2003) who documented variable profiles of *Y.*

enterocolitica and *Y. pseudotuberculosis* strains in migratory birds. Thus, PFGE, besides being a molecular technique for epidemiological survey of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* strains, is also a valuable tool for strain typing. It is both highly sensitive and discriminatory.

The *NotI* profile displayed four different bands which according to Tenover *et al*, (1995) means that the isolates are closely related and probably part of the same outbreaks in Jos metropolis. These findings tallied with the reports of Tenover *et al*, (1997) who documented the molecular typing methods for epidemiological studies of bacterial infections. Human infection with *Y. pseudotuberculosis* is suspected to be primarily associated with consumption of food or unchlorinated water contaminated by wild animal wastes (Fukushima *et al*, 1991). In previous studies differences in the rates of detection of *Y. pseudotuberculosis* and *Y. enterocolitica* depending on host species have been documented (Okwori *et al*, 2005).

All *Y. pseudotuberculosis* strains were of bioserotype 1/0:2, 0:1. These serotypes were most commonly isolated from birds in Europe, United States and Canada (Niskanen *et al*, 2003). They are typically found in healthy birds but have also been reported to cause disease in both birds and humans (Teritti *et al*, 1984). The PFGE results based on the *NotI* profiles, differentiated *Y. pseudotuberculosis* strains into 7 variants (Plate 13).

These results provided the first knowledge on the occurrence and characterization of these largely unknown human pathogens in food, animal and human samples in Nigeria and may be regarded as a first step in understanding the epidemiology of yersiniosis in this country.

Data herein reported highlight genetic variability or diversity among *Y. enterocolitica* and *Y. pseudotuberculosis* strains using PFGE typing Technology in Jos - Nigeria.

In conclusion the PFGE applied in the present study was shown to be an efficient tool for identification of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* isolates. The only major problem was that the technique required the handling of large quantities of materials and had no stability of profiles when compared with PCR after long frozen storage.

5.10 SUMMARY OF RESULTS

1. It was established that the different strains of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* exist in Jos, Plateau State, Nigeria.
2. The biotype varies among human and animal isolates with similar genotypic profiles.
3. *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* were confirmed to be zoonotic in nature with same serotype affecting both human and animals.
4. Using molecular biology technique it was revealed that Pulsed Field Gel Electrophoresis is the best tool for the characterization of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*.
5. Susceptible experimental animals for the diagnosis of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* were identified as rabbits and guinea pigs respectively.
6. The prevailing serotypes of *Yersinia enterocolitica* in animal, human and food were revealed to be Ni.

7. The prevailing genotype of *Yersinia pseudotuberculosis* in animals, human and food was noted to be Na.
8. Pre-enrichment, selective enrichment broths and selective agar inoculation for the isolation of *Yersinia* species are more effective and reliable.
9. *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* were susceptible to a number of commonly available antibacterial agents.

5.11 CONTRIBUTIONS TO KNOWLEDGE / FINDINGS

1. The study elucidated the epidemiology of yersiniosis in Jos, Plateau State, Nigeria.
2. Both *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* were confirmed as agents of gastroenteritis in the study areas.
3. *Yersinia enterocolitica* was confirmed an agent of lower abdominal discomfort that mimicks the clinical symptoms of appendicitis.
4. Both *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* are wide spread and are aetiological agents of food borne diseases (mostly from food contaminated with animal wastes eg milk).
5. It was discovered that only serotype O:9 of *Yersinia enterocolitica* and O:1 of *Yersinia pseudotuberculosis* exist in Jos, Nigeria.
6. Pigs were noted as reservoir host for both *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*.

7. There is a seasonal variation in the occurrence of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in human and animal infections.
8. It was observed that Nigerian and French isolates of *Yersinia enterocolitica* differ geographically with virulent marker inter changed at position 320 of the nucleotide bond (where arginine was replaced with guanine).

5.12 CONCLUSION/RECOMMENDATION

Y. enterocolitica and *Y. pseudotuberculosis* are zoonoses of high pathogenicity and virulence to man and animals. These organisms have been found distributed amongst animals and humans in Jos and its environ. Prevention depends upon avoiding ingestion of the organisms in food or water. When animals are kept together in a place, access of wild birds and rodents to food supplies should be prevented. Fresh vegetables should be carefully cleaned before being fed to animals and overcrowding and poor hygiene should be avoided. Elimination of reservoirs and minimizing the contamination of food products are of immense importance. In hospital settings, enteric precautions in the care of patients hospitalized with the infection should be institutionalized. There are no specific measures from the prevention of *Y. enterocolitica* infection, even if vaccines were available, the sporadic nature of the disease would make it difficult to identify those at risk. Also prompt treatment of infected cases in animals and humans should be adopted. Many cases could be prevented by avoiding the consumption of uncooked meats, especially pork, proper treatment of milk and adequate chlorination or boiling of drinking water. Direct contact with infected animals and person-to-person spread probably account for a small proportion of cases. Center for Disease Control (CDC) has collaborated in an educational campaign to increase public awareness about prevention of *Y. enterocolitica* infections.

Further epidemiological studies are recommended to increase our understanding of the distribution and pathogenesis of *Yersinia* species in man and animals in Nigeria. There is therefore the need to establish the actual prevalence of *Yersinia* species through co-ordinated nation-wide community based studies with particular reference to age and sex distributions in apparently healthy and ill members of the community. It would also be desirable to produce *Yersinia* typing antisera at commercial quantities so as to make the identification of isolates easier and less time consuming particularly in the developing African countries for routine and epidemiological studies. On the other hand, a central regional reference laboratory could be established, for example, in a laboratory with the most adequate facilities in the country, for the purposes of collation and helping to develop other smaller laboratories to identify, biotype or serotype *Yersinia* isolates from human, animal and environmental sources. Pathogenicity test such as the “animal model” described in this thesis should be further studied and standardized with a view to recommending a simple, cheap and reproduceable model for routine use. Lastly the PCR and the PFGE methods applied in this study have been shown to be efficient molecular techniques for the characterization of *Y. enterocolitica* and *Y. pseudotuberculosis*.

In view of the fact that the incidences of tuberculosis in pigs and humans have been reduced to a very low level in many parts of the world, it may be possible to reconsider regulations that require incision of the submaxillary lymph to detect tuberculosis nodes by meat inspectors. To reduce *Yersinia* infections, removal of pig head containing the highly contaminated tonsils and tongue should be made

mandatory at all Abattoirs in Nigeria. Small-scale backyard animal farming particularly piggery, should be standardized, as these animals have been found to harbour pathogenic *Yersinia* species.

5.13 RECOMMENDATION FOR FURTHER RESEARCH

Yersinia enterocolitica is a heterogenous genus which is reflected in recurrent suggestion of alterations in the taxonomy of the *Yersinia* genus and by the inadequacy of the methods available for the analysis and determination of *Yersinia* species. The method available for analysis of *Yersinia enterocolitica* 0:3 which is the most common serotype in Europe and an emerging human pathogen, probably under estimate the presence of the bacterium. Further more, relatively few genotypically based methods have been developed for the determination of pathogenic *Yersinia*. Thus practical diagnostic tools based on genomic analysis are needed to further unravel the epidemiological behaviour of the bacterium, hence the dearth need for further research studies.

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APPENDIX A**FORMULAR FOR THE PREPARATION OF SOME CULTURE MEDIA AND REGEANTS USED IN THIS PROJECT****MACCONKEY AGAR (OXOID CM7)**

<u>Compound (designation/manufacturer)</u>	<u>GRAM/LITRE Distiled water</u>
Peptone (Oxoid L37)	20g
Lactose	10g
Bile salts (oxoid L55)	5g
Sodium chloride	5g
Neutral red	0.075g
Agar No. 3 (Oxoid L13)	12g
Distilled water	1 litres

pH 7.4

DESOXYCHOLATE CITRATE AGAR (OXOID CM35)

Lab-lemco powder (Oxoid L29)	5.0 g
Peptone (Oxoid L37)	5.0 g
Lactose	10.0 g
Sodium citrate	5.0g
Sodium thiosulphate	5.0g
Ferric citrate	1.0 g
Sodium desoxycholate	2.5 g
Sodium thiosulphate	5.0 g
Ferric citrate	1.0 g
Sodium desoxycholate	2.5 g
Neutral red	0.025g
Agar No. 3 (Oxoid L13)	15.0 g
Distilled water	1 litre

pH 7.0

DIFFERENTIAL AND SELECTIVE MEDIUM FOR *YERSINIA ENTEROCOLITICA* (DYS)

Bacteriological peptone (Oxoid, L37)	15
Casein hydrolysate (Oxoid, L41)	5
Sodium desoxycholate (Oxoid, L57)	2
Sodium chloride (M & B)	5
L (+) Arabinose (BDH)	10
L-arginine (BDH)	6.5
L-lysine (BDH)	6.5
Neutral red (BDH)	0.04
Agar bacteriological, No. 1 (Oxoid, b11)	12

pH 7.4 ± 0.1

UREA AGAR SLOPE

Basal Medium

Peptone (rich in tryptophane)	30.0g
KH ₂ P04	1.0g
Sodium chloride	5.0g
Agar	4.0
Phenol red - alcoholic solution	2.9ml
Distilled	1.0litre

Urea Solution

Prepare 20% urea solution in water. Add 10ml. of 20% sterile (filtered) urea solution to 90ml of basal agar (molten at 50°C)

ANDRADES INDICATOR

Acid fuchsin	0.15g
NaCl (IN)	16.00ml
Distilled water	100.00ml

TRYPTONE SOYA BROTH WITH 0.6% YEAST EXTRACT (OXOID CM 129)

Pancreatic Digest of Casein (Oxoid L42)	17.0g
Papaic Digest of soyabean Meal (Oxoid L44)	3.0g
Sodium chloride	5.0g
Dibasic Potassium proosphate	2.5g
Dextrose	2.5g
Yeast extract (Oxoid L21)	6.0g
Distilled water	1 litre
pH 7.3	

TRYPTONE SOYA AGAR

Tryptone (xoid L42)	15.0g
Soya peptone (Oxoid 444)	5.0g
Sodium chloride	5.0g
Agar No. 3 (Oxoid L13)	15.0g
Distilled water	1 litre
pH. 7.3	

CEFSULODIN IRGASAN NOVOBIOCIN (CIN) AGAR

Bacto Yeast extract	2.0
Bacto peptone	7.0
Proteose peptone, Difco	3.0
Mannitol	20.0
Sodium deoxycholate	0.5
Sodium chloride	0.1
Sodium cholate	0.5
Sodium pyruvate	2.0
Magnesium sulphate hypatohydrate	10.0
Bacto Agar	13.5
Bacto crystal violet	1.0
Irgasan	4.0mg

pH 7.4

The ingredients were steamed to dissolve in 1 litre of distilled water and heated to boiling to dissolve completely. It was then sterilized by autoclaving at 121°C for 15 minutes and allowed to cool to 45°C – 50°C. 10ml of the Bacto. Yersinia antimicrobial supplement CN was added aseptically and mixed thoroughly avoiding air bubbles and dispensed into sterile petridishes.

TAE buffer

Solutions

1 × TAE buffer 40 mM Tris/HCl, pH=8.2

20 mM Acetic acid

2 mM EDTA, pH 7.6

10 x Loading buffer for agarose gels 0.25% (w/v) Bromophenol blue

25% (v/v) Ficoll 400

Agarose gel

0.8%-2% Agarose in 1x TAE

Ethidium bromide staining solution 1µg Ethidium bromide per ml H₂O.

Dilution for Sequence reactions

	Forward(F)	Reverse(R)
Big dye	2 µl	2 µl
Buffer	3 µl	3 µl

Water	5 μ l	5 μ l
Primers	1 μ l	1 μ l
Purified DNA	5 μ l	5 μ l

Thermocycler program-BD-3

Denaturation	96 °C for 1 minute	} 25 cycles
Hybridation	96 °C for 10 seconds	
Elongation	50 °C for 5 seconds	
Final elongation	60 °C for 3 minutes	

GROWER'S MASH (1000KG)

Maize	510g
Groundnut cake	380g
Wheat offal	50g
Bone meal	45g
Oyster Shell	25g
Common Salt	2kg
Minvit (VM201)	2kg
Amprolmix	-
Fishmeal	-
Methionine	-

COLE'S HAEMATOXYLIN

Preparation:

Haematoxylin	1.5g
1% iodine in 95% alcohol	50ml
Saturated Aq ammonium alum	700ml
Distilled Water	250ml

Dissolve the haematoxylin in the warmed distilled water before adding and mixing with the iodine. Add the alum solution and bring to the boil. Cool immediately and filter before use.

EOSIN SOLUTION:

Eosin powder	1g
Distilled water	100ml
Thymol	1 crystal

COLE'S HAMATOXYLIN & EOSIN METHOD

Staining procedure

1. Dewax section in xylene
2. Hydrate in 100%, 80%, 70% and 50% alcohol and immerse in water
3. Stain with cole's haematoxylin solution for 10 minutes
4. Wash thoroughly in water
5. Differentiate in 1% acid alcohol briefly
6. Blue the nuclei in Scott tap water for 4 minutes
7. Counterstain in 1% eosin for 4 mins
8. Wash thoroughly in water
9. Dehydrate in 50%, 70%, 80%, 100%
10. Clear in xylene
11. Mounting with Canada balsam

HAEMATOXYLIN SOLUTION

Haematoxylin powder	-	1.5g
Iodine (1% in 95% alcohol)	-	50ml
Ammonium alum (saturated ag. Solution)	-	700ml
Distilled water	-	250ml

EOSIN SOLUTION

1g eosin powder	-	1g
D/W	-	100ml
Thymol	-	1 crystal

PHOSPHATE BUFFERED SALINE (PBS, M/15 (OXOID) BR4A)

For the cold-temperature enrichment for *Yersinia enterocolitica*, 1 tablet was dissolved in 223ml of distilled water and autoclaved at 121°C for 15 minutes after dispensing into 10ml amount. pH 7.3

CAPTURED MULTIPLE AND ALIGNMENT SEQUENCES

[NPS@](#) is the [IBCP](#) contribution to [PBIL](#) in Lyon, France

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Thursday, February 3rd 2005 : InterProScan v4.0 / InterPro 8.1 available ([see news](#))
 Monday, January 3rd 2005 : Best wishes for new year 2005 ([see news](#))

Job **MULTALIN** (ID: 4b32067ffcd1) is running on **NPS@** server (started on 20050226-203630).

Results will be shown below. **Please wait and don't go back.**

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NPS@: Network Protein Sequence Analysis
 TIBS 2000 March Vol. 25, No 3 [291]:147-150
 Combet C., Blanchet C., Geourjon C. and Deléage G.

MULTALIN multiple alignment

[Abstract](#) F. CORPET Multiple sequence alignment with hierarchical clustering , 1988, Nucl. Acids Res., 16 (22), 10881-10890 [[HELP](#)]

alignment with width :
 residues.

Conservation level of % (works with "using conservation level of" option in list above, negative value will hide).

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                    10          20          30          40
                    |          |          |          |
18yp1143  -----
-----
46YP1143
NTCAAAGACCAGACTTTTAAATAGCTCAAGTYNGGCAKANCCGGTATGTTAAAGCG
TGAA
19yp1143  -----
NCGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGG---
25yp1143  -----
TACGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGG---
26yp1143  -----
ACGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGG---
32yp1143  -----
ACGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGG---
57yp1143  -----
ACGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGG---
35yp1143  -----
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37yp1143  -----
ACGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGG---
39yp1143  -----
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51yp1143  -----
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49yp1143  -----
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48yp1143  -----
ACGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGG---
47yp1143  -----
ACGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGG---
42yp1143  -----
ACGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGG---
43YP1143  -----
CGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGG---
31yp1143  -----
ACGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGGG--

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53yp1143      -----
ACGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGG--
41yp1143      -----
ACGCGCCCTATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGG---
52yp1143      -----
ACGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGG---
55yp1143      -----
ACGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGG---
24yp1143      -----
ACGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAGG---
20yp1143      -----
-----
38yp1143      -----
ATTTAATTTCACTCCAAGTCTCGTCTACTTGAAGTTGCAGGGGGGGG
40YP1143      -----
TCAATCTGAGCGACAGTCCATCAGCAGGCTCCTGTAGGCGG
Consensus
acgcgccatatttcataatcAatTTTGCCagattcatcaatgccataaGg
Prim.cons.
NTCAA3ACGCGCCATATCTTCATAATCAATTTTGCCAGATTCATCAATGCCATAAG
GGG

                                70          80          90          100
110          120          |          |          |          |
|          |
18yp1143      -----
GAGCAAGAAGAGGGTGCGT
46YP1143
ATGAACATTGCCGATTATGATGCAGATCTATGGCGTGCAATGGAGCAAGAAGTGGT
GCGT
19yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
25yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
26yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
32yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
57yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
35yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
37yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
39yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC

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51yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
49yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
48yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
47yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
42yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
43YP1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
31yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
53yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
41yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
52yp1143      -----AACGATATTGTACAGTTTACCGGAG-----
AAGTTA-----ACCGGTGAAC
55yp1143      -----AACGATATTGTACAGTTTGCCAGAG-----
AAGTTA-----ACCGGTGAGC
24yp1143      -----AACGATATTGTACACTGTTTACCTG-----
GAGTAAGTTGCAACCGGTGAAC
20yp1143      -----TCGGATTTGTACAGTTT-CCGGAG-----
AAGTTA-----CCGGTGTGC
38yp1143      GCGCTGTACTCACTCACCTGAATCACTTACTGGTT-----
GTTGACCTTAAAGTAAATAAG
40YP1143      TGGAATAACAACCGGACAATAGCAACCCCTTGT-----
TAACTACCAGCACTATCCTTG
Consensus      aaCgatattgtaCAgtttaccggag
aagtta      AccGGTgaac
Prim.cons.
33GAA3TA33AACGATATTGTACAGTTTACCGGAGTGCAAAGTTAC2AGAACCGGT
GAAC

          130          140          150          160
170          180          |          |          |          |
|          |
18yp1143
CAGGAAGAGCACATCGAGCTGATTGCCCTGAGAACTACACCAGCCCGCGTGATAT
GCAG
46YP1143
CAGGAAGAGCACATCGAGCTGATTGCCCTGAGAACTACACCAGCCCGCGTGTTAT
GCAG

```


19yp1143 CGTGGGTCAACAAATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 25yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 26yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 32yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 57yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 35yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 37yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 39yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 51yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 49yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 48yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 47yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 42yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 43YP1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 31yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 53yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 41yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 52yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 55yp1143 CGTGGGTCAA---ATGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CACCTGG
 24yp1143 CGTGGGTCAA---
 ATGGCCGCCGTGCGCCAAAATTCATCCCAGAACAGTGTGCACCTGG
 20yp1143 CTTGGGTCAA----TGGCCGCCGTGCGCCAAA-
 TTCATCCCAGAACAGTGT-CGCTGG
 38yp1143 CTCATCGGGATGAGTTCACTGGCTGCCTACCTGTAACAC----
 -----CAA
 40YP1143
 CTCAACAACTCAATAGCCGATCCAGCACACAGCTTCACCACCTAACCCGATCTCTT
 TCAG

Consensus CgtGggtcaa atGgcCgcccTGCgCCaaA
TtCatCcCCAGaaCagtgt cacctgG

Prim.cons.

CGTGGGTCAA22AATGGCCGCCGTGCGCCAAAGTTCATCCCCAGAACAGTGTTTAC
CTGG

	190	200	210	220
230				
240				
18yp1143				-
CTCAGGGTTCTCAGTTGACCAACAAACATGCTGAAGGCTATCCAGGCAAGCGTTAC				
TAC				
46YP1143				
GCTCAGGGTTCTCAGTTGACCAACAAATATGCTGAAGGCTATCCAGGCAAGCGTTA				
CTAC				
19yp1143				
TTGCAGCAATGCTGAGTAAACGGCCACGCTTCGCCCTGAGAACCGGAATTGCGGCT				
GAAC				
25yp1143		TTGCAGCAATGCTGAGTAAACGGCCACGTTTCG--		
		CCTGAGAACCGGAAT-GCGGCTGAAC		
26yp1143		TTGCAGCAATGCTGAGTAAACGGCCACGTTTCG--		
		CCTGAGAACCGGAAT-GCGGCTGAAC		
32yp1143		TTGCAGCAATGCTGAGTAAACGGCCACGTTTCG--		
		CCTGAGAACCGGAAT-GCGGCTGAAC		
57yp1143		TTGCAGCAATGCTGAGTAAACGGCCACGTTTCG--		
		CCTGAGAACCGGAAT-GCGGCTGAAC		
35yp1143		TTGCAGCAATGCTGAGTAAACGGCCACGTTTCG--		
		CCTGAGAACCGGAAT-GCGGCTGAAC		
37yp1143		TTGCAGCAATGCTGAGTAAACGGCCACGTTTCG--		
		CCTGAGAACCGGAAT-GCGGCTGAAC		
39yp1143		TTGCAGCAATGCTGAGTAAACGGCCACGTTTCG--		
		CCTGAGAACCGGAAT-GCGGCTGAAC		
51yp1143		TTGCAGCAATGCTGAGTAAACGGCCACGTTTCG--		
		CCTGAGAACCGGAAT-GCGGCTGAAC		
49yp1143		TTGCAGCAATGCTGAGTAAACGGCCACGTTTCG--		
		CCTGAGAACCGGAAT-GCGGCTGAAC		
48yp1143		TTGCAGCAATGCTGAGTAAACGGCCACGTTTCG--		
		CCTGAGAACCGGAAT-GCGGCTGAAC		
47yp1143		TTGCAGCAATGCTGAGTAAACGGCCACGTTTCG--		
		CCTGAGAACCGGAAT-GCGGCTGAAC		
42yp1143		TTGCAGCAATGCTGAGTAAACGGCCACGTTTCG--		
		CCTGAGAACCGGAAT-GCGGCTGAAC		
43YP1143		TTGCAGCAATGCTGAGTAAACGGCCACGTTTCG--		
		CCTGAGAACCGGAAT-GCGGCTGAAC		
31yp1143		TTGCAGCAATGCTGAGTAAACGGCCACGTTTCG--		
		CCTGAGAACCGGAAT-GCGGCTGAAC		

53yp1143 TTGCAGCAATGCTGAGTAAACGGCCACGTTCG--
 CCTGAGAACCGGAAT-GCGGCTGAAC
 41yp1143 TTGCAGCAATGCTGAGTAAACGGCCACGTTCG--
 CCTGAGAACCGGAAT-GCGGCTGAAC
 52yp1143 TTGCAGCAATGCTGAGTAAACGGCCACGTTCG--
 CCTGAGAACCGGAAT-GCGGCTGAAC
 55yp1143 TTGCAGCAATGCTGAGTAAACGGCCACGTTCG--
 CCTGAGAACCGGAAT-GCGGCTGAAC
 24yp1143
 TTGCAGCAATGCTGAGTAAACGGCCACCGTTCGCCTGCAGAACCGGAAT-
 GCGGCTGAAC
 20yp1143 TTGCAGCAATGCTGAGTATCCGGCCTCGTTCG---
 CCTAGAACCGGAAT-GCGGCTGAAC
 38yp1143
 TTACTTTGAGAAATATAAATATGGTAATAGCTCTCTTGTACTAGCTCAATGATAC-
 --GT
 40YP1143
 TTTACTTTTTTAATCTAAAAAATGCTGGTTCAGGCGATATTC AATTACAACCGAAC-
 --AC
 Consensus TTgCAGcaaTgcTgagTAAaCgGCcAcgtTcg
 cctgagAaCCgGaAt GCGgCtgaAC
 Prim.cons.
 TTGCAGCAATGCTGAGTAAACGGCCACGTTCCGGCCCTGAGAACCGGAAT2GCGGCT
 GAAC

	250	260	270	280
290	300			

18yp1143 GGTGGCTGTGAGTATGTCTG-
 ATATCGTTGAGCAGTTGGCTATTGACCGCGCAAAAGAGTT
 46YP1143 GGTGGCTGTGAGTATGTCTG-
 ATATCGTTGAGCAGTTGGCTATTGACCGCGCAAAAGAGTT
 19yp1143
 GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
 CGAT
 25yp1143
 GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
 CGAT
 26yp1143
 GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
 CGAT
 32yp1143
 GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
 CGAT

57yp1143

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

35yp1143

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

37yp1143

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

39yp1143

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

51yp1143

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

49yp1143

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

48yp1143

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

47yp1143

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

42yp1143

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

43YP1143

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

31yp1143

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

53yp1143

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

41yp1143

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

52yp1143

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

55yp1143

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

24yp1143

GTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

20yp1143

GTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

38yp1143

GGTGGC-----
GATGAAACTGTAAACAGGTGAATATGCTTATGTGGA-----

40YP1143

CGAACCAAGACTAACCACCTGACCAAACTATCCACATAAGCATATTCATCTGTTTA-

Consensus

GtTGGCgtaatcTgcGcCGaAcAAcTCTtTtgcGcgGtCaATAGcCaaCTGctcAa
cGaT

Prim.cons.

GTTGGCGTAATCTGCGCCGAACAACCTCTTTTGC GCGGTCAATAGCCAACCTGCTCAA
CGAT

	350	360	310	320	330	340
18yp1143						
46YP1143						
19yp1143						
25yp1143						
26yp1143						
32yp1143						
57yp1143						
35yp1143						
37yp1143						
39yp1143						
51yp1143						
49yp1143						

48yp1143 ATCGAC-
 ATACTCACAGCCACCGTAGTAACGCTTGCCTGGATAGCCTTCAGCATATTTGT
 47yp1143 ATCGAC-
 ATACTCACAGCCACCGTAGTAACGCTTGCCTGGATAGCCTTCAGCATATTTGT
 42yp1143 ATCGAC-
 ATACTCACAGCCACCGTAGTAACGCTTGCCTGGATAGCCTTCAGCATATTTGT
 43YP1143 ATCGAC-
 ATACTCACAGCCACCGTAGTAACGCTTGCCTGGATAGCCTTCAGCATATTTGT
 31yp1143 ATCGAC-
 ATACTCACAGCCACCGTAGTAACGCTTGCCTGGATAGCCTTCAGCATATTTGT
 53yp1143 ATCGAC-
 ATACTCACAGCCACCGTAGTAACGCTTGCCTGGATAGCCTTCAGCATATTTGT
 41yp1143 ATCGAC-
 ATACTCACAGCCACCGTAGTAACGCTTGCCTGGATAGCCTTCAGCATATTTGT
 52yp1143 ATCGAC-
 ATACTCACAGCCACCGTAGTAACGCTTGCCTGGATAGCCTTCAGCATATTTGT
 55yp1143 ATCGAC-
 ATACTCACAGCCACCGTAGTAACGCTTGCCTGGATAGCCTTCAGCATATTTGT
 24yp1143 ATCGACTCT--
 TCACAGCCACCGTAGTAACGCTTGCCTGGATAGCCTTCAGCATATTTGT
 20yp1143 ATCGAC-
 ATACTCACAGCCACCGTAGTAACGCTTGCCTGGATAGCCTTCAGCATGTTTGT
 38yp1143 -----
 TAGTTTGGTCAGTGTTAGTCTTGGTTCGGTGTTCG----GTTGTAAATTGAA
 40YP1143 -----
 CAGTTTCATCGCCACCACGTATCATTTGAGCTAGTACAAGAGAGCTATTACCA
 Consensus aTcgac
 atAcTcacaGCCAcCGTAgtaaCGCtTgCCtGgaTagccttcagcataTTTgT
 Prim.cons.
 ATCGACCATACTCACAGCCACCGTAGTAACGCTTGCCTGGATAGCCTTCAGCATAT
 TTGT

		370	380	390	400
410	420				

18yp1143 -AGGCGAACGAGGCCGGATACTCAGCATTGC-----
 -TGCAACCAGGCGACAC
 46YP1143 CAGGCGAACGTGGCCGTTTACTCAGCATTGC-----
 -TGCAACCAGGTGACAC
 19yp1143
 TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
 GCAA
 25yp1143
 TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
 GCAA

26yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

32yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

57yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

35yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

37yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

39yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

51yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

49yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

48yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

47yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

42yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

43YP1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

31yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

53yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

41yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

52yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

55yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

24yp1143

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

20yp1143

TGGTCAACTGAGAACCCTGAGC-
TGCATATCACGCGGGCTGGTGTAGTTCTCAGGGCAA

38yp1143

TATCGCCTGAACCAGCATTTTTTAGATTAAAAAGTAAACTGAAAGAGATCGGGTTA
GGTG

40YP1143

TATTATATATTCTCAAAGTAATTGGTGTACAGGTAGGCAGCCAGTGAACTCATCC
CGAT

Consensus

TgGtCaActGAGaaCccTgAgcctGCATaaCAcGcgggCtGgtgTaGttCtcaGaG
gCAa

Prim.cons.

TGGTCAACTGAGAACCCTGAGCCTGCATAACACGCGGGCTGGTGTAGTTCTCAGAG
GCAA

		430	440	450	460
470	480				

18yp1143 TGT-CTGGGGATGAATTTGGCGCACGGCGGCCATT-
GACCCAAGGCACACCGGTA ACTTC

46YP1143
TGTTCTGGGGATGAATTTGGCGCACGGCGGCCATTTGACCCACGGTTACCGGTTA
ACTT

19yp1143
TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

25yp1143
TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

26yp1143
TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

32yp1143
TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

57yp1143

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

35yp1143

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

37yp1143

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

39yp1143

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

51yp1143

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

49yp1143

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

48yp1143

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

47yp1143

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

42yp1143

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

43YP1143

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

31yp1143

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

53yp1143

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

41yp1143

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

52yp1143

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

55yp1143

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
TC-T

24yp1143
 TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
 TCGT

20yp1143 TCAGCTCGATGTGCTCTTCCTGACGCACCTCTTCTTGCTC---

38yp1143 GTGAAGCTGTGTGCTGGATCGGCTA-

TTGAGTTGTTGAGCAAGGATAGTGCTGGTAGTTA

40YP1143 GAGCTTATTTACTTTAAGGTCAACA-

ACCAGTAAGTGATTCAAGGTGAGTGAGTACAGC--

Consensus

TcagCTcGaTgTGcTcTTcctGacgcaCcaCTtcTTGctCCAttgcAcgcCatagA
 tc t

Prim.cons.

TCAGCTCGATGTGCTCTTCCTGACGCACCACTTCTTGCTCCATTGCACGCCATAGA
 TCTT

	530	540	490	500	510	520
18yp1143		TCC---GGAAACTGTA-----				
	CAAATCCGA-----					
46YP1143		ACTCCGGTAAACTGTA-----				
	CAATATCGTTCCTTATGGCATTGATG					
19yp1143		GCATCATAA-				
	TCGGCAATGTTTCATTTACAGCTTTAACATCCGGCTCTCCTGACTTAG---					
25yp1143		GCATCATAA-				
	TCGGCAATGTTTCATTTACAGCTTTAACATCCGGCTCTCCTGACTTAG---					
26yp1143		GCATCATAA-				
	TCGGCAATGTTTCATTTACAGCTTTAACATCCGGCTCTCCTGACTTAG---					
32yp1143		GCATCATAA-				
	TCGGCAATGTTTCATTTACAGCTTTAACATCCGGCTCTCCTGACTTAG---					
57yp1143		GCATCATAA-				
	TCGGCAATGTTTCATTTACAGCTTTAACATCCGGCTCTCCTGACTTAG---					
35yp1143		GCATCATAA-				
	TCGGCAATGTTTCATTTACAGCTTTAACATCCGGCTCTCCTGACTTAG---					
37yp1143		GCATCATAA-				
	TCGGCAATGTTTCATTTACAGCTTTAACATCCGGCTCTCCTGACTTAG---					
39yp1143		GCATCATAA-				
	TCGGCAATGTTTCATTTACAGCTTTAACATCCGGCTCTCCTGACTTAG---					
51yp1143		GCATCATAA-				
	TCGGCAATGTTTCATTTACAGCTTTAACATCCGGCTCTCCTGACTTAG---					
49yp1143		GCATCATAA-				
	TCGGCAATGTTTCATTTACAGCTTTAACATCCGGCTCTCCTGACTTAG---					
48yp1143		GCATCATAA-				
	TCGGCAATGTTTCATTTACAGCTTTAACATCCGGCTCTCCTGACTTAG---					

```

47yp1143      GCATCATAA-
TCGGCAATGTTTCATTTACGCTTTAACATCCGGCTCTCCTGACTTAG---
42yp1143      GCATCATAA-
TCGGCAATGTTTCATTTACGCTTTAACATCCGGCTCTCCTGACTTAG---
43YP1143      GCATCATAA-
TCGGCAATGTTTCATTTACGCTTTAACATCCGGCTCTCCTGACTTAG---
31yp1143      GCATCATAA-TCGGCAATGTTTCATTTAC-
CTTTAACATCCGGCTCTCCTGACTTAG---
53yp1143      GCATCATAA-TCGGCAATGTTTCATTTACGCTTTAAC-
CCGGCTCTCCTGACTTAG---
41yp1143      GCATCATAA-TCGGCAATGTTTCATTTACGCTTTAT-
ATTAAAAAGTCTGGTCTTTGA--
52yp1143      GTATCAAGA-
ATTTCCGGACTGGGTGCAACGCTTTCAACAATCGG-----
55yp1143      GCATCAT-----
-----
24yp1143      GCATCAAAAATCGGCCATGTTTCATTTACGCTTTAACATCCGGCTCTCCTGA----
-----
20yp1143      -----
-----
38yp1143      ACAAGGGGGGTTGCTATTGTCCGTTGTTATTTCCACCAGCCTACAGGAGCCTGCTG
ATGG
40YP1143      -----
GCCCCCCCCTGCAACTTCAAG
Consensus      gcatcataa
tcggcaatgtttcatttcacgctttaacatccggctctcctgacttag
Prim.cons.
GCATCATAAATCGGCAATGTTTCATTTACGCTTTAACATCCGGCTCTCCTGACTTA
GA3G

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550

|

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18yp1143      -----
46YP1143      A-----
19yp1143      -----
25yp1143      -----
26yp1143      -----
32yp1143      -----
57yp1143      -----
35yp1143      -----
37yp1143      -----
39yp1143      -----
51yp1143      -----
49yp1143      -----
48yp1143      -----

```

```

47yp1143      -----
42yp1143      -----
43YP1143      -----
31yp1143      -----
53yp1143      -----
41yp1143      -----
52yp1143      -----
55yp1143      -----
24yp1143      -----
20yp1143      -----
38yp1143      ACTGTCGCTCAGATTGAG
40YP1143      TACGACGAGAAGTTTG--
Consensus
Prim.cons.    A22G2CG222AG2TTGAG

```

Alignment data :

Alignment length : 558

Residues conserved for 90 % or more (upper-case letters) : 156 is 27.96 %

Residues conserved for 50 % and less than 90 % (lower-case letters) : 342 is 61.29 %

Residues conserved less than 50 % (white space) : 60 is 10.75 %

IV conserved positions (!) : 0 is 0.00 %

LM conserved positions (\$) : 0 is 0.00 %

FY conserved positions (%) : 0 is 0.00 %

NDQEBZ conserved positions (#): 0 is 0.00 %

Sequence 0001 : 18yp1143 (365 residues).

Sequence 0002 : 46YP1143 (492 residues).

Sequence 0003 : 19yp1143 (505 residues).

Sequence 0004 : 25yp1143 (499 residues).

Sequence 0005 : 26yp1143 (498 residues).

Sequence 0006 : 32yp1143 (498 residues).

Sequence 0007 : 57yp1143 (498 residues).

Sequence 0008 : 35yp1143 (498 residues).

Sequence 0009 : 37yp1143 (498 residues).

Sequence 0010 : 39yp1143 (498 residues).

Sequence 0011 : 51yp1143 (499 residues).

Sequence 0012 : 49yp1143 (498 residues).

Sequence 0013 : 48yp1143 (498 residues).

Sequence 0014 : 47yp1143 (498 residues).

Sequence 0015 : 42yp1143 (498 residues).

Sequence 0016 : 43YP1143 (497 residues).

Sequence 0017 : 31yp1143 (498 residues).

Sequence 0018 : 53yp1143 (498 residues).

Sequence 0019 : 41yp1143 (498 residues).

Sequence 0020 : 52yp1143 (484 residues).

Sequence 0021 : 55yp1143 (449 residues).

Sequence 0022 : 24yp1143 (503 residues).
Sequence 0023 : 20yp1143 (365 residues).
Sequence 0024 : 38yp1143 (491 residues).
Sequence 0025 : 40YP1143 (476 residues).

MULTALIN options used :

c=dna
g=5
k1=90
k2=50
order=optA
s=abs

Result files (text) :

[MULTALIN](#)

User : public@194.199.241.5. Last modification time : Sat Feb 26

20:36:34 2005. Current time : Sat Feb 26 20:36:34 2005 This service is

supported by [Ministère de la recherche](#) (ACC-SV13), [CNRS](#) (IMABIO, COMI,

GENOME) and [Région Rhône-Alpes](#) (Programme EMERGENCE) .

[Comments.](#)

DISTRIBUTION OF QUERRIED BLAST SEQUENCES CAPTURED

BLASTN 2.2.10 [Oct-19-2005]

Reference:

Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25:3389-3402.

RID: 1109440903-25106-156784972405.BLASTQ4

Query= 54yp1143

(324 letters)

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, environmental samples or phase 0, 1 or 2 HTGS sequences)
2,910,708 sequences; 13,358,659,363 total letters

If you have any problems or questions with the results of this search please refer to the [BLAST FAQs](#)

[Taxonomy reports](#)

[Distribution of 9 Blast Hits on the Query Sequence](#)

Mouse-over to show defline and scores. Click to show alignments

E	Sequences producing significant alignments:	Score
Value		(bits)
gi 45435511 gb AE017130.1 	Yersinia pestis biovar Medievali...	464 e-
128		
gi 21959948 gb AE013905.1 	Yersinia pestis KIM section 305 ...	456 e-
125		
gi 15979072 emb AJ414146.1 	Yersinia pestis strain CO92 com...	375 e-
101		
gi 51587641 emb BX936398.1 	Yersinia pseudotuberculosis IP3...	357
1e-95		
gi 38524371 emb BX813325.2 	Mouse DNA sequence from clone R...	40
3.3		
gi 55734182 emb BX510332.10 	Zebrafish DNA sequence from cl...	40
3.3		
gi 21212365 emb AL683847.12 	Mouse DNA sequence from clone ...	40
3.3		

Alignments



—



>[gi|45435511|gb|AE017130.1|](#) Yersinia pestis biovar Medievalis str.
91001 section 4 of 16 of the
complete genome
Length = 290510

Score = 464 bits (234), Expect = e-128
Identities = 241/322 (74%), Gaps = 6/322 (1%)
Strand = Plus / Minus

Query: 1 cattgttttttgcctccggcgggaaacagttattgtcatagaatagctaaatggcataggt
60

Sbjct: 224114 cattgttttttgcctccggcgggaaacagttattgtcataga-tagctaaatggcatag--
224058

Query: 61 atagctaaatggnn
120

Sbjct: 224057 atagctaaacaacatagatagctaaacaacatagatagctaaacaacatagatagataaa
223998

Query: 121 nnnnnnnnnnnnnnnntcgcacgtttggcaggttacannnnnnnatcgctcggttaa
180

Sbjct: 223997 caacatagatagataaatcgcacgtttggcaggttacattttttatcgctcggttaa
223938

Query: 181 aacagggttaattcattgctgatatcatttacagtaaatagataggcagcagtggtgcgtt
240

|||||
Sbjct: 223937 aacagggttaattcattgctgatatcatttacagtaaatagataggcagcagtggtg-gtt
223879

Query: 241 attgtgataatgtgggttatcggctcacatgggcagttatgagaggctagcatgtttgaa
300

|||||
Sbjct: 223878 attgtgataatgtgggttatc-gctca-atgggcagttatgagaggctagcatgtttgaa
223821

Query: 301 ctattaanaaagtctgggtctttg 322

|||||
Sbjct: 223820 ctattaanaaagtctgggtctttg 223799

☐ gi|21959948|gb|AE013905.1 Yersinia pestis KIM section 305 of
415 of the complete genome
Length = 12521

Score = 456 bits (230), Expect = e-125
Identities = 241/322 (74%), Gaps = 6/322 (1%)
Strand = Plus / Minus

Query: 1 cattgttttttgctccggcgggaaacagttattgtcatagaatagctaaatggcataggt
60

|||||
Sbjct: 4272 cattgttttttgctccggcgggaaacagttattgtcataga-tagctaaatggcatag--
4216

Query: 61 atagctaaatggnnn
120

|||||
Sbjct: 4215 atagctaaacaacatagatagctaaacaacatagatagataaacaacatagatagataaa
4156

Query: 121 nnnnnnnnnnnnnnnntcgcacgtttggcaggttacannnnnnnatcgctcggttaaaat
180

|||||
Sbjct: 4155 caacatagatagataaatcgcacgtttggcaggttacattttttatcgctcggttaaaat
4096

Query: 181 aacagggttaattcattgctgatatcatttacagtaaatagataggcagcagtggtgcgtt
240

|||||
Sbjct: 4095 aacagggttaattcattgctgatatcatttacagtaaatagataggcagcagtggtg-gtt
4037

Query: 241 attgtgataatgtgggttatcggctcacatgggcagttatgagaggctagcatgtttgaa
300

|||||

Query: 1 cattgttttttgctccggcgggaaacagttattgtcatagaatagctaaatggcataggt
60

||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 171096 cattgttttttgctccggcgggaaacagttattgtcatag-atagctaaatggcatag--
171152

Query: 61 atagctaaatggnn
120

|||||||||
Sbjct: 171153 atagctaaacaacatagatagctaaacaacatagatagctaaacaacatagatagctaaa
171212

Query: 121 nnnnnnnnnnnnnnnnn 137

Sbjct: 171213 caacatagatagataaa 171229

☐ [>gi|51587641|emb|BX936398.1|](#) Yersinia pseudotuberculosis IP32953
genome, complete sequence
Length = 4744671

Score = 357 bits (180), Expect = 1e-95
Identities = 175/233 (75%), Gaps = 3/233 (1%)
Strand = Plus / Plus

Query: 90
nnntcgcacgtttg 149

|||||||||
Sbjct: 1402672
catagatagctaaatgacatagatagctaaacaacatagatagataaatcgcacgtttg 1402731

Query: 150
caggttacannnnnnnatcgtcgggttaaataacagggttaattcattgctgatattcatt 209

|||||||||
||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1402732
caggttacatttttttatcgtcgggttaaataacagggttaattcattgctgatattcatt 1402791

Query: 210
tacagtaaataatgataggcagcagtggttgcggttattgtgataatgtgggttatcggctcaca 269

||||||||||||||||||||||||||||||||||||||||||||||||||||
|
Sbjct: 1402792 tacagtaaataatgataggcagcagtggtg-gttattgtgataatgtgggttatc-gctca-
a 1402848

Query: 270 tgggcagttatgagaggctagcatggttgaactattaaaagtctggtctttg 322

||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1402849 tgggcagttatgagaggctagcatggttgaactattaaaagtctggtctttg
1402901

Score = 95.6 bits (48), Expect = 7e-17
 Identities = 65/87 (74%), Gaps = 3/87 (3%)
 Strand = Plus / Plus

Query: 1

cattgttttttgctccggcggggaaacagttattgtcatagaatagctaaatggcataggt 60
 |||
 Sbjct: 1402637 cattgttttttgctccggcggggaaacagttattgtcatag-atagctaaatgacatag-
 - 1402693

Query: 61 atagctaaatggnnnnnnnnnnnnnnnn 87

|||||
 Sbjct: 1402694 atagctaaacaacatagatagataaat 1402720

☐ [>gi|38524371|emb|BX813325.2|](#) Mouse DNA sequence from clone RP23-319D11 on chromosome X, complete sequence
 Length = 62720

Score = 40.1 bits (20), Expect = 3.3
 Identities = 23/24 (95%)
 Strand = Plus / Plus

Query: 26 cagttattgtcatagaatagctaa 49

|||||
 Sbjct: 25622 cagttattgtccttagaatagctaa 25645

☐ [>gi|55734182|emb|BX510332.10|](#) Zebrafish DNA sequence from clone CH211-225M7, complete sequence
 Length = 171212

Score = 40.1 bits (20), Expect = 3.3
 Identities = 20/20 (100%)
 Strand = Plus / Plus

Query: 53 gcataggtatagctaaatgg 72

|||||
 Sbjct: 118047 gcataggtatagctaaatgg 118066

☐ [>gi|21212365|emb|AL683847.12|](#) Mouse DNA sequence from clone RP23-109A3 on chromosome 4, complete sequence
 Length = 212973

Score = 40.1 bits (20), Expect = 3.3
 Identities = 23/24 (95%)
 Strand = Plus / Plus

[gi|31335543|emb|AL954345.11|](#) Mouse DNA sequence from clone ... [40](#)
3.3

Alignments

--

[>gi|45435511|gb|AE017130.1|](#) Yersinia pestis biovar Medievalis str.
91001 section 4 of 16 of the
complete genome
Length = 290510

Score = 79.8 bits (40), Expect = 4e-12
Identities = 43/44 (97%)
Strand = Plus / Minus

Query: 194 atgagaggctagcatggttagaactattaaaaagtctggtctttg 237
|||||
Sbjct: 223842 atgagaggctagcatggttagaactattaaaaagtctggtctttg 223799

Score = 63.9 bits (32), Expect = 2e-07
Identities = 47/52 (90%)
Strand = Plus / Minus

Query: 97 gttaaaataacaagattcattcattgctgatataaattacagtaaattgatag 148
|||||
Sbjct: 223945 gttaaaataacagggttaattcattgctgatattcattacagtaaattgatag 223894

Score = 44.1 bits (22), Expect = 0.21
Identities = 22/22 (100%)
Strand = Plus / Minus

Query: 61 atcgcacgtttggcagggttaca 82
|||||
Sbjct: 223981 atcgcacgtttggcagggttaca 223960

[>gi|21959948|gb|AE013905.1|](#) Yersinia pestis KIM section 305 of
415 of the complete genome
Length = 12521

Score = 79.8 bits (40), Expect = 4e-12
Identities = 43/44 (97%)
Strand = Plus / Minus

Query: 194 atgagaggctagcatggttagaactattaaaaagtctggtctttg 237
|||||
Sbjct: 4000 atgagaggctagcatggttagaactattaaaaagtctggtctttg 3957

[>gi|51587641|emb|BX936398.1|](#) *Yersinia pseudotuberculosis* IP32953
 genome, complete sequence
 Length = 4744671

Score = 79.8 bits (40), Expect = 4e-12
 Identities = 43/44 (97%)
 Strand = Plus / Plus

Query: 194 atgagaggctagcatgttagaactattaaaaagtctggtctttg 237
 |||
 Sbjct: 1402858 atgagaggctagcatgtttgaactattaaaaagtctggtctttg 1402901

Score = 63.9 bits (32), Expect = 2e-07
 Identities = 47/52 (90%)
 Strand = Plus / Plus

Query: 97 gttaaaataacaagattcattcattgctgatataaattacagtaaattgatag 148
 ||| | || |
 Sbjct: 1402755 gttaaaataacagggttaattcattgctgatattacagtaaattgatag
 1402806

Score = 44.1 bits (22), Expect = 0.21
 Identities = 22/22 (100%)
 Strand = Plus / Plus

Query: 61 atcgcacgtttggcaggttaca 82
 |||
 Sbjct: 1402719 atcgcacgtttggcaggttaca 1402740

[>gi|41529689|emb|BX247884.6|](#) Zebrafish DNA sequence from clone
 CH211-174M1 in linkage group 1,
 complete sequence
 Length = 108530

Score = 40.1 bits (20), Expect = 3.3
 Identities = 20/20 (100%)
 Strand = Plus / Minus

Query: 129 taaattacagtaaattgatag 148
 |||
 Sbjct: 39973 taaattacagtaaattgatag 39954

[>gi|52353895|gb|AC122737.7|](#) *Mus musculus* chromosome 3, clone RP23-
 30203, complete sequence
 Length = 179544

Score = 40.1 bits (20), Expect = 3.3
 Identities = 20/20 (100%)
 Strand = Plus / Minus

Query: 120 ttgctgatataaattacagt 139
 |||
 Sbjct: 118804 ttgctgatataaattacagt 118785

[>gi|51988057|gb|AC123647.9|](#) Mus musculus chromosome 3, clone RP23-105J10, complete sequence
 Length = 202415

Score = 40.1 bits (20), Expect = 3.3
 Identities = 20/20 (100%)
 Strand = Plus / Minus

Query: 120 ttgctgatataaattacagt 139
 |||
 Sbjct: 3836 ttgctgatataaattacagt 3817

[>gi|3449324|dbj|AB016883.1|](#) Arabidopsis thaliana genomic DNA, chromosome 5, TAC clone:K12G2
 Length = 45878

Score = 40.1 bits (20), Expect = 3.3
 Identities = 23/24 (95%)
 Strand = Plus / Plus

Query: 96 agttaaataacaagattcattca 119
 |||
 Sbjct: 30014 agttaaataacaagattcattca 30037

[>gi|4225899|gb|AC006525.1|](#) Homo sapiens chromosome 5 clone RP1-4017, complete sequence
 Length = 90604

Score = 40.1 bits (20), Expect = 3.3
 Identities = 20/20 (100%)
 Strand = Plus / Minus

Query: 111 attcattcattgctgatata 130
 |||
 Sbjct: 46871 attcattcattgctgatata 46852

[>gi|59481431|gb|CP000021.1|](#) Vibrio fischeri ES114 chromosome II, complete sequence
 Length = 1332022

Score = 40.1 bits (20), Expect = 3.3
 Identities = 20/20 (100%)
 Strand = Plus / Plus

Query: 122 gctgatataaattacagtaa 141
 |||
 Sbjct: 25226 gctgatataaattacagtaa 25245

[>gi|51571806|emb|BX511079.9|](#) Zebrafish DNA sequence from clone
 CH211-57G18 in linkage group 2,
 complete sequence
 Length = 149877

Score = 40.1 bits (20), Expect = 3.3
 Identities = 20/20 (100%)
 Strand = Plus / Plus

Query: 124 tgatataaattacagtaaat 143
 |||
 Sbjct: 52204 tgatataaattacagtaaat 52223

[>gi|31335543|emb|AL954345.11|](#) Mouse DNA sequence from clone RP23-
 79N6 on chromosome X, complete
 sequence
 Length = 79546

Score = 40.1 bits (20), Expect = 3.3
 Identities = 20/20 (100%)
 Strand = Plus / Minus

Query: 98 ttaaataacaagattcatt 117
 |||
 Sbjct: 76017 ttaaataacaagattcatt 75998

--
 -

Distribution of 20 Blast Hits on the Query Sequence

Mouse-over to show defline and scores. Click to show alignments

Sequences producing significant alignments: Value	Score	E (bits)
gi 45435511 gb AE017130.1 Yersinia pestis biovar Medievali... 5e-10		73
gi 21959948 gb AE013905.1 Yersinia pestis KIM section 305 ... 5e-10		73
gi 15979072 emb AJ414146.1 Yersinia pestis strain C092 com... 5e-10		73
gi 51587641 emb BX936398.1 Yersinia pseudotuberculosis IP3... 5e-10		73
gi 41529689 emb BX247884.6 Zebrafish DNA sequence from clo... 2.2		41
gi 52353895 gb AC122737.7 Mus musculus chromosome 3, clone... 2.2		41
gi 51988057 gb AC123647.9 Mus musculus chromosome 3, clone... 2.2		41
gi 3449324 dbj AB016883.1 Arabidopsis thaliana genomic DNA... 2.2		41
gi 4225899 gb AC006525.1 Homo sapiens chromosome 5 clone R... 2.2		41
gi 59481431 gb CP000021.1 Vibrio fischeri ES114 chromosome... 2.2		41
gi 51571806 emb BX511079.9 Zebrafish DNA sequence from clo... 2.2		41
gi 31335543 emb AL954345.11 Mouse DNA sequence from clone ... 2.2		41

Alignments



[>gi|45435511|gb|AE017130.1|](#) Yersinia pestis biovar Medievalis str.
 91001 section 4 of 16 of the
 complete genome
 Length = 290510

Score = 72.9 bits (36), Expect = 5e-10
 Identities = 42/44 (95%)
 Strand = Plus / Minus

Identities = 22/22 (100%)
Strand = Plus / Minus

Query: 58 atcgcacgtttggcaggttaca 79
|||||
Sbjct: 4139 atcgcacgtttggcaggttaca 4118

☐ [>gi|15979072|emb|AJ414146.1|](#) Yersinia pestis strain CO92 complete genome; segment 6/20
Length = 210050

Score = 72.9 bits (36), Expect = 5e-10
Identities = 42/44 (95%)
Strand = Plus / Plus

Query: 191 atgaggggctagcatggttagaactattaaagctctggtctttg 234
||||| |||||
Sbjct: 171402 atgagaggctagcatggttgaactattaaagctctggtctttg 171445

Score = 64.9 bits (32), Expect = 1e-07
Identities = 47/52 (90%)
Strand = Plus / Plus

Query: 94 gttaaataacaagattcattcattgctgatataaattacagtaaattgatag 145
||||| | || |||||
Sbjct: 171299 gttaaataaacagggttaattcattgctgatatacatttacagtaaattgatag 171350

Score = 44.7 bits (22), Expect = 0.14
Identities = 22/22 (100%)
Strand = Plus / Plus

Query: 58 atcgcacgtttggcaggttaca 79
|||||
Sbjct: 171263 atcgcacgtttggcaggttaca 171284

☐ [>gi|51587641|emb|BX936398.1|](#) Yersinia pseudotuberculosis IP32953 genome, complete sequence
Length = 4744671

Score = 72.9 bits (36), Expect = 5e-10
Identities = 42/44 (95%)
Strand = Plus / Plus

Query: 191 atgaggggctagcatggttagaactattaaagctctggtctttg 234

||||| ||||||||||||||| ||||||||||||||||||||||||
 Sbjct: 1402858 atgagaggctagcatgtttgaactattaaagctctggtctttg 1402901

Score = 64.9 bits (32), Expect = 1e-07
 Identities = 47/52 (90%)
 Strand = Plus / Plus

Query: 94 gttaaaataacaagattcattcattgctgatataaattacagtaaattgatag 145
 ||||||||||||| | || ||||||||||||||||| | |||||||||||||||||
 Sbjct: 1402755 gttaaaataacagggttaattcattgctgatatacatttacagtaaattgatag
 1402806

Score = 44.7 bits (22), Expect = 0.14
 Identities = 22/22 (100%)
 Strand = Plus / Plus

Query: 58 atcgcacgtttggcaggttaca 79
 ||||||||||||||||||||||||
 Sbjct: 1402719 atcgcacgtttggcaggttaca 1402740

☐ [>gi|41529689|emb|BX247884.6|](#) Zebrafish DNA sequence from clone
 CH211-174M1 in linkage group 1,
 complete sequence
 Length = 108530

Score = 40.7 bits (20), Expect = 2.2
 Identities = 20/20 (100%)
 Strand = Plus / Minus

Query: 126 taaattacagtaaattgatag 145
 ||||||||||||||||||||
 Sbjct: 39973 taaattacagtaaattgatag 39954

☐ [>gi|52353895|gb|AC122737.7|](#) Mus musculus chromosome 3, clone RP23-
 30203, complete sequence
 Length = 179544

Score = 40.7 bits (20), Expect = 2.2
 Identities = 20/20 (100%)
 Strand = Plus / Minus

Query: 117 ttgctgatataaattacagt 136
 ||||||||||||||||||||
 Sbjct: 118804 ttgctgatataaattacagt 118785

Sbjct: 25226 gctgatataaattacagtaa 25245

☐ [gi|51571806|emb|BX511079.9|](https://gi.ncbi.nlm.nih.gov/record/51571806) Zebrafish DNA sequence from clone
CH211-57G18 in linkage group 2,
complete sequence
Length = 149877

Score = 40.7 bits (20), Expect = 2.2
Identities = 20/20 (100%)
Strand = Plus / Plus

Query: 121 tgatataaattacagtaa 140
|
Sbjct: 52204 tgatataaattacagtaa 52223

☐ [gi|31335543|emb|AL954345.11|](https://gi.ncbi.nlm.nih.gov/record/31335543) Mouse DNA sequence from clone RP23-
79N6 on chromosome X, complete
sequence
Length = 79546

Score = 40.7 bits (20), Expect = 2.2
Identities = 20/20 (100%)
Strand = Plus / Minus

Query: 95 ttaaaataacaagattcatt 114
|
Sbjct: 76017 ttaaaataacaagattcatt 75998

Lambda	K	H
1.37	0.711	1.31

Gapped

Lambda	K	H
1.37	0.711	1.31

Matrix: blastn matrix:1 -3
Gap Penalties: Existence: 5, Extension: 2
Number of Sequences: 2910708
Number of Hits to DB: 17,962,725
Number of extensions: 986591
Number of successful extensions: 58931
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's gapped: 58931
Number of HSP's successfully gapped: 1
Number of extra gapped extensions for HSPs above 10.0: 58928
Length of database: 13,358,659,363

A: 0
 X1: 11 (21.8 bits)
 X2: 15 (30.0 bits)
 X3: 25 (50.0 bits)
 S1: 13 (25.0 bits)

Results interpretation

Here is an example of one pattern obtained with FPSPD

```
REFERENCE      FPSPD00215
FAMILY         ATP-gua_Ptrans
PATTERN        [VM]-X-[DS]-G-[VI]-X(2)-[LM]-X(3)-E-X(5)-[GY]-X(4)-[IP]-X(4)-
P-X(2)-[QKCP]
1PATTERN       [VM]-X(29)-[QKCP]
POSITION       374
LENGTH         31
SEED VALUE     226
S.F.INDEX      44.013172
M.F.INDEX      26.535751
FUNCTIONAL     3 - Very high functionality
STRUCTURES     1crka:2crk:1bg0:
```

Eleven fields are always present. The meaning of every field is:

```
REFERENCE
Meaning ----- Reference of every pattern in FPSPD.
Use ----- The reference is useful in order to find a pattern using
              the "Find the pattern from a FPSPD-ID" option.
Interest ----- The reference can be mention when citing a pattern to make
                  easier to other people to find the pattern in the
database.
```


APPENDIX B
STATISTICAL ANALYSIS

(Microsoft package for Social Sciences version 14 program, 2006)

Table 4: Comparison of yersiniosis among different age groups of diarrhoeic subjects

Number of subjects					
	Sum of Squares	df	Mean Square	F	P value
Between Groups	34.267	5	6.853	4.673	.004
Within Groups	35.200	24	1.467		
Total	69.467	29			

Post Hoc Tests

Duncan ^a				
Subset for alpha = .05				
Age group	N	1	2	3
51-Above	5	.2000		
41-50	5	.8000	.8000	
31-40	5	1.0000	1.0000	
0-10	5	1.4000	1.4000	
21-30	5		2.4000	2.4000
11-20	5			3.4000

a. Uses Harmonic Mean Sample Size = 5.000.

Table 5: Comparison of occurrence of *Y. enterocolitica* and *Y. pseudotuberculosis* among diarrhoeic patients studied

	Status	N	Mean	Std. Deviation
Number of diarrhoeic	<i>Y. enterocolitica</i>	5	4.2000	2.38747
	<i>Y. pseudotuberculosis</i>	5	5.0000	2.54951

t-test for Equality of Means

	t	df	P value
Number of diarrhoeic patients	-.512	8	.622

Table 6: Comparison of yersiniosis among sex groups of diarrhoeic patients

	Gender	N	Mean	Std. Deviation
Number of diarrhoeic	Male	5	4.2000	2.58844
	Female	5	5.0000	2.54951

t-test for Equality of Means			
	t	df	P value
Number of diarrhoeic Patients	-.492	8	.636

Table 7: Comparison prevalence of yersiniosis among different animals studied

Number of animals					
	Sum of Squares	df	Mean Square	F	P value
Between Groups	2271.350	3	757.117	1.319	.303
Within Groups	9183.600	16	573.975		
Total	11454.950	19			

Post Hoc Tests

Duncan ^a		
Species of animal	N	Subset for alpha = .05
		1
Dogs	5	25.2000
Poultry	5	32.6000
Sheep	5	37.8000
Pigs	5	54.2000

a. Uses Harmonic Mean Sample Size = 5.000.

Table 8: Comparison of *Y. enterocolitica* and *Y. pseudotuberculosis* among different animals studied

	Status	N	Mean	Std. Deviation
Number of animals	Positive YE isolate	4	145.2500	70.85372
	Positive YP isolate	4	32.7500	37.73924

t-test for Equality of Means

	t	df	P value
Number of animals	2.803	6	.031

Table 9: Comparison of prevalence of *Y. enterocolitica* and *Y. pseudotuberculosis* among animals in the study areas

	Status	N	Mean	Std. Deviation
Number of Animals	Positive YE	5	86.2000	52.51857
	Positive YP	5	63.6000	34.26806

t-test for Equality of Means

	t	df	P value
Number of Animals	.806	8	.444

Table 11: Comparison of seasonal distribution of *Y. enterocolitica* and *Y. pseudotuberculosis* in humans

	Status	N	Mean	Std. Deviation
Number in humans	Positive YE	2	12.5000	6.36396
	Positive YP	2	10.5000	7.77817

t-test for Equality of Means

	t	df	P value
Number in humans	.281	2	.805

Table 12: Comparison of seasonal distribution of *Y. enterocolitica* and *Y. pseudotuberculosis* in animals

	Status	N	Mean	Std. Deviation
Number in animals	Positive YE	2	266.0000	90.50967
	Positive YP	2	108.5000	12.02082

t-test for Equality of Means

	t	df	P value
Number in animals	2.440	2	.135

Table 13: Comparison of the distribution of Pathogenic *Y. enterocolitica* (YE) and *Y. pseudotuberculosis* (YP) strains screened by elongated PFGE.

Number of strains					
	Sum of Squares	df	Mean Square	F	P value
Between Groups	473.667	5	94.733	1.446	.330
Within Groups	393.000	6	65.500		
Total	866.667	11			

Post Hoc Tests

Duncan ^a			
Origin	N	Subset for alpha = .05	
		1	
Lettuce	2		1.0000
Sheep	2		1.5000
Fish	2		3.0000
Fermented milk	2		5.5000
Pig	2		10.0000
Human	2		19.0000

a. Uses Harmonic Mean Sample Size = 2.000.

Table 14: Comparison of the distribution of Pathogenic *Y. enterocolitica* (YE) and *Y. pseudotuberculosis* (YP) strains screened by elongated PFGE

	Number of	N	Mean	Std. Deviation
Number of strains	YE	6	9.6667	12.04436
	YP	6	3.6667	2.58199

t-test for Equality of Means

	t	df	P value
Number of strains	1.193	10	.260

APPENDIX C

LIST OF PAPERS PUBLISHED FROM THIS WORK

1. **OKWORI A. E. J., G.O.A. AGADA., A.O. OLABODE., S.E. AGINA., E. S. OKPE., J. OKOPI (2007) The prevalence of pathogenic *Yersinia enterocolitica* among diarrhea patients in Jos, Nigeria. African Journal of Biotechnology Vol. 6 (8): 1031-1034.**
2. **OKWORI, AEJ; A.O. OLABODE., S.E. AGINA., M.O. ODUGBO (2007) Experimental *Yersinia pseudotuberculosis* enteritis in laboratory animals. African Journal of Biotechnology Vol.6 (20) : 2411-2414**
3. **OKWORI A. E. J., AGINA, S.E. OLABODE, A.O (2005). Faecal carriage of *Yersinia* species in pigs, sheep and poultry on display for sale in Vom and Bukuru areas of Jos LGA, Plateau State. Nigeria. *Nig. J. of Microbiol.* Vol. 19:230-234**

LIST OF PAPERS IN PRESS

4. **OKWORI AEJ., DRANCOURT, M., AGINA, S.E., RAOULT, D., TOIDI, E (2005). Multispacer typing of *Yersinia enterocolitica* isolates from Nigeria and France.**
5. **OKWORI, A.E.J., PILAR, O.M., AGINA, S.E., HANNU, K (2006) Molecular characterization of Nigerian *Y. enterocolitica* and *Y. pseudotuberculosis* strains by pulsed field gel electrophoresis (PFGE).**
6. **Okwori A.E.J., Agina, S.E., Olabode, A.O., Ochapa, A.A., Turay, A.A., Lombin, L.H(2008) Profiles of *Yersinia enterocolitica* isolated from apparently healthy pigs in Jos, Nigeria**
7. **Okwori, A.E.J., Olabode, A.O., Yusuf, A.O., Agina, S.E., Okpe, E. S., Onovoh, E.M (2008) Occurrence of *Yersinia enterocolitica* in post appendiceal tissues in Jos, Nigeria**

8. AEJ Okwori., S.E. Agina., S.C. Chollom., A.O. Olabode., R.R. Ashi., M.O. Odugbo., A.O. Owolodun (2007) **Experimental pathogenicity of *Yersinia enterocolitica* strains in laboratory animals**

9. A.E.J. OKWORI., A.O. OLABODE., O.M PILAR., S.E. AGINA ., C. OBIORA., F.C. ONWULIRI., H. KORKEALA (2007) **Occurrence of *Yersinia enterocolitica* in fermented cow milk in Nigeria**

SEMINAR PRESENTATION

1. **Faecial carriage of *Yersinia* species in pigs, sheep and poultry on display for sale in Vom and Bukuru areas of Jos South Local Governments, Plateau State, Nigeria.** A paper presented at the 27th annual conference & general meeting of Nigerian society for Microbiology in Bauchi, December 2003.
2. **Phenogenotypic variability of *Y. enterocolitica* strains isolated in Vom.** A paper presented to Members of staff National Veterinary Research Institute, Vom on the 21st Sept, 2006.
3. **Experimental pathogenicity of *Yersinia enterocolitica* strains in laboratory animals.** Presented at the 31st Annual National Conference, Nigeria Society for Biotechnology, held in Sokoto, October, 2007
4. **Molecular diversity of *Yersinia enterocolitica* strains.** Presented at the 27th Annual conference, The Nigeria Society of Biochemistry and molecular Biology (NSBMB) held in Owerri, November, 2007.
5. **Genotypic variability of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* strains.** Presented at the 20th Annual

Conference, Biotechnology Society of Nigeria (BSN) held in Abakaliki, November, 2007.