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THE PATHOGENIC MECHANISMS OF *YERSINIA ENTEROCOLITICA* DISEASE

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ABSTRACT

Several aspects of the pathogenesis of *Yersinia enterocolitica* were studied. They fall mainly in the following four categories:

(a) Invasion potential and toxin production.

(b) Aspects of host-pathogen interaction.

(c) Salmonella-Yersinia synergy.

(d) The role of iron in the pathogenesis of *Yersinia enterocolitica* infections.

(a) Invasion potential and toxin production.

Various animal models such as rabbits (ileal loops), mice and guinea pigs, as well as HeLa cells, were compared for invasiveness. Of these the rabbit ileal loop and HeLa cell models proved to be the most sensitive. A good correlation was obtained with these two models when *Y. enterocolitica* strains were shown to invade more than 80% of HeLa cells and were also strongly invasive for rabbit mucosa. The former cell culture model can therefore be used as a screening test for invasiveness of this organism.

The sequence of invasive events in the intestinal epithelium was studied histopathologically by making use of light and electron microscopy. From these studies it became clear that:

(i) *Y. enterocolitica* first adhered to the mucous membrane.

(ii) Goblet cells discharged their contents into the intestinal lumen.

(iii) The bacteria penetrated into the mucosa with little damage to the tissues.

(iv) Multiplication of *Y. enterocolitica* occured inside epithelial cells.

The elaboration of toxins was examined by using various models such as the rabbit ileal loop, chinese hamster ovary cells, Y1 mouse adrenal cells and infant mice. Only the latter gave positive results when some *Y. enterocolitica* strains were grown in trypticase soy broth at 25°C for 48 hours. These experiments indicated that a heat-stable toxin was elaborated by the bacteria. However, as this toxin is only produced at incubation temperatures well below 37°C, its role in the pathogenicity of *Y. enterocolitica* could not be ascertained.

(b) Aspects of host-pathogen interactions.

Since no surface adherence factors such as the K88 antigen of certain *Escherichia coli* strains could thus far be demonstrated in *Y. enterocolitica*, it was postulated that the electric charge of the O-antigen may play a role in this respect. Immuno-electrophoretic studies were performed on the O-antigens of various serotypes of this bacterium and it was found that strains showing high invasive potential, exhibited slow migrating O-antigens in an electric field. This characteristic may aid the bacterium in penetrating the mucus barrier and adhering to the mucous membrane.

After it was found that a non-virulent strain was more rapidly cleared from the blood of rats than a virulent one, phagocytosis and killing of these two strains by human granulocytes and macrophages were examined *in vitro*. It was demonstrated that granulocytes phagocytised the bacteria more rapidly than macrophages but that the latter were more effective in killing the organisms. The virulent strain showed a higher resistance to killing by phagocytes which may account for the longer survival of these bacteria in the blood, liver and spleen compared with a non-virulent strain.

(c) Salmonella-Yersinia synergy

In a controlled study, *Y. enterocolitica* and *S. typhimurium* were compared alone and in combination in the rabbit ileal loop model. Prior to inoculation into the loops, *Y. enterocolitica* was incubated at either 25°C or 37°C, while *S. typhimurium* was grown at 37°C only. Examination at 18 hours revealed appreciably more fluid secretion with the *S. typhimurium* - *Y. enterocolitica* combination, when the latter was grown at 25°C, than in the other experiments.

In another experiment it was demonstrated that mice inoculated with *S. typhimurium* 48 hours before challenge with *Y. enterocolitica*, succumbed more readily to the infection than the controls which did not receive salmonellae prior to inoculation with *Y. enterocolitica*.

(d) The role of iron in the pathogenesis of *Y. enterocolitica* infections.

Another factor which may influence the outcome of infection with *Y. enterocolitica* is iron overload. Batches of mice were injected intraperitoneally with 240 μg ferric ammonium citrate and then challenged intragastrically with suspensions of *Y. enterocolitica*. These animals showed a much higher fatality rate than the controls which were not pretreated with iron.

In summary the following conclusions may be drawn from the investigations reported in this thesis:

(i) *Y. enterocolitica* is an invasive pathogen of the salmonella type.

(ii) The electric charge of the O-antigen was shown to correlate with the invasive ability of strains and it was postulated that this

may relate to the ability of the organisms to penetrate mucus barriers and possibly assist adhesion to the mucous membranes.

(iii) Although it was shown that some *Y. enterocolitica* strains are able to elaborate a heat-stable enterotoxin, its role in the pathogenesis of this bacterium is still uncertain.

(iv) Organs such as the liver, spleen and lung, assist in clearing the blood of bacteria.

(v) Yersinia-Salmonella synergy was demonstrated and iron overload was shown to enhance the virulence of *Y. enterocolitica* and/or diminish host resistance.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the Faculty of Medicine, University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.



_____ day of _____, 19__.

ABBREVIATIONS

ATP = Adenosine triphosphate
BP = Butterfield's phospate buffer
BHI = Brain heart infusion
CT = Cholera toxin
cAMP = Cyclic Adenosine-monophosphate
CHOK1 = Chinese hamster ovary K1
DNase = Deoxyribonuclease
EMB = Eosine-Methylene Blue
EDTA = Ethylene-diamine-tetra-acetic acid
FCS = Foetal calf serum
H-E = Hektoen-Enteric
HBSS = Hanks' balanced salt solution
KIA = Kligler's iron agar.
KCN = Potassuim cyanide
LT = Heat-labile toxin
Mdal = Megadalton
MRB = Modified Rappaport's broth
MEM = Minimal essential medium
NA = Nutrient agar.
PBS = Phosphate buffered saline
PF = Vascular permeability factor
PML = Polymorphonuclear leukocyte
R-strains = Typical *Yersinia enterocolitica* except for rhamnose fermentation
RM-strains = *Yersinia enterocolitica* strains atypically positive for melibiose, raffinose, citrate and other reactions
S-strains = Typical *Yersinia enterocolitica* except for negative sucrose reaction.
ST-strains = Extremely inactive *Yersinia enterocolitica* strains
SS-agar = Salmonella-Shigella agar
SSD-agar = Salmonella-Shigella deoxycholate agar
ST = Heat-stable toxin
TSI = Triple sugar iron
TSB = Trypticase soy broth
XLD = Xylose lysine decarboxylase.

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1. HISTORICAL REVIEW OF *YERSINIA ENTEROCOLITICA* INFECTIONS

1.1 INTRODUCTION

The genus *Yersinia* comprises three Gram-negative coccobacillary species, *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* (Bergey's Manual of Comparative Bacteriology, 1975).

One of the earliest descriptions of plague (caused by *Y. pestis*) is found in I Sameul 6:4 (New International Version): "The Philistines asked, 'What guilt offering should we send him?' They replied, 'Five gold tumors and five gold rats, according to the number of the Philistine rulers, because the same plague has struck both you and your rulers ...'" Since then this disease has been described in countless scientific publications as well as historical and popular literature. At present outbreaks in various countries, including South Africa, continue to occur and public health measures against this scourge are being carried out unabatedly. In the Witwatersrand area the Reef Field Plague Committee meets once every three months to discuss the extermination of rodents and fleas in the various areas as well as any signs of new outbreaks of the disease.

Y. pseudotuberculosis was first isolated by Malassez and Vignal in 1883 from guinea pigs displaying tuberculosis-like lesions. In 1953 Masshoff described a purulent abscess-forming mesenteric lymphadenitis in children who had been operated on for acute appendicitis. The following year Knapp and Masshoff established the relationship between this entity and the recovery of *Y. pseudotuberculosis* from mesenteric lymph nodes, thereby confirming its role as a human pathogen.

1.2 ISOLATION OF *Y. ENTEROCOLYTICA* IN THE USA

With regard to *Y. enterocolitica* Schleifstein and Coleman (1939) discussed five bacteria isolated between 1923 and 1939, two from ulcers on the face of adult patients, two from children with acute enterocolitis, and one from a four year old girl where the disease was initially diagnosed as typhoid fever, and later as acute appendicitis.

They described these organisms as having certain biochemical characteristics in common with *Bacillus lignieri* and *Pasteurella pseudotuberculosis*.

In 1964 Frederikson proposed the name *Yersinia enterocolitica* to describe this new bacterial species, including two of the strains described by Schleifstein and Coleman.

While most of the early investigations on yersiniosis were conducted in Europe, relatively little work was done in America until 1968. All the isolations from the United States of America discussed by Sonnenwirth in 1968 came from patients suffering from septicaemia. Further cases were reported by Sonnenwirth in 1970. In 1971 Braunstein *et al* discussed the isolation of *Y. enterocolitica* from a five month old boy with mesenteric lymphadenitis. This bacterium was now isolated in the United States with greater frequency and further cases were reported by Vilinskas *et al* (1971), and Sedgwick and Tilton (1971). In 1973 new isolates, one from the spleen of a patient at autopsy, and twenty-nine other isolations from various sources, were reported by Gutman *et al* and Weaver and Jordan. Bottone *et al* (1974) described thirteen clinical cases of *Y. enterocolitica* infection, only one of which was associated with mesenteric lymphadenitis and belonged to serotype O 8. The twelve remaining strains belonged to serotype O 17 and were isolated from eye, stool, trachea, throat, wound, and urine specimens . These strains were the first isolates in the U.S.A. found to ferment rhamnose and raffinose.

A case of epidemiological interest was described by Keet in 1974. The patient was a 75 year old man with *Y. enterocolitica* septicaemia who claimed that all his food came from tins, and the author came to the conclusion that the patient must have acquired the infection from a mountain stream from which the patient drank water and a similar serotype was isolated. In view of the findings of other investigators (Morris and Feeley, 1976; Schiemann and Toma, 1978) he might however have overlooked another

aspect, viz that the source of infection could have been the contents of one of the tins.

In another report from the U.S.A. in 1975, Jacobs discussed a case of arthritis, with diarrhoea and rash caused by *Y. enterocolitica* in a six month old girl. Subsequently Greenwood *et al* (1975) reported the use of cold enrichment procedures for the isolation of *Y. enterocolitica* from stool specimens.

Until 1975 most laboratory animals were found to be resistant to infection with *Y. enterocolitica*-strains. Carter (1975) however demonstrated the pathogenic potential of a strain in mice infected intragastrically.

In 1976 Chester and Stotsky compared the biochemical and cultural characteristics of rhamnose-positive isolates of *Y. enterocolitica* with typical rhamnose-negative strains and found that unlike rhamnose-negative isolates the former strains could also utilise raffinose and citrate.

Up to that time most investigators examined humans and animals exclusively as reservoirs for *Y. enterocolitica*. However, Morris and Feeley (1976) investigated various foodstuffs for the presence of this bacterium and subsequently isolated it from ice-cream, mussel, oyster, and water. Most of these strains though, belonged to serotypes not ordinarily associated with disease in man (Bergstrand and Windblad, 1974). Highsmit *et al* (1977) incriminated well-water as the source of a water-borne outbreak of yersiniosis. Most of the strains recovered from environmental sources, with the exception of serotype O 3, were non-typable and rhamnose-positive.

In 1978 Schieman and Toma reported 42 isolates of *Y. enterocolitica* from 131 samples of raw milk. Of these isolates only thirteen could be typed serologically and they belonged to seven different serotypes, with type O 5 occurring most frequently. The presence of *Y. enterocolitica* in milk might account for its isolation from ice cream, especially if pasteurization is inadequate.

1.3 ISOLATION OF *Y. ENTEROCOLITICA* IN EUROPE

From an early stage workers in Europe were intensely active in their investigations on the occurrence of *Y. enterocolitica*, at that stage still an unnamed species, in various animal hosts. After the first report of the isolation of this bacterium in man in the United States by Schleifstein and Coleman (1939), Becht (1962) and Daniels (1963) reported its recovery from chinchillas, and Mollaret (1965) from hares, whereas Dickinson and Mocquat (1961) established pigs as a reservoir. The latter two workers named the organism *Pasteurella pseudotuberculosis b*, whilst Hässig *et al* (1949) called it *Pasteurella* X, Mollaret, Germe X, and Schleifstein and Coleman used the name *Bacterium enterocoliticum*.

From 1972 to 1974 Aldová and Limm (1974) investigated the presence of *Y. enterocolitica* in small rodents near Prague. Different serotypes seemed to be area-specific and only type O 8, found in voles *(Microtus arvalus)* is associated with disease in man.

In 1964 Carlsson *et al* described a case of acute terminal ileitis caused by *P. pseudotuberculosis* X, and during the following two years Mollaret and Destombes (1964), Wauters and Mollaret (1965), and Windblad *et al* (1966), reported the isolation of this organism from humans in France and Sweden. During the years that followed, in contrast to the rest of the world, numerous reports on the isolation of *Y. enterocolitica* emanated from Europe (Windblad *et al*, 1966; Niléhn and Sjöström, 1967; Niléhn, 1967). Most of these strains were associated with enteritis and mesenteric lymphadenitis.

In 1967 Ahvonen *et al* reported on *Yersinia*-associated polyarthritis from Finland. About half of the sero-positive patients also had marked erythema nodosum, and only

a few presented with diarrhoea. During the previous year a case of *Y. enterocolitica* liver abscesses was reported from Holland (Bloquiaux, 1968). This condition was only noticed once before (Hässig *et al*, 1949) and was later confirmed by Rabson *et al* (1972).

It is evident from the above that isolations of *Y. enterocolitica* were gradually increasing in Europe during the 1960's, most probably because more investigators were interested in this organism.

Mollaret's (1971) comprehensive paper reviewing 642 confirmed cases of yersiniosis from different sources over the previous four years introduced the literature on this subject in the 1970's, and from then onwards many reports accumulated from France, Sweden, and Finland as well as from Norway, Belgium, and Czechoslovakia.

Thus, Mygind and Thulin (1970) reported on three *Y. enterocolitica*-associated cases of erythema nodosum, and Blum *et al* (1970) discussed a case of a thallasaemic baby from whom *Y. enterocolitica* was isolated. The latter workers speculated on the possibility of the above disease being a predisposing factor to the infection with *Yersinia*.

In Finland 117 strains of *Y. enterocolitica* were isolated by Ahvonen from 6 030 patients as opposed to only three *Y. pseudotuberculosis* strains during the period up till 1972 (Ahvonen, 1972). The difference in the number of isolations of these two species clearly indicates the relative importance of *Y. enterocolitica* as a human pathogen in that country as opposed to *Y. pseudotuberculosis*.

During the same year reports also came from Hällström *et al* (1972) and Laitinen *et al* (1972) in Finland. The former discussed the isolation of *Y. enterocolitica*, mainly serotypes O 3 and O 9, during the period 1965 to 1970.

Most of the patients presented with mesenteric lymphadenitis, a few with gastroenteritis, while some of these also suffered from arthritis and erythema nodosum. The nine patients described by Laitinen *et al* all presented with arthritis, their ages ranging from 22 to 49 years. In most of these cases the arthritis was preceded by fever, diarrhoea, abdominal pain, headache, tiredness, and a feeling of weakness.

The dramatic and explosive interest in *Y. enterocolitica*, especially in Europe, was reviewed in Canada in 1973 in an editorial entitled "The Spectacular Rise of *Yersinia enterocolitica*".

Toivanen *et al* heralded 1973 with a report from Finland on a hospital outbreak of yersiniosis affecting nursing staff. The original source of infection was a young female patient from whom *Y. enterocolitica*, serotype O 9 was isolated. The infection was most probably transmitted sequentially from person to person, although the possibility of infection originating from the patient herself as a single source, cannot be excluded. This is one of the few cases recorded where the source of the outbreak was evident.

Olsovsky *et al* (1975) discussed an outbreak of yersiniosis in two nursery schools in Czechoslovakia. The same kitchen served both schools but, apart from speculation on this common factor, the source of the outbreak was not established.

From Sweden, Windblad (1973) reported on the various disease patterns associated with *Y. enterocolitica*, stressing the importance of serotype O 3 as a very common cause of intenstinal infection in Sweden. In a subsequent paper Bergstrand and Windblad (1974) discussed 31 isolated cases of *Y. enterocolitica* infection in childeren caused by serotype O 3.

In most cases of septicaemia due to Y. enterocolitica, underlying disease such as thallassaemia major, cirrhosis

of the liver, African siderosis, etc, (Mollaret *et al*, 1971; Rabson *et al*, 1972) can be demonstrated. The systemic infection caused by this organism have been described by other workers in elderly patients. Josefsson and Lindberg (1975) reported a fatal case of septicaemia in an 81 year old woman where no underlying disease was apparent.

The role of *Y. enterocolitica* as a cause of arthritis was emphasized by Windblad (1975) when he reported on 74 cases of arthritis associated with *Y. enterocolitica* infection. Of these cases 42 were female and the rest male, and in most cases the acute arthritic stage was preceded by acute enteritis. *Y. enterocolitica* serotype O 3 was isolated from all these patients.

Usually atypical infection involving unusual sites such as the skin, wounds, urinary tract and the eye, are caused by strains other than serotypes O 8 and O 9 (Bottone, 1978), but in 1976 Niemi *et al* discussed the histopathology of skin lesions in 20 patients infected with serotypes O 3 and O 9. The principal histopathological changes in erythema nodosum were septal or diffuse, mild panniculitis and in some cases also necrotizing vasculitis. Perivascular lymphocytic infiltration without vasculitis was the most prominant feature in erythema figuratum.

Most of the reports from France came from Mollaret (1965, 1971, 1972), Wauters and Mollaret (1965), Cornelis *et al* (1973), Alonso *et al* (1975), Alonso and Bercovier (1975) and by 1972 Mollaret had acummulated data on 1 000 cases at the Institut Pasteur and together with Chevalier and De Planche (1964) and Lucas (1965) he also used the strains from his collection to study the biochemical characteristics of *Y. enterocolitica*.

Hagen *et al* (1974) reported from Norway on an ery-

sipelas-like lesion caused by *Y. enterocolitica* serotype O 3 in a 71 year old man. The organism was isolated in pure culture from an abscess on the calf.

Another European country from where the isolation of *Y. enterocolitica* had been reported, was Belgium (Wauters *et al*, 1965, 1970). In 1977 Van Trappen *et al* discussed 36 patients with yersiniosis. The most frequent symptoms were abdominal pain, diarrhoea, and fever. Some of these cases presented with erythema nodosum. In 1978 De Bois *et al* discussed 54 cases of erythema nodosum with raised antibody titres in 21 of these patients. Further evidence of an association between the erythema nodosum and *Y. enterocolitica* was the presence of gastrointestinal symptoms about 2 weeks before the appearence of the skin lesions.

Relatively little work on yersiniosis has emanated from Britain. Chessum *et al* (1971) reported a case of *Y. enterocolitica* infection in a 69 year old man who presented with fever and general malaise and later developed mild tenderness of the left calf. Ampicillin treatment was started, but was discontinued two days later on his admittance to hospital. Forty-eight hours after admission, with no treatment, his temperature became normal, and he was discharged from hospital. From a blood specimen taken the day after the patient was admitted to hospital, *Y. enterocolitica* serotype O 3 was isolated. When the patient was seen 3 weeks later as an outpatient, he was well with no symptoms or abnormal findings.

1.4 ISOLATION OF *Y. ENTEROCOLITICA* IN OTHER COUNTRIES

Hewstone and Davidson (1972) discussed the first isolation of *Y. enterocolitica* in Australia. The patient was a 9 year old boy suffering from thallassaemia major. He presented with abdominal pain and the bacterium was isolated from blood and an abscess on the heel.

From 1973 onwards fairly frequent reports emanated from Canada. Abramovitch and Butas (1973) discussed a case of septicaemia due to *Y. enterocolitica* in a 71 year old woman suf-

fering from alcoholic cirrhosis and iron deficiency anaemia. In 1974 DeLorme *et al* reported 35 cases of yersiniosis in children aged 1 to 15 years, the majority being between 1 and 5 years of age. In two of these cases salmonellae were also isolated. Other reports came from Schieven and Randall (1974), Randall and Bannatyne (1975), and Narasimhan *et al* (1978). The latter authors described a case of bacteraemia in a 71 year old woman in whom no underlying disease could be demonstrated.

South Africa is one of the few countries outside Europe and America exhibiting a sustained interest in *Y. enterocolitica*. The first isolation of this bacterium in the RSA was reported by Koornhof in 1967, and this case was subsequently described in more detail by Rabson and Koornhof and Rabson *et al* in 1972. The first isolations of *Y. enterocolitica* in Japan were also reviewed by Zen-Yoji and Maruyama in this year.

A further report by Rabson *et al* (1975) described 13 cases of generalized *Y. enterocolitica* infection, 6 of whom presented with a septicaemic picture and the rest with subacute localizing infections usually producing hepatic or splenic abscesses. Of the former cases all recovered after antibiotic treatment, whereas 5 of the latter cases died despite appropriate therapy.

Another 14 cases of generalized yersiniosis were described by Robins-Browne *et al* in 1979(b). Eight of these patients presented with rigors. In the differential diagnosis typhoid fever or malaria was usually considered. The remaining 6 patients were all black males with a subacute form of the disease and abdominal pain was a common complaint. Most patients in this group were initially diagnosed as suffering from amoebic hepatitis. Robins-Browne *et al* further reported in 1979(a) on the enterotoxigenicity of three *Y. enterocolitica* strains isolated from children.

From the above it is evident that there was a dra-

matic increase in the isolations of *Y. enterocolitica* during the last decade. This could possibly be ascribed to a genuine increase of the infection (Windblad 1973). An alternative and more probable explanation is that this organism had been prevalent for a long time, but that neither the disease nor the organisms had been recognised. Some of these bacteria might have been erroneously identified as *Y. pseudotuberculosis* (Ahvonen 1972).

1.5 REFERENCES

Abramovitch, H. and Butas, C.A. (1973). Septicaemia due to *Yersinia enterocolitica*. Can. Med. Assoc. J. *109* : 1112-1115.

Ahvonen, P. (1972). Human yersiniosis in Finland. Ann. Clin. Res. *4* : 39-48.

Ahvonen, P., Sievers, K. and Aho, K. (1969). Arthritis associated with *Yersinia enterocolitica* infection. Acta Rheumatol. Scand. *15* : 232-253.

Aldová, E. and Lim. D. (1974). *Yersinia enterocolitica* in small rodents. Communication 1 : Pilot study in two wildlife areas. Zbl. Bakt. Hyg. I. Abt. Orig. A *226* : 491-496.

Alonso, J.M., Bejot, J., Bercovier, H. and Mollaret, H.H. (1975) Sur un groupe de souches de *Yersinia enterocolitica* fermentant le rhamnose. Méd. Mal. Infect. *5* : 480-492.

Alonso, J.M. and Bercovier, H. (1975). Premiers isolements en France de *Yersinia enterocolitica* chez micromammiféres sauvages. Med. Mal. Infect. *5* : 180-181.

Becht, H. (1962). Untersuchungen über die Pseudotuberculose beim Chinchilla. Dtsch. Tieraerztl. Wochenschr. *69* : 626-627.

Bergstrand, C.G. and Windblad, S. (1974). Clinical manifestations of infection with *Yersinia enterocolitica* in children. Acta Paedr. Scand. *63* : 875-877.

Bloquiaux, W. (1968). Multiple leverabscessen door *Yersinia enterocolitica*. T. Gastro-ent. *11* : 578-583.

Blum, D., Viart, P. and Dachy A. (1970). Septicémie à *Yersinia enterocolitica* chez deux enfants atteints de thallassémie majeure. Arch Franq. Pédiat. *27* : 445

Bottone, E.J. (1978). Atypical *Yersinia enterocolitica* : clinical and epidemiological parameters. J. Clin. Microbiol. *7* : 562-567.

Bottone, E.J., Chester, B., Malowany, M.S. and Allerhand, J. (1974). Unusual *Yersinia enterocolitica* isolates not associated with mesenteric lymphadenitis Appl. Microbiol. *27* : 858-861.

Braunstein, H., Tucker, E.B. and Gibson, B.C. (1971). Mesenteric lymphadenitis due to *Yersinia enterocolitica* : report of a case. Am. J. Clin. Pathol. *55* : 506-510.

Buchanan, R.E. and Gibbons, N.E. (Ed.) (1975). *Bergey's Manual of Determinative Bacteriology*. (8th Ed.) The Williams and Wilkins Company, Baltimore.

Carlsson, M.G., Ryd, H. and Sternby, N.H. (1964). A case of human infection with *Pasteurella pseudotuberculosis* X. Acta Pathol. Microbiol. Scand. *62* : 128-132.

Carter, P.B. (1975). Pathogenicity of *Yersinia enterocolitica* for mice. Infect. Immun. *11* : 164-170.

Chessum, B., Frengly, J.D., Fleck, D.G. and Mair, N.S. (1971). Case of septicaemia due to *Yersinia enterocolitica*. Br. Med. J. *3* : 466-468.

Chester, B. and Stotsky, G. (1976). Temperature-dependent cultural and biochemical characteristics of rhamnose-positive *Yersinia enterocolitica* J. Clin. Microbiol. *3* : 119-127.

Cornelis, G., Wauters, G. and Vanderhaeghe. (1973). Présence de β-lactamase chez *Yersinia enterocolitica*. Ann. Microbiol. (Inst. Pasteur). *124* (B) : 139-152.

Daniëls, J.J.H.M. (1963). Untersuchungen an als *Pasteurella pseudotuberculosis* diagnostizierten Stämmen von Chinchillas. Zentralbl. Veterinaermed. *10* : 413-419.

De Bois, I. *et al* (1978). *Yersinia enterocolitica* as a cause of erythema nodosum. Dermatologica. *156* : 65-78.

DeLorme, J., Laverdiere, M., Marineau, B. and Lafleur, L. (1974). Yersiniosis in children. Can. Med. Assoc. J. *110* : 281-284.

Dickinson, A.B. and Mocquat, G. (1961). Studies on the bacterial flora of the alimentary tract of pigs. I. *Enterobacteriaceae* and other Gram-negative bacteria. J. Appl. Bact. *24* : 252-284.

Editorial (1973). The spectacular rise of *Yersinia enterocolitica*. Can. Med. Assoc. J. *108* : 1097.

Frederiksen, W. (1964). A study of some *Yersinia pseudotuberculosis*-like bacteria (*Bacterium enterocoliticum* and *Pasteurella)*. in Proc 14th Scand. Congr. Path. Microbiol, Oslo. Universiteitsforaget Trykningssentral pp 103-104.

Greenwood, J.R., Flanagan, S.M., Pickett, M.J. and Martin, W.J. (1975). Clinical isolation of *Yersinia enterocolitica* : cold temperature enrichment. J. Clin. Microbiol. *2* : 559-560.

Gutman, L.T., Ottesen, E.A., Quan, T.J., Noce, P.S. and Katz, S.L. (1973). An interfamilial outbreak of *Yersinia enterocolitica* enteritis. N. Engl. J. Med. *288* : 1372-1377.

Hagen, A.G., Lassen, J. and Berge, L.N. (1974). Erysipelas-like disease caused by *Yersinia enterocolitica* Scand. J. Infect. Dis. *6* : 101-102.

Hällstrom, K., Sairanen, E. and Ohela, K. (1972). A pilot clinical study on yersiniosis in South-Eastern Finland. Acta Med. Scand. *191* : 485-491.

Hässig, A. Karrer, J. and Pusteria, F. (1949). Über Pseudotuberculose beim Menschen. Schweiz. Med. Wschr. *79* : 971-973.

Hewstone, A.S. and Davidson, G.P. (1972). *Yersinia enterocolitica* septicaemia with arthritis in a thallassaemic child. Med. J. Aust. *1* : 1035-1038.

Highsmith, A.K., Feeley, J.C., Skaliy, P., Wells, J.G. and Wood, B.T. (1977). Isolation of *Yersinia enterocolitica* from well water and growth in distilled water. Appl. Microbiol. *34* : 745-750.

Jacobs, J.C. (1975). *Yersinia enterocolitica* arthritis. Pediatrics *55* : 236-238.

Josefsson, K. and Lindberg, A. (1975). Fatal *Yersinia enterocolitica* septicaemia. Scand. J. Infect. Dis. *7* : 76-77.

Keet, E.E. (1974). *Yersinia enterocolitica* septicaemia N.Y. State J. Med. *74* : 2226-2230.

Knapp, W. and Masshoff, W. (1954). Zur Ätiologie der abszedierenden retikulozytären Lymphadenitis. Dtsch. Med. Wochenschr. *79* : 1266-1271.

Koornhof, H.J. (1967). Pasteurella 'X' (*Yersinia enterocolitica*) infection in man. Abstract. Med. Proc. *13* : 352.

Laitinen, O., Tuuhea, J. and Ahvonen, P. (1972). Polyarthritis associated with *Yersinia enterocolitica* infection, clinical features and laboratory findings in nine cases with severe joint symptoms. Ann. Rheum. Dis. *31*:34.

Mollaret, H.H. (1965). Le laboratoire dans le diagnositc d'infection humaine à bacilli de Malassez et Vignal. Gas mèd. Fr. Vol XXX, 3457-3478.

Mollaret, H.H. (1971). L'infection humaine A "Yersinia Enterocolitica" en 1970 a la lumiére de 642 cas r écents. Aspects cliniques et perspectives epidemiologiques. Pathol. Microbiol. *19* : 189-205.

Mollaret, H.H. (1972). Un domaine pathologique nouveau: l'infection á *Yersinia enterocolitica*. Ann. Biol. Clin. (Paris). *30* : 1 - 6.

Mollaret, H.H., Chevallier, A. and DePlanche, M.C. (1964). Contribution a l'étude d'un nauveau groupe de germes proches du bacille de Malassez et Vignal. I. Characteres culturaux et biochimiques. Ann. Inst. Pasteur (Paris). *107* : 121-127.

Mollaret, H.H. and Destombes, P. (1964). Les germe "X" en pathologie humaine. Presse Med. *72* : 2913-2915.

Mollaret, H.H. and Lucas, A. (1965). Sur les particularités biochemiques des souches de *Yersinia enterocolitica* isoleés chez les lièvres. Ann. Inst. Pasteur. *108* : 121-125.

Morris, G.K. and Feeley, J.C. (1976). *Yersinia enterocolitca:* a review of its role in food hygiene. Bull. World Health Organ. *54* : 79-85.

Mygind, V. and Thulin, H. (1970). *Yersinia enterocolitica:* a new cause of erythema nodosum. Br. J. Dermatol. *82* : 351-354.

Narasimhan, S.L., Schieven, B.C. and Campsall, E.W.R. (1978). Septicemia caused by *Yersinia enterocolitica*. C.M.A. Journal *118* : 682.

Niléhn, B. (1967). Studies on *Yersinia enterocolitica*. Characterization of 28 strains from human and animal sources. Acta Pathol. Microbiol. Scand. *69* : 83-91.

Niléhn, B. Sjöström, B. (1967). Studies on *Yersinia enterocolitica*. Acta Pathal. Microbiol. Scand. *71* : 612-628.

Niemi, K.M., Hannuksela, M. and Salo, O.P. (1976). Skin lesions in human yersiniosis. A histopathological and immunohistological study. Br. J. Dermatol. *94* : 155-160.

Olsŏvsky, Z., Olsăková, V., Chobot, S. and Sviridov, V. (1975). Mass occurrence of *Yersinia enterocolitica* in two establishments of collective care of children. J. Hyg. Epidemiol. Microbiol. Immunol. (Praha) *19* : 22-29.

Rabson, A.R., Hallett, A.R. and Koornhof, H.J. (1975). Generalized *Yersinia enterocolitica* infection. J. Infect. Dis. *131* : 447-451.

Rabson, A.R., Koornhof, H.J., Notman, J. and Maxwell, W.G. (1972). Hepatosplenic abscesses due to *Yersinia enterocolitica*. Br. Med. J. *4* : 341.

Randall, C. and Bannantyne, R.M. (1975). Experience with *Yersinia enterocolitica* at The Hospital for Sick Children, 1972-1974. Can. Med. Assoc. J. *113* : 542-545.

Robins-Browne, R.M., Jansen van Vuuren, C.J., Still, C.S., Miliotis, M.D. and Koornhof, H.J. (1979a). The pathogenesis of *Yersinia enterocolitica* gastroenteritis. In Contr. Microbiol. Immunol. Vol. 5, ed by P.B. Carter, L. Lafleur and S. Toma, S. Karger, Basel. pp 324-328.

Robins-Browne, R.M. Rabson, A.R. and Koornhof, H.J. (1979b) Generalized infection with *Yersinia enterocolitica* and the role of iron. In Contr. Microbiol. Immunol. Vol. 5, ed. by P.B. Carter, L. Lafleur and S. Toma, S. Karger, Basel. pp. 277-282.

Schiemann, D.A. and Toma, S. (1978). Isolation of *Yersinia enterocolitica* from raw milk. Appl. Environ. Microbiol. *35* : 54-58.

Schieven, B.C. and Randall, C. (1974). Enteritis due to *Yersinia enterocolitica*. J. Pediat. *84* : 402-404.

Schleifstein, J.J. and Coleman, M.B. (1939). An unidentified microorganism resembling *B. lignieri* and *Past. pseudotuberculosis*, and pathogenic for man. N.Y. State J. Med. *39* : 1749-1753.

Sedgwick, A.K. and Tilton, R.C. (1971). Biochemical and serological characteristics of a *Yersinia enterocolitica* isolate. Appl. Microbiol. *21* : 383-384.

Sonnenwirth, A.C. (1970). Bacteraemia with and without meningitis due to *Yersinia enterocolitica*, *Edwardsiella tarda*, *Comamonas terrigena*, and *Pseudounonas maltophilia*. Ann. N.Y. Acad. Sci. *174* : 488-502.

Toivanen, P., Toivanen, A., Olkkonen, L. and Aantaa, S. (1973). Hospital outbreak of *Yersinia enterocolitica* infection. Lancet. *1* : 801-803.

Vantrappen, G., Agg. H.O., Ponette, E., Geboes, K. and Bertrand, P.H. (1977). Yersinia enteritis and enterocolitis: gastroenterological aspects. Gastroenterology *72* : 220-227.

Vilinskas, J., Tilton, R.C. and Kriz, J.J. (1971). A new clinical entity : human infection with *Yersinia* presenting as an acute abdomen. Am. Surg. *37* : 568-572.

Wauters, G. and Mollaret, H.H. (1965). Un nouveau cas d'infection humaine a' *Yersinia enterocolitica*. Rev. Belge Pathol. Med. Exp. *31* : 328-

Weaver, R.E. and Jordan, J.C. (1973). Recent human isolates of *Yersinia enterocolitica* in the United States. in Contrib. Microbiol. Immunol. Vol. 2 ed. by S. Windblad, S. Karger Basel, pp. 120-125.

Windblad, S. (1973). The clinical panorama of human yersiniosis. in Contr. Microbiol. Immunol. Vol. 2 ed by S. Windblad. S. Karger, Basel; pp. 129-132.

Windblad, S. (1975). Arthritis associated with *Yersinia enterocolitica* infections. Scand. J. Infect. Dis. *7* : 191-195.

Windblad, S., Niléhn, B. and Sternby, N.H. (1966). *Yersinia enterocolitica (Pasteurella* X) in human enteric infections. Br. Med. J. *2* : 1363-1366.

Zen-Yoji, H. and Maruyama, T. (1972). The first successful isolation and identification of *Yersinia enterocolitica* from human cases in Japan. Jpn. J. Microbiol. *16* : 493-500.

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2. TAXONOMY AND NOMENCLATURE

The name *Yersinia* was proposed by Van Lochem in 1944 for a new genus to separate some members of the *Pasteurella* genus from the haemorrhagic septicaemia group of bacteria. This name was proposed in honour of the French bacteriologist A.J.E. Yersin, who first isolated the plague bacillus *Yersinia (Pasteurella) pestis* in 1894.

Schleifstein and Coleman (1939) called the bacterium now known as *Y. enterocolitica, Bacterium enterocoliticum,* Dickinson and Mocquot (1961) preferred *Pasteurella pseudotuberculosis* type *b,* Hässig *et al* (1949), *Pasteurella X* and *P. pseudotuberculosis* X, while Mollaret and Destombes (1964) used the name Germe X.

In 1954 Thal proposed the inclusion of those organisms comprising the genus *Yersinia (Y. (Pasteurella) pestis* and *Y. (Pasteurella) pseudotuberculosis)* into the family *Enterobacteriaceae.* The validity of this proposal was substantiated by Sneath and Gowan (1958) and Talbot and Sneath (1960) who, in computer analyses of various bacterial species, showed a high degree of similarity between these species and the family *Enterobacteriaceae.*

Frederiksen (1964) was of the opinion that the characteristics of the bacterium discussed by the above authors, were sufficiently distinct to separate it from *Y. pseudotuberculosis,* but on the other hand it resembles *Y. pseudotuberculosis* to such an extent that its classification in the genus *Yersinia* (within the family *Enterobacteriaceae)* as a seperate species, *Y. enterocolitica,* was justified.

In a numerical study of the pasteurellae, Smith and Thal (1965) agreed with Frederiksen's assignment of *"Pasteurella X"* to the genus *Yersinia* and further emphasized its similarity to *Y. pseudotuberculosis* and its distinctiveness from *Y. pestis.* However, because some of the strains examined by Frederiksen were indole-positive, Smith and Thal favoured the less inclusive term *Yersinia X,* until the status of the indole-positive strains could

be established.

Y. enterocolitica is currently classified in the genus *Yersinia* within the family *Enterobacteriaceae*, a residence of dubious distinction according to Knapp and Thal (1973) who stated: "At present there are many strains wandering from collection to collection, labelled *'Yersinia enterocolitica*, but which, in our opinion, cannot be considered as such". They also asked the question: "What is *Yersinia enterocolitica?"* In the opinion of these authors there is sufficient diversity in the biochemical reactions of species designated *Y. enterocolitica* to warrant the establishment of two different species, *Y. enterocolitica* for indole-positive strains, and *Y. enteritidis* for indole-negative strains. They also mentioned that at the sessions of the 'Subcommittee on the taxonomy of *Yersinia, Pasteurella,* and *Francisella* (ICNB)' in Mexico City in 1970, the following statement was recorded in the report of the Subcommittee: "In the future the Subcommittee plans to intensify study of *'Pasteurella X' (Y. enterocolitica),* particularly the biochemical, serological, and genetic aspects of this species; at present there is not enough evidence to permit placing of this species in the genus *Yersinia* together with *Y. pestis* and *Y. pseudotuberculosis."* At a subsequent meeting in 1972, Mollaret and Knapp emphasized the need for genetic studies.

Stevens and Mair (1973) reported on a numerical taxonomic study of *Y. enterocolitica* strains. The principal aim of this work was to discover if a numerical study of 191 strains designated *Y. enterocolitica* disclosed a homogeneous species, or whether certain strains were sufficiently different to form distinct groups. They also included representatives of the family *Enterobacteriaceae,* strains of *Y. pesudotuberculosis, Y. pestis,* and various *Pasteurella* species, primarily as reference or "marker" strains. They found that on the basis of overall simi-

larity, 2 major groups of *Y. enterocolitica* strains exist: the classical variety consisting of 2 subgroups (isolated from humans, pigs, monkeys, chinchillas, and various other sources), and another group of strains isolated from hares. The first two subgroups were called A_1 and A_2 and belong to biotypes three and four respectively, whereas subgroup A_3, the strain found in hares, belongs to biotype five, serotype O 2.

Moore and Brubaker (1975) examined DNA-relatedness among *Y. enterocolitica* strains and showed that those giving both positive and negative reactions concerning indole, xylose, esculin, and gas from glucose fermentation were closely related with DNA-hybridization homologies ranging from 79 % to 100 %. The 11 strains tested were representatives of 9 serotypes. Using a lower temperature of reassociation ($60^{o}C$) than Brenner *et al* (1976) Moore and Brubaker showed a low degree of relatedness (18 - 24 %) between *Y. enterocolitica* and the two recognised members of the genus *Yersinia, Y. pestis* and *Y. pseudotuberculosis*. These data approached those obtained for homology studies among 10 selected isolates of seven genera within the family *Enterobacteriaceae* (2 - 16 %). These investigators suggested that the proposal to maintain *Y. enterocolitica* within the genus *Yersinia* required further evaluation. However, Brenner *et al* (1976) conducted their studies at $60^{o}C$ and $75^{o}C$ (only closely related sequences will associate at the latter temperature) and showed a 40 - 60 % relatedness to *Y. pseudotuberculosis*.

In 1976 Brenner and colleagues performed studies characterizing *Y. enterocolitica* and *Y. pseudotuberculosis* by DNA-hybridization in order to resolve the taxonomic dilemma. Examining biochemically typical and atypical reference strains of *Y. enterocolitica* which gave both positive and negative results for indole, xylose, esculin, and salicin, these investigators, basing their fin-

dings on DNA-relatedness data, concluded that they saw no reason to separate indole positive and indole negative strains from *Y. enterocolitica.*

In their study they recognized three known *Y. enterocolitica* DNA-relatedness groups and added a 4th relatedness group all of which could be biochemically defined. One DNA-relatedness group corresponds to typical *Y. enterocolitica* strains, both indole-positive and negative; the second is composed of rhamnose-positive, raffinose-negative, melibiose-negative, and α-methylglucoside-positive isolates; the third is composed of *Y. enterocolitica* strains that are rhamnose-, melibiose-, α-methylglucoside-, and raffinose-positive; the fourth hybridization consists of sucrose-negative strains. These investigators favoured the retention of all these groups in the genus *Yersinia,* but specified that only the first group is *Y. enterocolitica.* Their data further indicated that *Y. enterocolitica* is 40 - 50 % related to *Y. pseudotuberculosis* and that both *Yersinia* species were distantly but significantly related to other *Enterobacteriaceae,* but not more distant than has already been encountered among many genera of enterobacteria. The *Yersinia* species showed no relationship to *P. multocida* from a taxonomic point of view.

Two of the reactions used to distinguish *Y. enterocolitica* and *Y. pseudotuberculosis* are rhamnose and sucrose. Most strains of *Y. enterocolitica* are sucrose-positive and rhamnose-negative. However, some rhamnose-positive and sucrose-negative strains have been included in *Y. enterocolitica* or referred to as *Y. enterocolitica*-like organisms.

In a further study on DNA-relatedness among strains of *Y. enterocolitica* Brenner *et al* (1977) separated this bacterium into five different groups. There are two types of rhamnose-positive strains. One type is typical except for rhamnose (R). The other is also atypically positive for melibiose, raffinose, citrate, and other re-

actions (RM); There are also two types of sucrose-negative strains. One type is typical except for the negative sucrose reaction (S) but the other, an extremely inactive strain, is also negative for trehalose, ornithine decarboxylase, ortho-nitrophenyl-galactosidase, and nitrate reductase (ST); The sucrose-negative and the rhamnose-positive strains are usually isolated from the environment or from healthy animals (Alonso *et al*,1975). RM strains have been associated with disease (Bottone *et al*, 1974) but not with mesenteric lymphadenitis or enterocolitis which are usually found in infections with typical *Y. enterocolitica*.

DNA data indicate that R, RM, S, and ST strains are more closely related to yersinae than to any other group within the *Enterobacteriaceae* (Brenner *et al*, 1977).

RM strains form a single DNA relatedness group that is about 60 % related to *Y. enterocolitica*, but they show substantial biochemical differences (Bottone *et al*, 1974; Alonso *et al*, 1975; Brenner *et al*, 1976) (table 2-1). These strains are also distinguishable from *Y. enterocolitica* serologically and clinically. Brenner proposed that the name *Y. intermedia* be adopted for these strains.

The R strains are about 60 % related to *Y. enterocolitica*, *Y. intermedia*, and S strains. They are seperable from *Y. enterocolitica* by their positive rhamnose reaction and from *Y. intermedia* by negative reactions for melibiose, raffinose, and α-methyl glucoside (table 2-1). These organisms are isolated mainly from healthy human and animal carriers and from water and food (Alonso *et al*, 1975). The R strains are a seperate species and the name *Y. frederiksenii*, was proposed for them (Brenner, *et al* 1977).

The two groups of sucrose-negative strains, S and ST, have markedly different biochemical characteristics (table 2-1). S strains are most frequently isolated from water and ST strains from hares.

Table 2-1. Biochemical differentiation of *Y. enterocolitica*, *Y. intermedia* (RM strains), *Y. frederiksenii* (R strains), S strains, and ST strains at 37°C.*

Test	*Y. entero-colitica*	*Y. inter-media*	*Y. fred-eriksinii*	S strains	ST strains
Rhamnose	-	+	+	-	-
Raffinose	-	+	-	-	-
Melibiose	-	+	-	-	-
∝-Methyl glucoside	-	+	-	-	-
Sucrose	+	+	+	-	-
Trehalose	+	+	+	-	-
Ornithine decarboxylase	+	+	+	-	-
Nitrate reductase	+	+	+	-	-

* *Y. intermedia* is more active metabolically at 22 °C.

Preliminary DNA-relatedness data indicate that S strains are seperable from *Y. enterocolitica*. Data are not yet available on ST strains. Therefore, any decision on whether S and ST strains are biogroups of *Y. enterocolitica* or one or two seperate species, must await the results of further studies.

2.1 REFERENCES

Alonso, J.M., Bejot, J. Bercovier, H. and Mollaret, H.H. (1975). Sur un groupe de souches de *Yersinia enterocolitica* fermentant le rhamnose. Med. Mal. infect.*5:* 480 - 492.

Bottone, E.G., Chester, B., Malowany, M.S. and Allerhand, J. (1974). Unusual *Yersinia enterocolitica* isolates not associated with mesenteric lympadenitis. Appl. Microbiol. *27:*858-861.

Brenner, D.J. (1979). Speciation in *Yersinia.* in Contr. Microbiol. Immunol. vol. 5 ed. by P.B. Carter *et al*, S. Karger, Basel. pp. 33-43.

Brenner, D.J., Steigerwalt, A.G., Falcao, D.P., Weaver, R.E. and Fanning, G.R. (1976). Characterization of *Yersinia enterocolitica* and *Y. pseudotuberculosis* by DNA-hybridization and by biochemical reactions. Int. J. Syst. Bacteriol. *26:* 180-194.

Dickinson, A.B. and Mocquot, G. (1961). Studies on the bacterial flora of the alimentary tract of pigs. I. *Enterobacterioceae* and other Gram-negative bacteria. J. Appl. Bact. *24 :* 252-284.

Frederiksen, W. (1964). A study of some *Yersinia pseudotuberculosis*-like bacteria *(Bacterium enterocoliticum* and *Pasteurella)* in Proc. 14th Scand. Congr. Path. Microbiol, Oslo. Universiteitsforaget Trykiningssentral. Oslo. pp. 103-104.

Hässig, A., Karrer, J. and Pusteria, I. (1949). Über Pseudotuberculose beim Menschen. Schweiz, med. Wschr. *79 :* 971-973.

Knapp, W. and Thal, E. (1973). Differentiation of *Yersinia enterocolitica* by biochemical reactions. in Contr. Microbiol. Immunol.vol. 2. ed. by S. Windblad, S. Karger, Basel. pp. 10-16.

Mollaret, H.H. and Destombes, P. (1964). Les germe "X" en pathologie humaine. Presse Med. *72* : 2913-2915.

Moore, R.L. and Brubaker, R.R. (1975). Hybridization of deoxyribonucleotide sequences of *Yersinia enterocolitica* and other selected members of the *Enterobacteriaceae*. Int. J. Syst. Bacteriol. *25* : 336 - 339.

Schleifstein, J.J. and Coleman, M.B. (1939). An unidentified micro-organism resembling *B. lignieri* and *Past. pseudotuberculosis*, and pathogenic for man. N.Y. State J. Med. *39* : 1949 - 1753.

Smith, J.E. and Thal, E. (1965). A taxonomic study of the genus *Pasteurella* using a numerical technique. Acta Pathol. Microbiol. Scand. *64* : 213-223.

Sneath, P.H.A. and Cowan, S.T. (1958). An electrotaxonomic survey of bacteria. J. Gen. Microbiol. *19* : 551-565.

Stevens, M. and Mair, N.S. (1973). A numerical taxonomic study of *Yersinia enterocolitica* strains in Contr. Microbiol. Immunol. vol. 2 ed. by S. Windblad, S. Karger, Basel. pp. 17-22.

Talbot, J.M. and Sneath, P.H.A. (1960). A taxonomic study of *Pasteurella septica*, especially of strains isolated from human sources. J. Gen. Microbiol. *22* : 303-311.

Thal, E. (1954). Untersuchungen über *Pasteurella pseudotuberculosis*. Berlingska Baktryckeriet, Lund.

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3. BACTERIOLOGICAL AND CULTURAL CHARACTERISTICS

Schleifstein and Coleman already in 1939 described many of the morphological features of *Y. enterocolitica* recognized at present, viz a motile (at 25° but not at 37°C), Gram-negative rod (figure 3-1), with a tendency to bipolar staining. Considerable morphological variation is exhibited on different artificial media. Coccoid forms about the size of staphylococci and short rods are found in 18 hour cultures on blood agar (Schleifstein and Coleman, 1943; Bottone, 1977). Bacillary forms with definite polar bodies predominate in 18 hour cultures of modified Endo agar. Stained with Loeffler's methylene blue, *Y. enterocolitica* bears a striking resemblance in size and form to corynebacteria (figure 3-3) when cultured on Endo, MacConkey, and Salmonella-Shigella (SS) agar. Schleifstein and Coleman (1943) also noted that on beef infusion agar the rods were thicker than on Endo agar, with rounded ends and some tendency to bipolar staining.

Irrespective of biotype or serotype, the flagellar anatomy of *Y. enterocolitica* has been described as peritrichous at 25°C (Schleifstein and Coleman, 1939; Frederiksen, 1964; Nilehn,1969). Few, or no flagellae are present in poorly motile cells or in cultures grown at 37°C. In broth cultures incubated at 22°C it is actively motile, displaying a tumbling, spinning motion in contrast to the absence of motility or the sluggish movement of a few cells when grown at 37°C. Temperature-dependent motility, as well as other temperature-related phenomena, such as toxin production (Pai *et al*, 1978; Pai and Mors, 1978; Robins-Browne *et al*, 1979 a and b), are hallmarks of this species.

Schleifstein and Coleman (1943) also found that cultures developed readily under anaerobic conditions on plating media for the isolation of intestinal pathogens, viz beef infusion agar without enrichment, desoxycholate citrate, Eosin Methylene Blue (EMB), and modified Endo

agar. After 18 hours incubation, smooth opaque colonies, varying from the size of a pinpoint to about 0,5 mm in diameter, and from 1 to 2 mm after longer incubation, were observed (figure 3-4). Rough colonies with crenated edges were found to develop readily and were larger and more translucent than smooth colonies.

Bottone (1977) found that *Y. enterocolitica* had a propensity towards pleomorphism that was tempered by both age and media composition. When viewed microscopically after 18 hours incubation at either 22 or 37°C on blood agar, the organism presented as a small *Pasteurella*-like coccobacillus with rounded ends. Short rods with apparent bipolar staining may also be observed (figure 3-2).

In older cultures, particularly those incubated at 37°C, pleomorphism may prevail, even on an enriched medium such as blood agar. In liquid media coccobacillary forms and cells displaying modest pleomorphism predominate.

Capsules have not been demonstrated in smears prepared from growth on artificial media, but Bottone (1977) discussed the appearence of capsules in *Y. enterocolitica* in smears made from peritoneal exudates from experimentally infected mice.

Y. enterocolitica grows well on a variety of artificial media (Schleifstein and Coleman, 1939; Niléhn, 1969; Bottone, 1977). Some strains do however, show a marked degree of variability in their ability to grow on the so-called "enteric media", eg while most strains belonging to serotype O 3, O 8, and O 9 (the most frequently encountered serotypes in human infections) grow on Endo, Eosin Methylene Blue (EMB), MacConkey, desoxycholate, Hektoen-Enteric (H-E), SS, and Xyloselysine-decarboxylase (XLD) agars, rhamnose-positive strains of serotype O 14, O 16, and O 17 fail to develop on H-E and SS agars and grow poorly on XLD, even after incubation at 22°C.

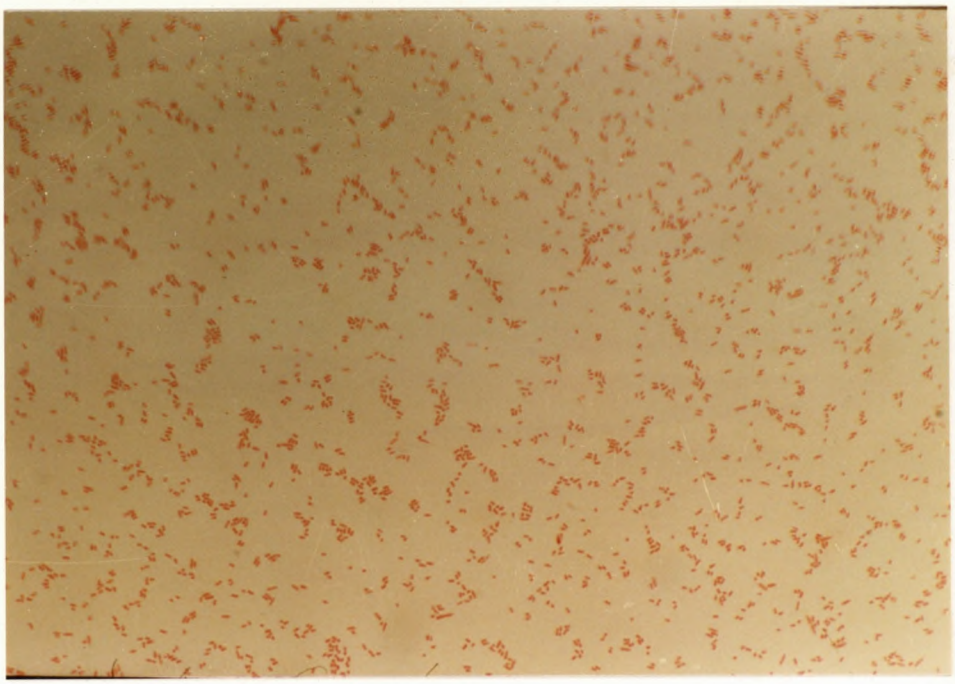

Figure 3-1. Gram stain of an 18-hour culture of *Y. enterocolitica* grown on nutrient agar and incubated at 22°C. X 1200

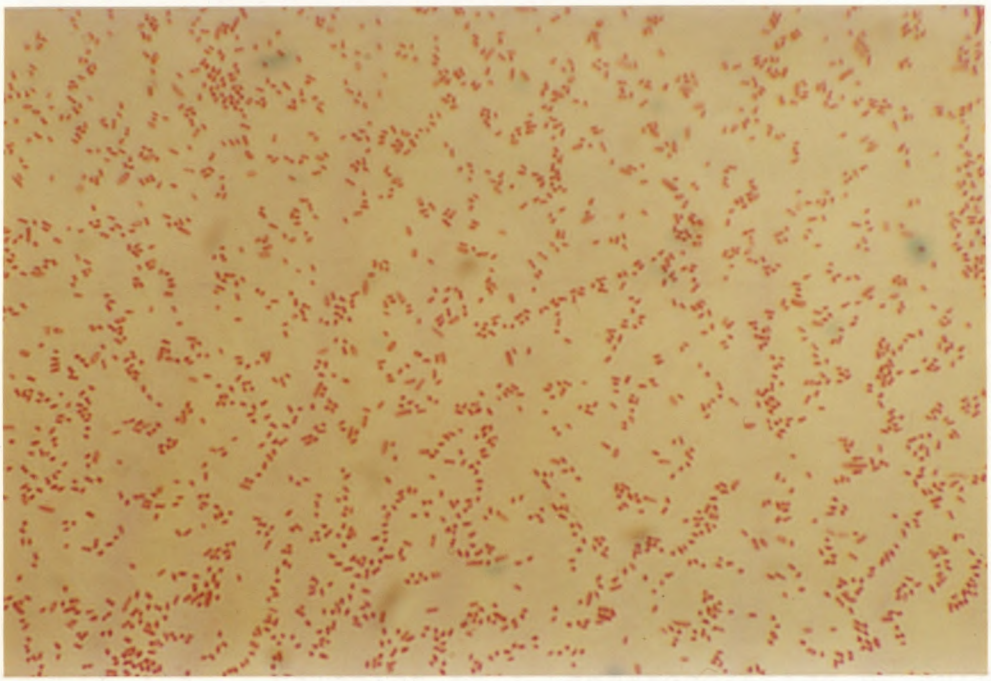

Figure 3-2. Gram stain of *Y. enterocolitica* grown on blood agar for 18 hours at 22°C. X 1200

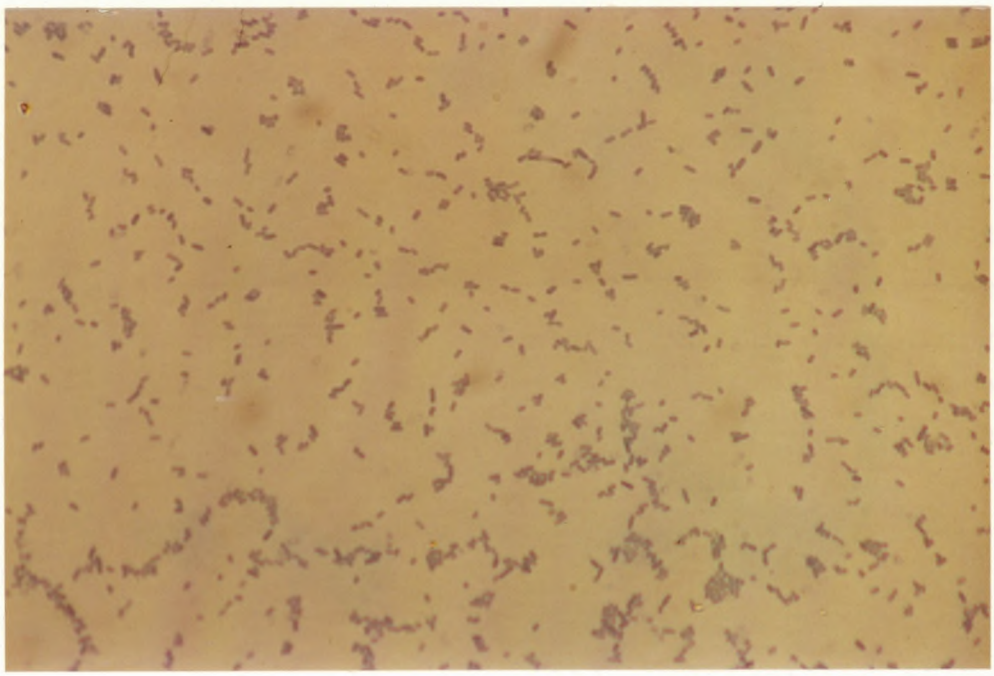

Figure 3-3. Smear of *Y. enterocolitica* stained with Loeffler's methylene blue after 18 hours growth on SS agar at 22°C. x 1200

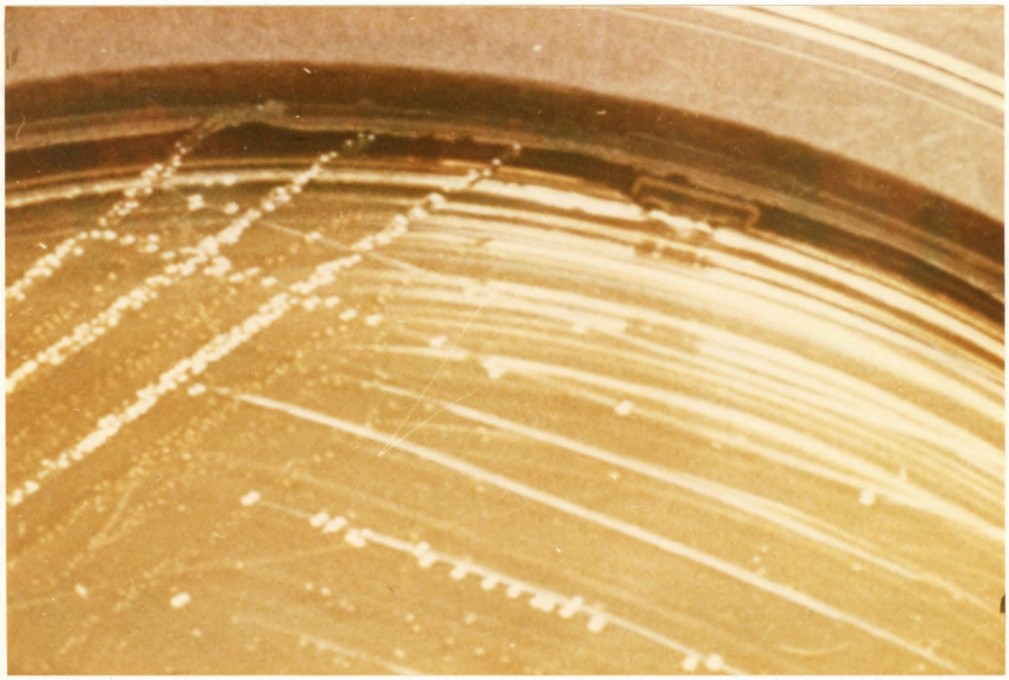

Figure 3-4. Colonies of *Y. enterocolitica* on nutrient agar after 24-hour incubation at 22°C.

The recovery of *Y. enterocolitica* from clinical sources in which it is the sole infecting agent (ie blood and mesenteric lymph nodes) does not present a problem. It is however difficult to isolate this bacterium from specimens containing large numbers of other microorganisms, such as faeces. Apart from the mere presence of these other species, their generation time is only approximately half that of *Y. enterocolitica* (Nilehn, 1969) so that the latter is completely overgrown in culture. The following factors must constantly be borne in mind for the isolation of this species: (i) several "enteric" media, such as MacConkey agar with Crystal Violet, SS agar, SSD (deoxycholate) agar, and Selenite broth, must be utilized; (ii) recognition of *Yersinia* colonies on these media; (iii) the effect of incubation temperature and the length of incubation; (iv) the need for cold enrichment; and (v) when isolated, awareness of the marked similarity of biochemical reactions compared with other members of the family *Enterobacteriaceae*.

Although *Y. enterocolitica* is usually lactose-negative, those strains that grow on XLD and H-E agars may be mistaken for coliforms because they ferment sucrose and xylose in XLD and salicin and sucrose in H-E agar, which renders their colonies indistinguishable from those of *Escherichia, Enterobacter,* and *Klebsiella* species (Toma, 1973).

On enteric media capable of supporting the growth of *Y. enterocolitica* strains, colonies develop slowly. In contrast to the pinpoint colonies of *Yersinia,* those of *E. coli* and *Salmonella* species attain sizes of 4 to 5 mm after the same incubation time. The organism may thus easily be overlooked when present in small numbers and surrounded

by colonies of other enteric organisms, such as found in stool cultures. *Y. enterocolitica* colonies may be disregarded on Eosin Methylene Blue (EMB), agar because of their small size and lavender colouration, which is reminiscent of the growth of faecal streptococci on this medium (Bottone, 1977). To facilitate the isolation of *Y. enterocolitica* from enteric media, Toma (1973) suggested scanning the agar surfaces with the aid of a stereoscopic microscope (magnification X 13) using oblique illumination. Pinpoint colonies may then be selected as potential *Y. enterocolitica*.

Many modified media have been used and proposed by various workers for the isolation if *Y. enterocolitica*. Two such media for the recovery of this bacterium from foods have been described by Lee (1977). In one medium 10g/ℓ Tween 80 and 0,2g/ℓ calcium chloride were added to MacConkey agar. After incubation on this medium for 48 hours at 25°C, lipolytic strains of *Y. enterocolitica* produce flat, wrinkled colonies surrounded by a concentric multizoned area of precipitate which imparts a sheen to the agar medium. While this medium is useful for detecting lipolytic *Y. enterocolitica* of biotypes one and two, not all biotypes have this capability in equal intensity and other enteric organisms may produce identical reactions. The second medium described by Lee to be used in conjunction with cold enrichment procedures consists of 10g/ℓ Tween 80, 10g/ℓ sorbitol, 0,3g/ℓ sodium lauryl sulphate, and 0,2g/ℓ calsium chloride added to deoxyribonuclease (DNase) agar. Following sterilization and cooling, 40mg/ℓ acridine orange and 25mg/ℓ triphenyltetrazolium-chloride are added. On this medium *Y. enterocolitica* colonies are typically colourless or pink and, because of the inhibitory effects of sorbitol, show little or no lipolytic activity.

Temperature and duration of incubation markedly influence the rate of growth of *Yersinia* strains on enteric

media. Nilehn (1969) showed that strains of human and animal origin (including strains of varied bio- and serotypes) differed significantly in their ability to grow on a particular medium at 25°C and 37°C. It may be concluded from the above observations that initial recovery of *Y. enterocolitica*, particularly from faecal material, requires inoculation on duplicate sets of enteric media followed by incubation at both 25°C and 37°C for at least 48 hours.

Cold (4°C to 7°C) enrichment procedures are of value in the recovery of *Y. enterocolitica* from faecal sources, and according to many workers (Nilehn, 1969; Wetzler, 1970; ZenYoji and Maruyama, 1972; Toma *et al*, 1972; Ahvonen, 1972; Jacobs, 1975; Greenwood *et al*, 1975; Eiss, 1975) it gives better results than methods which include subculture of holding broths after incubation for 24 or 48 hours at 37°C. Of the more widely used procedures, one was described by Wetzler (1970) who advocated inoculating isotonic saline with or without 25g/ℓ potassium tellurite, followed by subculture after 24 hours at 4°C to 7°C. Another procedure proposed by Paterson and Cook (1963) utilized 0,067 M phosphate buffered saline (PBS) (pH 7,6) with subculture at intervals ranging up to 28 days. In 1975 Toma and Deidrich chose the method of Paterson and Cook for the examination of 544 faecal samples collected from pigs at an abattoir in Toronto after having established that it yielded the highest isolation rate when compared with three other fluid media.

With cold enrichment procedures *Y. enterocolitica* continues to multiply while most other vegetative microbial species die slowly, thereby enhancing recovery of this bacterium. Ahvonen (1972) reported that of 120 successful isolations of *Y. enterocolitica* from specimens collected from patients suffering from enteritis, 52 were positive only after cold enrichment.

Schiemann and Toma (1978) reported on the isolation of *Y. enterocolitica* from raw milk using four different enrichment procedures, viz:

(i) modified Rappaport's broth (MRB) incubated at room temperature for five days;

(ii) Butterfield's phosphate buffer (BP) pH 7,2, incubated at $4^{o}C$ for 14 days;

(iii) MRB inoculated with 1 mℓ of PB previously incubated at $4^{o}C$ for 14 days and then incubated at $23^{o}C$ for an additional five days; and

(iv) MRB inoculated with 1 mℓ of cooked meat broth incubated initially at $23^{o}C$ for 28 days with subsequent incubation at $23^{o}C$ for five days. The largest number of isolates of *Y. enterocolitica* from the raw milk samples was obtained by using the third method mentioned above. These workers however, also stressed the use of more than one selective medium for the recovery of *Y. enterocolitica*.

During a prevalence study at Chamdor Abattoir, Krugersdorp, the following media were used for the isolation of *Y. enterocolitica:* MacConkey agar with crystal violet, MacConkey agar with a carbenicillin disc, SS agar, SSD agar, Modified Rappaport's agar, Modified Rappaport's Broth and Selenite Broth. In a subsequent study Butterfield's phosphate buffer was also used for cold enrichment.

Four strains of *Y. enterocolitica* serotype O 3 were isolated, of which one came from a healthy gatekeeper and the rest from ceacal contents of pigs. The media that proved most useful were SS agar, SSD agar and selenite broth, subcultured onto SSD agar.

These findings corresponded with the results of a previous study to determine the effectiveness of the above mentioned media. Normal stool samples were mixed with various concentrations of *Y. enterocolitica* and the media were inoculated and incubated at $4^{o}C$, $25^{o}C$ and $37^{o}C$.

3.1 REFERENCES

Ahvonen, P. (1972). Human yersiniosis in Finland. Ann. Clin. Res. *4* : 39-48.

Bottone, E.J. (1977). *Yersinia enterocolitica:* a panoramic view of a charismatic microorganism. CRC. Crit. Rev. Microbiol. *5* : 562-567.

Eiss, J. (1975). Selective culturing of *Yersinia enterocolitica* at a low temperature. Scand J. Infect. Dis. *7* : 249-251.

Frederiksen, W. (1964). A study of some *Yersinia pseudotuberculosis*-like bacteria ("*Bacterium enterocoliticum* and *Pasteurella).* in Proc. 4th. Scand. Congr. Path. Microbiol., Oslo. Universiteitsforaget Trykiningssentral. Oslo. pp. 103-104.

Greenwood, J.R., Flanagan, S.M., Pickett, M.J. and Martin, W.J. (1975). Clinical isolation of *Yersinia enterocolitica* : cold temperature enrichment. J. Clin Microbiol. *2*: 559-560.

Jacobs, J.C. (1975). *Yersinia enterocolitica* arthritis. Pediatrics *55* : 236-238.

Lee, W.H. (1977). Two plating media modified with Tween 80 for isolating *Yersinia enterocolitica.* Appl. Environ. Microbiol. *33* : 215-216.

Niléhn, B. (1969). Studies on *Yersinia enterocolitica.* Acta Pathol. Microbiol. Scand. Suppl. *206* : 1-48.

Pai, C.H. and Mors, V. (1978). Production of enterotoxin by *Yersinia enterocolitica.* Infect. Immun. *19* : 908-911.

Pai, C.H., Mors, V. and Toma, S. (1978). Prevalence of enterotoxigenicity in human and non-human isolates of *Yersinia enterocolitica.* Infect. Immun. *22* : 334-338.

Paterson, J.S. and Cook, R. (1963). A method for the recovery of *Pasteurella pseudotuberculosis* from faeces. J. Pathol. Bacteriol. *85* : 241-242.

Robins-Browne, R.M., Jansen van Vuuren, C.J., Still, C.S., Miliotis, M.D. and Koornhof, H.J. (1979a). The pathogenesis of *Yersinia enterocolitica* gastroenteritis. in Contr. Microbiol. Immun. vol. 5. ed. by P.B. Carter, L. Lafleur and S. Toma. S. Karger, Basel. pp. 324-328.

Robins-Browne, R.M. Still, C.S., Miliotis, M.D. and Koornhof, H.J. (1979b). Mechanism of action of *Yersinia enterocolitica* enterotoxin. Infect. Immun. *25* : 680-684.

Schieman, D.A. and Toma, S. (1978). Isolation of *Yersinia enterocolitica* from raw milk. Appl. Environ. Microbiol. *35* : 54-58.

Schleifstein, J.J. and Coleman, M.B. (1939). An unidentified microorganism resembling *B. lignieri* and *Past. pseudotuberculosis* and pathogenic for man. N.Y. Stad. J. Med. *39* : 1749-1753.

Schleifstein, J.J. and Coleman, M.B. (1943). *Bacterium enterocoliticum*. Annu. Rep. Division of Laboratories and Research, New York, Department of Health, Albany. p. 56.

Toma, S. (1973). Survey on the incidence of *Yersinia enterocolitica* in the province of Ontario. Can. J. Publ. Health *64* : 477-478.

Toma, S. and Deidrick, V.R. (1975). Isolation of *Yersinia enterocolitica* from swine. J. Clin. Microbiol. *2* : 478-481.

Toma, S., Lior, H., Quim-Hill, M., Sher, N. and Walker, W.A. (1972). *Yersinia enterocolitica* infection. Report of two cases. Can. J. Public Health *63* : 433-

Wetzler, T.F. (1970). Animal diseases transmissable to man, pseudotuberculosis. in Diagnostic Procedures for Bacterial, Mycotic, and Parasitic Infections. 5th ed. ed. by H.L. Bodily and E.L. Updyke. Am. Public Health Ass., Washington, D.C. pp. 449-468.

Zen-Yoji, H. and Maruyama, T. (1972). The first successful isolation and identification of *Yersinia enterocolitica* from human cases in Japan. Jpn. J. Microbiol. *16* : 493-500.

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4. BIOCHEMICAL AND SEROLOGICAL CHARACTERISTICS AND PHAGE TYPING

4.1 BIOCHEMICAL CHARACTERISTICS

Apart from the fact that *Y. enterocolitica* strains are diverse in their biochemical characteristics, the utilization of different substrates is also dependent on incubation temperatures. This must always be taken into account when characterizing an isolate of *Y. enterocolitica*. Furthermore the more consistent characteristics must be utilized to establish the identity of this species and to permit its differentiation from other *Enterobacteriaceae*.

The more frequent serotypes associated with disease in man can assimilate a variety of carbohydrates (table 4-1). Glucose, sucrose, arabinose, galactose, mannitol, sorbitol, trehalose, xylose and cellobiose are utilized at 22°C as well as 37°C within 24 hours with slight variations. Rhamnose, raffinose, dulcitol and adonitol are not utilized by these strains, acid from salicin and esculin hydrolysis is strain variable and maltose is utilized best at 22°C. Lactose fermentation usually requires prolonged incubation (up to 30 days) (Niléhn, 1969).

The utilization of the above-mentioned substances may vary slightly with temperature, whereas the Voges-Proskauer reaction, growth in KCN and motility, are completely temperature-dependent and are positive at 22°C and not at all at 37°C (Niléhn, 1969; Bottone, 1977).

Y. enterocolitica does not produce gelatinase, oxidase, phenylalanine deaminase, citratase, lysine carboxylase and arginine dehydrolase enzymes, but possesses catalase, β-galactosidase, orinithine decarboxylase, nitrate reductase type B and urease, while extracellular deoxyribonuclease activities vary from strain to strain (Brenner *et al*, 1976). Urease activity may be delayed or even absent on initial isolation, but it is evident on subculturing within 5 hours (Niléhn, 1969; Bottone *et al*, 1974). Because urease acti-

vity is of fundamental importance in characterizing *Y. enterocolitica*, it is necessary to keep these tests for at least 48 hours before discarding them as negative, or to repeat the test from a subculture of the isolate.

Indole production is variable (Coleman,1950; Niléhn, 1969; Toma *et al*, 1972; Knapp and Thal, 1973) with most European (Windblad *et al*, 1966; Niléhn, 1969; Wauters, 1973) and South African (Rabson and Koornhof, 1972; Robins-Browne *et al*, 1979 b) isolates being reported as indole-negative in contrast to the USA where most isolates are indole-positive (Sonnenwirth, 1968). The first two indole-positive strains of *Y. enterocolitica* isolated in South Africa were reported by Robins-Browne *et al* in 1979(a). The testing for indole production is dependent on the availability of substrate in the media, incubation temperature and also the reagents, such as Ehrlich's or Kovac's, used for the test (Bottone *et al*, 1974). Niléhn (1969) reported strong reactions with Kovac's reagent using tryptone broth incubated at 37°C, and Ahvonen (1972) found that all isolates of serotype O 9 were weakly indole-positive, particularly at 25°C, using the same medium and reagent as Niléhn.

It was shown by Bottone (1977) that *Y. enterocolitica* colonies can easily be mistaken for colonies of other *Enterobacteriaceae*, especially when observed on Hektoen-Enteric (H-E) and xylose-lysine-decarboxylase (XLD) agars. According to him it is common practice in most diagnostic laboratories to pick off lactose-negative colonies for transfer to triple sugar iron (TSI) agar as the initial screening process for the recovery of enteric pathogens from stool specimens. As most *Y. enterocolitica* rapidly ferment sucrose in this agar slant medium (which also contains glucose and lactose as fermentable substances) they render the slant and butt entirely acid (yellow). *Citrobacter*, *Escherichia*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Serratia*, *Aeromonas*, (Bergey's Manual, 1975) or any other bacterial species capable of utilizing either lactose or sucrose, display the

same reaction. Therefore it remains critical when using TSI to further examine any isolate resembling a "urease-positive *E. coli*" as a potential *Y. enterocolitica* by testing for lactose and sucrose independently (Sonnenwirth, 1969).

Bottone (1977) recommends the screening of all stool specimens with Kligler's iron agar (KIA) which lacks sucrose and therefore excludes the confusion engendered by fermentation of this carbohydrate. When an alkaline slant (lactose-negative) and an acid butt (glucose-positive) is found in this medium the isolate is always further tested for *Salmonella* and *Shigella*, but as *Y. enterocolitica* gives the same reaction, it should not be excluded from the organisms under consideration.

The temperature-dependent features of *Y. enterocolitica*, eg. motility, acetylmethylcarbinol production, and growth in KCN are all well known, but in 1974 Bottone *et al* reported on the recovery of 12 unusual isolates of *Y. enterocolitica* whose cultural and biochemical characteristics were markedly influenced by incubation temperature. Among the characteristics displayed at 22°C and either delayed or absent at 37°C were β-haemolysis, citrate utilization (after 48 hours), scant growth on XLD after incubation for 48 hours, and prompt fermentation of rhamnose and raffinose. Further studies of these same strains by Chester and Stotsky (1976) revealed that fermentation of lactose (6 days), melibiose, cellobiose, and β-galactosidase activity were all increased at the lower temperature. These authors also showed that resistance to certain antibiotics were enhanced at 22°C. Since these reports, five more isolates from the Mount Sinai Hospital displaying the same temperature-dependent characteristics were studied by Bottone (1978). Seven of these isolates were inagglutinable with existing antisera, while eight were of serotype O 17, and one each of serotypes O 14 and O 16.

Alonso *et al* (1975) showed that rhamnose-positive strains recovered in Europe differed from typical *Yersinia enterocolitica* and also that the seventeen Mount Sinai strains mentioned above differed from those from Europe in several respects. The European strains all possessed type A nitrate reductase, which was only observed in five of the other rhamnose-positive strains studied. In typical *Y. enterocolitica* strains as well as in most of the rhamnose-positive strains type B nitrate reductase is found and this was absent in the Mount Sinai strains. (See Table 4-1).

Another unusual feature associated with the rhamnose-positive isolates was the agglutination of 15 of the 53 strains with *Y. pseudotuberculosis* antisera. These isolates can be distinguished from *Y. pseudotuberculosis* by the production of indole and ornithine decarboxylase activity, the fermentation of sucrose (Bottone *et al*, 1974; Chester and Stotsky, 1976) and the absence of lysis by specific *Y. pseudotuberculosis* bacteriophage (Alonso *et al*, 1975). According to Alonso and co-workers rhamnose fermentation (although a constant negative characteristic of typical *Y. enterocolitica)* does not exclude the possibility of this bacterium when observed in isolates which have other recognized features of *Y. enterocolitica*.

Rhamnose-positive strains have been recovered before from animals and water sources (Lassen, 1972; Toma and Laufleur 1974; Kapperud and Jonsson, 1976) but since they have been found to infect man, the prompt fermentation of rhamnose and raffinose at 22°C serves to distinguish these unusual isolates.

Because of the biochemical heterogeneity that exists among strains of *Y. enterocolitica* Niléhn first proposed in 1969 that the strains be arranged according to their biochemical reactions into groups or "biotypes". Keeping methodologies and incubation temperatures constant, she investigated 330 *Y. enterocolitica* isolates and, on the

basis of positive characteristics, delineated five biotypes (table 4-2). In the following year Wauters (1970) using lecithinase activity as an additional characteristic and omitting some of Niléhn's substrates, devised a similar classification scheme. On the other hand, Knapp and Thal (1973) separated *Y. enterocolitica* and *Y. enterocolitica*-like organisms into four groups on the basis of production of indole and of acid in xylose, esculin, and salicin. Strains belonging to the first group show typical *Y. enterocolitica* reactions and are either completely negative for the grouping characteristics or variable for acid in xylose. Strains of the second group represent indole-positive *Y. enterocolitica*. Group 3 strains are positive with all the distinguishing tests and they also produce gas in glucose, whereas strains of group 4 are variable in their reactions. The latter two groups are biochemically atypical and these authors expressed the opinion that they do not belong to the species *Y. enterocolitica*. They stated that they do not oppose the creation of biotypes within the framework of a clearcut biochemical pattern, but that they do oppose the creation of new biotypes without such a pattern. They proposed that indole-positive variants be assigned to *Y. enterocolitica* and indole-negative strains be designated *Y. enteritidis* (Knapp and Thal, 1973). As discussed in chapter 2 Brenner and colleagues (1976), however, could find no justification for such division when they studied DNA-relatedness. On the other hand they showed that the atypical strains comprising group 4 of Knapp and Thal are less closely related to typical *Y. enterocolitica* and form a second, and even possibly a third, relatedness group distinct from typical *Y. enterocolitica*.

Table 4-1 Biochemical characteristics of typical *Y. enterocolitica* (Niléhn, 1969).

Test	Result	Test	Result
TSI	Acid/Acid	Urease	+
KIA	Alkaline/Acid	β-Galactosidase	+
Indole	V	Catalase	+
Citrate	0	Oxidase	0
Voges-Proskauer		Gelatinase	0
25°C	+	Nitrate reductase (B)	+
37°C	0	Deaminase	
Fermentation		Phenylalanine	0
Glucose	+	Decarboxylase	
Arabinose	+	Lysine	0
Cellobiose	+	Ornithine	+
Galactose	+	Dihydrolase	
Maltose	+	Arginine	0
Mannitol	+	Deoxyribonuclease	V
Sorbitol	+	Hydrolysis	
Sucrose	V	Esculin	V
Trehalose	+	Tween 80	V
Xylose	V	Growth, KCN	
Lactose	0(L)	25°C	+
Adonitol	0	37°C	0
Dulcitol	0	Motility	
Raffinose	0	25°C	+
Rhamnose	0	37°C	0
Melibiose	0	Hydrogen sulphide	
Salicin	V	TSI	0
		KIA	

TSI : triple sugar iron agar; L : late
KIA : Kligler's iron agar;
V : variable;
+ : posisitve;
0 : negative;

Table 4-2. Biotypes of *Y. enterocolitica* according to Niléhn, Wauters, and Knapp and Thal.

Tests	*Y. enterocolitica* biotypes													
	Niléhn					Wauters					Knapp and Thal			
	1	2	3	4	5	1	2	3	4	5	1	2	3[a]	4[b]
Lecithinase			nt			+	0	0	0	0				
Salicin (acid)	+	0	0	0	0			nt			0	0	+	V
Esculin hydrolysis	+	0	0	0	0			nt			0	0	+	V
Indole production	+	+	0	0	0	++	(L)	0	0	0	0	+	+	V
Xylose (acid)[c]	+	+	+	0	0	+	+	+	0	0	V	+	+	V
Lactose (acid)[c]	+	+	+	0	0	+	+	+	0	0				
Nitrate reduction	+	+	+	+	0	+	+	+	+	0				
Trehalose (acid)	+	+	+	+	0	+	+	+	+	0				
Sorbitol (acid)	+	+	+	+	0			nt						
Ornithine decarb.[c]	+	+	+	+	0	+	+	+	+	0				
Voges-Proskauer[c]	+	+	+	+	0			nt						
β-Galactosidase[c]	+	+	+	+	0	+	+	+	+	0				
Sucrose[c]	+	+	+	+	0			nt						
Sorbose	+	+	+	+	0			nt						

\+ : positive;
0 : negative;
V : variable;
(L) : late;
nt : not tested
a : may produce gas in glucose
b : may show atypical reactions, i.e. lactose, rhamnose-positive
c : test performed at 25°C

It is the opinion of Bottone *et al* (1974) that those bacteria possessing the salient temperature-related biochemical and physiological characteristics of *Y. enterocolitica* (with the possible exccption of the rhamnose positive strains) although varying in some biochemical markers, still belong to this species. In contrast to typical *Y. enterocolitica*, the rhamnose-positive strains show a more extended profile of temperature-dependent biochemical and physiological responses and are agglutinable in some instances with *Y. pseudotuberculosis* antisera (Bottone *et al*, 1974; Alonso *et al*,1975; Chester and Stotsky, 1976). Because representative rhamnose-positive strains have also been found to be genetically distinct from typical *Y. enterocolitica* (both indole-positive and negative strains) (Brenner *et al*, 1976), the question remains as to whether or not these strains should be assigned separate species status within the genus *Yersinia* (See chapter 2).

4.2 SEROLOGICAL CHARACTERISTICS

Y. enterocolitica possesses lipopolysaccharide O-antigens, similar to other Gram-negative bacteria. Experimental immunization of rabbits with O-antigen results in the production of agglutinins. It must be kept in mind that such immunization is experimental and not identical to an infection with living bacteria (Windblad, 1967; Windblad, 1973).

Experimental immunization also produces an O-agglutinin against other Gram-negative bacteria, probably because of a common "gram-negative" antigen. This is illustrated by the cross-agglutination of salmonellae of different groups, *E. coli*, *Proteus*, *Y. pseudotuberculosis* and *Y. pestis*, although the titres of agglutinins against these Gram-negative bacteria are not as high as against *Y. enterocolitica*.

When sera against *Y. enterocolitica* O are absorbed out with Salmonella species or with *E. coli*, the non-specific cross-reacting agglutinins against Gram-negative bacteria disappear completely, whereas low cross-reacting titres

remain which react with the different serotypes of *Y. enterocolitica*. After immunization of rabbits with formalin- or phenol-killed cultures of *Y. enterocolitica*, no cross-reacting agglutinins of the "Gram-negative" type are observed against salmonellae or *E. coli*. In the same way, immunization of rabbits with living cultures of *Y. enterocolitica* results in a rather specific agglutinin with very few cross-reacting antibodies. This correlates with the observation that no cross-reacting O-agglutinins can be demonstrated in humans or animals with *Y. enterocolitica* infection (Windblad, 1973a).

Based on OH (phenol-killed) antigens as well as living bacteria, Windblad, in 1967 introduced a system based on cross-absorption techniques, to determine the O-serotypes of *Y. enterocolitica*. Using this technique, nine serotypes were found, but when more strains were tested, further serotypes came to light (Wauters, 1973; Niléhn, 1973).

From the serological studies of Windblad it also became evident that different serotypes possess different host ranges: chinchilla strains belong to serotype O 1, strains from hares to type O 2 and strains predominent in humans belong to types O 3, O 8 and O 9, although other serotypes have also been isolated from man (Ahvonen, 1972; Windblad, 1973a; Bottone *et al*, 1974; Robins-Browne *et al*, 1979 a). Serotype O 3 has also been found in pigs in many countries (Rabson and Koornhof, 1972; Vandepitte *et al*, 1973; Esseveld and Goudzwaard, 1973, Rabson and Koornhof, 1973; Rakovský *et al*, 1973; Rabson *et al*, 1973) which might indicate a reservoir for human infection.

Aldová and Lim (1974) isolated mainly serotypes O 2 and O 3 from small rodents in one area of Southern Bohemia and O 16 in another region. Biotype 5 predominated in the first area and biotype 1 in the second.

The human serotypes also seem to be country-specific. O 3 is the most common serotype associated with human

infection in Europe (Windblad, 1967; Niléhn, 1969; Hallström *et al*, 1972; Windblad, 1973; Windblad 1973b) and South Africa (Koornhof, 1967; Rabson and Koornhof, 1972; Rabson *et al*, 1972; Rabson *et al*, 1975; Robins-Browne *et al*, 1979 a), whereas serotype O 9 is highly prevalent in countries such as Finland, Belgium and Holland (Ahvonen, 1969; Niléhn and Ericsen, 1969; Szita and Rédey, 1971; De Bois *et al*, 1978) and the predominant serotype in the USA is O 8 (Bottone *et al*, 1974; Keet, 1974; Brenner *et al*, 1976; Bottone 1978; Black *et al*, 1978). Other much less common serotypes infecting man are sometimes found in these countries (Bottone *et al*, 1978; Robins-Browne *et al*, 1979 b).

Wauters grouped *Y. enterocolitica* isolates according to O-factors related to O-serotypes and nineteen different H-factors (Wauters, 1973). The H-patterns are constant in most serogroups and also well defined, whereas in others they can be very complex (tables 4-5 and 4-6). There exists a biochemical homogeneity inside a given serological group and several biochemical characteristics show a close parallelism with the antigenic pattern (table 4-3). Instead of all the varied substrates used by Niléhn (1969), indole-production and lecithinase activity were used in table 4-3 as distinguishing tests for the subdivision of *Y. enterocolitica* into different biotypes. As can be seen the division of lecithinase and indole-negative strains demonstrate a close correlation between individual serotypes and biotypes. Furthermore the strains belonging to this division are also highly host-specific (table 4-4).

The second division consists of lecithinase-negative strains which are weakly indole-positive. These correspond to biotype 2, serogroups O 5,27 and O 9. Serotype O 9 is host-specific but O 5,27 is not.

Table 4-3 Correlation between biotypes and serological groups of *Y. enterocolitica* (Wauters, 1973)

Lecithinase	-Indole	-	Biotype 3	Serotype O : 1
			5	2
			4	3
				15
			2	5B
		+		9
	+Indole	++	1	most others

Table 4-4 Lecithinase-negative strains of *Y. enterocolitica* (Wauters, 1973)

	Biotype	O-factors	H-factors	Origin
Indole -	3	1,2a,3	a,b,c,	chinchilla
	5	2a,2b,3	b,c,	hare
	4	3	a,b,c,	man, pig
	4	15	(b),c	man
Indole +	2	5,27	a,b,c	various
	2	9	a,b	man, pig

Table 4-5 Lecithinase-positive strains of *Y. enterocolitica* (Wauters, 1973).

O-groups	H-factors	Origin
8	b,e,f,i	man
4,5A,6,7,8	complex a-k	various
10,18,19,20,21,22,23,24	complex a-k	man
13,14,16,29	n,m,p	man

Table 4-3 Correlation between biotypes and serological groups of *Y. enterocolitica* (Wauters, 1973)

Lecithinase	-Indole	-	Biotype 3	Serotype O : 1
			5	2
			4	3
				15
			2	5B
		+		9
	+Indole	++	1	most others

Table 4-4 Lecithinase-negative strains of *Y. enterocolitica* (Wauters, 1973)

	Biotype	O-factors	H-factors	Origin
Indole -	3	1,2a,3	a,b,c,	chinchilla
	5	2a,2b,3	b,c,	hare
	4	3	a,b,c,	man, pig
	4	15	(b),c	man
Indole +	2	5,27	a,b,c	various
	2	9	a,b	man, pig

Table 4-5 Lecithinase-positive strains of *Y. enterocolitica* (Wauters, 1973).

O-groups	H-factors	Origin
8	b,e,f,i	man
4,5A,6,7,8	complex a-k	various
10,18,19,20,21,22,23,24	complex a-k	man
13,14,16,29	n,m,p	man

The third division consists of lecithinase-positive and indole strongly positive strains, corresponding approximately to Niléhn's biotype 1. On the other hand these strains display considerable diversity in their O and H antigenic patterns. Moreover, most of the O serogroups are represented by a limited number of known strains, the H antigens of which are often different and very complex; the H-patterns may vary within a given O-group and always contain factors different from a, b and c (Table 4-5). The only common serotype present in biotype 1, is O 8 which has a constant H-pattern consisting of factors b, e, f, i. It seems that host-specificity correlates fairly well with the presence of specific H antigens within the O-groups.

Considering its complex antigenic structure it is not surprising that cross-reactions exist between *Y. enterocolitica* and other bacteria, eg. *Brucella abortus* (Hurvell and Lindberg 1973; Asakawa *et al* 1973; Hurvell, 1975). It was also demonstrated that patients with thyroid disorders showed raised antibody titres against *Y. enterocolitica* (Shenkman and Bottone, 1976; Becht *et al*, 1977), comparable to the cross-reacting antigens observed between other bacteria and human tissue (Kaplan and Meyeresian, 1965; Springer 1971).

To distinguish clearly between *Y. enterocolitica* and other members of the *Enterobacteriaceae*, especially in experimental infections, immunofluorescent techniques have been used (Fribourg-Blanc, 1971; Karlsson *et al*, 1973).

Furthermore the O-group antigens of *Y. enterocolitica* are used extensively in clinical investigations where, in many cases, positive serological findings are the only proof of infection with *Y. enterocolitica* (Windblad *et al*, 1966; Szita *et al*, 1971; Esseveld and Goudzwaard, 1973; Asakawa *et al*, 1973; Toivanen *et al*, 1973; Gutman *et al*, 1973; Maeland and Digranes, 1973; Granfors *et al*, 1978).

4.3 PHAGE TYPING

Phage types of *Y. enterocolitica* were discussed by various authors (Mollaret and Nicolle, 1965; Niléhn and Ericsen, 1969; Niléhn, 1969, Nicolle *et al*, 1973; Niléhn, 1973). Most of the *Y. enterocolitica* strains are typable in this way. Human isolates of serotypes O 3 and O 8 seem to be specific for certain regions eg. the phage typing done by Niléhn (1973) showed that isolates from the USA belong mainly to phage type 8, those from Canada to type Ic, and those from South Africa to type Ia. The strains from Zaïr, Finland, Belgium, Hungary and Czechoslovakia belong mainly to type 3. Although the phage types often are country-specific, they are not species-specific (Niléhn, 1973; Nicolle *et al*, 1973). Strains isolated in the RSA are sent to the Institute Pasteur for typing and so far all the strains belonging to serotype O 3 were phage type IX (Mollaret) (Rabson and Koornhof, 1972; Rabson *et al*, 1972; Rabson and Koornhof, 1973; Robins-Browne *et al*, 1977).

Two strains isolated in 1978, belonging to serotypes O 5 and O 6, were both phage type X (Mollaret) (Robins-Browne *et al*, 1979b).

From the above it is evident that *Y. enterocolitica* is a very diverse organism and that much research still has to be done to clearly define its structural and biochemical heterogeneity in relation to its environment including host specificity and pathogenesis.

4.4 REFERENCES

Ahvonen, P. (1972). Human yersiniosis in Finland. Ann. Clin. Res. *4* : 39-48.

Aldová, E. and Limm. D. (1974). *Yersinia enterocolitica* in small rodents. Communication 1 : Pilot study in two wildlife areas. Zbl. Bakt. Hyg. I. Abt. Orig. A*226* : 491-496.

Alonso, J.M., Bejot, J., Bercovier, H., and Mollaret, H.H. (1975). Sur un groupe de souches de *Yersinia enterocolitica* fermentant le rhamnose. Méd. Mal. infect. *5* : 480-492.

Asakawa, Y., Akahane, S., Kagata, N., Noguchi, M., Sakazaki, R. and Tamura, K. (1973). Two community outbreaks of human infection with *Yersinia enterocolitica*. J. Hyg. (Camb.) *71* : 715-723.

Becht, K., Clemmensen, O., Larsen, J.H. and Bendixen, G. (1977). Thyroid disease and Yersinia. Lancet. 1977: 1060-1061.

Bergey's Manual of Determinative Bacteriology. (1975) Ed. by R.E. Buchanan and N.E. Gibbons. The Williams and Wilkens Co. Baltimore. pp 330-333.

Black, R.E., Jackson, R.J., Tsai, T., Medvesky, M., Shayegani, M., Feeley, J.C., Macleod, K.I.E. and Wakelee, A.M. (1978). Epidemic *Yersinia enterocolitica* infection due to contaminated chocolate milk. N. Engl. J. Med. *298* : 76-79.

Bottone, E.J., Chester, B., Malowany, M.S. and Allerhand, J. (1974). Unusual *Y. enterocolitica* isolates not associated with mesenteric lymphadenitis. Appl. Microbiol. *27* : 858-861.

Bottone, E.J. (1977). *Yersinia enterocolitica*, a panoramic view of a charismatic microorganism. CRC. Crit. Rev. Microbiol. *5* : 211-241.

Bottone, E.J. (1978). Atypical *Yersinia enterocolitica:* Clinical and epidemiological parameters. J. Clin. Microbiol. *7 :* 562-567.

Brenner, D.J., Steigerwalt, A.G., Falcãs, D.P., Weaver, R.E. and Fanning, G.R. (1976). Characterization of *Yersinia enterocolitica* and *Y. psendotuberculosis* by DNA hybridization and by biochemical reactions. Int. J. Syst. Bacteriol. *26 :* 180-194.

Chester, B. and Stotsky, G. (1976). Temperature - dependent cultural and biochemical characteristics of rhamnose-positive *Yersinia enterocolitica.* J. Clin. Microbiol. *3 :* 119-127.

Coleman, M.B. (1950). *Bacterium enterocoliticum.* Annu. Rep. Div. Res. N.Y. State Dept. Health. p. 68.

BeBois, J. *et al* (1978). *Yersinia enterocolitica* as a cause of erythema nodosum. Dermatologica. *156 :* 65-78.

Esseveld, H. and Goudzwaard, C. (1973). On the epidemiology of *Yersinia enterocolitica* infections. Pigs as the source of infection in man. in Contr. Microbiol. Immunol. vol. 2, ed. by S. Windblad, S. Karger, Basel. pp. 99-101.

Fribourg-Blanc, A. (1971). Immunofluorescence study of antigen relationships between *Brucella* and some strains of *Yersinia enterocolitica.* Am. N.Y. Acad. Sc. *177 :* 37-38.

Granfors, K., Viljonen, M.K., Ahvonen, P. and Toivanen, P. (1978). Measurement of IgM and IgG antibodies to *Yersinia* by solidphase radio-immunoassay. J. Infect. Dis. *138 :* 232-236.

Gutman, L.T., Ottesen, E.A., Quan, T.J., Noce, P.S. and Katz, S.L. (1973). An inter-familial outbreak of *Yersinia enterocolitica* enteritis. N. Engl. J. Med. *288 :* 1372-1377.

Hällstrom, K., Sairanen, E. and Ohela, K. (1972). A pilot clinical study on yersiniosis in South-Eastern Finland. Acta Med. Scand. *191* : 485-491.

Hurvell, B. and Lindberg, A.A. (1973). Immuno chemical studies on the cross-reactions between *Brucella* species and *Yersinia enterocolitica* type 9. in Contr. Microbiol. Immunol. vol. 2. ed. by S. Windblad, S. Karger, Basel. pp. 159-168.

Hurvell, B. (1975). Differentiation of cross-reacting anti-bodies against *Brucella abortus* and *Yersinia enterocolitica* by electroimmuno assay. Acta Vet. Scand. *16* : 318-320.

Kaplan, M.H. and Meyeresian, M. (1962). An immunologic cross-reaction between group A streptococcal cells and human heart tissue. Lancet. *1* : 706-710.

Kapperud, G. and Jonsson, B. (1976). *Yersinia enterocolitica* in brown trout (*Salmo trutta L*) from Norway. Acta Pathol. Microbiol. Scand. *84* : 66-68.

Karlsson, K.A., Hurvell, B. and Thal, E. (1973). On the detection of *Yersinia enterocolitica* by the immunofluorescent method. in Contr. Microbiol. Immunol. vol. 2. ed. by S. Windblad, S. Karger, Basel pp. 71-75.

Keet, E.E. (1974). *Yersinia enterocolitica* septicaemia. N.Y. State J. Med. *74* : 2226-2230.

Knapp, W. and Thal, E. (1973). Differentiation of *Yersinia enterocolitica* by biochemical reactions in Contr. Microbiol. Immunol. vol. 2, ed. by S. Windblad, S. Karger, Basel, pp. 10-16.

Koornhof, H.J. (1967). Pasteurella 'X' *Yersinia enterocolitica*] infection in man. Abstract. Med. Proc. *13* : 352.

Lassen, J. (1972). *Yersinia enterocolitica* in drinking water. Scand. J. Infect. Dis. *4* : 125-127.

Maeland, J.A. and Digranes (1975). Human serum antibodies against heat-stable antigens from *Yersinia enterocolitica*. Acta Pathol. Microbial. Scand. Sect. B. *83* : 451-456.

Mollaret, H.H. and Nicolle, P. (1965). Sur la frequence de la lysogéniédans léspèe nouvelle *Yersinia enterocolitica*. C.R. Acad. Sci. *260* : 1027-1029.

Nicolle, P., Mollaret, H. and Brault, J. (1973). Recherches sur la lysogénie la lysosensibilité la lysotypie et la sérologie de *Yersinia enterocolitica* in Contr. Microbiol. Immunol. vol. 2. ed. by S. Windblad, S. Karger, Basel. pp. 54-58.

Niléhn, B. (1969). Studies on *Yersinia enterocolitica*. Acta, Pathol. Microbiol. Scand. Suppl. *206* : 1-48.

Niléhn, B. and Ericsen, C. (1969). Studies on *Yersinia enterocolitica*. Bacteriophages liberated from chloroform treated cultures. Acta Pathol. Microbiol. Scand. *75* : 177-187.

Niléhn, B. (1973). Host range, temperature characteristics and serologic relationships among *Yersinia* phages in Contr. Microbiol. Immunol. vol. 2 ed. by S. Windblad, S. Karger, Basel. pp. 59-67.

Rabson, A.R. and Koornhof, H.J. (1972). *Yersinia enterocolitica* infections in South Africa. S. Afr. Med. J. *46* : 798-803.

Rabson, A.R. and Koornhof, H.J. (1973). *Yersinia enterocolitica* infections in South Africa. in Contr. Microbiol. Immunol. vol. 2. ed. by S. Windblad, S. Karger, Basel. pp. 102-105.

Rabson, A.R., Koornhof, H.J., Notman, J. and Maxwell, W.G. (1972). Hepatosplenic abscesses due to *Yersinia enterocolitica*. Br. Med. J. *4* : 341.

Rabson, A.R., Hallett, A.R. and Koornhof, H.J. (1975). Generalized *Yersinia enterocolitica* infection. J. Infect. Dis. *131* : 447-451.

Rakovsky, J., Paucková, V. and Aldová, E. (1973). Human *Yersinia enterocolitica* infections in Czechoslovakia. in Contr. Microbiol. Immunol. vol. 2, ed. by S. Windblad, S. Karger, Basel. pp. 93-98.

Robins-Browne, R.M. and Koornhof, H.J. (1977). Recent advances in infantile gastroenteritis research. S. Afr. J. Sci. *73* : 105-108.

Robins-Browne, R.M., Jansen van Vuuren, C.J., Still, C.S., Miliotis, M.D. and Koornhof, H.J. (1979a). The pathogenesis of *Yersinia enterocolitica* gastroenteritis. in contr. Microbiol. Immunol. vol. 5. ed. by P.B. Carter, L. Lafleur and S. Toma. pp. 324-328.

Robins-Browne, R.M., Jacobs, M.R., Koornhof, H.J. and Mauff, A.C. (1979b). *Yersinia enterocolitica* biotype 1 in South Africa. S. Afr. Med. J. *55* : 1057-1059.

Shenkman, L. and Bottone, E.J. (1976). Antibodies to *Yersinia enterocolitica* in thyroid disease. Ann.Int. Med. *85* : 735-739.

Sonnenwirth, A.C. (1968). Bacteraemia with and without meningitis due to *Yersinia enterocolitica, Edwardsiella tarda, Comamonas terrigena,* and *Pseudomonas maltophilia.* Ann. N.Y. Acad. Sci. *174* : 488-502.

Sonnenwirth, A.C. (1969). *Yersinia enterocolitica* as an etiologic agent in maningitis. Bact. Proc. *128* : 87.

Springer, G.F. (1971). Blood-group and Forssman antigenic determinants shared between microbes and mammalian cells. Proc. Allergy. *15* : 9-77.

Szita, J., Kâli, M. and Redey, B. (1971). Serological diagnosis of *Yersinia enterocolitica.* Acta Microbiol. Acad. Sci. Hung. *18* : 113-118.

Toivanen, P., Toivanen, A., Olkkonen, L. and Aantaa, S. (1973). Hospital outbreak of *Yersinia enterocolitica* infection. Lancet. *1* : 801-803.

Toma, S. and Lafleur, L. (1974). Survey on the incidence of *Yersinia enterocolitica* infection in Canada. Appl. Microbiol. *28* : 469-473.

Vandepitte, J., Wauters, G. and Isebaert, A. (1973). Epidemiology of *Yersinia enterocolitica* infections in Belgium. in Contr. Microbiol. Immunol. vol. 2. ed. by S. Windblad, S. Karger, Basel. pp. 111-119.

Wauters, G. (1970). Contribution a lêtude da *Yersinia enterocolitica.* Catholic University of Louvain. Vander, Louvain, Belgium.

Wauters, G. (1973). Correlation between ecology, biochemical behaviour and antigenic properties of *Yersinia enterocolitica.* in Contr. Microbiol. Immunol. vol. 2. ed. by S. Windblad, S. Karger, Basel. pp. 38-41.

Windblad, S., Nilêhn, B. and Sternby, N.H. (1966). *Yersinia enterocolitica* (Pasteurella X) in human enteric infections. Br. Med. J. *2* : 1363-1366.

Windblad, S. (1967). Studies on serological typing of *Yersinia enterocolitica.* Acta Pathol. Microbiol. Scand. Suppl. *187* : 115.

Windblad, S. (1973a). Studies on the O-serotypes of *Yersinia enterocolitica.* in Contr. Microbiol. Immunol. vol. 2. ed. by S. Windblad, S. Karger, Basel. pp. 27-37.

Windblad, S. (1973b). The clinical panorama of human yersiniosis. in Contr. Microbiol. Immunol. vol. 2. ed. by S. Windblad, S. Karger, Basel. pp. 129-132.

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5. EPIDEMIOLOGY AND PATHOGENESIS

5.1 EPIDEMIOLOGY

In spite of the intense interest in yersiniosis in recent years, including many efforts to uncover its mode of transmission, relatively little is known about the epidemiology of *Y. enterocolitica*. It has been isolated from various animal species (Mollaret, 1971; Hubbert, 1972; Kapperud, 1975; Toma and Deidrick, 1975; Kapperud and Johnson, 1976). eg. rodents (Kapperud, 1975; Alonso and Bercovier, 1975), hares (Mollaret and Lucas, 1965), chinchillas (Becht, 1962; Hubbert, 1972; Vandepitte *et al* 1973) and also from water (Lassen, 1972; Toma, 1973). These isolations, however are mostly strains of sero- and biotypes not usually associated with human disease. On the other hand sero- and phage types of *Y. enterocolitica* identical to those causing disease in man have been isolated from pigs in various countries (Mollaret, 1971; Rabson and Koornhof, 1972; Ahvonen *et al*, 1973; Tsubokura *et al*, 1973; Esseveld and Goudzwaard, 1973; Bottone *et al*, 1974; Zen-Yoji *et al*, 1974; Toma and Deidrick, 1975). Household pets have also been incriminated as sources of infection (Ahvonen *et al*, 1973). Although the reservoir of infection could only be established in very few instances, patients suffering from infection with *Y. enterocolitica* often gave a history of contact with pork or pigs (Rábson *et al*, 1972; Szita *et al*, 1973; Rakovský *et al*, 1973; Rabson and Koornhof, 1973).

In some European countries it has been shown that outbreaks of yersiniosis coincide with the domestic slaughter of pigs in households during festive seasons from December to March (Rakovský *et al*, 1973), with the majority of cases reported during the late autumn and winter months (Arvastson *et al*, 1971; Mollaret, 1972; Vandepitte *et al*, 1973; Rakovský *et al*, 1973; DeLorme *et al*, 1974). In South Africa the incidence of yersiniosis coincides mainly with late summer and autumn (Robins-Browne *et al*, 1979).

Although absolute proof is still lacking, there is little doubt that *Y. enterocolitica* can spread from person to person via direct or indirect contact. This is corroborated by the many cases of yersiniosis in young children where there had been no history of animal contact (Albert and Laffleur, 1971; Zen-Yoji and Maruyama, 1972; DeLorme *et al*, 1974; Bergstrand and Windblad, 1974), and by spread in closed establishments (Zen-Yoji *et al*, 1973; Toivanen *et al*, 1973; Olsovsky *et al*, 1975) and families as well as by interfamilial outbreaks (Ahvonen and Rossi, 1970; Gutman *et al*, 1973; Szita *et al*, 1973). In the latter instances the outbreaks were not explosive in nature. Various individuals became ill one after another, which is more compatible with person-to-person transmission rather than spread through animal or food sources. This is further supported by the discovery of asymptomatic carriers (Mollaret 1972).

The possibility of wild animals being "natural" carriers of *Y. enterocolitica* cannot be excluded because strains able to cause disease in humans, have been isolated from wild animals (Wetzler and Hubbart, 1968; Hubbart, 1972). In his search for a source of infection in a case of septicaemia attributed to *Y. enterocolitica*, Keet (1974) isolated a strain, identical to the causative organism in the patient, from a mountain stream. The possibility thus exists that the water could have become contaminated by deer or other animal faeces.

From the above it can be concluded that although the reservoir has been found in a few cases of yersiniosis and that the mode of transmission was also clear in some instances, much has still to be learnt about the epidemiology of *Y. enterocolitica*.

5.2 PATHOGENICITY

The pathogenicity of *Y. enterocolitica* for experimental animals, was for the first time discussed by Schleifstein and Coleman in 1939. They found it highly virulent

for mice, infections having been established intraperitoneally, subcutaneously, or orally with cultures or organ emulsions from infected animals. After intraperitoneal infection, death usually ensued within two to three days, whereas mice fed with organ emulsions died within nine to twenty-six days. These authors also found that pathogenicity of *Y. enterocolitica* for other laboratory animals such as white rats, rabbits, and guinea pigs was variable. The white rat was moderately susceptible, the guinea pig was easily infected via the intraperitoneal route, but not via the subcutaneous and oral routes, whereas only one of three rabbits showed any signs of disease.

At autopsy wasting and emaciation were observed in the mouse. Local induration was frequently noticed at the site of injection. Inguinal and peritoneal lymph glands were usually enlarged and the peritoneum showed a granular, whitish exudate. Characteristic were the numerous miliary necrotic nodules varying in size from pinpoint to 3 to 5 mm in diameters. While these were scattered throughout the peritoneal cavity and on the diaphragm, nodules were always found beneath the capsule of the liver and spleen and throughout the parenchyma of these organs. Minute nodules were occasionally observed in the lungs and kidney, the intestines, and the adrenal gland. An abscess commonly formed at the site of injection. Mice infected via the oral route usually had ulceration of the intestinal mucosa with extension throughout all the layers. Films made of the nodules and peritoneal exudates showed large numbers of thick, Gram-negative bacilli, usually in clumps, occasionally as diplobacilli. Some of the bacilli showed a clear halo, but no definite capsule could be demonstrated. Bottone (1977), however demonstrated capsules in *Y. enterocolitica* from peritoneal exudates.

Schleifstein and Coleman also established another well-known characteristic of *Y. enterocolitica*, viz that cultures maintained on artificial media tend to lose their virulence within a comparatively short time.

For many years after 1939 no strains of *Y. enterocolitica* pathogenic for animals could be found (Akkermans and Terpstra, 1963; Mollaret and Guillon, 1965). In 1972 however, Keet isolated a strain of *Y. enterocolitica* from a man with septicaemia which proved highly pathogenic for mice via the intravenous as well as the oral route (Carter, 1973; Carter, 1975). Further experimental work with this WA strain (ATCC no. 27729) during 1974 showed an infection in mice comparable to that found in man (Carter and Collins, 1974; Carter 1975). In the same year another two isolates which were pathogenic for mice, were found by Quann and co-workers. The typical lesions produced by these strains included splenomegaly, enlargement of salivary glands, cervical and mesenteric lymph nodes, and occasionally necrotic foci involving the spleen and liver.

Rakovský (1973) managed to infect guinea pigs and rabbits with *Y. enterocolitica* strains of serotype O 3. The guinea pigs were infected intraperitoneally with suspensions washed from blood agar plates or cultured in broth. The blood agar cultures established infection and the guinea pigs died within 24 to 48 hours. However, those that were infected subcutaneously or with the broth culture, did not die, nor did they show any signs of illness. In the rabbits only the blood agar cultures were used for infection via the intravenous route and they died within 5 to 11 days. *Y. enterocolitica* was isolated from the faeces as early as 24 hours post-infection. Marked inflammation was noted in the liver with lymphocytes penetrating into the parenchyma and in the appendix haemorrhagic necrotic inflammation with ulcerations was observed. *Y. enterocolitica* was cultivated from the blood, liver, spleen and contents of the intestines.

Recently many more strains of *Y. enterocolitica* were found with pathogenic properties for experimental animals. Like many of its other characteristics, Niléhn (1973) showed that the virulence of *Y. enterocolitica* can be tempera-

ture dependent. This observation was further elucidated by the determination of the bactericidal effect of normal human serum on strains of *Y. enterocolitica* cultured at 25°C which were found to be more resistant to human serum than those cultured at 37°C. The same effect was noted in mice infected intraperitoneally with strains cultured at the above two temperatures.

In 1975 Alonso *et al* tested pathogenic strains in cyclophosphamid treated mice and athymic nude mice. Highly pathogenic strains killed conventional as well as cyclophosphamid treated mice, whereas many strains non-pathogenic for these mice, killed athymic nude mice. These authors demonstrated by their experiments the importance of T-lymphocytes in *Y. enterocolitica* infection. The absence of T-lymphocytes in this case lead to diminished natural as well as aquired resistance. This resulted in a disease in the mice comparable to that in immunodeficient humans. In another paper Bercovier *et al* (1976) discussed a strain of *Y. enterocolitica* which was non-pathogenic for holoxenic mice, but caused disease, similar to the natural human infection, in C_3H/He mice.

Various experimental models are now in use for the testing of the pathogenicity of *Y. enterocolitica*, eg. rabbits (Une, 1977) guinea pigs (Obwolo, 1977) and tissue cultures (Une *et al*, 1977; Lee, 1977; Obwolo, 1977). From these experiments it became clear that *Y. enterocolitica* is an invasive pathogen, but it was only in 1978 that the production of a heat-stable toxin was demonstrated in this organism (Pai *et al*, 1978; Pai and Mors, 1978; Robins-Browne *et al*, 1979a). This toxin is however, not formed in detectable amounts at an incubation temperature of 37°C, so that its role in establishing the disease, has not yet been fully evaluated.

In experiments not reported in this thesis, the effect of *Y. enterocolitica* toxin was tested in HeLa cells and mice. The cell cultures and animals were challenged with *Y. enterocolitica* WA together with previously prepared crude toxin. The controls received only bacteria. No difference in infection between the test cells or animals and the controls could however be demonstrated (Van Vuuren and Koornhof, unpublished data).

The V and W antigens of *Y. pestis* and *Y. pseudotuberculosis* (Brubaker 1979) may provide protection against phagocytosis by leukocytes possessing O_2- dependent killing mechanisms (Burrows, 1963) or serve to permit intracellular growth within host cells lacking these mechanisms (Brubaker, 1972). It was shown by Carter *et al* (1980) that *Y. enterocolitica* strains were also able to produce these antigens and that their presence directly correlated with the virulence of the bacteria for mice. From work done by Zink *et al* (1980), Gemski *et al* (1980) and Portnoy *et al* (1981) it is clear that the production of these antigens is mediated by a 42 megadalton (Mdal) plasmid. The loss of this plasmid would therefore result in loss of virulence, a phenomenon often encountered in strains of *Y. enterocolitica* kept on artificial media over extended periods.

5,3 REFERENCES

Ahvonen, P. and Rossi, T. (1970). Familial accurrence of *Yersinia enterocolitica* and acute arthritis. Acta Paldiatr. Scand. Suppl. *206* : 121-122.

Ahvonen, P., Thal, E. and Vasenius, H. (1973). Occurrence of *Yersinia enterocolitica* in animals in Finland and Sweden. in Contr. Microbiol. Immunol. Vol. 2 ed. by S. Windblad, S. Karger, Basel. pp. 135-136.

Akkermans, J.G. and Terpstra, J.I. (1963). Pseudotuberkulose bij chinchillas veroorzaakt door een bijzondere species. tijdschr. Dierengeneesk. *88* : 91-95.

Alonso, J.M. and Bercovier, H. (1975). Premiers isolements en France de *Yersinia enterocolitica* chez des micromammifères sauvages. Med. Malad. Infect. *5* : 180-181.

Albert, G. and Lafleur, L. (1971). *Yersinia enterocolitica* in children : Presentation of 24 cases observed at Hospital Sainte-Justine (Montreal). Can. J. Publ. Health. *62* : 70-

Arvastson, B., Damgaard, K. and Windblad, S. (1971). Clinical sympoms of infection with *Yersinia enterocolitica*. Scand. J. Inf. Dis. *3* : 37-40.

Becht, H. (1962). Untersuchungen über die Pseudotuberculose beim Chinchilla. Dtsch. Tieraerztl. Wochenschr. *69* : 626-627.

Bercovier, H., Alonso, J.M., Destombes, P. and Mollaret, H.H. (1976). Infection experimentale de souris axeniques par *Yersinia enterocolitica*. Ann. Microbiol (Inst. Pasteur). *127* : 493-501.

Bergstrand. C.G. and Windblad, S. (1974). Clinical manifestations of infection with *Yersinia enterocolitica* in children. Acta Paediatr. Scand. *63* : 875-877.

Bottone, E.J. (1977). *Yersinia enterocolitica*: a panoramic view of a charismatic microorganism. CRC Crit. Rev. Microbiol. *5*: 211-241.

Bottone, E.J., Chester, B., Malowaney, M.S. and Allerhand, J. (1974). Unusual *Y. enterocolitica* isolates not associated with mesenteric lymphadenitis. Appl. Microbiol. *27*: 858-861.

Brubaker, R.R. (1972). The genus *Yersinia*: biochemistry and genetics of virulence. Curr. Top. Microbiol. Immunol. *57*: 111-158.

Brubaker, R.R. (1979). Expression of virulence in yersiniae. in Microbiology, ed. by D. Schlessinger, Am. Soc. Microbiol., Washington. pp 168-171.

Burrows, T.W. (1963). Virulence of *Pasteurella pestis* and immunity to plague. Ergebn. Mikrobiol. Immun. Forsch. Exp. Ther. *37*: 59-113.

Carter, P.B. (1975). Pathogenicity of *Yersinia enterocolitica* for mice. Infect. Immun. *11*: 164-170.

Carter, P.B. and Collins, F.M. (1974). Experimental *Yersinia enterocolitica* in mice. Kinetics of growth. Infect. Immun. *9*: 851-857.

Carter, P.B., Varga, C.F. and Keet, E.E. (1973). A new strain of *Yersinia enterocolitica* pathogenic for rodents. Appl. Microbiol. *26*: 1016-1018.

Carter, P.B., Zahorchak, R.J. and Brubaker, R.R. (1980). Plague virulence antigens from *Yersinia enterocolitica*. Infect. Immun. *28*: 638-640.

DeLorme, J., Laverdière, M., Martieau, B. and Lafleur, L. (1974). Yersiniosis in children. Can. Med. Assoc. J. *110*: 281-284.

Esseveld, H. and Goudzwaard, C. (1973). On the epidemiology of *Yersinia enterocolitica* infections. Pigs as the source of infection in man. in Contr. Microbiol. Immunol. Vol 2, ed. by S. Winblad, S. Karger, Basel. pp 99-101.

Gemski, P., Lazere, J.R. and Casey, T. (1980). Plasmid associated with pathogenicity and calcium dependency of *Yersinia enterocolitica*. Infect. Immun. *27*: 682-685.

Gutman, L.T., Oltesen, E.A. Quan, T.J., Noce, P.S. and Katz, S.L. (1973). An interfamilial outbreak of *Yersinia enterocolitica* enteritis. N. Engl. J. Med. *228*: 1372-1377.

Hubbart, W.T. (1972). Yersiniosis in ammmals and birds in the United States. Am. J. Trop. Med Hyg. *21*: 458-463.

Kapperud, G. (1975). *Yersinia enterocolitica* in smaal rodents from Norway, Sweden, and Finland. Acta Pathol. Microbiol. Scand. Sect. B. *83*: 335-342.

Kapperud, G. and Jonsson, B. (1976). *Yersinia enterocolitica* in brown trout (*Salmo trutta* L) from Norway. Acta Pathol. Microbiol. Scand. *84*: 66-68.

Keet, E.E. (1974). *Yersinia enterocolitica* septicaemia. N.Y. State J. Med. *74*: 2226-2230.

Lassen, J. (1972). *Yersinia enterocolitica* in drinking water. Scand. J. Infect Dis. *4*: 125-127.

Mollaret, H.H. (1971). L'infection humaine A "Yersinia enterocolitica" en 1970 a la lumière de 642 cas r ècents. Aspects cliniques perspectives epidemiologiques. Pathol. Microbiol. *19*: 189-205.

Mollaret, H.H. (1972). Un domaine pathologique noveau: l' infection a' *Yersinia enterocolitica*. Ann. Biol. Clin. (Paris). *30*: 1-6.

Mollaret, H.H. and Lucas, A. (1965). Sur les particularités biochemiques des souches de *Yersinia enterocolitica* isolees ches les kiévres. Ann. Inst. Past. *108*: 121-125.

Mollaret, H.H. and Guillon, J.C. (1965). Contribution à l' etude d'un noveau groupe de germes (Yersinia enterocolitica) proches du bacille de Malassez et Vignal II. Pouvoir pathogène expérimental. Ann. Inst. Pasteur. *109*: 608-613.

Nilêhn, B. (1973). Host range, temperature characteristics and serologic relationships among *Yersinia* phages. in Contr. Microbiol. Immunol. Vol. 2, ed. by S. Winblad, S. Karger, Basel. pp 85-92.

Obwolo, M.J. (1977). The pathology of experimental yesiniosis in guinea pigs. J. Comp. Pathol. *87*: 213 221.

Olsovsky, Z., Olsakova, V., Chobot, S. and Sviridov, V. (1975). Mass occurrence of *Yersinia enterocolitica* in two establishments of collective care of children. J. Hyg. Epidemiol. Microbiol. Immunol. (Praha). *19*: 22-29.

Pai, C.H. and Mors, V. (1978). Production of enterotoxin by *Yersinia enterocolitica*. Infect Immun. *19*: 908-911.

Pai, C.H., Mors, V. and Toma, S. (1978). Prevalence of enterotoxigenicity in human and nonhuman isolates of *Yersinia enterocolitica*. Infect. Immun. *22*: 334-338.

Portnoy, D.A., Moseley, S.L. and Falkow, S. (1981). Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. *31:* 775-782.

Quan, T.J., Meek, J.L., Tsuchiya, K.R., Hudson, B.W. and Barnes, A.M. (1974). Experimental pathogenesis of recent North American isolates of *Yersinia enterocolitica*. J. Infect. Dis. *129*: 341-344.

Rabson, A.R. and Koornhof, H.J. (1972). *Yersinia enterocolitica* infections in South Africa. S. Afr. Med. J. *46*: 798-803.

Rabson, A.R. and Koornhof, H.J. (1973). *Yersinia enterocolitica*infections in South Africa. in Contr. Microbiol. Immunol. Vol. 2, ed. by S. Winblad, S. Karger, Basel. pp 102- 105.

Rabson, A.R., Koornhof, H.J., Notman, J. and Maxwell, W.G. (1972). Hepatosplenic abscesses due to *Yersinia enterocolitica*. Br. Med. J. *4*: 341.

Rakovsky, J., Paucková, V. and Aldová, E. (1973). Human *Yersinia enterocolitica* infections in Czechoslovakia. in Contr. Microbiol. Immunol. Vol 2, ed. by S. Winblad, S. Karger, Basel. pp 93-98.

Robins-Browne, R.M., Jansen van Vuuren, C.J., Still, C.S., Miliotis, M.D. and Koornhof, H.J. (1979). The pathogenesis of *Yersinia enterocolitica* gastroenteritis. in Contr. Microbiol. Immunol. Vol. 5, ed. by P.B. Carter, L. Lafleur and S. Toma, S. Karger, Basel. pp 324 328.

Schleifstein, J.J. and Coleman, M.B. (1939). An unidentified microorganism resembling *B. lignieri* and *Past. pseudotuberculosis*, and pathogenic for man. N.Y. State J. Med. *39*: 1749-1753.

Szita, J., Kâli, M. and Redey, B. (1973). Incidence of *Yersinia enterocolitica* infection in Hungary. Observations on the laboratory diagnosis of yersiniosis. in Contr. Microbiol. Immunol. Vol. 2, ed. by S. Winblad, S. Karger, Basel. pp106-110.

Toivanen, P., Toivanen, A., Olkkonen, L. and Aantaa, S. (1973). Hospital outbreak of *Yersinia enterocolitica* infection. Lancet. *1*: 801-803.

Toma, S. (1973). Survey on the incidence of *Yersinia enterocolitica* in the province of Ontario. Can. J. Publ. Health. *64*: 477-487.

Toma, S. and Deidrick, V.R. (1975). Isolation of *Yersinia enterocolitica* from swine. J. Clin. Microbiol. *2*:478-481.

Tsubokura, M., Otsuki, K. and Itagaki, K. (1973). Isolation of *Yersinia enterocolitica* from swine. Jap. J. Vet. Sci.*35*: 419-424.

Une, T. (1977). Studies on the pathogenicity of *Yersinia enterocolitica* II. Interaction with cultured cells *in vitro*. Microbiol. Immunol. *21*: 365-377.

Une, T., Zen-Yoji, H., Maruyama, T. and Yanagawa, Y. (1977). Correlation between epithelial cell infectivity *in vitro* and O-antigen groups of *Yersinia enterocolitica*. Microbiol. Immunol. *21*: 727-729.

Vandepitte, J., Wauters, G. and Isebaert, A. (1973). Epidemiology of *Yersinia enterocolitica* infections in Belgium. in Contr. Microbiol. Immunol. Vol. 2, ed. by S. Winblad, S. Karger, Basel. pp 111-119.

Wetzler, T. and Hubbart, U.T. (1969). *"Yersinia enterocolitica"* in North America. Symp. Ser. Immunobiol. Scand. *9*: 343-356.

Zen-Yoji, H. and Maruyama, T. (1972). The first successful isolation and identification of *Yersinia enterocolitica* from human cases in Japan. Jap. J. Microbiol. *16*: 493-500.

Zen-Yoji, H., Maruyama, T. , Sakai, S., Kimura, T., Mizuno, T. and Mamose, T. (1973). An outbreak of enteritis due to *Yersinia enterocolitica* occurring at a junior high school. Jap. J. Microbiol. *17*: 220- 222.

Zen-Yoji, H., Sakai, S., Maruyama, T. and Yamagawa, G. (1974). Isolation of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from swine, cattle and rats at an abattoir. Jap. J. Microbiol. *18*: 103-105.

Zink, D.L., Feeley, J.C., Wells, J.G., Vanderzant, C., Vickery, J.C., Roof, W.D. and O'Donovan, G.A. (1980). Plasmid-mediated tissue invasiveness in *Yersinia enterocolitica*. Nature. *283*: 224-226.

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6. IMMUNOELECTROPHORETIC PATTERNS OF *YERSINIA ENTEROCOLITICA* ANTIGENS

6.1 INTRODUCTION

For a microbial infection to occur there must be interaction between the pathogen and the host. In most situations the pathogen makes contact with the host at the cellular level, resulting in an interaction between the surfaces of the microbe and the host cell respectively. The microbial cell surface must therefore have some characteristic which enables it to adhere to the cytoplasmic membrane of the host cell, and without adherence the microbe cannot penetrate and multiply (Smith, 1977).

Although some microbes enter the host directly via a vector bite or trauma, most infections begin on the mucus membranes of the respiratory, alimentary or urogenital tracts. These membranes are protected by diverse mechanisms such as moving lumen contents, surface mucus which contain various immunoglobulins, such as IgA, IgM and IgG, or by naturally occuring commensal organisms and inflammatory cells (Smith, 1977). Early attack on mucus membranes can take one of four forms :

(i) attachment and multiplication without significant penetration, followed by enterotoxin secretion, such as cholera;

(ii) attachment with subsequent penetration of the mucosa together with destruction of the tissue, such as in shigellosis;

(iii) attachment with penetration of the mucosa into the deeper layers without apparent damage to the mucosal tissues, such as in salmonellosis (Plotkin *et al*, 1979); and

(iv) attachment and multiplication without penetration or toxin secretion such as certain so-called enteropathogenic *E. coli* (Levine *et al*, 1978).

Among the enteropathogenic members of the *Enterobacteriaceae* adherence to the mucous membrane of the intestinal tract is a prerequisite for pathogenesis (Richardsand Douglas, 1978). Some of the *E. coli* strains causing diarrhoea in piglets attach themselves to the microvilli of the mucosa by means of a plasmid-mediated surface protein, the K88 entigen (Hohmann and Wilson, 1975; Riising *et al*, 1975; Nagy *et al*, 1976); On the other hand K88 negative strains can also be enteropathogenic in piglets, indicating that other surface-adhesive factors may be involved in *E. coli* infections (Hohmann and Wilson, 1975; Nagy *et al*, 1976). Tebbutt *et al* (1976) also showed that both pilated and non-pilated gonococci adhere to the epithelial cells of human endocervix *in vitro*.

In 1971 Ørskov *et al* found two main immunoelectrophoretic patterns with O antigens of *E. coli* strains obtained by water extraction at 60°C. The one pattern is a slow cathodally migrating antigen and in this group are found most of the strains associated with infantile diarrhoea. The other, to which strains associated with dysentery-like disease belong, show an anodally migrating antigen, which also does not migrate very far from the wells. Some of the strains belonging to this latter category were tested for the sugar composition of the polysaccharide part of the lipopolysaccharide (Ørskov *et al*, 1977). Chemical analysis showed that most of these lipopolysaccharides contain either hexuronic acid or N-acetyl neuraminic acid and it is likely that these constituents convey the negative charge to the O antigen.

The abovementioned findings prompted our department to extend immunoelelectrophoretic studies to other bacteria, including *V. cholorae*, *Salmonella* and *Shigella* species and *Y. enterocolitica*. In this study only the findings related to *Y. enterocolitica* will be reported.

6.2 MATERIALS AND METHODS

6.2.1 Bacterial strains (See Appendix B)

The following strains of *Y. enterocolitica* were tested :

M1, M2, 4257, M3, 5122, Thompson, Beukes, M4,33 M5,27, M6,31, M7,8 and M8.

6.2.2 Preparation of watery extracts of the antigens

This was done according to the technique described by Ørskov *et al*, (1971). Strains of *Y. enterocolitica* were grown on modified D_5 medium (Schlecht and Westphal, 1966) at 37°C for 24 hours. Growth from these plates were washed off with sterile normal saline (0,9% NaCl) to form a thick suspension of at least 10^{10} bacteria/mℓ . This was then heated in a water bath at 60°C for 20 minutes, followed by centrifugation at 8 000 X G for 15 minutes. The supernatants were stored at 4°C until used and the pellet discarded. In an attempt to exclude the possible effect of proteins which may be bound to O-antigens, part of each extract was boiled for one hour and stored at 4°C.

6.2.3 Preparation of antisera

Antisera were prepared in rabbits according to conventional methods (Edwards and Ewing, 1962; Kauffmann, 1966). Autoclaved suspensions of *Y. enterocolitica* serotypes O 1 to O 8 were injected intravenously into adult rabbits, starting with 0,5 mℓ and increasing the dosage by 0,5 mℓ every five days to a maximum of 2,5 mℓ . A total of seven injections was given and the antibody titres were determined by tube agglutination before exsanguination of the rabbits by cardiac puncture. Antibody titres varied between 1/256 and 1/1 024 for the various serotypes.

6.2.4 The immunoelectrophoretic technique (Ørskov *et al*, 1971; Ørskov and Ørskov, 1972).

The experiments were performed using a Biorad Model 500 power pack and tank (T & C Scientific Supplies). Six glass microscope slides were put in a plastic holder and coated with 18 mℓ of a 1% agarose in Barbitone-TRIS-Barbitone buffer, pH 8,6 ionic strength 0,05. Wells and troughs were cut with a Biorad Puncher (T & C Scientific Supplies). The agar from the wells was sucked off and the wells were filled with 1 to 1,5 $\mu\ell$ antigen extract. In duplicate experiments 60°C extracts were put into wells on one side of the trough and boiled extracts in the wells on the other. The electrophoretic separation was performed at 8 volts/cm for 90 minutes, using the same buffer as above. The agarose was removed from the troughs and replaced with approximately 150 $\mu\ell$ undiluted homologous antisera. The slides were left in a moist chamber for 24 to 48 hours to allow diffusion and precipitation, washed in four changes of isotonic saline to remove unprecipated protein, and then rinsed in distilled water for 24 hours to remove the NaCl. The slides were then removed from the holder after they were dried, and stained with Coomassie Blue R250 (Biorad cat. no. 161.0400) for 1 hour. Background stain was removed with 10% acetic acid.

6.3 RESULTS

The results of the immunoelectrophoretic studies that were performed on eight different serotypes of *Y. enterocolitica*, can be seen in figure 8-1.

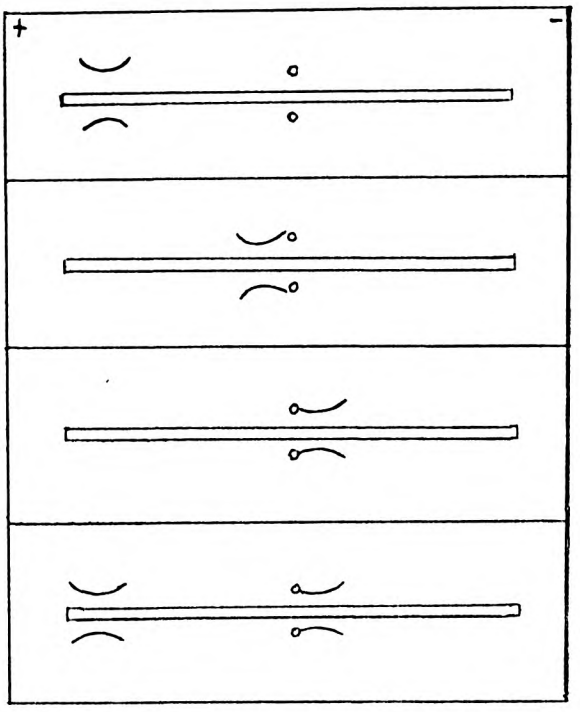

Figure 6-1. Immunoelectropherogram of O-antigens of various serotypes of *Y. enterocolitica*. Homologous antisera in top wells; boiled extracts in bottom wells.

The immunoelectrophoretic pattern of serotypes O 1; O 4,33; O 5,27; O 6,31 and O 7,8 is the same: a fast migrating antigen, similar to that of *E. coli* strains associated with dysentery-like disease (Ørskov *et al*, 1971), whereas serotype O 3 shows the opposite, viz a slow cathodally migrating O antigen. Serotype O 2 has an O antigen with two components, one migrating fairly fast anodally, and another slowly migrating component directed cathodally.

6.4 DISCUSSION

Of all the serotypes tested, the types mainly associated with gastroenteritis in man are O 3 and O 8, and although their clinical presentation is similar, they show exactly opposite immunoelectrophoretic patterns. Ørskov *et al* (1971) speculated on the invasiveness of *E. coli* strains presenting with negatively charged O antigens. As was shown in chapter 7, both serotypes O 3 and O 8 of *Y. enterocolitica* are invasive for rabbit intestine

and no difference could be demonstrated in the way the mucosa was penetrated, nor with regard to the subsequent multiplication inside the tissues.

The same immunoelectrophoretic pattern as that of serotype O 8 was demonstrated by *Shigella boydii*, *Sh. dysenteria*, *Sh. sonnei* (Robins-Browne, 1978), and invasive *E. coli* (Ørskov *et al*, 1971). It was subsequently shown in our laboratory that strain WA (serotype O 8) was invasive for guinea pig and rabbit conjunctivae.

As mentioned in chapter 11, one of the resistance mechanisms of the host in gastrointestinal infections is the secretion of mucus into the lumen which may trap and flush the pathogens from the intestine. No specific properties such as the K88 antigen of *E. coli*, could so far be demonstrated in *Y. enterocolitica*. Before contact can be made with the microvilli of the mucous membrane, penetration of the mucus layer is essential. The only known property that may play a role in enabling *Y. enterocolitica* to penetrate the mucus layer, is the electric charge of the O antigen. The speed by which the O antigen migrates in an electric field, may also have an effect on its penetrative abilities. Serotypes O 1; O 2; O 4,33; O 5,27; O 6,31 and O 7,8 all have fast migrating antigens and they have only rarely been incriminated as causative agents of gastroenteritis and only serotype O 5,27 could be demonstrated as having invasive properties (chapter 11). Most of the serotypes other than O 3, O 8 and O 9 have been isolated from extraintestinal sites, such as the eye and skin (Ahvonen, 1972; Niemi *et al*, 1976; Bottone, 1977). There is a better correlation between the *Y. enterocolitica* serotypes O 3 and O 8, whose O antigens migrate slowly, and invasive potential. It is therefore possible that *Y. enterocolitica* strains with the slowly migrating antigens can interact better or in a different way with mucus and mucous membranes for adherence, than those with rapidly migrating antigens. Alternatively they may contribute to the inter-

action between the surface of the pathogen and the cell membrane.

6.5 REFERENCES

Ahvonen, P. (1972). Human yersiniosis in Finland. Ann. Clin. Res. *4* : 39-48.

Bottone, E.J. (1977). *Yersinia enterocolitica:* a panoramic view of a charismatic microorganism. CRC. Crit. Rev. Microbiol. *5* : 211-241.

Edwards, P.R. Ewing, W.H.L. (1962). Identification of *Enterobacteriaceae*. U.S. Publ. Health Service, Burgess Publ. Co.

Hohmann, A. and Wilson, M.R. (1975). Adherence of enteropathogenic *Escherichia coli* to intestinal epithelium *in vivo*. Infect. Immun. *12* : 866-880.

Kauffmann, F. (1966). The bacteriology of *Enterobacteriaceae*. Munksgaard, Copenhagen.

Levine, M.M., Nalin, D.R., Hornick, R.B., Berquist, E.J., Waterman, D.H., Yound, C.R., and Scotman, S. (1978). *Escherichia coli* strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. Lancet *i* : 1119 - 1122.

Nagy, B., Moon, H.W. and Isaacson, R.E. (1976). Colonization of porcine small intestine by *Escherichia coli:* ileal colonization and adhesion by pig enteropathogens that lack K88 antigen and by some acapsular mutants. Infect. Immun. *13* : 1214-1220.

Niemi, K.-M., Hannuksela, M. and Salo, O.P. (1976). Skin lesions in human yersiniosis. A histopathological and immunohistological study. Br. J. Dermatol *94* : 1155-160.

Ørskov, F., Ørskov, I., Jann, B. and Jann, K. (1971). Immunoelectrophoretic patterns of extracts from all *Escherichia coli* O and K antigen test strains. Correlation with pathogenicity. Acta Pathol. Microbiol. Scand. Sect. B. *79* : 142-152.

Ørskov, F. and Ørskov, I. (1972). Immunoelectrophoretic patterns of extracts from *Escherichia coli* test strains O1 to O157 examinations in homologous OK sera. Acta Pathol. Microbiol. Scand. Sect. B. *80* : 905-910.

Ørskov, I. *et al* (1977). Serology, chemistry, and genetics of O and K antigens of *Escherichia coli*. Bacteriol. Rev. *41* : 667-710.

Plotkin, G.R., Kluge, R.M. and Waldman, R.H. (1979). Gastroenteritis: etiology, pathophysiology and clinical manifestations. Medicine *58* : 95-114.

Richards, K.L. and Douglas, S.D. (1978). Pathophysiological effects of *Vibrio cholerae* and enterotoxigenic *Escherichia coli* and their enterotoxins on eukaryotic cells. Micro. Rev. *42* : 592-613.

Riising, H.-J., Svendsen, J. and Larsen, J.L. (1975). Occurrence of the K88-negative *Escherichia coli* serotypes in pigs with post-weaning diarrhoea. Acta Pathol. Microbiol. Scand. Sect. B. *83* : 63-64.

Robins-Brown, R.M. (1978). Personal communication.

Schlecht, S. and Westphal, O. (1966). Wachstum Und Lipopolij-saccharid (O-antigen)-Gehalt von Salmonellen bei Züchtung auf Agar-Nährböden. Znl. Bakt. I. Abt. Orig. *200* : 241-259.

Smith, H. (1977). Microbial surfaces in relation to pathogenicity. Bact. Rev. *41* : 475-500.

Tebbutt, G.M., Vealle, D.R., Hutchinson, J.G.P. and Smith, H. (1976). The adherence of pilate and non-pilate strains of *Neisseria gonorrhoeae* to human and guinea pig epithelial tissues. J. Med. Microbiol. *9* : 263-274.

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7. EFFECT OF BACTERIAL SYNERGY ON THE VIRULENCE OF *YERSINIA ENTEROCOLITICA*

7.1 INTRODUCTION

The bowel of man, like that of all other animals, contains its own characteristic symbiotic and indigenous flora which act in various ways to protect the host from infection. The antagonism towards intruders can be affected by sheer numbers, i.e. direct overcrowding of the pathogens or by competition for nutrients. Furthermore the gut flora can secrete bactericidal substances such as colicins. When two different pathogens invade the intestinal tract they may act antagonistically or synergistically towards each other, or their combined effect may be additive only.

Rabson and Koornhof (1973) and Robins-Browne *et al* (1979) found that often *Y. enterocolitica* was isolated together with another enteropathogen such as salmonellae or *E. coli*, or mixed infections were suspected based on raised serological titres.

In order to examine the effects of synergism in the laboratory, rabbits and mice were infected with *Y. enterocolitica* and *S. typhimurium*, separately and together and the results compared.

7.2 MATERIALS AND METHODS

7.2.1 Fluid production in rabbit ileum

(a) Bacterial strains (see appendix B)

S. typhimurium W118 was grown on nutrient agar (NA) at 37°C for 24 hours, as was *Y. enterocolitica* 2705 except that the latter bacterium was also cultured at 25°C. Live *V. cholerae* was grown on syncase agar for 24 hours at 37°C as positive control. The bacteria were washed of the plates with Brain-heart infusion (BHI) broth (Difco) and the concentrations were adjusted to 1×10^7 cfu/mℓ .

(b) Procedure

The rabbit ileal loop technique employed was the same as described in chapter 11. Seven loops were ligated per rabbit with the negative controls at both ends and the positive control in the middle. The two loops on either side of the positive control served as test loops. *S. typhimurium* was injected into the loops of four animals, *Y. enterocolitica* into four others and four rabbits received both bacteria (1 mℓ + 1 mℓ) into each loop. The rabbits were sacrificed after 18 hours, the abdomens were opened up and the loops were examined for fluid accumulation.

7.2.2 The effect of bacterial synergy on virulence in mice.

(a) Bacterial strains (See Appendix B)

The same strains mentioned in 11.1.1 above were used, except *V. cholerae* was left out.

(b) Procedure

Balb/c mice weighing 18 to 20 g were divided into batches of six and inoculated intragastrically with the bacteria as set out in table 11-2. They were first infected with *S. typhimurium* and 48 hours later with *Y. enterocolitica*. The mice were kept under surveillance for 30 days and deaths were recorded as they occurred.

7.3 RESULTS

Each organism was tested in duplicate loops for fluid accumulation in three different rabbits, with live *V. cholerae* as positive control and BHI broth as negative control. The results can be seen in table 7-1. The fatality rate of mice, demonstrating the synergistic effect of the two bacterial strains is given in table 7-2.

Table 7-1 Average ratio's of fluid accumulation in rabbit ileal loops infected with *Y. enterocolitica* and *S. typhimurium* separately and together.

Bacterial strains	Average ratio's: fluid in mℓ/length of loop in cm
V. cholerae	0,98
BHI broth	0
S. typhimurium W118	0
Y. enterocolitica	
grown at 25°C	0
grown at 37°C	0
S. typhimurium (37°C) plus *Y. enterocolitica*	0
grown at 25°C	0,56
grown at 37°C	0

Table 7-2 Mortality of mice infected orally with varying doses (cfu) of *Y. enterocolitica* and *S. typhimurium* W118. All deaths occurred between the second and fourth weeks after infection.

	Y. enterocolitica cfu					
S. typhimurium cfu	10^{10}	10^{9}	10^{8}	10^{7}	10^{6}	0
5×10^{5}	6/6	1/6	1/6	0/6	0/6	0/12[a]
5×10^{4}	2/6	0/6	2/6	0/6	2/6	0/6
5×10^{3}	2/6	1/6	0/6	0/6	1/6	0/6
5×10^{2}	1/6	0/6	1/6	0/6	0/6	0/6
0	0/12[a]	1/6	0/6	0/6	0/6	0/6

[a] half the mice in these groups received 10^{10} heat-killed bacteria.

7.4 DISCUSSION

A ratio of 0,9 and higher, based on the amount of fluid in mℓ to the length of the loop in cm, is accepted as positive for fluid production. This is elicited by *V. cholerae* and therefore it is normally used as positive control in tests for fluid accumulation in rabbit ileal loops.

S. typhimurium was not able to cause fluid accumulation, nor was *Y. enterocolitica* when cultured at either 25°C or 37°C. The same negative results were found when these two organisms were introduced together into the loops with *Y. enterocolitica* grown at 37°C for 24 hours. When the latter bacterium was however, cultured at 25°C for 24 hours and then injected into the loops together with *S. typhimurium*, a fluid ratio of 0,56 was measured (table 7-1). Although this ratio is not regarded as positive for fluid accumulation, it is nevertheless substantially greater than the zero findings for single challenges, suggesting that a synergestic mechanism may operate.

Table 7-2 shows the increased virulence of *Y. enterocolitica* for mice when they were infected with *S. typhimurium* 48 hours prior to inoculation with *Y. enterocolitica*. The highest inoculum of the latter bacterium, viz 10^{10} cfu failed to kill 12 mice when administered alone, but prior infection with *S. typhimurium* caused 11 mice out of 24 to die, a mortality of nearly 50%. Furthermore, the synergy appeared to be related to increasing inocula of the respective organisms.

In a further experiment by Robins-Browne *et al* (1979) *S. typhimurium* and *Y. enterocolitica* were injected simultaneously intraperitoneally into mice, and no enhanced virulence could be demonstrated. When viewed in conjunction with the above two experiments, it may be concluded that salmonellae can lower

gut resistance, thereby enabling *Y. enterocolitica* to penetrate and cause invasive disease.

7.5 REFERENCES

Rabson, A.R. and Koornhof, H.J. (1973). *Yersinia enterocolitica* infections in South Africa. in Contr. Microbiol. Immunol. vol. 3. ed. by S. Windblad, S. Karger, Basel. pp. 102-105.

Robins-Browne, R.M., Rabson, A.R. and Koornhof, H.J. (1979). *Yersinia enterocolitica* in South Africa. in *Yersinia enterocolitica*. ed. by E.J. Bottone, Florida CRC Press. in press.

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8. THE EFFECT OF IRON ON THE VIRULENCE OF *YERSINIA ENTEROCOLITICA*

8.1 INTRODUCTION

Enterobacteria synthesize a number of organic iron-chelating substances which are apparently necessary for their uptake of environmental iron. These chelators include phenolic acids such as 2,3-dihydroxybenzoic acid and the catechol 2,3-dihydroxybensoyl serine and its cyclic trimer, enterochelin. The latter is the most effective in the transport of iron into the bacterial cells (Pollack *et al*, 1970). These compounds are of interest in the assessment of microbial pathogenicity, since the growth of pathogenic organisms *in vivo* depends in part on their capacity to obtain the iron firmly held by host substances like plasma transferrin, a major potential source of the iron that they need (Bullen *et al*,1971).

It has been shown that iron overload may play a role in increasing the host's susceptibility to infections with *Y. pestis* (Jackson and Burrows, 1956), *Y. pseudotuberculosis* (Bradley and Skinner, 1974), *Pasteurella septica* and *E. coli* (Bullen and Rogers, 1969), all bacteria fairly closely related to *Y. enterocolitica*. Infections with the latter organism also tend to be more severe when the possibility of iron overload exists, eg in patients with alcoholic cirrhosis (Abramovitch and Butas, 1973), thallassaemia (Blum *et al*, 1970), and Bantu siderosis (Rabson *et al*, 1972).

Robins-Browne *et al* (1979) showed that the addition of iron to fresh serum destroys its bactericidal activity, and also that the parenteral administration of iron in the form of ferric ammonium citrate, heightened the susceptibility of mice to fatal infection with *Y. enterocolitica*.

8.2 MATERIALS AND METHODS

8.2.1 Bacterial strains (see Appendix B)

In the two preliminary experiments *Y. enterocolitica* 5122 was used as test organism, but in the final experiment the WA strain was employed. The bacteria were grown on Nutrient Agar (NA) at 25°C for 24 hours, washed off into sterile phosphate buffered saline (PBS) and the concentrations were adjusted to approximately 1 X 10^{10} cfu/mℓ.

8.2.2 Procedure of preliminary experiments

In the first experiment 50 adult outbred mice were injected intraperitoneally with 200 μg iron in the form of ferric ammonium citrate. Immediately after this the mice in the test group were challenged intragastrically with 0,1 mℓ of the bacterial suspension. The control group received sterile PBS intragastrically.

In the second preliminary experiment the mice were divided into batches of five and injected intravenously with 0,1 mℓ iron dextran (Imferon) (Fison's Chemicals) containing 5 mg available iron. The animals were infected directly afterwards with 0,1 mℓ of tenfold dilutions of the bacterial suspension as set out in table 8-2. In contrast to the first experiment, the control group did not receive any iron, but was challenged with the same numbers of bacteria.

8.2.3 Procedure of final experiment

(a) Mouse mortality

Adult white outbred mice were divided into two groups, the first group containing five and the second group four batches. In each batch there were 25 animals. The first group received intraperitoneal (ip) injections of 80 μg iron in the form of ferric amonium citrate on three consecutive days, totalling 240 μg of

iron per mouse. On the second day, immediately following the iron injections, the mice in both groups were challenged intragastrically (ig) with 0,1 mℓ bacterial suspension in tenfold dilutions, starting with 1 x 10^9 and ending with 1 x 10^6 cfu. One batch of mice which were injected with iron, received only sterile PBS as toxicity control. The mice were observed for 30 days and the mortality rate recorded.

(b) Blood clearance in rats.

White rats were injected ip with 0,5g iron in the form of ferric ammonium citrate on three consecutive days, totaling 1,5g iron per animal. The control group received an equivilent amount of ammonium citrate. On the third day the rats were challenged via the tail vein with 0,1 mℓ of a bacterial suspension containing 10^8 cfu/mℓ. The rats were sacrificed in triplicate at intervals of 0,5, 4, 24 and 48 hours after infection. The rest of the experiment was performed as described in chapter 10.

(c) Phagocytosis and killing

Rats were injected with iron as described under 9.2.3 (b). Controls received the equivalent amount of ammonium citrate. Macrophages were harvested as follows: 10 mℓ HBSS containing 5 units Heparin (Wellcome) per mℓ were injected ip. The rats were turned over a few times to facilitate mixing of the macrophages with the HBSS after which the suspension was withdrawn using a 10 mℓ syringe with an 18 gauge needle. The macrophage suspension was about 80% pure, the rest of the cells being mainly lymphocytes, with a few epithelial cells. The numbers of macrophages were adjusted to 1 x 10^6 per mℓ and the bacterial suspension to 2 x 10^7 cfu per mℓ giving a ratio of 1 : 20 macrophages : bacteria.

Before harvesting the cells 5 mℓ blood was

obtained from each rat via cardiac puncture. The blood was allowed to clot and the serum was removed.

Polymorphonuclear leukocytes (PML) were harvested as described in chapter 10 and adjusted to 1:20 PMLs : bacteria.

Phagocytosis and killing were examined according to the technique described by Smith and Rommel (1977) (see chapter 10). Instead of fresh AB-serum, serum from rats with and without iron overload was used.

8.3 RESULTS

(a) Preliminary experiments

The results of the two preliminary experiments are given in tables 8-1 and 8-2 and those of the final experiment in table 8-3.

In the first preliminary experiment fifteen mice died within 12 to 18 hours, and three more 36 hours after infection. Four out of 25 mice in the control group died within 6 to 10 hours, presumably as result of the high dose of ferric ammonium citrate.

Table 8-1 Death rate of mice challenged intragastrically with *Y. enterocolitica* 5122 after receiving 200 μg iron intraperitoneally.

Inoculum *Y. enterocolitica* 5122	Numbers of mice	
	Challenged	Deaths
5×10^9 cfu	25	18
Sterile PBS	25	4

Because of the higher tolerance of the mice to iron dextran this compound was used in the second preliminary experiment. According to the manufacturers (Fison's Chemicals) Imferon has an LD_{50} of more than 1 mg/g body weight for mice. The

results of this experiment are given in table 9-2.

Table 8-2. Death rate of mice injected intravenously with 5 mg of iron in the form of iron dextran, before intragastric challence with *Y. enterocolitica* 5122.

Y. enterocolitica cfu	Number of Deaths	
	With iron	Without iron
$2,5 \times 10^9$	4/5 (80%)	1/5 (20%)
$2,5 \times 10^8$	2/5 (40%)	1/5 (20%)
$2,5 \times 10^7$	0/5	0/5
$2,5 \times 10^6$	0/5	0/5
$2,5 \times 10^5$	0/5	0/5

Deaths occured after 9, 10, 11 and 12 days. The mice were observed for 24 days.

(b) Mouse mortality

In the final experiment using mice iron was used as in the first preliminary experiment. To counteract the toxicity of the ferric ammonium citrate, only 80 μg of iron was injected at a time, but by repeating this for three consecutive days, each mouse received a final dose of 240 μg.

Both iron toxicity and microbial pathogenic controls were incorporated in this experiment. One batch of 25 mice received iron only and no bacteria, whereas one group was challenged with bacteria and no iron (table 8-3).

Using the Wilcoxin (Breslan) survival test (life tables and survival functions, Health Sciences computing facility, University of California, Los Angeles 90024) the cummulative proportion of surviving animals with and without iron was compared (fig. 8-2). The survival proportion for mice not pre-treated with iron is clearly much higher than for those that did receive iron.

Table 8-3. Mortality of mice challenged with *Y. enterocolitica* WA, with or without intraperitoneal iron.

Days after infection	*Y. enterocolitica* cfu								
	with 240 μg Iron					Without Iron			
	10^9	10^8	10^7	10^6	0	10^9	10^8	10^7	10^6
5		1					1		
11						1			
13	2	2	1				1		
14	3					2			
15	2	1				2			
16	2					1			
17	5	2				1			
18	3								
19	1					1			
20	1								
21	1	1							
22		1							
TOTAL	20	8	1	0	0	8	2	0	0

Table 8-4. SUMMARY OF THE RESULTS FROM TABLE 8-3

Inoculum		Number of Deaths	% Mortality	Mean time of days
With Iron	Without Iron			
10^9		20/25	80	17
10^8		8/25	32	15,5
total ($10^9 + 10^8 + 10^7$)		28/100	29	11,23
	10^9	8/25	32	15,33
	10^8	2/25	8	9
	total ($10^9 + 10^8 + 10$)	10/100	10	13,75

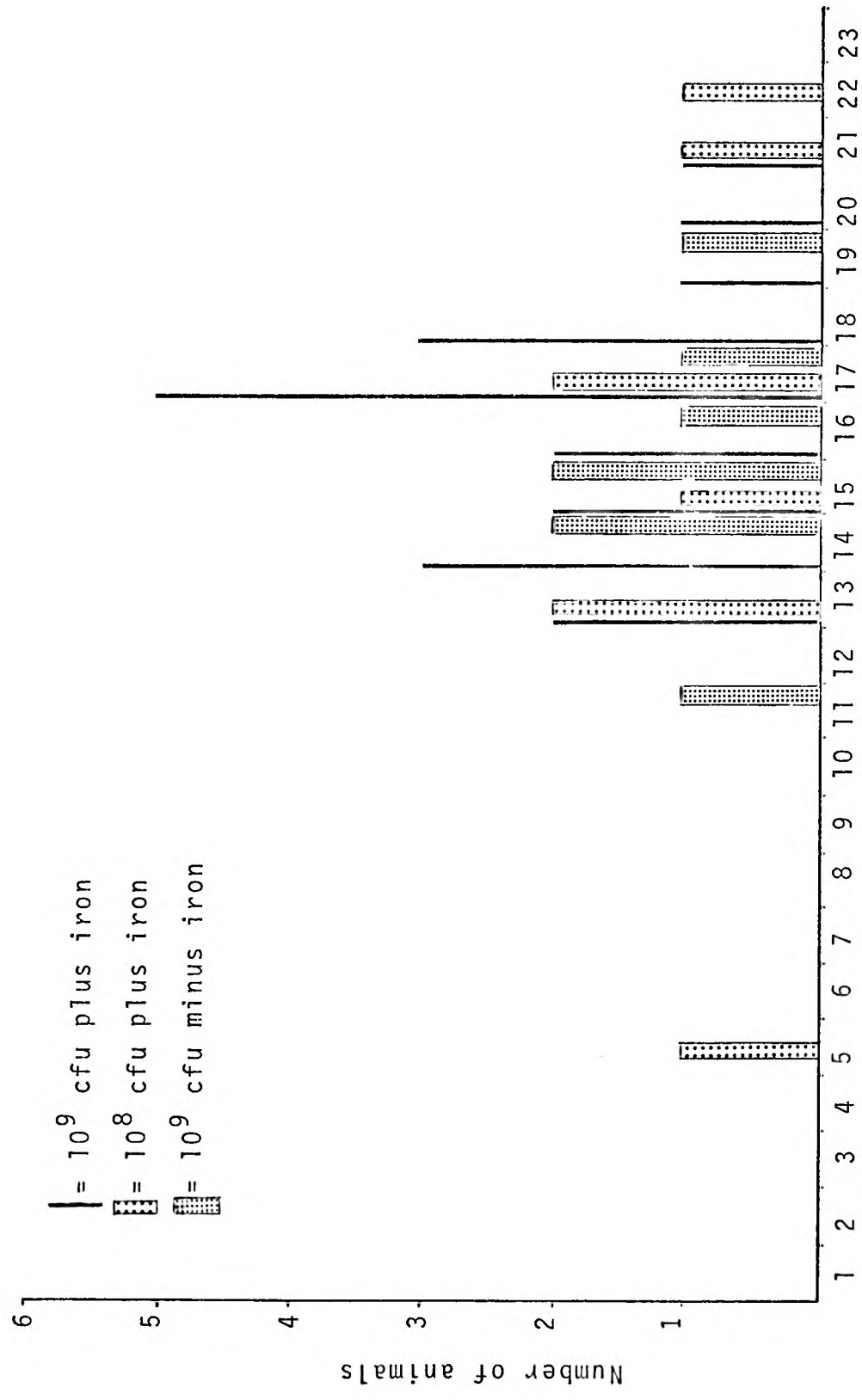

Figure 8-1. Comparison of mortality rate of mice infected with various concentrations of *Y. enterocolitica*

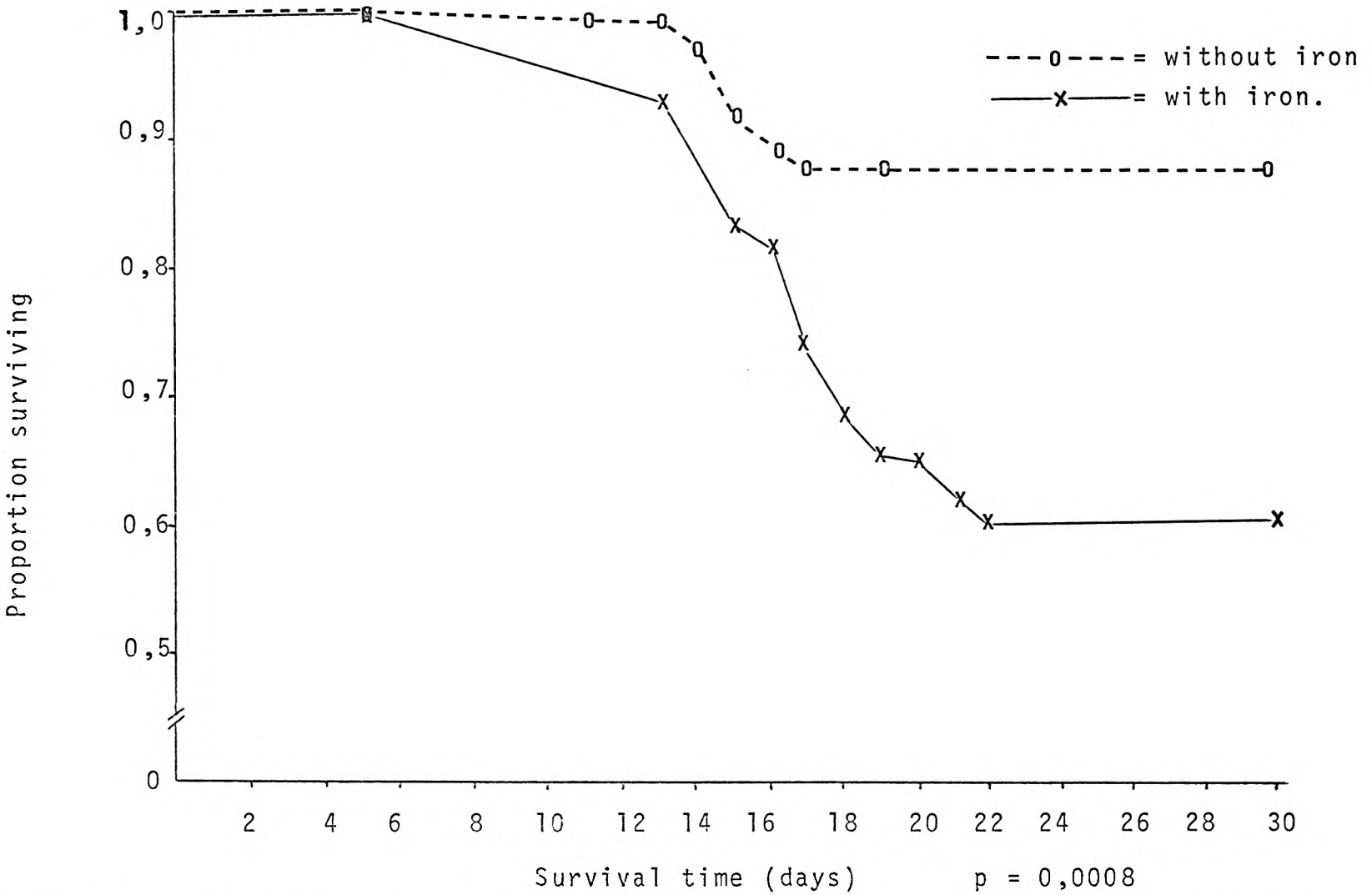

Figure 8-2. Survival curve of mice challenged with *Y. enterocolitica* after intraperitoneal injection with iron. The control group did not receive iron.

(c) Blood clearance

The results of the rat experiment are recorded in table 8-5 and figures 8-3 to 8-5.

Table 8-5. Results of plate counts of liver, spleen, lung and blood from rats challenged intravenously with *Y. enterocolitica* WA.

	Time interval after infection in hours	*Y. enterocolitica* cfu/g tissue			
		Liver	Spleen	Lung	Blood
With iron	0,5	2×10^6	4×10^6	4×10^6	$<10^2$
	1	3×10^6	6×10^6	$1,5 \times 10^7$	$<10^2$
	4	8×10^5	3×10^5	3×10^6	$<10^2$
	24	1×10^5	1×10^5	1×10^6	$<10^2$
	48	1×10^6	4×10^7	7×10^7	$<10^2$
Without iron	0,5	2×10^6	5×10^6	2×10^6	$<10^2$
	1	4×10^6	6×10^6	4×10^6	$<10^2$
	4	8×10^4	7×10^4	2×10^6	$<10^2$
	24	6×10^4	5×10^4	3×10^5	$<10^2$
	48	1×10^6	4×10^7	$6,5 \times 10^7$	$<10^2$

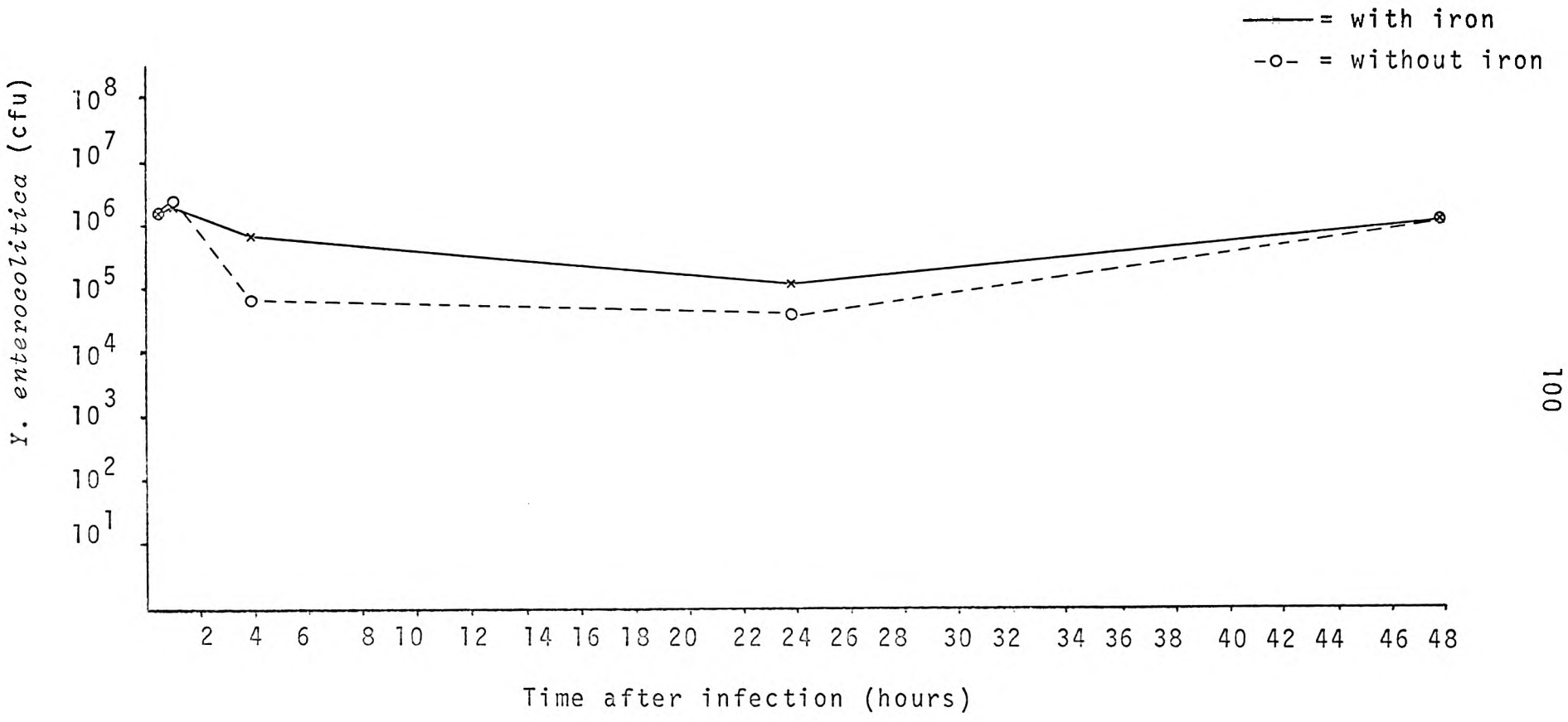

Figure 8-3. Numbers of *Y. enterocolitica* isolated from the liver of rats with and without iron overload.

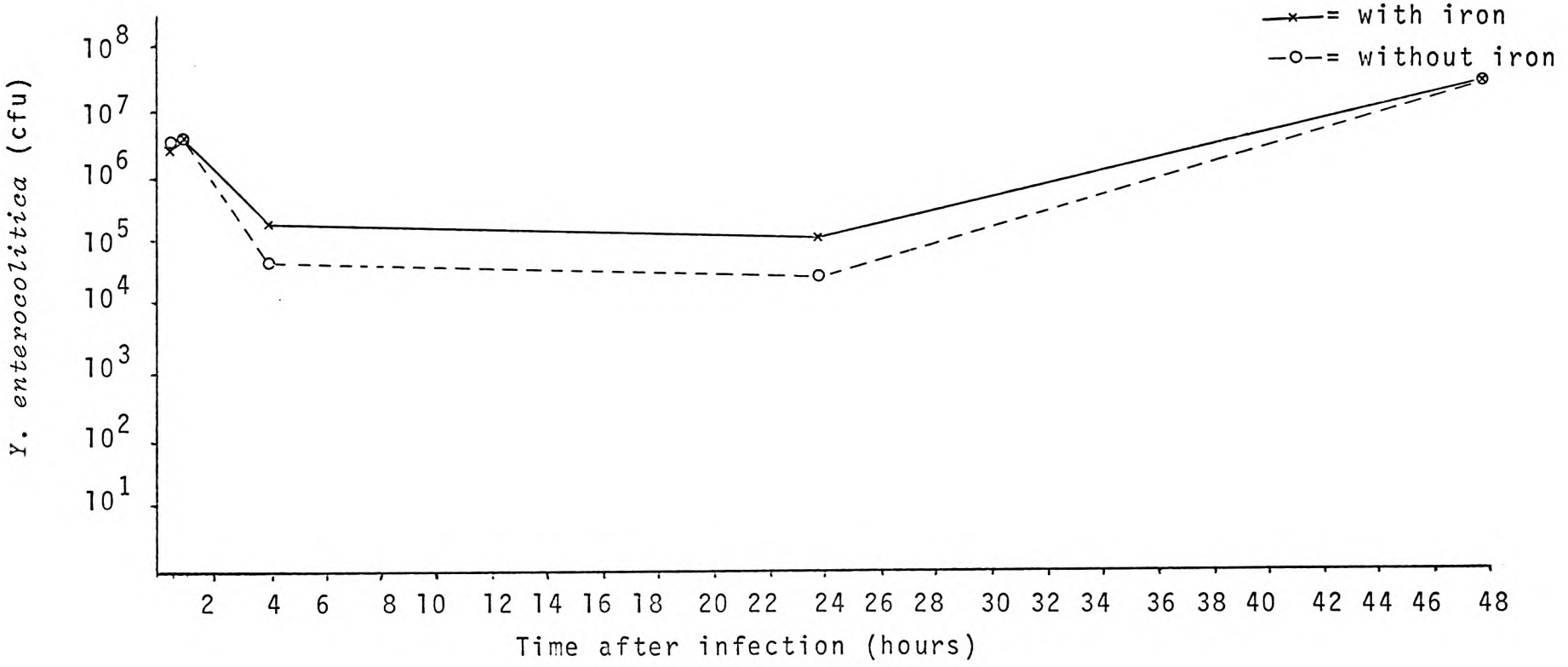

Figure 8-4. Numbers of *Y. enterocolitica* isolated from the spleen of rats with and without iron overload.

At 48 hours post infection, the numbers of bacteria isolated from various organs from both groups of animals were approximately the same (table 8-5, figures 8-3 to 8-5), indicating that the organisms in the control group overcame the natural resistance of these rats and started to multiply extensively.

(d) Phagocytosis and killing

Table 8-6. Summary of percentage phagocytosis and killing of *Y. enterocolitca* WA by rat PML's and macrophages.

Incubation time	Type of leukocyte	Pretreatment with iron	% Phagocytosis		% Killing	
			c̄Fe	c̄oFe	c̄Fe	c̄oFe
0,5 hours	PMLs	+	40	33	2,5	3
		-	40	53	5	1,9
	Macrophages	+	17	20	0	0
		-	20	10	0	0
3 hours	PMLs	+	80	70	45	45
		-	65	60	62,5	85,7
	Macrophages	+	45	30	22,2	30
		-	40	30	30	33,5

\+ = Rats pretreated with 1,5g iron as ferric ammonium citrate.
c̄Fe = Serum from rats with iron overload.
\- = Controls injected with ammonium citrate.
c̄oFe = Serum from rats without iron.

From table 8-6 and figures 8-5 and 8-6 the following are noted:

(i) No set pattern is established after 30 minutes incubation.

(ii) After 3 hours incubation the suspensions with serum from rats with iron overload showed a higher percentage phogocytosis but lower killing than the cultures that received normal serum.

(iii) In the 3 hour incubation group phagocytosis was higher for leukocytes harvested from rats injected with iron than for the controls.

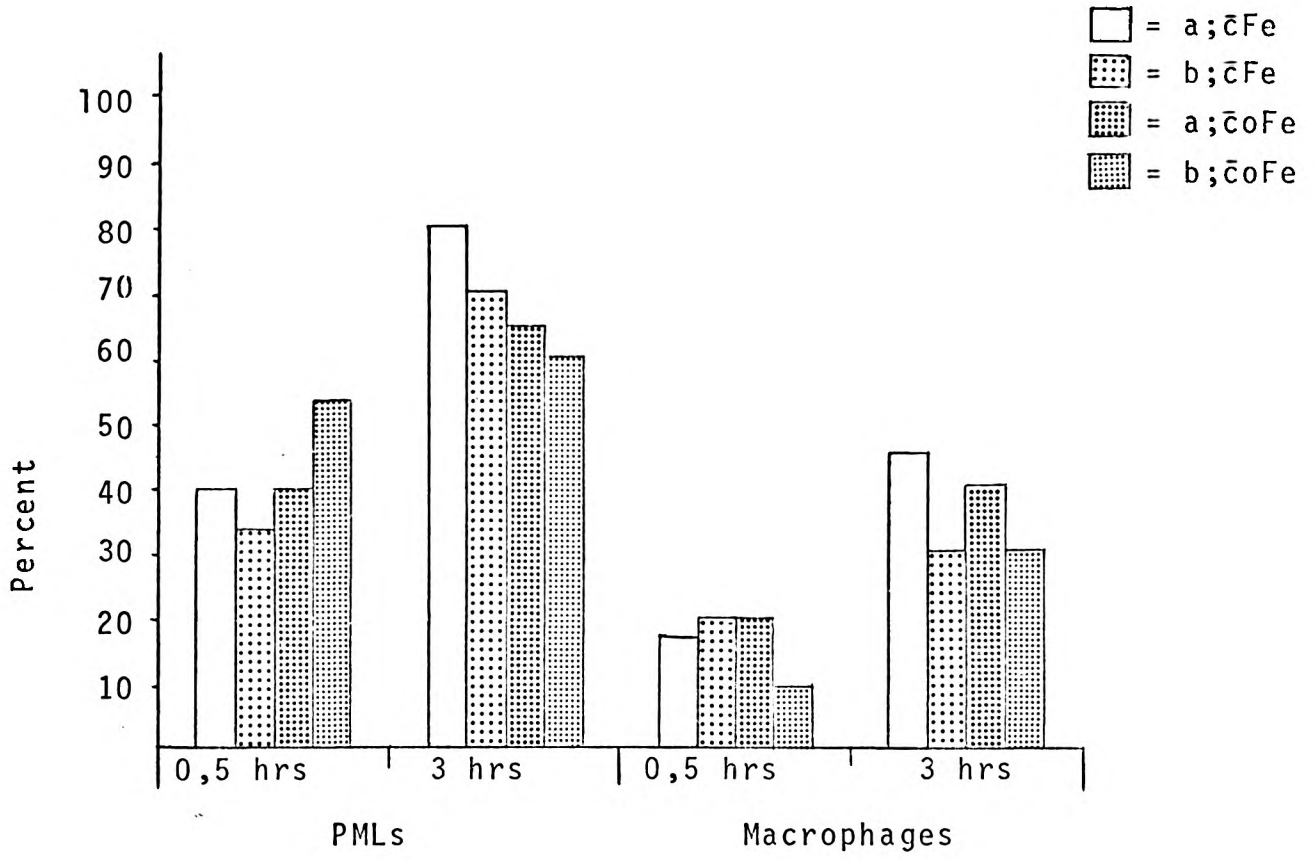

Figure 8-5. Percentage phagocytosis of *Y. enterocolitica* by polymorphonuclear leukocytes and macrophages at two different incubation times.

a = serum from rats with iron overload.
b = serum from rats without iron overload.
c̄Fe = leukocytes from rats pretreated with iron.
c̄oFe = leukocytes from rats without iron pretreatment.

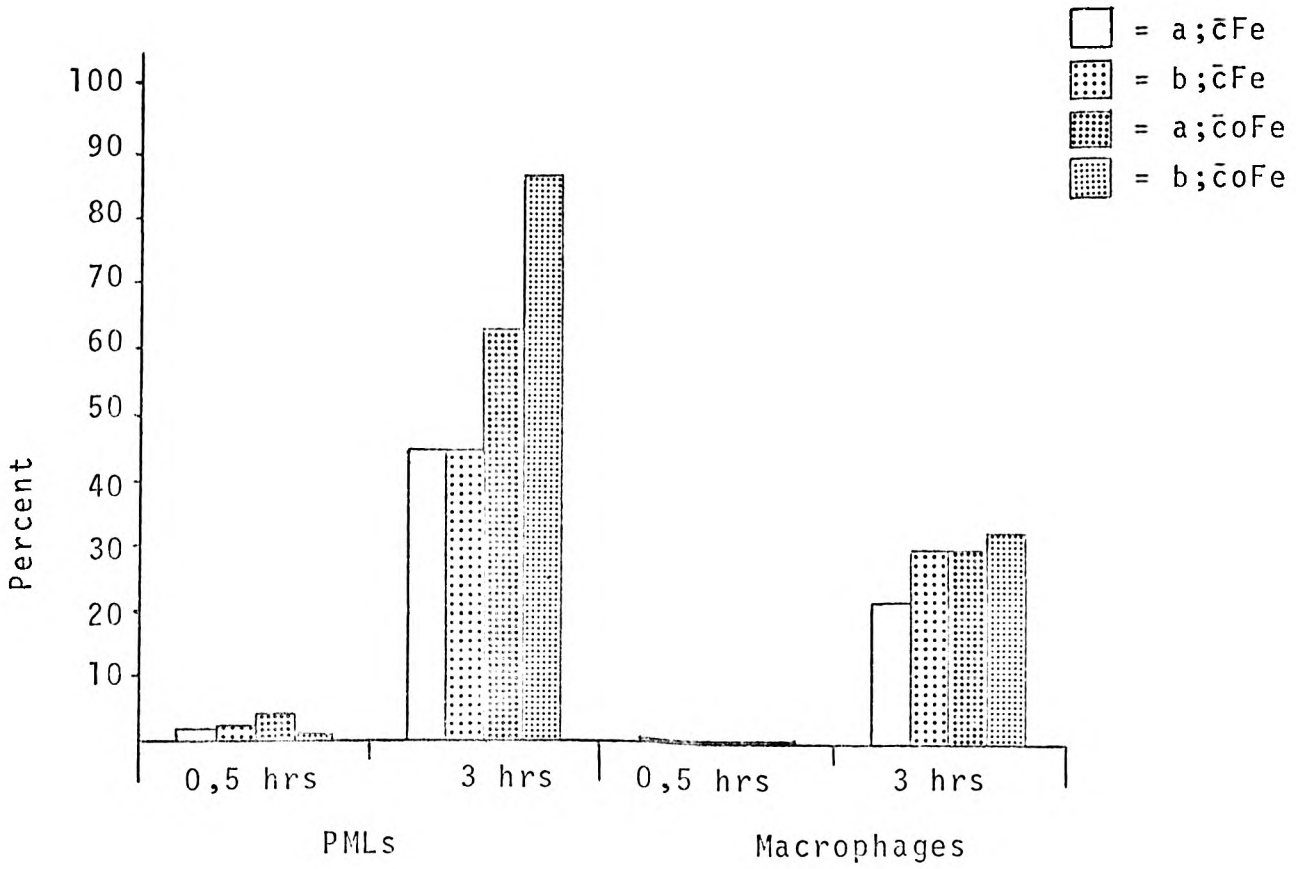

Figure 8 - 6. Percentage killing of *Y. enterocolitica* by polymorphonuclear leukocytes and macrophages at two different incubation times.

a = serum from rats with iron overload.

b = serum from rats without iron overload.

c̄Fe = leukocytes from rats pretreated with iron.

c̄oFe = leukocytes from rats without iron pretreatment.

8.4 DISCUSSION

It is clear that mice pretreated with a high dose of iron showed greater susceptibility when challenged with large inocula of *Y. enterocolitica*, compared with controls not having received iron.

The high mortality rate of the mice within 36 hours after infection in the first experiment may be ascribed to the toxic action of the ferric ammonium citrate. In the second and third experiments, the lowered early fatality rates encountered with the use of iron dextran or by administering smaller doses of ferric ammonium citrate over a period of three days are almost definitely the result of lower tissue concentrations of free iron as a result of slow release or staggering of the doses of iron.

In a similar experiment Robins-Browne *et al*, (1979) infected mice 24 hours after pretreatment with 200 μg ferric ammonium citrate, and achieved comparable results. However, unlike the findings described here, the mice in Robins Browne's experiments did not die within the first nine days after challenge. This can be ascribed to the fact that the acute toxicity of iron did not play a role in the latter experiments, as the iron was administered 24 hours before bacterial challenge and only survivors of the initial iron administration were used.

Rats were chosen for the second and third experiments, mainly because they are bigger than mice and therefore easier to handle, especially for intravenous infection. To compensate for their size, the amount of iron was increased about fourfold.

Initial studies performed by Carter in 1973 on the WA strain showed it to be highly virulent, eg an oral LD_{50} of 3×10^8 *Y. enterocolitica* WA was established for mice. Furthermore this strain also proved to be virulent for rats via the intravenous route (unpublished data from Carter, 1973). Considering the experiments reported here, it is possible that this strain lost

much of its virulence after two years of storage and repeated subculturing. As can be seen in table 8-3, a challenge dose of 10^9 bacteria was required to establish a fatal infection in about 32% of the animals. In mice treated with iron however, a mortality rate of 80% was recorded with that dose. Thus, in spite of relatively low virulence of the WA strain, the excess iron led to a very substantial increase in mouse mortality.

The increased susceptiability of mice to fatal infection by *Y. enterocolitica* in the presence of iron overload is also clearly demonstrated in figure 8-2. Unlike the controls, the survival curve for mice pretreated with iron falls sharply after the thirteenth day.

In the experiment on blood clearance in rats the initial effect of iron overload in *Y. enterocolitica*, rather than the final outcome, was examined. The rats were sacrificed at various intervals after challenge with the bacteria and plate counts of the liver, spleen and lung were made to monitor the systemic spread of the organisms up to 48 hours after challenge. From figures 8-3 and 8-4 it is evident that the bacteria survived better up to 24 hours in animals which were pretreated with high doses of iron. This correlates with the finding that survival of *Y. enterocolitica* within leukocytes is increased in the presence of excess iron (figure 8-6). At 48 hours post infection the numbers of bacteria recovered from the various organs were approximately the same for both the test and control groups, indicating that the organisms in the latter group was overcoming the natural defence systems of the host.

Iron overload in rats affects phagocytosis *in vitro* (table 8-6, figure 8-5). An increase in the numbers of *Y. enterocolitica* phagocytosed by both types of leukocytes is noted when serum from animals, injected

with high doses of iron, is added to the leukocyte-bacterial suspensions. Furthermore, combinations of leukocytes and serum from animals with iron overload showed increased phagocytosis. It is therefore possible that phagocytosis *in vivo* could be enhanced in the presence of excess iron, leading to increase in systemic spread of the bacteria.

In addition to this the bacteria seem to survive better inside these cells (table 8-6, figure 8-6) a factor which could contribute to the establishment of a fatal infection.

In a recent study Smith *et al* (1981) evaluated the enhancing effect of iron dextran on the virulence of *Y. enterocolitica* serotype O 3 in mice. They used the intraperitoneal route for both the iron dextran and the organisms. Their results further substantiated the role of iron on the pathogenicity of *Y. enterocolitica* and showed that their model could be used for the assessment of virulence of serotype O 3 strains.

The role of iron was also studied extensively in infections other than yersiniosis. Thus Kellogg *et al* (1968) found that both virulent and non-virulent gonococci required ferric ions for growth. Stimulation began at 5 ug%, and increased to a maximum at approximately 280 ug%, where the colony size had doubled compared with that of the controls. Iron compounds significantly influenced the fate of 11 day old chicken embryos inoculated intravenously with viable avirulent T3 or T4 gonococci but not with killed cells or sonicates. In the presence of added iron however, fewer organisms were needed to establish lethal infection (Payne and Finkelstein, 1975). These findings correlate with the *Y. enterocolitica* experiments reported here. The same workers also found that added iron did not influence the outcome of infection with fully virulent organisms. It was suggested that virulent organisms possess a mechanism by which they are able to acquire sufficient iron for growth.

It is interesting to note that in the case of *Y.*

enterocolitica, iron overload did not reduce the survival time of the infected mice but rather the number of mice that succumbed. In fact, in the group that received 10^9 bacteria, the mean number of days after challenge was slightly longer in the iron-treated animals (19 to 15 days respectively) (table 8-4). This finding suggested that host susceptibility played a more important role than the innate virulence of the organisms. This may relate to the protein binding of iron in the affected animal. The best known iron binding protein is transferrin which is found in the plasma of the majority of vertebrates as well as in secretions such as milk, nasal and bronchial mucus, tears, saliva, gastrointestinal fluid, hepatic bile, synovial fluid, urine, cervical mucus, and seminal fluid (Weinberg, 1978). It was shown that in mice infected with *Listeria monocytogenes* the plasma transferrin increased remarkably (Sword, 1966), thereby increasing the iron binding capacity of the host. It is likely therefore that with the administration of excessive doses of iron, the iron binding proteins such as transferrin, are saturated so that enough free iron is available to the pathogen.

Other host mechanisms which may have played a role concern the inhibition of chemotoxis (Becroft *et al*, 1977) and the bacterial action of leukocytes (Gladstone and Walton, 1970, 1971; Walton and Gladstone, 1976). Phagocytosis by neutrophils and opsonization by serum factors were however not affected directly (Burrows, 1963; Bullen *et al*, 1967; Gladstone and Walton, 1970; Becroft *et al*, 1977). It was also found that excess iron neutralized basic lysosomal proteins by precipitation (Gladstone and Walton, 1970, 1971; Walton and Gladstone, 1976) and might also impair leukocyte killing by reducing hydrogen peroxide to harmless water (Kaplan *et al*, 1975).

From these experiments one may conclude that iron

overload profoundly affects many diverse bacteria, including *Yersinia enterocolitica* and that the impairment of the host defences are probably related to the function of transferrin as well as leukocyte activity.

8.5 REFERENCES

Abramovitch, H. and Butas, C.A. (1973). Septicaemia due to *Yersinia enterocolitica*. Can. Med. Assoc. J. *109* : 1112-1115.

Becroft, D.M.O., Dix, M.R. and Farmer, K. (1977). Intramuscular iron-dextran and susceptibility of neonates to bacterial infections. Arch. Dis. Child. *52* : 778-781.

Blum, D., Viart, P. and Dachy, A. (1970). Septicémic à *Yersinia enterocolitica* chez deux enfants atteints de thallassémic majeure. Arch. Franq. Pédiat. *27* : 445.

Bradley, J.M. and Skinner, J.I. (1974). Isolation of *Yersinia pseudotuberculosis* serotype V from the blood of a patient with sickle-cell anaemia. J. Med. Microbiol. *7* : 383-386.

Bullen, J.J., Cushnie, G.H. and Rogers, H.J. (1967). The abolition of the protective effect of *Clostridium welchii* type A antiserum by ferric iron. Immunology *12* : 303-312.

Bullen, J.J. and Rogers, H.J. (19). Bacterial iron metabolism and immunity to *Pasteurella septica* and *Escherichia coli*. Nature (London) *224* : 380-382.

Bullen, J.J., Rogers, H.J. and Leigh, L. (1972). Iron binding protein in milk and resistance to *Escherichia coli* infection in infants. Br. Med. J. *1* : 303-312.

Burrows, T.W. (1963). Virulence of *Pasteurella pestis* and immunity to plague. Curr. Top. Microbiol. Immunol. *37* : 59-113.

Gladstone, G.P. and Walton, E. (1970). Effect of iron on the bactericidal proteins from rabbit polymorphonuclear leucocytes. Nature (London) *227* : 849-851.

Gladstone, G.P. and Walton, E. (1971). The effect of iron and haematin on the killing of staphylococci by rabbit polymorphs. Br. J. Exp. Pathol. *52* : 452-464.

Jackson, S. and Burrows, T.W. (1956). The virulence-enhancing effect of iron on non-pigmented mutants of virulent strains of *Pasteurella sepsis*. Br. J. Exp. Pathol. *37* : 577-583.

Kaplan, S.S., Quie, P.G. and Basford, R.E. (1975). Effect of iron on leukocyte function: inactivation of H_2O_2 by iron. Infect. Immun. *12* : 303-308.

Kellogg, D.S. (Jnr.), Cohen, I.R., Norins, L.C., Schroeter, A.L. and Reising, G. (1968). *Neisseria gonorrhoeae* II. Colonial variation and pathogenicity during 35 months *in vitro*. J. Bact. *96* : 596-605.

Payne, S.M. and Finkelstein, R.A. (1975). Pathogenesis and immunology of experimental gonococcal infection : Role of iron in virulence. Inf. Immun. *12* : 1313-1318.

Pollack, S., Aisen, P., Lasky, F.D. and Vanderhoff, G. (1976). Chelate mediated transfer of iron from transferrin to desferrioxamine. Br. J. Haematol. *34* : 231-235.

Rabson, A.R., Koornhof, H.J., Notman, J. and Maxwell, W.G. (1972). Hepatosplenic abscesses due to *Yersinia enterocolitica*. Br. Med. J. *4* : 341.

Robins-Browne, R.M., Rabson, A.R. and Koornhof, H.J. (1979). Generalized infection with *Yersinia enterocolitica* and the role of iron. in Contr. Microbiol. Immunol. vol. 5 ed. by. P.B. Carter, L. Lafleur and S. Toma, S. Karger, Basel, pp. 277-282.

Smith, R.E., Carey, A.M., Damare, J.M., Hetrick, F.M. Johnston, R.W. and Lee, W.H. (1981). Evaluation of iron dextran and mucin for enhancement of the virulence of *Yersinia enterocolitica* serotype O 3 in mice. Inf. Immun. *34* : 550-560.

Sword, C.P. (1966). Mechanisms of pathogenesis in *Listeria monocytogenes* infection. I. Influence of iron. J. Bact. *92 :* 536-542.

Walton, E. and Gladstone, G.P. (1976). Factors affecting the susceptibility of staphyloccocci to killing by teh cationic proteins from rabbit polymorphonuclear leucocytes: the effects of alteration of cellular energetics and of various iron compounds. Br. J. Exp. Pathol. *57 :* 560-570.

Weinberg, E.D. (1978). Iron and infection. Microbiol. Rev. *42 :* 45-66.

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9. BLOOD CLEARANCE OF *YERSINIA ENTEROCOLITICA*

9.1 INTRODUCTION

Carter and Collins (1974) showed that salmonellae and yersiniae penetrate the small intestine preferably in the distal ileum from where they spread to several discrete, rather than to single lymph nodes. As was shown in chapter 6 the bacteria are not retained in the lymph nodes for very long. It is therefore possible that they either reach the blood circulation via the lymphatic system, or by direct penetration of the blood vessels from the lamina propria. In experiments with white mice *Y. enterocolitica* was isolated from lung and liver 48 hours after infection via the oral route. Blood cultures at this stage, however, proved negative. Carter (1975) found that a rapidly fatal systemic infection ensued when *Y. enterocolitica* WA was administered intravenously, especially with organisms grown at 26°C. These organisms were cleared from the blood more slowly than those grown at 37°C.

The question arose as to what role the liver, spleen and lung play in the clearance of bacteria from the blood. The following experiments were devised in an attempt to gain better insight into this question.

9.2 MATERIALS AND METHODS

9.2.1 Bacterial strains (see Appendix B)

Two strains of *Y. enterocolitica* were chosen for this study, M2 a non-pathogenic and WA, a pathogenic strain for rodents. The bacteria were cultured at 25°C on nutrient agar (NA) for 24 hours, washed off into phosphate buffered saline (PBS) and the concentrations were then adjusted to 1×10^9 cfu/mℓ.

9.2.2 Procedure (see Appendix A)

Male outbred rats were aneasthetized with Halothane (ICI Pharmaceuticals) and the abdomens were

incised along the mid-ventral line. Of the bacterial suspensions 0,1 mℓ was injected into the mesenteric vein. The rats were sutured and returned to their cages. After the time intervals set out in table 9-1 they were sacrificed and parts of the liver, lung and spleen were removed aseptically. Each part was placed in a pre-weighed conical plastic tube (Falcon 2095) containing 5 mℓ PBS and glass beads. The tubes were weighed again to determine the mass of each piece of tissue. The tissues were then broken up by shaking and spot plate counts were performed. The 0-hour animals served as controls. From each animal a drop of heart blood was put into nutrient broth and incubated at 25°C overnight for the detection of *Y. enterocolitica* in the blood stream.

9.3 RESULTS

For this experiment one *Y. enterocolitica* strain pathogenic for rodents (WA, Carter, 1975) and another non-pathogenic strain (M2) were chosen. Three of the four rats infected with the WA strain died between 24 and 48 hours after challenge, compared with one out of four infected with the M2 strain. The other three of the latter group showed no outward signs of illness.

At autopsy, shortly after the death of the rats, or on sacrifice at 48 hours, all the animals infected with *Y. enterocolitica* WA, and two challenged with M2, presented with yellowish-white necrotic foci in the liver and spleen. These rats also presented with a greenish diarrhoea, but *Y. enterocolitica* WA could only be isolated from the stool of one.

Table 9-1 Results of plate counts of liver, spleen, and lung and isolations from blood in rats infected with *Y. enterocolitica* strains.

Strain (2 each)	Time interval after infection in hours	*Y. enterocolitica*, cfu/g tissue			
		Spleen	Liver	Lung	Isolation from heart blood
WA	0	$8,7X10^6$	$1,6X10^7$	$2X10^4$	+
WA	0,5	$1,6X10^6$	$1,8X10^7$	$4X10^2$	+
WA	4	$2,8X10^6$	$8,6X10^6$	$2,5X10^6$	+
WA	24	$3X10^4$	$2,1X10^4$	$1X10^4$	+
WA	48	$2,7X10^7$	$2,8X10^7$	$2,8X10^6$	-
M2	0	$1,9X10^6$	$3,2X10^6$	a	+
M2	0,5	$1,5X10^6$	$2,1X10^5$	a	+
M2	4	a	a	a	-
M2	24	$1,5X10^4$	a	a	-
M2	48	$5,6X10^4$	a	a	-

\- = negative;

\+ = positive;

a = $<10^2$

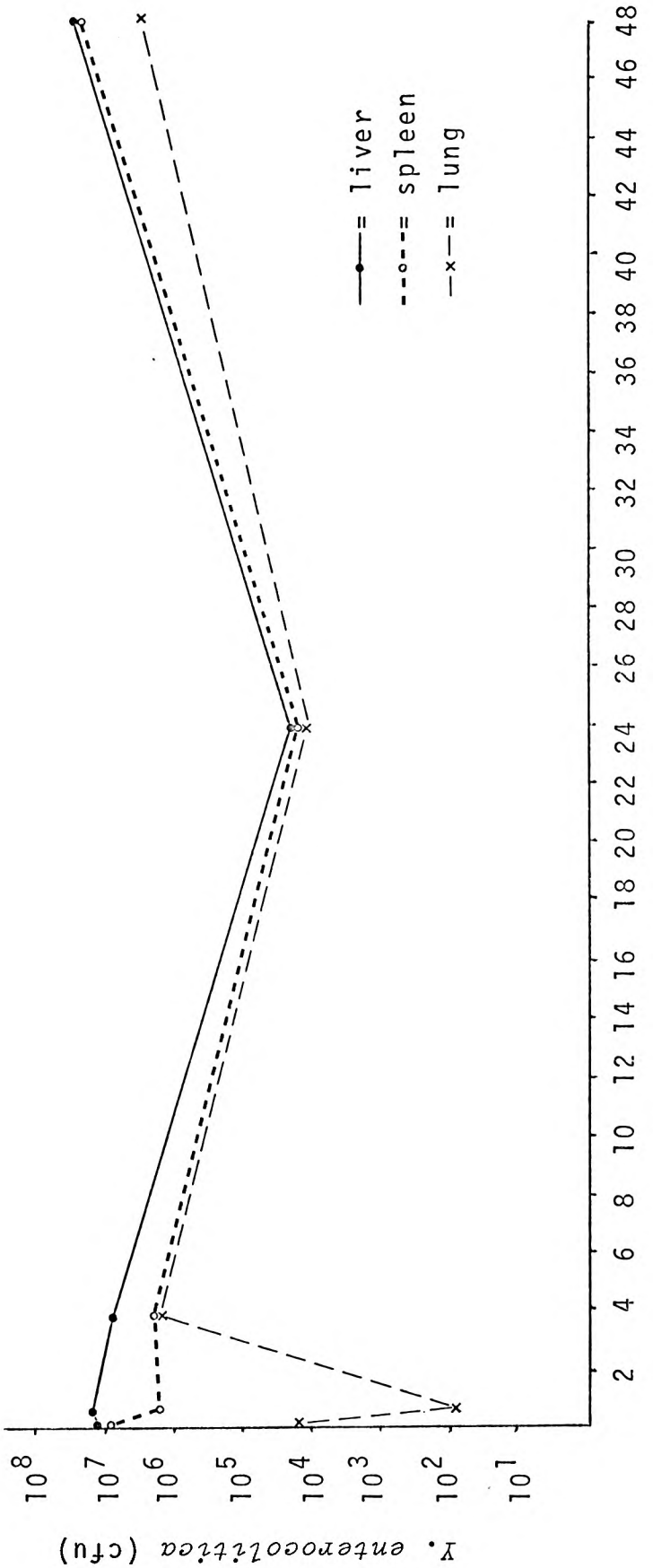

Figure 9-1. Numbers of *Y. enterocolitica* WA isolated from the spleen, liver and lung of animals infected mice the hepatic portal system.

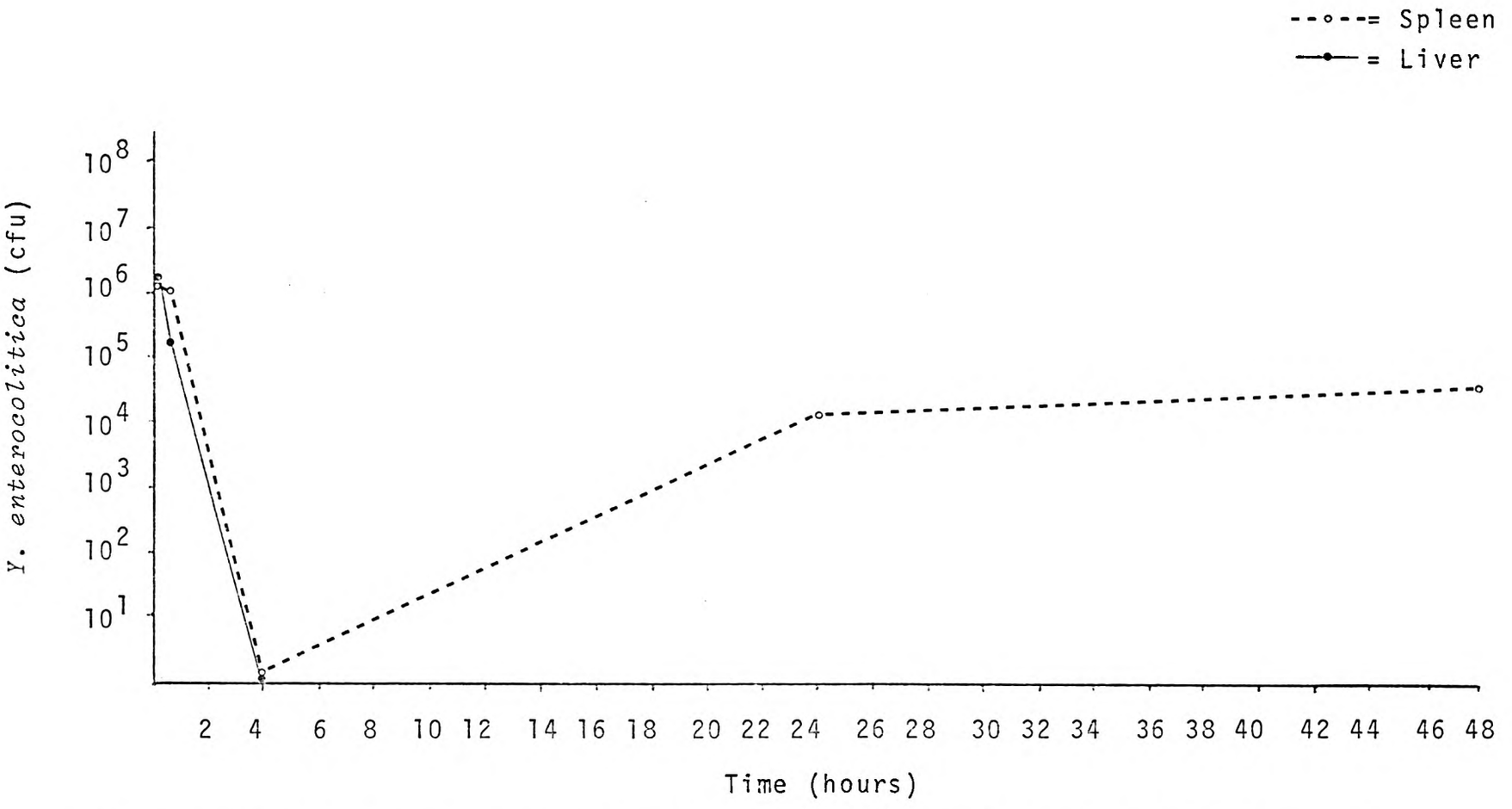

Figure 9-2. Numbers of *Y. enterocolitica* M2 isolated from the spleen and liver of rats infected via the hepatic portal system.

9.4 DISCUSSION

Positive blood cultures were obtained from rats infected with the WA strain up to 24 hours after infection (table 9-1) whereas the M2 strain could only be isolated from the blood up to 30 minutes after infection. It is thus clear that the M2 strain was cleared from the blood much faster than WA which only disappeared from the circulatory system between 24 and 48 hours after initiation of infection. The less virulent bacteria were removed from the blood stream at a much faster rate than the more virulent organisms.

It is evident that both the spleen and liver play a major role in clearing the blood of the invading pathogens (figures 9-1 and 9-2), although the lungs also appeared to play a role in the case of the WA strain (table 9-1, figure 9-1). After an initial decline in the numbers of bacteria up to 24 hours, there was a steep rise up to 48 hours after infection. The bacteria were however, totally cleared from the blood between 24 and 48 hours after challenge.

With regard to the relative role of the spleen, liver and lung, it appears that initially all three organs played an important role in the clearance of both the virulent and avirulent strain. However, it appears that definite infection with the virulent WA strain was only established after 24 hours and none of the three organs were able to completely destroy the organisms for the duration of the experiment.

In the case of the non-virulent strain M2, it appears as if the liver and lungs were more effective in either the early destruction of the bacteria, or that they allowed the organisms to escape to be subsequently trapped in the spleen. It is interesting to note that the numbers of bacteria increased in the spleen up to 24 h and then remained unchanged. It is obvious that even with the non-virulent organisms, the spleen was not able to completely

destroy all the trapped bacteria immediately.

It is also clear that initially the bacterial numbers decreased in these organs and then started to increase rapidly. The explanation for this must be sought in the action of the reticulo-endothelial cells in these organs which managed to trap the bacteria, although not very effectively, if one considers the persistance of the bacteria in the blood (table 9 -1). Only between 24 and 48 hours post infection, could the organs clear the blood of bacteria, but by that time the virulence of *Y. enterocolitica* WA had already overcome the host defence mechanisms and a fatal infection was established, mirrored in the death of three out of four rats.

Y. enterocolitica M2 was only found in the blood at 0 and 0,5 hours, which demonstrates the rapid clearing of the blood by the liver and spleen. The flattening of the curve for M2 isolations from the spleen indicates that the non-specific resistance of this organ was beginning to overcome the infection.

A factor that cannot be ignored in an experiment like this, is the serum killing effect of the blood (Nilehn, 1969; Robins-Browne *et al*, 1979). The WA strain was originally isolated from a patient with bacteraemia (Keet, 1974; Carter, 1975), so that it is likely that the resistance of this strain to serum killing is much higher than that of most other strains. This was borne out by the much longer persistance of WA in the blood stream in the present experiment.

In conclusion it is obvious that both liver and spleen play a role in clearence of the blood of *Y. enterocolitica* organisms, but the liver is probably the more important of the two organs as evidenced in the persistance of M2 in the spleen. One of the factors playing a part here could be the liver to extract and bind excess iron from the blood.

9.5 REFERENCES

Carter, P.B. and Collins, F.M. (1974). The route of enteric infection in normal mice. J. Exp. Med. *139* : 1189-1203.

Keet, E.E. (1974). *Yersinia enterocolitica* septicaemia. N.Y. State J. Med. *74* : 2226-2230.

Miles, A.A. and Mizra, S.S. (1938). The estimation of the bactericidal power of the blood. J. Hyg. *39* : 732-749.

Nilehn, B. (1969). Studies on *Yersinia enterocolitica*. Acta Pathol Microbiol. Scand. Suppl. *206* : 1-48.

Robins-Browne, R.M., Jansen van Vuuren, C.J. Still, C.S., Miliotis, M.D. and Koornhof, H.J. (1979). The pathophysiology of *Yersinia enterocolitica* in Contr. Microbiol. Immunol. vol. 5. ed. by P.B. Carter, L. Lafleur and S. Toma, S. Karger, Basel. pp. 324-328.

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10. PHAGOCYTOSIS AND KILLING OF *YERSINIA ENTEROCOLITICA* BY GRANULOCYTES AND MACROPHAGES *IN VITRO*

10.1 INTRODUCTION

Une (1977) showed that *Y. enterocolitica* had to penetrate epithelial linings to establish infection and also that pathogenic strains not only survived inside macrophages but also started multiplying after a short latent period.

In the study of blood clearance of *Y. enterocolitica* (chapter 9) similar observations were made when it was noted that the pathogenic strain first decreased in numbers and then increased rapidly up to 48 hours when the animals were sacrificed. These bacteria also persisted in the blood stream for 24 hours and longer, whereas the non-pathogenic strain could only be demonstrated in the circulation for up to 30 minutes after inoculation. This strain disappeared from the liver after 4 hours, but persisted in the spleen, increasing in numbers up to 24 hours, and stabilizing after that.

Une discussed phagocytosis and killing only by macrophages, but from the above experiment it was clear that the circulating granulocytes could also play a role in clearing the blood of bacteria. It was therefore decided to examine phagocytosis and killing of *Y. enterocolitica* strains by macrophages as well as granulocytes *in vitro*.

10.2 MATERIALS AND METHODS

10.2.1 Bacterial strains (see Appendix B)

Two strains of *Y. enterocolitica*, M2 and WA, were used. The bacteria were grown on nutrient agar (NA) at 25°C and 37°C for 24 hours, washed twice in phosphate buffered saline (PBS) and resuspended in Hanks' Balanced Salt Solution (HBSS)

(Wellcome). The bacterial concentration was then adjusted to approximately 1 x 10^9 cfu/mℓ (see appendix A).

Spot plate counts (Miles and Mizra, 1938) were done to determine the number of viable bacteria. Serial dilutions to 10^{-9} were made in saline and 10 µℓ of the 10^{-4} to 10^{-8} dilutions were "spotted" in triplicate onto NA plates. The plates were incubated at room temperature overnight and counted the next day.

10.2.2 Isolation of Leukocytes

(a) Monocytes

Thirty millilitre of heparinized (10 µg/mℓ) whole human blood was layered onto 20 mℓ Hypague-Ficoll (Pharmacia, Uppsula, Sweden) in a 50 mℓ conical plastic tube (Falcon 2095) and centrifuged at 200 x g for 40 minutes. The inter-phase, containing monocytes, was transferred to another 50 mℓ tube using a sterile disposable 10 mℓ syringe. The tube was filled with saline and centrifuged for 5 minutes at 200 x g. The supernatant was then discarded and the pellet washed twice with HBSS, resuspended in the same medium with 10% fresh AB serum and transferred to sterile glass petri dishes to allow the monocytes to adhere. The petri dishes were incubated at 37° C for 4 hours and the non-adhering cells (lymphocytes) were removed with HBSS which was first heated to 37° C in a water bath. The adhering macrophages were removed from the plates by adding ethylenediamine-tetra-acetic acid (EDTA) diluted to 1 in 5 000 at 37°C for 15 minutes. This suspension was centrifuged for 5 minutes at 100 x g, resuspended in HBSS in conical disposable 15 mℓ centrifuge tubes (Falcon 0075) and counted, using an Improved Neubauer Haemocytometer (Protea Laboratory Service). The number of cells was adjusted to 1 x 10^7

cells/mℓ. From this suspension 0,1 mℓ was transferred to each of a series of plastic tubes containing round glass coverslips for adherence of the macrophages. To each tube 1 mℓ HBSS was added and the tubes were then incubated for 2 hours at 37° C to allow for adhesion. Duplicate tubes were prepared for each organism at both temperatures as well as one control containing only HBSS.

(b) Granulocytes

After removal of the monocytes, as described above, the pellet was resuspended in 30 mℓ physiological saline and a 3% gelatin solution was added to give a final volume of 50 mℓ. The suspension was then incubated at 37°C for 30 minutes, after which the granulocyte interphase was removed with a sterile pasteur pipette and transferred to another 50 mℓ tube made up with saline to a volume of 50 mℓ. The suspension was centrifuged at 100 x g for 5 minutes and washed twice with HBSS at the same speed. The pellet was resuspended in 3 mℓ HBSS and transferred to 15 mℓ disposable conical tubes (Falcon). Any erythrocytes still present at this stage were lysed at 4°C for 5 minutes with a 0,83% NH_4Cl solution containing 1 g $KHCO_3$ and 37,5 mg EDTA per 1000 mℓ. After this the suspension was centrifuged at 50 x g for 5 minutes and the pellet resuspended in HBSS. The number of granulocytes per mℓ was determined as above and the concentration was adjusted to 1 x 10^7 cells/mℓ.

10.2.3 Phagocytosis and Killing

(a) Granulocytes

To four 5 mℓ disposable plastic tubes, the following was added for each serotype grown at 25°C and 37°C :

0,5 mℓ granulocyte suspension
0,1 mℓ bacterial suspension
0,3 mℓ HBSS
0,1 mℓ fresh human serum

giving an approximate ratio of 1:20 granulocytes: bacteria per mℓ. To the control tubes 0,8 mℓ HBSS was added, but without the granulocytes. The tubes were put onto a vertical rotating disc at 30 rpm and incubated at 37°C. Out of each group three tubes were tested at 10 minute-intervals and the fourth after 2 hours. The suspensions were centrifuged at 75 x g for 5 minutes and bacterial counts, using the spot-plate technique described above, were done on NA to determine the rate of phagocytosis. The pellet was resuspended in cold distilled water to give the white cells an osmotic shock and the released bacteria were counted to determine the rate of killing. The experiment was duplicated as described under (c).

(b) Macrophages

After washing the macrophage layers with PBS, 0,8 mℓ fresh HBSS, 0,1 mℓ bacterial suspension, and 0,1 mℓ fresh AB serum were added to the tubes. The suspensions were then incubated at 37°C with shaking by hand every 30 minutes to enhance contact between the bacteria and the leukocytes. After incubation, spot plate counts were done to determine the number of bacteria not phagocytised. The media were then poured off and the coverslips washed twice with PBS to remove non-adherent bacteria. One set of coverslips were fixed in methanol for 10 minutes and stained for three minutes with 5% Giemsa stain (Gurr SMM Limited). The duplicate set was kept in cold distilled water for 10 minutes to give the white cells an osmotic shock and bacterial counts were done to determine the number of viable bacteria released from the cells.

(c) Acridine orange staining technique

This technique of Smith and Rommel (1977) was also used to examine phagocytosis and killing of *Y. enterocolitica* WA by leukocytes. The white cell suspensions were prepared as described under 10.2.2 (a). The leukocyte-bacterial suspensions were prepared as follows: 0,02 mℓ fresh AB serum and 0,5 mℓ bacterial suspension were added to 0,5 mℓ of the granulocyte and macrophage suspensions respectively to give granulocyte-bacterial ratios of approximately 1:20. After mixing 300 uℓ of mixture was layered on coverslips 22 x 40 mm, supported on applicator sticks in Petri dishes lined with moist filter paper. The Petri dishes were incubated in a humidified CO_2 incubator at $37^{o}C$. Coverslips were removed at 30 minute and 3 hour intervals and stained as follows: they were rinsed carefully in HBSS (pH 7,2) and then stained for 45 seconds with 0,01% acridine orange (BDH Chemicals) in HBSS. The monolayers were then rinsed again with HBSS and the extracellular bacteria quenched with 0,05% crystal violet (BDH Chemicals) in normal saline (0,85% NaCl) for 45 seconds. The coverslips were finally rinsed with HBSS and mounted, monolayer side on the microscope slides and sealed with nail varnish. Slides were examined using a fluorescent microscope with incidence ultra-violet light. Live phagocytosed bacteria fluoresced bright green, extracellular bacteria pale green and dead ones bright red. The numbers of bacteria inside the cells were counted in about 100 cells and the average bacteria per cell was calculated. The rest of the

calculations were done as follows:

$$\text{Percentage phagocytosis} = \frac{\text{Average number of bacteria per cell}}{\text{Average number of bacteria available per cell}} \times 100$$

$$\text{Percentage killing} = \frac{\text{Average number of dead bacteria per cell}}{\text{Average number of dead + alive bacteria per cell}} \times 100$$

10.3 RESULTS

10.3.1 Results of first experiment

Giemsa-stained slides showed very few bacteria, some adhering to the membranes of the macrophages and others seemingly inside the cells. No vacuoles could be demonstrated around the intracellular bacteria.

The granulocytes phagocytosed both *Y. enterocolitica* strains very rapidly; within 30 minutes after initial contact more than 90% of the bacteria were engulfed. Two hours after inoculation the percentage phagocytosis of *Y. enterocolitica* WA was still the same, whereas in the case of M2 the percentage rose by a further 4% to 5% (table 10-1, figure 10-1). No difference in phagocytosis could be demonstrated between organisms grown at 25°C and 37°C.

Phagocytosis by macrophages (table 10-3, figure 10-1) was less efficient. In initial experiments not recorded in this thesis, the AB serum was omitted, resulting in very low phagocytosis. The addition of fresh AB serum enhanced phagocytosis but still not to the same extent observed in granulocytes.

A significant difference was observed between killing of M2 (non-pathogenic) and WA (pathogenic)

by both the granulocytes and macrophages (tables 10-2 and 10-3: figure 10-2). Macrophages were somewhat more effective than granulocytes in killing WA, but less against M2, although the rate of killing of the latter was higher than that of WA by both types of leukocytes.

Table 10-1 Results of Phagocytosis by Granulocytes. Bacteria incubated at 25°C and 37°C.

M2 (25°C)

Time	Inoculum[a] cfu/mℓ	Test count (b)cfu/mℓ	No. bacteria therefore phagocytosed	% Phagocytosis	Control[a] cfu/mℓ
10 min.	2,5 x 10	1,8 x 10^8	7 x 10^7	28	ND
20 min.	2,5 x 10^8	8 x 10^7	1,7 x 10^8	68	ND
30 min.	2,5 x 10^8	2 x 10^7	2,3 x 10^8	92	2,3 x 10^8
2 hours	2,5 x 10^8	1 x 10^7	2,4 x 10^8	96	ND

M2 (37°C)

10 min.	1,3 x 10^8	9,7 x 10^7	3,3 x 10^7	25,4	ND
20 min.	1,3 x 10^8	4,8 x 10^7	8,2 x 10^7	63,1	ND
30 min.	1,3 x 10^8	4,8 x 10^6	1,21 x 10^8	93,1	1,1 x 10^8
2 hours	1,3 x 10^8	2 x 10^6	1,28 x 10^8	98,5	ND

WA (25°C)

10 min.	2 x 10^8	1,4 x 10^8	6,2 x 10^7	31	ND
20 min.	2 x 10^8	7,1 x 10^7	1,3 x 10^8	65	ND
30 min.	2 x 10^8	1 x 10^7	1,9 x 10^8	95	1,7 x 10^8
2 hours	2 x 10^6	9 x 10^6	1,91 x 10^8	95,5	ND

WA (37°C)

10 min.	2,6 x 10^8	1,9 x 10^8	7 x 10^7	26,9	ND
20 min.	2,6 x 10^8	9,2 x 10^7	1,7 x 10^8	65,3	ND
30 min.	2,6 x 10^8	3 x 10^7	2,3 x 10^8	92	2,1 x 10^8
2 hours	2,6 x 10^8	3 x 10^7	2,3 x 10^8	92	ND

(a) = Mean of three counts;

(b) = Mean of three samples;

ND = Not Done

Table 10-2 Results of killing by granulocytes. Bacteria grown at 25°C and 37°C

M2 (25°C)

Time	No. Bacteria(a) phagocytosed (cfu/mℓ)	No. viable (b) bacteria after release by white cells (cfu/mℓ)	No. Bacteria therefore killed	% Killing
10 min.	7×10^7	$6,9 \times 10^7$	1×10^6	1,4
20 min.	$1,7 \times 10^8$	$1,6 \times 10^8$	5×10^6	2,9
30 min.	$2,3$ s 10^8	$2,1 \times 10^8$	2×10^7	8,7
2 hours	$2,4 \times 10^8$	1×10^7	$2,3 \times 10^8$	95,8

M2 (37°C)

10 min.	$3,3 \times 10^7$	$3,2 \times 10^7$	8×10^5	2,4
20 min.	$8,2 \times 10^7$	$7,9 \times 10^7$	3×10^6	3,7
30 min.	$1,21 \times 10^8$	$1,1 \times 10^8$	$1,1 \times 10^6$	9
2 hours	$1,28 \times 10^8$	$1,1 \times 10^7$	$1,17 \times 10^8$	91,4

WA (25°C)

10 min.	$6,2 \times 10^7$	$6,1 \times 10^7$	1×10^6	1,6
20 min.	$1,3 \times 10^8$	$1,27 \times 10^8$	3×10^6	2,3
30 min.	$1,9 \times 10^8$	$1,33 \times 10^8$	7×10^6	3,6
2 hours	$1,91 \times 10^8$	$1,1 \times 10^8$	$8,1 \times 10^7$	42,4

WA (37°C)

10 min.	7×10^7	$6,9 \times 10^7$	1×10^6	1,4
20 min.	$1,7 \times 10^8$	$1,63 \times 10^8$	7×10^6	4,1
30 min.	$2,3 \times 10^8$	$2,25 \times 10^8$	5×10^6	2,2
2 hours	$2,3 \times 10^8$	$1,4 \times 10^8$	9×10^7	39,1

(a) = From table 10-1

(b) = Mean of 3 samples

TABLE 10-3 Phagocytosis and killing by macrophages 4 hours after inoculation with *Yersinia enterocolitica*.

Strain	1. Inoculum(a) (cfu/mℓ)	2. Bacterial count of medium(b) (cfu/mℓ)	3. No. Bacteria ∴ Phagocytosed (1-2)	4. % Phagocytosis	5. Bacterial count after release by Macrophages.(b) (cfu/mℓ)	6. No. Bacteria ∴ killed (3-5)	% Killing
M2(25°C)	$1,5 \times 10^8$	$1,2 \times 10^8$	3×10^7	20	$1,3 \times 10^7$	$1,7 \times 10^7$	56,7
M2(37°C)	$1,8 \times 10^8$	$1,5 \times 10^8$	$3,6 \times 10^7$	20	$1,4 \times 10^7$	$2,2 \times 10^7$	61
WA(25°C)	$1,7 \times 10^8$	$1,4 \times 10^8$	3×10^7	17,6	$1,6 \times 10^7$	$1,4 \times 10^7$	46,7
WA(37°C)	2×10^8	$1,6 \times 10^8$	$4,8 \times 10^7$	24	$2,3 \times 10^7$	$2,5 \times 10^7$	52,7

(a) = Mean of three counts

(b) = Mean of three samples.

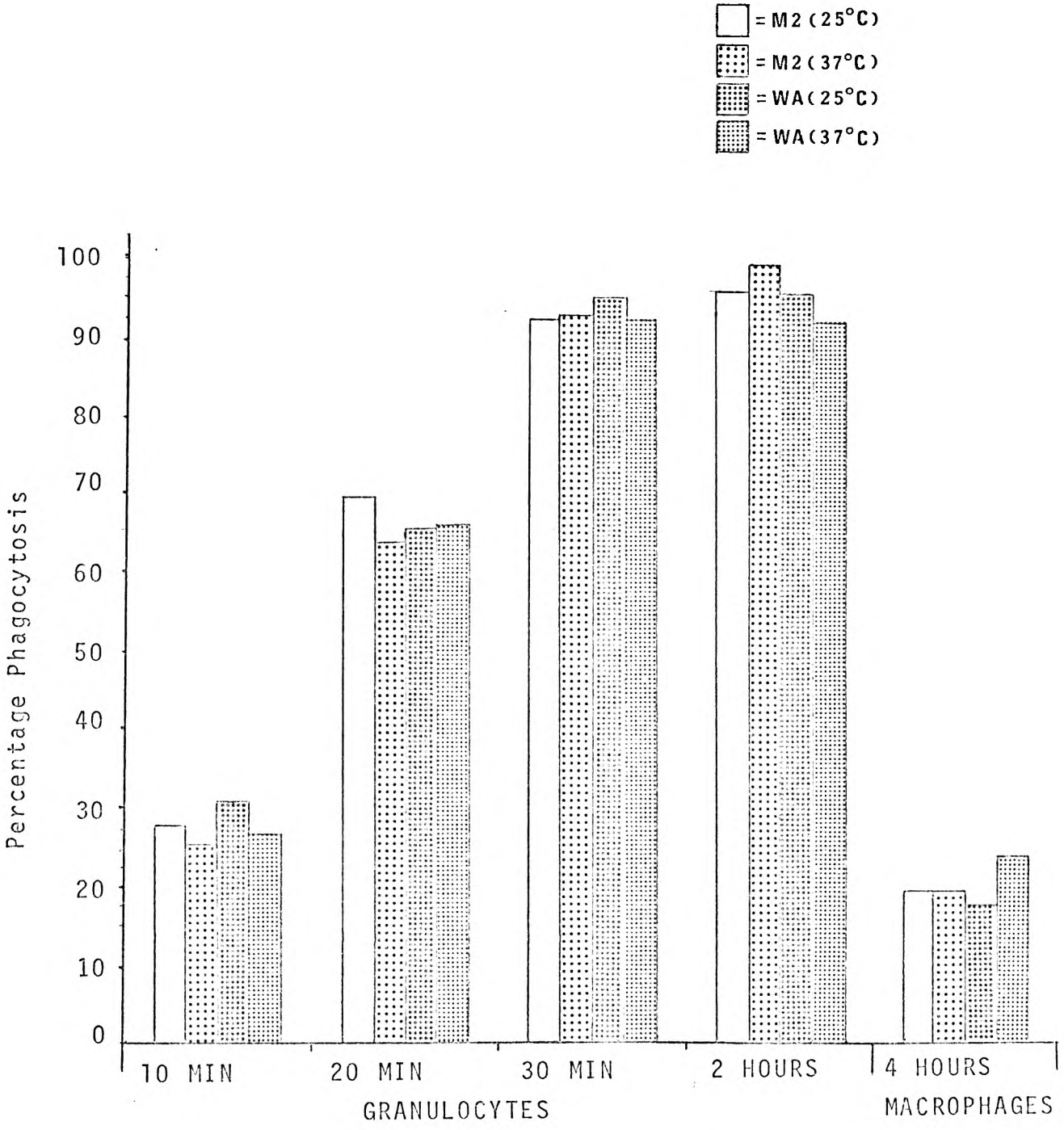

Figure 10-1. Comparison of phagocytosis of 2 *Y. enterocolitica* strains, grown at different temperatures, at various time intervals, by granulocytes and macrophages.

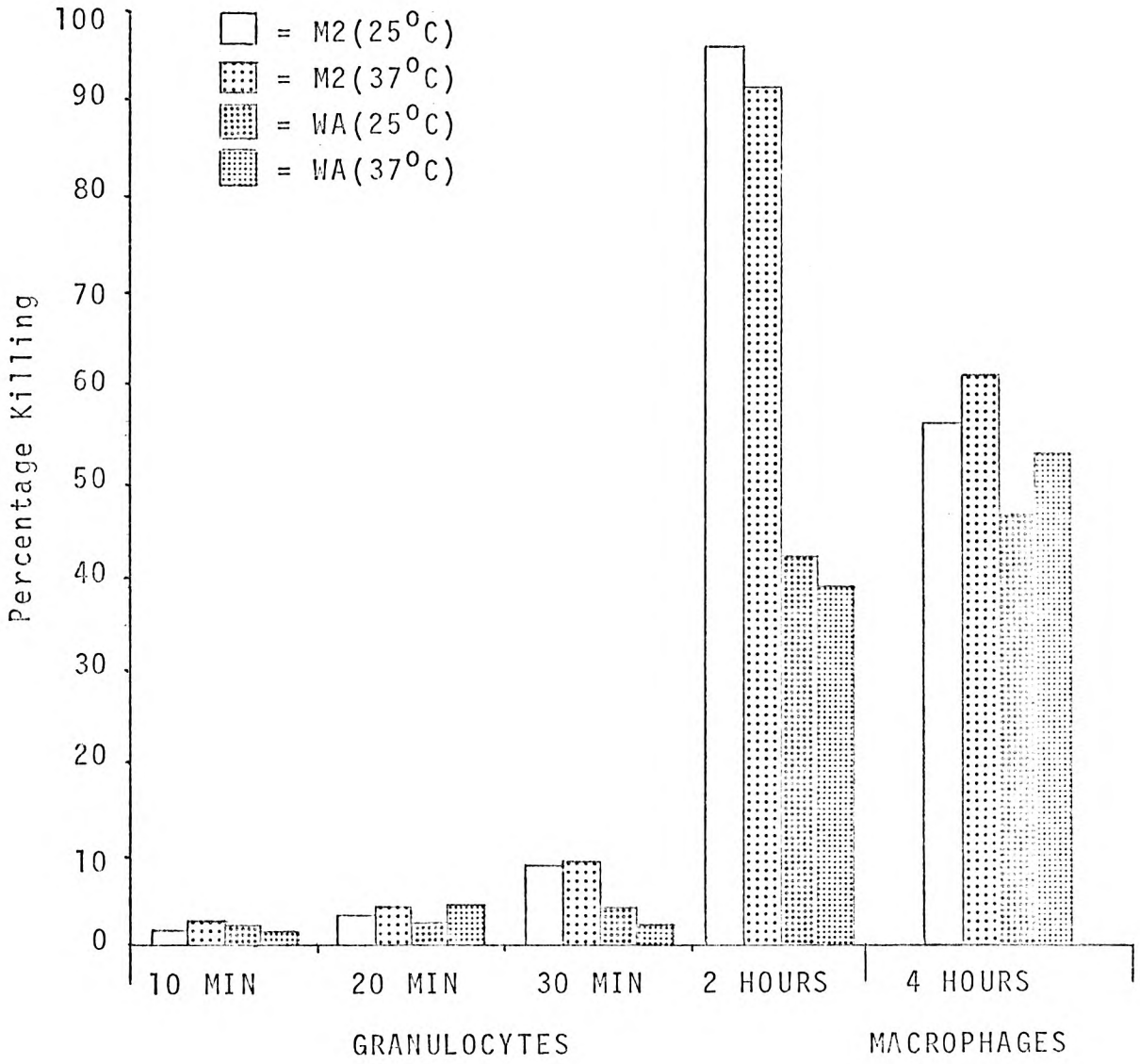

Figure 10-2. Comparison of killing of 2 *Y. enterocolitica* strains, grown at different temperatures, at various time intervals, by granulocytes and macrophages.

10.3.2. Results of second experiment.

The principle behind this test is that on staining cells, infected with bacteria, with acridine orange, dead material will fluoresce red and live material green under incidence ultraviolet radiation. This can be seen clearly in figure 10-3.

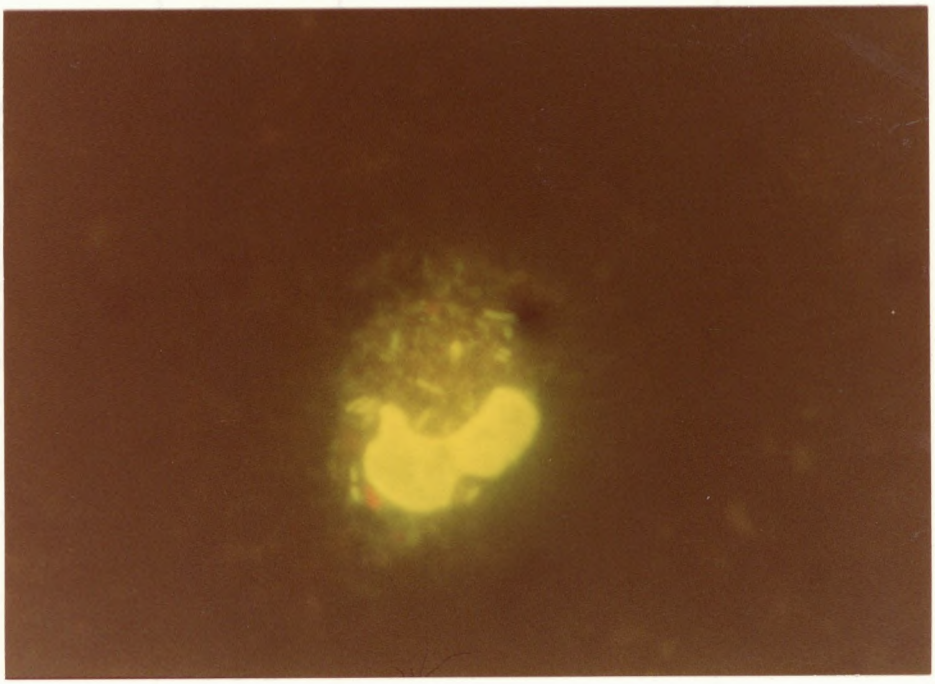

Figure 10-3. Acridine orange stained granulocyte with intracellular bacteria. X 1200.

Percentage phagocytosis by macrophages was appreciably lower than by granulocytes, especially after 30 minutes incubation (table 10-4 , figure 10-4). Killing by granulocytes was also about 15% higher than by macrophages (table 10-4 figure 10-4).

TABLE 10-4. Phagocytosis and killing of *Y. enterocolitica* WA by granulocytes and macrophages using the acridine orange staining technique.

Leukocytes	Average no bacteria available per cell	Average no bacteria phagocytosed	% Phagocytosis	Average no dead bacteria per cell	% killing
Granulocytes 0,5 hrs.	24	13,4	55,8	0,5	3,7
Granulocytes 3 hrs.	24	15,1	62,9	7,8	51,7
Macrophages 0,5 hrs.	24	2,7	11,3	0	0
Macrophages 3 hrs.	24	7,9	32,9	2,8	35,4

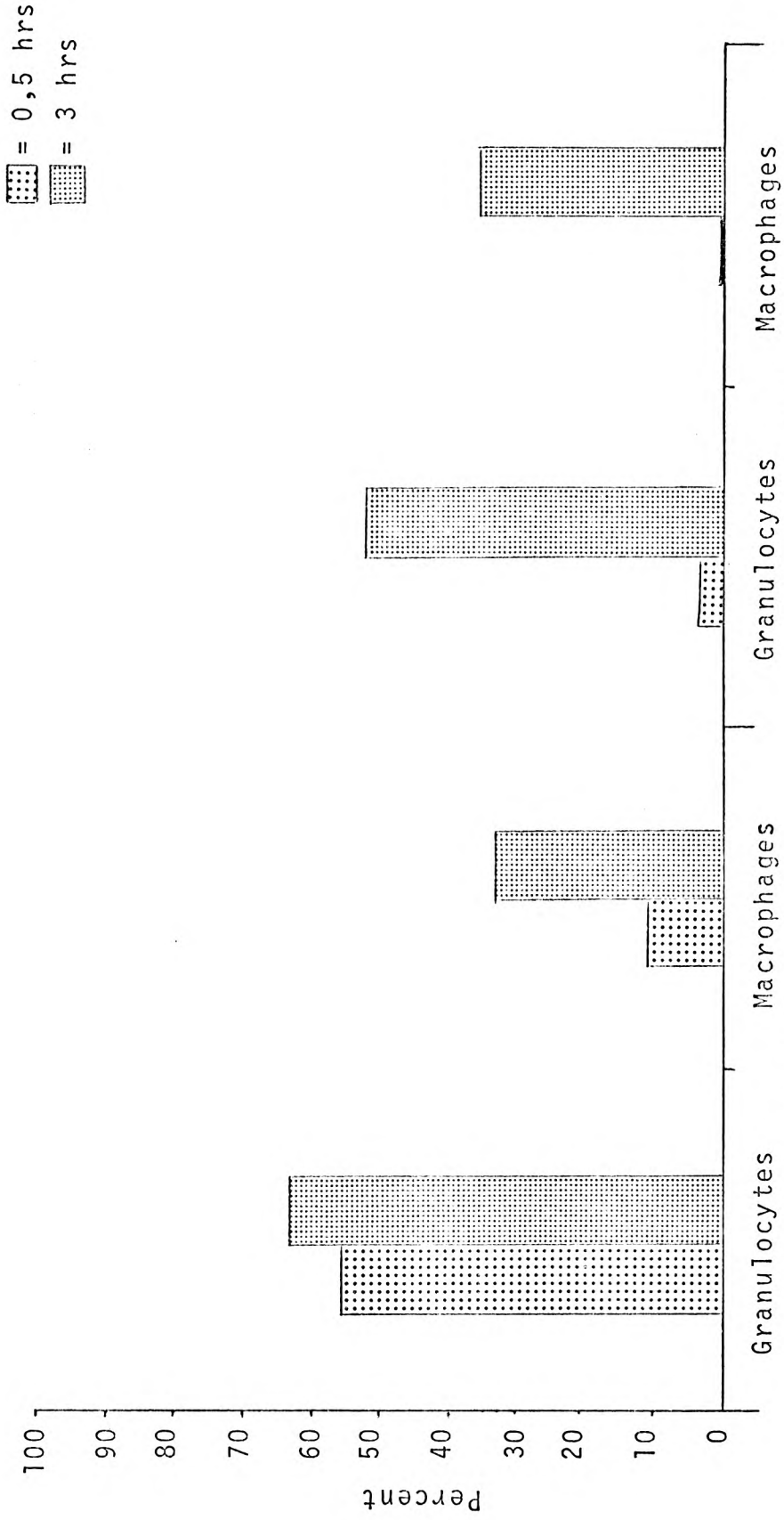

Figure 10-4. Phagocytosis and killing of *Y. enterocolitica* WA by granulocytes and macrophages as demonstrated by using acridine orange staining.

10.4. DISCUSSION

Une (1977) described the correlation between HeLa cell infectivity and phagocytosis of *Y. enterocolitica* by rabbit peritoneal macrophages. It was found that those strains which showed an ability to infect HeLa cells were phagocytosed by macrophages at a higher rate than those strains which lacked this ability.

The M2 strain used in this study, was shown to be unable to invade HeLa cells (table 12-1) as well as rabbit intestinal mucosa (chapter 11). On the other hand, the WA strain invaded both mucosal and HeLa cells. These two strains were therefore chosen to examine the effect of phagocytosis and killing by human granulocytes and macrophages. The WA strain was also used in phagocytosis experiments with granulocytes and peritoneal macrophages from rats with acute iron overload (chapter 9).

Une only tested the action of macrophages, but the findings in chapter 13 suggested that granulocytes also played a role as a first line of defence against organisms invading the blood stream. In these experiments it was found that the non-pathogenic M2 strain was cleared from the blood and also disappeared from the liver much faster than the pathogenic WA strain. From tables 10-1 and 10-3 and figure 10-1 it is evident that there is little difference in the rate of phagocytosis of M2 and WA, by granulocytes. These two strains were however, found to be phagocytosed at different rates by macrophages; the percentage phagocytosis of WA was higher, bearing out the findings of Une.

In the first experiment it was found that phagocytosis of both strains was more than 90% complete

after 30 minutes exposure to granulocytes, whereas only about 20% of the bacteria were phagocytosed by macrophages after 4 hours incubation.

In the second experiment both types of leukocytes were tested for phagocytosis at 30 minutes and 3 hours incubation. The percentage phagocytosis by granulocytes was much lower than in the first experiment at both time intervals. It may be that not all the bacteria are released from the cells by osmotic shock, thus giving false counts, whereas with acridine orange staining actual numbers of bacteria are counted microscopically.

Comparing the two techniques for phagocytic power of macrophages, the second one showed a higher percentage phagocytosis for *Y. enterocolitica* WA. This may be explained by the mixing of the bacteria and macrophages before allowing the cells to adhere to the coverslips, thereby increasing the contact between the phagocytes and micro-organisms.

The granulocytes as well as the macrophages were able to kill M2 more efficiently than WA. This finding correlates with the results discussed in chapter 9, where M2 disappeared completely from the liver and lungs in the test animals, as apposed to WA, which not only persisted, but started to multiply after 24 hours. Une also found that when non-pathogenic *Y. enterocolitica* were used, they slowly decreased in numbers after phagocytosis by rabbit peritoneal macrophages, but in the case of pathogenic strains they first decreased and then started to increase in numbers, supporting the above mentioned findings.

From the results given in tables 10-2 and 10-3, and figure 10-2, the granulocytes seem to be more

effective in killing M2 than the macrophages. From the same tables and figure it seems that it is the other way around concerning the WA strain, even if the difference is slight. Considering the results recorded in table 10-4 and figure 10-4 it is clear however that granulocytes are also more efficient in killing WA. Kagan *et al* (1975) found that granulocytes killed 90 to 96% *Staphylococcus aureus* in 2 hours. These phagocytes therefore play an important role in the defence of the body against pathogens invading the blood stream.

Another conclusion which can be drawn from these findings is that pathogenic *Y. enterocolitica* are able to survive inside leukocytes and in this way may allow for systemic dissemination of infection.

10.5 REFERENCES

Kagan, E., Soskolne, C.L., Zwi, S., Hurwitz, S., Maier, G.M.G., Ipp, T. and Rabson, A.R. (1975). Immunologic studies in patients with recurrent bronchopulmonary infections. Am. Rev. Respir. Dis. *111 :* 441-451.

Miles, A.A. and Mizra, S.S. (1938). The estimation of the bactericidal power of the blood. J. Hyg. *39 :* 732-749.

Smith, D.L. and Rommel, F. (1977). A rapid micro-method for the simultaneous determination of phagocytic-microbicidal activity of human peripheral blood leukocytes *in vitro*. J. Immunol. Methods. *17:* 241-247.

Une, T. (1977). Studies on the pathogenicity of *Yersinia enterocolitica II*. Interaction with cultures cells *in vitro*. Microbiol. Immunol. *21 :* 265-377.

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11. INVASIVENESS OF *YERSINIA ENTEROCOLITICA*

11.1 INTRODUCTION

There are three basic mechanisms by which bacteria may cause diarrhoea in humans. These categories are determined by the characteristic interaction of the bacteria with the intestinal mucosa (Plotkin *et al*, 1979). *Vibrio cholerae* is the classical example of the first group together with some strains of *Escherichia coli*. These bacteria multiply in the small intestine releasing an exotoxin (enterotoxin) which causes loss of fluid and electrolytes. Although the organisms adhere to the intestinal mucosa there is no invasion and no obvious morphological changes (Sprinz, 1969; Du Pont and Hornick, 1973).

Of the second group of enterophathogens, *Shigella* is the classical example and some *E. coli* strains also show this type of pathogenic mechanism, viz invasion of the mucosa (Takeuchi *et al*, 1965; Du Pont *et al*, 1971; Kent *et al*, 1976). In shigellosis there is penetration of the colonic mucosa with subsequent multiplication of the bacteria and destruction of the tissues (Labrec *et al*, 1964; Ogawa *et al*, 1967), although many patients develop a watery diarrhoea before classical dysenteric picture of blood and mucus in the stool, indicating an initial involvement of the small intestine (Du Pont and Hornick, 1973). In some patients a watery diarrhoea may be the only manifestation of the disease (Du Pont *et al*, 1971).

The salmonellae constitute the third important group of intestinal pathogens (Sprinz, 1969). These bacteria are also invasive in the small intestine and colon, but there is very little destruction of the mucosa (Takeuchi, 1967; Du Pont and Hornick, 1973).The epithelial lining remains intact and the organisms invade the lamina propria where an inflammatory response is elicited. *S. typhi* causes a predominantly mononuclear cell inflammatory response, while non-typhoid salmonellae result in a predominantly polymorphonuclear leucocyte reaction in which the bacteria are phagocytosed and contained in the lamina pro-

pria (Du Pont and Hornick, 1973). Unlike *S. typhi* these salmonellae are destroyed by neutrophils (Plotkin *et al*, 1979). Salmonellae also cause fluid secretion in the small intestine, but it is usually much less in comparison with the amount of fluid secreted in diarrhoea caused by *V. chlolerae*. The fluid secreted into the small intestine is not due to the elaboration of a free enterotoxin demonstrable *in vitro*, but is more likely to be associated with the invasion of the bacteria and subsequent inflammation (Gianella *et al*, 1973). However, a bacterial property or factor seems to contribute to this fluid loss in addition to the invasion of the mucosa. This factor may induce fluid secretion through the accumulation of cyclic adenosine monophosphate indirectly by stimulating the prostaglandin system and direct activation of adenyl cyclase (Gianella *et al*, 1973; Gots *et al*, 1974; Gianella *et al*, 1975).

Before enteric pathogens can elicit their effects, either by elaboration of an enterotoxin or by invasion into the tissues, it is necessary for the bacteria to adhere to the mucosal surfaces (Sack, 1975; Guentzel *et al*, 1977; Richards and Douglas, 1978). The adherence phenomenon is probably essential for the colonization of enteropathogenic bacteria.

As will be discussed in greater detail later, the mechanism of *Y. enterocoltica* infections resembles that of salmonellosis most closely. Infection with this organism presents with diverse symptoms, of which non-bloody diarrhoea (watery, sometimes greenish mucoid), abdominal pain, nausea and vomiting are the most common (Gutman *et al*, 1973; Finlayson *et al*, 1973; Schieven and Randall, 1974; Bradford *et al*, 1974). Evidence of gastroenteritis is, however not always present (Aryastson *et al*, 1971). In addition to the role of *Y. enterocolitica* as a recognised gastrointestinal pathogen, the isolation of this bacterium from mesenteric lymph nodes, which in most cases is associated with terminal ileitis (Windblad *et al*, 1966; Nilehn and Sjöström, 1967, Jansson *et al*, 1968), bacteraemia (Blum *et al*, 1970; Chessum *et al*, 1971; Hewstone and Davidson,

1972; Jacobs, 1975; Kent *et al*, 1976), and hepatosplenic abscesses (Mollaret, 1965; Rabson *et al*, 1972), provide ample evidence for its invasive potential. Using mice as experimental animals, Carter (1975), demonstrated invasion by *Yersinia enterocolitica* of the ileal mucosa, primarily involving Peyer's patches as well as mesenteric lymph nodes, lung, spleen and liver. Invasiveness for HeLa cells by strains of *Y. enterocolitica* has also been established (Une *et al*, 1977; Lee, 1977).

The following experiments were designed to study the mechanism of invasion by *Y. enterocolitica:*

11.2 MATERIALS AND METHODS

11.2.1 Ligated Rabbit Ileal Loops (De and Chatterje, 1953)

(a) Bacterial strains (See appendix B)

The following *Y. enterocolitica* strains were tested: M1; M2; M3; 5122; Constantinides; Thompson; 4257; M4,32; M4,33; M5,27; M6,30; M6,31; M7,8; and M8. They were grown on nutrient agar (NA) for 24 hours at 25°C and 37°C, washed off the plates into brain heart infusion (BHI) broth and the concentrations of bacteria were determined by means of spectrophotometric methods (Appendix A) and spot plate counts (Miles and Mizra, 1938). The working concentrations were adjusted to 3×10^7 colony forming units (cfu)/mℓ. The positive control for invasion, *Salmonella typhimurium* W118 was grown on NA for 24 hours, washed off into BHI broth and the bacterial concentration adjusted to 3×10^6 cfu/mℓ. As positive control for fluid accumulation live *Vibrio cholerae* was used. These organisms were grown on syncase agar (Finkelstein *et al*, 1966) for 24 hours at 37°C, washed off into BHI broth and the bacterial concentration was adjusted to 1×10^5 cfu/mℓ. Syncase- and BHI broths were used as negative controls.

(b) Procedure

Outbred rabbits weighing 2 to 3 kg were starved for 48 hours with water *ad lib*, prior to inoculation with the bacteria. The rabbits were anaesthetized with pentobarbitone sodium (Maybaker (SA) (Pty) Limited) about 30 minutes after an intramuscular injection of 1 mℓ chlorpromazine (largactil) (Maybaker) which acted as a sedative. The abdomen was incised on the mid-ventral line and ileal loops of approximately 15 cm each, were ligated, starting at the end proximal to the caecum. Three ligatures were tied between each loop, the first two about 1 cm apart to form the loop, and the third to seal off the point of injection of the bacterial suspension. Two mℓ of each suspension were injected into each loop. The top and bottom loops were used for the negative controls and the middle of five or seven loops were used for the positive control. After infection, the abdomen was sutured and the rabbit returned to its cage. Animals were sacrificed at intervals of 4, 6 and 18 hours by intracardiac injection of 5 mℓ pentobarbitone (Euthobarb) (Maybaker). Immediately after death, the abdomen was opened, the ileum removed and placed on ice. From each loop the following was removed aseptically :

(i) any fluid accumulated in the loop into a sterile container;

(ii) two small pieces of tissue, one frozen immediately for cryostat sectioning, and the other fixed in 10% formol saline for wax embedding;

(iii) part of the contents for smears; and

(iv) a piece of the tissue was transferred to nutrient broth for culturing.

From four animals small pieces of tissue were placed in cold 5% gluteraldehyde for electron microscopy. Blood samples for culturing were obtained by cardiac puncture.

11.2.2 Infection of mice

(a) Bacterial strains (see Appendix B)

The following bacterial strains were used in this experiment:

Y. enterocolitica strain Thompson and a *Y. pseudotuberculosis* strain. The bacteria were grown on NA at 25°C for 24 hours after which they were washed off in phosphate buffered saline (PBS). The organisms were washed twice in PBS and then resuspended in the same buffer. The concentrations of the bacteria were then adjusted to 4×10^9 cfu/mℓ.

In another experiment strain 5122 was used, cultured in the same way as described above.

(b) Procedure

White, outbred mice, weighing 18 to 20 g, were put together in four batches of eight mice per batch. Each group was challenged intragastrically with a 0,1 mℓ suspension of the two bacteria. *Y. pseudotuberculosis* was used as positive and PBS as negative control. Mice were sacrificed in pairs at intervals of 6, 24, 48 and 192 hours after inoculation. At necropsy the iliac lymph nodes were removed aseptically, ground with a sterile mortar and pestle in 1 mℓ PBS and plate counts were done. Small pieces of ileum were fixed in 10% formol saline for histological examination.

In another experiment ten mice were inoculated intragastrically with 0,1 mℓ of a suspension of *Y. enterocolitica*, containing 1×10^9 cfu/mℓ.

The mice were sacrificed after four days and pieces of liver, lung, spleen and intestine were placed into nutrient broth for 96 hours at $4^{o}C$, after which the culture were incubated at room temperature for 24 hours. Nutrient agar plates were streaked from these cultures and incubated at room temperature for 24 hours to isolate *Y. enterocolitica.*

11.2.3 Infection of guinea pigs

(a) Bacterial strains (see Appendix B)

Y. enterocolitica 5122 and *S. typhimurium* W118 were employed in this experiment. The bacteria were cultured as in11.2. and concentrations were adjusted to 1 x 10^{8} cfu/mℓ in PBS.

(b) Procedure

Adult guinea pigs were starved for 48 hours with water *ad lib,* prior to infection. Intestinal movement was slowed down by intraperitoneal injection of 0,1 mℓ of tincture of opium and the animals were inocultated intragastrically with 2 mℓ of each of the bacterial suspensions after 30 minutes. The test was performed in duplicate with the two negative controls receiving 2 mℓ sterile PBS each. The guinea pigs were sacrificed 48 hours later by intracardial injection of euthobarb (pentobarbitone) (Maybaker). The abdominal cavity was opened immediately after death and the small intestine was infused carefully with 10% formol saline so as not to disfigure the villi. The intestine was then removed and placed into a glass jar containing 10% formol saline. After fixation the intestine was cut longitudinally into two halves of which one half was processed and the other half retained for subsequent study, if required. The flattened intestine was carefully rolled onto an applicator stick before embedding into wax. This "swiss roll" pro-

cedure permits the examination of the whole length of the intestine in a short time.

11.2.4 Histological Procedures

(a) Sectioning

Wax-embedded tissues were sectioned to 4 μm thickness using a rotary microtome. The sections were floated out in a water bath at 50°C, fished out onto clean slides and fixed on a hot plate. Xylol was used as clearing agent after which the sections were rehydrated through a series of diminishing alcohol concentrations.

Frozen tissues were sectioned to 5 μm thickness using a cryostat microtome. Sections were put onto clean glass slides and stored at -20°C until stained.

Gluteraldehyde-fixed tissues were processed and sectioned for electron microscopy by the Department of Histopathology, SAIMR.

(b) Staining

Most of the sections from wax-embedded tissues were stained with 10% Giemsa's stain (Gurr) for 10 minutes, differentiated with a 0,1% acetic acid and mounted in DePeX (Gurr) under glass coverslips.

Some of the wax-embedded sections were trypsinised for 2 hours at 37°C in a Coplin jar containing 0,1% trypsin and 0,1% calcium chloride ($CaCl_2$) in distilled water, dried and stained using the indirect immunofluorescent method (Weller and Coons, 1954). The frozen sections were fixed in cold acetone (-20°C) for 10 minutes and stained in the same way. The sections were washed in two changes of PBS (pH7,2) for 20 minutes to remove all non-adherent protein and bacteria, then coated with a 1:20 dilutions of specific rabbit

antiserum for 30 minutes in a moist chamber. The rabbit antiserum was washed off in two changes of PBS for 30 minutes. The sections were then stained with a 1:30 dilution of fluorescent iso-thiocianate conjugated sheep-anti-rabbit serum (Burroughs-Wellcome) for 30 minutes in a moist chamber and washed again in two changes of PBS, During the last wash a few drops of a 20% Evans' blue solution were added to the PBS to counteract background fluorescence. The slides were then dried and mounted in a 10 + 1 glyserol+PBS solution under glass coverslips. They were viewed with as little delay as possible, using a Leitz phase-contrast fluorescent microscope.

11.2.5 Guinea pig keratoconjunctivitis test (Serény, 1955, 1957)

(a) Bacterial strains (see Appendix B)

The same strains of *Y. enterocolitica* as in 11.2.1 was used with *Sh. flexneri* 1251 as positive control. The bacteria were also grown in the same way as above, but the concentrations were adjusted to at least 1 x 10^{10} cfu/mℓ.

(b) Procedure

Adult guinea pigs were kept one to a cage. A drop of bacterial suspension was placed into one eye of each animal with a sterile Pasteur pipette and the animals were examined twice daily for signs of keratoconjunctivis, over a period of 6 days. The experiment was repeated, using adult rabbits as experimental animals.

11.3 RESULTS

11.3.1 Rabbit ileal loop experiments

(a) Giemsa-stained sections

Sections of the negative control loops ap-

peared normal (fig. 11-1). The microvilli were intact without any adherent bacteria, although the latter could be seen in the lumen. In some 18-hour loops a small number of polymorphonuclear leucocytes (PML's) was observed in the lamina propria and blood vessels, but they were not nearly as numerous as in the infected loops.

Live *V. cholerae* were employed in positive control loops which were used to demonstrate fluid accumulation. Adherence of bacteria to the microvilli were very prominent in sections of these loops (fig. 11-2). There was evidence of mild inflammatory changes and although the villi generally looked perfectly normal, in some sections extensive flattening was observed. There was evidence of dilatation of blood vessels with oedema and a few PML's were present in the lamina propria. No invading bacteria could be demonstrated.

In three rabbits *S. typhimurium* strain W118 was used in positive control loops for the demonstration of invasion. In sections of these loops large numbers of epithelial cells could be seen in the lumen, as well as extensive invasion of the mucosa by bacteria. The PML infiltration in the deeper layers was not prominent, but many leucocytes could be seen at the bases of the villi in the lamina propria. In the majority of loops infected with *Y. enterocolitica* serotype O 3, small, rodlike bacteria were seen adhering to the microvilli (fig. 11-3), with some having penetrated into the tissues (fig. 11-4). Adherence was best demonstrated in 4 and 6 hour loops, with some bacteria invading the mucous membrane, but not as deep by as could be demonstrated in 18-hour loops, where they extended up to the muscularis mucosae. In

most cases the mucosa seemed intact, although fairly large numbers of epithelial cells were demonstrated in Giemsa-stained smears of luminal contents. The goblet cells could be seen emptying their contents into the lumen, and in some of these, clumps of small bacilli were noted (fig. 11-5). Inflammation was evident by the dilatation of blood vessels and the increased numbers of PML's inside the vessels as well as in the lamina propria.

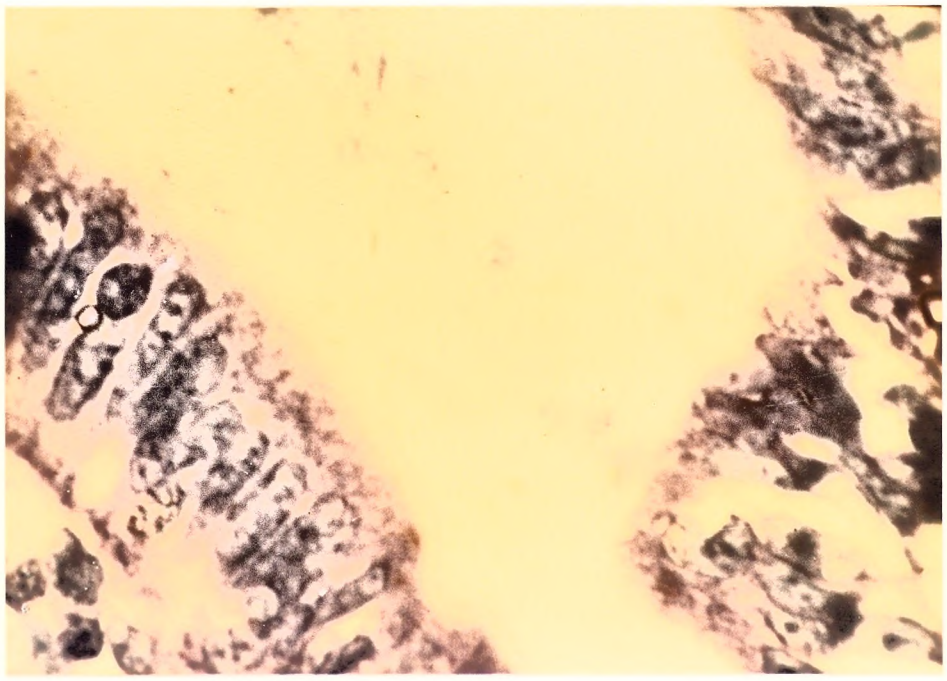

Figure 11-1. Giemsa-stained section of negative control loop injected with BHI broth, X 1 200.

In some sections large numbers of PML's were also observed in the proximal vicinity of the basement membrane and in the lacteal ducts. In the majority of sections numerous bacteria, of which approximately 70% were Gram-negative rods, were noted in the lumen. Erythrocytes and PML's were also present in the lumen, sometimes in fairly large numbers, and in many cases bacteria were adhering to the erythrocytes. In only one section could bacteria be seen inside a PML which was located in one of the villi. In most sections the villi appeared normal, although some seemed to be flattened without any other damage. More debris could be observed in the lumina of infected as opposed to the negative loops.

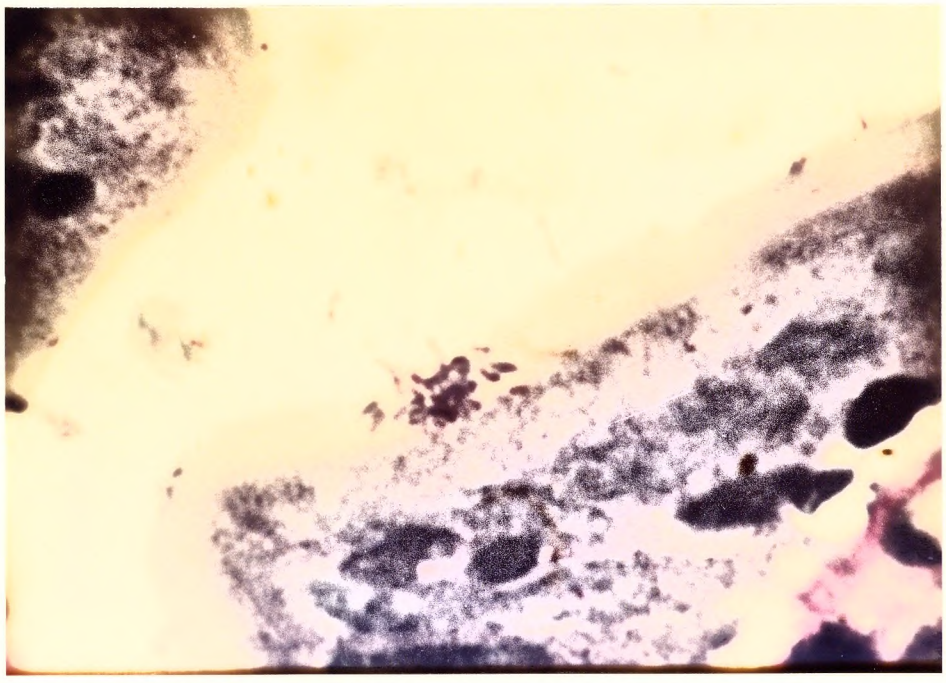

Figure 11-2. Giemsa-stained section of loop with *V. cholerae*. Bacteria are seen adhering to microvilli. X 1 200.

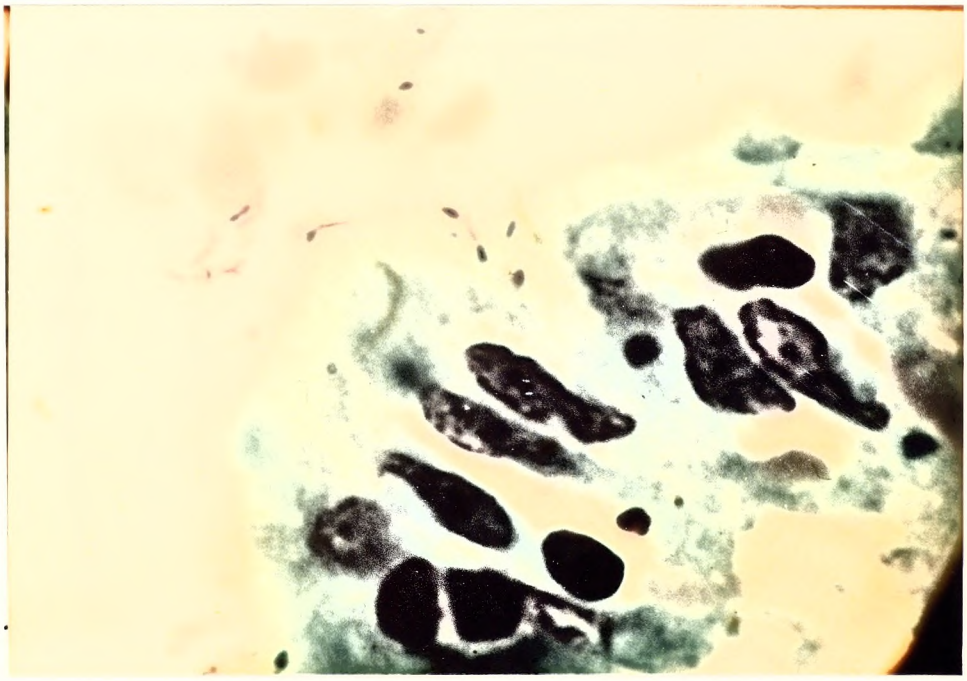

Figure 11-3. Giemsa-stained section of loop infected with *Y. enterocolitica* serotype O 3. Bacteria are seen adhering to the microvilli. X 1200.

In sections of loops challenged with a strain of serotype O 8, the same picture as discussed above, could be seen. Serotypes O 5,27 and O 6,31 were also invasive, but the effects on the ileum were not as pronounced as those elicited by the other two serotypes mentioned. Although the inflammatory response was similar to that caused by serotype O 3, the number of bacteria adhering to the microvilli was less, and very few could be seen penetrating the mucosa.

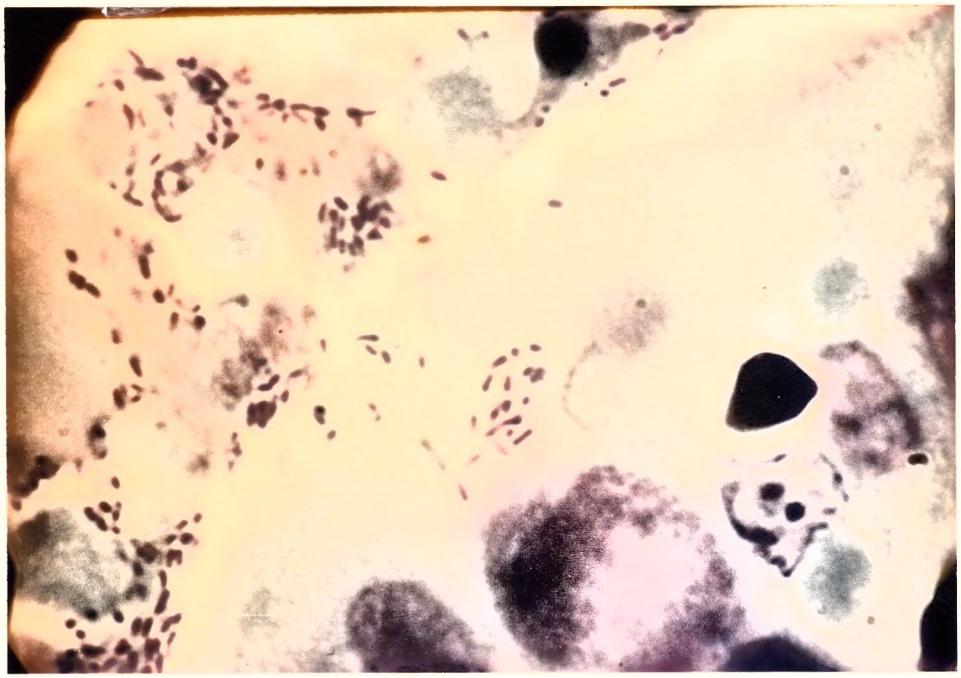

Figure 11-4. Giemsa-stained section of loop infected with *Y. enterocolitica* serotype O 3. Bacteria can be seen inside the tissues. X 1 200.



Figure 11-5. Giemsa-stained section of loop infected with *Y. enterocolitica* serotype O 3. Bacteria are seen inside empty goblet cell. X 1 200.

After the same time-interval their penetration into the mucosa was also not as deep as was seen with serotypes O 3 and O 8.

Sections taken from ileum which was challenged with serotypes O 1; O 2; O 4,32; O 4,33; O 6,30 and O 7,8 appeared normal when compared with negative controls.

(b) Immunofluorescence

This method was used in duplicate sections where invasion was present. In immunofluorescent-stained sections of loops challenged with *S. typhimurium*, these bacteria could be seen to invade deeply, in some cases even as far as the muscular layers. Very few bacteria were seen in the lumen. Loops of the same animals challenged with *Y. enterocolitica* serotype O 3 also showed invasion, but fewer bacteria could be demonstrated in the tissues and invasion was only as deep as the lamina propria. *Y. enterocolitica* were present in abundance in the lumen.

(c) Electron microscopy

In sections of loops infected with invasive strains, the damage to the tissues was better illustrated in electron micrographs than in the sections viewed with the light microscope. The debris noticed in the lumen in the latter sections was shown in electron micrographs to consist mainly of microvilli (fig.11-6). Bacteria could be visualised in the lumen, with some adhering to the microvilli. In one of the sections of a loop challenged with serotype O 3, a clump of bacteria could be observed inside a cell (fig. 11-7). In some sections ephithelial cells were seen inside the lumen, although the mucous membrane showed very little damage.

11.3.2 Infection of mice

In mice infected intragastrically, the following results were obtained:

Plate counts of iliac lymph nodes demonstrated 6 X 10^2 bacteria 6 hours after the intragastric inoculation of *Y. enterocolitica*. The *Y. pseudotuberculosis* strain yielded 2,5 X 10^3 organisms from iliac lymph nodes 6 hours after infection. From the mice sacrificed at 24, 48 and 192 hour intervals, neither *Y. enterocolitica* nor *Y. pseudotuberculosis* could be isolated from the lymph nodes.

Giemsa-stained sections of the small intestine showed abundant debris in the lumen. One *Y. enterocolitica* strain showed adhesion to the mucous membrane but no invasion, whereas with another strain, as well as with *Y. pseudotuberculosis*, adherence and invasion were evident. Clear signs of inflammation, such as dilated blood vessels and PML's spreading into the lamina propria, could also be demonstrated.

The results of the other experiment is given in table 11-1.

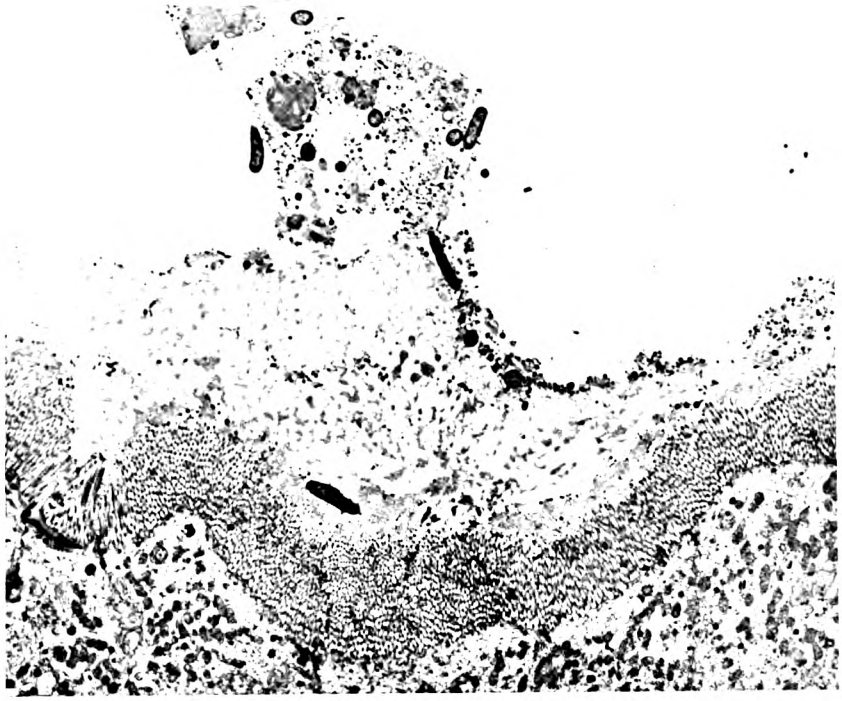

Figure 11-6. Electron micrograph of *Y. enterocolitica* adhering to the microvilli of rabbit ileum. X 12000.

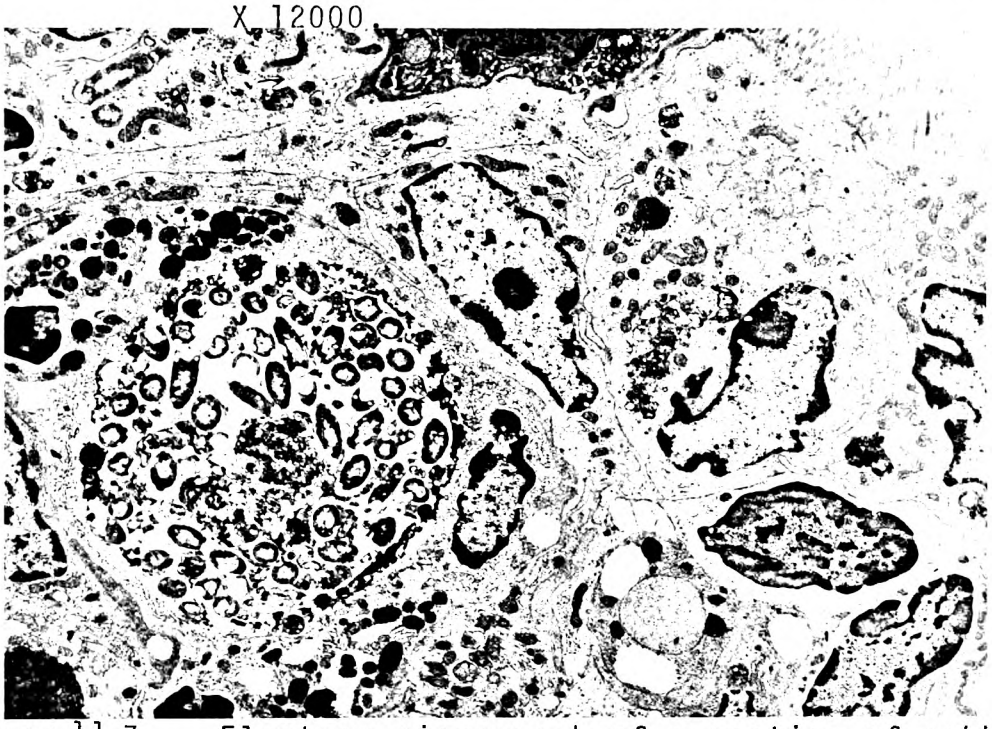

Figure 11-7. Electron micrograph of a section of rabbit ileum with a clump of *Y. enterocolitica* seen inside a cell. X 15000.

Table 11-1. Results of isolations of *Y. enterocolitica* serotype 03 from various organs of mice challenged intragastrically with a 0,1 mℓ suspension of PBS containing 1 x 10^9 cfu/mℓ.

Isolations from organs				
Mouse no.	Liver	Lung	Spleen	Intestine
1	+	+	+	+
2	+	+	+	-
3	+	-	-	-
4	-	-	-	-
5	+	-	-	+
6	+	+	+	+
7	+	+	+	+
8	-	+	+	-
9	+	+	+	-
10	+	+	+	+
TOTAL	8/10	7/10	7/10	5/10

\+ = positive

\- = negative

11.3.3 Guinea pig infections

None of the guinea pigs infected with *Y. enterocolitica* and *Salmonella* showed evidence of invasive disease. The "swiss roll" method of embedding allowed long pieces of intestine to be viewed on one slide. Giemsa-stained sections showed moderate amounts of organic material in the lumen of the intestine, but bacterial invasion could not be demonstrated. Using Giemsa and immunofluorescent antibody techniques, small numbers of bacteria were seen in the lumen. The presence of inflammation was equivocal. Some flattening of the villi was noted.

11.3.4 Serény's guinea pig keratoconjunctivitis test

Of all the strains of *Yersinia enterocolitica* tested, only WA showed naked eye evidence of invasion in the guinea pig conjunctiva. Both *Yersinia pseudotuberculosis* and *Sh. flexneri* caused keratoconjunctivitis in guinea pigs as well as in rabbits.

11.4 DISCUSSION

11.4.1 Choice of experimental animals

It is well known that in many cases inbreeding tends to impair the immune response. This is a good reason why this type of animal may be preferred for experimental work involving infective processes. Because inbred animals share a wide range of characteristics and therefore react in much the same way to external stimuli, the use of such animals are likely to yield more uniform results. In spite of these advantages, it was decided to use outbred animals as they more closely emulate natural conditions.

11.4.2 General considerations concerning animal models for invasive studies

The invasive potential of *Y. enterocolitica* strains of various serotypes was examined in rabbits, mice and guinea pigs. The first two models were used more extensively for examination of various serotypes, ranging from type O 1 to O 8. In the mouse and guinea pig only strains of serotype O 3 were employed, except for the Serény test where one strain of each of serotypes O 1 to O 8 was examined.

The guinea pig was found to be a poor model for invasive studies of *Y. enterocolitica;* no invasiveness could be demonstrated in the intestine and the Serény test was also negative for all the strains except those of serotype O 8.

In the mouse model invasion was more apparent. Apart from penetration of bacteria demonstrated in stained sections of the intestine *Y. enterocolitica* was also isolated from lymph nodes, spleen, liver and lung.

The rabbit proved to be the best animal for adherence and invasive studies and the use of the ligated loop model has the added advantage of per-

mitting several bacterial strains to be tested simultaneously. At the same time this model tended to eliminate host differences that are encountered when one animal is being used for each microbial strain. Furthermore the prolonged fasting of 48 hours in the rabbit resulted in much cleaner intestinal lumina which were better suited for microscropical study.

11.4.3 Invasive potential of *Y. enterocolitica* in guinea pigs.

The fact that most *Y. enterocolitica* strains failed to invade in the Serény test in guinea pigs compared with the positive controls *(Shigella* and *Y. pseudotuberculosis)* could possibly be attributed to a natural resistance of guinea pigs to *Y. enterocolitica* infection. It is, however, more likely that the *Y. enterocolitica* strains could have lost their virulence as a result of the repeated subculturing to which they were subjected before they were used for this experiment. The WA strain was later received from Carter and this strain invaded guinea pig as well as rabbit conjunctivae. This lack of invasion of epithelial cells by most *Y. enterocolitica* serotypes further correlated with the finding of no invasion in the ileum of guinea pigs infected intragastrically with this organism. This correlation however, was not observed in the rabbit. In this animal *Y. enterocolitica* was shown to invade the ileum but not the conjunctivae. In contrast to these findings shigellae as well as *Y. pseudotuberculosis* invaded both guinea pig and rabbit conjunctivae and ileal mucosae.

Another explanation for the lack of invasion of guinea pig conjunctivae may be found in the loss of the 41 Mdal plasmid by bacteria. This plasmid is essential for a positive Serény test (Zink *et al*, 1980).

11.4.4 Invasive potential of *Y. enterocolitica* in mice.

In an article by Carter (1975) it is mentioned that the intestinal lymph of mice drains mainly into three sets of lymph nodes of which the iliac node is the nearest to the terminal ileum. This node was therefore used to determine the lymphatic spread of bacteria from the intestine. Following an intragastric inoculum of 4×10^8 *Y. enterocolitica*, 6×10^2 organisms were isolated from this lymph node six hours after infection, but no bacteria could be demonstrated after this period. The same phenomenon was observed in mice infected with *Y. pseudotuberculosis* when $2{,}5 \times 10^3$ bacteria were recovered from the iliac lymph node after six hours. From this it is obvious that the organisms were rapidly and very effectively cleared from the lymph node at a very fast rate.

In another experiment *Y. enterocolitica* was isolated from the liver, spleen and lungs after intragastric challenge, demonstrating the systemic spread of this bacterium from the intestine (table 11-1).

In chapter 10 it is shown that phagocytosis of *Y. enterocolitica* by granulocytes occurs rapidly. *In vitro* experiments showed that non-pathogenic strains were killed at a faster rate by both granulocytes and macrophages, compared with pathogenic strains (table 10-2 and 10-3; figure 10-2). Une (1977) achieved similar results with rabbit macrophages *in vitro*. This

is a likely explanation for the rapid clearance of these organisms from the blood as discussed earlier in chapter 9. The pathogenic strain survived in the circulation much longer than its non-pathogenic counterpart. In the liver and spleen the numbers of pathogenic *Y. enterocolitica* decreased at first and then increased dramatically within 48 hours after infection.

11.4.5 Invasive potential of *Y. enterocolitica* in rabbit ileum.

As was mentioned earlier, the same rabbits were used for both control and infected loops. As expected, inflammatory changes such as dilated blood vessels and infiltration of PML's were observed in the infected loops. Not surprisingly, similar but much less marked changes were found in the negative control loops. These minimal changes were almost certainly the result of the ligation procedure and its effect on the blood supply of the loop. However, these negative control loops still served as useful comparisons for the assessment of the extent and severity of infections in the test loops.

The small rod-like bacteria seen in Giemsa-stained sections to adhere to and invade inoculated loops, were positively identified by immunofluorescent staining as predominantly *Y. enterocolitica*. Of the eight serotypes that were available, serotypes O 3 and O 8, which are characteristically associated with infection in man, proved to be more invasive for rabbit mucosa than the other serotypes.

Like salmonellae, *Y. enterocolitica* causes minimal mucosal damage. In electron micrographs of control sections microvilli could be seen in the lumen, but were not as numerous as in the infected loops. Even then the damage caused by

Y. enterocolitica was so slight that it could not be convincingly demonstrated by light microscopy.

Florey (1933) suggested that salmonellae:

(i) pass through or between epthelial cells, and
(ii) are possibly transported by phagocytes.

The first suggestion was proved to be correct by Takeuchi in 1975. By means of electron microscopic observations this worker showed that *Salmonella typhimurium* penetrated through the brush border resulting in degeneration of the microvilli. The same phenomenon was elicited by invasive *Y. enterocolitica*. Furthermore *Y. enterocolitica* were, like salmonellae, found to be contained in vesicles inside the cell, in some cases even multiplying (fig. 11-7). Gianella *et al*, (1973) also found that salmonellae multiply within epithelial cells.

Concerning the second suggestion by Florey, no direct proof is available, but in both salmonellae (Fromm *et al*, 1974) and *Y. enterocolitica* infections of the ileum, an influx into the lamina propria of PML's could be demonstrated. Furthermore Une (1977) showed that salmonellae could survive in mononucleated cells. In chapters 10 and 12 evidence of survival of *Y. enterocolitica* within granulocytes as well as macrophages, is discussed in greater detail.

In Giemsa-stained smears of the contents of infected loops, especially those challenged with serotypes O 3 and O 8, large numbers of epithelial cells could be seen, together with PML's and a few erythrocytes. In one of the electron micrographs epithelial cell sequestration was demonstrable in the lumen. The finding therefore

indicate a limited degree of damage is present, although not enough to cause ulceration and subsequent blood loss, as is found in infection with invasive shigellae, and *E. coli* (Takeuchi *et al*, 1965, Du Pont *et al*, 1971; Du Pont and Hornick, 1973; Kent *et al*, 1976).

In sections of infected loops, emptying of the majority of the goblet cells into the lumen was observed. This coincides with the findings of Gianella *et al*, (1973) in work with salmonellae. In infection with most enteropathogens a certain amount of mucus is present in the stool (Ogawa *et al*, 1967; Bradford *et al*, 1974), although in the case of *V. cholerae* and enterotoxigenic *E. coli* it is masked by the diluting effect of the excessive secretion of water (Robins-Browne and Koornhof, 1972; Bradford *et al*, 1974). The discharging of mucus by goblet cells is probably a non-specific defence mechanism of the intestine and is aimed at getting rid of the bacteria before they can adhere to the microvilli (Florey, 1933). It is proposed that the bacteria are trapped in the mucus and because of the increased peristalsis accompanying the diarrhoea, they are removed rapidly from the intestine. In the control loops the discharge of mucus from the goblet cells is not as marked as in the infected loops. On the other hand, the non-invasive strains of *Y. enterocolitica* still stimulate the goblet cells to empty into the lumen. Adherence *per se* can therefore not be regarded as the primary stimulus for excessive mucus secretion. It may, however act as secondary stimulus, enhancing the rate of discharge of goblet cells. The nature and mechanism of primary stimulation of goblet cells are not known.

From this study it is evident that *Y. entero-*

colitica is an invasive enteropathogen, similar in its action on mucosal surfaces to salmonellae. The degree of pathogenicity varies between the different serotypes, the human types being more invasive for the mucosal surfaces of rabbit and mice intestine as well as for HeLa cells (Chapter 12) than the other serotypes.

11.5 REFERENCES

Arvastson, B., Damgaard, K. and Windblad, S. (1971). Clinical symptoms of infection with *Yersinia enterocolitica*. Scand. J. Infect. Dis. *3* : 37-40.

Blum, D., Viart, P. and Dachy, A. (1970). Septicémie à *Yersinia enterocolitica* chez deux enfants atteints de thallassémie majeure. Arch. Franq. Pédiatr. *27* : 445

Bradford, W.D. Noce, P.S. and Gutman, L.T. (1974). Pathologic features of enteric infection with *Yersinia enterocolitica*. Arch. Pathol. *98* : 17-22.

Carter, P.B. (1975). Pathogenicity of *Yersinia enterocolitica* for mice. Infect. Immun. *11* : 164-170.

Chessum, B., Frengly, J.D., Fleck, D.G. and Mair, N.S. (1971). Case of septicaemia due to *Yersinia enterocolitica*. Br. Med. J. *3* : 466-468.

De, S.N. and Chatterje, D.N. (1953). An experimental study of the mechanisms of action of *Vibrio cholerae* on the intestinal mucous membrane. J. Pathol. *66* : 559-562.

DuPont, H.L., Formal, S.B., Hornick, R.B., Snyder, M.J., Libonati, J.P., Sheanan, D.G., LaBrec, E.H. and Kalas, J.P. (1971). Pathogenesis of *Escherichia coli* diarrhoea. N. Engl. J. Med. *285* : 1-9.

DuPont, H.L. and Hornick, R.B. (1973). Clinial approach to infections diarrhoeas. Medicine *52* : 265-270.

Finlayson, M.H., Coldrey, N.A., Street, B. and Brede, H.D. (1973). *Yersinia enterocolitica* in the Western Cape. S. Afr. Med. J. *47* : 111-112.

Florey, H.W. (1933). Observations on the functions of mucus and the early stages of bacterial invasion of the intestinal mucosa. J. Pathol. Bacteriol *37* : 283-289.

Fromm, D., Gianella, R.A., Formal, S.B., Quijano, R. and Collins, H. (1974). Active ion transport across isolated ileum invaded by salmonella. Gastroenterology. *66 :* 215-225.

Gianella, R.A., Formal, S.B., Dammin, G.J. and Collins, H. (1973a). Studies of fluid secretion, mucosal invasion, and morphologic reaction in the rabbit ileum. J. Clin. Invest. *52 :* 441-453.

Gianella, R.A., Washington, O., Gemski, P. and Formal, S.B. (1973b). Invasion of HeLa cells by *Salmonella* J. Infect. Dis. *128 :* 69-75.

Gianella, R.A., Gots, R.E., Charney, A.N., Greenough, W.B. and Formal, S.B. (1975). Pathogenesis of Salmonella-mediated intestinal fluid secretion. Activation of adenyl cyclase and inhibition by indomethacin. Gastroenterology. *69 :* 1233-1245.

Gots, R.E., Formal, S.B. and Gianella, R.A. (1974). Salmonella-mediated ileal secretion: stimulation of adenyl cyclase, inhibition by indomethacin and possible participation of prostaglandins. Clin. Res. *22 :* 358-

Gutman, L.T., Ottesen, E.A., Quan, T.J., Noce, P.S. and Katz, S.L. (1973). An interfamilial outbreak of *Yersinia enterocolitica* enteritis. N. Engl. J. Med. *288 :* 1372-1377.

Hewston, A.S. and Davidson, G.P. (1972). *Yersinia enterocolitica* septicaemia with arthritis in a thaelasaemic child. Med. J. Aust. *1 :* 1035-1038.

Jacobs, J.C. (1975). *Yersinia enterocolitica* arthritis. Pediatrics *55 :* 236-238.

Jansson, E., Wallgren, G.R. and Ahvonen, P. (1968). *Yersinia enterocolitica* as a cause of acute mesenteric lymphadenitis. Acta Paediatr. Scand. *57 :* 448

Kent, T.H., Formal, S.B. and LaBrec, E.H. (1966). Acute enteritis due to *Salmonella typhimurium* in opium-treated guinea pigs. Arch. Pathol. *81 :* 501.

LaBree, E.H., Schneider, H., Magnani, T.J. and Formal, S.B. (1964). Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. *88* : 1503-1518.

Lee, W.H. (1977). Two plating media modified with Tween 80 for isolating *Yersinia enterocolitica.* Appl. Environ. Microbiol. *33* : 215-216.

Mollaret, H.H. (1965). Les formes cliniques de l'infection humaine à bacille de Malassez et Vignal. Pathol. Biol. (Paris) *13* : 554-

Niléhn, B. and Sjöström, B. (1967). Studies on *Yersinia enterocolitica.* Acta Pathol. Microbiol. Scand. *71* : 612-628.

Ogawa, H., Nakamura, A., Nakaya, R., Mise, K., Honjo, S., Takasaka, M., Fujiwara, T. and Imaizumi, K. (1967). Virulence and epithelial cell invasiveness of dysentery bacilli. Jpn. J. Med. Sci. Biol. *20* : 315-328.

Pai, C.H. and Mors, V. (1978). Production of enterotoxin by *Yerinia enterocolitica.* Infect. Immun. *19:* 908-911.

Plotkin, G.R., Kluge, R.M. and Waldman, R.H. (1979). Gastroenteritis: etiology, pathophysiology and clinical manifestations. Medicine *58* : 95-114.

Portnoy, D.A., Moseley, S.L. and Falkow, S. (1981). Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. *31* : 775-782.

Rabson, A.R., Koornhof, H.J., Notman, J. and Maxwell, W.G. (1972) Hepatosplenic abscesses due to *Yersinia enterocolitica.* Br. Med. J. *4* : 341.

Richards, K.L. and Douglas, S.D. (1978). Pathophysiological effects of *Vibro cholerae* and enterotoxigenic *Escherichia coli* and their exotoxins on eukaryotic cells. Micro. Rev. *42:* 592-613.

Robins-Browne, R.M. and Koornhof, H.J. (1977). Recent advances in infantile gastro-enteritis research. S. Afr. J. Sci. *73* : 105-108.

Robins-Browne, R.M., Jansen van Vuuren, C.J., Still, C.S., Miliotis, M.D. and Koornhof, H.J. (1979). The pathogenesis of *Yersinia enterocolitica* gastroenteritis. in Contr. Microbiol. Immunol. vol. 5 ed. by P.B. Carter, L. Lafleur and S. Toma. S. Karger, Basel, pp 324-328.

Sack, R.B. (1975). Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. Annu. Rev. Micro. *29* : 333-353.

Schieven, B.C. and Randall, C. (1974). Enteritis due to *Yersinia enterocolitica*. J. Pediatr. *84* : 402-404.

Serény, B. (1955). Experimental *Shigella* keratoconjunctivitis: a preliminary report. Acta Microbiol. Acad. Sci. Hung. *2* : 293-296.

Serény, B. (1957). Experimental keratoconjunctivitis Shigellosa. Acta Microbiol. *4* : 367-376.

Sprinz, H. (1969). Pathogenesis of intestinal infections. Arch. Pathol. *87* : 556-562.

Takeuchi, A. (1967). Electron microscopic studies of experimental salmonella infection. 1. Penetration into the intestinal epithelium by *Salmonella typhimurium*. Am. J. Pathol. *50* : 109-136.

Takeuchi, A. (1975) Electron microscope observations on penetration of the gut epithelial barrier by *Salmonella typhimurium*. In Microbiology - 1975. Ed. by D. Schlessinger, Am. Soc. Microbiol. pp 174-181.

Takeuchi, A., Sprinz, H., LaBrec, E.H. and Formal, S.B. (1965). Experimental bacillary dysentery. An electron microscopic study of the response of the intestinal mucosa to bacterial invasion. Am. J. Pathol. *47* : 1011-1044.

Une, T. (1977). Studies on the pathogenicity of *Yersinia enterocolitica* II. Interaction with cultured cells *in vitro*. Microbiol. Immunol. *21* : 365-377.

Une, T., Zen-Yoji, H., Maruyama, T. and Yanagawa, Y. (1977). Correlation between epithelial cell infectivity *in vitro* and O-antigen groups of *Yersinia enterocolitica*. Microbiol. Immunol. *21* : 727-729.

Windblad, S., Niléhn, B. and Sternby, N.H. (1966). *Yersinia enterocolitica* (Pasteurella X) in human enteric infections. Br. Med. J. *2* : 1363-1366.

Zink, D.L., Feeley, J.C., Wells, J.G., Vanderzant, C., Vickery, J.C., Roof, W.D. and O'Donovan, G.A. (1980). Plasmidmediated tissue invasiveness in *Yersinia enterocolitica*. Nature (London) *283* : 224-226.

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12. INVASION OF HeLa CELLS BY *YERSINIA ENTEROCOLITICA*

12.1 INTRODUCTION

Because of the homogeneity of HeLa cells, they lend themselves to quantitative assessment of invasion (see table 12-1 and figure 12-4). It is also a convenient, inexpensive and quick procedure for examining invasive potential of pathogenic bacteria.

The invasive potential of *Y. enterocolitica* for HeLa cells has been tested by Une (1977), Une *et al* (1977) and Lee *et al* (1977). These authors have however, only examined the serotypes more commonly associated with disease in man, viz serotypes O 3, O 8, O 9 and also O 5. Pedersen *et al* (1979) examined strains from serotypes O 1 to O 26. They found that the interaction of *Y. enterocolitica* with HeLa cells was not only restricted to viable bacteria, since bacterial cells inactivated by formalin or ultraviolet light could also gain entrance to HeLa cells. Furthermore they demonstrated in the same study that monolayer cultures of porcine kidney cells were invaded by the same strains which invaded HeLa cells.

In this study the same strains of *Y. enterocolitica* which were used for invasive testing in the rabbit ileum (See chapter 11) were examined for invasive potential in HeLa cells. Positive demonstration of invasion was achieved by using electron and fluorescent microscopy.

12.2 MATERIALS AND METHODS

(a) Bacterial strains (see Appendix B)

Strains M1; 5122; Thompson; R38; M4,32; M4,33; M5,27; M6,31; M7,8; M8 and WA of *Y. enterocolitica* were grown on Nutrient agar at 25°C and 37°C for 24 hours. The positive controls, *Escherichia coli* 1314 and *Shigella flexneri* 1251 were incubated only at 37°C. Growth was washed off the plates with 20% Minimal

Essential Medium (MEM) (Wellcome Reagents) containing 2% foetal calf serum (FCS) (Wellcome) without antibiotics. The bacterial concentrations were adjusted to 1 x 10^7 cfu/mℓ in MEM.

(b) Culture of HeLa cells

HeLa A-2 cells were obtained from the Ophthalmology Department, Witwatersrand Medical School. Cells were maintained in 20% MEM with 10% FCS containing 50 µg/mℓ gentamicin in 75 cm^2 Falcon tissue culture flasks. The day before the experiment, the monolayers were trypsinized with 0,25% trypsin (Wellcome) and the concentration adjusted to 2 x 10^5 cells/mℓ in MEM plus 10% FCS and antibiotics. One mℓ of this suspension was inoculated into each of a number of flat-bottomed 5 mℓ disposable tubes (Searle Diagnostics) containing circular glass coverslips. The tubes were incubated at 37°C in an atmosphere containing 5% CO_2 for 24 hours.

(c) Procedure

The technique of Une (1977) was used with a few modifications. The HeLa cell monolayers were washed three times with warm (37°C) PBS to remove the antibiotics. Monolayers were then challenged in triplicated with 3 mℓ of the bacterial suspensions and incubated at 37°C for 90 minutes in 5% CO_2. The cells were washed three times with warm PBS to remove all the non-adhering bacteria and after the last wash 3 mℓ of 20% MEM solution containing 50 µg/mℓ gentamicin were put into each tube. The monolayers were then incubated at 37°C for 5 hours without additional CO_2. After incubation, they were washed in two changes of PBS, fixed for 10 minutes in methanol and stained with 10% Giemsa's stain for 1 hour. The cells were differentiated in 70% ethanol and water. The coverslips were removed from the tubes and mounted onto glass slides in DePeX. Microscopic observation was performed on five continuous fields of

each specimen. Invasion was considered positive if more than fifteen bacteria could be seen inside a cell and the percentage invasion was calculated as follows:

$$\text{Percentage Invasion} = \frac{\text{number of invaded cells}}{\text{total number of cells in 5 fields}} \times 100$$

(d) Electron microscopy

Infected HeLa cell monolayers were scraped off into MEM and centrifuged at 2 000 x g for 10 minutes. The MEM was poured off and the button was fixed in cold ($4^{o}C$) gluteraldehyde. The sectioning and photography was performed by the Histopathology Department of the SAIMR.

(e) Fluorescent staining with acridine orange

The acridine orange technique of Smith and Rommel (1977) was used. Monolayers were stained with a 0,01% acridine orange (BDH Chemicals) in Hank's Balanced Salt Solution (HBSS) (Wellcome), pH 7,2 for 45 seconds, rinsed with HBSS and extracellular bacteria were quenched with 0,05% crystal violet (BDH chemicals) in saline for 45 seconds. The slides were rinsed again with HBSS and mounted still wet. The sides of the coverslips were sealed with nail varnish. The slides were viewed with a fluorescent microscope using incident ultra violet. Viable organisms fluoresce green and dead ones red.

12.3 RESULTS

To evaluate the invasive potential of *Y. enterocolitica* for HeLa cells quantitatively, monolayers were stained with Giemsa stain (fig. 12-2) and the percentage invasion could be determined by direct microscopical count. This method does not however enable one to distinguish between bacteria that

are actually inside the cell and those that merely adhere to the cell membrane. In order to establish whether *Y. enterocolitica* did in fact invade the cells, sections were cut for electron microscopy and in figure 12-1 the bacteria can be seen inside the cell. Monolayers were also stained with acridine orange. The quenching effect of the crystal violet of extracellular bacteria enables one to determine which organisms are inside the cell (fig. 12-3).

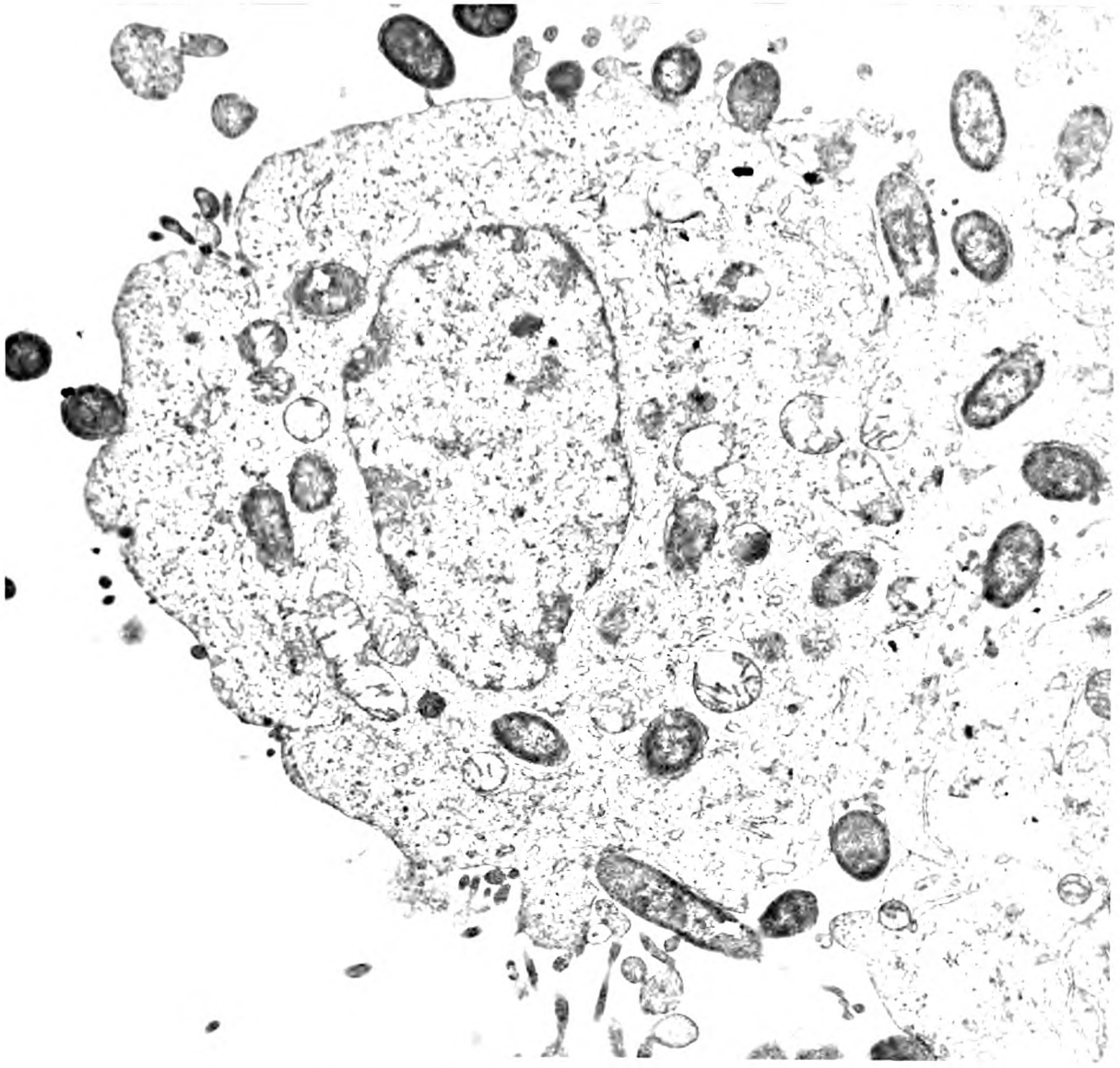

Figure 12-1. Electron micrograph of HeLa cell with intracellular *Y. enterocolitica* WA X 15 000

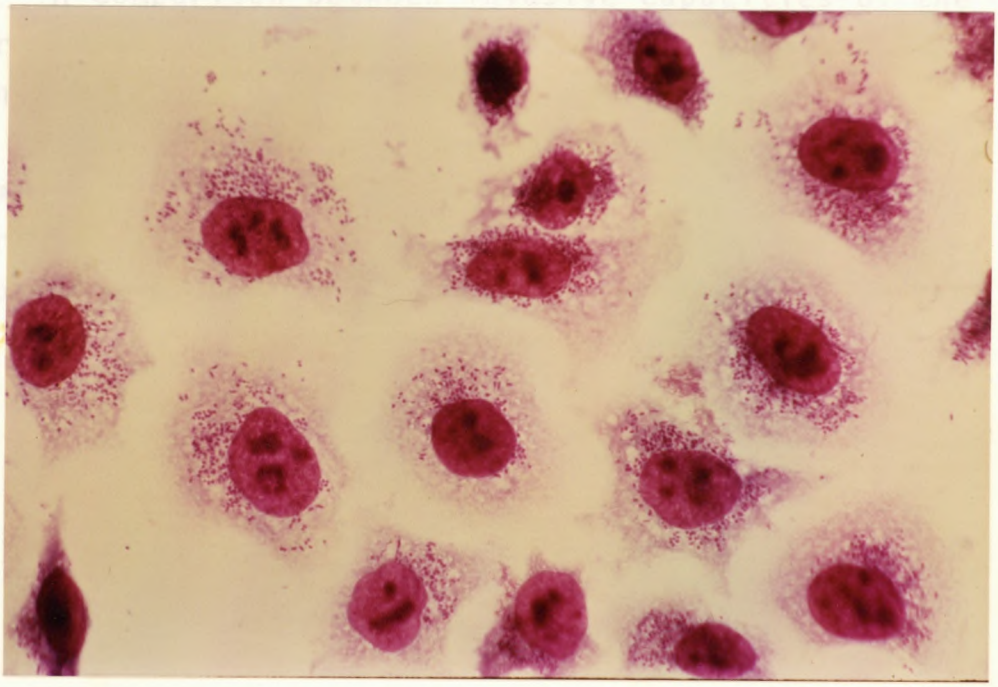

Figure 12-2. Photomicrograph of *Y. enterocolitica* invading HeLa cells. Bacteria can also be seen adhering to the cell membranes. X 1200.

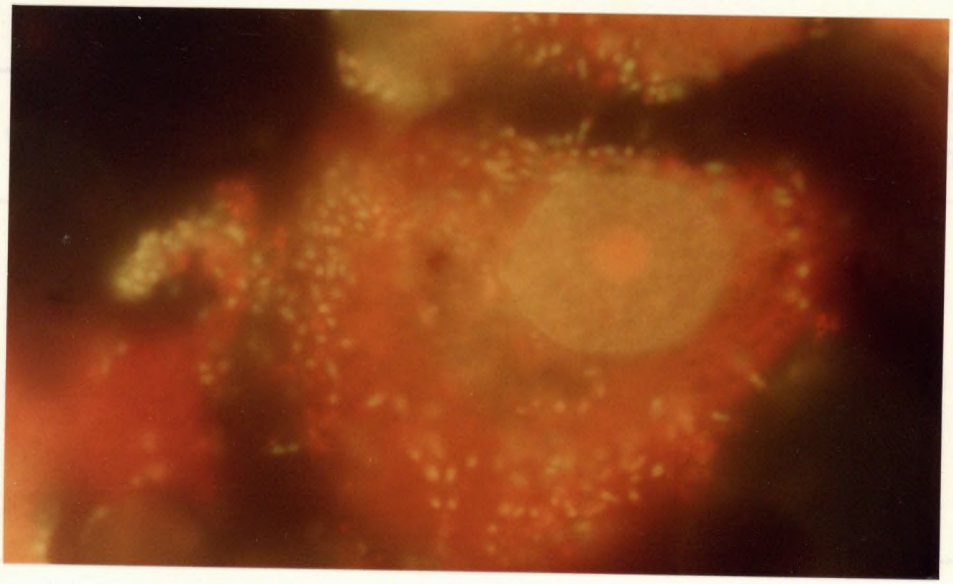

Figure 12-3. Photomicrograph of *Y. enterocolitica* invading HeLa cells. Stained with acridine orange. X 1200.

A comparison between invasive capacities of the various serotypes of *Y. enterocolitica* for HeLa cells can be seen in table 12-1 and figure 12-4. Of the 12 strains tested, M4,33 and M7,8 were shown to be completely non-invasive for these cells. All other strains, except 5122, produced more extensive invasion when grown at 25°C than when cultured at 37°C.

Table 12-1. Percentage of HeLa cells invaded by various *Y. enterocolitica* serotypes after cultivation at 25°C and 37°C.

Strain	Invasion (%)			Invasion (%)	
	25°C	37°C		25°C	37°C
M1	65	45	M5,27	25	15
M2	0	0	M6,31	54	23
5122	35	62	M7,8	0	0
Thompson	90	80	M8	100	20
R38	90	15	WA (Strain Ye2705)	100	89
M4,32	69	25	*E. coli* I314	ND*	65
M4,33	0	0	*Sh. flexneri* 1251	ND*	68

ND* = Not done.

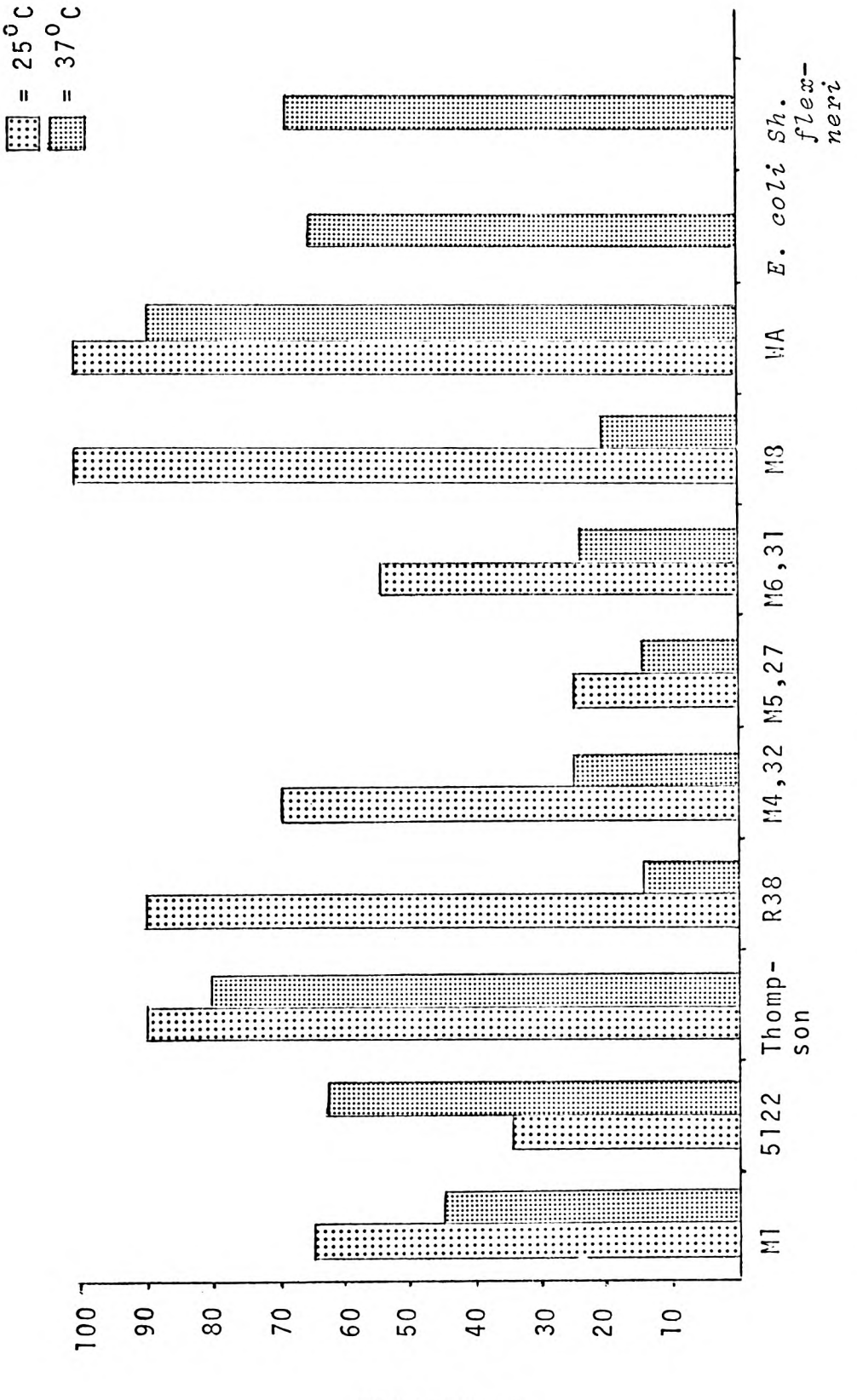

Figure 12-4. Histogram comparing the percentage invasion of HeLa cells by various serotypes of *Y. enterocolitica*, grown at two different temperatures. Two positive controls are included.

12.4 DISCUSSION

The strains of serotypes O 3 and O 8, the serotypes mostly associated with disease in humans, were all positive for invasion in HeLa cell monolayers (table 12-1 and fig. 12-4). Furthermore, invasion was proved by making use of electron microscopy (fig. 12-1) and acridine orange staining (fig. 12-3).

The production of flagellae at incubation temperatures of 25°C, with resulting motility, could conceivably lead to closer interaction between bacteria and cells, thereby explaining the higher invasive percentages of bacteria grown at this temperature. There is however, no additional experimental evidence to support this possibility.

It is interesting that the enterotoxin of *Y. enterocolitica* is also secreted at the lower temperature (Pai and Mars, 1978; Robins-Browne *et al*, 1979). Although most of the enterotoxin is disseminated into the environment, some of it remains cell wall associated. It was speculated that this may contribute to the adherence of the bacteria to cell surfaces. In experiments not reported here it was however later shown that the presence of free enterotoxin in no way altered adherence or invasion of *Y. enterocolitica* for HeLa cells.

The paradoxical behaviour of strain 5122 may be related to repeated subculturing as it was more extensively used in experimental work than the others. Portnoy *et al* (1981) discussed a plasmid which was shown to be associated with the pathogenic properties of *Y. enterocolitica*. According to them the plasmid associated properties did not appear to enhance adherence but rather the pathogenesis following invasion and involving intracellular multiplication.

These properties were also found to be expressed only at 37°C. Furthermore, Zink *et al* (1980) demonstrated that this plasmid was essential for *Y. enterocolitica* strains to produce a positive Serény test. It is therefore possible that the repeated subculturing of strain 5122 led to a loss of these virulence-determining factors.

The strains of serotypes O 3 and O 8 which were found to be positive for invasion in HeLa cells, also proved to be more invasive for rabbit ileal mucosa (chapter 11) than the other serotypes tested. Serotype O 5,27 also showed invasion for both these systems, but to a lesser extent. While types O 2; O 4,33; O 6,30 and O 7,8 were non-invasive for both rabbit ileum and HeLa cells, serotypes O 1; O 4,32 and O 6,31 showed invasion in the latter system.

Gianella *et al* (1973) found a correlation of more than 90% between HeLa cells and rabbit mucosal cell invasiveness by *S. typhimurium*. In the experiments recorded in this thesis it is evident that invasiveness in HeLa cells, using as criterion invasion of 80% of the cells, correlates well with invasion in the rabbit ileal loop (compare chapter 11). This suggests that a quantitative HeLa cell assay may prove to be extremely useful as an *in vitro* screening procedure for *Y. enterocolitica* invasiveness. If however, invasion in HeLa cells is low, invasion for the rabbit intestinal mucosa *in vitro* cannot be entirely excluded.

12.5 REFERENCES

Gianella, R.A., Washington, O., Gemski, P. and Formal, S.B. (1973). Invasion of HeLa cells by *Salmonella*. J. Infect Dis. *128 :* 69-75.

Lee, W.H., McGrath, P.P., Carter, P.H. and Gide, E.L. (1977).The ability of some *Yersinia enterocolitica* strains to invade HeLa cells. Can. J. Microbiol. *23 :* 1714-1722.

Pai, C.H. and Mors, V. (1978). Production of enterotoxin by *Yersinia enterocolitica*. Infect. Immun. *19 :* 908-911.

Pedersen, K.B., Winblad, S. and Bitsch, V. (1979). Studies on the interaction between different O-serotypes of *Yersinia enterocolitica* and HeLa cells. Acta Path. Microbiol. Scand. Sect. B *87 :* 141-145.

Portnoy, D.A., Moseley, S.L. and Falkow, S. (1981). Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. *31 :* 775-782.

Robins-Browne, R.M., Jansen van Vuuren, C.J., Still, C.S., Miliotis, M.D. and Koornhof, H.J. (1979). The pathogenesis of *Yersinia enterocolitica* gastroenteritis. in Contr. Microbiol. Immunol. vol. 5 ed. by P.B. Carter, L. Lafleur and S. Toma. S. Karger, Basel. pp 324-328.

Smith, D.L. and Rommel, F. (1977). A rapid micromethod for the simultaneous determination of phagocytic-microbicidal activity of human peripheral blood leukocytes *in vitro*. J. Immunol. Methods. *17 :* 241-247.

Une, T. (1977). Studies on the pathogenicity of *Yersinia enterocolitica* II. Interaction with cultured cells *in vitro*. Microbiol. Immunol. *21 :* 365-377.

Une, T., Zen-Yoji, H., Maruyama, T. and Yanagwa, Y. (1977). Correlation between epithelial cell infectivity *in vitro* and O-antigen groups of *Yersinia entero-*

colitica. Microbiol. Immunol. *21* : 727-729.

Zink, D.L., Feeley, J.C., Wells, J.G., Vanderzant, C., Vickery, J.C., Roof, W.D. and O'Donovan, G.A. (1980). Plasmidmediated tissue invasiveness in *Yersinia enterocolitica*. Nature (London) *283* : 224-226.

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13. GENERAL DISCUSSION AND CONCLUSIONS

13.1 INTRODUCTION

Various aspects of the pathogenic mechanisms by which *Yersinia enterocolitica* can cause disease were examined in this thesis, such as adherence of the bacteria to cell surfaces, penetration and multiplication inside cells and tissues, and toxigenicity, as well as other factors such as the survival of strains inside leukocytes and the locallized inflammatory response of the body.

13.2 ADHERENCE OF *Y. ENTEROCOLITICA* TO CELL SURFACES

Before enteric pathogens can elicit their effects on the intestinal tract, either by elaboration of an enterotoxin or by invasion of the tissues, it is necessary for the bacteria to adhere to the mucosal surfaces (Takeuchi, 1967; Silverblatt, 1974).

In chapter 11 it was shown that virulent strains of *Y. enterocolitica* adhere to the mucous membrane before penetrating into the tissues of the rabbit ileum (figures 11-3 and 11-6). The same phenomenon was noticed in invasive studies using HeLa cells as model (figure 12-8). Only pathogenic strains were able to adhere to cell surfaces, viz those of serotypes O 3 and O 8 and to a certain extent O 5,27.

Good correlation was found between adherence of more than 80% in HeLa cells and adherence and subsequent penetration of rabbit ileum.

Mucus secretion by the intestine is an important barrier to be overcome by pathogens and in order to interact with the mucous membrane, some mechanism must exist which enables the bacteria to penetrate this barrier. One possible mechanism operating in the case of *Y. enterocolitica* to this end may be the electric charge of its O-antigen. It was found that serotypes O 3 and O 8 possess O-antigens that

move in opposite directions to each other in an electric field, but in both cases these antigens were migrating slowly in contrast to the fast migrating antigens of the other non invasive serotypes studied. It is therefore tempting to propose that the electric charge of the bacteria as exemplified by the slow migrating O-antigens may play a role in the penetration of the mucus barrier or alternatively in adherence to the mucosal surface of the small intestine.

Miliotis (1981) demonstrated enhanced adhesion of *Y. enterocolitica* strains for human buccal cells in the presence of D-mannose. Certain sugars or related substances may therefore enhance adherence of these bacteria, possibly by possessing attachment sites for both bacteria and cell surfaces.

13.3 INVASIVENESS OF *Y. ENTEROCOLITICA*

It was shown in chapter 11 that pathogenic strains, expecially those of serotypes O 3 and O 8, invaded both rabbit intestinal mucosa and HeLa cells. A good correlation was demonstrated between HeLa cell invasiveness (invasion of more that 80% of cells in this system) and rabbit ileum. Little damage to the intestine could be demonstrated with light microscopy, although compared with controls, a somewhat increased number of erythrocytes, leukocytes and epithelial cells were seen in smears of the luminal contents. The major intestinal changes exihibited when challenged with virulent strains of *Y. enterocolitica*, were:

(i) A moderate degree of flattening of the villi;

(ii) A high influx of polymorphonuclear leukocytes (PML's) into the lamina propria, combined with

(iii) dilatation of blood vessels, signifying inflammation;

(iv) discharge of the contents of the majority of goblet cells into the lumen;

(v) bacteria adhering to the mucous membrane with some aggregating in the empty goblet cells; and

(vi) bacteria penetrating into the mucosa and submucosal layers.

The cell-associated events were more clearly demonstrated in electronmicrographs expecially the extent of the damage to the superficial mucosa. Most of the debris noticed in the lumen in Giemsa-stained sections was shown to be sloughed-off microvilli. Although sequestrated epithelial cells were demonstrated in electronmicroscope sections, no ulceration was evident.

The above-mentioned findings with *Y. enterocolitica* in the rabbit ileum model compare well with those of Takeuchi (1967) and Gianella *et al* (1973) on the invasiveness of salmonellae, suggesting that similar mechanisms operate.

13.4 TOXIGENICITY OF *Y. ENTEROCOLITICA*

It was found by various authors that many strains of *Y. enterocolitica* were able to secrete a heat-stable enterotoxin, comparable to the *Escherichia coli* stable toxin (ST) (Pai and Mors, 1978; Robins-Browne *et al*, 1979). However, the former organism can only elaborate its toxin at the temperature range of 20°C - 28°C. The pathological significance of this characteristic of *Y. enterocolitica* is therefore still obscure, although it may lead to food poisoning. This however, has not yet been recorded.

13.5 FACTORS INFLUENCING THE VIRULENCE OF *Y. ENTEROCOLITICA*

Iron overload is undoubtedly an important factor related to the invasive potential of *Y. enterocolitica*. It was demonstrated by various workers that patients with iron overload associated with diseases such as thallassaemia, alcoholic cirrhosis and Bantu siderosis (Blum *et al*, 1970; Abramovitch and Butas, 1973; Rabson *et al*, 1972), suffered from a more serious form of yersiniosis which was usually accompanied with a bacteraemia. These patients also responded less favourably to appropriate therapy than patients without iron overload.

Robins-browne *et al* (1979) showed that the addition of free iron to fresh serum inhibited serum killing of *Y. enterocolitica*. It was also demonstrated that mice pretreated with high doses of iron, succumbed more readily to intragastric challenge with these bacteria than those that did not receive any iron.

Iron overload probably leads to oversaturation of ironbinding proteins, so that the body is no longer able to compete with the bacteria for free iron, thereby allowing them to obtain this growth factor.

A phenomenon which is associated with enhanced virulence is bacterial synergy, which was discussed in chapter 7, where prechallenge with salmonellae led to a higher mortality rate than when *Y. enterocolitica* alone was administered.

Like Miliotis (1981) certain sugars, such as D-mannose were found to enhance the adherence of *Y. enterocolitica* to cell surfaces. However in an experiment not reported here no difference in the outcome of infection could be demonstrated when live mice were challenged with this organism in the presence or absence of D-mannose (Van Vuuren and Koornhof, unpublished data).

13.6 SYSTEMIC DISSEMINATION OF *Y. ENTEROCOLITICA*

After intragastric administration of *Y. enterocolitica* to mice, these bacteria could be isolated from various organs, such as lungs, liver, spleen and lymph nodes. It was shown that they reached the iliac lymph nodes within six hours after challenge. In only a few cases could *Y. enterocolitica* be isolated from the blood.

Une (1977) demonstrated the survival and multiplication of these bacteria in mononuclear leukocytes. In experiments challenging PMLs, as well as macrophages with virulent and avirulent strains of *Y. enterocolitica* it was found that the nonvirulent strain was destroyed by both these types of leukocytes, with the macrophages being more bactericidal than the PMLs, whereas the virulent strain tested was able to resist killing by both types of phagocytes (Chapter 10).

There are three possible mechanisms of systemic dissemination of *Y. enterocolitica*, viz:

(i) Direct penetration into the blood vessels;

(ii) direct spread via lymphatics to the blood stream or

(iii) dissemination by either of these routes after phagocytosis of *Y. enterocolitica* by PMLs and macrophages which transport them to the various organs of the body.

From the experiments described in chapter 11 it is clear that *Y. enterocolitica* reaches the blood stream mainly via regional lymph nodes. This mode of spread is similar to those of many other infections including typhoid fever and *Y. pseudotuberculosis* infections.

13.7 THE HOST RESPONSE TO INFECTION WITH *Y. ENTEROCOLITICA*

The first reaction of the body to intestinal infection of *Y. enterocolitica* is inflammation. This was evident by the influx of PML's into the lamina propria and submucosa.

Another reaction is the discharge of goblet cell contents into the intestinal lumen. It was postulated that this represents an attempt of the body to trap foreign bacteria in the mucus and expell them through increased peristalsis. Even non-adhering bacteria stimulated this reaction. As was demonstrated in chapter 11, the serotypes mostly associated with disease in man, differed from the others concerning the electric charge of their O-antigens, a factor which may enable them to penetrate the mucus barrier and aid in adherence to the epithelial membrane of the intestine.

Phagocytosis of *Y. enterocolitica* without complete killing is readily demonstrable *in vitro* by both PMLs and macrophages (chapter 10). It can be inferred that the cells in the inflammatory exudate in the lamina propria are likely to be instrumental in the spread of the organisms to the lymph nodes and beyond. In the latter case the stationary macrophages may play a more important role as was shown in chapters 10 and 12. Of the liver, spleen and lungs, the former played the most important part in clearing the blood of bacteria. Non-virulent strains did not survive for longer than 24 hours, whereas the numbers of virulent strains first decreased and then increased, showing their ability to overcome host defence mechanisms.

13.8 REFERENCES

Abramovitch, H. and Butas, C.A. (1973). Septicaemia due to *Yersinia enterocolitica*. Can. Med. Assoc. J. *109* : 1112-1115.

Blum, D., Viart, P. and Dachy, A. (1970). Septicémie à *Yersinia enterocolitica* chez deux enfants atteints de thallasémic majeure. Arch. Franq. Pédiat. *27* : 445.

Gianella, R.A., Formal, S.B., Dammin, G.J. and Collins, H. (1973). Studies of fluid secretion, mucosal invasion and morphologic reaction in the rabbit ileum. J. Clin. Invest. *52* : 441-453.

Miliotis, M.D. (1981) Adhesive and invasive properties of enteropathogenic bacteria. MSc Dissertation. Faculty of Science, University of the Witwatersrand, Johannesburg.

Pai, C.H. and Mors, V. (1978). Production of enterotoxin by *Yersinia enterocolitica*. Infect. Immun. *19* : 908-911.

Rabson, A.R., Koornhof, H.J., Notman, J. and Maxwell, W.G. (1972). Hepatosplenic abscesses due to *Yersinia enterocolitica* Br. Med. J. *4* : 341.

Richards, K.L. and Douglas, S.D. (1978). Pathophysiological effects of *Vibro cholerae and enterotoxigenic Escherichia coli* and their enterotoxins on enkarytic cells. Micro. Rev. *42* : 592-613.

Robins-Browne, R.M., Jansen van Vuuren, C.J., Still, C.S., Miliotis, M.D. and Koornhof, H.J. (1979). The pathogenesis of *Yersinia enterocolitica* gastroenteritis. in Contr. Microbiol. Immunol. vol. 5. ed. by P.B. Carter, L. Lafleur and S. Toma. S. Karger, Basel. pp 324-328.

Robins-Browne, R.M., Rabson, A.R. and Koornhof, H.J. (1979). Generalized infection with *Yersinia enterocolitica* and the role of iron. in Contr. Microbiol. Immunol. Vol. 5, ed. by P.B. Carter, L. Lafleur and S. Toma, S. Karger, Basel. pp277-282.

Robins-Browne, R.M. and Koornhof, H.J. (1977). Recent advances in infantile gastroenteritis research. S. Afr. J. Sci. *73* : 105-108.

Sack, R.B. (1975). Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. Ann. Rev. Microbiol. *29* : 333-335.

Silverblatt, F.J. (1974). Host-parasite interaction in the vat venal pelvis. J. Exp. Med. *140* : 1696-1711.

Takeuchi, A. (1967). Electron microscope studies of experimental salmonella infection 1. Penetration into the intestinal epithelium by *Salmonella typhimurium* Am. J. Pathol. *50* : 109-136.

Une, T. (1977). Studies on the pathogenicity of *Yersinia enterocolitica II*. Interaction with cultured cells *in mitro*. Microbiol. Immunol. *21* : 356-377.

APPENDIX A

DETERMINATION OF BACTERIAL NUMBERS BY MEANS OF SPECTROPHOTOMETRY

Y. enterocolitica was grown on Nutrient Agar (NA) for 24 hours and washed off in 0,9% saline. The suspension was divided into two parts after thorough mixing and both parts were washed twice in saline. The one part was resuspended in saline and serial tenfold dilutions were made up to 10^{-10}. Plate counts were done.

The other part was resuspended in 10% Formol saline and serial two-fold dilutions were made. These dilutions were read in a spectrophotometer for percentage transmission at 520 nm.

From the plate counts the number of bacteria in the original suspension was determined. From this figure the numbers of bacteria were calculated for the various two-fold dilutions in Formol saline. The results were plotted out in the graph in figure A1.

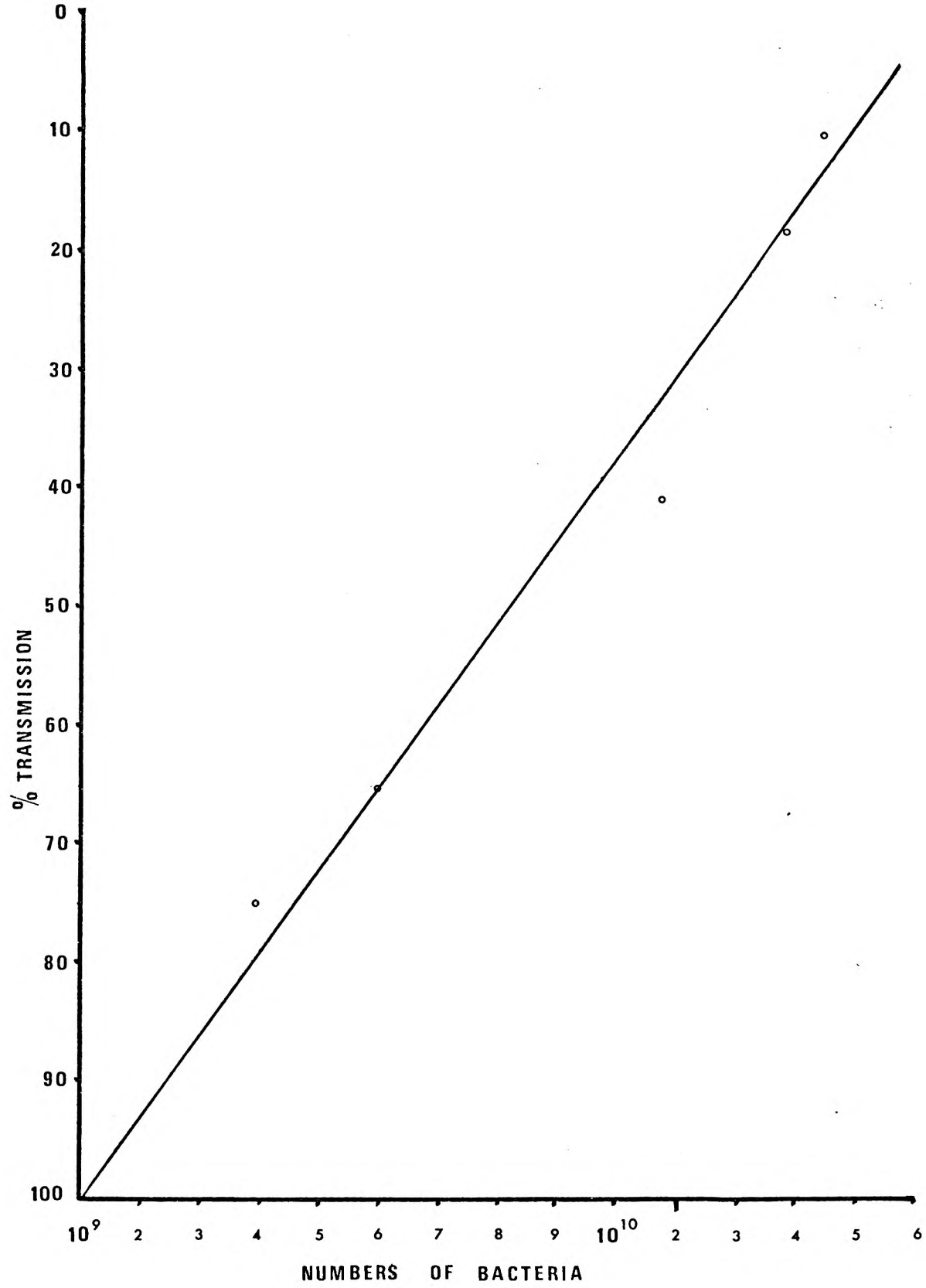

Figure A1. Determination of bacterial numbers by means of spectrophotometry. Percentage transmission of different dilutions read at 520 nm.

APPENDIX B Summary of *Y. enterocolitica* isolates.

A. Specimens received from Professor H H Mollaret, Centre National des Yersinia, Institute Pasteur, Paris.

	Our Number	Serotype	Biotype (Wauters & Le Minor)		Institute Pasteur Number
1	M1	1 (1,2a,3)	biotype 3	Ye	64
2	M2	2 (2a,2b,3)	biotype 5	Ye	178
3	M3	3	biotype 4	Ye	134
4	M4, 32	4, 32		Ye	96
5	M4, 33	4, 44		Ye	1476
6	M5	5	biotype 1	Ye	124
7	M5, 27	5, 27		Ye	885
8	M6, 30	6, 30		Ye	102
9	M6, 31	6, 31		Ye	1477
10	M7, 8	7, 8		Ye	106
11	M8	8		Ye	161

B. Specimens received from P.B. Carter (1976) (Trudeau Institute, Saranac Lake, New York).

	Our Number	
12	WISC 70746	Isolated from patient G. Sillaugh. Isolate from Wisconsin State Laboratory. Serotype O:7.
13	WA strain	Serotype 8, Ye 2705. Blood of septicaemic human male, William Allen. November 1972.

C. Specimens received from Professor A.R. Rabson, Department of Immunology, School of Pathology, SAIMR, Johannesburg.

(i) Isolates for which no clinical information is available.

	Our code	Serotype	Date of isolation
14	Robert	O:3	23/12/1970
15	Hallett (Kwashiorkor child)	b4:S3:L9a	27/04/1973
16	MacNab	O:3	NA

Appendix B (Continued)

	Our code	Serotype	Date of isolation
17	21/4	0:3	NA
18	1568	0:3	15/04/1974
19	108015	0:3	NA
20	15/1	0:3	NA
21	Ye W	0:3	NA
22	Ransom	0:3	03/01/1975
23	Beneke	0:3	NA
24	Melrose	0:3	NA
25	K.G. Nel	0:3	NA
26	Darne	b4:S3:L9a	1974

(ii) Isolates with limited clinical information.

	Our code	
27	M Henderson	Boy ± 5 years old. Bilateral aplastic kidneys. Renal failure. Developed fever. Organism isolated from peritoneal cavity.
28	Pretorius	Two year old female with mild diarrhoea for three weeks. Organism isolated from faeces 20/04/71. b4:S3:L9a (Ye 1327).
29	Prins	Preliminary diagnosis *Y. pseudotuberculosis*. Septicaemia for two months. *Y. enterocolitica* O 3 isolated from blood on 17/07/74.
30	Kuhl	Child with gastroenteritis. *Y. enterocolitica* biotype 4:S3:L9a isolated on 15/05/74 from stool.
31	Lessing	Vague abdominal pain. At laparotomy ileum was reddened and oedematous. Large mesenteric lymph nodes, 1½" in diameter. *Y. enterocolitica* b4:S3:L9a was isolated on 28/05/74.

Appendix B (continued)

	Our code	
32	Fortuin	Ten year old female admitted to Cape Red Cross Hospital, with a diagnosis of septicaemia, possibly typhoid. *Y. enterocolitica* b4:S3:L9a was isolated on 26/11/73 from blood.
33	Klockenberg	Child with gastroenteritis. *Y. enterocolitica* b4:S3:L9a was isolated on 15/05/74 from faeces.
34	Methwane	63 year-old male with cirrhosis and septicaemia. *Y. enterocolitica* b4:S3:L9a was isolated on 18/01/73 from blood.
35	Mgangua	Thirteen year old female presented with mesenteric adenitis, vomiting, mild pyrexia, no diarrhoea. *Y. enterocolitica* O 3 was isolated from stool on 28/10/71. YH 1/200:YO 1/100.
36	McIntyre	Patient presented with diarrhoea and vomiting. *Y. enterocolitica* O 3 and *Shigella sonnei* isolated from stool on 5/03/74.
37	Wilson	A five year-old child with diarrhoea. *Y. enterocolitica* b4:S1,2a, 3:8a was isolated on 12/10/73 from stool.
38	Smith	Eight year old child *Y. enterocolitica*, O 3 was isolated on 26/09/74 from faeces .
39	Coller	Two and a half year old male with diarrhoea and vomiting for a few weeks. *Y. enterocolitica* b4:S3:L9a was isolated from stool on 25/05/70.
40	94257	*Y. enterocolitica* O 3 was isolated from a pus swab on 11/09/76.

Appendix B (continued)

	Our code	
41	Smith	*Y. enterocolitica* O 3 was isolated on 23/09/74. YH 1/50.
42	A de Beer	European girl of 11 months with a history of vomiting and diarrhoea. Recurrent attacks of diarrhoea over the previous 3 months. *Y. enterocolitica* b4:S3:L9a and *Salmonella montevideo* were isolated on 15/03/68.
43	Swartz	A 2½ year old child with acute gastroenteritis. *Y. enterocolitica* b4:S3:L9a was isolated on 25/05/74.
44	Mhetwa	Normal food handler at a Johannesburg Prison. *Y. enterocolitica* b4:S3:L9a was isolated on 2/05/70 from stool.
45	Francina	*Y. enterocolitica* O 3 was isolated from stool on 4/07/79.
46	Polonsky	Diarrhoea only. *Y. enterocolitica* b4:S3:L9a was isolated from stool on 24/03/73.
47	Paiva	Three year old girl with gastroenteritis. *Y. enterocolitica* b4:S3:L9a was isolated from stool on 28/10/72.
48	Nel	Five and a half year old girl. Had a heart operation. Suffering from tonsilitis, and was on penicillin therapy. Symptoms of diarrhoea and vomiting. *Y. enterocolitica* b4:S3:L9a (Ye 1328) was isolated on 1/06/71 from stool.
49	80179	Coy. Food handler with no history of illness. Kept pigs. *Y. enterocolitica* isolated on 20/21/71 from stool.

Appendix B (continued)

(ii) Specimens for which more clinical information is available.

Our code		
50	Thompson	Presented with diarrhoea for several days. *Y. enterocolitica* was isolated from stool on 5/02/74. YH 1/50. Treated with furoxan, with no effect.
51	5122	Stephen Urqhart, 7 months old. 3/12/75: slightly loose stools, vomiting, odd fever and very irritable. Suggested cause was teething. *Y. enterocolitica* was isolated on 13/01/76. Proved sensitive to streptomycin, gentamicin, tetracycline, kanamycin, chloramphenicol, polymixin and cotrimoxazole. O and H agglutination titre was 1150.
52	Constantinides	A 19 year-old male was admitted complaining of fever, right iliac fossa pain and diarrhoea. The patient was a known case of thalassaemia major and had received over ninety units of blood during the previous three years. He had been on desferroxamine for 6 years. On examination the patient was extremely toxic with a fever of 105.6°F. His skin showed marked hyperpigmentation, his liver was enlarged to 7 fingers breadths below the costal margin and his spleen extended into the pelvis. The patient was passing frequent watery stools and had marked tenderness in the right iliac fossa. Blood and faecal cultures were positive for *Y. enterocolitica* b4:S3:L9a.

Appendix (continued)

	Our code	
52	Constantinides	*Clinical course.* The patient developed an acute arthritis of the right knee and ankle, and complained of pain over the spleen following which a splenic rub became audible. He was treated initially with gentamicin and cephaloridine, but when *Y. enterocolitica* was identified these antibiotics were discontinued and a course of streptomycin begun. On this treatment the diarrhoea, abdominal pain, and arthiritis gradually settled and the patient was afebrile 2 weeks later.
53	Beukes	Ten year old female. Admitted to outpatients at the Transvaal Memorial Hospital on 16/05/72. Complained of abdominal pain and not feeling well for about four months prior to admittance. She was physically active, but with a poor appetite. Frequent epistaxis. Readmitted on 20/07/73 with abdominal pain, loose stool and nausea. Abundant growth of *Staphylococcus pyogenes*, *Proteus morgani* and *Y. enterocolitica* was found on 22/07/73. *Y. enterocolitica* b4:S3:L9a (Ye 6808) was isolated. Antibiotic sensitivity tests were positive for tetracycline, chloramphenicol, colistin, gentamicin, furazolidone, nalidixic acid and kanamycin.

Appendix B (continued)

Our code		
54	Wolmarans	Four months old girl with diarrhoea, vomiting and pyrexia. Culture of stool grew *Y. enterocolitica* serotype O 3 and enteropathogenic *Escherichia coli*. Isolated 26/03/70. Antibody titres were: YO 1:50. YH 1:200.
55	Purcell	(Case 5 reported by Rabson and Koornhof 1972). A 23 year old male, presented at the Johannesburg General Hospital with a history of chronic diarrhoea, blood in the stools, and abdominal pain for 10 years. At sigmoidoscopy numerous shallow ulcers were noted in the rectum, and a biopsy was done on one. On histological examination a non-specific chronically inflamed superficial ulcer was noted in the base of which a small amount of amorphous material was seen, the nature of which could not be determined. *Y. enterocolitica* b4:S3:L9a was isolated from the stool and antibodies against this organism were detected in the serum (YO 1:100, YH 1:50). The patient was treated with a course of tetracycline and 9 months later his symptoms had not reappeared.
56	Leeuw	(Case 4 reported by Rabson and Koornhof 1972). A 69 year-old African male presented with pain over the upper abdomen and was found to have a pyrexia, hepatomegaly and evidence of recent weight loss. Special in-

Appendix B (continued)

	Our code	
56	Leeuw	vestigations showed anaemia (haemoglobin 7.2 g/100 mℓ), an erythrocyte sedimentation rate of 100 mm/hour and deranged liver function tests. The patient developed a left-sided pleural effusion with a productive cough and a chest X-ray revealed in addition an opacity in the posterior segment of the lower lobe. Further investigation showed a splenic abscess extending to the diaphragm, and after drainage and splenectomy, a cystic swelling appeared over the left parietal bone which radiologically showed the features of an osteitis. Aspiration was performed and *Y. enterocolitica* b4:S3:L9a was isolated on 20/6/70. Serological investigation yielded specific titres of 1:50 and 1:400 for O and H agglutinins respectively.

D. Specimens received from S Crips, Enterobacteriology Routine, Microbiology Department, SAIMR, Johannesburg.

	Our code	
57	A808392	Ashley Straker (child). *Y. enterocolitica* b4:S3:L9a (Ye 6811) and *S. pyogenes* were isolated from stool on 9/09/77. Negative for enteropathogenic *E. coli*. Antibiotic sensitivity pattern: streptomycin, gentamicin, tetracycline, kanamycin, chloramphenicol, polymixin, contrimoxazole, furozolidone and nalidixic acid.

Appendix B (continued)

58	017632	Magdalena van der Westhuizen. *Y. enterocolitica* 4:S3:L9a (Ye 7022) was isolated from stool on 21/02/78.
59	20609	Olga Dreyer. *Y. enterocolitica* b4:S3:L9a (Ye 7023) was isolated from stool on 27/02/78.
60	1120	Christiaan Jacobs. 16 month old male. The provisional diagnosis on admission to Fever's Hospital, Johannesburg, was yersiniosis. *Y. enterocolitica* b4:S3:L9a (Ye 6810) was isolated on 24/03/77 from stool. The main symptom was persistent gastroenteritis lasting 14 days. The child was on clear fluids and weighed 25 lbs 7 oz. There was no fever or vomiting. By 25/03/77 the child was recovering. The organism was sensitive to streptomycin, gentamycin, tetracycline, kanamycin, chloramphenicol, sulphonamides, polymixin and contrimoxazole. Serology was:YO 1:400, YH 1:800.
61	7409	Amanda J Feher. Admitted to Fever Hospital, Johannesburg with a provisional diagnosis of amoebic dysentry. Aged 1 year and weighing 19 lbs 15½ oz. On 14/01/77 stool was green, unformed and microscopy showed yeast cells. Culture yielded *E. coli* not of an enteropathogenic serotype. 15/01/1977. Stool yielded *Y. enterocolitica* b4:S3:L9a (Ye 6809) with the following sensitivity pattern: cephalosporin, streptomycin, gentamicin, tetracycline, kanamycin, chloramphenicol,

Appendix B (continued)

	Our code	
61	7409	sulphonamides, polymixin and contri-moxazole. 17/01/1977. An 08h00 stool yielded *Klebsiella* species. The patient was given half strength Darrows at 50 mℓ/hr and trichloryl syrup.18/01/1977: From a first subculture of blood, *Staphylococcus epidermidis* was cultured. The stool was brown, unformed, with no parasites. 19/01/1977. Serology was YO 1:1600 and YH 1:200. The titre to the O antigen of O 3 in both the mother and father was 1:1600. Serology against the H antigen was negative. 20/01/1977. The child's condition was satisfactory, with one loose stool and the patient was discharged.

NA = Not available.