GROWTH, SURVIVAL, ISOLATION AND INCIDENCE OF Y. ENTEROCOLITICA IN FOODS AND OTHER MATERIALS

A Thesis

Submitted by

MARGARET KENDALL

for the Degree of

MASTER OF PHILOSOPHY

in the

UNIVERSITY OF SURREY

Department of Microbiology,
University of Surrey.

September 1982

ABSTRACT

It was demonstrated that Brain Heart Infusion was a reliable broth medium both for growth of Y.enterocolitica from a minimal inoculum and for maximum recovery of the organism and was therefore suitable for use as a reference medium for the subsequent studies.

Growth and survival studies showed that in buffered Brain Heart Infusion broth Y. enterocolitica could survive at pH 4.2 without any significant change in numbers for at least 72 h. The threshold of inhibition/growth was pH 4.2 - pH 4.4. At the higher pH the lag period was prolonged but growth did occur after 38 h incubation. Yersinia enterocolitica grew readily between pH 4.6 and pH 7.8.

Concentrations of sodium chloride greater than 3% had an inhibitory effect on the growth of Y. enterocolitica. In 4% sodium chloride the colony count was 50% less than that in 3% after 48 h incubation. In 5% sodium chloride there was a more marked inhibition and colony counts rose slowly to a maximum of only 7×10^5 organisms/ml after 48 h.

Yersinia enterocolitica inoculated into boiled fish, egg, potato, rice, roast chicken and chocolate milk grew readily at all temperatures from 4° - 37°C. The organism survived without any change in count, for at least 8-9 weeks in these foods whilst stored at -20°C. Yersinia enterocolitica did not effect the pH of the food in which it was growing. The organism did not grow when inoculated into six mayonnaise sauces, with pH values of 2.9 - 4.8.

Evaluations were made of agar and broth media and methods for the isolation of Y. enterocolitica from foods and other materials. Six-teen media commonly used for the isolation of other pathogens were compared with some eight media specially formulated for Y. entero-colitica. No medium was shown to be ideal. In an attempt to formulate or modify a new isolation medium the effects of dyes and other selective agents on the growth of Y. enterocolitica and other organisms was studied. Malachite green (0.004%) incorporated into deoxycholate citrate sucrose agar was found to be inhibitory for many enterobacteria but not for Y. enterocolitica. This effect was shown to be related to the ratio of malachite green to sodium deoxycholate.

Five studies involving 1004 foods and other materials were carried out to further evaluate various media and methods for the isolation of Y. enterocolitica as well as to investigate the incidence of the

organism. The highest isolation rate was obtained from buffered peptone water incubated at 4°C and sub-cultured to lactose sucrose urea agar.

Biochemical characterization and antibiotic sensitivity patterns were determined for all the presumptive Y. enterocolitica strains and the isolation of Y. enterocolitica sensu stricto was confirmed from 53 of 1004 (5.3%) samples including 13/67 pasteurized milk, 7/119 beef, 16/159 pork, 6/40 sausages, 4/101 raw chicken, 4/14 sewer swabs, 2/140 animal feed and 1/1 cooked ham. Yersinia intermedia was isolated from seven samples and Y. frederiksenii from three samples.

There was no significant difference in the antibiotic sensitivity patterns of the three species.

Fourteen different serotypes were identified of which serotype 0:5 was the most commonly isolated. Other serotypes included 0:5,27, 0:6,30 and 0:8 each of which have been implicated in human illness. A few strains were rough and could not be serotyped and 17% of the isolates were not typable with the available antisera. Some foods contained multiple serotypes.

TABLE OF CONTENTS

List of Figures

List of Plates

List of Tables

ACKNOWLEDGEMENTS

I wish to thank Dr. R. Gilbert and Dr. B. Lloyd for their interest and guidance throughout the work. I am also grateful for the support of all my colleagues in the Food Hygiene Laboratory, Central Public Health Laboratory, especially Mr. E. Leeson and Mrs. C. Putman, for their technical assistance.

I am indebted to Mr. E. Fox of the Yersinia Reference Laboratory and Dr. B. Rowe and the staff of the Division of Enteric Pathogens for serotyping the food isolates; to Mrs. K. Saunders of the Media Production Department for her interest and assistance in the preparation of some of the media; to Miss B. Whyte and the staff of the Library, especially Mrs. P. Bradley and Mrs. J. Vurdien, for their patience and diligence in supplying me with the references I required and to the Management, Staff and Environmental Health Officers at the Slaughterhouse, Vicarage Road, Watford for their assistance.

I wish to thank also Mrs. O. Murphy for her encouragement; Mrs. S. Mole, Miss P. Davies, Mrs. H. Westmancoat, Dr. J. Steiner and Mr. and Mrs. G. Dighero for assistance with translation; Mr. J. Gibson for the photography; Mrs. D. Gracias, Mrs. D. Sturgeon and Mrs. C. Rawlinson for typing the drafts and the manuscript; Mrs. I. Cruickshank, Miss N. Cockman and others for reading and commenting on the manuscript and last but not least, all my friends for their prayerful support during its preparation.

Praise the Lord !

INTRODUCTION AND REVIEW

A. Yersinia enterocolitica in its association with human infection

Yersinia enterocolitica as a cause of human disease has been recognized and reported with increasing frequency from many parts of the world, especially since the early 1960's. Although the principal clinical manifestation is gastroenteritis the organism has been isolated from septicaemias, wounds, eyes and other sites.

The true geographical distribution of Y. enterocolitica is still very incomplete as isolation and identification has depended solely on there being an interested and competent laboratory available.

There is published evidence to show that Y. enterocolitica may be spread both from person to person and from a common source such as animal, food or water (Toivanan et al. 1973; Gutman et al. 1973; Black et al. 1978).

1. Clinical manifestations

Mair (1977) reviewed the clinical manifestations of Y. enterocolitica infections and showed that they can be divided into primary and secondary states. The former includes enteritis, acute mesenteric lymphadenitis, terminal ileitis and septicaemia. The secondary complications include erythema nodosum, arthritis and various clinical conditions such as Reiter's syndrome, meningitis, subcutaneous abscess and erysipelas-like illness.

Enteritis is the most common type of illness caused by Y. enterocolitica, and mainly affects young children. The symptoms of abdominal pain, fever, diarrhoea, nausea and vomiting make it indistinguishable from infections by the organisms of the Salmonella or Shigella groups or by pathogenic Escherichia coli. The incubation time is three to seven days and duration of the illness one to three days.

Zen-Yoji and co-workers describe an incident in which 198 of 1086 pupils suffered typical symptoms of abdominal pain (76%), fever (61%), headache (60%), diarrhoea (36%), malaise (33%) and vomiting (12%). Half of the patients experienced severe pain in the umbilical region or lower right quadrant and three cases underwent appendectomy (Zen-Yoji et al. 1973). Acute mesenteric lymphadenitis and terminal ileitis resemble acute or sub-acute appendicitis. There is pain in the middle or right lower quadrant of the abdomen, vomiting is frequent and the temperature rises to 38-40°C and diarrhoea is frequently present. Normally at laparotomy the appendix looks normal although the mesenteric

lymphnodes may be inflamed and swollen. A clear or slightly purulent peritoneal exudate is often present and sometimes the terminal ileum and caecum show gross hyperaemic swelling and oedema.

Septicaemia is rare and usually occurs in elderly patients and those suffering from some other disorder such as thalassaemia, aplastic anaemia or leukaemia. Despite antibiotic treatment septicaemia results in a 50% mortality rate.

Erythema nodosum as a complication of Y. enterocolitica infection occurs predominantly in females over 20 years old following enteritis symptoms. The eruption reaches its maximum in a few days and is accompanied by moderate fever and fatigue.

Polyarthritis may also occur following gastrointestinal symptoms. Several joints become affected and the pain and swelling fluctuate from one joint to another. The knees and ankles are the most commonly attacked followed by the fingers, toes, wrists, elbows and shoulders and occasionally the lumbar spine, hips and sacro-iliac joints. These symptoms usually subside after one to four months but stiffness may persist for much longer.

Besides the clinical states described there are reports of Y. enterocolitica being the causative organism of conjunctivitis (Chin & Noble 1977; Crichton 1978), wound infection (Greenstein & Dreiling 1974) and lung abscess and osteomyelitis of a rib (Sebes et al. 1976).

2. World distribution of infections

The isolation of Y. enterocolitica from cases of human infection has been reported in 37 countries throughout the world. These are listed in Table 1 together with the year that the first isolations were made and the total number of cases recorded. Much of the information in this table was compiled at the W.H.O. Reference Centre in Paris (Mollaret et al. 1979). Since their summary was published the first isolations have been reported in Austria (Stanek et al. 1979), Guatemala (Gini & Torres 1979) and Nigeria (Anjorin et al. 1979). The world distribution of Y. enterocolitica is also shown pictorially (Plate 1).

The total number of isolates does not reflect the size or population of a country but rather the presence of interested bacteriologists. For example Belgium has reported more than 2000 cases since 1964 but the USSR less than 100 cases since 1967. It is thus almost certain that yersiniosis has a worldwide distribution and that the absence of

Table 1

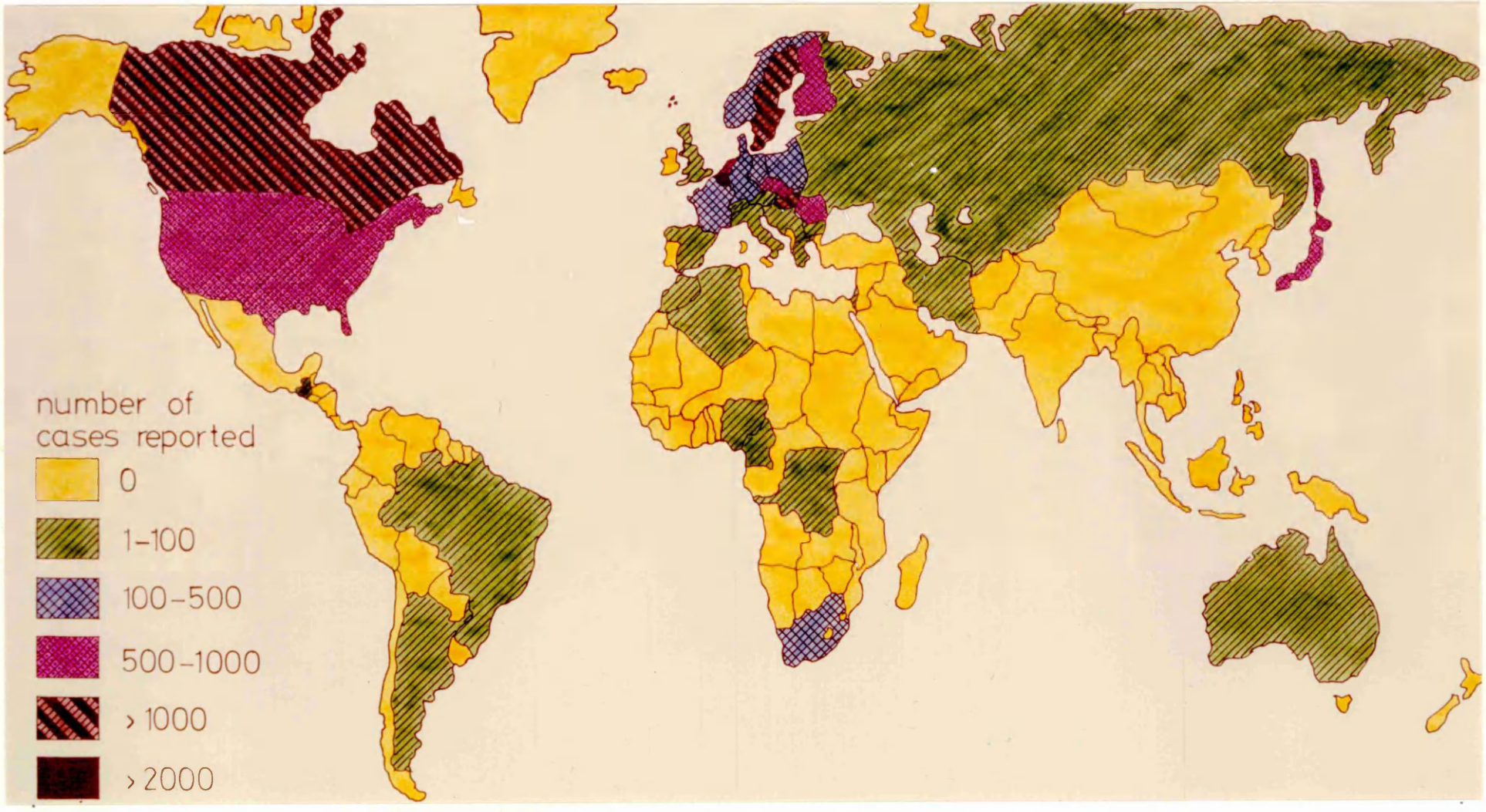
Countries where Y. enterocolitica has been isolated from human infections

(modified from Mollaret et al. 1979)

Country	Year first isolated	Number of cases	Country	Year first isolated	Number of cases
Algeria	1959	+	Iran	1976	+
Argentina	1976	+	Israel	1972	+
Australia	1977	+	Italy	1968	+
Austria	1979*	+	Japan	1971	++++
Belgium	1964	++++++	Morocco	1976	+
Brazil	1968	+	Netherlands	1963	++++
Bulgaria	1975	+	Nigeria	1979*	+
Cameroun	1967	+	Norway	1967	++
Canada	1960	+++++	Poland	1971	+++
Czechoslovakia	1967	++++	Rumania	1968	++++
Denmark	1932	+++	South Africa	1966	+++
East Germany	1973	++	Spain	1971	+
Faroe Islands	1960	+	Sweden	1963	+++++
Finland	1965	++++	Switzerland	1948	+
France	1958	++	United States	1932	++++
Great Britain	1943	+	USSR	1967	+
Greece	1974	+	West Germany	1962	++
Guatemala	1979*	+	Yugoslavia	1972	+
Hungary	1969	+++++	Zaire	1968	+

Number of cases: + = 1-100; ++ = 100-200; +++ = 200-500; ++++ = 500-1000; +++++ = >1000; ++++++ = >2000; * = date paper published, year of first isolation not known.

Plate 1. Worldwide distribution of Y.enterocolitica infections



reported infections in countries reflects deficiences in surveillance.

The most common serotypes isolated vary from country to country. Serotype 0:3 (biotype 4) is prevalent in Europe, Canada, South Africa and Japan. In the USA serotype 0:8 (biotype 1) is the most common serotype isolated and in Scandinavia both serotypes 0:3 and 0:9 are frequently found. Other serotypes including 0:5, 27 and 0:6,30 have been implicated occasionally in human infections (Toma & Lafleur 1974; Robins-Browne et al. 1979).

During the period 1975-1981 187 cases of Y. enterocolitica infection were reported in England and Wales (PHLS Communicable Disease Surveillance Centre, unpublished data). The organism was isolated from faeces, blood culture and from a variety of other sites including liver abscesses, pus, peritoneum, an hysterectomy wound and an eye. Serotype 0:3 was the most frequently isolated with smaller numbers of serotypes 0:1, 0:5, 0:5,27, 0:6,30, 0:8 and 0:9, and many strains were not typable.

3. Incidents of intestinal infections

The mode of spread of Y. enterocolitica is still uncertain but the evidence obtained in 16 outbreaks which have occurred since 1973 indicates that infection from a common food, water or animal source and/or person to person spread can and does take place. Some clues of possible causes or sources of the organism may be found from the following incidents which have occurred in Japan, USA, Canada, Europe, Scandinavia and England, and which are summarised in Table 2.

Four community outbreaks have occurred in Japan, all involving junior and primary schools. In the first outbreak 132 strains of Y. enterocolitica serotype 0:3 were isolated from 198 cases, two more were serotype 0:5 and six serotype 0:9, and another patient carried both types 0:3 and 0:9 (Zen-Yoji et al. 1973). Two other incidents (Asakawa et al. 1973) were similar to the one described above and affected a total of 733 children and teachers. All the strains isolated were serotype 0:3.

The fourth outbreak (Sakazaki et al., unpublished and cited by Asakawa et al. 1973) involved another primary school. Unfortunately bacteriological examination for Y. enterocolitica was not performed during the outbreak, but high agglutinin titres to serotype 0:3 were demonstrated in the patients convalescent sera taken two months later.

Table 2

Incidents of intestinal infections caused by Y. enterocolitica

Location	Number of persons ill	Number of persons at risk	Serotypes isolated from patients	Suspected vehicle of infection and serotype isolated	Reference
Mixed school, Japan	189	441	0:3	Unknown.	Asakawa et al. 1973
Primary school, Japan	544	1042	0:3	Unknown.	Asakawa et al. 1973
Interrelated families, USA	16	21	0:8	Dog (bitch) and a litter of puppies.	Gutman et al. 1973
Hospital, Finland	6	Unknown	0:9	Person to person.	Toivanen et al. 1973
Primary school, Japan	Unknown	Unknown	0:3 (serology)	Unknown.	Sakazaki et al. cited by Asakawa et al. 1973
Junior high school, Japan	198	1086	0:3 0:5 0:9	Unknown	Zen-Yoji et al. 1973
Schools and Community, USA	>179	>2000	0:8	Chocolate milk 0:8	Black et al. 1978

Continued/.....

Table 2 (continued)

Location	Number of persons ill	Number of persons at risk	Serotypes isolated from patients	Suspected vehicle of infection and serotype isolated	Reference
Nursery school, Czechoslovakia	15	142	0:3	Unknown.	Olšovský et al. 1975
School A, Canada	58	346	0:5,27	Raw milk.	de Grace et al. 1976
School B, Canada	80	485	0:5,27	Raw milk.	de Grace et al. 1976
Baby, USA	1	7	0:20	Family dog 0:20	Wilson et al. 1976
Ski resort, USA	100	185	?	Well water. 0:3	Eden et al. 1977
Baby, Denmark	1	?	0:3	Well water.	Christensen 1979
School, England	17	135	Not typable	Coleslaw	PHLS Communicable Disease Surveillance Centre, unpublished.
Family, England	1	2	0:3	Unknown and ? person to person.	PHLS Communicable Disease Surveillance Centre, unpublished.
School, England	96	200	0:3	Pigs and person to person.	Bartlett et al. 1982

Four outbreaks have been reported in the American literature, the first involved 16 of 21 persons in four related and neighbouring families. The illness led to two appendectomies and two deaths. Yersinia enterocolitica serotype 0:8 was isolated at autopsy from a baby which died and raised antibody titres were demonstrated in other patients. A dog (bitch) had whelped a litter of nine puppies, five of which had died from diarrhoeal disease just prior to the human outbreak. The surviving animals were destroyed after the onset of the incident and without cultural examination (Gutman et al. 1973).

The second incident in the USA is the only proven foodborne Y. enterocolitica outbreak in the world. This occurred in a village where 37 of 119 people reporting ill with abdominal pain and fever were found to be infected with Y. enterocolitica type 0:8. The same serotype was also isolated from 1 of 60 people reporting diarrhoea without abdominal pain and fever. Twenty-three of the culture positive patients, 9 of 40 culture negative and one symptomless child had a raised antibody level (>128). Sixteen patients underwent appendectomy. The common food source of the organism was identified as a chocolate milk drink. This was purchased from a small dairy which was the exclusive supplier of milk to the local schools. The chocolate milk was prepared by adding chocolate syrup to previously pasteurised milk in an open vat and mixing by hand with a perforated metal stirring rod. Yersinia enterocolitica type 0:8 was isolated from 1 of 4 unopened cartons of chocolate milk obtained from the school cafeteria (Black et al. 1978).

The third North American incident was again associated with a family of dogs. Eight of a litter of eleven puppies had died from what was described as "wasting away" but there was no evidence of diarrhoea. However a four month old baby girl was found to have enlarged inguinial nodes and Y. enterocolitica serotype 0:20 was isolated from an aspirate. On bacteriological examination four of seven members of the baby's family yielded serotype 0:6 but the three surviving puppies all yielded Y. enterocolitica serotype 0:20 (Wilson et al. 1976).

The fourth outbreak occurred in 1975, it was reported that many visitors and staff of a ski resort in Montana had experienced predominantly nausea, vomiting, diarrhoea and abdominal pain either during or after visiting the resort. Within a two month period 76% of the staff had experienced gastrointestinal illness. An epidemiological

survey showed a significant association between the drinking water and illness (P = 0.0001) but no association between illness and room location or where the guests ate or drank. Yersinia enterocolitica was isolated from two of the wells which supplied the drinking water. Four different serotypes were identified including serotypes 0:3 and 0:5 and some strains were non-typable. After the isolation of Y. enterocolitica from the water 100 persons who had been ill were examined bacteriologically but the organism was not found (Eden et al. 1977).

In Canada two associated outbreaks have occurred. The source was never proved and the bacteriological evidence obtained was poor but the epidemiological evidence did implicate raw milk. Groups of children from two separate schools attended "sugaring off" parties at a sugar bush near Montreal at various times during a four week period. A total of 831 persons were exposed to infection and 138 became ill. However, unlike other outbreaks diarrhoea was the predominant symptom. Yersinia enterocolitica serotype 0:5,27 was isolated from the only two children examined and serotype 0.6,30 was found in the raw milk. The possibility of other etiological agents being responsible could not be ruled out (de Grace et al. 1976).

The one outbreak which occurred in Czechoslovakia (Olšovský et al. 1975) centred on two establishments for the collective care of children, both of which received their food from a single kitchen. Mass sampling of stools took place nine days after the explosive occurrence and Y. enterocolitica serotype 0:3 was isolated from 6 of 15 patients. Follow-up serological examination showed raised agglutinin titres in these persons.

The Finnish outbreak described by Toivanen et al. (1973) began with the hospitalization of a schoolgirl with suspected appendicitis. She had been transferred to a second ward when it was established that she was infected with Y. enterocolitica. Six members of staff in the two wards became infected. Yersinia enterocolitica serotype 0:9 was isolated from the girl and a member of staff and all infected persons had raised antibody titres. The incubation time in all cases was approximately ten days after presumed exposure.

Well water appeared to be the source of yersinosis in a Danish baby (Christensen 1979). The child was diagnosed as having acute gastroenteritis and a raised antibody titre to Y. enterocolitica serotype 0:3 was demonstrated. The same serotype was isolated from a

well which provided the only water supply to the household and which was used in the preparation of the baby's feed. Blood samples from the rest of the family did not reveal raised titres to Y. enterocolitica.

The three outbreaks which have occurred in England have as yet not been reported in the literature. In the first incident 17 of 135 persons suffered nausea, vomiting, abdominal pain and diarrhoea $2\frac{1}{2}$-26 h after consumption of a school meal which included coleslaw prepared with dressing from a jar opened a week previously. Yersinia enterocolitica was isolated from 4 of 17 persons with symptoms (including one food handler) and 1 of 3 asymptomatic food handlers. The strains were all serologically non-typable using live suspensions but agglutinated serotype O:5,27 using steamed suspensions. None of the food was available for examination.

The second case involved a farmer's wife who developed symptoms after a visit to London. She had eaten smoked salmon and scampi in lobster sauce 48 h before and chicken sandwiches 12 h before, onset of illness. Yersinia enterocolitica serotype O:3 was isolated from the patient and also from her husband who had not been to London and had no symptoms. The organism was not isolated from the couple's son or their nine pet dachshounds (PHLS Communicable Disease Surveillance Centre, unpublished data).

The third outbreak was in a boys' boarding school in the south of England. During the autumn and spring terms some 96 of 200 boys and 2 of 35 staff were ill, some more than once. Abdominal pain and malaise were the principal clinical features but some boys presented with abdominal pain and diarrhoea or diarrhoea alone. Two of the 96 boys had suspected appendicitis and one was operated on. Yersinia enterocolitica serotype O:3 was isolated from ten boys and a raised antibody titre to serotype O:3 was demonstrated in 36 boys. A detailed epidemiological investigation showed that infection was closely associated ($P = 0.0002$) with attendance to the pigs which the school kept on its farm. There was also some suggestion of person to person spread particularly within individual classes. Yersinia enterocolitica has so far not been isolated from the pigs (Bartlett et al. 1982).

The incidents referred to in this section suggest a variety of causes and sources of infection rather than a primary etiological

agent. Where a specific source of infection has been implicated the commonest causes would appear to be foodstuffs, animals and water.

B. Yersinia enterocolitica - the organism

The organism now known as Yersinia enterocolitica has been called a variety of names, Pasteurella "x", Pasteurella pseudotuberculosis b, and Pasteurella pseudotuberculosis-like bacterium. The designation Y. enterocolitica was proposed by Frederiksen in 1964 and the organism has recently been classified as a member of the family Enterobacteriaceae. Yersinia enterocolitica is morphologically and culturally related to Y. pseudotuberculosis, but differs from it with regard to serology, pathogenicity to animals, bacteriophage sensitivity and certain biochemical reactions. It was first isolated between 1933-43 in New York State from two patients with cervical adenitis and three patients with acute enteritis and terminal ileitis (Sonnenwirth 1976). There were very few cases recognized between 1943 and the early 1960's since when numerous human cases have been diagnosed in Europe, North America, Asia and Australasia.

1. Morphological, colonial and biochemical characteristics

Yersinia enterocolitica is a Gram-negative, oxidase-negative, catalase-positive facultatively anaerobic, fermentative rod that grows on common laboratory media (e.g. nutrient agar) and reduces nitrates to nitrites (except for Wauter's biotype 5). It is rarely pigmented, is non-sporing and does not produce a capsule when grown in vitro. It is motile (at 22°C only) by peritrichous flagella. It is on the basis of these characteristics that it has been admitted to the family Enterobacteriaceae.

On nutrient agar incubated at 22°-37°C for 24 h Y. enterocolitica produces colonies 0.5-1 mm in diameter. On bile salt selective media used to detect enteric pathogens from stool samples the organism grows much better at 22° than at 37°C (Nilehn 1969b; Wauters 1970). The reported growth range of Y. enterocolitica strains is from 4° to 41°C.

The morphological, cultural and biochemical characteristics of Y. enterocolitica have been reviewed by many workers (Bottone 1977; Nilehn 1969b; Wauters 1970; Sonnenwirth 1976; Feeley et al. 1976). However, the biochemical classification and differentiation of Y. enterocolitica and Y. enterocolitica-like organisms has remained confused. One of the reasons why there is confusion in the literature about the characteristics of the organism may be because some positive biochemical reactions are only obtained at temperatures below 30°C.

These tests include Voges-Proskauer, β-galactasidase, fermentation of lactose, raffinose, rhamnose, Simmon's citrate and ornithine decarboxylase.

Recently an international group of workers defined Y. enterocolitica sensu stricto and the Y. enterocolitica-like organisms (Bercovier et al. 1980a, 1980b; Brenner et al. 1980; Ursing et al. 1980), but this classification has not yet been officially accepted by the International Committee on Systematic Bacteriology. The biochemical characteristics of Y. enterocolitica sensu stricto are described by Bercovier and co-workers following a study of 7000 strains at the National Reference Centre in Paris are shown in Table 3. In addition to those already listed the following tests gave positive reactions for all the strains examined, catalase, methyl red (37°C), fermentation in O-F test, acid production from D-glucose, glycerol, ribose, D-fructose, D-mannose, D-mannitol and N-acetyl-glucosamine. Negative reactions were obtained with all the strains for the following tests:- oxidase, motility at 37°C, Voges-Proskauer at 37°C, Simmon's citrate at 37°C, malonate, mucate, potassium cyanide, gas from glucose, hydrogen sulphide (Kliegler's), phenylalanine deaminase, tryptophan deaminase, lysine decarboxylase, arginine decarboxylase, β-xylosidase (PNPX 37°C), gelatin, and acid production from erythritol, L-xylose, adonitol, L-rhamnose, dulcitol, D-melizitose, alpha-methyl-xyloside, alpha-methyl-D-mannoside, alpha-methyl-D-glucoside, inulin, amylose and glycogen.

Several different schemes have been described for the biotyping of Y. enterocolitica (Nilehn 1969b; Wauters 1970; Knapp & Thal 1973). A fourth scheme which is a slight modification of Wauters' scheme has since been published by Bercovier et al. (1980a). The four schemes are summarized in Table 4. The basis of the schemes are the different reactions of Y. enterocolitica strains in such tests as lecithinase (lipase), indole, xylose, aesculin and salicin. Even with these schemes there has been and still is considerable disagreement as to which of the various biotypes are true Y. enterocolitica. Further work is continuing on the serology, genetic and deoxyribonucleic acid homology at the request of the sub-committee on Pasteurella, Yersinia and Francisella.

The proposed classification of Y. enterocolitica sensu stricto and the Y. enterocolitica-like organisms includes the naming of three new species, Y. frederiksenii (Ursing et al. 1980), Y. intermedia (Brenner et al. 1980) and Y. kristensenii (Bercovier et al. 1980b). The

Biochemical characteristics of Y. enterocolitica sensu stricto (Bercovier et al. 1980a)

Test	Reaction at 28°C	%+	%(+)	Neotype Strain 161
Motility	+ or (+)	88	10	+
Urease	+	99	0	+
Indole	V*	27	10	+
Methyl red	+ or (+)	60	35	+
Voges Proskauer	+**	90	8	+
Simmon's citrate	-	<1	0	-
Christensen's citrate	V	65	0	+
Nitrate to Nitrite/type	+**/B	97/B	0	+/B
Tetrathionate reductase	V*	35	0	+
Ornithine decarboxylase	+**	97	0	+
β-galactosidase (ONPG) (37°C)	+**	90	0	+
Lipase (Tween 80)	V*	21	9	+
Deoxyribonuclease	V*	40	28	-
Polypectate	(+)	0	100	(+)
Acid production from:				
D-arabinose	-	<1	0	-
L-arabinose	+	99	0	+
D-xylose	V*	26	15	+
Galactose	+	99	0	+
L-sorbose	+**	90	4	+
D-cellobiose	+	99	0	+
Maltose	+	99	0	+
Lactose	-	8	15	-
D-melibiose	-	<1	0	-
Sucrose	+**	98	0	+
D-trehalose	+**	97	0	+
D-raffinose	-	<1	0	-
i-inositol	+ or (+)**	70	21	+
D-sorbitol	+**	97	0	+
Aesculin	V	31	25	+
Salicin	V	15	16	+
Amygdalin	V	15	70	+
Arbutin	V	60	16	+
Dextrin	-	5	25	-
Starch	(+)	22	78	(+)

+ = 90% or more positive within 72 h; (+) = 90% or more positive between 4 and 7 days; V = 10.1 - 89.9% positive; - = <10% positive after 72 h; * = reaction varies with biotype; ** = most negative strains biotype 5.

Table 4

Biotyping schemes for Y. enterocolitica

Reaction	Biotypes																			
	Nilehn (1969b) a.					Wauters (1970) a.					Knapp & Thal (1973) a.				Bercovier et al. (1980a) b.					
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	1	2	3	4	5	
Lecithinase						+	-	-	-	-					+	-	-	-	-	
Salicin	+	-	-	-	-						-	-	+	V						
Aesculin	+	-	-	-	-						-	-	+	V						
Indole	+	+	-	-	-	+	(+)	-	-	-	-	+	+	V	+	+	-	-	-	
D-xylose	+	+	+	-	-	+	+	+	-	-	V	+	+	V	+	+	+	-	-*	
Nitrate-reduction	+	+	+	+	-	+	+	+	+	-					+	+	+	-	-	
Trehalose	+	+	+	+	-	+	+	+	+	-					+	+	+	+	-	
D-sorbitol	+	+	+	+	-															
Ornithine decarboxylase	+	+	+	+	-	+	+	+	+	-										
Voges-Proskauer (25°C)	+	+	+	+	-															
B-galactosidase (25°C)	+	+	+	+	-	+	+	+	+	-										
Sucrose	+	+	+	+	-										+	+	+	+	V	
Sorbose	+	+	+	+	-															
DNase															-	-*	-*	+	+	

+ = positive reaction; - = negative reaction; ( ) = delayed reaction; V = variable reaction; * = some strains positive reaction after 72 h; a = tests were done at 35° and 37°C unless otherwise stated; b = tests were done at 28°C.

differentiation of Y. enterocolitica sensu stricto and these other Yersinia spp. is shown in Table 5.

2. Serological identification

Yersinia enterocolitica can be serotyped by both "O" and "H" antigens. However, as many of the "O" antigens share common "H" antigens the serotyping scheme is based solely on the somatic "O" antigen. The serotyping scheme was originally developed by Winblad (1968) who identified 17 "O" and 16 "H" antigens. It was later extended to 34 "O" types by Wauters et al. (1971, 1972) and at the present time 57 "O" antigens have been recognized.

The relationship between serological type and pathogenicity in humans is as confused as the biochemical classification. Nevertheless, serotypes O:3 (Wauters' biotype 3 or 4) and O:9 (Wauters' biotype 2) are predominantly associated with human disease, but several other types including O:5,27; O:6 and O:8 (which may be Wauters' biotypes 1, 2 or 3) have also been implicated with increasing frequency. There is also a distinct geographic distribution of the different serotypes. Serotype O:3 is prevalent in Europe, Japan, South Africa and Canada, serotype O:9 is found mainly in Europe, and O:5,27; O:6 and O:8 in Australia, Europe, Japan and the USA.

3. Phage typing

Mollaret & Nicolle (1965) reported that bacteriophage had been obtained from strains referred to as Y. enterocolitica and that they had lytic activity against other strains within the group. In a later study Nicolle and co-workers investigated 116 strains of Y. enterocolitica from man and animals and found a high percentage to be lysogenic. There also appeared to be some host specificity of the different typing patterns (Nicolle et al. 1967). Host specificity was confirmed by Nilehn (1969b) and Nilehn & Ericson (1969). Nilehn (1973) also showed that there was a variable geographic distribution of phage types. The majority of her human isolates from Scandinavia belonged to phage group II and those from Europe to phage group III. Nineteen of 57 animal strains were also the European type III and the remainder of the strains were divided into another ten different patterns. The serological and phage type geographical distribution

Table 5

Differentiation of Y. enterocolitica sensu stricto and other Yersinia spp. (Bercovier et al. 1980a)

Test at 28°C	Y. enterocolitica biotypes 1-4	Y. enterocolitica biotypes 5	Y. kristensenii	Y. frederiksenii	Y. intermedia	Yersinia species X1	Yersinia species X2	Y. pseudotuberculosis
Nitrate reduction	+	-	+	+	+	+	+	+
Voges-Proskauer	+	+	-	+	+	-	+	-
D-cellobiose	+	+	+	+	+	+	-	-
Sucrose	+	V	-	+	+	-	-	-
D-trehalose	+	-	+	+	+	+	+	+
L-rhamnose	-	-	-	+	+	-	+	+
D-melibiose	-	-	-	-	+	-	-	+
α-methyl-D-glucoside	+	-	-	-	+	-	-	-
Ornithine decarboxylase	+	V	+	+	+	-	+	-
Indole	V	-	V	+	+	-	-	-
L-sorbose	+	V	+	+	+	-	-	-
D-sorbitol	+	-	+	+	+	-	+	-
D-raffinose	-	-	-	-	+	-	-	V (11%)
Citrate (Simmon's)	-	-	-	V	+	-	+	-
Maltose	+	+	+	+	+	+	-	+
β-xylosidase (PNPX)	-	-	-	V	-	-	-	+

+ = 90% or more positive within 72 h; V = 10.1-89.9% positive; - = < 10% positive after 72 h.

is summarized in Table 6.

Table 6

Geographical distribution of serological and phage types of Y. enterocolitica

Serotype 'O'	Phage	Geographic distribution
5,27	X_2	Australia
6	or	Europe, Japan
8	X_o	USA
9	X_3	Europe
3	VIII	Europe, Japan
	IXa	South Africa
	IXb	Canada

4. Antibiotic sensitivity

It has been suggested that there might be a correlation between specific biochemical characteristics and antibiotic susceptibility patterns but there is no evidence to support this. Nilehn (1967) reported that the majority of 28 rhamnose-negative strains of Y. enterocolitica from both human and animal sources and which she tested using the paper disc method on blood agar plates were sensitive to streptomycin 50 mcg, tetracycline 50 mcg, oxytetracycline 50 mcg,

chloramphenicol 30 mcg, nitrofurantoin 30 mcg, colistin 900 IU, nalidixic acid 30 mcg, neomycin 50 mcg and sulphonamide 2.5 mcg, but resistant to methicillin 30 mcg, oleandomycin 50 mcg, novobiocin 50 mcg and fucidin 50 mcg. Most strains were slightly sensitive to ampicillin 20 IU and 50% of the strains were resistant to erythromycin 50 mcg and benzyl penicillin 20 IU. Chester & Stotzky (1976) also using the disc diffusion method found all their rhamnose-positive strains of Y. enterocolitica were sensitive to ampicillin 10 µg and cephalothin 30 µg but Raevuori et al. (1978) found that only 33% of their rhamnose-positive Y. enterocolitica strains and only 22% rhamnose-negative strains were sensitive to ampicillin 8 µg and 1 and 7% respectively sensitive to cephalothin.

However, there does appear to be some temperature related variation in the susceptibility to antibiotics of different strains of Y. enterocolitica. Kouwatli et al. (1979) have shown that, generally, strains were more sensitive to cephalexin, cephalothin, cephapirin, cephexitin and cephazolin at 37° than at 22°C and a few strains were sensitive to ampicillin at 37° and not at 22°C. Likewise these workers also showed that some strains of Y. enterocolitica were more resistant to gentamicin, kanamycin and rifampicin at 37° than 22°C.

5. Pathogenicity

General. The mechanisms of pathogenicity of Y. enterocolitica are still not completely clear. In a human volunteer experiment 3.5×10^9 Y. enterocolitica/ml were taken orally and resulted in enterocolitis with a mild (37.8°C) fever. Symptoms lasted for four weeks and the organism was cultured from faeces in almost pure culture during this period (Szita et al. 1973). Carter (1975) produced laboratory infections in mice which were claimed to resemble closely a naturally acquired human infection. Pathogen free male mice were challenged intragastrically with approximately 10^8 Y. enterocolitica. Within five days the Peyer's patches in the distal ileum showed signs of swelling and hyperaemia and the mesenteric lymph nodes became swollen. By the twelfth day lesions were seen on the liver, spleen and lungs. Sera taken from mice surviving 30 days exhibited significant agglutination titres.

Une (1977a) demonstrated that in the rabbit, pathogenic strains of Y. enterocolitica easily penetrated the epithelial lining of the

intestinal mucous membrane into the target reticuloendothelial tissues and multiplied within mononuclear cells and produced granuloma. In view of these findings Une suggested that Y. enterocolitica should be included in the category of the invasive type bacteria such as Shigella and some Salmonella.

The same worker (Une 1977b) also showed that those strains of Y. enterocolitica which had produced experimental enterocolitis in rabbits also had the ability to penetrate HeLa cells and to survive or multiply within macrophages. Except for one strain tested all those which failed to produce pathological changes in the rabbit also failed to penetrate HeLa cells. Similar findings are also reported by Zen-Yoji (1978).

Enterotoxin. Several workers have studied an enterotoxin produced by some strains of Y. enterocolitica and found it to be heat stable at 100°C for 15 min, active in the suckling mouse and rabbit ileal loop system, but not in the Y1 adrenal cell assay and also as active in the 6 h rabbit ileal loop as in the 18 h assay. These characteristics suggest that the enterotoxin of Y. enterocolitica is similar to that of E. coli (Pai & Mors 1978). Boyce et al. (1979) also found that Y. enterocolitica enterotoxin was similar to that of E. coli enterotoxin in its ability to resist a low pH (pH 1.0) but whereas E. coli enterotoxin was destroyed at pH 11.0 that of Y. enterocolitica was unaffected. Enterotoxins from both organisms were methanol soluble.

In a later study Mors & Pai (1980) examined 88 strains of Y. enterocolitica for heat stable toxin production and ability to penetrate HeLa cells and evoke keratoconjunctivitis in guinea pigs (Sereny test). They were able to classify the strains in to five groups according to their potential pathogenic properties (Table 7). Most of serotypes 0:3, 0:9 and 0:5,27 belonged to group 4 and none to groups 1, 2 or 5, but serotype 0:8 strains were divided between groups 4 and 5. Other serotypes were primarily groups 1 and 2. All isolates belonging to serotypes 0:3, 0:8, 0:9 and 0.5,27 and only 5 of 39 strains of other serotypes were HeLa positive. Only strains of serotype 0:8 were Sereny positive and heat stable enterotoxin was produced by all serotypes. The ability of only certain strains to invade HeLa and other mammalian epithelial cells is further described by Lee et al. (1977), Pederson et al. (1979), Kapperud (1980b) and Okamoto et al. (1980). These latter workers also reported that the adhesion of Y. enterocolitica

Table 7

Pathogenic classification of Y. enterocolitica

according to Mors & Pai (1980)

Group	Properties of enterotoxin		
	Heat stable	HeLa invasive	Sereny test
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	+	+	+

+ = positive reaction; - = negative reaction

to various cell tissues including HeLa, FL, Y 1 adrenal, human intestine and human conjunctiva was temperature dependent and occurred at 25° but not at 37°C. Kapperud (1980a) also reported some temperature dependent variations in toxin production.

It has been shown that invasiveness as indicated by the Serény test may be plasmid associated as the presence of a particular species of plasmid DNA has been shown to correlate with a positive Serény test (Zink et al. 1978; Gemski et al. 1980; Portnoy et al. 1981).

Pai et al. (1978) found that enterotoxin production was more prevalent in strains isolated from humans than those isolated from food, water or animals but that serotypes 0:3, 0:8, 0:5,27, 0:6:30 and 0:9 which are often isolated from human infection were almost always enterotoxigenic.

Following the Y. enterocolitica serotype 0:8 outbreak in the USA (Black et al. 1978) in which milk was found to be the vehicle of infection, seven of eight of the strains isolated were found to be toxigenic and the question of pre-formed toxin was raised (Boyce et al. 1979). Francis et al. (1980) examined 36 strains of Y. enterocolitica (isolated from raw milk) for enterotoxin production in milk and reported that 3 of 36 strains produced enterotoxin at 25° but not at 4°C. However, Kapperud & Langeland (1981) have reported that some of their strains of Y. enterocolitica and Y-enterocolitica-like organisms did produce toxin at refrigeration temperature and therefore may be capable of causing food intoxication after food storage at refrigeration temperature.

C. Distribution of Yersinia enterocolitica in foods, faeces, animals and the environment

It is not yet clear just how widespread Y. enterocolitica is in foods, faeces, animals and the environment. But there have been an increasing number of reports from Europe, Asia, The Americas and Australasia of the isolation of the organism from these sources. However, there have only been two published reports of the isolation of this organism in the UK, from a bush baby (Mair et al. 1970) and budgerigars (Giles & Carter 1980).

Since 1974 the World Health Organization Reference Centre in Paris has been collecting data and cultures of Y. enterocolitica from sources throughout the world but it is obvious that not all strains have been deposited with them as many isolations reported in the literature are not cited in their summary (Mollaret et al. 1979).

1. Foods

The largest number of isolations of Y. enterocolitica have been made from raw meats. Leistner et al. (1975) in Germany examined 215 samples of meat including poultry (121), pork (29), beef meat (37) and raw frying sausage (Rohwurst) (28) and isolated Y. enterocolitica and Y. enterocolitica-like organisms from 56 (46%), 10 (35%), 6 (16%) and 1 (4%) respectively. Fifty of the 73 strains isolated were Y. enterocolitica "in the strict sense". Nine different serotypes were identified, the most frequent type being serotype 0:6 followed by 0:5 and many of the strains were serologically non-typable. A 4% isolation rate of Y. enterocolitica from comminuted turkey meat has been reported in the USA (Guthertz et al. 1976). Hanna et al. (1976) isolated 10^3-10^5 Y. enterocolitica per cm^2 from 10/105 (9.5%) cuts of vacuum packed beef and 2/34 (6%) lamb cuts which had been stored at 1°-3°C for up to 35 days. Most of these strains were serologically non-typable.

Norberg (1981) isolated Y. enterocolitica from 20 of 82 frozen whole chicken examined. Three of the isolates belonged to serotype 0:6 and three more to serotypes 0:4, 0:5b and 0:8. The remaining strains were not typable.

Yersinia enterocolitica-"resembling" organisms have been recovered at levels of 10^7/g on high pH (> pH 6.0) vacuum packaged beef strip loins held for six weeks at 0°-2°C but only at levels below 10^5/g on

normal pH (pH<6.0) strip loins held for ten weeks (Seelye & Yearbury 1979). In a Canadian survey of retail pork products 5/69 (7%) processed and 63/128 (49%) raw pork products were found to contain Y. enterocolitica. Thirty-two of 80 (40%) isolates were serotypable with types 0:3 and 0:5 occurring most frequently. Serotype 0:3 was isolated only from the raw meats (Schiemann 1980a).

Schiemann & Toma (1978) isolated Y. enterocolitica from 29/131 (22%) raw milk samples examined. Thirteen of these isolates were serotypable and belonged to seven different "O" serotypes including 0:6,30, 0:14 and 0:15 and 0:5, with 0:5 occurring most frequently. Schiemann (1978a) has also isolated the organisms from pasteurized fluid dairy products (0.4%), cheese curd (9.2%) and a further study of raw milk samples produced an 18.2% Y. enterocolitica isolation rate. Most of the curd samples showed a positive phosphatase test indicating their production was from raw milk. One sample of cheese yielded Y. enterocolitica after four weeks storage at 4°C but was negative after eight weeks. The four different serotypes isolated included 0:6,30 but not 0:5.

Yersinia enterocolitica has also been isolated from both raw and pasteurized milk and a dairy factory holding vat in Australia. Many of the strains isolated were serotype 0:5. Some were serotypes 0:5b, 0:6, 0:13/15 and 0:23/15, and the remainder were non-typable (Hughes 1979, 1980).

More recently Y. enterocolitica has been isolated from Australian raw goats milk (Hughes & Jensen 1981). Many of the isolates fermented rhamnose but some resembled biotypes 1 or 2.

Sixty-one of 71 raw milks from a dairy in Eastern France were found contaminated with Y. enterocolitica, of which 40 of 61 isolates were biotype 1 and serotype 0:5 predominated (Vidon & Delmas 1981).

In Czechoslovakia, Aldová et al. (1975) found Y. enterocolitica in various foods including cream, ice cream, processed meat products, Italian type salad and egg mayonnaise. Many strains were serologically non-typable but those which were identified included serotypes 0:3 (from paté), 0:5, 010, K1 and 0:14. Serotype 0:5 being found most frequently.

From time to time seafood has been shown to be contaminated with Y. enterocolitica. Spadaro & Infortuna (1968) isolated the organism

from 1% of mussels taken from the stagnant waters of an Italian lake and which was badly drained to the sea by a narrow canal. Kapperud & Jonsson (1976) isolated Y. enterocolitica from 3/20 Norwegian brown trout and Peixotto et al. (1979) detected the organism in 6/45 (13%) oysters, 2/50 (4%) shrimps and 12/50 (21%) crab samples.

In addition to these reports the WHO Reference Centre lists chocolate milk, which was responsible for a proven outbreak in the USA, whipped cream, black pudding and various vegetables including carrots, tomatoes, green salads, coarse salads, beetroot and mushrooms as having been found contaminated with Y. enterocolitica (Mollaret et al. 1979).

2. Faeces of healthy humans

Potentially pathogenic bacteria are not infrequently carried by healthy humans without any apparent signs or symptoms and, as the few studies which have been carried out have shown, Y. enterocolitica is no exception.

Asakawa et al. (1979) collected 4673 faecal samples from healthy humans including 3643 food handlers and 1030 school children. The highest carriage rate was amongst the kindergarten age group of children with 1.4% (9/627) followed by butcher food handlers 0.87% (5/571), primary school age children 0.49% (2/403) and the lowest rate was amongst other food handlers 0.06% (2/3072). None of these carriers showed any symptoms of clinical illness. The 18 isolates belonged to serotypes 0:3 (3), 0:4 (1), 0:5 (4), 0:6 (4), 0:7 (2) and 0:14 (4).

In Norway, Kapperud (1980c) sampled 397 presumed healthy students during April-September 1979. Twenty strains of Y. enterocolitica and Y. enterocolitica-like organisms were isolated from ten (3%) of these students. Six of the students harboured two to four antigenically distinct strains. The 14 typical Y. enterocolitica strains represented five different serotypes which were 0:4, 0:5, 0:6, 0:7, 8 and 0:13,7; some strains were not typable. The remaining six strains showed atypical reactions of one or more of the following characteristics; rhamnose, sucrose, cellobiose and sorbose, and were designated Y. enterocolitica-like. The strains recovered in this survey were similar to strains previously isolated from wild living mammals, fish and water in Scandinavia.

An ecological study of Y. enterocolitica in France included the examination of healthy humans from rural areas. Two surveys were carried out in which a total of 182 faeces from children in two schools were examined. Although it is not shown how many different children these 182 samples represented it is stressed that the six isolates in the first survey were from different children to the two isolated in the second survey (Alonso et al. 1979).

In the inter-familial outbreak described by Gutman et al. (1973) 2/31 control persons examined were found to be excreting Y. enterocolitica which were serologically distinguishable from the outbreak strain. In a family outbreak in the UK the husband of the patient was found to be symptomlessly excreting the same serotype (0:3) as the patient (PHLS Communicable Disease Surveillance Centre, unpublished data).

3. Animals

The WHO Reference Centre in Paris has received cultures and reports of the isolation of Y. enterocolitica from a wide range of animals all over the world. The animals from which the organism has been isolated were frequently healthy carriers and included dogs (35), cats (41), swine (250), goats (60), cattle (48), elk (22), monkeys (21), hares (201), rabbits (15), chinchilla (180) and small numbers (<10 of each) of horses, foxes, mink, ocelot, racoon, sheep, camel, deer and guinea pigs. A total of 500 wild rodents, representing 20 different species, from Czechoslovakia, Sweden, Norway, Finland, Denmark, France, Japan, Poland and Romania have also been reported infected with Y. enterocolitica. The organism has been isolated to a lesser extent from chickens, geese, ducks, pigeons, sparrows, goldfish, canaries, grouse, bunting, lapwing and finch (36 in all) and also from trout, perch, roach, snails, frogs, oysters, seagulls and fleas (47 total) (Mollaret et al. 1979). In addition Y. enterocolitica has been isolated from mussels (Spadaro & Infortuna 1968), shrimp and crab (Peixotto et al. 1979) and butterclams (Munger et al. 1980). In the UK there are published reports of infection in a bush baby (Galago) (Mair et al. 1970) and when 25 of 26 budgerigars in an aviary were found dead Y. enterocolitica serotype 0:6,30 was isolated from the six birds examined (Giles & Carter 1980).

The role of the fly in transmission of Y. enterocolitica has been investigated by Fukushima et al. (1979) who isolated the organism from flies captured on fly papers which had been hung in five farm piggeries and also from similar papers and pieces of ham hung in the kitchens of the same farms.

It is not possible to compare the results of different workers using both different sampling techniques and isolation methods. Mollaret et al. (1979) state clearly that because of the wide variety of techniques used for isolation and the nature of the samples examined that it is impossible to give any accurate estimate of the carriage of Y. enterocolitica in animals, small wild mammals or birds. These workers even felt that there was no proof of the animal origin of human infections. However, the predominant human serogroup 0:3 has been detected frequently in swine and many investigators have speculated that this animal might be the chief reservoir and an important source of human Y. enterocolitica infection (Ahvonen & Rossi 1970; Esseveld & Goudzwaard 1973; Rabson & Koornhof 1973; Tsubokura et al. 1973; Zen-Yoji et al. 1974).

4. Soil and water

The WHO Reference Centre (Mollaret et al. 1979) have reported the isolation of Y. enterocolitica from the soil of a dairy in Czechoslovakia, the prairies in France, rivers and ponds (100) in Norway, Czechoslovakia, USA, Denmark and France, sewers (250) and drinking water (20) in Norway, Japan and Czechoslovakia. Saari & Jansen (1979) examined 231 river samples taken in Colorado and Wisconsin and isolated 140 strains of Y. enterocolitica. Twenty-three serotypes were identified including 0:3, 0:6,30 and 0:8 although the most frequently occurring types were 0:4,32, 0:4,33 (13%) followed by 0:2,3 (9%), 0:16 (4%) and 0:17 (4%) and 43% were serologically non-typable.

In Canada Y. enterocolitica has been isolated from 43 surface and well waters and one municipal supply. Most of the isolates were rhamnose-positive. Eighteen strains (38%) were serotypable, representing nine different types with 0:4 occurring most frequently followed by 0:6,30. The water isolates of serotypes 0:6,30 resembled human strains of the same type (Schiemann 1978b).

Yersinia enterocolitica has also been isolated from water supplies

during investigations for suspected waterborne outbreaks of gastro-intestinal disease. Highsmith et al. (1977) investigated the water supply to a mountain ski resort after 750 cases of acute gastro-enteritis occurred and isolated Nilehn's biotype 1 from several well waters sampled over three days. Five of the 19 strains isolated were serotypable and included type 0:3. It was noted that the presence or absence of coliforms was not a reliable indication of the presence or absence of Y. enterocolitica.

In Denmark serotype 0:3 was isolated from well water which had been used for the preparation of a baby's feed. The baby had been diagnosed serologically to have been infected with the same type (Christensen 1979).

D. Media and methods for the isolation of Yersinia enterocolitica

As with most pathogens which are associated with food poisoning the most extensive work on methods and media for isolation of Y. enterocolitica has been carried out on clinical specimens. The procedures which are successful for isolation from clinical samples are not necessarily, and in fact seldom are, the best for isolation from foods. In the human disease situation large numbers of organisms are expected to be present at least during the early stages of the illness and isolation is usually possible using the direct plating technique. Unless the enteric pathogens contaminating a food have been able to grow to large numbers broth media must be used which will be capable of promoting the growth of as few as one or two organisms in 25-50 g of sample. The majority of foods contain a wider variety of non-pathogenic bacteria than clinical specimens, and these will multiply in the broth media and may overgrow any pathogens present unless a selective agent is employed. Many workers have used to advantage the ability of Y. enterocolitica to grow at 4°C as an aid to the enrichment of this organism.

A number of the agar and broth media commonly used for the isolation of Salmonella, Shigella and other enteric pathogens have been tried, with varying success, for the isolation of Y. enterocolitica. Recently several workers have attempted to formulate media specifically for the purpose of isolating this organism. These new media have either been of the differential type, depending on a biochemical or metabolic characteristic of Y. enterocolitica for identification, or the selective type incorporating one or more selectively inhibitory agents.

The success or failure of a new medium or method depends on the correct choice of the selective agents. In making that choice factors such as the pH of the medium and the effect that other ingredients may have on the action of the selective agents or on the growth of the sought after organism must be taken into account.

It is also advantageous to make a medium as specific as possible for the sought after organism as even members of the same bacterial family may be antagonistic in mixed culture. It has been shown that Klebsiella aerogenes [Aerobacter aerogenes], Enterobacter cloacae [Aerobacter cloacae] and E. coli can inhibit organisms of the Shigella and Salmonella groups as well as some of the Gram-positive bacteria including Staphylococcus aureus and Bacillus spp. (Wynne 1947; Halbert 1948; Hentges & Fulton 1964; Hentges 1967, 1969). Barclay (1981)

experienced difficulty in isolating Y. enterocolitica from a mixed culture of E. coli which may have been due to antagonism.

1. Selective agents

Numerous types of selective agents have been incorporated into media including metallic and other salts, acids, phenol, aniline dyes, surface active agents and chemotherapeutic reagents. As early as 1887 phenol was being used to suppress the growth of "indifferent bacteria" in the search for ways to isolate Salmonella typhi [Bacterium typhosus] (Garrod 1933). In his original paper on the discovery of penicillin, Fleming (1929) advocated its use as a selective substance for the isolation of Haemophilus influenzae and antibiotics have since been incorporated successfully into many media. In the majority of present day media selectivity is obtained using combinations of agents.

Many of the basic systematic studies of the inhibitory action of chemical agents were undertaken 60-80 years ago. Kitasato (1888) and Fermi (1898) studied the action of acids and alkalis and the latter worker defined methods whereby hydrochloric and lactic acids might be used to isolate a single organism from a mixture of two. The principle of this technique has been developed almost solely over the years for the isolation of Mycobacterium tuberculosis from sputum.

Dyes. The use of dyes was explored by Drigalski & Conradi (1902) and Churchman (1912). Drigalski and Conradi showed that when crystal violet was incorporated into agar for culturing faeces "cocci" and other saprophytic bacteria were suppressed. However Churchman determined that bacterial susceptibility to crystal violet was linked to the Gram reaction, and that incorporation of this dye enabled a Gram-negative organism to be recovered in pure culture from a mixture of both Gram-positive and Gram-negative bacteria. Following the publication of these findings many other workers investigated the effects of a wider range of dyes and a greater variety of bacteria (Krumweide & Pratt 1914; Simon & Wood 1914; Stearn & Stearn 1926; Sartorius 1928). The only information of practical value to emerge from all this work was that the violet dyes will entirely eliminate Gram-positive bacteria or in extreme dilutions differentiate certain Gram-positive types and that the green dyes will eliminate Gram-positive bacteria and in high dilutions differentiate certain Gram-negative organisms (Garrod 1933). Of the green dyes, malachite and brilliant green have been found to be

the most useful in the media for the isolation of the enteric pathogens. Brilliant green is an example of a dye which has been and still is used successfully in different media to both suppress and enhance the growth of a Gram-negative bacterium, that is E. coli. The variable selectivity of this agent and probably others, is related very closely to the pH of the medium into which it is incorporated, a relationship first described by Killian (1924). Fung and Miller (1973) studied the effect of 42 dye compounds on 30 bacterial species but Y. enterocolitica was not included.

Salts. Eisenberg (1918) carried out a detailed systematic study of 240 salts. Although he did not evaluate the practical possibilities of the use of these salts but defined principles and provided data from which more specialized work might follow, he did classify the salts into two types, the "dyestuff type" to which Gram-negative bacteria are more resistant and the "inverse type" to which Gram-positive bacteria are more resistant. The salts which are most frequently utilized in media which have been developed for enteric pathogens include sodium citrate, ferric ammonium citrate, bismuth sulphite, ferrous sulphate, magnesium chloride, potassium tetrathionate, sodium hydrogen selenite and reducing or oxidizing agents such as sodium thiosulphate.

Surface active agents. The properties of surface active agents in the form of bile and bile salts have been known for almost 100 years. When MacConkey (1908) reviewed the literature he showed their first recorded use to be circa 1889. MacConkey had been working with bile salts for several years and had identified sodium taurocholate and demonstrated its inhibitory effect on Gram-positive bacteria whilst allowing Gram-negative bacteria such as S. typhi [B. typhosus] and E. coli [Bacterium coli] to grow.

Of the numerous other bile salts identified probably the best known is sodium deoxycholate. Leifson (1935) investigating this agent with the purpose of providing an improved medium for salmonella isolation found that its effect on the growth of bacteria was dependent upon several factors. If the base medium was below pH 7.5 none of the Gram-positive bacteria tested showed any appreciable growth after 24 h in the presence of 0.1% or more sodium deoxycholate but there was no inhibition of the Gram-negative bacteria. Above pH 7.5 Gram-positive bacteria began to grow. Leifson also found that in the presence of an increased concentration of sodium chloride there was an inhibitory effect produced against various Gram-negative bacteria including

"lactose fermenting colon bacilli".

Other surface active agents include sodium lauryl sulphate and Teepol 619 which have similar properties to those of sodium taurocholate but are more reliable products and have been used as substitutes for bile salt (Mallmann & Darby 1941; Jameson & Emberley 1956). Tergitol 7 is also a surface active agent but has been utilized mainly for its ability to emulsify fat (Galton et al. 1954). It is non-inhibitory to Gram-negative bacteria but it has been suggested that there may be some inhibition of Gram-positive bacteria.

Selective agents for Y. enterocolitica. Most of the early investigations into the selectivity of the various chemicals were carried out because of the importance of finding effective methods for isolating S. typhi [B. typhosus] and Vibrio cholerae. Escherichia coli [Bacterium coli] was also often included in these studies, but as Y. enterocolitica had not even been recognized at that time it could not receive any attention from the researchers. It is only recently that dyes and selective agents including anti-bacterial drugs have been investigated specifically in an attempt to find suitable substances for incorporation into media for the isolation of Y. enterocolitica (Schiemann 1979a, 1980b).

Schiemann (1979a) incorporated 69 dyes at different concentrations into a tryptone glucose agar and studied their effects on the quantitative recovery of five strains representing five serotypes (0:3, 0:5,27, 0:6,30, 0:8, 0:9) of Y. enterocolitica. He also included one strain each of Pseudomonas aeruginosa and Bacillus cereus for comparative purposes. The results showed that Y. enterocolitica was relatively resistant to dyes compared to B. cereus but not when compared with Ps. aeruginosa. He suggests that the dye resistance of Y. enterocolitica is similar to that observed for other Gram-negative bacteria. A further five strains of Y. enterocolitica together with the Pseudomonas and Bacillus spp. and in addition Enterobacter spp. were utilized to evaluate some of the dyes in more detail. Only one dye, metanil yellow, showed any potential as a selective agent for Y. enterocolitica. The study also revealed strain variation in sensitivity to certain dyes, in particular brilliant green, neutral red and basic fuchsin each of which is used in different media for enteric pathogens and which have been recommended for the recovery of Y. enterocolitica.

In the first part of his second study Schiemann (1980b) incorporated graded concentrations of 28 chemical agents into a tryptone mannitol agar

and recorded their effect on the growth of a second set of five strains of Y. enterocolitica (serotypes 0:3, 0:5,27, 0:6,30, 0:8, 0:9). The results showed a high tolerance (>80% recovery rate) of all strains for surface-active agents such as bile salts, sodium deoxycholate, sodium taurocholate, Teepol 610 and Tergitol 7 and also for magnesium. In contrast, Cetrimide, paranitrophenol, potassium tellurite and sodium azide were inhibitory at very low concentrations but Irgason (2,4,4'-tri-chloro-2-hydroxydiphenyl ether, Ciba-Giegy) was tolerated at concentrations inhibitory to some other members of the Enterobacteriaceae.

The second part of Schiemann's study was on the effect of 57 antibacterial agents on the growth of 18 strains of Y. enterocolitica. The minimum inhibitory concentrations (MIC) were determined and two agents were found to be tolerated by Y. enterocolitica at concentrations which are active against certain other Gram-negative bacteria, viz. SCE-129 (cefsulodin) which is highly active against Ps. aeruginosa, and novobiocin which is especially effective against Proteus. In a further evaluation of SCE-129 (cefsulodin) against 52 additional strains of Y. enterocolitica it was found that 100% tolerated 10 µg/ml and 98% were unaffected by 20 µg/ml. Although there is no other known report of the effect SCE-129 on Y. enterocolitica, resistance to novobiocin has been reported (Raevuori et al. 1978).

Apart from these studies by Schiemann little other work has been reported. Soltész et al. (1980) investigated sodium oxalate incorporated into a bile salt agar and found that Y. enterocolitica could tolerate a 1% concentration. Aulisio et al. (1980) developed the use of potassium hydroxide treatment on mixed cultures and samples of food and they showed that Y. enterocolitica could tolerate 0.5% potassium hydroxide for at least 5 min whereas strains of Y. pseudotuberculosis, E. coli, S. typhimurium, Shigella sonnei and Shigella flexneri failed to survive 1 min.

Temperature. In addition to the use of chemicals and dyes as selective agents the principle of using an incubation temperature which whilst allowing the desired organism to grow hinders the growth of other bacteria in a mixed bacterial flora is an old and proven method and was referred to by MacConkey (1908). Many pathogens will grow at temperatures well above their optimum and by increasing the incubation temperature of enrichment media it is possible to improve selectivity,

for example, the incubation at 43° rather than 37°C of enrichment cultures for Salmonella gives significantly higher isolation rates (Harvey & Price 1968).

Because of the ability of Y. enterocolitica to grow at 4°C, a rare characteristic amongst the Enterobacteriaceae, incubation of media at this temperature has been advocated for the selective isolation of this organism. Eiss (1975) reports that whilst he was able to demonstrate growth of Y. enterocolitica at 4°C, 13 of 14 strains of other enterobacteria including Enterobacter cloacae, Proteus, Klebsiella and Salmonella spp. failed to show any increase in growth. The remaining strain of E. cloacae showed a large increase in count. Eiss also reported that isolation rates of Y. enterocolitica from clinical specimens were improved by as much as 44% when 4°C was used for incubation of the enrichment cultures. Many other workers have also used 4°C both for clinical specimens (Pai et al. 1979; Weissfeld & Sonnenwirth 1980) and for foods (Inoue & Kurose 1975; Black et al. 1978).

2. Selective agars

As a consequence of Y. enterocolitica being able to tolerate high concentrations of bile salt many of the common enteric media have been used for the isolation of this organism.

Agar media. The common enteric agar media which have been suggested as suitable for the isolation of Y. enterocolitica include MacConkey (MAC), lactose sucrose urea (LSU) (Juhlin & Ericson 1961), Salmonella-Shigella (SS), eosin methylene blue (EMB), deoxycholate citrate (DCA), hektoen enteric (HEK), endo (ENDO), xylose lysine deoxycholate (XLD) and bismuth sulphite (BS) agars.

Several workers have compared the growth characteristics of different strains of Y. enterocolitica on some of these media. Nilehn (1969a) studied the growth of 35 strains, which were mostly of human origin, on LSU, SS, DCA and ENDO agars incubated at 37° and 25°C with the purpose of finding suitable media for the isolation of Y. enterocolitica from human faeces by direct plating. She found that the strains of human origin (serotypes 0:3 and 0:9) usually gave reasonably good growth on all media at both temperatures, but older strains from different origins gave very varied results both with the individual media and at the different incubation temperatures. When the same media were streaked

with a mixed culture there was very little inhibition of unwanted Gram-negative bacteria, especially Proteus, on ENDO agar but the remaining three media when incubated at 25°C showed increased inhibition of undesired faecal bacteria, which proved to be advantageous in the isolation of Y. enterocolitica. This organism's distinct colonial characteristics on LSU also made differentiation somewhat easier than on the other media. Whichever medium was used incubation for at least two days was essential.

Mehlman et al. (1978) studied the recovery from pure cultures of four strains of Y. enterocolitica on MAC, SS, HEK, EMB and BS agars incubated at 35°, 26° and 22°C. Considerable variation in sensitivity of the cultures to SS, EMB, HEK and BS agars was encountered. The greatest recovery was obtained at 26°C. MacConkey agar was least influenced by temperature and EMB and BS agars tended to be toxic at 35°C.

Falcao et al. (1979) made a study of 43 strains of Y. enterocolitica on SS, HEK, XLD, BG and EMB agars also incubated at 35° and 22°C. Their results were the reverse of those of Nilehn (1969a) in that they found 35°C to be the better temperature for incubation. None of the strains grew on BG but all produced satisfactory colonies on MAC, SS and EMB agars. However some strains were inhibited on HEK and XLD agars. It was also observed that colonial variation occurred on EMB, XLD, HEK but not on MAC or SS agars.

The most recent study by Barclay (1981) compared MAC, HEK, XLD, SS, BG, DCA and EMB agars incubated at 35°C for up to 48 h. He confirmed that after 24 h incubation on these agars the largest colonies attained were less than 1 mm diameter and that each medium required 48 h at 35°C to obtain colonies of a workable size. Barclay also found on several occasions that whilst there was no inhibition of Y. enterocolitica in pure culture on MAC, from a culture mixed with E. coli, only E. coli was recovered. However Y. enterocolitica was recovered when the same cultures were seeded on to SS agar. Yersinia enterocolitica was difficult to differentiate from coliforms on HEK, XLD and EMB agars as these media contain sucrose which was fermented by all the organisms tested. On BG agar, which also contains sucrose, the concentration of BG inhibited the growth of Y. enterocolitica so that even after 48 h incubation colonies were still only 1 mm diameter and they could not be readily differentiated from other sucrose fermenting organisms.

Several batches of two different brands of BS agar were investigated for the development of Y. enterocolitica-like organisms by Hanna et al. (1977a). These workers found that there was variation brand to brand as well as batch to batch both in the growth of the Y. enterocolitica-like organisms, some of which were completely inhibited and also in the growth of the Pseudomonas strains which had been mixed with them. Similar brand and batch variations were reported by Kendall (1982a) when investigating this medium for growth of Salmonella spp. and other Gram-negative bacteria.

Recently several other agar media have been formulated especially for the isolation of Y. enterocolitica, for example, Salmonella-Shigella deoxycholate (SS + D) (Wauters 1973), MacConkey Tween (LEE) and MacConkey DNA (DNA) (Lee 1977), pectin (PEC) agar (Bowen & Kominos 1979), cellobiose-arginine-lysin (CAL) agar (Dudley & Shotts 1979), cefsulodin-irgasan-novobiocin (CIN) agar (Schiemann 1979b) and sodium oxalate (SO) agar (Soltész et al. 1980).

Wauters (1973) reported that Y. enterocolitica will tolerate as much as a 3% concentration of sodium deoxycholate. By incorporating 2% into SS agar he found that this gave a medium with good selectivity whilst allowing rapid growth of Y. enterocolitica. It was necessary to adjust the base medium to pH 7.0 or above to avoid precipitation of the sodium deoxycholate but by incubating below 37°, preferably at 29°C, the colonies of Y. enterocolitica were visible after 20 h. Wauters recommends the use of tenfold magnification on a stereo microscope to distinguish the colony type which is characteristic only of serotypes 0:3 and 0:9.

LEE and DNA agars were formulated by Lee (1977) for the differentiation of Y. enterocolitica from other lactose-negative bacteria. Lee found it necessary to incubate both agars at 25°C for 48 h. Although Serratia liquifaciens, Aeromonas and some Pseudomonas spp. produce a lipase-positive reaction on both media the colonial characteristics differ from those of Y. enterocolitica. Foods were inoculated with two clinical isolates. Whilst both the MAC media were as efficient as SS agar for the isolation of these clinical strains the SS agar inhibited the growth of many of the naturally occurring food strains which were also present.

Bowen & Kominos (1979) modified the pectin medium of Starr et al. (1977) by adding more agar to facilitate sub-culturing and by changing the indicator in order to enhance the appearance of the depresssion caused by pectin-positive colonies. It was found that of 118 enterobacteria tested, only 13 Y. enterocolitica and 3 Klebsiella oxytoca

produced colonies which sank into the agar. Yersinia enterocolitica could easily be identified in mixed cultures even in the presence of three times as many other enterobacteria. The recovery rates of Y. enterocolitica on PEC were compared to those on Müller-Hinton (MH), HEK, XLD, SS and MAC agars. Using MH as control (100% recovery) PEC gave 100% recovery with much lower rates on HEK 5%, XLD 15%, SS 7% and MAC 25%.

Cellobiose-arginine-lysine (CAL) agar (Dudley & Shotts 1979) was formulated as a differential medium for the recognition of Y. enterocolitica from other bacteria commonly found in water and faecal specimens. The differentiation being dependent upon Y. enterocolitica fermenting cellobiose and being arginine and lysine negative. Twelve strains of Y. enterocolitica were inoculated onto the agar, eleven produced characteristic colonies, and one failed to ferment the cellobiose. One of ten clinical isolates (from faeces) also failed to ferment this sugar. It was observed that the overall colony counts were similar on both the new medium and MAC. The CAL agar required 36-40 h incubation at 25°C.

Following his studies on selective agents, dyes and antibiotics Schiemann (1979b) formulated CIN agar. Using a basal medium specially synthesized to give optimum growth of Y. enterocolitica recovery of this organism was possible after only 24 h incubation at 32°C. This medium was found to be highly selective against Ps. aeruginosa, E. coli, Klebsiella pneumoniae and Proteus mirabilis. The reaction from fermentation of mannitol gave a characteristic colony which could be used to differentiate Y. enterocolitica from other Gram-negative bacteria.

Soltész et al. (1980) discovered that Y. enterocolitica could tolerate 1% sodium oxalate which they then incorporated into a specially formulated base medium. Two hundred and twenty-four faecal samples were artificially infected with 1 of 40 strains of Y. enterocolitica, including serotypes 0:3, 0:6, 0:8 and 0:9. The stools were subcultured to DCA, SS+D, MAC, LSU and SO agars. Incubation at 29° or greater was better than 22° but at 37°C there was a tendency for colonies to be rough. Using SO agar Y. enterocolitica was re-isolated from 99.5% of the faeces as opposed to 90% or less from the other media.

3. Broth media

A variety of broth media have been used for the enrichment of Y. enterocolitica. Amongst these are phosphate buffered saline (PS), Wauters (1973) broth (WB), Gram-negative broth (GN), peptone water (PW), cooked meat medium (CMM), various modifications of selenite broth and tetrathionate broth.

A report has been published in which the growth of Y. enterocolitica has been compared in different enrichment broths (Barclay 1981). Phosphate buffered saline, selenite F (SF), tetrathionate (TB) and GN broths to which a sterile faecal suspension had been added were inoculated separately with five strains of Y. enterocolitica (300-1100 organisms/ml) and mixed with five strains of E. coli (50,000 organisms/ml). Barclay found that all five strains of Yersinia grew well in PS incubated at 4°C, attaining a colony count of greater than 100,000 organisms/ml within nine days. The E. coli strains declined in numbers to less than 50 organisms/ml over the same period.

Three of the five Yersinia strains could not be recovered from SF incubated at 35°C for 1 day, however, a fourth strain was recovered after a further 4 days at room temperature. The highest colony count of Y. enterocolitica which was attained was 50,000 organisms/ml. Only one strain of E. coli declined in numbers, the rest remained above 50,000 organisms/ml.

Tetrathionate broth (without BG) was also incubated at 35°C for 1 day followed by 4 days at room temperature. Only 2/5 Y. enterocolitica strains could be recovered after 1 day (colony counts 300 and 15,000 organisms/ml) but after 4 days at room temperature all five strains had attained counts of greater than 50,000 organisms/ml. Five strains of E. coli were inhibited and not recovered.

All strains of Yersinia and E. coli grew well in GN broth but, because of the overgrowth of E. coli, Y. enterocolitica could not be recovered, after incubation for 1 day at 35°C, without the use of highly selective agars. After 4 days incubation at room temperature each strain of Y. enterocolitica had attained colony counts of 10,000 organisms/ml and were easily recovered.

Wauters (1973) modified Rappaport broth (Rappaport et al. 1956) by increasing the magnesium chloride, reducing the malachite green, thus raising the spontaneous pH and adding carbenicillin. He recommended this medium (WB) to be used in conjunction with SS+D agar and solely

for the isolation of the medically important serotypes 0:3, 0:9 and possibly 0:1 and 0:8. The isolation rate of Y. enterocolitica from WB was found to be as much as four times greater than that from selenite or Rappaport.

Inoue and Kurose (1975) prepared selenite with added novobiocin (SN), incubated at 37°C for 24 h and compared it with PS incubated at 5°C for 3 weeks. Both broths were inoculated with cow intestinal contents (115 samples) and beef meat (61 samples). Nine cow and 15 beef samples were found to be positive for Y. enterocolitica through PS but only one sample was positive through SN.

Mehlman et al. (1978) modified PS by the addition of 1% sorbitol and 0.15% bile salts. The results of their study, using artificially inoculated pork meat, suggested that Y. enterocolitica had to be present at a level greater than 1000 organisms/ml to be detectable through PS. In the presence of the sorbitol and bile salts not only was Yersinia isolated when the initial level was 1000 cells/ml but was also detected in the uninoculated control sample which had appeared not to contain Yersinia through PS. The results also indicated that using the modified medium the incubation time at 4°C need be no more than 3 weeks.

Two modified selenite broths formulated by Lee et al. (1980) were basically phosphate buffer, malachite green and carbenicillin with varying amounts of sodium selenite (0.15% and 0.25%). Beef and pork were artificially inoculated with Y. enterocolitica and incubated at 22°C for 2-3 days in WB and each of the selenite broths. Yersinia enterocolitica was recovered from 0.25% selenite enrichment in 10 of 11 tests and this medium was found to be less inhibitory than WB. One strain of Y. enterocolitica was inhibited by both malachite green and carbenicillin but omission of these agents made isolation of Y. enterocolitica more difficult because of the overgrowth of other bacteria.

4. Methods of isolation

Three basic methods namely, direct plating, direct enrichment and pre-enrichment, have been used for the isolation of enteric pathogens from foods and environmental samples. All three techniques have also been suggested for the isolation of Y. enterocolitica from similar specimens.

Direct plating method. Direct plating is employed where countable numbers of the pathogen could be expected to be found and would normally be used for clinical specimens. A small portion of the sampled is rubbed directly on to the surface of a suitable agar plate and the inoculum further spread with a sterile loop to give individual colonies.

Few workers have used direct plating for the isolation of Y. enterocolitica from foods and even fewer have compared this method with an enrichment technique.

Kapperud & Jonsson (1976) cultured swabs from the pyloric sacs of brown trout directly on to lactose bromothymol blue agar (Drigalski agar) and incubated at room temperature for 46 h. They recovered three strains of Y. enterocolitica from the 20 brown trout examined.

Seelye & Yearbury (1979) spread the surface of LEE agar with decimal dilutions of homogenates of beef meat which had been stored for up to 10 weeks. Yersinia enterocolitica was first isolated only after 4 weeks storage when approximately 10,000 organisms/ml were present.

Leistner et al. (1975) and Saari & Jansen (1979) compared direct plating and direct enrichment methods. Leistner et al. cultured 215 raw meat samples on to SS and MAC agars which were incubated at 25°C for 48 h, Y. enterocolitica was not detected in any samples. The same samples enriched in PS incubated at 4°C for 21 days yielded 50 strains of Y. enterocolitica.

Saari & Jansen compared direct plating on to deoxycholate citrate mannitol agar and enrichment in CMM incubated at room temperature for 3 and 21 days respectively for the isolation of Y. enterocolitica from unconcentrated river water. Direct plating yielded 89 isolates and enrichment in CMM an additional 51 strains.

Direct and pre-enrichment methods. Direct enrichment is usually used for samples likely to contain large numbers of non-pathogenic contaminating organisms. The sample is inoculated into enrichment broths possibly containing selective agents and after incubation at either, the optimum temperature of growth or a suitable selective temperature for the pathogen being investigated, the broth cultures are sub-cultured on to an agar medium.

The pre-enrichment method is generally used for isolation of pathogens which may have been stressed during food processing, e.g. by drying, freezing or heating. The samples are inoculated into nutrient,

non-selective type broths and incubated at the optimum temperature for growth of the pathogen. After incubation a portion of this broth is transferred to one or more selective enrichment media which are then incubated at either the optimum or a selective temperature before sub-culture to agar media.

Yersiniologists have generally used only the direct enrichment method, utilizing not just a variety of broth media but also a range of incubation times and temperatures.

Phosphate buffered saline has invariably been incubated at 4°C but incubation times suggested have varied from 3 weeks (Fukushima et al. 1979), 3-12 weeks (Alonso et al. 1979), 8-9 weeks (Peixotto et al. 1979) and one group of workers incubated pond water and snail samples for one year at 4°C (Botzler et al. 1976).

Occasionally other media have been incubated at 4°C, such as CMM and yeast extract-casein-cystine broth which was used to isolate Y. enterocolitica from ham and flies (Fukushima et al. 1979), PW for isolation from pig tongues (Alonso et al. 1979) and cold mannitol broth from seafoods (Peixotto et al. 1979).

Wauters' broth has been found to be most successful when incubated at 22-25°C (Christensen 1979; Schiemann 1978a, 1978b, 1980a; Schiemann & Toma 1978). The same temperature has also been suggested for various modified selenite media (Lee et al. 1980; Stern 1981). The higher incubation temperature of 35°C appears to have only been suggested for GN and SN broths (Guthertz et al. 1976; Inoue & Kurose 1975).

Some workers have considered the membrane filtration technique to be necessary for the examination of waters. The filter pad either being incubated in broth media or placed on to the surface of selective agars (Highsmith et al. 1977; Christensen 1979).

A series of studies have been carried out by Schiemann in which he has compared direct and pre-enrichment methods using various media and incubation times (Schiemann 1978a, 1978b, 1980a; Schiemann & Toma 1978). A summary of the methods and results of these studies is shown in Table 8. No one temperature or medium stood out as the best for all foods. The results of his study in which Schiemann (1980a) obtained 54/63 isolations of Y. enterocolitica from raw pork through direct enrichment in WB, were in direct contradiction to his earlier study in which the maximum isolation rate from raw milk was only obtained from WB after pre-enrichment in PS (Schiemann & Toma 1978).

Table 8

Comparison of enrichment methods for the isolation of Y. enterocolitica

Sample	Method of enrichment	Medium	Incubation time days	Incubation temperature °C	No. of samples positive by method-medium	No. of samples examined (total positive)
Bathing water [a]	Direct	Endo agar (membrane filter)	1	23	3	307 (6)
		Christensens cold enrichment buffer	14	4	2	
	Pre-	Christensens buffer plus Modified Rappaport broth	14 2	4 RT	3	
Raw milk [b]	Direct	Modified Rappaport broth	5	RT	6	131 (42)
		Butterfields phosphate buffer	14	4	8	
	Pre-	Cooked meat broth plus Modified Rappaport broth	28 5	23 23	3	
		Butterfields phosphate buffer plus Modified Rappaport broth	14 5	4 23	24	
Cheese, Cheescurd, Raw milk [c]	Direct	Modified Rappaport broth	5	22	9	180 (17)
		Christensens buffer	14	4	11	
	Pre-	Christensens buffer plus Modified Rappaport broth	14 2	4 22	7	

continued / .....

Table 8 (Continued)

Sample	Method of enrichment	Medium	Incubation time days	Incubation temperature °C	No. of samples positive by method-medium	No. of samples examined (total positive
Raw pork [d]	Direct	Modified Rappaport broth	7	22	54	128 (63)
	Pre-	Phosphate buffer plus Modified Rappaport broth	21	4		
			2	RT	16	
Processed pork [d]	Direct	Modified Rappaport broth	7	22	0	69 (5)
	Pre-	Phosphate buffer plus Modified Rappaport broth	21	4		
			2	RT	5	

RT = room temperature; a = Schiemann (1978b); b = Schiemann & Toma (1978); c = Schiemann (1978a); d = Schiemann (1980

Aulisio et al. (1980) suggested alkali treatment of enrichment cultures prior to sub-culture to agar plates as a means of obtaining pure cultures of Y. enterocolitica from foods. Artificially inoculated chicken meat was incubated at 4° (PS only) or 26°C for up to 10 days in PS, sorbitol bile broth, haemoglobin-oxalate bile broth, veal infusion broth and Brain Heart Infusion broth. One loopful of each broth culture was mixed with 0.1 ml of 0.5% potassium hydroxide in saline for a few seconds before sub-culture to MAC agar. The recovery rates after potassium hydroxide treatment compared to those after only sodium chloride treatment and after 3 days incubation at 4°C were 33%/6% respectively, after 7 days 46%/46% respectively, and after 10 days 53%/93% respectively. Even low levels (10-100 organisms/ml) of Y. enterocolitica were detected using the alkali method on enrichment broths incubated at 26°C for 1-2 days.

E. Factors affecting the growth and survival of Yersinia enterocolitica

Yersinia enterocolitica has been isolated from a variety of environments including food and water where they are subjected to many factors which may cause stress or injury. These include acid pH, high sodium chloride content, lack of nutrients and presence of chlorine. The organisms may also become stressed by heat or other treatment. These factors, which affect the bacteriologist's ability to isolate bacteria are reviewed here in relation to the growth and survival of Y. enterocolitica.

1. pH

Hanna et al. (1977b) studied the growth of Y. enterocolitica-like organisms in Brain Heart Infusion broth which had been adjusted with either sodium hydroxide or hydrochloric acid to pH values of 5.0, 6.0, 7.0, 8.0 and 9.0. They found that growth of the strains was better at pH 7.0 and 8.0 than at 6.0 and 9.0 with little or no growth occurring at pH 5.0 over a 24 h incubation period.

Since the work presented in this thesis was carried out Stern et al. (1980a) have reported similar studies to those already described but they incubated at 3° and 25°C for 10 days and 48 h respectively. In broth at pH 4.6 the number of organisms rose to $10^{7.6}$ cells per ml with 48 h incubation at 25°C and viable cells were demonstrated in the broth at pH 4.4 throughout the 48 h incubation. In broth incubated at 3°C the pH value of 4.4 was bacteriacidal or bacteriostatic to the different strains over 10 days incubation. These workers also found that some strains survived at the extreme pH values for up to 10 days.

2. Sodium chloride tolerance

When this project commenced no information was available on the effect of sodium chloride on the growth of Y. enterocolitica and very little has been published to date. However, Pruitt & Johnson (1978) reported that heat stressed cells of Y. enterocolitica grown on a rich medium showed no sensitivity to sodium chloride when incubated at 25°C whereas there was a 3 log decline in count when incubated at 3°C.

Other workers, (Stern et al. 1980), demonstrated that at 3°C the greatest increase in growth of Y. enterocolitica occurred in Brain Heart Infusion broth containing 0.5% sodium chloride and that there was

significantly greater growth in 5% compared to 7% sodium chloride over a 10 day incubation period. In 5% sodium chloride incubated at 25°C Y. enterocolitica multiplied from 10^2 cells/ml to $10^{5.5}$ cells/ml within 48 h. There was some inhibitory effect by 7% sodium chloride and 9% was bacteriocidal for the strains tested. The growth of Y. enterocolitica in 5% sodium chloride at 3°C was unusual as generally enterobacteria are inhibited at this concentration. The cell counts/ml of the clinical strains used in this study were found to be significantly greater than the cell counts of the environmental strains used.

3. Nutrients and temperature

Yersinia enterocolitica is able to survive and grow in foods incubated at both refrigerator and ambient temperatures. Most of the studies have been carried out on raw and cooked meats. Hanna et al. (1977e) studied the growth of five strains of Y. enterocolitica on both raw and cooked beef and pork held at 7°C for 10 days and 25°C for 24 h. At both temperatures the growth from an initial inoculum of 10^2-10^3 organisms/g of meat increased steadily to $>10^8$/g. At 25°C the increases in Y. enterocolitica counts were found to be somewhat greater on cooked meat than on raw meat; one strain failed to grow at 7°C on raw beef.

In another study the same workers (Hanna et al. 1977c) studied the effect of different methods of packaging in relation to the development of Y. enterocolitica on raw beef steaks. After inoculation the steaks were either vacuum packed in impermeable laminated nylon-saran-poly pouches (02 transmission rate 32 cc/m^2/24 h) or wrapped in polyvinyl chloride (PVC) (02 transmission rate 5.1-6.5 1/M^2/24 h) and stored at 1°, 2.5° and 5°C for 21-35 days. The counts of Y. enterocolitica and the total aerobic counts were consistently higher in the PVC wrapped steaks than in the vacuum packages. The differences in Y. enterocolitica counts of comparable steaks packaged in PVC and vacuum packs after 21 days at 5°C were in the order of 1.9-4.1 logs and at 1°C 2.6-4.6 logs. Yersinia enterocolitica was easily isolated and recognized in both studies despite a high background flora.

Stern et al. (1980b) examined the growth and competitive nature of Y. enterocolitica in whole milk. A combination of four strains were inoculated with and without five competing psychrophilic bacteria into

ultra heat treated milk and incubated at 3° and 25°C. In milk containing a pure culture of Y. enterocolitica and incubated at 3°C the count increased from 250 to more than 4.6×10^7 organisms per ml in less than 3 weeks. In the milk with competing organisms (Ps. fluorescens, Micrococcus varians, Alcaligenes faecalis and B. cereus) the total count after 10 days was 1.7×10^7 organisms /ml and Y. enterocolitica was only detectable when the milk was sub-cultured onto a selective medium.

In a further study, Hanna et al. (1977b) inoculated beef sirloin with 10^3-10^4 or 10^6-10^7 Y. enterocolitica per gram of meat and found that extensive reduction took place during 4 weeks frozen storage at -18 to -20°C. With an initial inoculum of approximately 10^4 cells / gram no survivors could be detected after 2-4 weeks. With the higher inoculum (10^6) the reduction of one strain was through 4 logs during the same period.

4. The environment

The survival of Y. enterocolitica in drinking and surface waters has been observed to be 35 and 12 days respectively (Pokorný 1974). In Belgium experiments have shown that at ambient temperature in shade Y. enterocolitica can survive in surface water as well as in a dried state. Survival time varied with the type of environment. In sea water some strains disappeared in a few days while others survived several weeks. In spring water with little organic matter the number of organisms diminished rapidly and none could be cultivated after $1\frac{1}{2}$ months. In dry chemically pure sand the number of living organisms began to fall off after 3 months and reached zero after 6 months. The longest survival time was observed in surface water such as pond water which is rich in organic matter, after more than 900 days living organisms could still be detected (Oye 1978).

Other studies by Schillinger & McFeters (1978) showed that Y. enterocolitica survived in stream water at 5.0-8.5°C for more than 14 days, by contrast, in chlorinated tap water, destruction (4 logs) occurred within 6 h.

5. Heat resistance and thermal injury

Foods are frequently subjected to heat treatments to aid or effect preservation.

Hanna and co-workers have shown that few if any Y. enterocolitica would be expected to survive in foods heated throughout to 60°C for 3 or more min. These workers noticed considerable variation in the heat resistance of their test cultures in skimmed milk especially at 50°C. After 10 min one strain was reduced by approximately 4 logs but the same treatment had little effect on one of the other strains used (Hanna et al. 1977d).

Buckeridge et al. (1980) studied the effect of thermal injury on recovery of Y. enterocolitica. They heated the organism at 50°C and 62°C for 30 min and found that 72 h instead of 48 h incubation of the plating out media was required before colonies appeared. Even then the colonies produced were variable in size and most were smaller than those of the corresponding untreated cells after an identical incubation period.

Other workers (Restaino et al. 1980) have found that the recovery rate of Y. enterocolitica after heat injury is directly related to the menstruum in which the heating took place. They also found that clinical isolates are slightly less sensitive to heat stress than environmental strains.

6. Radiation

Although not yet permitted in this country ionizing radiation has been proposed as a means of preserving foods but this process does still leave some surviving organisms and subsequent refrigeration is required. However, as Y. enterocolitica will grow at 4°C El-Zawahry & Rowley (1979) studied the effect of various potential processing conditions on the radiation resistance and recovery of this organism. They found Y. enterocolitica to be amongst the most radiation sensitive of the non-sporing foodborne bacteria. The D values of three strains of Y. enterocolitica irradiated at 25°C in trypticase soy broth ranged from 9.7-11.8 krad. When irradiated in ground beef at 25°C the D value of the one strain used was 19.5 krad and at -30°C 38.8 krad. The addition of 2.5% sodium chloride was found to have little or no effect on the sensitivity of Y. enterocolitica to radiation in either trypticase soy broth or ground beef. They also showed that Y. enterocolitica surviving radiation were no more sensitive to subsequent freezing than non-irradiated cells.

AIMS OF THE THESIS

Human infections with Y. enterocolitica have been recognized and reported with increasing frequency during the last 25 years. The principal manifestation is gastroenteritis but the organism has also been isolated from septicaemias, wounds, eye and other sites. The true epidemiological picture of Y. enterocolitica infection is still unclear as isolation of the organism has depended on there being an interested and competent laboratory available.

There is published evidence to show that spread of infection may be by person to person or, by implication, from a common animal, food or water source. However, only one outbreak involving chocolate milk in the USA has been proven to be foodborne following isolation of the same biochemical and serological type from both patients and food.

In many countries of the world isolation of Y. enterocolitica has been reported from a wide range of both domestic and wild animals as well as from raw meat and poultry, dairy products, water and seafood.

The isolation methods and media used for isolation of the organism have been too diverse for comparison and mostly the media used have been those formulated for the isolation of other pathogens. Although one or two workers have formulated broth and agar media specifically for Y. enterocolitica these have been for the isolation from clinical specimens rather than from foods.

Yersinia enterocolitica serotypes 0:3 and 0:9 have generally been considered to be the only types of medical importance, but other serotypes such as 0:5,27, 0:6,30 and 0:8 have been implicated with increasing frequency in human infections and these are also known to occur in foods.

The number of human infections recognized and reported in the UK has averaged 22 per year since 1975 but there have been no published reports of any isolations from foods or other materials.

The purpose of this thesis is to study the growth, survival, isolation and incidence of Y. enterocolitica in foods and other materials with a view to improving the methods of recovery of this pathogen so that the organism's incidence and possible sources in the UK could be assessed. The following studies were therefore undertaken.

1. Investigation of the growth and survival of Yersinia enterocolitica

   a) Effect of pH.
   b) Effect of sodium chloride.
   c) Growth of Y. enterocolitica in boiled fish, potato, rice, hard-boiled egg, roast chicken, chocolate milk and mayonnaise sauce, incubated at temperatures ranging from -20° to 37°C.

2. Evaluation of isolation media

   a) Comparison of several broth media for growth of Y. enterocolitica at 30°, 22° and 4°C.
   b) Comparative evaluation of agar media in frequent use for the isolation of other enteric pathogens as well as any new media as they become available during the course of the project.
   c) Investigation and possible development of new media specifically for the selective isolation of Y. enterocolitica.

3. Isolation, identification and incidence of Yersinia enterocolitica

   a) Comparison of isolation methods for Y. enterocolitica from artificially and naturally contaminated materials.
   b) Characterization of isolates from naturally contaminated foods and other materials.
   c) Incidence and sources of Y. enterocolitica in foods obtained in the UK.

MATERIALS AND METHODS - GENERAL

In this section the materials and methods used throughout the study are described. Methods which are applicable only to a specific section are described in that section. Where commercially available media and reagents were used the name and code are given and the materials prepared according to the manufacturer's instructions. Details of other media are described in full or the appropriate reference is given. The broth media were stored at room temperature and the agar plates (approximately 20 ml in each), unless otherwise stated were dried open and inverted at 37°C for 1 h and then stored at 4°C until required. Unless stated all the quantities quoted for the media recipes which follow are grams/ml per litre of distilled water.

A. Materials

1. Media

Bismuth sulphite agar (BS_1). BBL 11031. Plates contained approximately 25 ml of the medium and were used within 5 days of preparation.

Bismuth sulphite agar (BS_2). Difco B72. Plates contained approximately 25 ml of the medium and were used within 5 days of preparation.

Bismuth sulphite agar (BS_3). Lab M 13. Plates contained approximately 25 ml of the medium and were used within 5 days of preparation.

Bismuth sulphite agar (BS_4). Oxoid CM 201. Plates contained approximately 25 ml of the medium and were used within 5 days of preparation.

Blood agar - layered plates (BA) g/l nutrient broth No 2 (Oxoid CM 67). Agar 12; defibrinated horse blood 50. This medium was poured over a thin pre-set layer of peptone water agar.

Brain Heart Infusion broth (BHI). Difco B37.

Brain Heart Infusion broth (BHI OX). Oxoid CM 225.

Brain Heart Infusion agar (BHI agar). g/l Brain Heart Infusion (Difco B37). Agar 12.

Brain Heart Infusion agar plus dye (BHI + D). g/l Brain Heart Infusion (Difco B37). Agar 12; lactose 10; neutral red 5.

Brilliant green agar modified (BG). Oxoid CM 329.

Buffered Brain Heart Infusion (BBHI). Double strength Brain Heart

Infusion (Difco B37); equal volume 0.68M McIlvaines citric acid phosphate buffer solution of the required pH. (Giegy Scientific Tables 1962).

Buffered peptone water (BP) (Edel & Kampelmacher 1973). Peptone (Difco B118) 10; sodium chloride 5; disodium hydrogen orthophosphate ($12H_2O$) 9; potassium dihydrogen orthophosphate 1.5. The medium was sterilized at 115°C for 10 min. Final pH 7.2 ± 0.1.

Citrate agar (modified from Simmons 1926). Sodium chloride 5; magnesium sulphate ($MgSO_4$ $7H_2O$) 0.2; ammonium hydrogen phosphate 1; dipotassium hydrogen phosphate 1; citric acid 2; 0.2% bromothymol blue 40; agar 20. The citric acid was added to the other ingredients in the water, the reaction adjusted to pH 6.8, the medium was filtered, distributed, sterilized at 115°C for 20 min and allowed to set as slopes.

Decarboxylase broth (Decarb). Peptone (Oxoid L37) 5; Lab Lemco (Oxoid L29) 5; pyridoxal hydrochloride 0.005; glucose 0.5; 1% bromocresol purple 1 ml; 0.2% cresol red 2.5 ml; appropriate L-amino acid 10. The medium was distributed into test tubes and covered with at least ½" heavy liquid paraffin before sterilization at 108°C for 30 min.

Deoxycholate citrate agar (DCA) (modified from Leifson 1935).
Base agar Yeastrel 5; peptone (Oxoid L37) 5; lactose 10; agar (Davis New Zealand) 11; 1% neutral red solution, aqueous 0.75 ml.

Solution A tri-sodium citrate 170; sodium thiosulphate 170; ammonium ferric citrate (brown) 20.

Solution B sodium deoxycholate standardized by PHLS (Kendall 1982b) normally 100.

For use base agar 400 ml; solution A 20 ml; solution B 6 ml.

Deoxycholate citrate sucrose agar (DCSA) (modified from Leifson 1935).
Base agar Yeast extract 5; peptone (Oxoid L37) 5; lactose 10; sucrose 10; agar (Davis New Zealand) 13; 1% neutral red 0.75 ml; distilled water 900 ml.

Solution A as DCA.

Solution B as DCA.

For use as DCA.

Deoxyribonuclease sorbitol agar (DNA) (Lee 1977). g/l DNA test agar (Difco 0632). Tween 80 10; sorbitol 10; sodium lauryl sulphate 0.3; calcium chloride 0.2; acridine orange 40 mg; triphenyl tetrazolium

chloride 25 mg.

Diagnostic sensitivity test agar (DST). Oxoid CM 261.

Eosin methylene blue agar (EMB). Oxoid CM 69.

Gelatin stabs. Difco B11.

Glucose phosphate broth (GPB). Peptone 5; potassium dihydrogen phosphate (KH_2PO_4) 5; glucose 5. The ingredients were mixed until dissolved, distributed in 5 ml quantities in tubes and sterilized at 115°C for 10 min.

Gram-negative broth (GN) (Hajna 1955). Difco 0486.

Hartley digest broth (HD) (modified from Hartley 1922). Minced meat 555; sodium carbonate 7.3; pancreatic extract (Cole & Onslow 1916) 18; chloroform 18; hydrochloric acid 15.

Hedley Wright broth (HW) (modified from Wright 1933). Minced fat-free meat 455; peptone (Lab M No 1) 10; sodium chloride 5.

Hektoen enteric agar (HEK). Oxoid CM 419.

Hugh and Leifson's OF agar (H & L) (Hugh & Leifson 1953). Peptone 2; sodium chloride 5; dipotassium hydrogen phosphate 0.3; agar 3; 0.2% bromothymol blue 15 ml; 10% sterile glucose 10. The ingredients were mixed, the reaction adjusted to pH 7.1, filtered, distributed into tubes to give a column of medium 1½" high and then sterilized at 115°C for 20 min.

Lactose sucrose urea agar (LSU) (modified from Juhlin & Ericson 1961).
Base agar g/850 ml of distilled water. Lactose 40; sucrose 40; beef extract (Difco 0126) 5; proteose peptone No 3 (Difco 0122) 3; peptone (Difco 0118) 7; disodium hydrogen phosphate ($Na_2HPO_4 2H_2O$) 6.2; potassium dihydrogen phosphate (KH_2PO_4) 4.34; agar 15. The agar was dissolved in the water by soaking overnight at room temperature followed by heating at 115°C for 15 min. This method dissolved the agar rapidly and ensured that free oxygen was removed from the water before the addition of the remaining ingredients which then received minimal heating during subsequent sterilization at 115°C for 15 min.

Solution A g/53 ml of distilled water.
Potassium tetrathionate (Merck Art 5169) 0.5; urea 5; sodium deoxycholate 2; tri-sodium citrate 2. The ingredients were dissolved by heating at 80°C for 20 min.

Solution B g/100 ml of distilled water.
Water blue (Merck Art 1279) 2. Sterilized at 121°C for 15 min.

Solution C g/40 ml of distilled water.
Phenol red (Merck Art 7241) 1; absolute alcohol 60. The phenol red was dissolved in the absolute alcohol and then heated in a boiling water bath until seething. The distilled water was heated to boiling and added to the phenol red/alcohol mixture.

Solution D g/40 ml of distilled water.
Alpha-naphthal phthalein (Merck Art 6246) 1; absolute alcohol 60. The alpha-naphthal phthalein was dissolved in the absolute alcohol and then heated in a boiling water bath until seething. The distilled water was heated to boiling and added to the seething mixture.

For use To the molten base agar was added solution A (all); solution B 3 ml; solution C 10 ml and solution D 10 ml. The pH was 6.6 without adjustment. Where possible the ingredients were Analytical Reagent grade.

Lee agar (LEE) (Lee 1977). g/l MacConkey agar (Oxoid CM 7). Tween 80 10; calcium chloride 0.2.

MacConkey agar (MAC). Oxoid CM 7.

Malonate broth. 1% bromothymol blue (alcoholic) 2.5 ml; ammonium sulphate 2; dipotassium hydrogen orthophosphate 0.6; potassium dihydrogen orthophosphate 0.4; sodium chloride 2; sodium malonate 3; DL-phenylalanine 2; yeastrel 1.

Motility agar (based on Bacto-motility G1 medium, Difco 8069). Brain Heart Infusion (Difco B37) 25; gelatin 53.4; agar 3. The medium was sterilized at 121°C for 15 min and distributed into 50 mm petri dishes to give a layer 3-4 mm deep.

Nitrate broth (NO_3). g/l nutrient broth No 2 (Oxoid CM 67). Potassium nitrate 1. The nitrate was dissolved in the nutrient broth, distributed into tubes containing an inverted Durham's tube and sterilized at 115°C for 20 min.

Nutrient agar (NA). g/l nutrient broth No 2 (Oxoid CM 67). Agar 12.

Nutrient broth (ND). Difco B3.

Nutrient broth (NB). Oxoid CM 67.

Ortho-nitro phenyl-β-galactopyranoside broth (ONPG) (Lowe 1962).

ONPG solution — O-nitro-phenyl-β-galactopyranoside 6 grams dissolved in 0.01 M disodium hydrogen phosphate pH 7.5, sterilized by filtration and stored at 4°C in the dark.

For use — ONPG solution (250 ml) added aseptically to peptone water (750 ml), distributed in 2.5 ml volumes and stored at 4°C.

Pectin agar (PEC) (Bowen & Kominos 1979). Yeast extract (Difco B127) 5; 10% aqueous calcium chloride 6; agar 7; phenol red 0.14; sodium salt polygalacturonic acid (Sigma P1879) 30. The pH must not exceed 7.3 and the medium must not be allowed to set during preparation because it can not be re-melted.

Peptone water (PW). Peptone 10; sodium chloride 5.

Peptone water agar (PWA). Peptone 10; sodium chloride 5; agar 12.

Peptone water sugars (PWS). Peptone (Oxoid L34) 10; sodium chloride 5; Andrades indicator 10; 10% sugar solution 50.

Andrades indicator (modified from Andrade 1906). Acid fuchsin 5; normal sodium hydroxide 150–180. The acid fuchsin was dissolved in water and 150ml sodium hydroxide added, mixed and left to stand for 24h with frequent shaking. If necessary more alkali was added until the required colour change from red to brown was attained.

Sugar solutions — Required sugar 100. Dissolved in water and sterilized by steaming at 100°C for 30 min on 3 consecutive days.

Phenylalanine agar (PPA) (Ewing et al. 1957). DL-phenylalanine 2; yeast extract 3; disodium hydrogen phosphate 1; sodium chloride 5; agar 20. The ingredients were dissolved by heating in the water, filtered, tubed, sterilized at 115°C for 20 min and solidified in a slanting position to give a long slope.

Phosphate buffered saline (PS). Potassium dihydrogen phosphate 9.12; disodium hydrogen phosphate 9.5; sodium chloride 8.5. The solution was sterilized at 115°C for 10 min.

Ringer solution - quarter-strength (RS). Oxoid tablets BR52.

Selenite F broth (SF). Sodium hydrogen selenite 4; peptone (Oxoid L37) 5; mannitol 4; disodium hydrogen phosphate 7.5; sodium dihydrogen phosphate 5.3. The ingredients were dissolved at 60°C and sterilized by filtration.

Selenite cystine broth (SC). Difco 0687.

Salmonella-Shigella agar (SS). Oxoid CM 99.

Salmonella-Shigella plus deoxycholate agar (SS + D) (Wauters 1973). g/l SS agar (Oxoid CM 99). Sodium deoxycholate 20. The pH adjusted to 7.6 to prevent the precipitation of the bile salt.

Sodium oxalate agar (SO) (Soltész et al. 1980). Bacteriological peptone (Oxoid L37) 15; casein hydrolysate (Oxoid L41) 5; lactose 10; sodium oxalate (BDH) 8; sodium deoxycholate 16; sodium chloride 5; bile salts (Oxoid L55) 5; neutral red 0.08; agar (Difco B140) 20.

Supplemented phosphate buffered saline (S) (Mehlman et al. 1978) Disodium hydrogen phosphate 8.23; sodium dihydrogen orthophosphate 1.2; sodium chloride 5; sorbitol 10; bile salts (Oxoid L55) 1.5. Sterilized at 121°C for 15 min, final pH 7.6 ± 0.1.

Thiosulphate citrate bile salt cholera agar (TCBS). Oxoid CM 333.

Todd Hewitt broth (TH) (Todd & Hewitt 1932). Minced fat free meat 454; neopeptone 20; sodium hydrogen carbonate 2; glucose 2; sodium chloride 2; disodium hydrogen orthophosphate 1; 12.5N sodium hydroxide 2.7.

Trypticase soy broth with dextrose (TS + D). BBL 11768.

Trypticase soy broth without dextrose (TS). BBL 11774.

Urea agar (Christensen 1946). Peptone 1; sodium chloride 5; sodium dihydrogen phosphate 2; glucose 1; 0.2% phenol red 6; 20% sterile aqueous urea solution 100; agar 20. The glucose and phenol red were added to the rest of the ingredients after sterilization. The medium was allowed to set as slopes.

Wauters broth (WB) (Wauters 1973).

Solution A Tryptone (Bacto 123) 10. Sterilized at 121°C for 15 min.

Solution B Disodium hydrogen phosphate 11.88. Sterilized by boiling for 1 h.

Solution C Magnesium chloride 400. Sterilized by boiling for 1 h.

Solution D g/100 ml. Malachite green 2.

Solution E mg/ml. Carbenicillin 1,000; dissolved in sterile distilled water.

For use The following were mixed together: solution A 620; solution B 160; solution C 208; solution D 6.4; solution E 1.2. The medium was stored at 4°C.

Xylose lysine deoxycholate agar (XLD). BBL 11838.

2. Reagents

Alpha naphthol solution. α-naphthol 5; ethanol 100 ml.

Ferric chloride. Ferric chloride 10; water 100 ml.

Kovac's reagent. p-dimethyl-aminobenzaldehyde 5; amyl alcohol 75 ml; concentrated hydrochloric acid 25 ml. Stored at 4°C and protected from the light.

Methyl red solution. Methyl red 0.04; ethanol 40; distilled water made up to 100 ml.

Nitrate test reagents

Solution A sulphanilic acid 0.8; 5N-acetic acid 100 ml. Dissolved by gentle heating.

Solution B alpha-naphthylamine 0:5; 5N-acetic acid. Dissolved by gentle heating and stored in a darkened bottle.

Phosphatase reagents

Buffer solution. Anhydrous sodium carbonate 3.5; sodium bicarbonate 1.5. Dissolved in distilled water and made up to 1 l.

Buffer substrate. Disodium p-nitrophenyl phosphate 0.15; made up to 100 ml with buffer solution. The buffer substrate was stored in the refrigerator and protected from light and discarded after 1 week.

3. Antibiotics and antibacterial agents

Multodisc Oxoid 30-44K. Ampicillin 25 μg; colistin sulphate 10 μg; sulphafurazole 500 μg; nitrofurantoin 200 μg; kanamycin 30 μg; streptomycin 25 μg; chloramphenicol 50 μg and tetracycline 50 μg.

Multodisc Oxoid U4. Ampicillin 25 μg; carbenicillin 100 μg; cephaloridine 25 μg; colistin sulphate 10 μg; gentamicin 10 μg; sulphafurazole 500 μg; sulfamethoxazole/treimethoprim (co-trimoxazole) 25 μg and tetracycline 50 μg.

Multodisc Oxoid 725E. Ampicillin 2 μg; cephaloridine 5 μg; chloramphenicol 10 μg; erythromycin 10 μg; kanamycin 5 μg; penicillin G 1.5 units; streptomycin 10 μg and tetracycline 10 μg.

Single sensitivity disc (Oxoid), nalidixic acid 30 μg.

Single sensitivity disc (Oxoid), furazolidone 100 μg.

Single sensitivity disc (Oxoid), neomycin 30 μg.

Single sensitivity disc (Oxoid), sulphatriad 300 μg.

4. Equipment

Containers. Sterile 4oz, 1lb and 2lb wide mouth screw-capped jars were used for all the growth studies.

Incubators. These were those in general use within the Food Hygiene Laboratory. With the exception of 12° and 22°C they could be regulated to within ± 1°C.

12°C. This was a refrigerator set to run at the highest temperature. There was a temperature fluctuation between 12° and 15°C.

22°C. This was not equipped with any means of cooling. The minimum temperature obtained was 22°C but the maximum temperature depended on that of ambient. Throughout the work the temperature as recorded each day was 24°C ± 1°C.

pH papers. Merck non-bleeding type; acilit, pH 0-6.0 (Art 9531); neutralit, pH 5.0-10.0 (Art 9533); alkalit pH 7.5-14.0 (Art 9532); universal indikator pH 0-14.0 (Art 9535).

Ultipette (Barky Instruments Ltd). An adjustable automatic pipette using pasteur pipettes instead of plastic tips and capable of delivering 0.1-2.0 ml volumes.

5. Reference organisms

A collection of five Yersinia enterocolitica, one Yersinia pseudotuberculosis and eleven strains representing other genera from the Enterobacteriaceae was assembled. Except for one strain of Y. enterocolitica they were originally isolated from either human or food sources. These basic reference organisms are listed in Table 9.

During the course of the investigations other strains were added to the original collection and these are shown in a supplementary list (Table 10).

Table 9

Reference organisms

Strain	Identity code	Source and remarks	
Yersinia enterocolitica	PHLS Leicester	Human	Serotype 0:3
Yersinia enterocolitica	PHLS Leicester	Human	Serotype 0:6,30
Yersinia enterocolitica	PHLS Leicester	Human	Serotype 0:9
Yersinia enterocolitica	NCTC 10460	Chinchilla	Denmark
Yersinia enterocolitica	NCTC 10598	Human	Blood
Yersinia pseudo-tuberculosis	NCTC 10216	Human	-
Enterobacter cloacae	NCTC 8155	Dried milk	Food Hygiene Lab.
Escherichia alkalescens	NCTC 7925	Unknown	-
Escherichia coli	QC 21043/75	Simulated faeces	PHLS quality control survey
Escherichia coli	NCTC 10418	Unknown	Antibiotic assay control
Hafnia alvei	NCTC 9540	Water	U.K.
Klebsiella aerogenes	NCTC 8846	Ice cream	PHLS Bradford
Proteus mirabilis	NCTC 2896	Minced heart	U.K.
Red Mouth* bacterium	NCTC 10476	Rainbow trout	Kidney
Salmonella typhimurium	F5621/75	Sausage	Food Hygiene Lab.
Shigella flexneri	NCTC 8519	Human	Serotype 2a
Shigella sonnei	F2040/76	Simulated faeces	PHLS quality control survey

* = reclassified as Yersinia ruckeri (Skerman et al. 1980); PHLS = Public Health Laboratory Service; NCTC = National Collection of Type Cultures; QC = Quality Control; F = Food Hygiene.

Table 10

Supplementary reference organisms

Strains from this supplementary list of reference organisms were included in some of the studies when it was thought that useful additional information could be obtained.

Strain	Identity code	Source and remarks	
Aerobic sporing bacillus	F4278/77	Cooked prawns	Routine isolate
Bacillus alvei	NCTC 3349	Bees	Foul brood
Bacillus cereus	F5089/75	Human	-
Gram-positive coccus A	F4278/77	Cooked prawns	Routine isolate
Gram-positive coccus B	F4278/77	Cooked prawns	Routine isolate
Pseudomonas aeruginosa	DHI		Habs serotype 1
Yersinia enterocolitica	Y46	Pork	Serotype 0:5,27
Yersinia enterocolitica	Y63	Pork	Serotype 0:6,30
Yersinia enterocolitica	Y136	Milk	Serotype 0:8
Proteus morganii	NCTC 235	Human	Summer diarrhoea
Proteus rettgeri	NCTC 7475	Human	Faeces
Proteus vulgaris	NCTC 4175	Unknown	-
Providencia sp.	NCTC 6344	Human	Bacillary dysentery

F = Food Hygiene; NCTC = National Collection of Type Cultures; DHI = Division of Hospital Infection; Y = Yersinia, food isolate.

B. Methods

1. Maintenance of reference strains

The basic and supplementary reference strains were maintained throughout this study on duplicate nutrient agar slopes in screw-capped bottles. These were stored at room temperature after initial incubation at 30°C for 24 h. One culture was retained unopened and kept as a stock culture and the other was used as a working culture. Only when necessary (in some cases after as long as three years) was the stock culture sub-cultured to Brain Heart Infusion agar plates to check purity and a new working culture prepared.

The reference organisms were sub-cultured from the working cultures to Brain Heart Infusion agar plates to check purity and ensure that each was growing readily before every experiment.

2. Preparation of inocula

Overnight Brain Heart Infusion broth cultures which had been incubated at 30°C were diluted with quarter-strength Ringer solution. Unless otherwise stated an initial dilution was made using a standard opacity tube equivalent to 1000×10^6 *E. coli*/ml for comparison. Further dilutions were made to give estimated concentrations of the organisms as required.

3. Surface colony counts

An Ultipette which had been calibrated and fixed to deliver 1 ml volumes was used to prepare ten-fold dilutions in quarter-strength Ringer solution. The surface colony counts were made using a modified surface drop technique (Thatcher & Clark 1968) on Brain Heart Infusion agar plus dye plates which were incubated at 30°C for 48 h.

Broth cultures. Duplicate samples of the broths were prepared for each study. One replicate jar of broth was inoculated with the test strains at the beginning of the working day and a surface colony count carried out immediately (0 h) and after 6-8, 24, 30, 48 and 72 h incubation as required.

The second replicate together with the inoculum was retained at 4°C for approximately 7 h, then allowed to warm to room temperature

(approximately 30 min). The test strains were inoculated into the broth and surface colony counts carried out immediately (0 h) and also after 14, 18, 22, 38 and 62 h as required. This procedure was followed in order to obtain the counts which would need to be done outside reasonable extended working hours.

Foods. Sufficient 10 g samples of each food were distributed to carry out all the surface colony counts required.

The jars of food required for surface colony counts after 6-8, 24, 30, 48, 72 h and 7, 10, 21, 23, 30, 42, 48, 56, or 63 days were inoculated with the test strains at the beginning of the working day. One jar of food was retained and a surface colony count carried out immediately (0 h).

The remaining jars of food together with the inocula were retained at 4°C for a further 7 h and then allowed to warm to room temperature (approximately 30 min). The remaining food was inoculated with the test strains and surface colony counts carried out after 14 and 18 h incubation as needed. One jar was retained and a surface colony count carried out immediately (0 h). This procedure was followed in order to obtain the counts which would need to be done outside reasonable extended working hours.

Where it was suspected that the surface colony counts would be < 50/g, and therefore beyond the lower limit of the counting method, 90 ml volumes of Brain Heart Infusion broth were added to the 10 g portions of food instead of quarter-strength Ringer solution. After using this as the 1/10 dilution for the surface colony counts these suspensions were incubated at 30°C for 48 h. Sub-cultures were then made on to Brain Heart Infusion agar plus dye plates and incubated at 30°C for 48 h.

If it became obvious from the early surface colony count results that there were contaminant organisms present besides the Y. enterocolitica test strains lactose sucrose urea agar and deoxycholate citrate sucrose agar plates were used as well as the Brain Heart Infusion plus dye plates for subsequent surface colony counts.

4. pH testing

Broth cultures. A Merck test paper was moistened with the broth culture and the colour change compared, whilst still wet, to the standard colour chart.

Foods. The ten gram samples were moistened with 1-2 ml quarter-strength Ringer solution and mixed thoroughly. A test paper was then moistened with the food and the colour change compared, whilst still wet, to the standard chart.

5. Biochemical tests

β-galactosidase. ONPG broth was inoculated and incubated at 22° or 30°C for up to 3 days. The appearance of a yellow colour indicated β-galactosidase activity.

Carbohydrate fermentation. All strains were sub-cultured, using a straight wire, into Andrades peptone water sugars; glucose (containing an inverted Durham's tube to show gas production), adonitol, arabinose, cellobiose, dulcitol, glycerol, inositol, inulin, lactose, maltose, mannitol, melibiose, raffinose, rhamnose, salicin, scrbitol, sucrose, trehalose and xylose. They were incubated at the required temperature for up to 7 days and a record made of the results after 1, 2, 5 and 7 days. The production of acid was shown by a colour change from pale straw coloured to pink or red.

Catalase production. The surface of a nutrient agar slope was heavily inoculated, incubated for up to 2 days, and 1 ml of 10 volume hydrogen peroxide run gently over the growth. The appearance of bubbles rising from the growth indicated catalase production.

Citrate utilization. Using a straight wire and a very light inoculum citrate agar was stabbed directly into the bottom of the butt and out over the surface of the slope in a single straight line. The tubes were incubated at 22° or 30°C for up to 7 days. Citrate utilization was indicated by growth and a colour change from green to blue.

Decarboxylase reactions. All strains were sub-cultured, using a straight wire, into a control (no L-amino acid), arginine, lysine and ornithine broths. Each tube was inoculated through the paraffin layer and incubated at the required temperature for 4 days. The media first became yellow due to acid production from the glucose and later if decarboxylation occurred they turned violet. The control remained yellow.

Gelatin liquifaction. Using a straight wire the cultures were inoculated in a single stab to the bottom of the tube and incubated at 22°C

for up to 14 days. Liquifaction was indicated by the presence of fluid culture.

Indole production. Peptone water was inoculated and incubated at 22°C for 1 day and 1 ml of Kovacs' reagent added. A positive reaction was shown by the development of a red colour in the reagent.

Lipase reaction. All strains were sub-cultured to Lee agar and incubated at 22°C for 48 h. Lipase production was indicated by the presence of an opaque shiny precipitate in the agar surrounding the growth.

Malonate utilization. Malonate broth was inoculated and incubated at the required temperature for 24 h. A positive malonate reaction was indicated by a blue colour, with a negative reaction the medium remained green.

Methyl red reaction (MR). Glucose phosphate broth was inoculated and incubated at 22° or 30°C for 5 days. Two drops of methyl red solution were added and the culture shaken. A red colour indicated a positive reaction, yellow a negative reaction. After reading, the same culture was used for the Voges-Proskauer test.

Motility. Two petri dishes of motility medium were stabbed to just below the surface of the agar with the test organism and incubated at 22° and 36°C. The growth of motile organisms spread across the medium from the point of inoculation, whereas growth was confined to the point of inoculation if the organisms were non-motile.

Nitrate reduction. Nitrate broth was inoculated and incubated at 22° or 30°C for 5 days. Any gas formation in the test tube was noted before adding 1 ml nitrate reagent A followed by 1 ml nitrate reagent B. A red colour showed that nitrite was present and that the nitrate had been reduced.

Oxidation/fermentation test. One tube of Hugh & Leifson's medium was inoculated, using a straight wire, to the bottom of the agar. A $\frac{1}{2}$ inch layer of molten Vaseline was poured on to the surface and allowed to set. The tubes were incubated for up to 2 days. Fermentation was indicated by a colour change to yellow and growth into the depths of the agar.

Phenylalanine deamination. Strains were heavily inoculated on to the surface of a phenylalanine agar slope using a straight wire. The tubes were incubated at 30°C for 3 days and 3-4 drops of 10% ferric chloride were run over the growth. A positive result was shown by the

development of a green colouration.

Urease production. Strains were sub-cultured in to Christensen's urea agar using a straight wire which was inoculated direct to the bottom of the butt and then drawn over the surface of the slope in a single straight line. The cultures were incubated at 30°C for up to 3 days. Urease production was indicated by a colour change from yellow to mauve.

Voges-Proskauer test. After completion of the MR test 0.6 ml of 5% α-naphthol solution and 0.2 ml 40% potassium hydroxide were added and the broth shaken. The tubes were sloped to increase the air/liquid interface and left at room temperature for 1 h. A positive reaction was shown by a strong red colour.

6. Antibiotic sensitivity testing

The surface of diagnostic sensitivity test agar plates were seeded with 4-6 h broth cultures, the surplus culture was removed and the plates were allowed to dry. Multodiscs together with any single sensitivity discs were deposited firmly on to each plate using sterile forceps. Control culture E. coli NCTC 10418 was included for each batch of tests. The plates were incubated at the appropriate temperature overnight, i.e. 18-20 h.

The zones of inhibition were measured and the results compared to those of the control organism and recorded as follows: Sensitive, a zone within 3 mm of that of the control; Resistant, a zone not more than 2 mm radius measured from the edge of the disc; Moderately resistant, a zone falling between the above limits.

INVESTIGATIONS

A. Preliminary Investigations

Introduction

The preliminary investigations included familiarization with: 1. biochemical and growth characteristics of the reference strains; 2. antibiotic sensitivity of the reference strains; 3. evaluation of the effect of quarter-strength Ringer solution on the recovery of the reference strains; and 4. a study to select a suitable nutrient broth for use as a reference medium throughout the project.

The biochemical characteristics of the reference organisms obtained from the National Collection of Type Cultures (NCTC) were fully documented, however, many of the other strains selected had not been characterized. Before proceeding with the main parts of the project it was necessary to become familiar with the biochemical reactions of all the strains, especially the Yersinia spp., in the media generally available within Colindale. (NCTC prepare their own media using different brands of ingredients to those used by the remainder of the Central Public Health Laboratory).

Antibiotics are incorporated as selective agents into some media. Two such media which have been recommended for the isolation of Y. enterocolitica are Wauters' (1973) broth which contains carbenicillin and selenite-novobiocin broth (Inoue & Kurose 1975). The antibiotic sensitivity patterns of the reference strains were determined because evaluation of the media in current use and the possible development of new media for the isolation of Y. enterocolitica was one of the aims of this thesis.

A diluent should enable a true assessment to be made of the bacterial population and death or revival of the organisms should not take place during the dilution process. Quarter-strength Ringer solution (RS) was the diluent of choice for this project as it was in constant use in the Food Hygiene Laboratory. Jayne-Williams (1963) reported that neither E. coli nor S. typhimurium were effected by RS but there were no reports of the effects, if any, on growth or survival of Y. enterocolitica. Delays do occur during counting procedures either by accident or design, therefore, a study was undertaken to assess the effects of prolonged storage in RS on Y. enterocolitica and some of the other reference strains.

A non-selective broth in which Y. enterocolitica would grow

reliably from a minimal inoculum and also give maximum recovery, was required for use as a reference medium. Martin et al. (1976) reported that the presence of the metabolic by-product hydrogen peroxide (H_2O_2) in media may inhibit the growth of small numbers of bacteria particularly if they have been injured by heat or freezing. However, the effect of the H_2O_2 on injured cells is neutralized either by catalase which is also produced by many bacteria during the growth cycle or, by additional nutrients, for example, sodium pyruvate. To ensure that the broth chosen as the reference medium was not inhibitory and capable of allowing growth of Yersinia spp. to occur even from very small numbers of organisms, nine media were compared by adapting the multiple tube technique described by Boyd (1956) for estimating the minimum haemolytic dose. The maximum recovery rates of Yersinia spp. from each broth medium were also compared.

Methods

1. The biochemical and growth characterization and antibiotic sensitivity patterns of the reference organisms

The reference strains studied were:-

Y. enterocolitica serotype 0:3	E. coli QC 21043/75
Y. enterocolitica serotype 0:6,30	H. alvei
Y. enterocolitica serotype 0:9	K. aerogenes
Y. enterocolitica NCTC 10460	Prot. mirabilis
Y. enterocolitica NCTC 10598	Red mouth (RM) bacterium
Y. pseudotuberculosis	S. typhimurium
E. cloacae	S. flexneri
E. alkalescens	S. sonnei

a. Biochemical and growth characterization

Using a straight wire the reference strains were inoculated into the following media:- peptone water sugars containing adonitol, arabinose, cellobiose, dulcitol, glucose, glycerol, inositol, inulin, lactose, maltose, mannitol, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose (acid production), nutrient agar (catalase), Simmon's citrate (utilization of citrate), arginine, lysine and ornithine

decarboxylase, gelatin, peptone water (indole), Lee agar (lipase), malonate broth, motility medium (37° and 22°C), glucose phosphate broth (MR and Voges Proskauer), nitrate broth (nitrate reduction), ONPG broth, Hugh and Leifson medium (oxidation/fermentation), phenylalanine agar (deamination of phenylalanine), urea medium (urease production), and Brain Heart Infusion broths (growth at 4°, 22°, 30°, 35° and 43°C). All the media (except Simmon's citrate, gelatin, nitrate broth, and the Brain Heart Infusion broths) inoculated with Yersinia and RM bacterium strains were incubated at 30°C for up to 7 days and those inoculated with the other enterobacteria were incubated at 37°C for up to 7 days. Simmon's citrate and nitrate broth were incubated at 30°C for 5 days and gelatin at 22°C for 14 days. The Brain Heart Infusion broths were incubated as shown for up to 14 days. The Yersinia strains were also inoculated into a second tube of lactose, raffinose, rhamnose, melibiose peptone water sugars and Simmon's citrate, all of which were incubated at 22°C for 7 days.

b. Antibiotic sensitivity

Using the method described on page 86 the sensitivity patterns of the reference strains were determined against Oxoid multodisc 30-44K (see page 77) and single sensitivity discs impregnated with nalidixic acid 30 µg and furazolidine 100 µg. The plates seeded with Yersinia and RM bacterium were incubated overnight at 30° and those seeded with the other enterobacteria overnight at 36°C. Two plates were also seeded with the antibiotic control E. coli NCTC 10418 and incubated at 30° and 36°C.

2. The effect of quarter-strength Ringer solution (RS) on the recovery of Yersinia spp. and other enterobacteria

The reference strains studied were:-

Y. enterocolitica serotype 0:3
Y. enterocolitica serotype 0:6,30
Y. enterocolitica serotype 0:9
Y. enterocolitica NCTC 10460
Y. enterocolitica NCTC 10598
E. cloacae
E. alkalescens
E. coli QC 21043/75
H. alvei
K. aerogenes
Prot. mirabilis
S. typhimurium
S. flexneri
S. sonnei

Overnight broth cultures of the test strains were diluted with RS in six tenfold stages up to 10^6. The dilutions were kept at room temperature and surface colony counts carried out by placing 2 drops of each dilution onto a segment of a blood agar plate after 0, 15, 30, 45, 60, 75 min and 6 h. The plates were incubated at 30°C for 48 h.

3. The evaluation of nine non-selective broth media to select one for use as a reference medium

The reference strains studied were:-

Y. enterocolitica serotype 0:3
Y. enterocolitica serotype 0:9
Y. enterocolitica serotype 0:6,30
Y. pseudotuberculosis

The non-selective broth media studied were:-

Brain Heart Infusion (BHI)
Hartley Digest (HD)
Hedley Wright broth (HW)
Nutrient broth, Difco (ND)
Nutrient broth, Oxoid (NB)
Peptone water (PW)
Todd Hewitt broth (TH)
Trypticase soy with dextrose (TS + D)
Trypticase soy without dextrose (TS)

a. Comparison of maximum recovery rates

Overnight broth cultures of the test strains were inoculated (0.06 ml) into 100 ml volumes of the broth media (approximately 10,000 organisms/ml) and incubated at 22°C for 72 h. Surface colony counts (as described on page 82) were carried out on Brain Heart Infusion agar plates (BHI agar) after 0, 24, 30 (Y. enterocolitica serotypes 0:3 and 0:9 only), 48 and 72 h incubation. The colony counts of each of the test strains were recorded and the nine media were ranked according to the colony counts of each strain after each incubation period. The highest counts were scored nine points, the next highest eight points and so on to the lowest scoring one point. The media were compared for performance after each incubation period and for strain variation.

b. Growth from a minimal inoculum

Overnight broth cultures of the test strains were diluted as described on page 82 and further diluted to approximately 100 cells/ml. Additional dilutions of 1:10, 1:2, 1:2, 1:2 and 1:2 were prepared to give a range of approximately <1 to 10 cells/ml and 1 ml of each inoculated into each of ten tubes containing 5 ml of the broth media. The

broths were incubated at 22°C for 48 h.

The number of tubes from each dilution in which growth was observed were recorded and graphs plotted using probit transference. The 50% end point of growth was determined for each organism in each medium. This method was based on that used for the immunological study of minimum haemolytic dose (Boyd 1956).

Results

1. The biochemical and growth characterization and antibiotic sensitivity patterns of the reference organisms

a. Biochemical and growth characterization

The biochemical and growth characteristics of the reference organisms are shown in Table 11. The five strains of *Y. enterocolitica* did not produce acid from rhamnose, raffinose or melibiose and were Simmons' citrate negative after 7 days incubation at 22° and 30°C. However *Y. enterocolitica* serotype 0:6,30 and NCTC 10598 both produced acid from lactose within 3 days incubation at 30°C.

The six *Yersinia* spp. and RM bacterium produced visible growth in BHI after 9 days incubation at 4°C but although the strains of *Y. enterocolitica* and RM bacterium failed to grow at 43°C after incubation for 14 days, *Y. pseudotuberculosis* grew within 24 h. *Hafnia alvei* and *K. aerogenes* were the only other organisms to grow at 4°C within 9 days, and both these and the other seven reference strains grew well at 43°C within 24 h.

b. Antibiotic sensitivity

The antibiotic patterns of the 16 reference organisms are shown in Table 12. Four of the five strains of *Y. enterocolitica* were resistant to ampicillin 25 μg. The fifth strain (NCTC 10598) which was of human origin but unknown serotype was sensitive to ampicillin. All five strains of *Y. enterocolitica* were sensitive to colistin 10 μg, tetracycline 50 μg, chloramphenicol 50 μg and nalidixic acid 30 μg but showed variable reactions with sulphafurazole 500 μg, nitrofurantoin 200 μg, kanamycin 30 μg, streptomycin 25 μg and furazolidone 100 μg. *Proteus mirabilis* and *E. coli* were resistant or moderately resistant to all the antibiotics available. *Enterobacter cloacae*, *H. alvei*, *K. aerogenes*

Table 11

Characterization of the reference organisms

Test	Y. enterocolitica 0:3	Y. enterocolitica 0:6,30	Y. enterocolitica 0:9	Y. enterocolitica NCTC 10460	Y. enterocolitica NCTC 10598	Y. pseudotuberculosis	E. cloacae	E. alkalescens	E. coli QC 2104/75	H. alvei	K. aerogenes	Prot. mirabilis	RM bacterium	S. typhimurium	S. flexneri	S. sonnei
Adonitol	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Arabinose	+	+	+	+	-	(+)	+	+	+	+	+	-	-	+	+	+
Cellobiose	+	+	+	+	+	-	+	-	-	-	+	(+)	-	(+)	-	-
Dulcitol	-	-	-	-	-	-	-	(+)	+	-	-	-	-	+	-	-
Glucose	+	+	+	+	+	+	+*	+	+*	+*	+*	+*	+	+	+	+
Glycerol	(+)	+	+	(+)	+	(+)	(+)	(+)	+	+	+	(+)	(+)	-	-	(+)
Inositol	(+)	(+)	(+)	(+)	(+)	-	+	-	-	-	+	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	(+)	-	-	(+)	-	+	-	+	-	+	-	-	-	-	(+)
Maltose	+	+	+	(+)	+	+	+	+	+	+	+	-	+	+	(+)	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Melibiose	-	-	-	-	-	(+)	-	-	+	-	+	-	-	(+)	-	-
Raffinose	-	-	-	-	-	-	+	-	+	-	+	-	-	-	(+)	(+)
Rhamnose	-	-	-	-	-	-	+	(+)	+	+	+	-	-	+	-	(+)
Salicin	-	+	-	(+)	(+)	(+)	+	-	+	-	+	+	-	-	-	-
Sorbitol	+	+	+	(+)	+	-	-	(+)	+	-	+	-	-	+	-	-
Sucrose	+	+	+	+	+	-	+	-	+	-	+	-	-	-	-	(+)
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(+)	+
Xylose	(+)	(+)	+	(+)	+	+	+	+	+	+	+	+	-	+	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate utilization	-	-	-	-	-	-	+	-	-	-	+	+	-	+	-	-

Continued....2/

Test	Y. enterocolitica 0:3	Y. enterocolitica 0:6,30	Y. enterocolitica 0:9	Y. enterocolitica NCTC 10460	Y. enterocolitica NCTC 10598	Y. pseudotuberculosis	E. cloacae	E. alkalescens	E. coli QC 21043/75	H. alvei	K. aerogenes	Prot. mirabilis	RM bacterium	S. typhimurium	S. flexneri	S. sonnei
Decarboxylase:																
arginine	-	-	-	-	-	-	+	+	+	-	-	-	+	+	-	-
lysine	-	-	-	-	-	-	-	+	+	+	+	-	+	+	-	-
ornithine	+	+	+	+	+	-	+	+	+	+	-	+	+	+	-	+
Gelatin	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
Indole	-	+	-	-	-	-	-	+	+	-	+	-	-	-	-	-
Lipase	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-
Malonate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility 22°C	+	+	+	+	+	+	+	-	+	-	-	+	+	+	-	-
37°C	-	-	-	-	-	-	+	-	+	-	-	+	+	+	-	-
MR 22°C	+	-	+	+	+	+	-	+	-	-	-	+	+	+	+	+
37°C	+	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ONPG	+	+	+	+	+	+	+	-	+	+	+	-	+	-	-	+
Oxidation/ fermentation	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
Phenylalanine deamination	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Urease	+	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-
Voges-Proskauer 22°C	+	+	+	+	+	-	+	-	-	+	-	-	+	-	-	-
37°C	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-
Growth at 4°C	++	++	++	++	++	++	0	0	0	++	++	0	++	0	0	0
22°C	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
30°C	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
36°C	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
43°C	0	0	0	0	0	++	++	++	++	++	++	++	0	++	++	++

+ = acid production 1-2 days; (+) = delayed acid production 3 days or more;
+* = acid + gas production; - = negative; ++ = growth; 0 = no growth;
F = fermentation

Table 12

Antibiotic sensitivity patterns of the reference organisms

Antibiotic	Y. enterocolitica 0:3	Y. enterocolitica 0:6,30	Y. enterocolitica 0:9	Y. enterocolitica NCTC 10460	Y. enterocolitica NCTC 10598	Y. pseudotuberculosis	E. cloacae	E. alkalescens	E. coli QC 21043/75	H. alvei	K. aerogenes	Prot. mirabilis	RM bacterium	S. typhimurium	S. flexneri	S. sonnei
Ampicillin 25 µg	R	R	R	R	S	S	S	S	M	M	R	R	S	S	S	S
Colistin sulphate 10 µg	S	S	S	S	S	R	M	S	M	M	M	R	S	S	S	S
Sulphafurazole 500 µg	R	S	S	R	S	R	R	S	M	S	R	R	S	S	S	S
Tetracycline 50 µg	S	S	S	S	S	S	M	S	M	S	M	R	S	S	S	S
Chloramphenicol 50 µg	S	S	S	S	S	S	M	S	M	S	S	R	S	S	S	S
Nitrofurantoin 200 µg	S	S	S	R	S	R	S	S	M	S	M	R	S	S	S	S
Kanamycin 30 µg	R	S	S	S	S	S	S	S	M	S	S	M	S	S	S	S
Streptomycin 25 µg	R	S	S	M	S	S	S	S	M	S	S	M	S	M	S	S
Nalidixic acid 30 µg	S	S	S	S	S	S	M	M	M	S	M	M	S	S	S	S
Furazolidone 100 µg	S	M	M	S	R	R	M	M	M	S	M	R	S	S	S	S

S = sensitive; R = resistant; M = moderately resistant.

and S. typhimurium showed a variable resistance and RM bacterium, S. flexneri and S. sonnei were sensitive to all the antibiotics used.

2. The effect of quarter-strength Ringer solution on the recovery of Yersinia spp. and other enterobacteria

The effects of quarter-strength Ringer solution on Y. enterocolitica and some other enterobacteria are shown in Table 13. There was neither a reduction nor an increase in the viable counts of any of the organisms tested during the 6 h experimental period.

3. The evaluation of nine non-selective broth media to select one for use as a reference medium

a. Comparison of maximum recovery rates

The results of the comparison of the nine non-selective broth media for the maximum recovery of Yersinia spp. according to rank order are shown in Table 14 and the growth (average colony counts) is shown in Table 15. The growth curves in Brain Heart Infusion broth (BHI) and nutrient broth-Difco (ND) are shown in Figure 1, trypticase soy broth plus dextrose (TS + D) in Figure 2 and Todd Hewitt (TH) in Figure 3.

Trypticase soy broth with dextrose attained the highest overall score (97), and rank order 1 both in the strain comparison and the comparison of incubation times (Table 14). Todd Hewitt and BHI broths were ranked 2 and 3, scoring 88 and 87 respectively.

There was slight variation in the rank order of the broths with the individual test strains. Trypticase soy broth with dextrose attained rank order 1 with Y. enterocolitica serotypes 0:3 and 0:6,30, rank order 3 with serotype 0:9 and rank order 2 with Y. pseudotuberculosis. Todd Hewitt ranked 1 with serotype 0:9, 2 with serotype 0:3 and 0:6,30 and 4 with Y. pseudotuberculosis, whilst BHI ranked 1 with Y. pseudotuberculosis, 3 with serotypes 0:3 and 0:6,30 and 2 with serotype 0:9.

The scores of the remaining six broth media were generally much lower with the exception of Hartley digest (HD) which attained a rank order of 2 with Y. pseudotuberculosis and equal 3 (BHI) with serotype 0:9, Hedley Wright (HW) ranked equal 3 (BHI) with serotype 0:6,30 and nutrient broth-Oxoid (NB) ranked equal 3 (TS + D) with Y. pseudotuberculosis.

Table 13

The effect of storage in quarter-strength Ringer solution on Yersinia spp. and other enterobacteria

Organism	Time of storage (min)						
	0	15	30	45	60	75	300 (6 h)
	$\log_{10}$ organisms/ml						
Y. enterocolitica 0:3	7.5	7.8	7.8	7.6	7.8	7.5	7.9
Y. enterocolitica 0:6,30	7.8	7.8	7.9	7.8	7.8	7.9	7.8
Y. enterocolitica 0:9	7.8	7.8	7.7	7.9	7.7	7.8	7.6
Y. enterocolitica NCTC 10460	7.5	7.7	7.3	7.8	7.6	7.8	7.5
Y. enterocolitica NCTC 10598	7.9	7.5	7.6	7.8	7.7	7.7	7.9
E. cloacae	8.7	8.9	8.7	8.8	8.7	8.9	8.6
E. alkalescens	8.4	8.3	8.4	8.3	8.3	7.9	8.1
E. coli QC 21043/75	8.0	8.4	8.5	8.4	8.0	8.0	8.0
H. alvei	8.3	8.0	8.0	7.7	7.9	8.3	8.0
K. aerogenes	8.3	7.7	8.1	7.9	7.8	7.7	7.8
Prot. mirabilis	8.7	8.6	8.7	8.7	8.6	8.4	8.2
S. typhimurium	8.6	8.6	8.1	8.5	8.0	8.4	8.1
S. flexneri	7.7	7.5	7.4	7.7	7.7	7.5	7.7
S. sonnei	7.9	7.9	7.7	7.9	8.0	7.7	7.7

Table 14

Comparison of nine non-selective broth media for the maximum recovery of Yersinia spp.

Medium	Numerical scores based on recovery rates										
	Total* score for each Yersinia strain				Total score all strains	Rank order	Total+ scores for each incubation period (h)			Total score all strains	Rank order
	A	B	C	D			24	48	72		
Brain Heart Infusion	21	19	22	25	87	3	30	29	28	87	3
Hartley Digest	13	13	22	18	66	4	21	23	22	66	4
Hedley Wright	19	19	15	10	63	5	26	21	16	63	5
Nutrient (Difco)	5	9	7	7	28	9	5	9	14	28	9
Nutrient (Oxoid)	6	10	10	23	49	7	18	13	18	49	7
Peptone water	10	14	7	10	41	8	11	18	12	41	8
Todd Hewitt	25	23	24	16	88	2	34	28	26	88	2
Trypticase Soy with dextrose	26	27	21	23	97	1	31	34	32	97	1
Trypticase Soy without dextrose	13	17	14	8	52	6	14	17	21	52	6

A = Y. enterocolitica serotype 0:3; B = Y. enterocolitica serotype -:6,30; C = Y. enterocolitica serotype 0:9; D = Y. pseudotuberculosis; * = total scored during entire incubation period, maximum possible 27; + = total scored by all four strains, maximum possible 36.

The rank order for the three incubation times also varied but to a lesser extent than with the strain comparison; TS + D ranked 2 after 24 h and 1 after 48 and 72 h incubation, TH ranked 1 after 24 h and 3 after 48 and 72 h and BHI ranked 3 after 24 h and 2 after 48 and 72 h. None of the remaining media matched these rankings.

The average colony counts of each of the test strains varied by approximately 0.5-0.9 log in the nine broth media (Table 15). However the difference in the average colony counts in TS + D (rank order 1), TH (rank order 2) and BHI (rank order 3) varied by only 0.1 log (Y. enterocolitica serotype 0:9), 0.2 log (serotype 0:3 and Y. pseudotuberculosis) and 0.3 log (serotype 0:6,30).

In the comparison of the incubation times the difference in the average counts in the TS + D, TH and BHI was even smaller, 0.1 log after 24 and 48 h and 0.2 log after 72 h incubation. The actual growth curves of TS + D (rank order 1), TH (rank order 2), BHI (rank order 3) and ND (rank order 9) are illustrated in Figures 2, 3 and 1 respectively.

b. Growth from a minimal inoculum

The 50% end points of growth of the Yersinia spp. in nine non-selective broth media are shown in Table 16. An analysis of variance revealed : a, that there was no significant difference between any of the broths for the growth of Yersinia spp. from a minimal inoculum and b, there was a very significant difference between the different strains of Yersinia in some of the broths (P = 0.01).

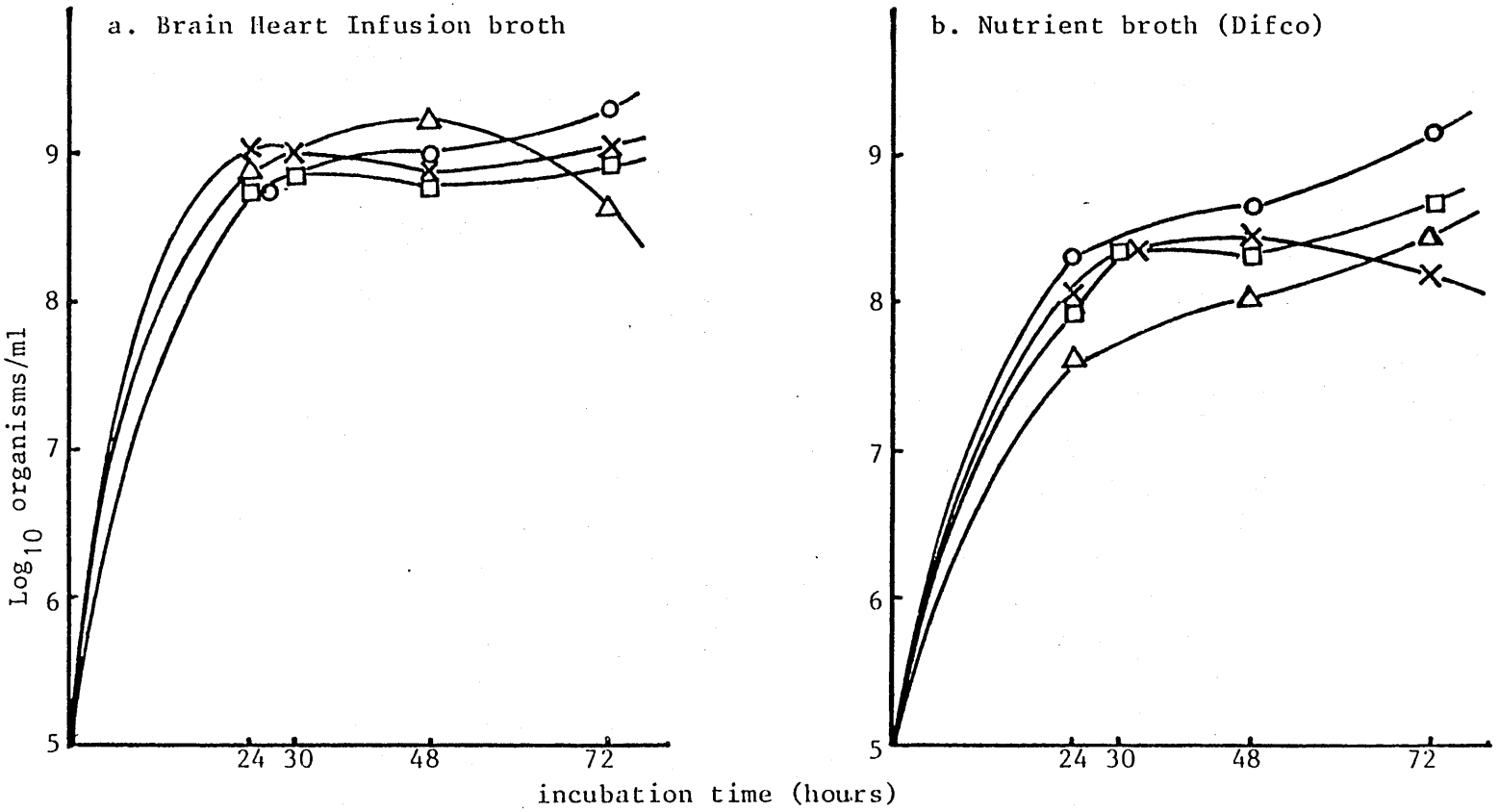
Table 15

Average recovery rates of four Yersinia spp. in nine non-selective broth media

		Medium								
		TS + D	TH	BHI	HD	HW	TS	NC	PW	ND
Average counts $\log_{10}$/ml of each strain	A	9.1	9.1	8.9	8.9	8.6	8.6	8.3	8.5	8.2
	B	9.4	9.1	9.1	9.0	8.8	9.1	8.7	9.0	8.9
	C	8.9	8.9	8.8	8.7	8.9	8.7	8.4	8.4	8.4
	D	8.7	8.6	8.9	8.4	8.6	8.3	8.7	8.3	8.1
Average counts $\log_{10}$/ml after incubation at	24 h	8.9	8.9	8.8	8.8	8.7	8.4	8.5	8.3	7.8
	48 h	9.1	9.0	9.0	8.8	8.8	8.7	8.5	8.8	8.4
	72 h	9.3	9.0	9.0	8.8	8.8	9.0	8.8	8.7	8.7
Overall rank order		1	2	3	4	5	6	7	8	9

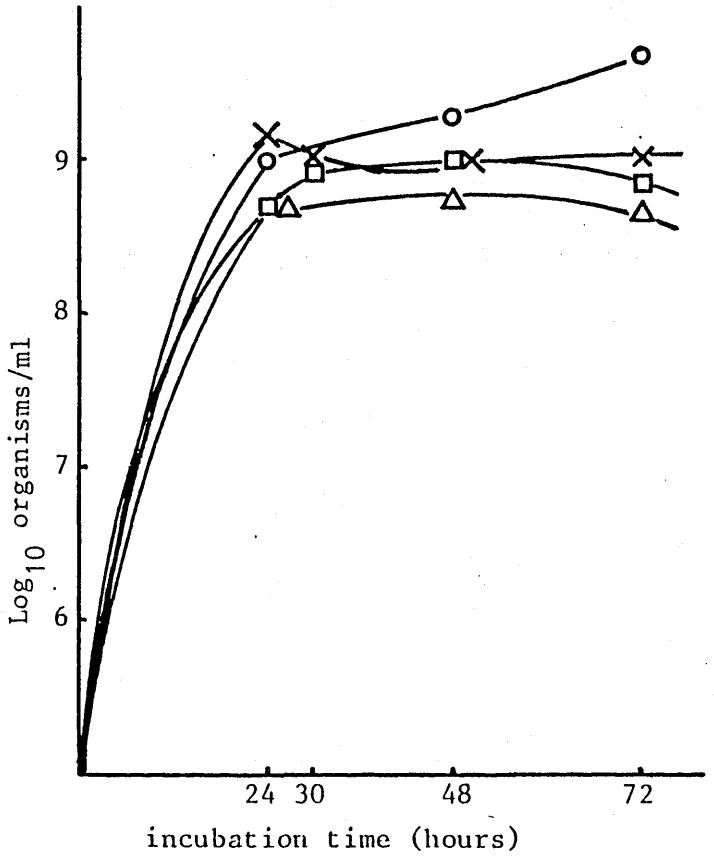
TS + D = trypticase soy plus dextrose; TH = Todd Hewitt broth; BHI = Brain Heart Infusion; HD = Hartley Digest broth; HW = Hedley Wright broth; TS = trypticase soy broth without dextrose; NC = nutrient broth (Oxoid); PW = peptone water; ND = nutrient broth (Difco).

Figure 1. Comparison of Brain Heart Infusion broth and nutrient broth (Difco) for the growth of Yersinia spp. at 22°C



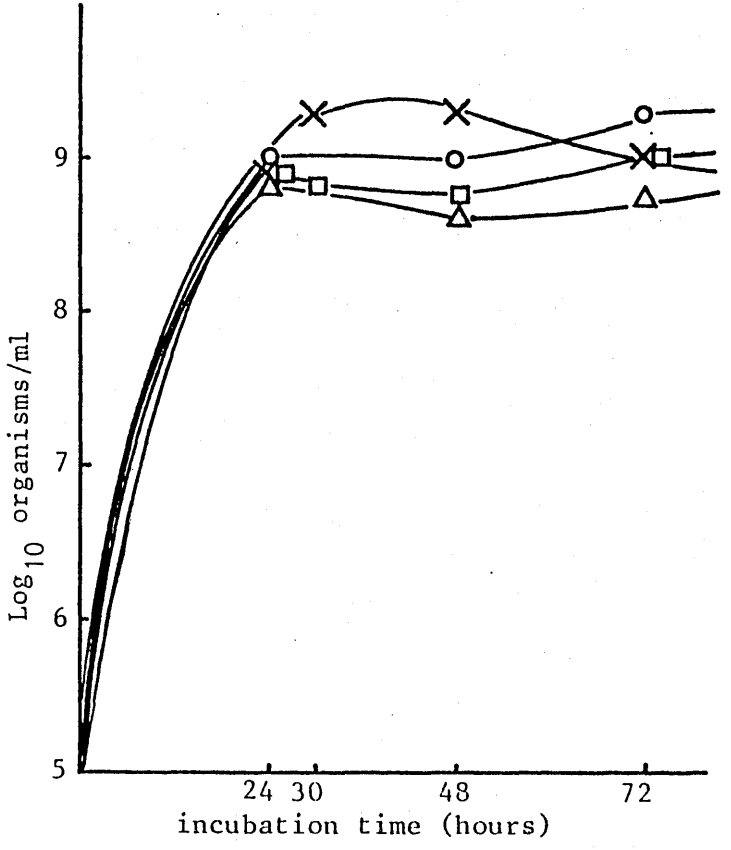
X = Y.enterocolitica serotype 0:3; O = Y.enterocolitica serotype 0:6,30; □ = Y.enterocolitica serotype 0:9;
Δ = Y.pseudotuberculosis

Figure 2. Growth of Yersinia spp.in trypticase soy broth with dextrose at 22°C



X= Y.enterocolitica serotype 0:3; O = Y.enterocolitica serotype 0:6,30; □ = Y.enterocolitica serotype 0:9; Δ = Y.pseudotuberculosis

Figure 3. Growth of Yersinia spp. in Todd Hewitt broth at 22°C



X = Y.enterocolitica serotype 0:3; O = Y.enterocolitica serotype 0:6,30; □ = Y.enterocolitica serotype 0:6,30; Δ = Y.pseudotuberculosis

Table 16

Comparison of the growth of Yersinia spp. from a minimal inoculum in nine non-selective broth media incubated at 22°C; 50% end points after 48 h

Broth	Organism				
	Y. enterocolitica Serotype 0:3	Y. enterocolitica Serotype 0:6,30	Y. enterocolitica Serotype 0:9	Y. pseudotuberculosis	Totals
Brain Heart Infusion	2.50	2.00	1.60	0.00	6.10
Hartley digest	2.90	2.50	2.30	0.00	7.70
Hedley Wright	1.80	2.85	2.00	1.50	8.15
Nutrient (Difco)	2.50	1.90	2.10	1.95	8.45
Nutrient (Oxoid)	1.90	2.65	1.60	1.95	8.10
Peptone water	2.15	2.50	1.25	0.00	5.90
Todd Hewitt	2.55	2.90	0.90	0.85	7.20
Trypticase soy with dextrose	1.90	1.70	1.85	0.10	5.55
Trypticase soy without dextrose	2.65	2.15	2.40	0.00	7.20
Totals	20.85	21.15	16.00	6.35	64.35

Discussion

Biochemistry. The ability of Y. enterocolitica to grow at 4°C is a characteristic not generally shared by other members of the Enterobacteriaceae, but it has been found that a few strains of the other species will grow at this temperature. Eiss (1975) reported that 1/14 strains of E. cloacae which he was studying grew profusely at 4°C. The reference strain of E. cloacae used in this project showed no sign of growth after 9 days incubation (Table 11). However these preliminary studies revealed that the two reference strains of H. alvei and K. aerogenes did grow at 4°C but no similar reports were found in the literature. Generally the biochemical results of this preliminary study agree with those described by Cowan & Steel (1974). However these workers do not show in their tables for Yersinia and related organisms the reactions to Voges-Proskauer, rhamnose and melibiose which are relevant to the new classification for Y. enterocolitica and Y. enterocolitica-like organisms. In addition most of their biochemicals were incubated at 37°C and those known to show temperature dependant variations are not indicated. However, Cowan and Steel's manual was not intended to be the definitive document for detailed classification but a guide which ensures a reasonable chance of an accurate diagnosis. Also until the new classification for Yersinia is internationally accepted the manual would not be expected to include the differentiation of the new species. For more detailed identification one must turn to other sources and the results in this preliminary study do agree with those reported by Bercovier et al. (1980a) for Y. enterocolitica sensu stricto.

RM bacterium which has recently been reclassified as Y. ruckeri (Skerman et al. 1980) varies in several characters from Y. enterocolitica by not producing acid from adonitol, cellobiose, inositol, sorbitol, sucrose, xylose and by not producing urease. This organism was also positive for arginine and lysine decarboxylase and gelatin liquifaction. These biochemical reactions are in agreement with those reported for RM bacterium by Ewing et al. (1978).

Antibiotic sensitivity. Most strains of Y. enterocolitica are resistant to ampicillin but occasionally sensitive strains are encountered such as Y. enterocolitica NCTC 10598. The reference strains of Y. enterocolitica were also sensitive to tetracycline (Table 12), an

antibiotic which is frequently used in the treatment of Yersinia infections. These results are similar to those obtained by Nilehn (1967) with the exception of ampicillin 20 μg and streptomycin 50 μg. Using a disc diffusion method most of Nilehn's strains were slightly sensitive and sensitive, respectively, whereas 4/5 reference strains of Y. enterocolitica were resistant to ampicillin and only 3/5 strains sensitive to streptomycin. More recently Kouwatli et al. (1979) have shown some temperature related variations in the susceptibility to antibiotics of different strains of Y. enterocolitica, and this factor may well account for some of the variations found by different workers.

Proteus is a particularly difficult organism to eliminate from isolation media and as expected the Prot. mirabilis reference strain was resistant to all the antibiotics used. This result is similar to the susceptibility reported by Garrod et al. (1973) who also showed the other species of Proteus to be variable in their reactions. The presence of Proteus spp. is especially a nuisance in broth and on agar media for the isolation of Y. enterocolitica because of the ability of both organisms to produce urease. The other pathogens such as E. coli and S. typhimurium also showed "typical" reactions, but as there is considerable strain variation amongst these species almost any pattern is typical. Although salmonellae are not normally present in foods in numbers large enough to cause any problems in the isolation of Y. enterocolitica, E. coli is frequently found in large numbers.

Unfortunately, novobiocin, which has been incorporated into media for the isolation of Y. enterocolitica was unavailable at the time of these preliminary studies.

The biochemical characterization and antibiotic sensitivity studies of the reference organisms revealed that none of the strains showed any unusual characteristics.

Effect of Ringer solution. The results obtained from the study of the effect of quarter-strength Ringer solution (RS) on the recovery of the reference strains (Table 13) are similar to those reported by Jayne-Williams (1963) who found that prolonged contact with RS had no effect on either E. coli or S. typhimurium. There are no reported studies of the effect of diluent on Y. enterocolitica. However, as there was no significant difference in the colony counts of the five test strains over the 6 h experimental period it was assumed that neither accidental delays nor planned storage in RS would have any appreciable effect on the recovery of these reference organisms.

Selection of reference broth. There were no reports of any comparisons of non-selective broth media for maximum recovery of Yersinia spp. with which to compare the results of this study. It is interesting to note, however, that in this comparison, the three media ranking 1, 2 and 3 (TS + D, TH and BHI respectively, Table 15), all contain small amounts of glucose but the other six media do not contain this or any other sugar.

There were also no other reported studies on the effect of non-selective broth media on the recovery of bacteria from a minimal inoculum with which to compare the results of this study (Table 16). Martin et al. (1976) reported only on the effect of the presence of hydrogen peroxide on the recovery from agar media. However, it does seem possible that catalase production in a broth medium is less likely to effect growth because of the dilution factor caused by the constant washing away of any by-products of growth, by the currents within the broth itself. On an agar medium the hydrogen peroxide produced would remain concentrated in close proximity to the organism and therefore be more likely to cause the inhibition reported.

There was no significant difference detected between the broth media available for this study so it was assumed that any of the nine media would be suitable for the growth of Yersinia from a minimal inoculum.

The results indicated that TS + D should be the medium of choice to obtain maximum recovery but at the time difficulties were being experienced in obtaining a ready supply of this product from the USA. It was therefore necessary to look more closely at TH and BHI. Both of these media obtained very similar rank scores. Todd Hewitt broth was prepared in Colindale from raw ingredients and thus potentially more prone to mistakes and variations than a dehydrated product. Brain Heart Infusion was readily available, already in regular use in the department and being a dehydrated product subject to rigorous quality control. Although dehydrated media do suffer batch variations these are usually minimal. As subsequent studies showed BHI proved to be very reliable with no significant batch to batch variation.

B. Growth and survival studies

Introduction

Many foods are handled after cooking and are therefore vulnerable to contamination by enteric pathogens from the food handlers. Salad ingredients such as cold meat, eggs, rice and mayonnaise (which is not cooked) are particularly hazardous as apart from receiving no further heating prior to being consumed they are frequently left in a warm kitchen for several hours during preparation of the final dishes. There have been an increasing number of reports of this type of food being implicated in food poisoning outbreaks particularly in the USA, where for instance a major outbreak of shigellosis was traced to salad dressing (Weissman et al. 1974). Yersinia enterocolitica presents a new problem because of its ability to grow at refrigeration temperatures ($4^{\circ}C$) which could, presumably, turn even the "safe" storage of food in a refrigerator, into a hazard.

Foods not only contain varying concentrations of sodium chloride but pH values vary considerably with each type of product. At the time of this study there were few published reports on either the effect of pH or sodium chloride on the growth and survival of Y. enterocolitica or indeed on growth and survival of the organism in foods. Hanna and co-workers (Hanna et al 1977b) had investigated the effects of heating, freezing and pH on Y. enterocolitica-like organisms from meat and found that extensive destruction occurred on beef during frozen storage. Using two inoculum levels, 1×10^3 to 1×10^4 and 1×10^6 to 1×10^7 organisms/g, and storage at $-20^{\circ}C$, one strain could not be detected after 2 weeks and two other strains could not be detected after 4 weeks. To study the effect of pH on the growth of Y. enterocolitica-like organisms, these workers adjusted the pH of BHI broth with sodium hydroxide or hydrochloric acid prior to sterilization and recorded the growth of the organisms over 24 h. They reported that the growth of the Y. enterocolitica-like organisms was better at pH 7.0 and pH 8.0 than at pH 6.0 or pH 9.0 and that little or no growth occurred at pH 5.0.

In a further study the same workers (Hanna et al. 1977e) demonstrated increases in the counts of Y. enterocolitica on artificially contaminated cooked and raw beef and pork incubated at 7° and $25^{\circ}C$

within 7-10 days and 24 h respectively. Only since the growth and survival studies presented in this thesis were carried out has Stern and his co-workers (Stern et al. 1980a) reported bacteriostatic and bacteriocidal inhibition of Y. enterocolitica by 7% sodium chloride in Brain Heart Infusion broth incubated at 3° and 25°C. In the same study they also observed that Y. enterocolitica could grow between pH 4.6-9.0.

The investigations presented in this section were aimed at studying the effect of pH and sodium chloride on the growth of Y. enterocolitica and the growth and survival of the organism in a variety of potentially hazardous foods stored at various temperatures.

Methods

1. The effect of pH on the growth and survival of Y. enterocolitica in Brain Heart Infusion broth

The reference strains used were:-

Y. enterocolitica serotype 0:3 Y. enterocolitica serotype 0:9

Buffered Brain Heart Infusion broth (BBHI) was prepared at pH 2.4, 3.6, 4.2, 4.4, 4.6, 4.8, 5.4, 6.6 and 7.8 and distributed in 20 ml volumes in 2 oz wide mouth screw capped jars.

Overnight BHI broth cultures of the test strains were diluted to an estimated concentration of 10^6 organisms/ml and inoculated (0.02 ml) separately into jars of each of the BBHI broths to give approximately 10^3 organisms/ml. The broth cultures were incubated at 22°C for 72 h and surface colony counts carried out after 0, 8, 14, 18, 22, 26, 30, 38, 48, 62 and 72 h incubation (see page82).

2. The effect of sodium chloride on the growth of Y. enterocolitica in Brain Heart Infusion broth

The reference strains used were:-

Y. enterocolitica serotype 0:3 Y. enterocolitica serotype 0:9

Brain Heart Infusion broth (BHI) to which had been added 0, 2.5, 3.5 and 4.5% sodium chloride to give final concentrations of 0.5, 3.0, 4.0 and 5.0% was distributed in 20 ml volumes in 2 oz wide mouth screw capped jars. Overnight broth cultures of the test strains were diluted

to an estimated concentration of 10^6 cells/ml and inoculated (0.02 ml separately into the BHI plus sodium chloride broths to give approximately 10^3 cells/ml. The broth cultures were incubated at 30°C for 48 h and surface colony counts carried out after 0, 8, 14, 18, 30 and 48 h incubation (see page 82).

3. The growth and survival of Y. enterocolitica in foods

The reference strains used were:-

Y. enterocolitica serotype 0:3 Y. enterocolitica serotype 0:9

Preparation of foods

Hard boiled egg, boiled fish, rice, potato and roast chicken were prepared by normal domestic methods. After cooking any non-edible parts were removed and where necessary the foods were chopped or finely homogenized.

Chocolate milk was prepared by adding 10% chocolate sauce to pasteurized milk.

Mayonnaise was prepared by normal domestic methods from six recipes (Table 17).

Table 17

Six recipes for mayonnaise sauce

Recipe		Egg	Mustard g	Salt g	Sugar g	Acid ml	Oil ml	Final pH
*	A	1 yolk	0.1	1.5	1.2	Lemon 40	142	2.9
*	B	1 whole	0.1	1.5	1.2	Lemon 40	142	3.3
**	C	1 whole	0.2	NS	None	Lemon 20	284	3.7
*	D	1 whole	0.1	1.5	1.2	Vinegar 40	142	4.0
**	E	1 whole	0.2	NS	None	Lemon 10	284	4.15
**	F	1 whole	0.2	NS	None	Vinegar 10	284	4.8

* = The Blender Book 1971. London, Elm Tree Books/Hamish Hamilton;

** = The Mixer Book 1972. London Elm Tree Books/Hamish Hamilton;

NS = "to taste", quantity not specified.

Inoculation of foods

Ten gram quantities of each food were distributed into sterile 1 lb screw capped jars. Overnight broth cultures of the test strains were diluted to give an estimated 3×10^5 organisms/ml (see page 82) and 0.06 ml volumes of the strains were then inoculated separately onto the surface of the 10 g samples to give approximately 10^3 organisms/ml.

Boiled fish and roast chicken, 120 x 10 g samples of each food were distributed and both test strains were inoculated separately onto the surface of 60 samples. Nine jars of food were each incubated at -20°C and 4°C and surface colony counts (see page 82) carried out after 1, 2, 3, 7, 10, 21, 35, 49 and 63 days. Ten jars of food were each incubated at 12°, 22°, 30° and 36°C and surface colony counts (see page 82) carried out after 5, 8, 14, 18, 24, 30, 48 and 72 h and 7 and 10 days. The pH of the food was noted after 24, 48 and 72 h incubation (see page 83).

Boiled potato and hard boiled egg, 84 x 10 g and 88 x 10 g samples of each food respectively, were distributed and both test strains were inoculated separately onto the surface of 42 boiled potato and 44 hard boiled egg samples. Eight jars of food were incubated at -20°C, and 4°C and surface colony counts (see page 82) carried out after 1, 2, 3, 7, 21, 35, 49 and 63 days. Six jars of food were incubated at 12°, 22°, 30° and 36°C and surface colony counts (see page 82) carried out after 5, 8, 14, 18, 24 and, in the case of hard boiled egg only, 30 h. The pH of the food was noted after 24, 48 and 72 h incubation (see page 83).

Boiled rice, 78 x 10 g samples of rice were distributed and both test strains were inoculated separately onto the surface of 39 samples. Six jars of rice were incubated at -20°C and surface colony counts carried out after 1, 2, 3, 23, 42 and 56 days. Eight jars were incubated at 4°C and surface colony counts (see page 82) carried out after 1, 2, 3, 7, 9, 23, 42 and 56 days. Eight jars were incubated at 12° and six each at 22°, 30° and 36°C and surface colony counts (see page 82) carried out after 5, 8, 14, 18, 24 and, in the case of 12°C also after 30, 48, and 72 h. The pH of the food was noted after 24, 48 and 72 h incubation (see page 83).

Chocolate milk, 80 x 10 g samples were distributed and both test strains were inoculated separately onto the surface of 40 samples. Four jars were incubated at -20° and 4°C and surface colony counts (see page 82) carried out after 1, 2, 3 and 7 days. Seven jars were incubated at

12°, 22°, 30° and 36°C and surface colony counts (see page 82) carried out after 8, 14, 18, 24, 30, 48 and 72 h. The pH of the food was noted after 24, 48 and 72 h incubation (see page 83).

Mayonnaise, 12 x 10 g samples of each recipe were distributed and the two test strains inoculated separately into 6 jars. Four jars were incubated at 30°C and surface colony counts (see page 82) carried out after 5, 8, 18 and 24 h. The diluent for the 24 h surface colony counts was BHI broth instead of RS and after use the 1:10 dilution was incubated at 30°C overnight and sub-cultured to BHI + D plates for incubation at 30°C for 48 h.

Results

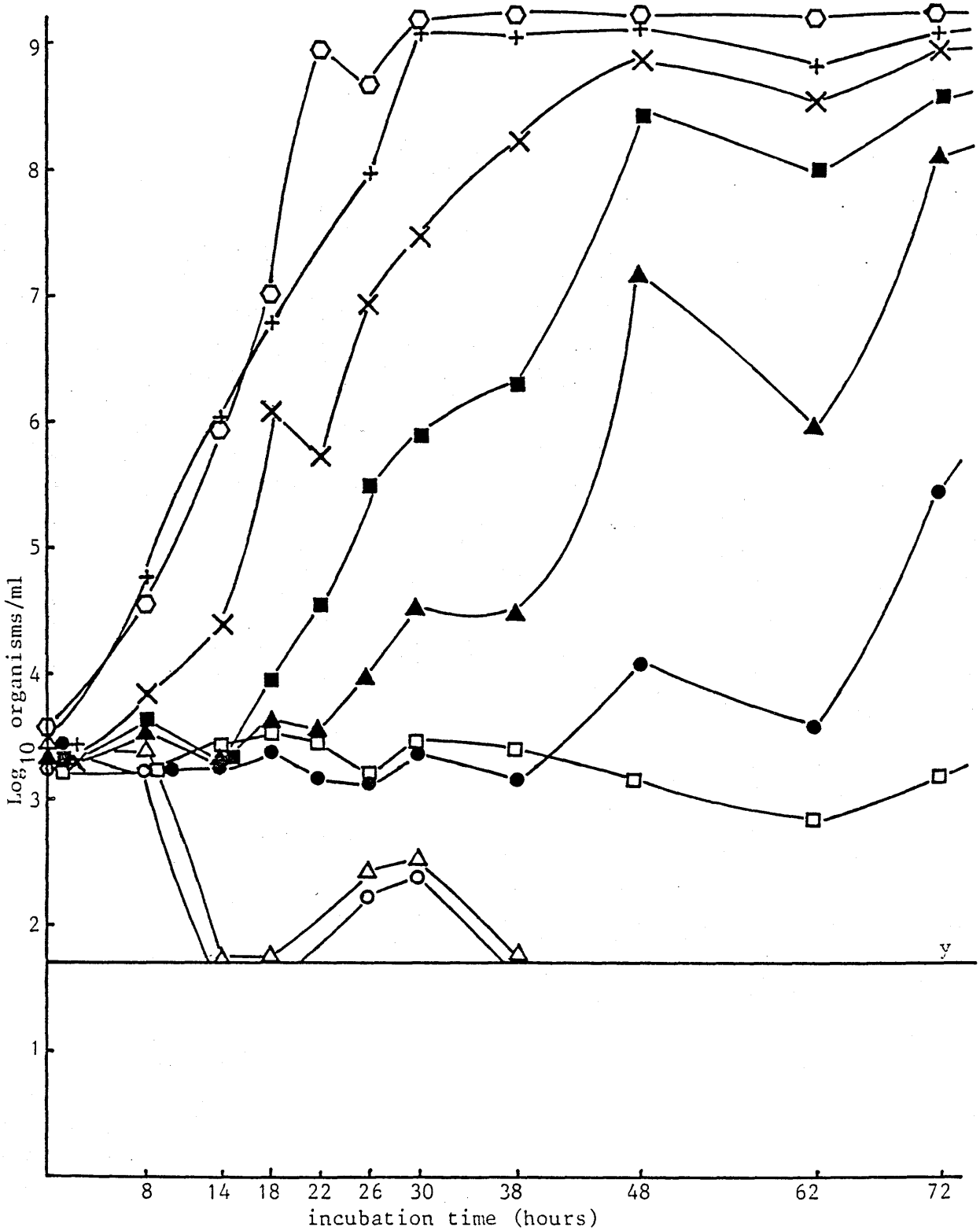
1. Effect of pH on the growth and survival of Y. enterocolitica in Brain Heart Infusion broth

The growth curves of Y. enterocolitica serotypes 0:3 and 0:9 at various pH values in BBHI broth are shown in Figures 4 and 5. The lower the pH value the more prolonged was the lag period. At pH 3.6 and below there was a rapid decrease in the number of Y. enterocolitica and within 48 h the number of both strains had diminished to below the limits of the counting method. At pH 4.2 both strains survived for 72 h with little or no variation from the original count. The pH values of 4.2 - 4.4 appeared to be the threshold of inhibition/growth and although at pH 4.4 the lag period was prolonged, growth did not occur after 38 Serotype 0:3 finally reached a level of 2.75×10^5 organisms/ml at 72 h and serotype 0:9 reached a level of 7.5×10^2 organisms/ml by 62 h; however, this strain appeared to have declined at 72 h. Growth of both strains occurred more readily at pH 4.6 and all values above this. For example at pH 6.6 and 7.8 levels of $>1 \times 10^9$ organisms/ml were attained within 30 h.

2. Effect of sodium chloride on the growth of Y. enterocolitica in Brain Heart Infusion broth

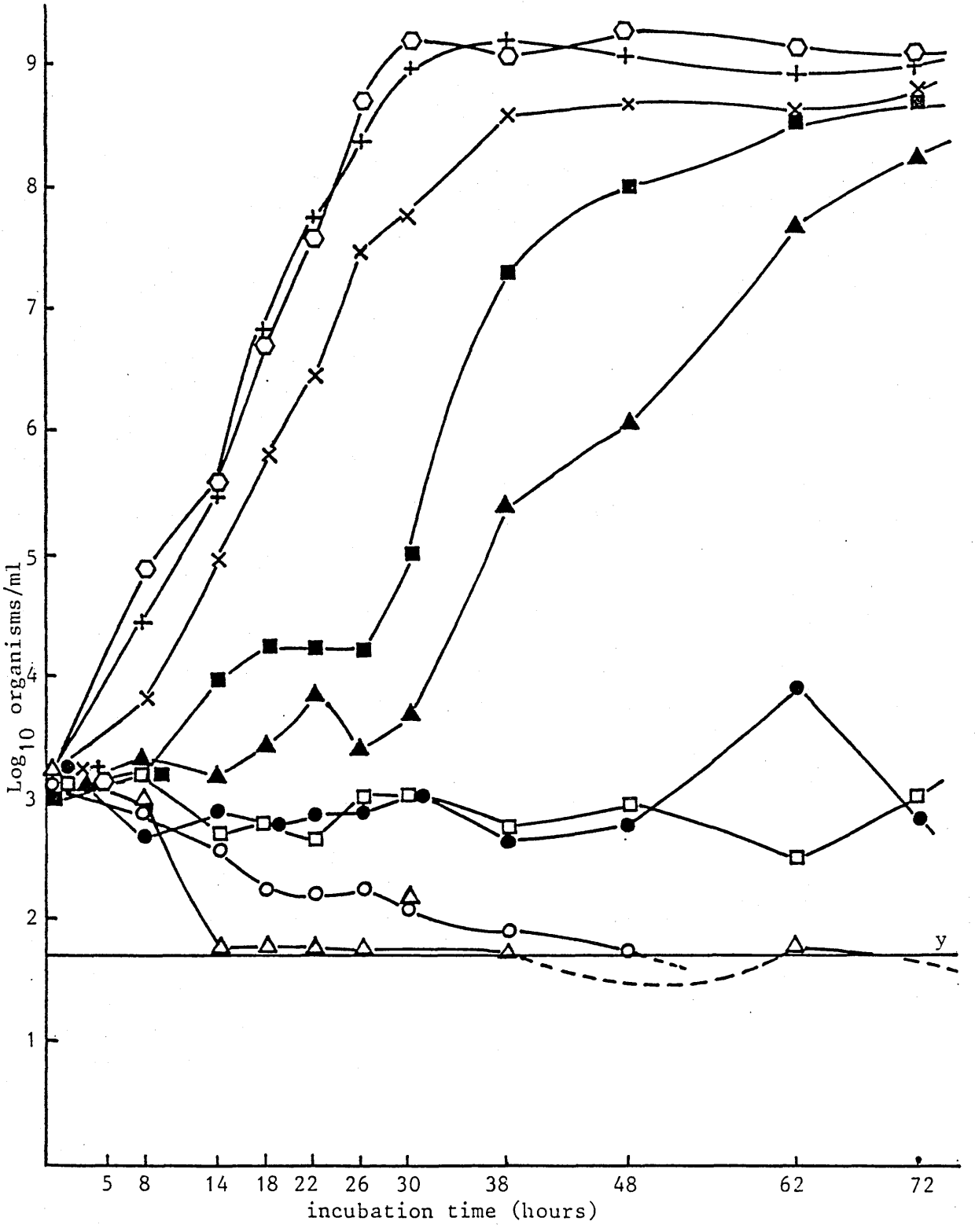
The effect of sodium chloride on the growth of Y. enterocolitica is shown in Figure 6. Both test strains attained colony counts of $>6.25 \times 10^8$ organisms/ml in 0.5% sodium chloride within 14-18 h

Figure 4. Effect of pH on the growth and survival of Y.enterocolitica serotype 0:3 in Brain Heart Infusion broth at 22°C



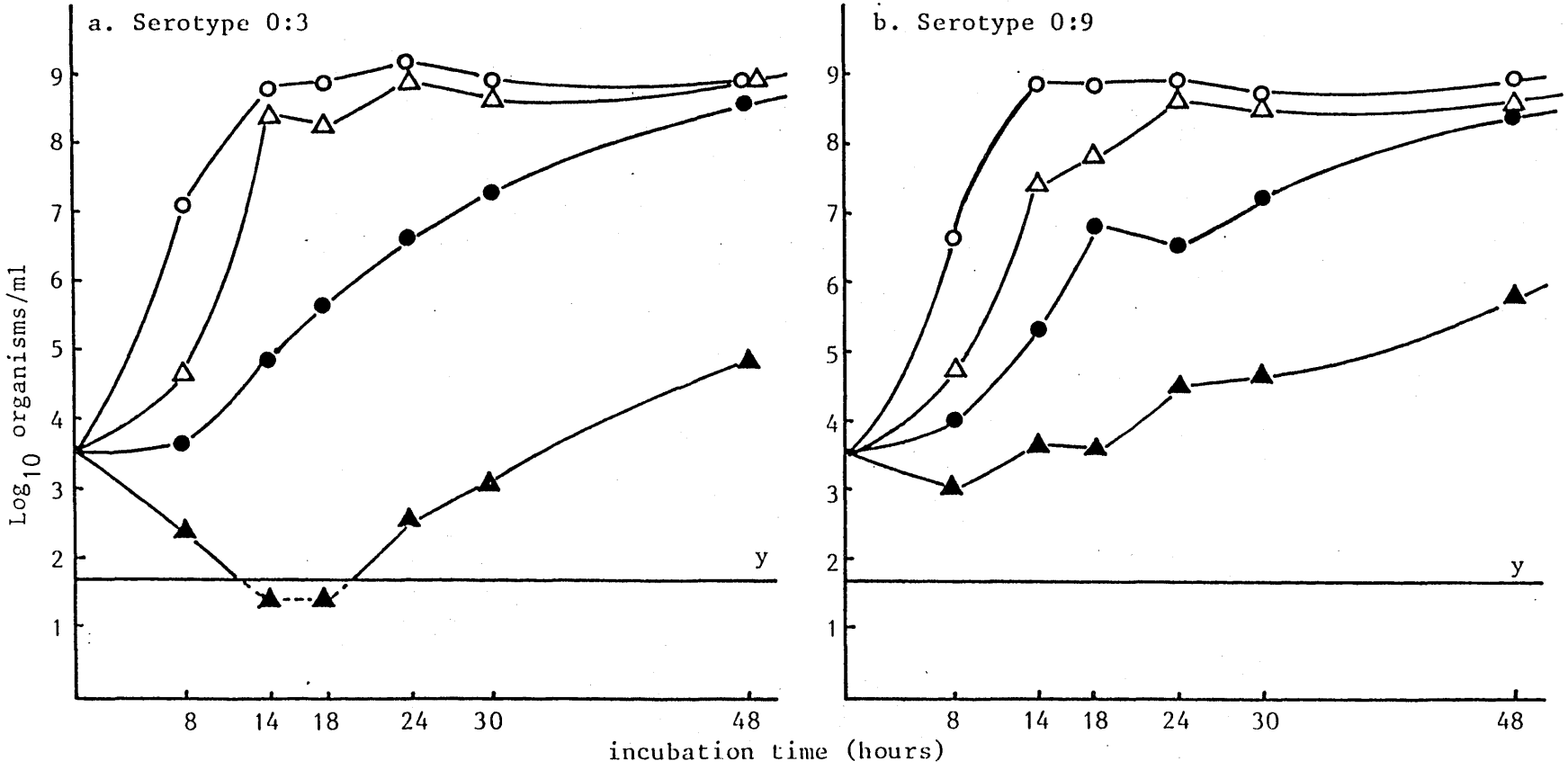
○ = pH 2.4; △ = pH 3.6; □ = pH 4.2; ● = pH 4.4; ▲ = pH 4.6; ■ = pH 4.8; × = pH 5.4; + = pH 6.6; ⬡ = pH 7.8; y = lower limit of counting method.

Figure 5. Effect of pH on the growth and survival of Y.enterocolitica serotype 0:9 in Brain Heart Infusion broth at 22°C



○ = pH 2.4; △ = pH 3.6; □ = pH 4.2; ● = pH 4.4; ▲ = pH 4.6; ■ = pH 4.8; × = pH 5.4; + = pH 6.6; ⬡ = pH 7.8; y = lower limit of counting method.

Figure 6. Effect of sodium chloride on the growth of Y.enterocolitica in Brain Heart Infusion broth at 30°C



○ = 0.5% ; △ = 3%; ● = 4%; ▲ = 5% sodium chloride; y = lower limit of counting method

incubation and in 3% sodium chloride similar counts were attained after 24 h. In 4% sodium chloride the rate of growth of both Y. enterocolitica serotypes 0:3 and 0:9 was inhibited and colony counts of 3×10^8 and 4×10^8 organisms/ml respectively were only attained after 48 h. In 5% sodium chloride this inhibitory effect was much greater. The colony counts of serotype 0:3 declined initially before rising to 8.5×10^4 organisms/ml after 48 h and although serotype 0:9 also showed a decline it was less marked than serotype 0:3 and the colony counts rose slowly to 7×10^5 organisms/ml after 48 h.

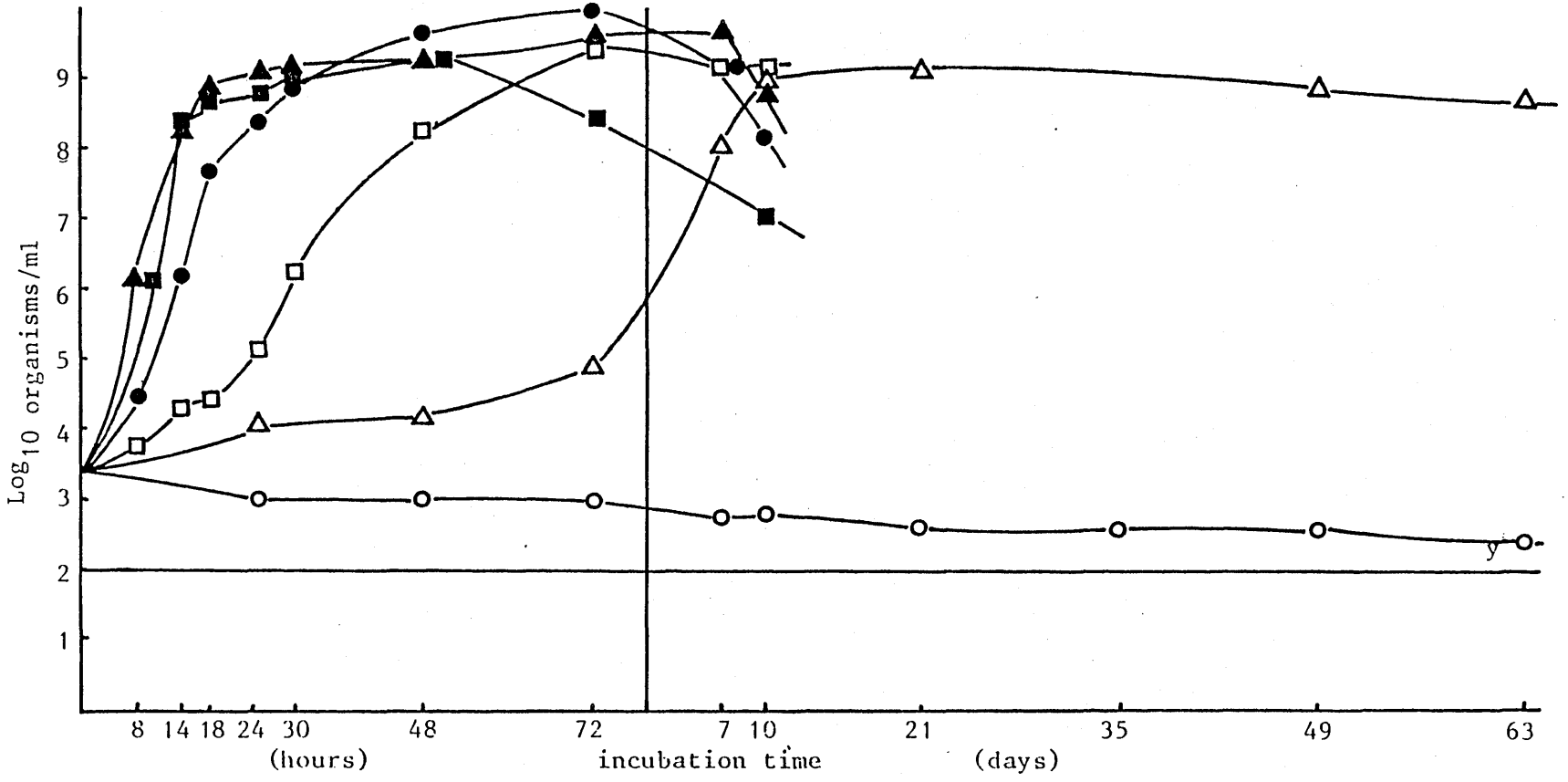
3. Growth of Y. enterocolitica in foods

Boiled fish, hard boiled egg, boiled potato, boiled rice and roast chicken. Growth patterns of Y. enterocolitica serotype 0:3 in boiled fish is shown in Figure 7. These results are representative of those obtained with both test strains in hard boiled egg, boiled potato, boiled rice and roast chicken. At 22°, 30° and 36°C the colony counts rose rapidly to $>1 \times 10^8$ organisms/ml within 30 h. At 12°C the same level was reached after 72 h and at 4°C there was a steady increase in count to $>1 \times 10^8$ organisms/ml over a period of 7-10 days. Both strains of Y. enterocolitica survived for at least 8-9 weeks storage at -20°C, the number of viable cells remaining at the same level as the initial count. The colony counts on the boiled fish and roast chicken incubated at 12°, 22°, 30° and 36°C and carried out after 7 and 10 days showed a distinct decline in the number of Y. enterocolitica present, the most rapid decrease being at 36°C. Boiled potato was the only food tested which became even slightly discoloured possibly by the growth of the Y. enterocolitica.

The roast chicken was found to be contaminated with an aerobic sporing bacillus which grew at the same rate as the test strain. It was impossible to read the results of the surface colony counts on BHI + D agar but the contaminant was inhibited on LSU agar. The growth of the test strains in the chicken did not appear to be inhibited by the bacillus.

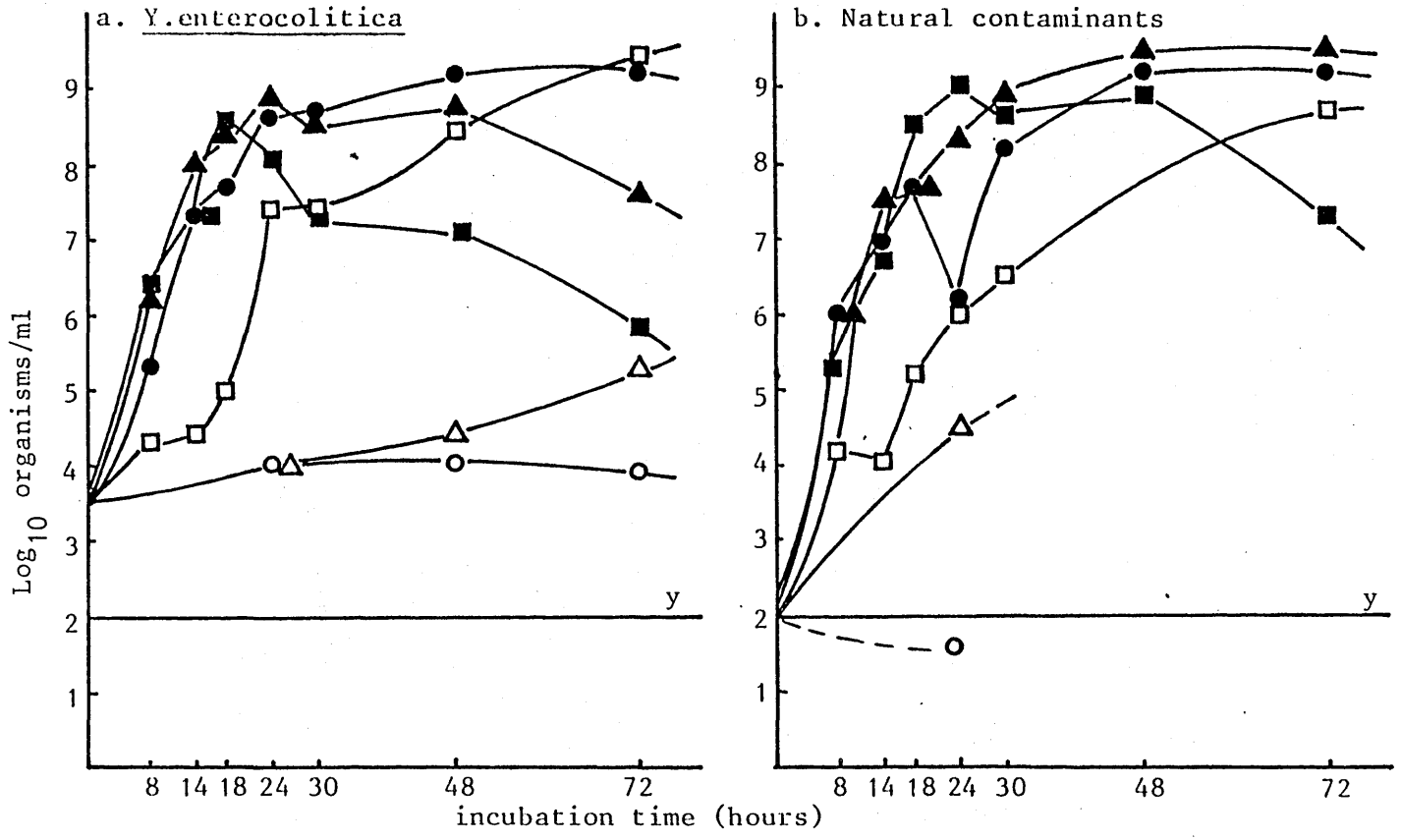
Chocolate milk. The growth patterns of Y. enterocolitica serotype 0:3 and competing organisms are shown in Figure 8. They are representative of the results obtained with both test strains. These results are not markedly different from those obtained with the other foods except there appears to be an unexplained and sudden constant fall and rise in the

Figure 7. Growth of Y.enterocolitica serotype 0:3 in boiled fish



○ = -20°; △ = 4°; □ = 12°; ● = 22°; ▲ = 30°; ■ = 36°C

Figure 8. Growth of Y.enterocolitica serotype 0:3 and natural contaminants in chocolate milk



○ = -20°; △ = 4°; □ = 12°; ● = 22°; ▲ = 30°; ■ = 36°C; y = lower limit of counting method.

number of viable organisms at each temperature. Both strains of Y. enterocolitica grew equally as well as the contaminant organisms.

Mayonnaise. The survival of both test strains is shown in Table 18. Both strains of Y. enterocolitica failed to grow in any of the six mayonnaise sauces examined and the death rate was rapid. Both test strains were recovered from recipe E (pH 4.15) after 8 h but not after 18 or 24 h. Serotype 0:9 was recovered from recipe F (pH 4.8) after 18 h but not after 24 h and serotype 0:3 was recovered from recipe D (pH 4.0) after 24 h but not after 5 or 18 h. Yersinia enterocolitica was isolated from only one (serotype 0:3, recipe D) of the 1:10 BHI dilutions incubated at 30°C overnight.

A portion of mayonnaise sauce, recipe E (pH 4.15) was mixed with boiled potato (pH 6.0) as for potato salad and it was noted that the finished product was pH 5.8.

The effect of the growth of Y. enterocolitica on the pH of the various foods after 24 h incubation is shown in Figure 9.

These results reflect the effect which Y. enterocolitica alone had on the pH of the foods. After 24 h incubation the other organisms were, in most cases, in low numbers whereas after 48 and 72 h they had multiplied to large numbers at most of the temperatures used and were themselves affecting the pH of the food.

The roast chicken, boiled potato and hard boiled egg were each contaminated with aerobic sporing bacilli which came from the inevitable handling that these particular foods had received during post-cooking preparation. The boiled rice did not contain any contaminants and the chocolate milk was naturally contaminated with many species of organisms. A wide range of colony counts (1.5×10^2 - 10.75×10^8 organisms/g) of the Y. enterocolitica test strains were obtained in the foods over the five incubation temperatures. The growth of Y. enterocolitica did not affect the pH of any of the foods. However, slight variations did occur in the pH of some of the samples incubated at the higher temperatures where the contaminating organisms grew more freely, for example, the boiled potato, hard boiled egg and chocolate milk. The aerobic sporing bacillus present in the roast chicken outgrew both strains of Y. enterocolitica at 36°C but there was no change in the pH of the food.

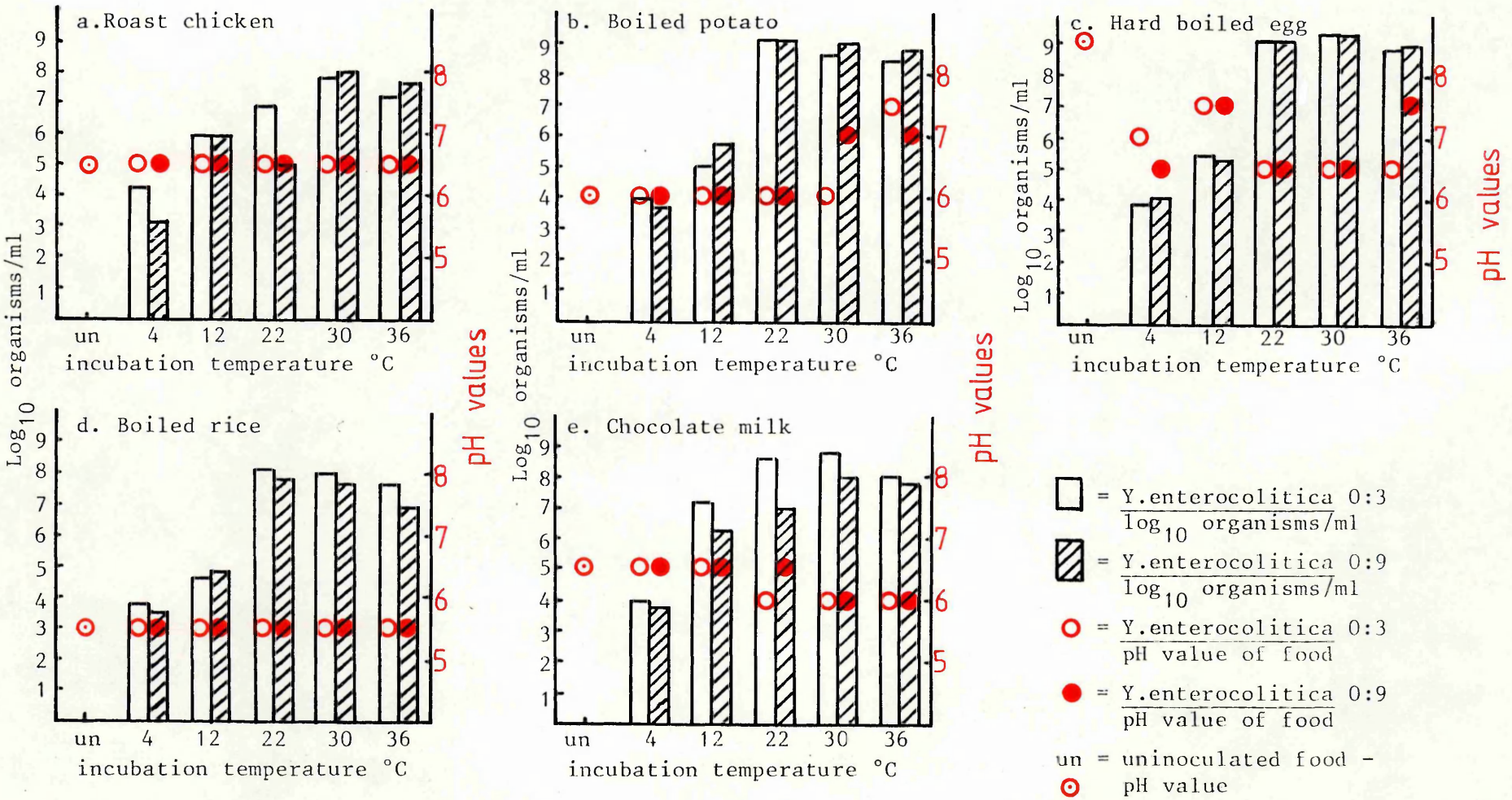
Table 18

Survival of Y. enterocolitica serotypes 0:3 and 0:9 in mayonnaise sauces

Strain	Mayonnaise recipe	pH	Incubation time (h) 0	5	8	18	24
				$\log_{10}$ organisms/ml			
Serotype 0:3	A	2.9	2.70	<1.70	<1.70	NT	NT
	B	3.3	2.18	1.70	<1.70	<1.70	NT
	C	3.7	3.18	1.70	<1.70	<1.70	<1.70*
	D	4.0	2.70	<1.70	NT	<1.70	1.70
	E	4.15	2.88	<1.70	1.70	<1.70	NT
	F	4.8	3.24	2.74	2.48	<1.70	<1.70*
Serotype 0:9	A	2.9	3.23	<1.70	<1.70	<1.70	NT
	B	3.3	2.85	<1.70	<1.70	<1.70	NT
	C	3.7	3.45	2.30	<1.70	<1.70	<1.70*
	D	4.0	3.38	<1.70	<1.70	<1.70	NT
	E	4.15	3.14	3.02	2.30	<1.70	<1.70*
	F	4.8	3.62	3.29	3.00	1.70	<1.70*

* = not recovered even after incubation of 1:10 dilution, BHI broth used instead of quarter strength Ringer solution; NT = not tested.

Figure 9. Effect of growth of Y.enterocolitica on the pH of various foods in relation to incubation temperature



Discussion

pH. The results obtained in this study (Figures 4 and 5) of the effects of pH of nutrient broths on the growth of Y. enterocolitica are comparable in many respects with those of Hanna et al. (1977b). The main difference between their results and those presented here is that they found no increase in growth over 24 h at pH 5.0 whereas these results show a definite increase in numbers within that period. This finding was confirmed by Stern et al. (1980a) who also showed that Y. enterocolitica could grow quite readily at pH 5.0.

Sodium chloride. At the time of the study of the effect of sodium chloride on the growth of Y. enterocolitica there was no other work with which to compare the results. The present study, however, does show that an increasing concentration of sodium chloride will cause increasing inhibition of Y. enterocolitica (Figure 6). These findings have been confirmed by Stern et al. (1980a).

Growth in foods. There were also few published reports of studies on the growth and survival of Y. enterocolitica in foods and none on the effect of the organism on foods when this project began. However, these results (Figures 7 and 8) are similar to those obtained by Hanna et al. (1977e) for growth of Y. enterocolitica on raw and cooked beef and pork. At 7°C they found that the main increase in numbers occurred between 3 and 7 days which is much the same as the results obtained in this study at 4°C. In another study by Hanna et al. (1977b) on the effect of freezing on the survival of Y. enterocolitica in cooked beef none of their strains of Y. enterocolitica could be detected after 4 weeks, whereas these results showed that very small numbers of Y. enterocolitica could still be demonstrated even after 9 weeks (63 days) at -20°C.

The results of these studies confirm that Y. enterocolitica is potentially a hazard as a food poisoning organism because of its ability not only to survive but grow on foods in what up to now has been regarded as "safe" conditions, at refrigerator temperatures (4°C).

The ability also of the organism to survive at pH 4.2 and to grow slowly at pH 4.4 creates the possibility that prolonged storage even of an acid food might be hazardous. However despite the ability of the test strains to grow at pH 4.4 in buffered BHI broth neither strains survived more than 18 h in mayonnaise sauce at pH 4.8 (Table 18). Even

so the potential hazard must not be ignored as Y. enterocolitica has been isolated from egg mayonnaise and Italian salad (Aldová et al. 1975).

The presence of Y. enterocolitica in a food cannot be suspected because of any changes in appearance or smell of the food. The clue to a bad food must still be the detection of spoilage by other organisms but these may not always be present, and indeed, in general food poisoning is caused by foods which look and smell normal and in some instances even taste extra delicious.

In summary it has been demonstrated that: Y. enterocolitica can survive at pH 4.2 and grow slowly at pH 4.4 after a prolonged log phase; the organism is able to grow as well in 3% sodium chloride as in 0.5% but is inhibited in 4% or more; and with the exception of mayonnaise sauces Y. enterocolitica is able to grow in foods at temperatures from 4°C - 36°C without affecting either the pH or the appearance of the food and is also able to survive for at least 63 days in foods stored at -20°C.

C. Evaluation of media and methods for the isolation of Yersinia enterocolitica

Introduction

A number of the agar and broth media commonly used for the isolation of Salmonella, Shigella and other enteric pathogens have been tried, with varying success, for the isolation of Y. enterocolitica. The agar media have included MacConkey (MAC), lactose sucrose urea (LSU), Salmonella-Shigella (SS), eosin methylene blue (EMB), deoxycholate citrate (DCA), hektoen enteric (HEK), Endo (ENDO), xylose lysine deoxycholate (XLD) and bismuth sulphite agars (BS).

At the time that this project was begun only one worker, Nilehn (1969a), had compared the growth characteristics of Y. enterocolitica on a variety of agar media, her aim to find a suitable medium for the isolation of the organism from human faeces by direct plating. She studied the growth of 35 strains mostly of human origin on LSU, SS, DCA and ENDO agars incubated at both 37° and 25°C. The strains of human origin, which were serotypes 0:3 and 0:9, gave reasonably good growth on all the media at both temperatures, although, older strains from different origins gave very varied results both with the individual media and at the different incubation temperatures. Nilehn also found that when the same media were streaked with a mixed culture there was very little inhibition of unwanted Gram-negative bacteria, especially Proteus spp. on ENDO agar, but that the remaining three media where incubated at 25°C showed increased inhibition of the undesired faecal bacteria which proved to be advantageous in the isolation of Y. enterocolitica. The distinct colonial characteristics of Y. enterocolitica on LSU made differentiation somewhat easier than on the other media. However, whichever medium was used, incubation for at least 2 days was found to be essential.

Wauters (1973) reported that Y. enterocolitica will tolerate as much as a 3% concentration of sodium deoxycholate and that by incorporating 2% into SS agar this medium (SS + D) gave good selectivity whilst allowing rapid growth of Y. enterocolitica. He found it necessary to adjust the pH of the base medium to 7.0 or above to avoid precipitation of the sodium deoxycholate. If incubated at 29°C the colonies of Y. enterocolitica were visible after 20 h. Wauters recommends the use of 10-fold magnification on a stereo microscope to distinguish the colony type which is characteristic only of serotypes 0:3 and 0:9.

As with the agar media a variety of broth media commonly used for the isolation of other pathogens have been suggested for the isolation of Y. enterocolitica, namely, Gram-negative broth (GN), peptone water (PW), cooked meat medium (CMM), various modifications of selenite broth, and tetrathionate broth. Only phosphate buffered saline (PS) and Wauters' (1973) broth (WB) were recommended specifically for the isolation of Y. enterocolitica.

Wauters (1973) modified Rappaport's (1956) broth by increasing the magnesium chloride, reducing the malachite green and adding carbenicillin. He recommended this medium (WB) to be used in conjunction with SS + D agar, and solely for the isolation of the serotypes 0:3 and 0:9 and possibly 0:1 and 0:8. He found the isolation rate of Y. enterocolitica from WB to be as much as four times greater than that from selenite or Rappaport broths.

Inoue & Kurose (1975) modified selenite by adding novobiocin (SN) which was incubated at 37°C for 24 h and compared it with PS incubated at 5°C for 3 weeks. Both broths were inoculated with cow intestinal contents (115) and beef meat (61). Nine cow and 15 beef samples were found to be positive for Y. enterocolitica through PS but only one sample was positive through SN.

Because of the lack of comparative studies, by other workers, of both agar and broth media for the isolation of Y. enterocolitica it was decided to include just such a study as part of this project. The comparisons reported in this section include some of the media commonly used, and readily available, for the isolation of the other enteric pathogens as well as some of those which have been specifically formulated for Y. enterocolitica. When this project began these latter media were few and far between. In view of this an attempt was also made to formulate a new medium, either agar or broth, and/or to modify an existing one to enable more effective isolation of Y. enterocolitica from foods and other materials. This was particularly relevant as most of the work so far had been on isolation from clinical specimens. As the success or failure of a new medium depends on the correct choice of the selective agents and as also in this field no work had been reported involving Yersinia spp. a study of the effect of various selective agents was undertaken.

Antagonism by other members of the same bacterial family when in mixed culture had also been reported. Klebsiella aerogenes [Aerobacter

aerogenes], E. cloacae [Aerobacter cloacae] and E. coli may inhibit organisms of the Salmonella and Shigella groups as well as some of the Gram-positive bacteria including Staphylococcus aureus and Bacillus spp. (Wynne 1947; Halbert 1948; Hentges & Fulton 1964; Hentges 1967, 1969). This project includes a study of the antagonistic effect of similar organisms on Y. enterocolitica.

During the last five years several more agar and broth media have been specially formulated for the isolation of Y. enterocolitica. These include MacConkey Tween (LEE) and deoxyribonuclease sorbitol (DNA) agars (Lee 1977), pectin (PEC) agar (Bowen & Kominos 1979), cellobiose arginine lysine (CAL) agar (Dudley & Shotts 1979), cefsulodin-irgasan-novobiocin (CIN) agar (Schiemann 1979b), sodium oxalate (SO) agar (Soltész et al. 1980), supplemented phosphate buffer (Mehlman et al. 1978) and two modifications of selenite broth by Lee et al. (1980). Where possible some of these media have been included in the studies reported in this section or in the comparisons of methods reported in section D.

Methods

1. Evaluation of agar media

Strains from the supplementary list of reference organisms were included in the following studies of agar media when it was thought that useful information could be obtained.

a) Colonial characteristics of Y. enterocolitica and other enterobacteria

The reference strains studied were:-

Y. enterocolitica serotype 0:3
Y. enterocolitica serotype 0:6,30
Y. enterocolitica serotype 0:9
Y. enterocolitica NCTC 10460
Y. enterocolitica NCTC 10598
Y. pseudotuberculosis
E. cloacae
E. alkalescens
E. coli QC 21043/75
H. alvei
K. aerogenes
Prot. mirabilis
RM bacterium
S. typhimurium
S. flexneri
S. sonnei

The supplementary strains studied were:-

Y. enterocolitica Y.46 serotype 0:5,27
Y. enterocolitica Y.63 serotype 0:6,30
Y. enterocolitica Y.136 serotype 0:8

The agar media studied were:-

Bismuth sulphate agar BBL (B1)
Difco (B2)
Lab M (B3)
Oxoid (B4)
Brilliant green agar, Oxoid (BG)
Deoxycholate citrate agar (DCA)
Deoxycholate citrate sucrose agar (DCSA)
Deoxyribonuclease sorbitol agar (DNA)
Eosin methylene blue agar (EMB)
Hektoen enteric agar (HEK)
Lactose sucrose urea agar (LSU)
Lee agar (LEE)
MacConkey agar (MAC)
Salmonella-Shigella agar (SS)
Salmonella-Shigella plus deoxycholate agar (SS + D)
Thiosulphate citrate bile salt agar (TCBS)
Xylose lysine deoxycholate agar (XLD)

The reference strains. Overnight BHI broth cultures of the reference strains were sub-cultured using a conventional streaking method on to all the test agar media except DCA. The agar plates were incubated at 30°C for 48 h except LEE and DNA agars which were incubated at 22°C for 48 h. The colonial characteristics of each organism on each medium were recorded.

The supplementary strains. Overnight BHI broth cultures of the supplementary strains and Y. enterocolitica serotype 0:3 were sub-cultured on to DCA, DCSA, LSU, LEE, MAC, SS and SS + D. The agar plates were incubated at 30°C for 48 h except LEE agar which was incubated at 22°C for 48 h. The colonial characteristics were recorded photographically.

b) Quantitative evaluation of the growth of Y. enterocolitica and other organisms

The reference strains studied were:-

Y. enterocolitica serotype 0:3
Y. enterocolitica serotype 0:6,30
Y. enterocolitica serotype 0:9
Y. enterocolitica NCTC 10460
Y. pseudotuberculosis
E. cloacae
E. alkalescens
E. coli QC 21043/75
H. alvei
K. aerogenes
Prot. mirabilis
S. typhimurium

The supplementary reference strains studied were:-

B. alvei	Gram-positive coccus A
B. cereus	Gram-positive coccus B
Bacillus sp.	Ps. aeruginosa

The media studied were:-

Brain Heart Infusion agar (BHI agar)	Salmonella-Shigella agar (SS)
Deoxycholate citrate sucrose agar (DCSA)	Salmonella-Shigella plus deoxycholate agar (SS + D)
Lactose sucrose urea agar (LSU)	Xylose lysine deoxycholate agar (XLD)
MacConkey agar (MAC)	

Overnight broth cultures of the test organisms were diluted 1:100 with quarter-strength Ringer solution and then further 10-fold dilutions were prepared and surface colony counts (see page 82) carried out on each of the test agars. The plates were incubated at 30°C for 48 h.

c) Notes on two additional agar media

i. Pectin agar (PEC)

The reference strains used were:-

Y. enterocolitica serotype 0:3	Y. enterocolitica NCTC 10460
Y. enterocolitica serotype 0:6,30	Y. enterocolitica NCTC 10598
Y. enterocolitica serotype 0:9	Y. pseudotuberculosis

Also used were 82 food isolates of Y. enterocolitica and six enrichment cultures from which Y. enterocolitica had been isolated by earlier sub-culture.

The test strains were streaked eight to a plate on to the pectin agar and incubated at 22°C for 48 h.

The enrichment cultures were streaked out using a conventional technique and also incubated at 22°C for 48 h.

ii. Sodium oxalate agar (SO)

Six enrichment cultures from which Y. enterocolitica had earlier been isolated were sub-cultured to the oxalate agar and incubated at 30°C for 48 h.

2. Evaluation of broth media

a) Qualitative evaluation of the growth of Yersinia spp. and other enterobacteria at various temperatures

The reference strains studied were:-

Y. enterocolitica serotype 0:3	E. coli QC 21043/75
Y. enterocolitica serotype 0:6,30	H. alvei
Y. enterocolitica serotype 0:9	K. aerogenes
Y. enterocolitica NCTC 10460	Prot. mirabilis
Y. enterocolitica NCTC 10598	RM bacterium
Y. pseudotuberculosis	S. typhimurium
E. cloacae	S. flexneri
E. alkalescens	S. sonnei

The broth media studied were:-

Brain Heart Infusion broth (BHI)	Selenite cystine broth (SC)
GN broth (GN)	Selenite F broth (SF)
Phosphate buffered saline (PS)	Wauters' broth (WB)

Overnight broth cultures of the test strains were inoculated (0.02 ml) into each of six tubes containing 5 ml of test medium and incubated at 4°, 12°, 22°, 30°, 36° and 43°C for 16 days.

b) Quantitative evaluation of the growth of Yersinia spp. and other enterobacteria

i. Growth of Yersinia spp. and other enterobacter at 30°, 22° and 4°C

The reference strains studied were:-

Y. enterocolitica serotype 0:3	E. coli QC 21043/75
Y. enterocolitica serotype 0:6,30	H. alvei
Y. enterocolitica serotype 0:9	K. aerogenes
Y. enterocolitica NCTC 10460	Prot. mirabilis
Y. enterocolitica NCTC 10598	RM bacterium
Y. pseudotuberculosis	S. typhimurium
E. cloacae	S. flexneri
E. alkalescens	S. sonnei

The broth media studied were:-

Brain Heart Infusion broth (BHI)	Selenite cystine broth (SC)
GN broth (GN)	Selenite F broth (SF)
Phosphate buffered saline (PS)	Wauters' broth (WB)

Growth at 30°C. Overnight broth cultures of the test strains were diluted to an estimated 3×10^4 organisms/ml and inoculated (0.06 ml) into 100 ml of each of the enrichment media, except phosphate buffered saline, and incubated at 30°C for 48 h. Surface colony counts (see page 82) were carried out after 0, 6, 24 and 48 h incubation and also after 72 h incubation where the organisms had shown little or no growth after 24 or 48 h.

Growth at 22° and 4°C. *Yersinia enterocolitica* serotypes 0:3 and 0:9 were also inoculated into further duplicate 100 ml volumes of all the broth media including phosphate buffered saline. One replicate was incubated at 22°C and colony counts (see page 82) carried out after 0, 6, 14, 18, 24, 30 and 48 h. The second replicate was incubated at 4°C and colony counts (see page 82) carried out after 0, 1, 2, 5, 9 and 13 days.

ii. Growth of Yersinia spp. from a minimal inoculum in five broth media incubated at 30° and 22°C

The reference strains studied were:-

Y. enterocolitica serotype 0:3	*Y. enterocolitica* NCTC 10460
Y. enterocolitica serotype 0:6,30	*Y. enterocolitica* NCTC 10598
Y. enterocolitica serotype 0:9	*Y. pseudotuberculosis*

The media studied were:-

Brain Heart Infusion broth (BHI)	Selenite F broth (SF)
GN broth (GN)	Wauters' broth (WB)
Selenite cystine broth (SC)	

Overnight broth cultures of the test strains were diluted to an estimated 100 cells/ml (see page 82). Additional dilutions of 1:10, 1:2, 1:2, 1:2, 1:2 were prepared to give a range of approximately <1-10 cells/ml and 1 ml of each inoculated into each of ten tubes containing 5 ml of the broth media. The broth media were incubated at 30° and 22°C for 48 h.

c) Evaluation of two additional broth media

The reference strains studied were:-

Y. enterocolitica serotype 0:3 Y. enterocolitica serotype 0:6,30

The broth media studied were:-

Buffered peptone water (BP) Supplemented phosphate buffer (S)

i. Growth at 30°, 22° and 4°C

Overnight BHI broth cultures of the test strains were diluted to an estimated 3×10^4 organisms/ml (see page 82) and 0.06 ml of each inoculated separately into triplicate 100 ml volumes of the broth media. Replicates were incubated at 30° and 22°C for 48 h and colony counts (see page 82) carried out after 6, 18, 24 and 48 h. The third replicate was incubated at 4°C for 13 days and surface colony counts (see page 82) carried out after 1, 2, 5, 9 and 13 days.

ii. Growth from a minimal inoculum

Overnight BHI broth cultures of the test strains were diluted to approximately 100 cells/ml (see page 82). Additional dilutions of 1:10, 1:2, 1:2, 1:2, 1:2 were prepared to give a range of approximately <1-10 cells/ml and 1 ml of each inoculated into each of ten tubes containing 5 ml of the broth media. The media were incubated at 30°C for 48 h.

d) Evaluation of batch to batch variation and effect of storage of broth media on the recovery of Y. enterocolitica

The reference strains studied were:-

Y. enterocolitica serotype 0:3 Y. enterocolitica serotype 0:6,30

The broth media studied are shown in Table 19.

Table 1)

Broth media evaluated for batch to batch variation and effect of storage on the recovery of Y.enterocolitica.

Broth medium and experiment code		Media production batch number	Manufacturer and code number	Storage time in laboratory after preparation (months)
	A	3702	Difco 669679	7
	B	5775	Difco 671085	4
Brain	C	NA	Difco 632733	<1
Heart	D	NA	Difco 671085	<1
Infusion	E	NA	Difco 666140	<1
	F	NA	Difco 673252	<1
	G	NA	Difco 669679	<1
Brain Heart Infusion	A	NA	Oxoid 27134	<1
	A	869	Difco 643400	22
	B	1951	Difco 654514	10
GN	C	346	Difco 654514	1
broth	D	NA	Difco 631023	<1
	E	NA	Difco 654514	<1
	F	NA	Difco 663943	<1
	A	1466	NA	10
Buffered	B	4375	NA	5
peptone	C	4804	NA	5
water	D	5662	NA	3
	E	7100	NA	1

NA = not applicable.

After preparation the broth media were stored at room temperature until required.

Overnight broth cultures of the test strains were diluted to an estimated 3×10^4 organisms/ml and 0.06 ml of each inoculated separately into 100 ml of the broth media, incubated at $30^{\circ}C$ for 48 h and surface colony counts (see page 82) carried out after 6,18, 24 and 48 h.

3. Investigations with a view to formulating a new medium

a) Investigations to determine antagonistic effects amongst the reference strains

The reference strains studied were:-

Y. enterocolitica serotype 0:3	E. coli QC 21043/75
Y. enterocolitica serotype 0:6,30	H. alvei
Y. enterocolitica serotype 0:9	K. aerogenes
Y. enterocolitica NCTC 10460	RM bacterium
Y. enterocolitica NCTC 10598	S. typhimurium
Y. pseudotuberculosis	S. flexneri
E. cloacae	S. sonnei
E. alkalescens	

i. Antagonism in mixed live cultures

Six hour Brain Heart Infusion (BHI) broth cultures of the test strains were each flooded onto the surface of individual BHI agar plates. The surplus culture was removed with a pipette and the surface of the plate allowed to dry for $\frac{1}{2}$-1 h. Overnight BHI cultures of each of the test strains were then spotted onto the surface of the inoculated plates using a multiple loop technique similar to that used for phage typing. The double inoculated BHI plates were then incubated at 30°C overnight.

ii. Antagonism by end-products of growth

Overnight BHI broth cultures of the test strains were inoculated in a single streak across the diameter of BHI agar and deoxycholate citrate sucrose agar (DCSA) plates. After incubation at 30°C for 48 h the growth was exposed to chloroform vapour for 30 min. The culture was scraped off the plate using a microscope slide and the plates re-exposed to chloroform for a further 30 min. The surface of the agar was then exposed to the air for 1 h to allow the chloroform vapour to disperse. Overnight BHI broth cultures of the test strains were streaked across the plates at right angles to the original growth line and incubated at 30°C for 24 h. Glass petri dishes were used for this work because the plastic variety are dissolved by chloroform.

b) The effect of dyes and other chemicals on the growth of Yersinia spp. and other organisms

i. Effect of dyes and other agents incorporated into Brain Heart Infusion agar and GN agar

The reference strains studied were:-

Y. enterocolitica serotype 0:3	Prot. mirabilis
Y. enterocolitica serotype 0:9	S. typhimurium
E. alkalescens	S. flexneri
H. alvei	

The supplementary strains studied were:-

Prot. morganii	Prot. vulgaris
Prot. rettgeri	Providencia sp.

The dyes studied were:-

Brilliant green	Fast green	Methylene blue
Crystal violet	Malachite green	Methyl red
Euflavine	Metachrome yellow	Tartrazine

The chemical agents studied were:-

Ammonium chloride	Potassium chromate	Sodium phosphate
Ammonium oxalate	Potassium permanganate	Sodium taurocholate
Caffeine citrate	Sodium azide	Sulphosalicyclic acid
Calcium chloride	Sodium borate	Tannic acid
Cetrimide	Sodium citrate, tri	Teepol 610
Chloral hydrate	Sodium deoxycholate	Tergitol 7
Lead acetate	Sodium lauryl sulphate	Tween 80

Sterile 1% solutions of all the dyes and chemicals were prepared by dissolving in distilled water and sterilizing for 10 min at 115°C.

Gram-negative agar (GN agar) was prepared by adding 1.2% powdered agar to distilled water containing dehydrated GN broth powder and the medium sterilized according to the manufacturer's instructions.

To 100 ml quantities of molten Brain Heart Infusion agar and GN agar was added aseptically 1 ml of each of the substrates to give a final concentration of 0.01%. The agars were poured into petri dishes and allowed to set before drying open and inverted at 37°C for 1 h.

Six hour BHI broth cultures of each of the test strains were streaked across the surface of the agars (four strains on each plate)

which were incubated at 30°C for 48 h. Resistant organisms grew heavily right across the streak, sensitive organisms did not grow and inhibited strains were recorded when growth was heavy at the site of original inoculum but was reduced to individual colonies within one cm.

ii. Effect of dyes and other agents incorporated as a gradient in deoxycholate citrate sucrose agar

The reference strains studied were:-

Y. enterocolitica serotype 0:3
Y. enterocolitica serotype 0:6,30
Y. enterocolitica serotype 0:9
Y. pseudotuberculosis
E. cloacae
E. alkalescens
E. coli QC 21043/75
H. alvei
K. aerogenes
Prot. mirabilis
S. typhimurium

The supplementary strains studied were:-

Prot. morganii
Prot. rettgeri
Prot. vulgaris
Providencia sp.
Ps. aeruginosa

The dyes studied were:-

Acid fuchsin
Alpha fast brilliant pink
Acriflavine
Basic fuchsin
Brilliant blue
Brilliant green
Crystal violet
Euflavine
Malachite green
Methyl orange
Methyl violet
Neutral red
Safranin O
Thiazine red
Toluidine blue O
Victoria blue
Water blue

The chemical agents studied were:-

Amino-acetanilide hydrochloride
Ammonium oxalate
Barbitone
Cobaltous sulphate
Iodine (Grams)
Lithium chloride
Mercurochrome
Phenol
Potassium chloride
Potassium iodide
Potassium permanganate
Sodium azide
Sodium bromide
Sodium hexametaphosphate
Sodium pyruvate
Sodium sulphite
Thymol
Triton X100
Vanillin
Zinc acetate

Sterile 1% solutions of all the dyes and chemicals were prepared by dissolving in distilled water and sterilizing for 10 min at 115°C.

The sterile solutions were added (0.5 ml) aseptically to 50 ml molten deoxycholate citrate sucrose agar. The agar was poured into plastic petri dishes which had been propped up on one side so that the agar formed a perfect wedge and was then left undisturbed to set. The plates were returned to the flat position and more molten deoxycholate citrate sucrose agar without any additions was poured in until the surface of the agar was level. The plates were allowed to set and then dried open and inverted at 37°C for 1 h.

Six h BHI broth cultures of each of the test strains were inoculated as single streaks across the surface of the plates beginning from the side containing the least amount of the test substrate. Four strains were placed on each plate which were incubated at 30°C for 48 h. The distance which the test strain grew along the inoculum streak indicated the degree of sensitivity to the test agent and was recorded as follows:- 1 = 25% of the length of the inoculum streak, 2 = 50%, 3 = 75% and 4 = growth along the entire length of the inoculum.

c) Further studies on the effect of malachite green on the growth of Y. enterocolitica and other organisms

i. The effect of varying concentrations of malachite green incorporated into deoxycholate citrate sucrose agar

The reference strains studied were:-

Y. enterocolitica serotype 0:3
Y. enterocolitica serotype 0:6,30
Y. enterocolitica serotype 0:9
Y. pseudotuberculosis
E. cloacae
E. alkalescens
E. coli QC 21043/75
H. alvei
K. aerogenes
Prot. mirabilis
S. typhimurium

The supplementary strains studied were:-

Prot. morganii
Prot. rettgeri
Prot. vulgaris
Providencia sp.
Ps. aeruginosa

Deoxycholate citrate sucrose agar (DCSA) was prepared and whilst still molten sterile 1% malachite green was added to give final concentrations of 0.002%, 0.004%, 0.006%, 0.008% and 0.01%. Plates were poured, allowed to set and dried open and inverted at 37°C for 1 h. Surface colony counts (see page 82) were carried out on 6 h BHI broth cultures of the test strains using the DCSA plus malachite green plates and also a normal DCSA plate. The plates were incubated at 30°C for 48 h.

ii. Identification of the agent combining with malachite green to enhance inhibition

The reference strains studied were:-

Y. enterocolitica serotype 0:3
Y. enterocolitica serotype 0:6,30
Y. enterocolitica serotype 0:9
Y. pseudotuberculosis
E. cloacae
E. alkalescens
E. coli QC 21043/75
H. alvei
K. aerogenes
Prot. mirabilis
S. typhimurium

The supplementary strains studied were:-

Prot. morganii	Providencia sp.
Prot. rettgeri	Ps. aeruginosa
Prot. vulgaris	

Four batches of deoxycholate citrate sucrose agar base were melted, allowed to cool to 60°C and the reaction adjusted to pH 7.5. To batch 1 was added solutions A and B as normal; to batch 2, 0.004% malachite green; to batch 3 solution A and 0.004% malachite green; to batch 4, solution B and 0.004% malachite green. Plates were poured, allowed to set and dried open and inverted at 37°C for 1 h. Surface colony counts (see page 82) of 6 h BHI broth cultures of the test strains were carried out on the four batches of agar media and the plates incubated at 30°C for 48 h.

iii. Determination of the range of the inhibitory property of malachite green/sodium deoxycholate mixture

The strains studied were:-

Y. enterocolitica serotype 0:3	Prot. rettgeri (supplementary list)

Deoxycholate citrate sucrose agar base was melted, the reaction adjusted to pH 7.5 and the standard quantity of solution A added. The agar was distributed into 120 lots of 100 ml each. To each of ten bottles was added the following amounts of sodium deoxycholate - solution B, 0.25, 0.5, 0.75, 1.0, 1.25, 1.50, 1.75, 2.0, 2.25, 2.50, 2.75, 3.0 ml. To one bottle only of each of the above batches of deoxycholate citrate sucrose agar was added the following quantities of 1% malachite green - 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 ml to give final concentration % of 0.001, 0.002, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009, 0.01. Plates were poured, allowed to set and dried open and inverted at 37°C for 1 h. Surface colony counts (see page 82) of 6 h BHI broth cultures of the test strains were carried out on each of the agar media. The plates were incubated at 30°C for 48 h.

After preparation and before use the colour of each of the agar plates was recorded and the pH determined using a flat bed electrode.

Results

1. Evaluation of agar media

a) Colonial characteristics of Y. enterocolitica and other enterobacteria

The major colonial characteristics of the reference strains on some enteric agar media are shown in Tables 20 to 23. The colonial characteristics of four strains of Y. enterocolitica on six agar media are shown in Plates 2 to 7. All six of the Yersinia reference strains failed to grow on thiosulphate citrate bile salt agar (TCBS).

In general all the reference strains grew in a regular shape with an entire edge. Any notable exceptions are mentioned in the following results.

Bismuth sulphite (BS) agar. The colonial characteristics of the reference strains on four brands of BS agar are shown in Table 20. The growth of the Yersinia strains was very poor on all the brands and the largest colonies were only 1 mm diameter. Lab M brand (B4) was the only one on which all six strains grew. Yersinia enterocolitica 0:9 failed to grow on Oxoid (B1), Y. pseudotuberculosis failed to grow on Difco (B2) and neither Y. enterocolitica 0:3 nor Y. pseudotuberculosis grew on BBL (B3). The colonies of the Yersinia spp. were generally a brown/green colour and did not blacken the agar or have a metallic sheen.

Where they grew the colonies of the other reference strains were larger (up to 2 mm diameter) and many were also brown/green in colour. Salmonella typhimurium produced typical colonies with blackening of the agar and a metallic sheen. A slight metallic sheen was also produced by E. cloacae on B3 and B4 and by Prot. mirabilis on B1 and B2.

Brilliant green agar (BG). Yersinia enterocolitica NCTC 10460 and NCTC 10598 did not grow on this medium (Table 21). Yersinia enterocolitica serotypes 0:3, 0:6,30 and 0:9 which fermented sucrose produced green colonies and Y. pseudotuberculosis which was sucrose negative produced a pink colony.

Escherichia alkalescens, E. coli QC 2104 3/75, H. alvei, RM bacterium, and the Shigella spp. failed to grow on this medium. The colonies of

Table 20

Comparison of the colonial characteristics of Y. enterocolitica and other enterobacteria on four brands of bismuth sulphite agar

Brand	Description of colonies	Y. enterocolitica 0:3	Y. enterocolitica 0:6,30	Y. enterocolitica 0:9	Y. enterocolitica NCTC 10460	Y. enterocolitica NCTC 10598	Y. pseudotuberculosis	E. cloacae	E. alkalescens	E. coli QC 21043/75	H. alvei	K. aerogenes	Prot. mirabilis	RM Bacterium	S. typhimurium	S. flexneri	S. sonnei
Oxoid	Size mm	1	<1	No growth	<1	<1	<1	No growth	1	No growth	No growth	1	1-2	No growth	2	No growth	No
	Colour	B/G	B/G		G	B/G	B/G		B/G			B/BL	B/G		B		
	Blackening of agar	-	-		-	-	-		-			-	-		+		
	Metallic sheen	-	-		-	-	-		-			-	(+)		+		
Difco	Size mm	1	<1	<1	<1	<1	No growth	No growth	1	<1	1	No growth	1-2	<1	2	1-2	1
	Colour	B/G	B/G	B/G	G	B/G			B/G	B/G	B/BL		B/G	B/G	B	G	G
	Blackening of agar	-	-	-	-	-			-	-	-		-	-	+	-	-
	Metallic sheen	-	-	-	-	-			-	-	-		(+)	-	+	-	-
BBL	Size mm	No growth	<1	<1	<1	<1	No growth	1	1	<1	No growth	1	1-2	<1	1	No growth	1
	Colour		B/G	B/G	G	B/G		B/BL	B/G	B/G		B/BL	Gr/G	B/G	Gr/G		G
	Blackening of agar		-	-	-	-		-	-	-		-	-	-	-		-
	Metallic sheen		-	-	-	-		(+)	-	-		-	-	-	-		-
Lab M	Size mm	1	<1	1	1	1	<1	1	1	1	1	1-2	1-2	<1	2	1-2	1-
	Colour	B/G	B/G	B/G	B/G	B/G	B/G	B/BL	B/G	B/G	B/BL	B/BL	Gr/G	B/G	B	G	G
	Blackening of agar	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
	Metallic sheen	-	-	-	-	-	-	(+)	-	-	-	-	-	-	+	-	-

B = brown; G = green; BL = black; Gr = grey; + = positive; (+) = weak reaction; - = no reaction.

E. cloacae and K. aerogenes were larger than those of the Yersinia spp., being 3 and 6 mm diameter respectively, but were also green. Salmonella typhimurium was similar in size, colour and elevation to Y. pseudotuberculosis and Prot. mirabilis colonies swarmed over the agar.

Deoxycholate citrate agar (DCA). The detailed colonial characteristics of the reference strains on this medium were not recorded but Plate 2 shows the colonial variations of four strains of Y. enterocolitica. All the strains produced smooth, regular shaped colonies with an entire edge. Serotypes 0:3 and 0:5,27 (Y46) were of low convex elevation but serotype 0:6,30 (Y63) was flatter and more effuse. The colonies of all three strains which did not ferment lactose were semi-translucent and colourless. Serotype 0:8 (Y136) which fermented lactose produced raised colonies which were more opaque and pale pink.

Deoxycholate citrate sucrose agar (DCSA). With the exception of Y. enterocolitica NCTC 10460 which was <1 mm diameter all the Yersinia spp. grew 1-3 mm diameter irregular shaped pink (sucrose and/or lactose fermenting) colonies (Table 21).

Only E. cloacae, E. coli QC 21043/75, K. aerogenes, and S. sonnei produced similar pink colonies but these were generally smaller, 1-2 mm, with the exception of E. cloacae which was 1-4 mm diameter. All the other strains produced colourless colonies.

Plate 3 illustrates the colonial variations of four Y. enterocolitica strains on DCSA. Each of the strains grew as irregular shaped, pale pink colonies (sucrose and/or lactose fermentation) and serotype 0:8 (Y136) had a marked crater-like surface.

Eosin methylene blue agar (EMB). The colonies of Yersinia spp. varied from 1-3 mm diameter and Y. enterocolitica NCTC 10460 and 10598 produced pink colonies whereas the other Yersinia strains were a definite mauve (Table 21).

The colonies of the other reference strains also grew to 1-3 mm diameter with the exception of E. cloacae which was from 2-8 mm diameter. Escherichia alkalescens, E. coli, H. alvei, K. aerogenes, S. typhimurium and S. sonnei also produced pink colonies (E. coli had a metallic sheen) and were usually of a low convex elevation.

Hektoen enteric agar (HEK). The Yersinia strains with the exception of Y. pseudotuberculosis produced orange, sucrose fermenting colonies 1-3 mm

Table 21

Comparison of the colonial characteristics of Y. enterocolitica and other enterobacteria on brilliant green, deoxycholate citrate sucrose and eosin methylene blue agars

Medium	Description of colonies	Y. enterocolitica O:3	Y. enterocolitica O:6,30	Y. enterocolitica O:9	Y. enterocolitica NCTC 10460	Y. enterocolitica NCTC 10598	Y. pseudotuberculosis	E. cloacae	E. alkalescens	E. coli QC 21043/75	H. alvei	K. aerogenes	Prot. mirabilis	RM bacterium	S. typhimurium	S. flexneri	S. sonnei
Brilliant green agar	Size mm	1	2	2	No growth	No growth	2	6	No growth	No growth	No growth	3	Swarming growth	No growth	2	No growth	
	Colour	G	G	G			P	G				G			P		
	Elevation	LC	LC	LC			LC	D				Rd			LC		
	Surface/density	S/T	S/O	S/O			S/O	S/O				R/O			S/T		
Deoxycholate citrate sucrose agar	Size mm	2	3	2	1	1	2	1-4	1	1	1	1	1	2	2	1	1
	Colour	P	P	P	P	P	P	P	-	P	-	P	-	-	-	-	P
	Elevation	Rd	U	Rd	Rd	D	LC	D	LC	LC	LC	D	LC	LC	LC	LC	C
	Surface/density	R/O	S/O	R/O	R/O	S/O	S/O	S/O	S/O	R/O	S/O	R/O	S/O	S/T	S/T	S/T	S/
Eosin methylene blue agar	Size mm	2	1-3	1-2	1-2	1	2	2-8	3	2-3	1-2	1-2	Swarming growth	1	2	1-2	1
	Colour	M	M	M	P	P	M	M	P	P/MS	P	P(B)		M	P	-	P
	Elevation	LC	U	U	Rd	Rd	LC	D	E	R	LC	Rd		LC	LC	LC	I
	Surface/density	S/O	S/O	R/O	S/O	S/O	S/T	S/O	S/T	S/O	S/T	R/O		S/T	S/T	S/O	S/

P = pink; M = mauve; (B) = black centre; MS = metallic sheen; Rd = raised; U = umbonate; D = domed; LC = low convex; C = convex; E = effuse; R = rough; S = smooth; O = opaque; T = translucent; - = no reaction.

Plate 2. Colonial variations of four strains of Y.enterocolitica on deoxycholate citrate agar

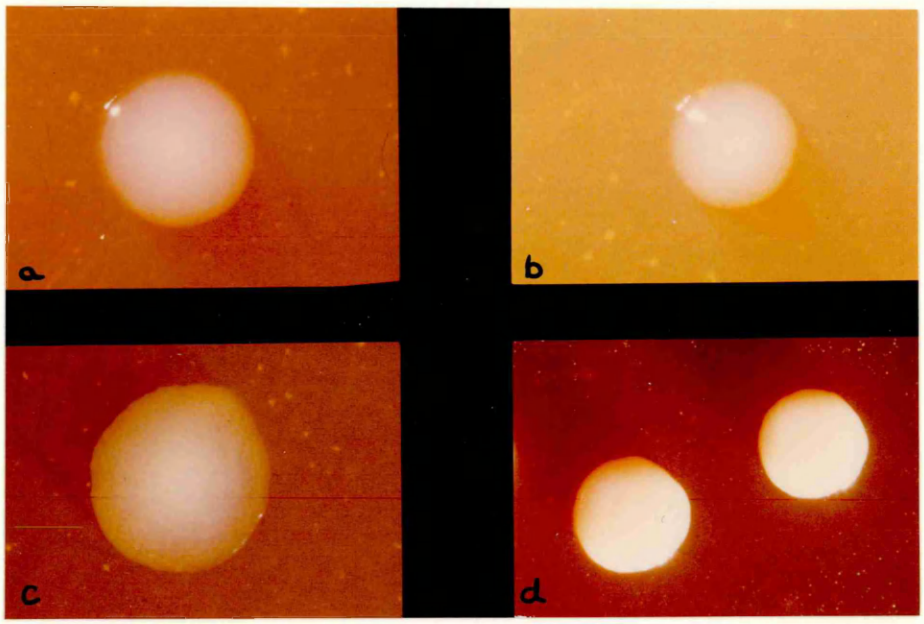
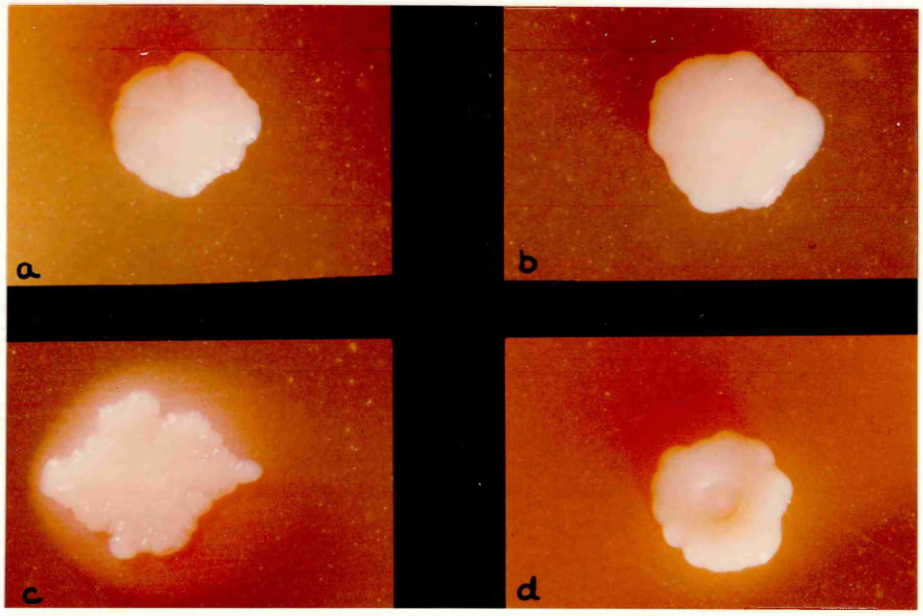


Plate 3. Colonial variations of four strains of Y.enterocolitica on deoxycholate citrate sucrose agar



Plates 2 and 3. a = serotype 0:3; b = serotype 0:5,27 (Y46); c = serotype 0:6,30 (Y63); d = serotype 0:8 (Y136).

diameter (Table 22). Yersinia pseudotuberculosis being sucrose negative was green in colour. The elevation of the Yersinia strains was raised or domed.

The other reference strains were slightly larger, 1-4 mm diameter and only E. cloacae, E. coli and K. aerogenes fermented sucrose and produced orange colonies but these organisms were, however, similar in elevation to the Yersinia spp. The remaining strains produced green colonies and were of low convex elevation.

Lactose sucrose urea agar (LSU). The five strains of Y. enterocolitica produced cream/green colonies from lactose and/or sucrose fermentation and were of domed elevation and Y. pseudotuberculosis produced a pink low convex colony (Table 22). All six strains were approximately 2-3 mm diameter and the underlying agar was pink. Older cultures were surrounded by a very narrow zone of pink in the agar (urease production). The Y. enterocolitica strains also had a characteristic strong sweet and sour smell.

The other reference strains varied from 1-2 mm diameter and all but H. alvei, RM bacterium and S. typhimurium (which were pink) produced variations of cream, green and white colonies which were generally not domed. Proteus mirabilis and RM bacterium also produced a pink zone in the agar round the colonies but the zone round Proteus sp. spread far into the agar eventually affecting the whole plate.

Plate 4 shows the colonial variations of four Y. enterocolitica strains on LSU. There was a slight variation in colony size but all the strains were smooth, domed, more opaque in the centre than on the periphery and had a soft butyricus appearance. Serotype 0:8 (Y136) was slightly more opaque at the centre than the other strains because of lactose and sucrose fermentation. All had the characteristic smell. The pink colouration of the agar is not visible in this photographic plate because of the lighting used to highlight the surface of the colony.

MacConkey agar (MAC). The Yersinia spp. had colonies between 1-4 mm diameter and with the exception of Y. enterocolitica 0:6,30 were colourless (Table 22). Yersinia enterocolitica 0:6,30 fermented lactose and produced a pink colony.

The colonies of the other reference strains were 1-4 diameter and all were colourless except E. cloacae, E. coli, K. aerogenes and S. sonnei.

Plate 5 shows the colonial variations of the four strains of Y. enterocolitica on MAC. There was a slight variation in colony size,

Table 22

Comparison of the colonial characteristics of Y. enterocolitica and other enterobacteria on Hektoen enteric, lactose sucrose urea and MacConkey agars

Medium	Description of colonies	Y. enterocolitica 0:3	Y. enterocolitica 0:6,30	Y. enterocolitica 0:9	Y. enterocolitica NCTC 10460	Y. enterocolitica NCTC 10598	Y. pseudotuberculosis	E. cloacae	E. alkalescens	E. coli QC 21043/75	H. alvei	K. aerogenes	Prot. mirabilis	RM bacterium	S. typhimurium	S. flexneri	S. sonnei
Hektoen enteric agar	Size mm	2	1-3	2	1	1	1	2-3	2	1-2	1-2	2	1-3	2-3	2	1-2	2-
	Colour	Or	Or	Or	Or	Or	G	Or	G	Or	G	Or	G(B)	G	G(B)	G	G
	Elevation	Rd	Rd	Rd	Rd	D	D	D	LC	U	LC	Rd	LC	LC	LC	LC	L
	Surface/density	R/O	S/O	S/O	R/O	S/O	S/T	S/O	S/O	R/O	S/T	R/O	S/O	R/O	S/T	S/O	S/
Lactose sucrose urea agar	Size mm	2	2	2	2-3	2	2-3	1-2	2	1-2	1	1-2	2	1	2	1-2	1-
	Colour	Cr/G	Cr/G	Cr/G	Cr/G	Cr/G	P	Cr	Gr/W	Cr/G	P	G	Gr	P	P	P/Gr	Cr
	Elevation	D	D	D	D	D	LC	D	U	LC	LC	LC	LC	LC	LC	LC	L
	Surface/density	S/O	S/O	S/O	S/O	S/O	S/T	S/O	S/O	S/O	S/O	R/O	S/O	S/O	S/T	S/T	S/
	Urease production	+	(+)	(+)	(+)	(+)	(+)	-	-	-	-	-	+	+	-	-	-
MacConkey agar	Size mm	2	3	2	1	1	2-4	3-4	2-3	3-4	1-3	2-3	2-3	2	3	1	2
	Colour	-	P	-	-	-	-	P	-	P	-	P	-	-	-	-	P
	Elevation	LC	LC	LC	LC	LC	E	D	LC	LC	LC	D	LC	LC	LC	LC	L
	Surface/density	S/T	S/T	S/T	S/T	S/T	R/T	S/O	S/O	R/O	S/T	S/O	S/O	S/O	S/T	S/O	R/

Or = orange; G = green; (B) = black centre; Cr = cream; P = pink; Gr = grey; W = white; Rd = raised; D = domed; LC = low convex; U = umbonate; E = effuse; R = rough; S = smooth; O = opaque; T = translucent; + = positive; (+) = weak reaction; - = no reaction.

Plate 4. Colonial variations of four strains of Y.enterocolitica on lactose sucrose urea agar

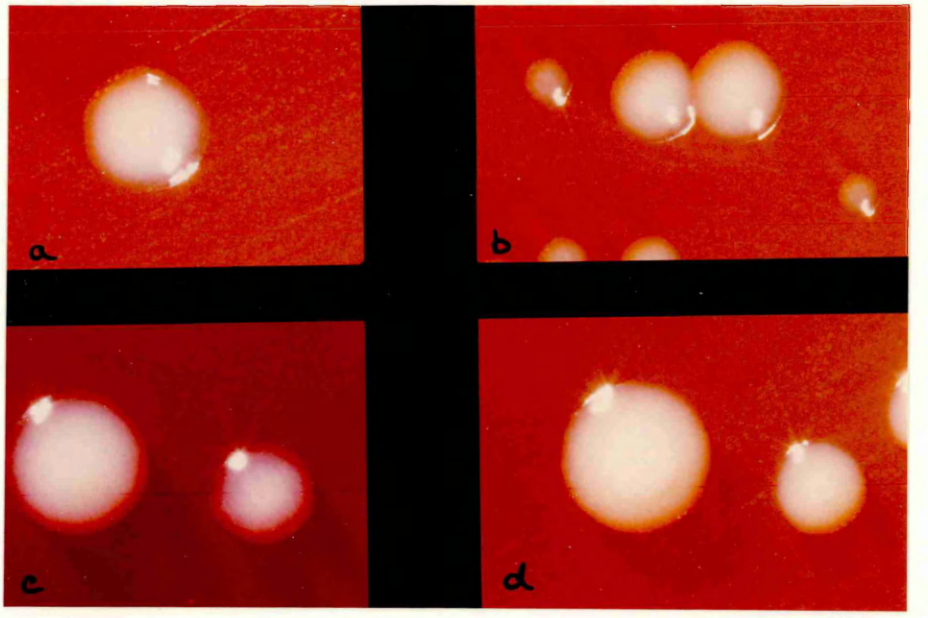
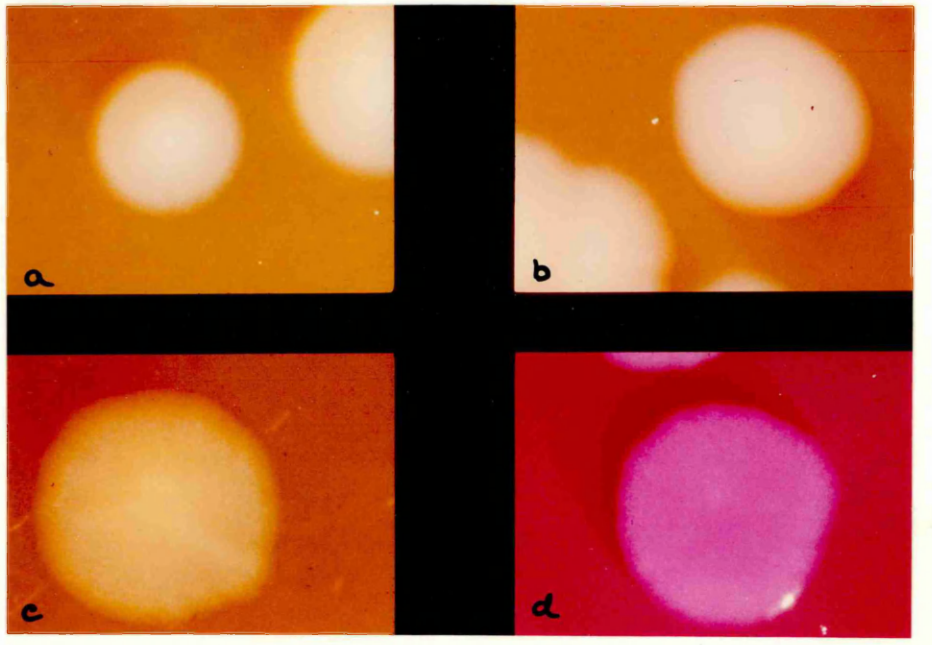


Plate 5. Colonial variations of four strains of Y.enterocolitica on MacConkey agar



Plates 4 and 5. a = serotype 0:3; b = serotype 0:5,27 (Y46); c = serotype 0:6,30 (Y63); d = serotype 0:8 (Y136).

serotypes 0:3, 0:5,27 (Y46) and 0:6,30 (Y63) were semi-translucent and colourless and serotype 0:8 (Y136) was slightly opaque and pink.

Salmonella-Shigella agar (SS). Yersinia enterocolitica 0:6,30 and Y. pseudotuberculosis failed to grow on this medium (Table 23). The remaining Yersinia strains had colourless colonies of 1 mm or less in diameter.

The colonies of the other reference strains were much larger, 1-2 mm and even up to 4 mm (E. cloacae) diameter. Escherichia alkalescens, H. alvei, Proteus, S. typhimurium and S. flexneri all had colourless colonies and the remaining strains were pink due to lactose fermentation, the Proteus colonies also had black centres. RM bacterium did not grow.

The colonial variations of the four strains of Y. enterocolitica are shown in Plate 6. Serotypes 0:5,27 (Y46) and 0:6,30 (Y63) grew considerably larger than serotype 0:3 or 0:8 (Y136). The colonies of serotypes 0:5,27, 0:6,30 and 0:8 were effuse and colourless although there was a slight pink tinge to serotype 0:5,27. Serotype 0:8 produced raised, opaque, bright pink colonies.

Xylose lysine deoxycholate agar (XLD). The Yersinia strains had small yellow colonies 1-2 mm diameter which in the main had a draughtsman-like appearance (Table 23).

The other reference strains produced larger colonies 1-4 mm which with the exception of Proteus (black centre) and RM bacterium were also yellow.

Deoxyribonuclease agar (DNA). All the reference strains were pink and did not produce DNA reaction (Table 24).

Lee agar (LEE). The colonies of the Yersinia reference strains were 1-2 mm diameter and colourless except for Y. enterocolitica 0:6,30 which was pink (lactose fermentation) (Table 24). Yersinia enterocolitica 0:3 and 0:9 and Y. pseudotuberculosis produced regular shaped colonies with an entire edge and were lipase negative. Yersinia enterocolitica 0:6,30 and NCTC 10598 had irregular filamentous shaped colonies and produced a precipitate in the surrounding agar which was reflective to light on the surface (Tween reaction indicating lipase production).

The other reference strains had larger colonies 1-4 mm diameter. Enterobacter cloacae, E. coli, K. aerogenes and RM bacterium were all lactose fermenting (pink colonies) and the latter produced a Tween

Table 23

Comparison of the colonial characteristics of Y. enterocolitica and other enterobacteria on Salmonella-Shigella and xylose lysine deoxycholate agars

Medium	Description of colonies	Y. enterocolitica 0:3	Y. enterocolitica 0:6,30	Y. enterocolitica 0:9	Y. enterocolitica NCTC 10460	Y. enterocolitica NCTC 10598	Y. pseudotuberculosis	E. cloacae	E. alkalescens	E. coli QC 21043/75	H. alvei	K. aerogenes	Prot. mirabilis	RM bacterium	S. typhimurium	S. flexneri	S. sonnei
Salmonella-Shigella agar	Size mm	1	No growth	1	<1	<1	No growth	1-4	2	1	1-2	1	1-2	No growth	2	1	1-2
	Colour	-		-	-	-		P	-	P	-	P	-(B)		-	-	P
	Elevation	LC		LC	LC	LC		D	LC	D	LC	D	LC		LC	LC	C
	Surface/density	S/T		S/T	S/T	S/O		S/O	S/O	S/O	S/O	S/O	S/O		S/T	S/T	S/T
Xylose lysine deoxycholate agar	Size nm	2	1	1	<1	1	2	1-4	2	2-3	1-3	2	2-4	2	2	1	1-2
	Colour	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	-(B)	-	Or(B)	P	P
	Elevation	Dr	Dr	Dr	LC	Rd	LC	D	LC	LC	LC	D	LC	LC	LC	LC	LC
	Surface/density	R/O	S/O	R/O	S/T	S/O	S/O	S/O	S/O	R/O	S/T	S/O	S/O	S/O	S/T	S/T	S/T

P = pink; (B) = black centre; Y = yellow; Or = orange; LC = low convex; Dr = draughtsman; Rd = raised; D = domed; C = convex; S = smooth; R = rough; O = opaque; T = translucent; - = no reaction.

Plate 6. Colonial variations of four strains of Y.enterocolitica on Salmonella-Shigella agar

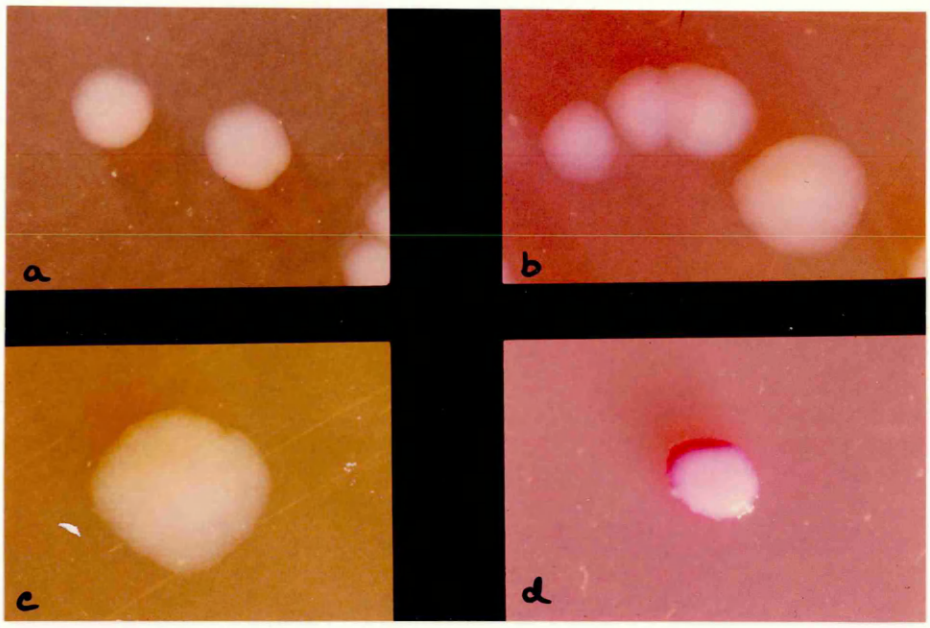
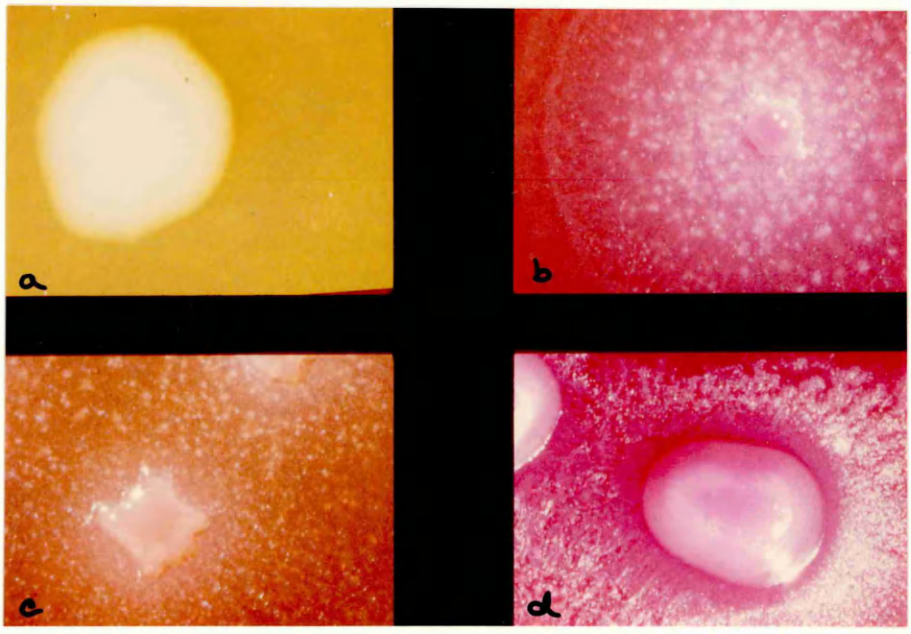


Plate 7. Colonial variations of four strains of Y.enterocolitica on Lee agar



Plates 6 and 7. a = serotype 0:3; b = serotype 0:5,27 (Y46); c = serotype 0:6,30 (Y63) d = serotype 0:8 (Y136).

Table 24

Comparison of the colonial characteristics of Y. enterocolitica and other enterobacteria on deoxyribonuclease, Lee and Salmonella-Shigella plus deoxycholate agars

Medium	Description of colonies	Y. enterocolitica 0:3	Y. enterocolitica 0:6,30	Y. enterocolitica 0:9	Y. enterocolitica NCTC 10460	Y. enterocolitica NCTC 10598	Y. pseudotuberculosis	E. cloacae	E. alkalescens	E. coli QC 21043/75	H. alvei	K. aerogenes	Prot. mirabilis	RM Bacterium	S. typhimurium	S. flexneri	S. sonnei
Deoxyribo-nuclease agar	Colour	P	P	P	P	P	P	P	P	P	P	P	Swarming growth	P	P	P	P
	DNase	+	+	-	+	+	-	+	+	-	+	+		+	+	-	+
	Lipase	-	+	-	-	-	+	+	+	+	+	+		+	+	-	+
Lee agar	Size mm	1	1	1	2	1-2	1	4	2-3	2-3	2-3	2	Swarming growth	1	2	1-2	3-4
	Colour	-	P	-	-	-	-	P	-	P	-	P		P	-	-	-
	Elevation	LC	D	LC	D	D	D	D	LC	LC	LC	D		D	LC	LC	LC
	Surface	S	S	S	S	S	S	S	S	S	S	S		S	S	S	R
	Lipase	-	+	-	-	+	+	+	-	+	-	-		+	-	-	-
Salmonella-Shigella plus deoxycholate agar	Size mm	1	<1	1	2	2	No growth	No growth	1-2	1	<0.5	2	No growth	1	<1	1	<1
	Colour	P	P	P	P	P			P	P	-	P		-	-	-	P
	Elevation	LC	LC	LC	LC	LC			LC	Rd	-	D		LC	LC	LC	LC
	Surface/density	S/T	S/T	S/T	S/O	S/T			S/T	R/O	-	S/O		S/T	S/T	S/T	S/T

P = pink; LC = low convex; Rd = raised; D = domed; S = smooth; R = rough; T = translucent; O = opaque; + = positive; - = no reaction.

reaction. Proteus colonies swarmed.

The colonial variations of the four strains of Y. enterocolitica on LEE are shown in Plate 7. The variations were diverse on this medium. Serotype 0:3 was of low convex elevation and colourless with no Tween (lipase) reaction. Serotype 0:5,27 (Y46) was bright pink and domed, almost as high as the colony was wide, had an irregular edge and was surrounded by a strong Tween reaction. Serotype 0:6,30 (Y63) was flatter than 0:5,27, irregular in shape, very pale pink and had a less strong Tween reaction. Serotype 0:8 (Y136) colonies were domed, a regular elongated shape with an entire edge and very bright pink (lactose fermentation) in colour with a strong Tween reaction.

Salmonella-Shigella plus deoxycholate agar (SS + D). Yersinia pseudotuberculosis did not grow and the other five Yersinia spp. produced pink colonies from <1-2 mm diameter (Table 24).

Enterobacter cloacae and Prot. mirabilis did not grow and the other strains were similar in size to the Yersinia spp., <0.5-2.0 mm diameter. Escherichia alkalescens, E. coli, K. aerogenes and S. sonnei all produced pink colonies and the rest of the strains were colourless.

b) Quantitative evaluation of the growth of Y. enterocolitica and other organisms

The results of the quantitative evaluation of the agar media are given in Table 25.

On the six media studied the five Yersinia spp., with the exception of Y. pseudotuberculosis were inhibited by less than 5% of the counts obtained on BHI agar. Yersinia pseudotuberculosis was only slightly inhibited on SS + D agar (6%).

Deoxycholate citrate sucrose agar (DCSA) inhibited completely the growth of all the Gram-positive organisms and that of E. alkalescens, E. coli and Prot. mirabilis by 23, 7 and 10% respectively. The remaining strains showed less than 5% reduction in growth.

Lactose sucrose urea agar (LSU) also inhibited all the Gram-positive strains. The growth of E. cloacae and E. coli was reduced by greater than 50% and that of E. alkalescens and K. aerogenes by 28 and 30% respectively. The other organisms were reduced by less than 5%.

MacConkey agar (MAC) inhibited 2/5 strains of the Gram-positive organisms only and with the exception of E. cloacae all the other reference strains

Table 25

Quantitative evaluation of the growth of Y. enterocolitica and other organisms on various agar media incubated at 30°C for 48 h

Agar medium	Surface colony count expressed as % of the count on Brain Heart Infusion agar																	
	Y. enterocolitica 0:3	Y. enterocolitica 0:6,30	Y. enterocolitica 0:9	Y. enterocolitica NCTC 10460	Y. pseudotuberculosis	E. cloacae	E. alkalescens	E. coli QC 2104 3/75	H. alvei	K. aerogenes	Prot. mirabilis	S. typhimurium	Ps. aeruginosa	B. alvei	B. cereus	Bacillus spp.	Gram-positive coccus A	Gram-positive coccus B
DCSA	99	98	105	99	101	96	77	93	96	99	90	105	95	0	0	0	0	0
LSU	96	98	96	99	97	48	72	42	98	70	96	108	95	0	0	0	0	0
MacConkey	97	99	100	98	102	86	100	101	99	99	100	98	97	0	0	84	108	92
SS agar	96	100	100	99	NT	94	75	46	98	99	99	100	95	0	0	0	0	0
SS + D	97	97	101	100	94	82	79	85	95	79	63	94	95	0	0	0	0	0
XLD agar	96	98	100	103	104	97	102	100	101	102	100	104	95	0	0	0	0	70

DCSA = deoxycholate citrate sucrose agar; LSU = lactose sucrose urea; SS = Salmonella-Shigella agar; SS + D = Salmonella-Shigella plus deoxycholate agar; XLD = xylose lysine deoxycholate agar; NT = not tested.

grew within 5% of the counts on BHI agar.

Salmonella-Shigella agar (SS) inhibited all the Gram-positive organisms and E. coli by greater than 50%, E. alkalescens by 25%, E. cloacae by 6% and the remaining strains grew to within 5% of the BHI agar counts.

Salmonella-Shigella plus deoxycholate agar (SS + D) inhibited all the Gram-positive strains and the other enterobacteria were inhibited between 5% (H. alvei) and 37% (Prot. mirabilis).

Xylose lysine deoxycholate agar (XLD) inhibited 4/5 Gram-positive organisms but all the other reference strains grew to within 5% of the counts on BHI agar.

c) Notes on two additional agar media

i. Pectin agar

Considerable difficulties were experienced in the production of this medium. The pectin formed lumps which had to be broken up by hand whilst keeping the mixture warm. If the medium set it could not be re-melted which also meant that sterilizer space had to be readily available on completion of the medium.

Pectinolytic colonies were, after 48 h incubation, depressed as if sinking into the agar. Several batches of medium had to be made before one was obtained on which Y. enterocolitica grew with the characteristic appearance. The tests described were carried out on this batch of medium.

Seventy-nine of 82 food isolates and 5/6 reference strains produced the pectinolytic reaction. Yersinia enterocolitica 0:3 was consistently negative. The sub-cultures from the enrichment cultures produced a very heavy growth of many organisms and it was impossible to distinguish any pectinolytic colonies.

ii. Sodium oxalate agar

Many organisms grew on this m edium from the enrichment cultures and it was not possible to distinguish Y. enterocolitica without further sub-culturing many colonies to screening media.

2. Evaluation of broth media

a) Qualitative evaluation of the growth of Yersinia spp. and other enterobacteria at various temperatures

The growth of Yersinia spp. and the other enterobacteria in Brain Heart Infusion broth, GN broth and phosphate buffered saline is shown in Table 26 and the growth in selenite cystine broth, selenite F broth and Wauters' broth is shown in Table 27.

Brain Heart Infusion broth (BHI). All the organisms studied grew well within the normal temperature ranges as expected for the different species. The six strains of Yersinia produced turbid growth at all the temperatures from 4°-36°C but only Y. pseudotuberculosis grew at 43°C. Hafnia alvei, K. aerogenes and RM bacterium grew at 4°C as well as the higher temperatures (Table 26).

GN broth (GN). The growth of all the strains in GN was identical to that obtained in BHI broth (Table 26).

Phosphate buffered saline (PS). None of the strains examined showed any sign of growth in PS during the 16 days incubation (Table 26).

Selenite cystine broth (SC). Yersinia enterocolitica 0:9 was the only Yersinia strain able to grow at 4°C in this medium. Hafnia alvei, K. aerogenes and RM bacterium also failed to grow at 4°C. RM bacterium did not grow either at 30°, 36° or 43°C but the growth of the other organisms was similar to that obtained in BHI (Table 27).

Selenite F broth (SF). None of the six Yersinia strains grew at either 4° or 12°C and Y. enterocolitica 0:3 failed to grow at any temperature in this medium. The other enterobacteria also failed to grow at 4° or 12°C with the exception of Prot. mirabilis which grew weakly at 12°C. The Shigella spp. grew only at the higher temperatures (Table 27).

Wauters' broth (WB). The Y. enterocolitica strains grew poorly in this broth and only at 12°, 22° and 30°C. Yersinia pseudotuberculosis did not grow at any temperature. The other strains studied also failed to grow at 4°, 36° or 43°C and at 12°, 22° and 30°C many grew only weakly or not at all (Table 27).

Table 26

Growth of Yersinia spp. and other enterobacteria in Brain Heart Infusion broth, GN broth, and phosphate buffered saline incubated at various temperatures for 16 days

Organism	Brain Heart Infusion broth						GN broth						Phosphate buffered saline					
	Temperature of incubation °C																	
	4	12	22	30	36	43	4	12	22	30	36	43	4	12	22	30	36	43
Y. enterocolitica 0:3	+	+	+	+	+	NG	+	+	+	+	+	NG	NG	NG	NG	NG	NG	NG
Y. enterocolitica 0:6,30	+	+	+	+	+	NG	+	+	+	+	+	NG	NG	NG	NG	NG	NG	NG
Y. enterocolitica 0:9	+	+	+	+	+	NG	+	+	+	+	+	NG	NG	NG	NG	NG	NG	NG
Y. enterocolitica NCTC 10460	+	+	+	+	+	NG	+	+	+	+	+	NG	NG	NG	NG	NG	NG	NG
Y. enterocolitica NCTC 10598	+	+	+	+	+	NG	+	+	+	+	+	NG	NG	NG	NG	NG	NG	NG
Y. pseudotuberculosis	+	+	+	+	+	+	+	+	+	+	+	+	NG	NG	NG	NG	NG	NG
E. cloacae	NG	+	+	+	+	+	NG	+	+	+	+	+	NG	NG	NG	NG	NG	NG
E. alkalescens	NG	+	+	+	+	+	NG	+	+	+	+	+	NG	NG	NG	NG	NG	NG
E. coli QC 21043/75	NG	+	+	+	+	+	NG	+	+	+	+	+	NG	NG	NG	NG	NG	NG
H. alvei	+	+	+	+	+	NG	+	+	+	+	+	NG	NG	NG	NG	NG	NG	NG
K. aerogenes	+	+	+	+	+	+	+	+	+	+	+	+	NG	NG	NG	NG	NG	NG
Prot. mirabilis	NG	+	+	+	+	+	NG	+	+	+	+	+	NG	NG	NG	NG	NG	NG
RM bacterium	+	+	+	+	+	NG	+	+	+	+	+	+	NG	NG	NG	NG	NG	NG
S. typhimurium	NG	+	+	+	+	+	NG	+	+	+	+	+	NG	NG	NG	NG	NG	NG
S. flexneri	NG	NG	+	+	+	+	NG	NG	+	+	+	+	NG	NG	NG	NG	NG	NG
S. sonnei	NG	+	+	+	+	+	NG	+	+	+	+	+	NG	NG	NG	NG	NG	NG

+ = growth; NG = no growth.

Table 27

Growth of Yersinia spp. and other enterobacteria in selenite cystine broth, selenite F broth and Wauters' broth incubated at various temperatures for 16 days

Organism	Selenite cystine broth						Selenite F broth						Wauters' broth					
	Temperature of incubation °C																	
	4	12	22	30	36	43	4	12	22	30	36	43	4	12	22	30	36	43
Y. enterocolitica 0:3	NG	+	+	+	+	NG	NG	NG	NG	NG	NG	NG	NG	(+)	(+)	(+)	NG	NG
Y. enterocolitica 0:6,30	NG	+	+	+	+	NG	NG	NG	+	+	+	NG	NG	(+)	(+)	(+)	NG	NG
Y. enterocolitica 0:9	+	+	+	+	+	NG	NG	NG	+	+	+	NG	NG	(+)	(+)	(+)	NG	NG
Y. enterocolitica NCTC 10460	NG	+	+	+	+	NG	NG	NG	+	+	+	NG	NG	(+)	(+)	NG	NG	NG
Y. enterocolitica NCTC 10598	NG	+	+	+	+	NG	NG	NG	+	+	+	NG	NG	(+)	(+)	(+)	NG	NG
Y. pseudotuberculosis	NG	+	+	+	+	NG	NG	NG	+	+	NG	NG	NG	NG	NG	NG	NG	NG
E. cloacae	NG	+	+	+	+	+	NG	NG	+	+	+	+	NG	+	NG	+	NG	NG
E. alkalescens	NG	+	+	+	+	+	NG	NG	+	+	+	+	NG	NG	+	NG	NG	NG
E. coli QC 21043/75	NG	+	+	+	+	+	NG	NG	+	+	+	+	NG	(+)	(+)	(+)	NG	NG
H. alvei	NG	+	+	+	+	+	NG	NG	+	+	+	(+)	NG	NG	(+)	NG	NG	NG
K. aerogenes	NG	+	+	+	+	+	NG	NG	+	+	+	+	NG	+	(+)	NG	NG	NG
Prot. mirabilis	NG	+	+	+	+	+	NG	(+)	+	+	+	+	NG	NG	NG	NG	NG	NG
RM bacterium	NG	+	+	NG	NG	NG	NG	NG	+	+	+	NG	NG	NG	NG	NG	NG	NG
S. typhimurium	NG	+	+	+	+	+	NG	NG	+	+	+	+	NG	+	(+)	+	NG	NG
S. flexneri	NG	NG	+	+	+	NG	NG	NG	NG	NG	NG	(+)	NG	NG	NG	NG	NG	NG
S. sonnei	NT	NT	NT	NT	NT	NT	NG	NG	NG	NG	+	(+)	NG	NG	NG	NG	NG	NG

+ = growth; (+) = weak growth; NG = no growth; NT = not tested.

b) Quantitative evaluation of the growth of Yersinia spp. and other enterobacteria

i. Growth of Yersinia spp. and other enterobacteria at 30°, 22° and 4°C

The growth of Y. enterocolitica and other organisms in Brain Heart Infusion, GN broth, selenite cystine broth, selenite F broth and Wauters' broth incubated at 30°, 22° and 4°C are shown in Figures 10 to 20.

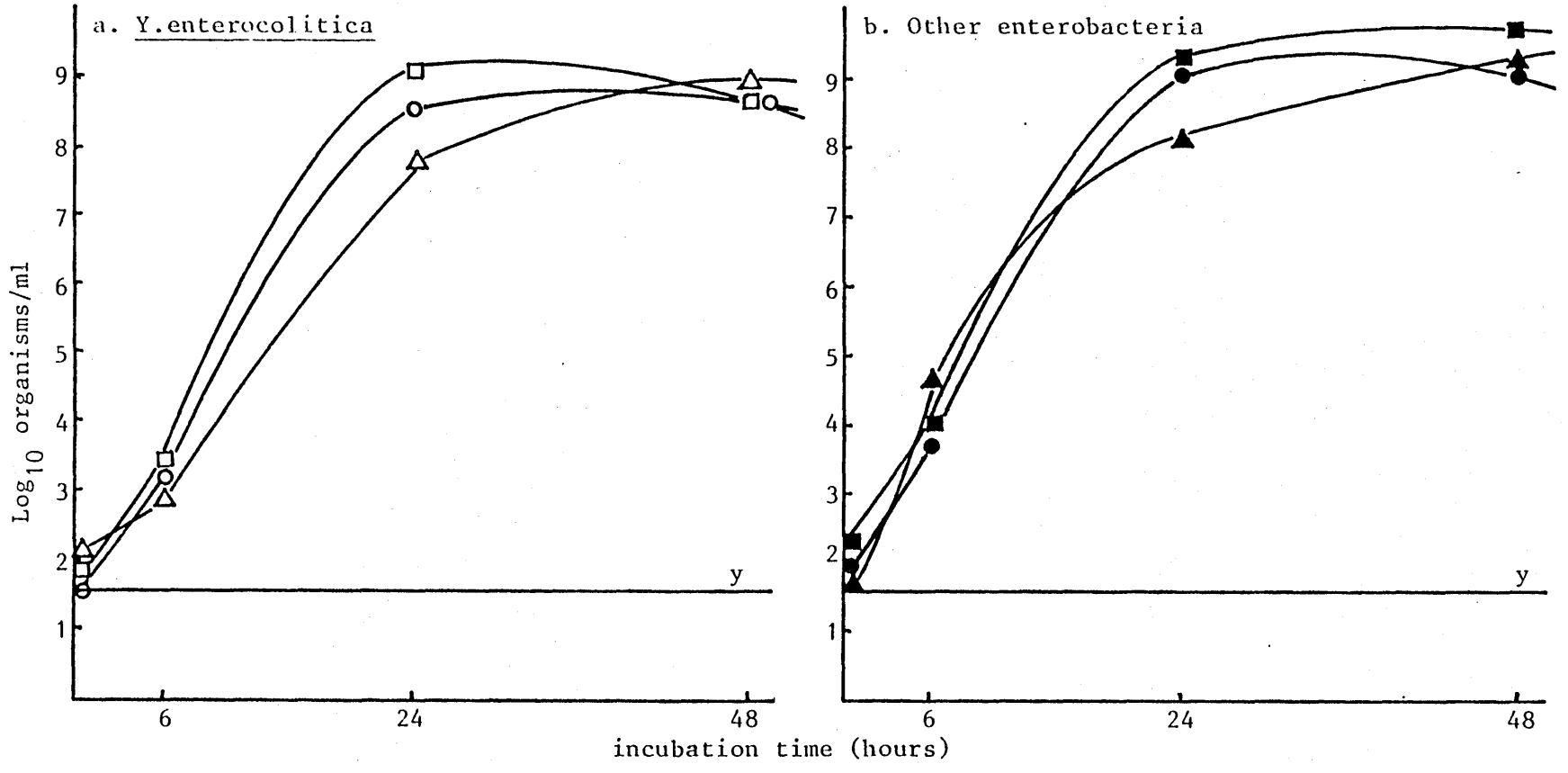
Brain Heart Infusion broth (BHI). The general trends of growth in BHI broth at 30°C are represented by the growth curves illustrated in Figure 10. At 30°C the six Yersinia strains grew rapidly (from an average 75 cells/ml) to between 7.5×10^7 (Y. enterocolitica 0:3) and 1.25×10^9 (Y. enterocolitica 0:6,30) cells/ml within 24 h. Yersinia enterocolitica 0:3 continued to grow steadily and attained 9.75×10^8 cells/ml after 48 h incubation. The numbers of the other Y. enterocolitica strains (not plotted) remained steady and Y. pseudotuberculosis (not plotted) was beginning to decline in numbers by 48 h.

The other enterobacteria grew rapidly from an average 125 cells/ml to between 1×10^8 (H. alvei) and 1.75×10^9 cells/ml (Prot. mirabilis) within 24 h incubation. (The growth curves of K. aerogenes, E. coli and Prot. mirabilis only are plotted). While the counts of H. alvei, E. coli and Prot. mirabilis continued to rise and attained 8×10^8, 1.5×10^9 and 5.75×10^9 cells/ml respectively by 48 h, the remaining enterobacteria, S. typhimurium, K. aerogenes, E. cloacae, E. alkalescens and RM bacterium, began to decline in numbers.

The growth of Y. enterocolitica 0:3 and 0:9 in BHI incubated at 22° and 4°C is shown in Figure 11. At 22°C both Y. enterocolitica 0:3 and 0:9 increased rapidly to 2×10^8 and 2.5×10^8 organisms/ml respectively in 24 h, rising to 1.35×10^9 and 9×10^8 organisms/ml respectively after 48 h incubation. At 4°C the strains attained 2×10^5 and 2×10^6 organisms/ml respectively within 5 days rising to 1.3×10^9 and 1×10^9 organisms/ml within 13 days incubation.

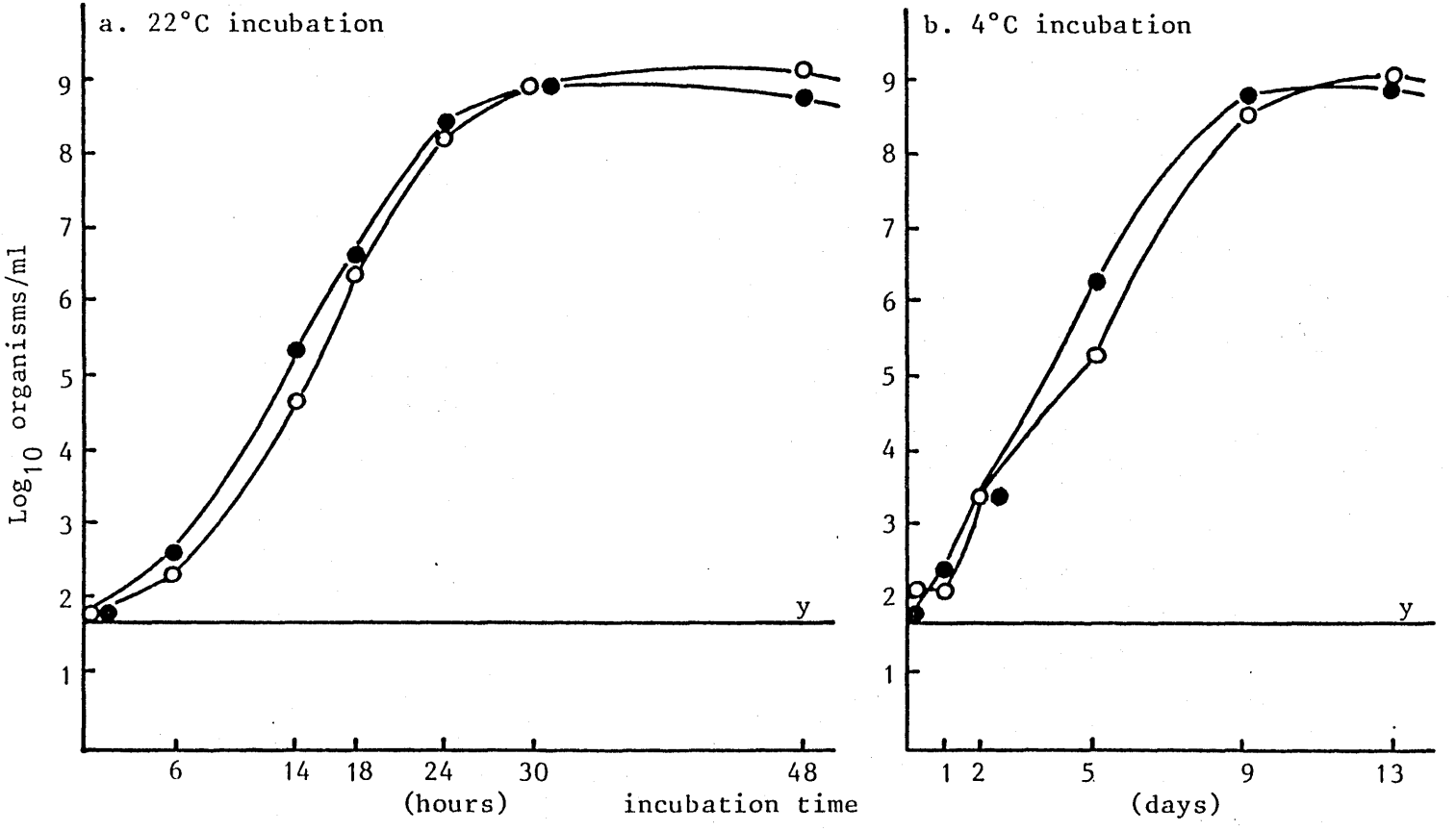
GN broth (GN). The general trends of growth in GN at 30°C are represented by the growth curves illustrated in Figure 12. At 30°C the colony counts of the Yersinia spp. rose rapidly to between x 1×10^8 (Y. enterocolitica 0:3) and 1.75×10^9 (Y. enterocolitica 0:6,30) organisms/ml. Although Y. enterocolitica 0:9 attained 2×10^8

Figure 10. Growth of Y.enterocolitica and other enterobacteria in Brain Heart Infusion broth at 30°C



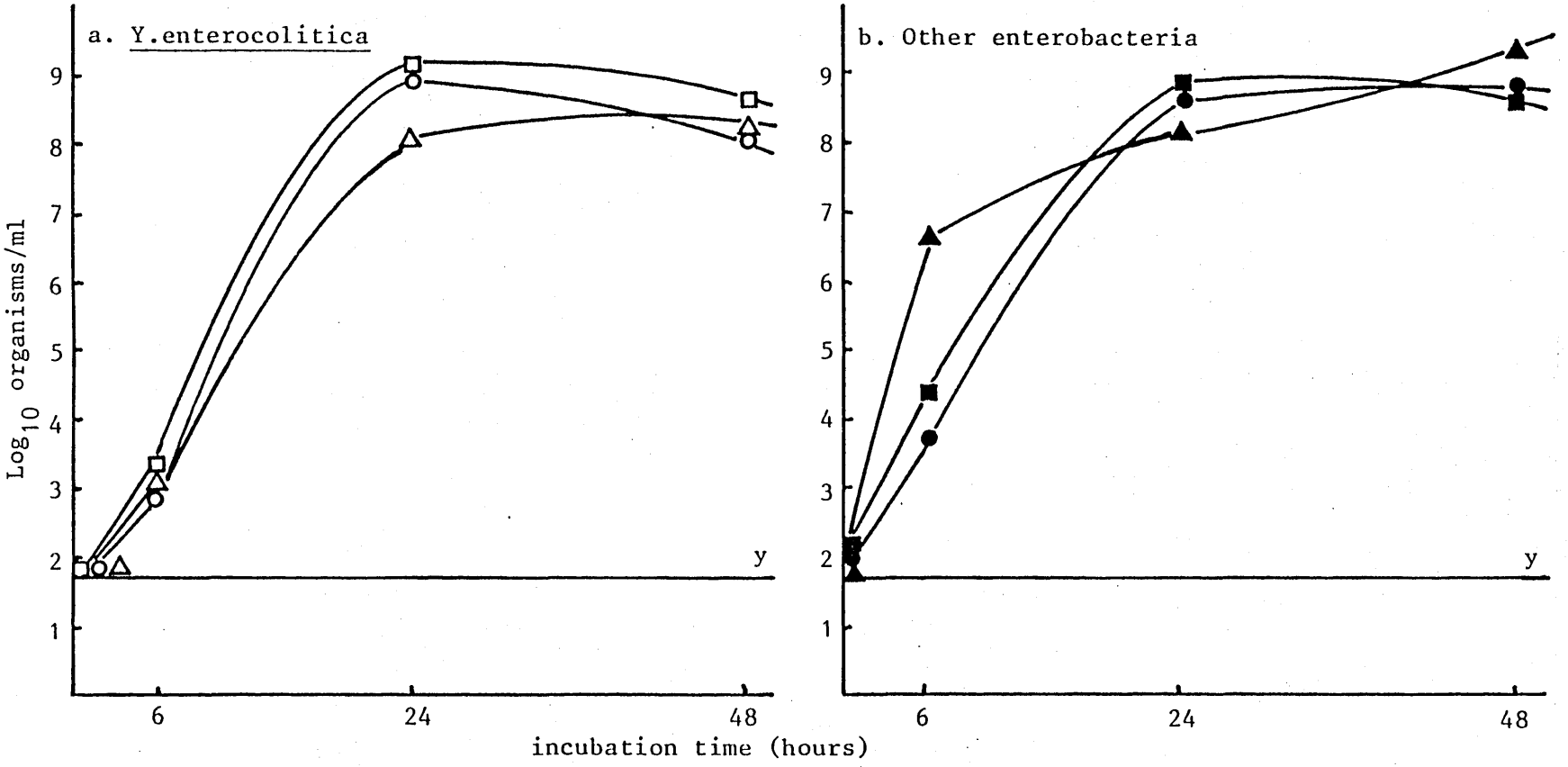
△ = Y.enterocolitica serotype 0:3; □ = Y.enterocolitica serotype 0:6,30; ○ = Y.enterocolitica serotype 0:9; ● = K.aerogenes; ▲ = E.coli; ■ = Prot.mirabilis; y = lower limit of counting method.

Figure 11. Growth of Y.enterocolitica serotypes 0:3 and 0:9 in Brain Heart Infusion broth at 22° and 4°C



O = serotype 0:3; ● = serotype 0:9; y = lower limit of counting method.

Figure 12. Growth of Y.enterocolitica and other enterobacteria in GN broth at 30°C



△ = Y.enterocolitica serotype 0:3; □ = Y.enterocolitica serotype 0:6,30; ○ = Y.enterocolitica serotype 0:9; ● = K.aerogenes; ▲ = E.coli; ■ = Prot.mirabilis; y = lower limit of counting method.

organisms/ml in GN, which was higher (33%) than the count attained in BHI, the colony counts of the other Yersinia strains (not plotted) were on average 36% lower than those attained in BHI after 24 h incubation. After 48 h incubation the numbers of all the Yersinia spp. had begun to decline to between 1.5×10^6 and 5×10^8 organisms/ml and were on average 59% lower than those attained in BHI after the same period.

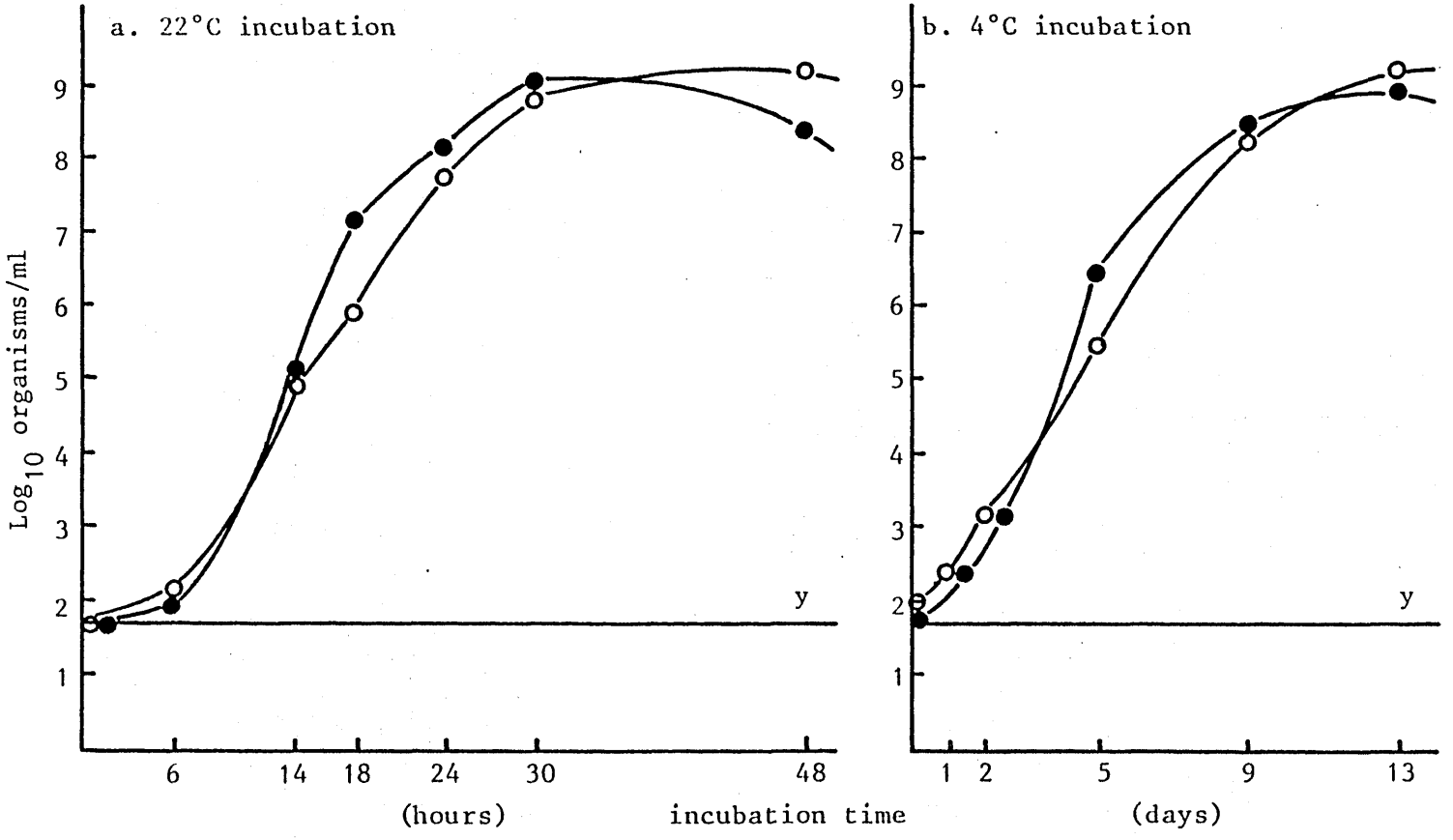
The other enterobacteria studied also increased in growth rapidly to between 1.5×10^8 (E. coli) and 9.75×10^9 organisms/ml (E. cloacae) within 24 h (the growth curves of K. aerogenes, E. coli and Prot. mirabilis only are plotted). Salmonella typhimurium and H. alvei grew better in GN than BHI and attained counts of 6×10^8 and 6.5×10^8 organisms/ml respectively, an average increase of 38%. The growth of the remaining strains was generally lower (average 41%) than those obtained in BHI. After 48 h incubation the colony count of S. sonnei had risen from 2×10^8 at 24 h to 4×10^8 organisms/ml but those of the other enterobacteria had begun to decline to between 4.25×10^7 and 9×10^8 organisms/ml which was on average 60% lower than the counts attained in BHI over the same period.

The growth of Y. enterocolitica 0:3 and 0:9 in GN incubated at 22° and 4°C is shown in Figure 13. At 22°C Y. enterocolitica 0:3 and 0:9 attained colony counts of 4.25×10^7 and 1.5×10^8 organisms/ml respectively in 24 h. Yersinia enterocolitica 0:3 continued to rise to 1.05×10^9 organisms/ml after 48 h but Y. enterocolitica 0:9 having increased in numbers to 9×10^8 organisms/ml after 30 h, declined to 2.25×10^8 organisms/ml after 48 h incubation. At 4°C the strains increased in numbers to 3×10^5 and 3×10^6 organisms/ml respectively in 5 days incubation and had increased to 1.4×10^9 and 7×10^8 organisms/ml after 13 days incubation.

Phosphate buffered saline (PS). This medium was not included in the evaluation of the growth of Yersinia spp. and other enterobacteria at 30°C. The growth of Y. enterocolitica 0:3 and 0:9 in PS incubated at 22° and 4°C is shown in Figure 14.

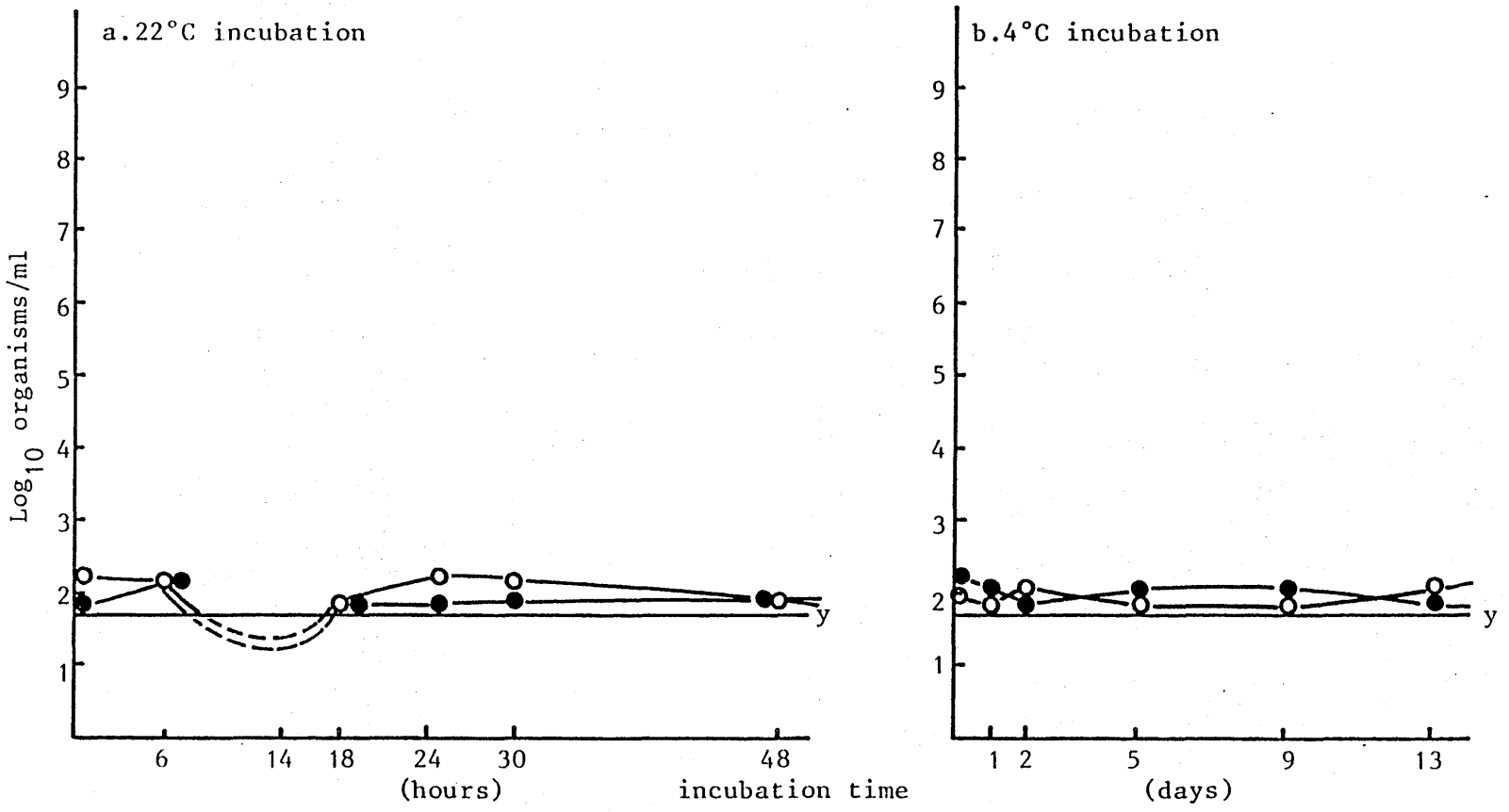
Neither of the Y. enterocolitica strains grew in this medium incubated at either 22° for 48 h or 4°C for 13 days. However, both strains did survive in numbers similar to the initial inoculum of 1×10^2 organisms/ml throughout the period of the experiment.

Figure 13. Growth of Y.enterocolitica serotypes 0:3 and 0:9 in GN broth at 22° and 4°C



○ = serotype 0:3; ● = serotype 0:9; y = lower limit of counting method

Figure 14. Growth of Y.enterocolitica serotypes 0:3 and 0:9 in phosphate buffered saline at 22° and 4°C



O = serotype 0:3; ● = serotype 0:9; y = lower limit of counting method.

Selenite cystine broth (SC). The general trends of growth in this medium at 30°C are represented by the growth curves illustrated in Figure 15. At 30°C Y. enterocolitica serotype 0:6,30 and NCTC strains 10460 and 10598 failed to grow during 72 h incubation. Yersinia enterocolitica 0:9, however, increased very slowly to 4.5×10^4 organisms/ml after 48 h rising to 2×10^7 organisms/ml after 72 h. The colony count of Y. enterocolitica 0:3 rose to 2×10^6 organisms/ml after 48 h incubation and Y. pseudotuberculosis (not plotted) attained 1.65×10^7 organisms/ml only after 72 h incubation.

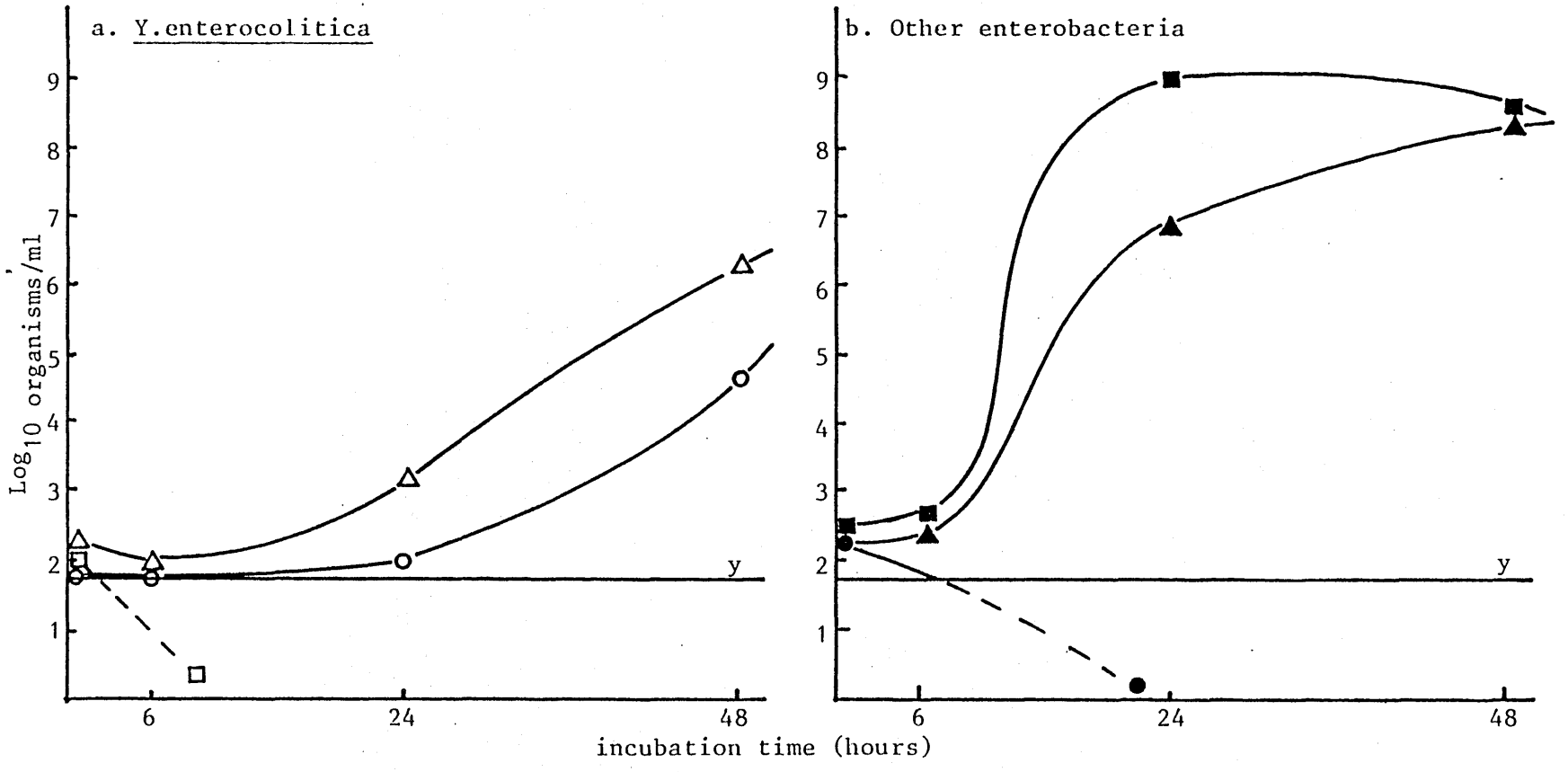
The colony counts of the other enterobacteria were also much reduced in SC and K. aerogenes, RM bacterium and E. alkalescens failed to grow during 72 h incubation (the growth of K. aerogenes, E. coli and Prot. mirabilis only are plotted). Shigella sonnei grew slowly, attaining 2×10^5 organisms in 48 h and 5×10^6 organisms/ml after 72 h incubation. Proteus mirabilis flourished and had attained a colony count of 1.25×10^9 organisms/ml after 24 h but had declined to 4.25×10^8 organisms/ml after 48 h. The remaining strains, H. alvei, E. cloacae, S. flexneri, E. coli and S. typhimurium, attained counts between 7.5×10^4 and 2×10^8 organisms/ml after 24 h rising to between 6×10^7 and 5×10^8 organisms/ml after 48 h incubation.

The growth of Y. enterocolitica 0:3 and 0:9 in SC incubated at 22° and 4°C is shown in Figure 16. At 22°C both Y. enterocolitica 0:3 and 0:9 grew steadily and attained 9×10^4 and 2×10^4 organisms/ml respectively in 24 h and 1×10^8 and 5×10^6 organisms/ml respectively after 48 h incubation. At 4°C these strains attained 2.75×10^6 and 5×10^5 organisms/ml respectively only after 13 days incubation.

Selenite F broth (SF). The general trends of growth in SF at 30°C are represented by those shown in Figure 17. At 30°C Y. enterocolitica 0:3 and Y. pseudotuberculosis (not plotted) failed to survive or grow during 72 h incubation. Yersinia enterocolitica NCTC 10598 (not plotted) did not grow during the first 24 h incubation but the colony count rose steadily to 1.3×10^7 organisms/ml after 48 h incubation. The numbers of Y. enterocolitica 0:3, 0:6,30 and NCTC 10460 (not plotted) increased to between 1.75×10^3 and 1.5×10^5 organisms/ml after 24 h and finally attained 1.75×10^8 - 2.5×10^8 organisms/ml after 48 h.

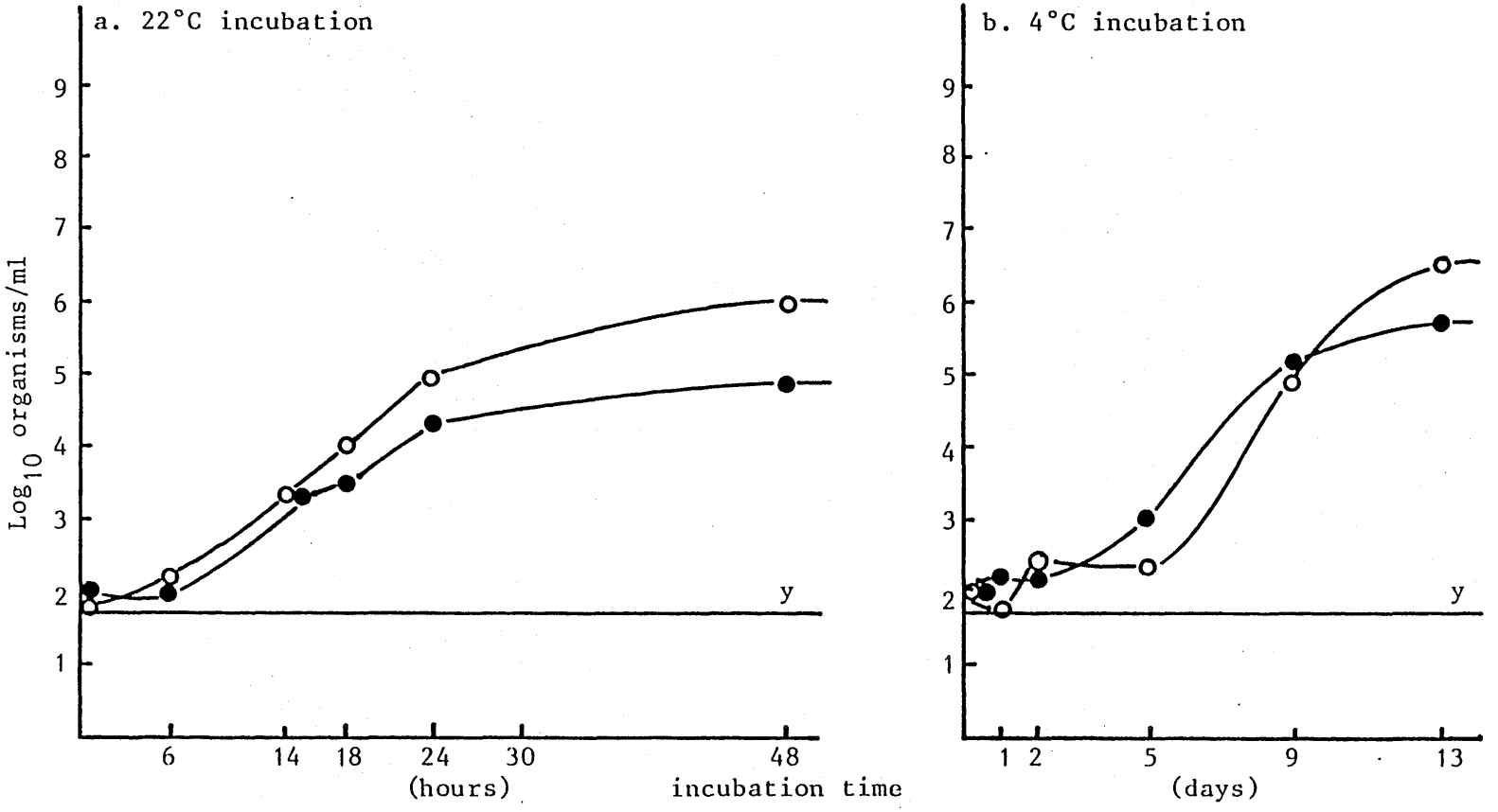
Enterobacter cloacae, E. alkalescens, RM bacterium, S. flexneri and S. sonnei all failed to survive or grow in SF. Hafnia alvei and Prot. mirabilis attained counts of 2×10^6 and 4.5×10^6 organisms/ml

Figure 15. Growth of Y.enterocolitica and other enterobacteria in selenite cystine broth at 30°C



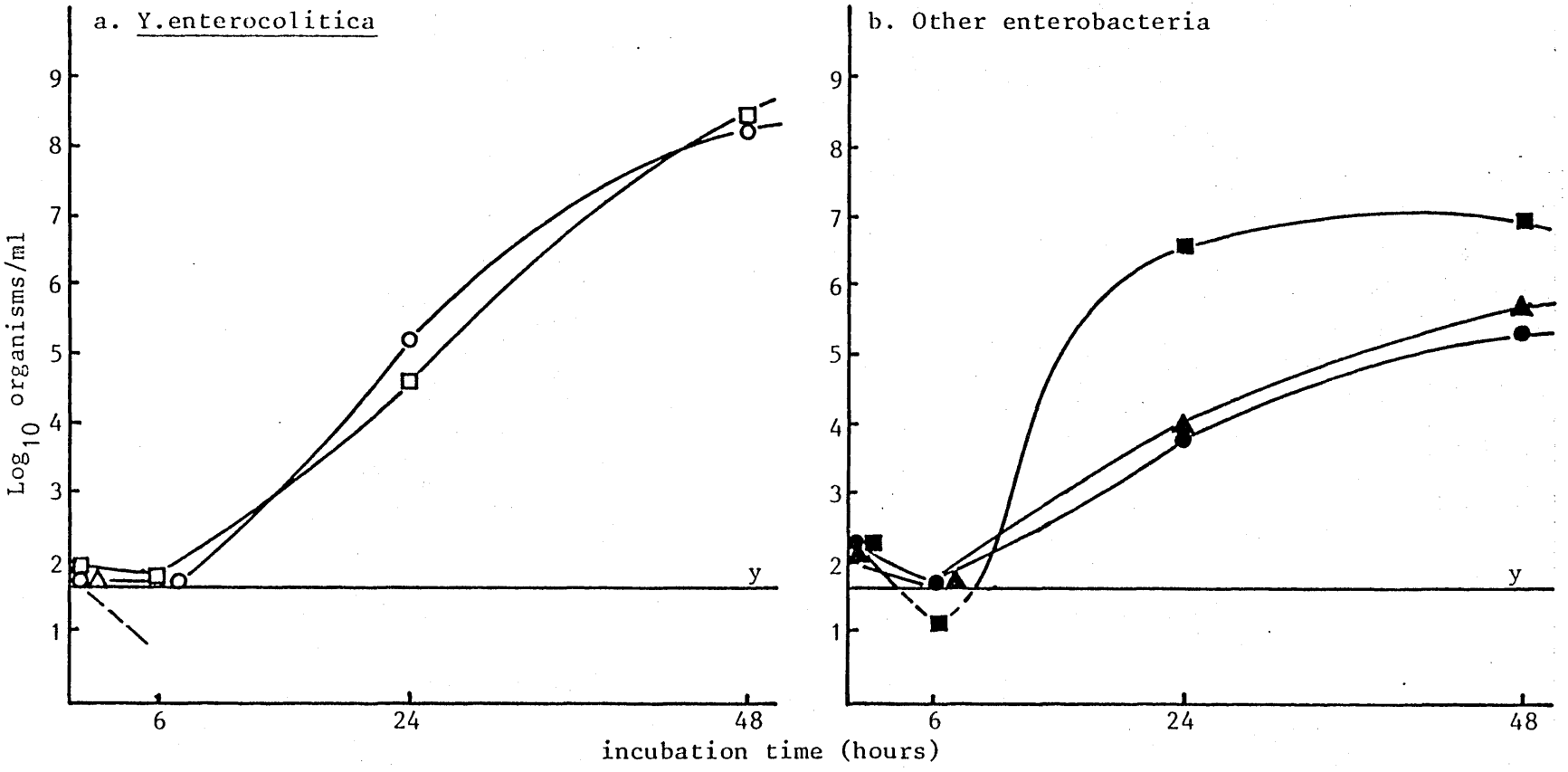
△ = Y.enterocolitica serotype 0:3; □ = Y.enterocolitica serotype 0:6,30; ○ = Y.enterocolitica serotype 0:9; ● = K.aerogenes; ▲ = E.coli; ■ = Prot.mirabilis; y = lower limit of counting method.

Figure 16. Growth of Y.enterocolitica serotypes 0:3 and 0:9 in selenite cystine broth at 22° and 4°C



○ = serotype 0:3; ● = serotype 0:9; y = lower limit of counting method.

Figure 17. Growth of Y.enterocolitica and other enterobacteria in selenite F broth at 30°C



△ = Y.enterocolitica serotype 0:3; □ = Y.enterocolitica serotype 0:6,30; ○ = Y.enterocolitica serotype 0:9; ● = K.aerogenes; ▲ = E.coli; ■ = Prot.mirabilis; y = lower limit of counting method.

respectively after 24 h, rising to 3.25×10^7 and 1×10^7 organisms/ml after 48 h incubation (the growth curves of K. aerogenes, E. coli and Prot. mirabilis only are plotted). Although S. typhimurium grew better than all the other strains and attained 3×10^7 organisms/ml after 24 h incubation the numbers rapidly declined to 4×10^6 organisms/ml after 48 h incubation. The remaining strains, E. coli and K. aerogenes, achieved maximum colony counts of 2.5×10^5 and 5×10^5 organisms/ml only after 48 h incubation.

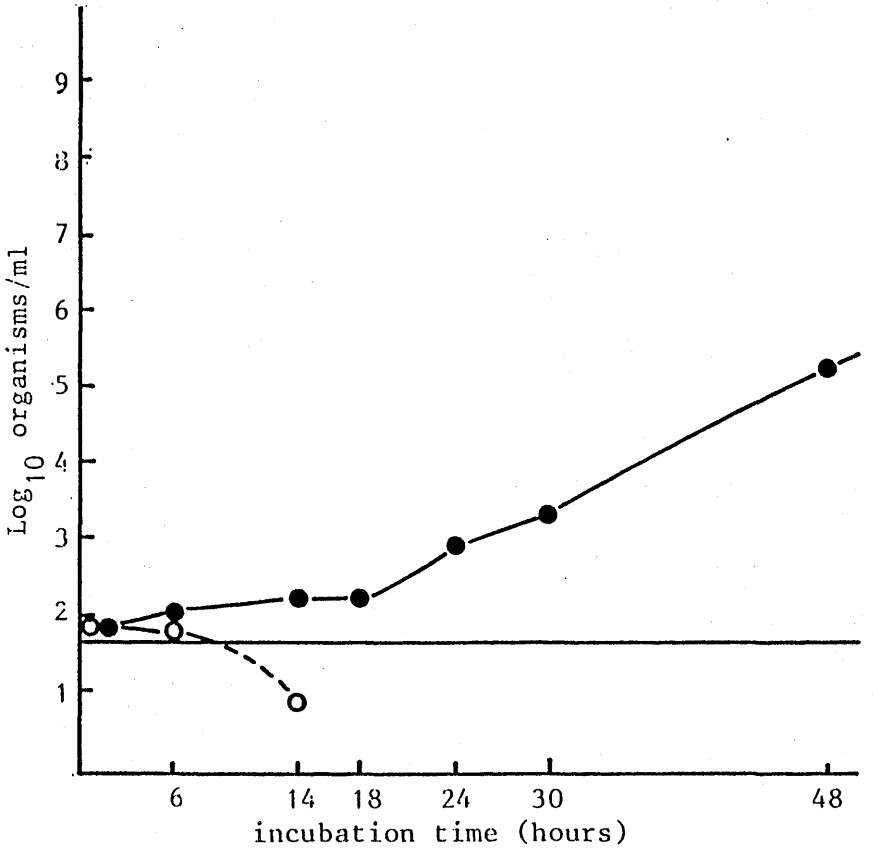
The growth of Y. enterocolitica 0:3 and 0:9 in SF incubated at 22° is shown in Figure 18. At 22°C Y. enterocolitica 0:3 was not recovered after 14 h incubation, whereas Y. enterocolitica 0:9 increased slowly and attained 1.25×10^5 organisms/ml after 48 h. At 4°C neither strain was recovered after the initial count carried out at the time of inoculation (0 h).

Wauters' broth (WB). The general trends of growth in WB at 30°C are shown in Figure 19. Yersinia pseudotuberculosis was the only Yersinia strain which did not grow in WB at 30°C. Yersinia enterocolitica 0:9 and NCTC 10460 (not plotted) grew very slowly and only attained counts of 5×10^4 and 1.25×10^4 organisms/ml respectively in 24 h whereas the other three strains attained counts of between 2.6×10^6 and 4×10^7 organisms/ml during the same period. After 48 h incubation all five strains had attained counts of between 4.75×10^7 and 2.5×10^8 organisms/ml.

Red mouth bacterium and S. sonnei were the only enterobacteria strains which did not grow in WB but Prot. mirabilis and S. flexneri were greatly inhibited (the growth curves of K. aerogenes, E. coli and Prot. mirabilis only are plotted). Shigella flexneri attained only 3×10^3 organisms/ml and the number of Prot. mirabilis remained the same as the initial inoculum throughout 72 h incubation. The remaining enterobacteria, E. cloacae, E. alkalescens, E. coli, H. alvei, K. aerogenes and S. typhimurium attained counts of between 5×10^6 and 1.25×10^8 organisms/ml after 24 h rising to between 6.5×10^6 and 2×10^8 organisms/ml after 48 h incubation.

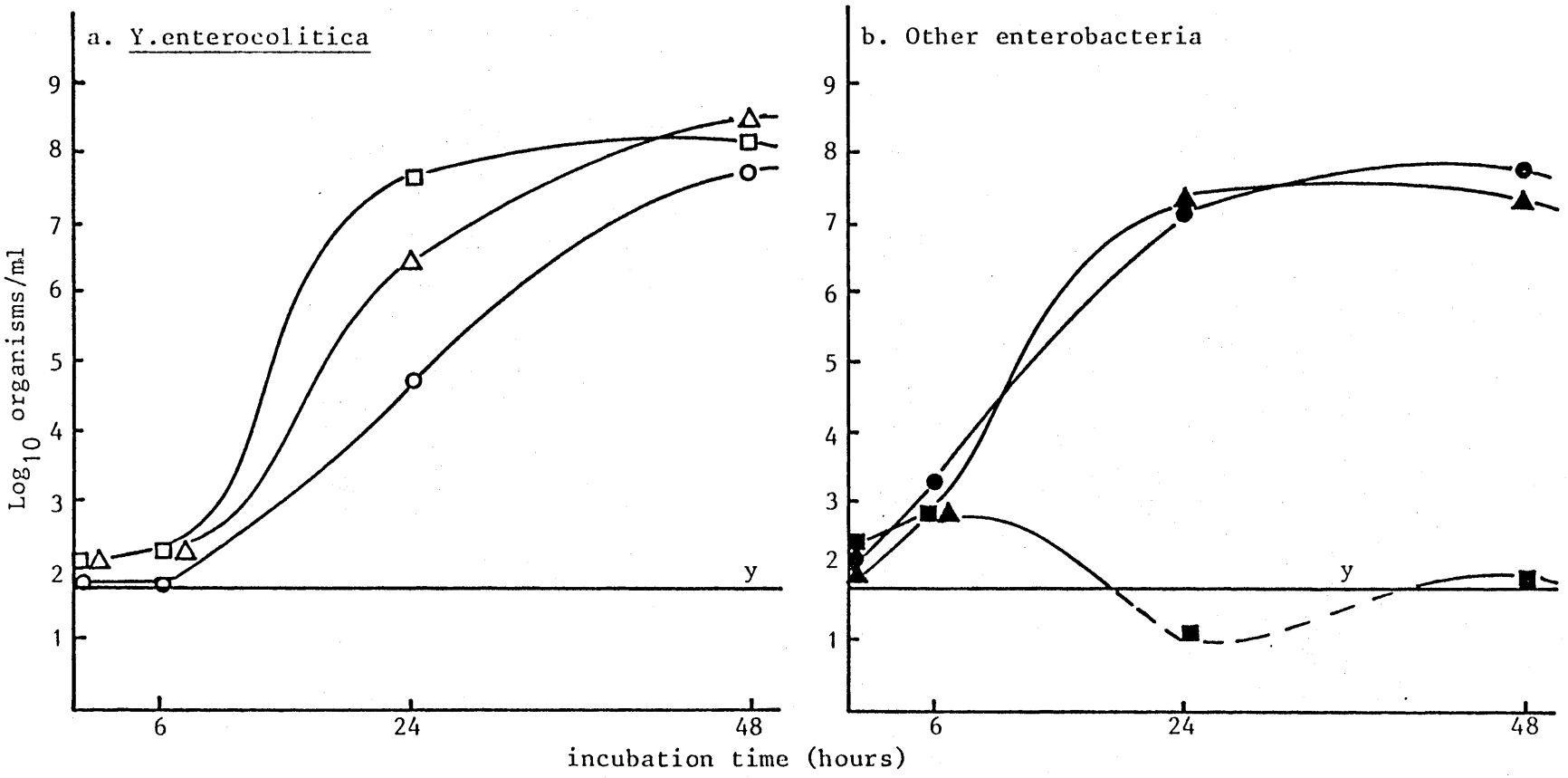
The growth of Y. enterocolitica 0:3 and 0:9 in WB incubated at 22° and 4°C is shown in Figure 20. At 22°C there was very little increase in numbers of either Y. enterocolitica 0:3 or 0:9 during the first 24 h incubation, the strains only attaining 5×10^3 and 1.5×10^4 organisms/ml respectively. Subsequently they increased more rapidly and attained

Figure 18. Growth of Y.enterocolitica serotypes 0:3 and 0:9 in selenite F broth at 22°



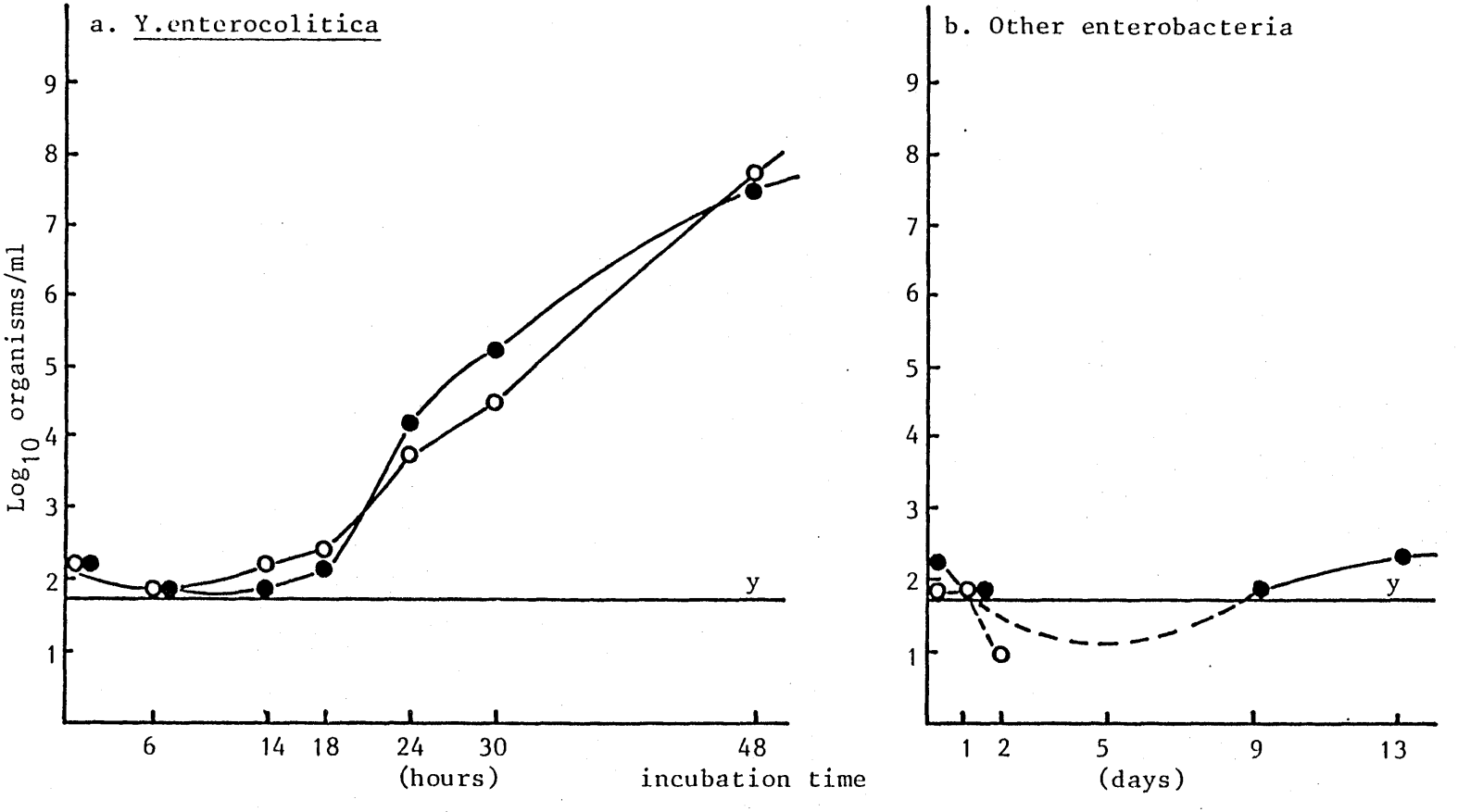
O = serotype 0:3; ● = serotype 0:9; y = lower limit of counting method

Figure 19. Growth of Y.enterocolitica and other enterobacteria in Wauters' broth at 30°C



△ = Y.enterocolitica serotype 0:3; □ = Y.enterocolitica serotype 0:6,30; ○ = Y.enterocolitica serotype 0:9; ● = K.aerogenes; ▲ = E.coli; ■ = Prot.mirabilis; y = lower limit of counting method.

Figure 20. Growth of Y.enterocolitica serotypes 0:3 and 0:9 in Wauters' broth at 22° and 4°C



O = serotype 0:3; ● = serotype 0:9; y = lower limit of counting method.

4.5×10^7 and 3×10^7 organisms/ml respectively after 48 h incubation. At 4°C Y. enterocolitica 0:3 was not recovered after 2 days incubation and Y. enterocolitica 0:9 survived 13 days incubation with only a slight increase in numbers to 2.5×10^2 organisms/ml between 9 and 13 days.

ii. Growth of Yersinia spp. from a minimal inoculum in five broth media incubated at 30° and 22°C

The growth of Yersinia spp. from a minimal inoculum in four enrichment media compared with BHI incubated at 30° and 22°C is shown in Table 28.

GN broth. GN broth was the only medium in which all the strains grew. With the exception of Y. enterocolitica NCTC 10460 and Y. pseudotuberculosis there was very little difference in the results obtained at the two temperatures. These results indicate that 22°C did favour slightly the growth of Y. pseudotuberculosis and 30°C favoured that of Y. enterocolitica NCTC 10460. Because of the very high growth of this latter strain at 30°C the experiment was repeated and similar results obtained.

Selenite cystine broth. Yersinia enterocolitica 0:3 and 0:6,30 were the only strains to grow from a minimal inoculum in this medium and only then at 30°C.

Selenite F broth. Yersinia enterocolitica 0:9 was the only strain to grow in this medium and again only at 30°C.

Wauters' broth. Yersinia enterocolitica 0:3, 0:9 and NCTC 10598 all grew in WB at 30°C but only the latter strain also grew at 22°C.

c) The evaluation of two additional broth media

i. Growth rates at 30°, 22° and 4°C

The growth of Y. enterocolitica 0:3 and 0:6,30 in buffered peptone (BP) and supplemented phosphate buffer (S) incubated at 30°, 22° and 4°C is shown in Figures 21 and 22 respectively.

Both strains grew rapidly in BP (Figure 21) incubated at both 30° and 22°C and attained $>1 \times 10^8$ organisms/ml within 24 h incubation and remained at the same level during 48 h. At 4°C serotype 0:6,20 attained similar levels to those attained at 22°C within 9 days and was

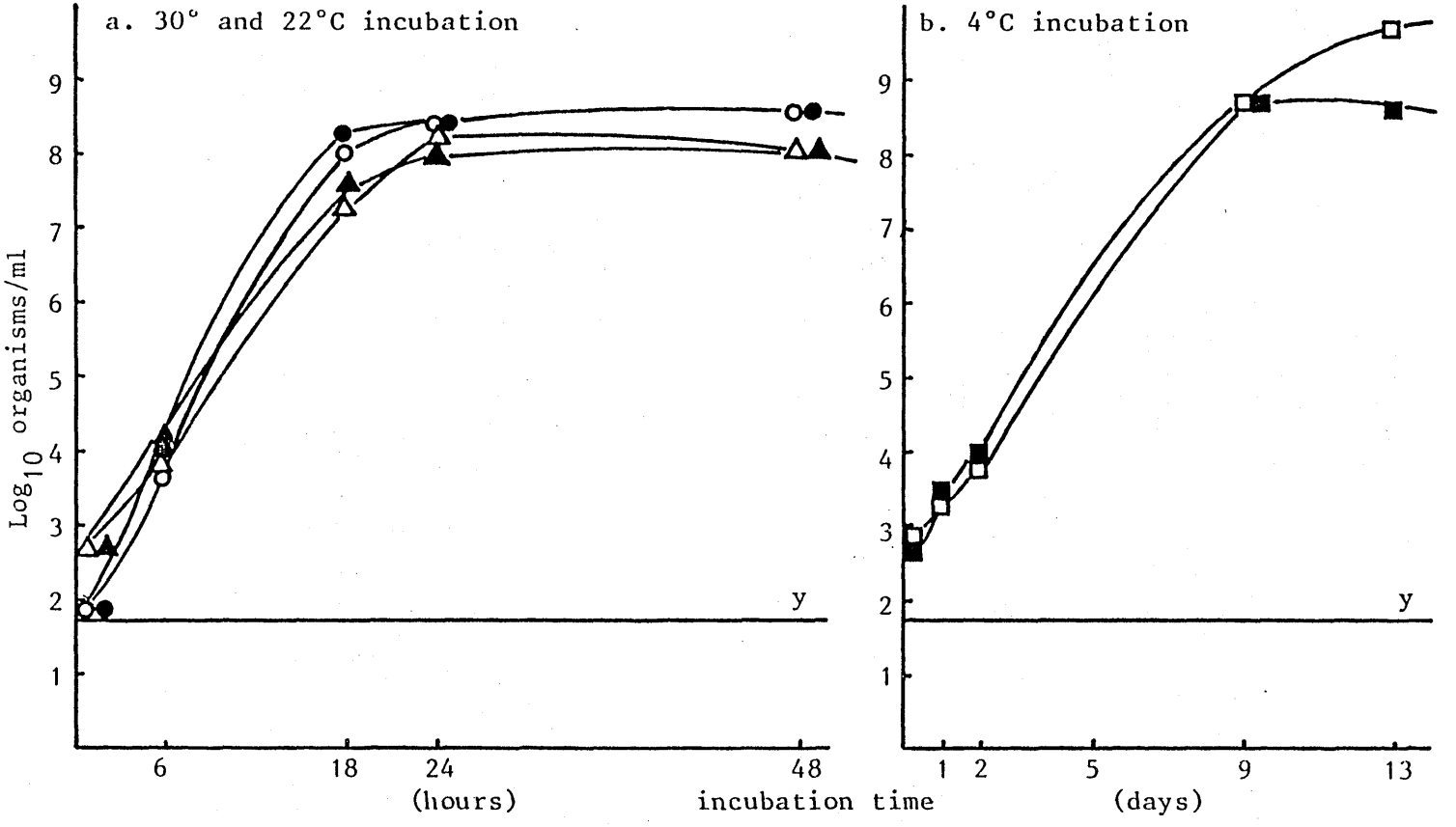
Table 28

Growth of Yersinia spp. from a minimal inoculum in four broth media compared with Brain Heart Infusion incubated at 30° and 22°C for 48 h

Broth medium	Incubation temperature °C	50% end points of growth expressed as a % of those obtained with BHI					
		Y. enterocolitica					Y. pseudo-tuberculosis
		serotype 0:3	serotype 0:6,30	serotype 0:9	NCTC 10460	NCTC 10598	
GN broth	30	97.3	86.8	101.7	1000	80.9	30
	22	90.3	87.0	105.8	122.5	86.8	69
Selenite cystine	30	53.6	52.8	0	0	0	0
	22	0	0	0	0	0	0
Selenite F broth	30	0	0	92.1	0	0	0
	22	0	0	57.6	0	0	0
Wauters' broth	30	13.1	0	32.1	0	92.9	0
	22	0	0	0	0	18.4	0

BHI = Brain Heart infusion

Figure 21. Growth of Y.enterocolitica serotypes 0:3 and 0:6,30 in buffered peptone water at 30°,22° and 4°C



○ = serotype 0:3 at 30°C; ● = serotype 0:6,30 at 30°C; △ = serotype 0:3 at 22°C; ▲ = serotype 0:6,30 at 22°C; □ = serotype 0:3 at 4°C; ■ = serotype 0:6,30 at 4°C; y = lower limit of counting method.

beginning to decline in numbers by 13 days. Serotype 0:3 continued to increase in numbers throughout the 13 days incubation.

In S (Figure 22) growth of both test strains was rather slower than that in BP, Y. enterocolitica 0:3 attained only 3.5×10^3 organisms/ml after 18 h incubation at 30°C and declined rapidly to 1×10^2 organisms/ml within 48 h. In contrast Y. enterocolitica 0:6,30 grew steadily and attained 5×10^7 organisms/ml after 48 h incubation. At 22°C both 0:3 and 0:6,30 grew steadily to $>1.5 \times 10^5$ organisms/ml in 24 h and rose to 2.5×10^7 and 1.5×10^8 organisms/ml after 48 h incubation. At 4°C similar counts of both strains were attained after 13 days incubation.

ii. Growth from a minimal inoculum

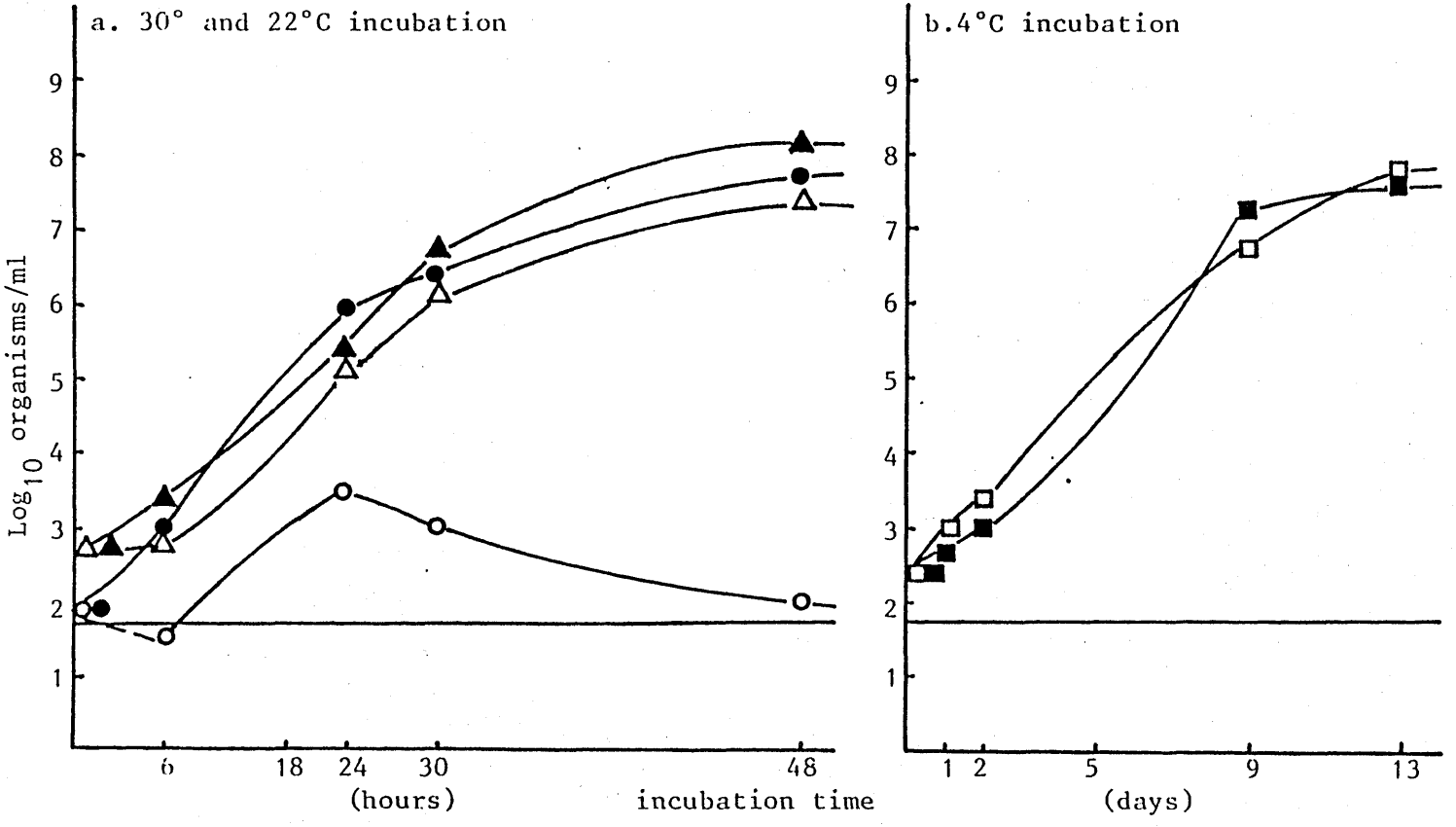
The growth of Y. enterocolitica 0:3 and 0:6,30 from a minimal incoulum in BP and S incubated at 30°C is shown in Table 29. Yersinia enterocolitica 0:3 showed a 17% reduction in growth in BP compared to that obtained in BHI and failed to grow in S,but Y. enterocolitica 0:6,30 grew equally in both media and showed only a 5% reduction of growth compared to that in BHI.

d) Evaluation of batch to batch variation and effect of storage of broth media on the recovery of Y.enterocolitica.

Brain Heart Infusion broth. The comparison of the growth of Y. enterocolitica 0:3 and 0:6,30 in four batches of Difco BHI and one batch of Oxoid BHI is shown in Figure 23. Difco batch G was freshly prepared from the same batch of dehydrated powder as Difco batch A which had been stored at room temperature for seven months. Similarly Difco batch D was freshly prepared from the same batch of dehydrated powder as Difco batch B which had been stored at room temperature for 4 months.

There was no significant difference between the maximum counts of either of the organisms in either the five freshly prepared or the two stored batches of broth. The colony counts ranged from 7.5×10^8 - 5×10^9 organisms/ml after 24 h incubation and 4.5×10^8 - 3.5×10^9 organisms/ml after 48 h incubation. There was a marked decline in the numbers of both strains in batches B and C (not plotted) after 48 h and Y. enterocolitica 0:3 and Y. enterocolitica 0:6,30 showed a similar decline in batches F and E respectively (not plotted). The colony counts of both reference strains in the Oxoid BHI batch A which was only

Figure 22. Growth of Y.enterocolitica serotypes 0:3 and 0:6,30 in supplemented phosphate buffer at 30°,22°and 4°C



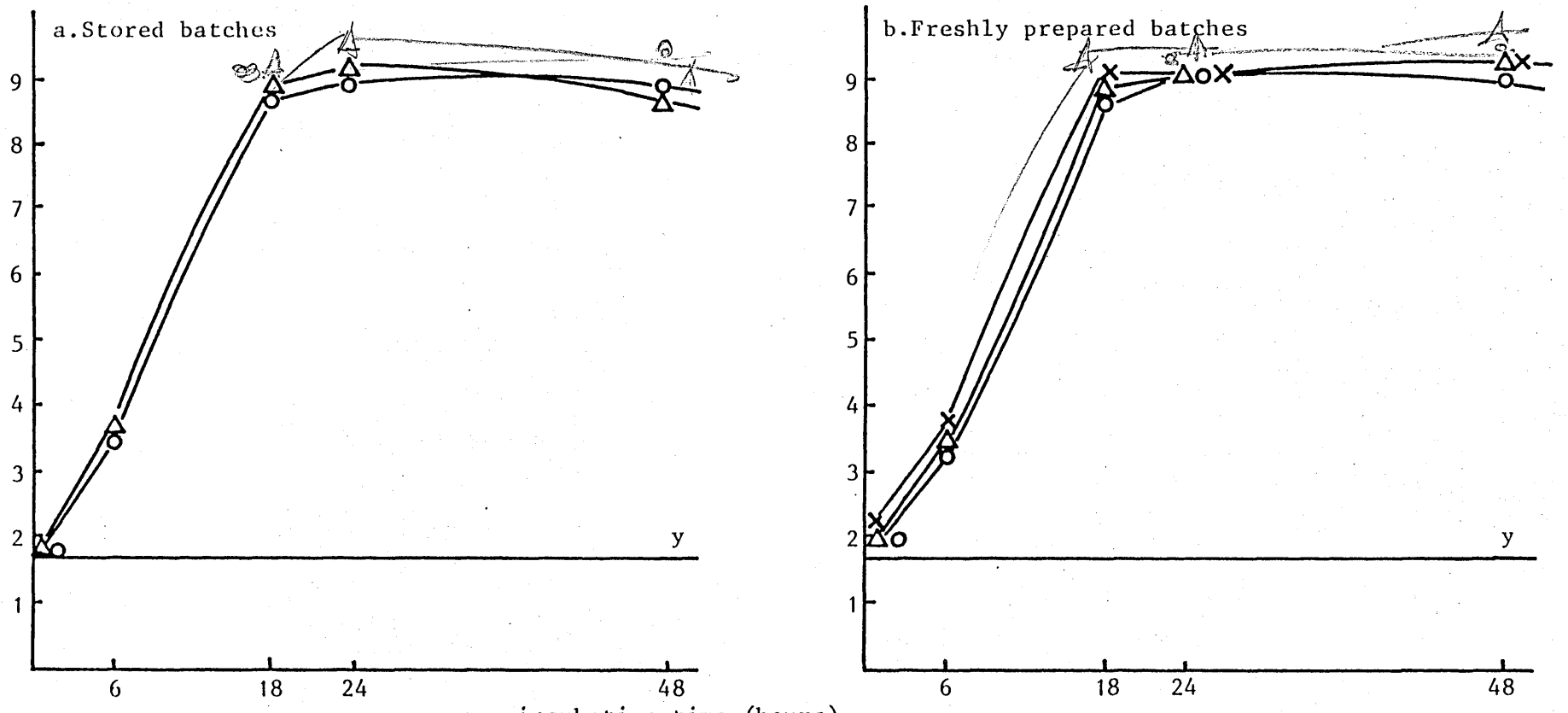
○ = serotype 0:3 at 30°C; ● = serotype 0:6,30 at 30°C; △ = serotype 0:3 at 22°C; ▲ = serotype 0:6,30 at 22°C; □ = serotype 0:3 at 4°C; ■ =serotype 0:6,30 at 4°C; y = lower limit of counting method.

Table 29

Growth of Y. enterocolitica from a minimal inoculum in two broth media as compared with Brain Heart Infusion incubated at 30°C

Medium	50% end points of growth expressed as a % of that obtained with Brain Heart Infusion	
	Y. enterocolitica	
	Serotype 0:3	Serotype 0:6,30
Buffered peptone water	83	95
Supplemented phosphate buffer	0	95

Figure 23. Growth of Y.enterocolitica serotypes 0:3 and 0:6,30 in different batches of Difco and Oxoid Brain Heart Infusion broth at 30°C



open symbols = serotype 0:3; solid symbols = serotype 0:6,30; O,● = Difco batch A(stored) and Difco batch G(fresh); Δ,▲ = Difco batch B(stored) and Difco batch D(fresh); X = Oxoid batch A(stored); y = lower limit of counting method

available freshly prepared were similar to those obtained in the freshly prepared Difco media.

GN broth. The growth of Y. enterocolitica 0:3 in different batches of Difco GN broth is shown in Figure 24. The GN batch E was freshly prepared from the same batch of dehydrated powder as batch B which had been stored for 10 months at room temperature and batch C which had been stored for 1 month. Neither Y. enterocolitica 0:3 nor 0:6,30 grew in batch A which had been stored at room temperature for 22 months. In batches B and C Y. enterocolitica 0:3 and 0:6,30 attained colony counts of between 1×10^9 and 2.25×10^9 organisms/ml after 24 h but showed a marked decline to between 1.5×10^8 and 6.5×10^8 organisms/ml after 48 h incubation. In the freshly prepared batches D, E and F the colony counts were in general slightly lower than those in the stored batches and attained between 5×10^8 and 5.5×10^9 organisms/ml after 24 h incubation and between 2.5×10^8 and 6.5×10^9 organisms/ml after 48 h. Both reference strains showed a notable decline in numbers in batch F after 48 h.

Buffered peptone water. The comparison of growth of Y. enterocolitica 0:3 in five batches of buffered peptone water is shown in Figure 25. The medium was prepared from raw ingredients and therefore it was not possible for a comparison to be made between stored and freshly prepared broths made from exactly the same ingredients. Both Y. enterocolitica 0:3 and 0:6,30 grew to greater than 1.5×10^8 organisms/ml within 24 h in all the broths with the exception of Y. enterocolitica 0:3 in batch A, which grew very slowly only attaining 4.5×10^6 organisms/ml. After 48 h incubation both strains had attained greater than 2×10^8 organisms/ml but Y. enterocolitica 0:3 showed a decline in numbers in batch C and Y. enterocolitica 0:6,30 a similar decline in batch B.

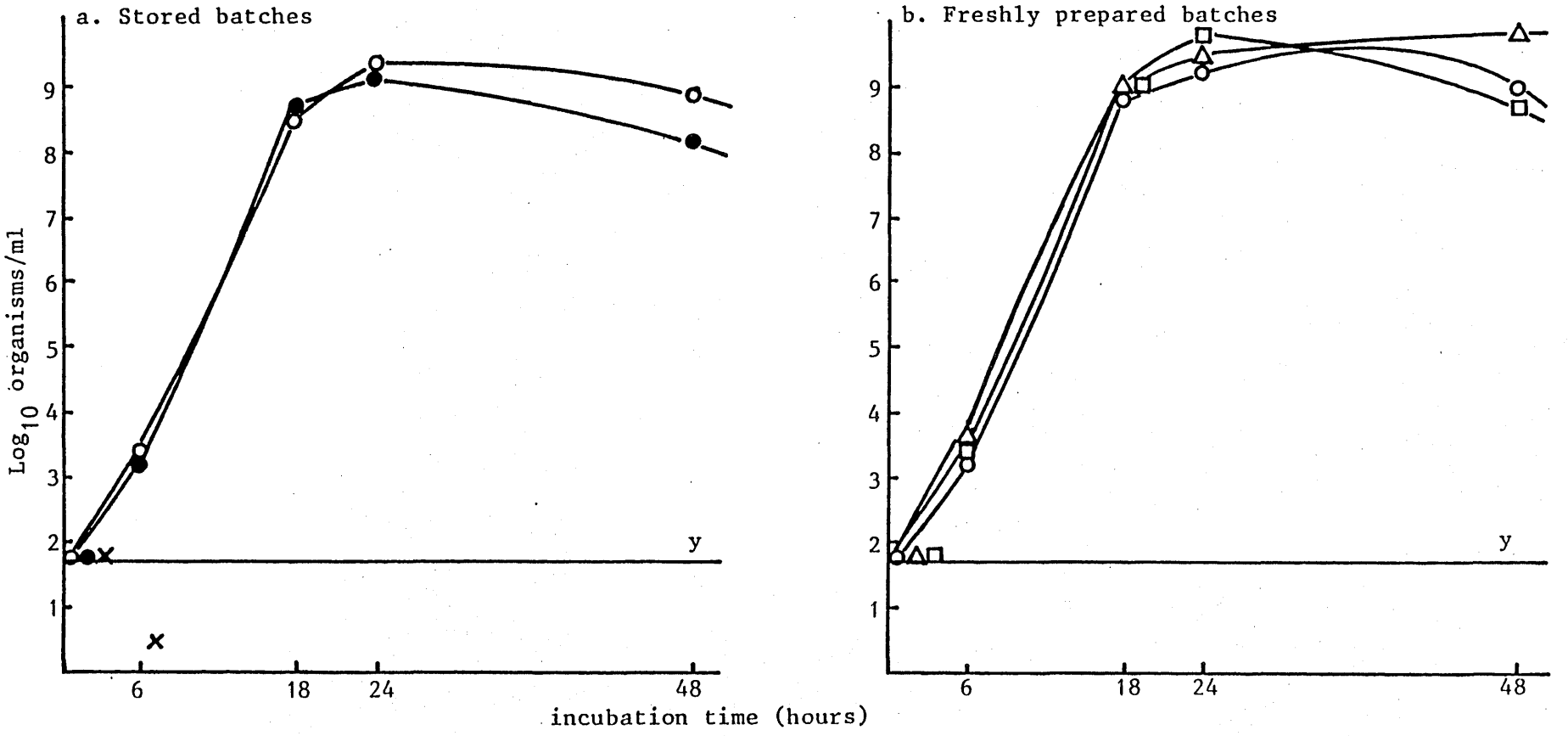
3. Investigations with a view to formulating a new medium

a) Investigations to determine antagonistic effects amongst the reference strains

i. Antagonism in mixed live cultures

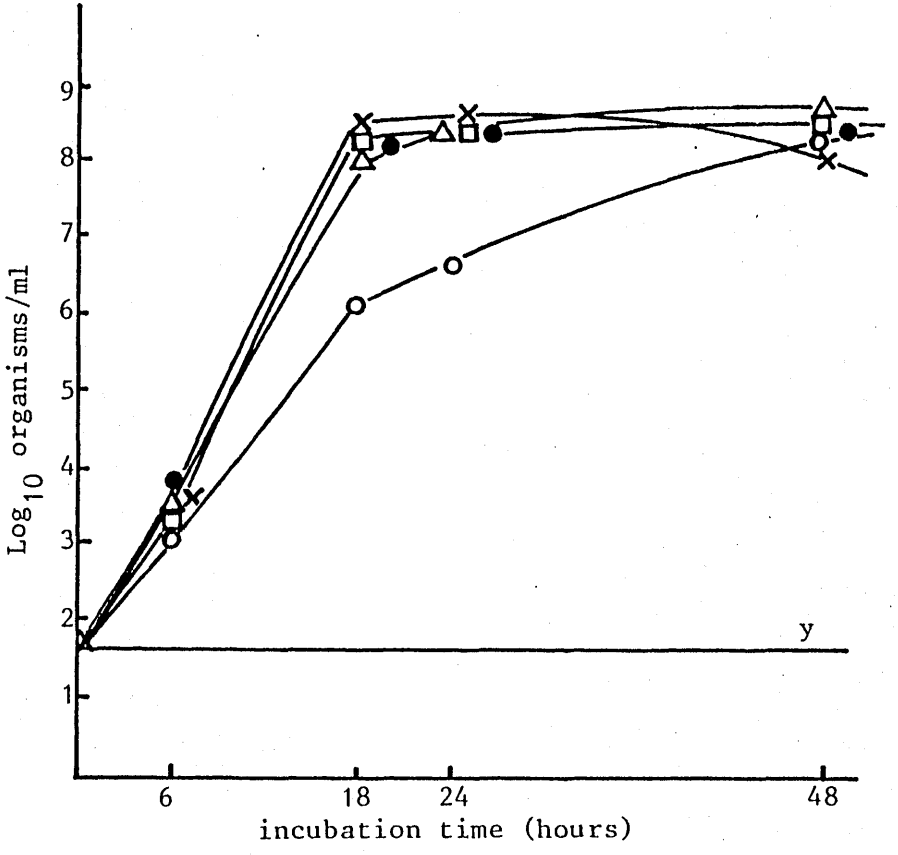
None of the reference strains showed any sign of inhibition either of themselves or each other in live mixed cultures.

Figure 24. Growth of Y.enterocolitica serotype 0:3 in different batches of Difco GN broth at 30°C



△ = batch A; □ = batch B; ○ = batch C; ▲ = batch D; ■ = batch E; ● = batch F; y = lower limit of counting method.

Figure 25. Growth of Y.enterocolitica serotype 0:3 in five batches of buffered peptone water at 30°C



○ = batch A; ● = batch B; ✕ = batch C; □ = batch D; △ = batch E; y = lower limit of counting method.

ii. Antagonism by end-products of growth

The inhibition of the reference strains on Brain Heart Infusion and deoxycholate citrate sucrose agars are shown in Tables 30 and 31 respectively.

Brain Heart Infusion agar. Yersinia enterocolitica 0:9, Y. pseudotuberculosis and S. sonnei were all slightly inhibited by themselves on BHI, each of the strains showed a reduction in growth where they crossed the growth area of the original killed strain (Table 30). There was weak inhibition of Y. enterocolitica 0:3, 0:6,30, 0:9, NCTC 10460, 10598, E. alkalescens, H. alvei and especially the Shigella spp. but this was caused by species other than themselves. Some of the reference strains were more definitely inhibited and no growth occurred where the "killed" and live strains crossed, S. flexneri was totally inhibited by Y. enterocolitica NCTC 10460, S. sonnei by E. alkalescens and both strains by H. alvei and S. typhimurium. Yersinia enterocolitica 0:3 was totally inhibited by Y. pseudotuberculosis and RM bacterium. The latter organism also inhibited Y. enterocolitica 0:9 and Y. pseudotuberculosis. Hafnia alvei was totally inhibited by E. alkalescens.

Deoxycholate citrate sucrose agar. There were very few weak inhibitory affects when the strains were grown on DCSA (Table 31). The inhibition occurred as a definite area of no growth where the live strain crossed the streak line of the "killed" organism. With the exception of Y. pseudotuberculosis all the Yersinia spp. exhibited inhibition of themselves as well as of each other. Yersinia enterocolitica 0:6,30, NCTC 10460 and 10598 affected, to varying degrees, the two Shigella strains. Escherichia coli, K. aerogenes, RM bacterium and S. typhimurium were also inhibited by themselves and without exception all the other strains were inhibited by E. coli which was the only organism not to be inhibited by K. aerogenes. Klebsiella aerogenes was the only strain not inhibited by RM bacterium and S. typhimurium.

b) The effect of dyes and other chemicals on the growth of Yersinia spp. and other organisms

i. Effect of dyes and other chemical agents incorporated into Brain Heart Infusion and GN agars

The effects of the dyes and chemicals incorporated into BHI agar and

Table 30

Antagonism of the reference strains on Brain Heart Infusion agar

Killed organism	Test strain: Y. enterocolitica 0:3	Y. enterocolitica 0:6,30	Y. enterocolitica 0:9	Y. enterocolitica NCTC 10460	Y. enterocolitica NCTC 10598	Y. pseudotuberculosis	E. cloacae	E. alkalescens	E. coli QC 21043/75	H. alvei	K. aerogenes	RM bacterium	S. typhimurium	S. flexneri	S. sonnei
Y. enterocolitica 0:3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y. enterocolitica 0:6,30	-	-	-	-	-	-	-	-	-	±	-	-	-	±	±
Y. enterocolitica 0:9	-	-	±	-	-	-	-	-	-	±	-	-	-	±	±
Y. enterocolitica NCTC 10460	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Y. enterocolitica NCTC 10598	-	-	-	-	-	-	-	-	-	-	-	-	-	±	±
Y. pseudotuberculosis	+	-	-	-	-	±	-	±	-	±	-	-	-	±	±
E. cloacae	±	-	-	-	-	-	-	±	-	-	-	-	-	-	-
E. alkalescens	±	-	-	-	-	-	-	-	-	+	-	-	-	±	+
E. coli QC 21043/75	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-
H. alvei	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
K. aerogenes	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-
RM bacterium	+	±	+	±	-	+	-	-	-	±	-	-	-	±	-
S. typhimurium	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
S. flexneri	-	±	±	±	±	-	-	-	-	-	-	-	-	-	-
S. sonnei	-	-	-	-	-	-	-	-	-	-	-	-	-	±	±

+ = inhibition; ± = weak inhibition; - = no inhibition.

Table 31

Antagonims of the reference strains on deoxycholate citrate sucrose agar

Killed organism	Test strain: Y. enterocolitica 0:3	Y. enterocolitica 0:6,30	Y. enterocolitica 0:9	Y. enterocolitica NCTC 10460	Y. enterocolitica NCTC 10598	Y. pseudotuberculosis	E. cloacae	E. alkalescens	E. coli QC 21043/75	H. alvei	K. aerogenes	RM bacterium	S. typhimurium	S. flexneri	S. sonnei
Y. enterocolitica 0:3	+	+	+	+	+	+	-	-	-	-	-	-	-	±	±
Y. enterocolitica 0:6,30	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+
Y. enterocolitica 0:9	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
Y. enterocolitica NCTC 10460	+	+	+	+	+	+	-	-	-	-	-	-	-	±	+
Y. enterocolitica NCTC 10598	+	+	+	+	+	+	+	-	-	±	-	-	-	+	+
Y. pseudotuberculosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±
E. cloacae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. alkalescens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli QC 21043/75	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H. alvei	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K. aerogenes	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
RM bacterium	+	+	+	+	+	+	±	+	+	±	-	+	+	+	+
S. typhimurium	+	+	+	+	+	+	±	+	+	-	-	+	+	+	+
S. flexneri	-	±	±	±	±	-	-	-	-	-	-	-	-	-	-
S. sonnei	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = inhibition; ± = weak inhibition; - = no inhibition.

GN agar are shown in Tables 32 and 33 respectively.

Brain Heart Infusion agar. The reference strains were resistant to all the dyes and chemicals incorporated into BHI agar with the exception of those shown in Table 32. All strains were sensitive to brilliant green and sodium azide with the exception of S. typhimurium which was partially inhibited by the brilliant green. The two Yersinia spp. were both resistant to malachite green and tannic acid but although Y. enterocolitica 0:3 was also resistant to crystal violet Y. enterocolitica 0:9 showed some sensitivity to this dye. Both Yersinia strains were inhibited by euflavine. Proteus vulgaris was the only organism to be even slightly sensitive to malachite green and all four Proteus spp. were inhibited by tannic acid.

GN agar. The test strains were also resistant to all the dyes and chemical agents incorporated into GN agar with the exception of those shown in Table 33. All the test strains were sensitive to both brilliant green and sodium azide. Both the Yersinia spp. were sensitive to crystal violet, malachite green and tannic acid, and resistant to euflavine and potassium chromate. Yersinia enterocolitica 0:3 was partially inhibited by methylene blue. The four Proteus spp. were inhibited by tannic acid and showed variable sensitivity to crystal violet, malachite green and potassium chromate, and were resistant to euflavine and methylene blue.

ii. Effect of dyes and other chemical agents incorporated as a gradient into deoxycholate citrate sucrose agar

All the test strains were resistant to the dyes and other agents except for those shown in Table 34. Scoring of results in Table 34 is different to that in Tables 32 and 33 because this method employed a dye/chemical gradient which produced a range of results.

Pseudomonas aeruginosa failed to grow and is recorded as not tested. Yersinia enterocolitica 0:3, 0:6,30 and 0:9 were all resistant to malachite green, methyl violet, neutral red and toluidine blue O. Malachite green was the sole agent which had any inhibitory effect on any of the other test strains. Although Y. pseudotuberculosis was inhibited by this dye so too were Providencia sp ., H. alvei, E. alkalescens, E. cloacae, E. coli and the four Proteus sp. The effects of malachite green on the test strains are illustrated in Plates 8-11.

Table 32

The effect of dyes and chemical agents, incorporated into Brain Heart Infusion agar, on the growth of Y. enterocolitica and other organisms

Agent	Y. enterocolitica 0:3	Y. enterocolitica 0:9	E. alkalescens	H. alvei	Prot. mirabilis	Prot. morganii	Prot. rettgeri	Prot. vulgaris	Providencia sp.	S. typhimurium	S. flexneri
Brilliant green	S	S	S	S	S	S	S	S	S	I	S
Crystal violet	R	I	R	I	I	S	S	I	I	R	S
Euflavine	I	I	S	R	R	S	R	S	I	R	S
Malachite green	R	R	R	R	R	R	R	I	R	R	S
Sodium azide	S	S	S	S	S	S	S	S	S	S	S
Tannic acid	R	R	R	R	I	I	I	I	S	R	R

S = sensitive; I = inhibited, reduced growth; R = resistant.

Table 33

Effect of various dyes and chemical agents, incorporated into GN agar, on the growth of Y. enterocolitica and other organisms

Agent	Y. enterocolitica 0:3	Y. enterocolitica 0:9	E. alkalescens	H. alvei	Prot. mirabilis	Prot. morganii	Prot. rettgeri	Prot. vulgaris	Providencia sp.	S. typhimurium	S. flexneri
Brilliant green	S	S	S	S	S	S	S	S	S	S	S
Crystal violet	S	S	R	I	S	I	S	S	I	R	S
Euflavine	R	R	I	R	R	R	R	R	I	R	S
Malachite green	S	S	S	S	S	S	I	S	I	I	S
Methylene blue	I	R	R	R	R	R	R	R	R	R	I
Potassium chromate	R	R	R	R	R	I	R	I	R	R	R
Sodium azide	S	S	S	S	S	S	S	S	S	S	S
Tannic acid	S	S	R	I	I	I	I	I	S	R	S

S = sensitive; I = inhibition, reduced growth; R = resistant.

Table 34

Effect of various dyes and chemical agents incorporated as a gradient into deoxycholate citrate sucrose agar on the growth of Y. enterocolitica and other organisms

Dye	Y. enterocolitica 0:9	Y. enterocolitica 0:3	Y. enterocolitica 0:6,30	Y. pseudotuberculosis	S. typhimurium	Providencia sp.	H. alvei	E. alkalescens	K. aerogenes	E. cloacae	Ps. aeruginosa	E. coli QC 21043/75	Prot. mirabilis	Prot. vulgaris	Prot. rettgeri	Prot. morganii
Acriflavine	<1	<1	<1	0	4	1	1	0	3	2	NR	1	<1	<1	1	1
Brilliant green	0	0	1	0	<1	0	0	0	4	1	NR	0	1	<1	0	<1
Crystal violet	2	1	3	1	3	2	3	1	4	4	NR	3	2	2	1	1
Euflavine	1	1	1	1	3	2	2	0	3	2	NR	1	2	1	1	<1
Malachite green	4	4	4	1	(4)	1	2	0	4	1	NR	0	1	1	2	2
Methyl violet	4	4	4	4	4	4	4	2	4	4	NR	4	4	4	4	2
Neutral red	4	4	4	3	4	4	4	4	4	4	NR	4	4	4	4	4
Toluidine blue 0	4	4	4	4	4	4	3	4	4	4	NR	4	4	3	4	4
Mercurochrome	1	1	1	1	2	2	1	1	2	2	NR	2	2	1	2	2
Sodium azide	2	2	2	2	4	4	4	0	1	1	NR	1	NT	NT	NT	NT

4 = resistant, growth along entire length of streak; (4) = some inhibition; 3 = growth 75% of streak; 2 = 50% of streak; 1 = 25% of streak; 1 = less than 25% of streak; 0 = sensitive, no growth; NR = no result available; NT = not tested.

Plates 8-11 Effects of malachite green incorporated as a gradient into deoxycholate citrate sucrose agar

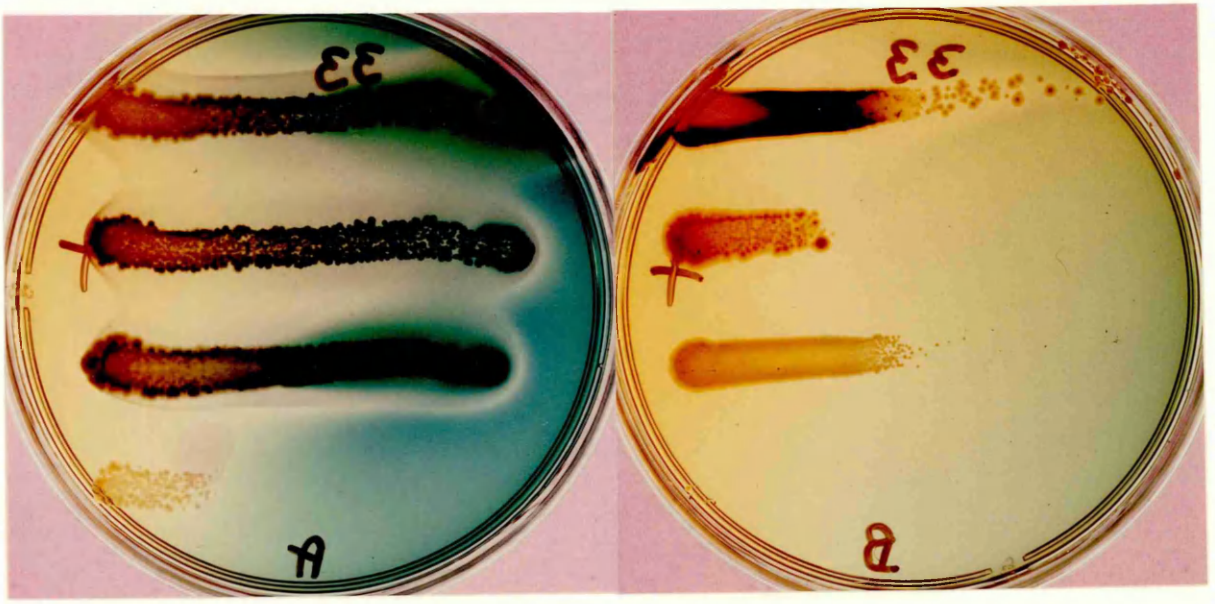


Plate 8. Y.enterocolitica 0:9
Y.enterocolitica 0:3
Y.enterocolitica 0:6,30
Y.pseudotuberculosis

Plate 9. S.typhimurium
Providencia sp.
H.alvei
E.alkalescens

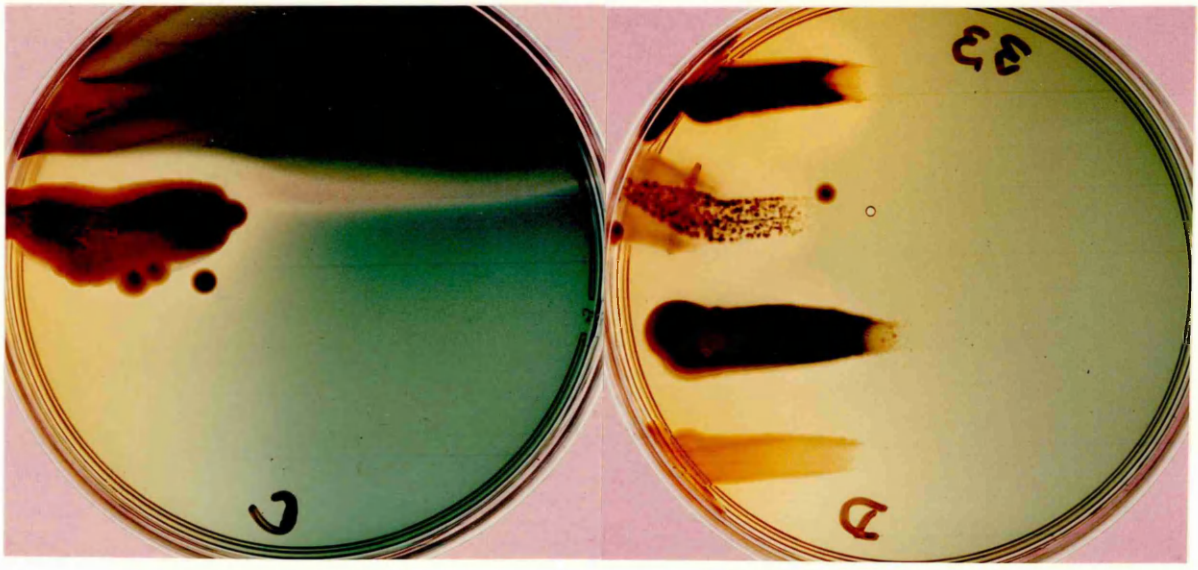


Plate 10. K.aerogenes
E.cloacae
Ps.aeruginosa
E.coli

Plate 11. Prot.mirabilis
Prot.vulgaris
Prot.rettgeri
Prot.morganii

c) Further studies on the effect of malachite green on the growth of Y. enterocolitica and other organisms

These investigations were made because of the potential selectivity for Y. enterocolitica revealed in the earlier experiments.

i. The effect of varying concentrations of malachite green incorporated into deoxycholate citrate sucrose agar

The effects of the varying concentrations of malachite green is shown in Table 35 and Plates 12-15.

The four strains of Yersinia spp. together with K. aerogenes all grew without any inhibition on each of the DCSA plus malachite green agars. In contrast whilst E. cloacae, E. alkalescens, E. coli, Prot. vulgaris, Providencia sp. and Ps. aeruginosa were noticeably inhibited on all the agars, H. alvei, Prot. mirabilis, Prot. morganii and Prot. rettgeri were inhibited only on DCSA containing 0.004% malachite green (D4 agar) and grew without the slightest inhibition on DCSA plus 0.002% and on DCSA plus 0.006% malachite green. Plates 12-15 clearly illustrate these effects of the malachite green agars on Y. enterocolitica 0:3, E. cloacae, Prot. rettgeri and Providencia sp. respectively. This experiment was repeated on two more occasions and the results were identical.

ii. Identification of the agent combining with malachite green to enhance inhibition

The results of the investigation are shown in Table 36. The DCSA agar base plus 0.004% malachite green had some effect on the growth of E. cloacae, E. alkalescens, E. coli, Providencia sp. and S. typhimurium and DCSA agar base plus solution A and 0.004% malachite green affected Providencia sp. only. However DCSA agar plus solution B (sodium deoxycholate) plus malachite green had a very inhibitory effect on the growth of all the test strains except the Yersinia spp., H. alvei, Prot. morganii and Ps. aeruginosa.

iii. The determination of the range of the inhibitory property of malachite green/sodium deoxycholate mixture

The effects of varying concentrations of malachite green and sodium deoxycholate on the growth of Y. enterocolitica 0:3 and Prot. rettgeri are shown in Tables 37 and 38 respectively. Table 37 shows that none

Table 35

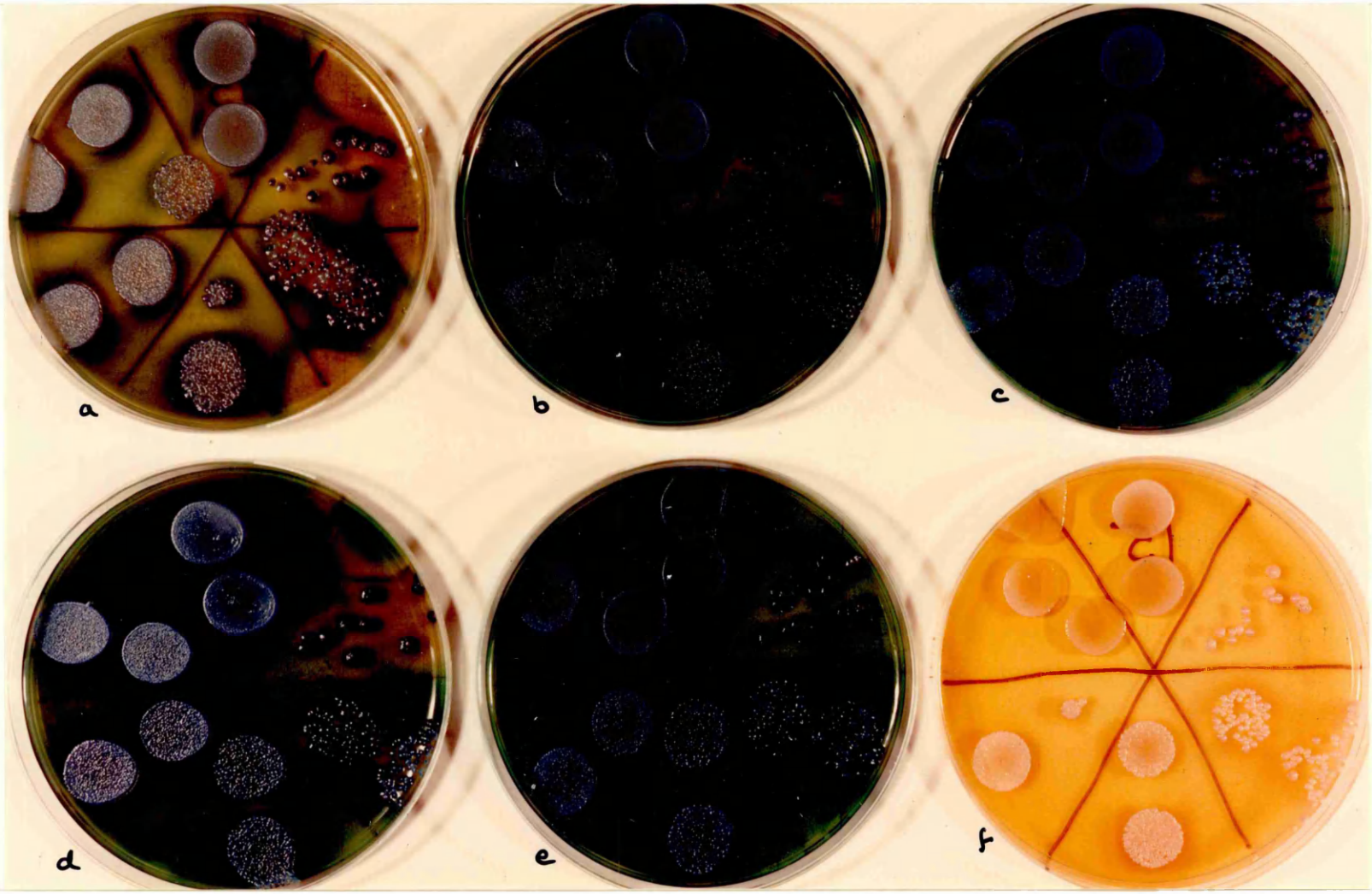
Effect of varying concentrations of malachite green incorporated into deoxycholate citrate sucrose agar on the growth of Y. enterocolitica and other organisms

Test organism	Log $_{10}$ number of organisms/ml Final concentration malachite green %					
	0	0.002	0.004	0.006	0.008	0.01
Y. enterocolitica 0:3	8.60	8.60	8.60	8.48	8.65	8.60
Y. enterocolitica 0:6,30	8.88	8.60	8.81	8.81	8.70	8.85
Y. enterocolitica 0:9	8.95	8.81	8.85	8.70	8.78	8.70
Y. pseudotuberculosis	8.18	8.18	8.00	8.00	8.30	8.30
E. cloacae	7.70	6.40	<2.70	3.65	6.60	6.60
E. alkalescens	7.78	<2.70	<2.70	<2.70	<2.70	<2.70
E. coli QC 21043/75	5.18	<2.70	<2.70	<2.70	<2.70	<2.70
H. alvei	8.98	9.00	4.78	8.70	9.18	8.90
K. aerogenes	8.81	8.60	8.60	8.18	8.30	8.30
Prot. mirabilis	8.48	8.78	3.00	8.60	8.70	8.81
Prot. morganii	8.74	9.00	5.18	8.81	9.00	9.00
Prot. rettgeri	8.78	8.88	3.74	8.30	9.00	8.70
Prot. vulgaris	8.81	3.00	<2.70	<2.70	3.00	<2.70
Providencia sp.	8.78	4.40	3.00	3.60	4.70	NT
Ps. aeruginosa	8.70	7.95	7.70	7.60	7.54	7.81
S. typhimurium	8.48	8.48	8.00	8.00	8.54	8.40

NT = not tested.

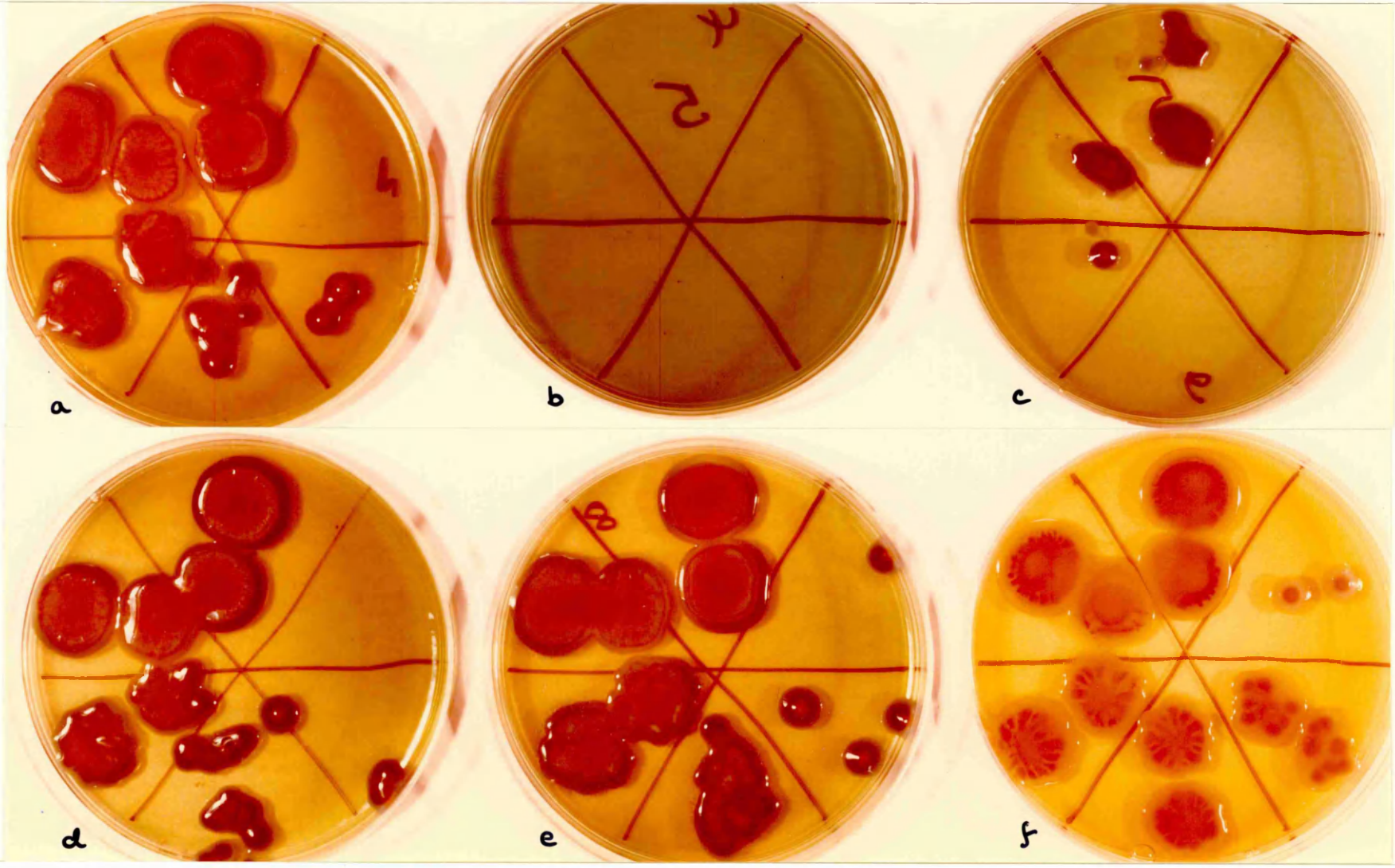
NB. Boxes emphasize significantly reduced populations. Note particularly column containing 0.004% malachite green.

Plate 12. Effect of deoxycholate citrate sucrose agar plus varying concentrations of malachite green on the growth of Y.enterocolitica 0:3



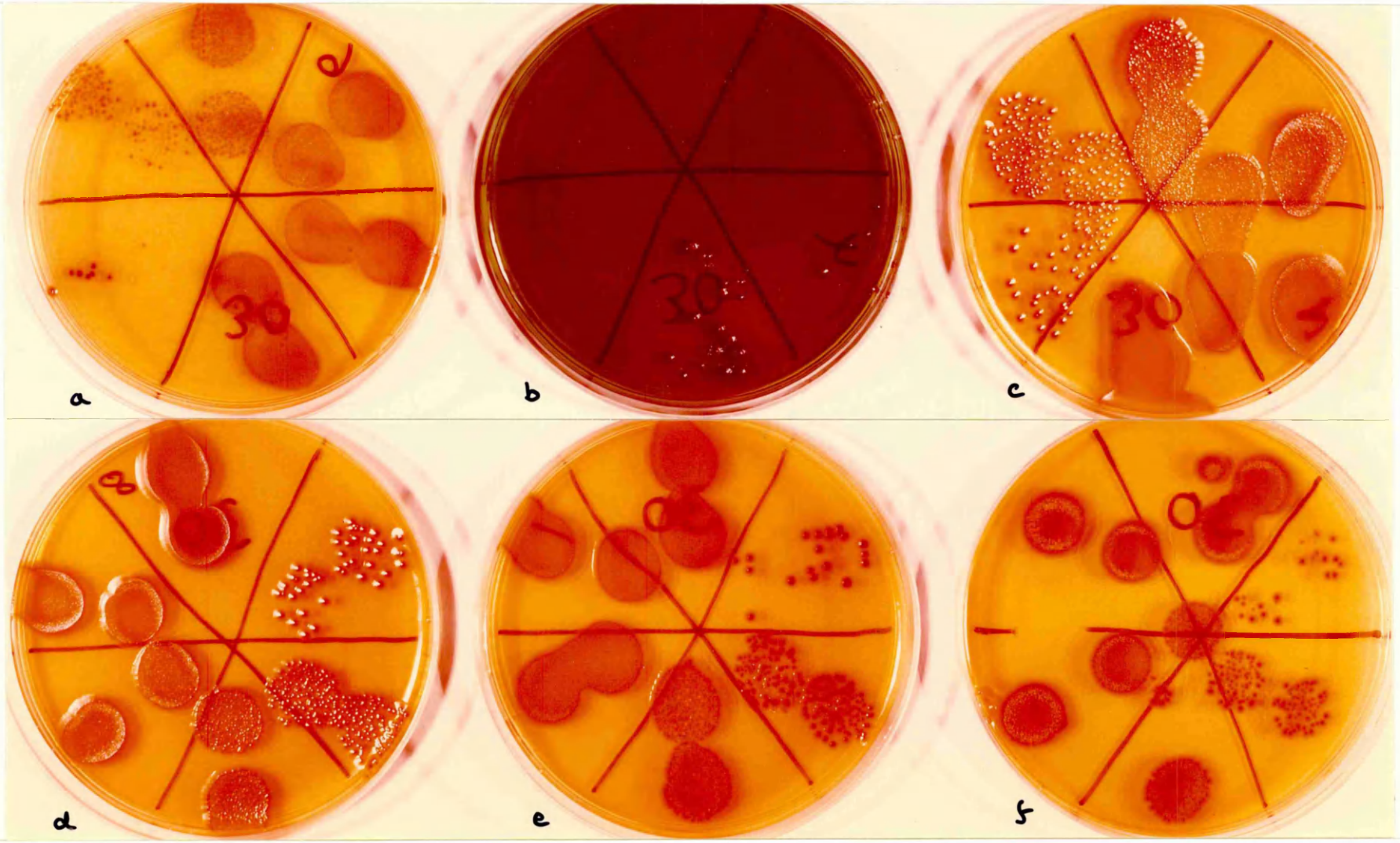
a = 0.002; b = 0.004; c = 0.006; d = 0.008; e = 0.01; f = 0% w/v malachite green

Plate 13. Effect of deoxycholate citrate sucrose agar plus varying concentrations of malachite green on the growth of E.cloacae



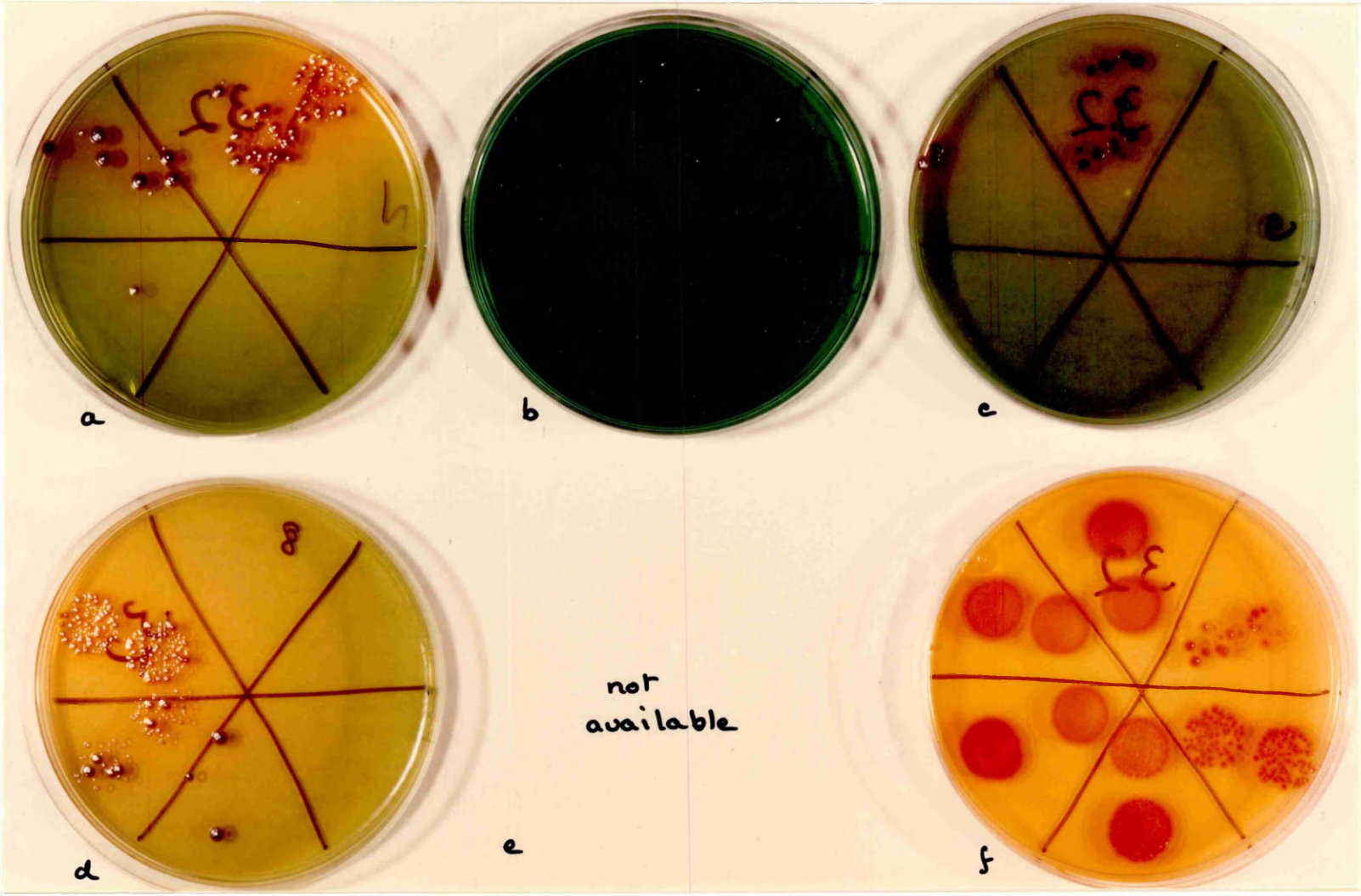
a = 0.002; b = 0.004; c = 0.006; d = 0.008; e = 0.01; f = 0% w/v malachite green

Plate 14. Effect of deoxycholate citrate sucrose agar plus varying concentrations of malachite green on the growth of Prot.rettgeri



a = 0.002; b = 0.004; c = 0.006; d = 0.008; e = 0.01; f = 0% w/v malachite green

Plate 15. Effect of deoxycholate citrate sucrose agar plus varying concentrations of malachite green on the growth of Providencia sp.



a = 0.002; b = 0.004; c = 0.006; d = 0.008; e = 0.01; f = 0% w/v malachite green

Table 36

Identification of the agent combining with malachite green to enhance the inhibitory effect

Test organism	Log $_{10}$ number organisms/ml			
		0.004% malachite green incorporated into		
	Deoxycholate citrate sucrose agar	Deoxycholate base only	Deoxycholate base + Solution A	Deoxycholate base + Solution B
Y. enterocolitica 0:3	9.08	8.86	8.88	8.78
Y. enterocolitica 0:6,30	8.95	8.90	8.95	8.70
Y. enterocolitica 0:9	9.00	8.95	8.98	8.78
Y. pseudotuberculosis	8.30	8.30	8.40	8.00
E. cloacae	8.70	7.00	8.78	6.00
E. alkalescens	8.48	7.98	9.08	7.60
E. coli QC 21043/75	8.85	5.54	8.81	3.30
H. alvei	8.95	8.85	9.06	8.18
K. aerogenes	8.60	8.60	8.60	7.54
Prot, mirabilis	8.95	9.10	8.19	2.70
Prot. morganii	8.78	9.10	9.11	9.04
Prot. rettgeri	8.81	8.00	8.88	3.70
Prot. vulgaris	9.18	8.81	8.95	2.70
Providencia sp.	8.85	5.65	5.74	3.30
Ps. aeruginosa	8.00	NT	8.00	8.30
S. typhimurium	8.81	7.70	8.70	5.30

NT = not tested; Solution A = tri-sodium citrate, sodium thiosulphate, ammonium ferric citrate scales; Solution B = sodium deoxycholate.

NB. Boxes emphasize significantly reduced populations.

Table 37

Effect of varying concentrations of malachite green and sodium deoxycholate on the growth of Y. enterocolitica serotype 0:3

Standardized sodium deoxycholate (solution B) /100 ml of medium	Log $_{10}$ number of organisms/ml Quantity of 1% malachite green/100 ml									
	ml									
ml	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
0.25	8.00	8.00	7.93	8.18	8.00	8.30	8.18	8.04	8.30	7.85
0.50	8.00	8.00	8.18	7.70	7.70	8.18	8.00	8.00	8.18	7.70
0.75	8.00	7.70	8.00	7.88	8.18	7.70	8.00	8.18	8.00	7.70
1.00	8.00	8.00	8.30	8.30	7.70	8.00	8.00	8.18	8.18	7.70
1.25	7.78	8.00	7.90	8.18	8.00	8.18	8.00	8.00	8.00	8.00
1.50	8.00	8.18	8.00	8.18	8.30	8.00	8.06	8.18	8.00	7.70
1.75	8.00	8.30	8.00	8.00	7.70	8.00	8.18	8.18	8.00	8.00
2.00	8.00	8.00	8.00	8.18	8.00	8.30	8.18	7.95	8.00	8.00
2.25	7.70	7.70	8.00	8.00	8.00	7.70	8.18	7.70	8.00	8.00
2.50	7.70	8.00	8.18	7.70	8.00	7.85	8.00	8.00	7.70	7.78
2.75	7.70	8.00	7.70	8.00	7.70	8.00	8.00	8.80	8.18	7.88
3.00	7.70	7.70	8.00	7.70	8.00	8.00	7.70	8.18	8.00	7.70

NB. The area bounded by thick lines indicates the agars on which the colony size was reduced to average 1.5 mm.

of the 120 malachite green/sodium deoxycholate combinations had any effect on the colony counts of Y. enterocolitica 0:3. There was no appreciable difference in the colony size obtained on the majority of the agars but those containing more than 0.6 ml malachite green and 2 ml sodium deoxycholate were on average 1.5 mm diameter as opposed to 2 mm on the rest of the agars.

The picture shown in Table 38 of the effects of the same malachite green/sodium deoxycholate combinations on the growth of Prot. rettgeri is quite different. There was a very distinct correlation between the ratio of malachite green to sodium deoxycholate and the inhibition of this strain. The normal concentration of sodium deoxycholate in DCSA (1.5 ml) combined with 0.3-0.4 ml of 1% malachite green was the threshold of the inhibition. On the edges of this band of inhibition many of the colonies were too small to count accurately.

The colour of the uninoculated agars varied with the combination of malachite green and sodium deoxycholate but despite this all were shown to be pH 7.5 (Table 39). The colonial characteristics of Y. enterocolitica 0:3 and 0:9 on DCSA + 0.004% malachite green are illustrated in Plates 16 and 17.

Table 38

Effect of varying concentrations of malachite green and sodium deoxycholate on the growth of Prot. rettgeri

Standardized sodium deoxycholate (solution B) /100 ml of medium	Log 10 number of organisms/ml Quantity of 1% malachite green/100 ml									
ml	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
0.25	8.70	8.70	8.70	8.65	8.78	8.48	8.85	8.70	8.70	8.48
0.50	8.54	8.54	8.78	8.48	8.70	8.18	8.70	8.48	8.60	8.70
0.75	8.48	8.48	8.40	8.74	8.54	8.70	8.48	8.54	8.78	8.40
1.00	8.70	8.18	8.54	8.60	8.48	8.60	8.60	8.60	8.65	8.30
1.25	8.40	7.70	8.30	8.54	8.48	8.54	8.65	8.48	8.48	8.60
1.50	8.54	8.54	4.00	(3.00)	8.40	8.48	8.65	8.48	8.65	8.48
1.75	8.40	8.65	(4.00)	3.48	3.40	8.54	8.54	8.60	8.81	8.60
2.00	8.40	8.60	8.60	(6.00)	3.18	3.00	(2.00)	(4.00)	8.48	8.54
2.25	8.48	8.40	8.70	8.54	4.40	2.70	2.70	2.70	2.70	(5.00)
2.50	8.30	8.40	8.48	8.30	(5.00)	(4.00)	2.70	2.70	2.70	2.70
2.75	8.48	8.78	8.48	8.65	8.40	8.60	(5.00)	3.78	3.18	2.70
3.00	8.40	8.48	8.40	8.48	8.54	8.40	8.54	(5.00)	(3.00)	3.18

( ) = approximate colony count only, colonies too small to count.

NB. The thick lines emphasize significantly reduced populations.

Table 39

Colour variations of the agars containing different combinations of malachite green and sodium deoxycholate

Standardized sodium deoxycholate (solution B) /100 ml of medium	Colour changes within the agar (pH) Quantity of 1% malachite green/100 ml									
	ml									
ml	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
0.25	Y (7.5)	Y	Y	Y	Y	Y	Y	Y	Y	Y (7.5)
0.50	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
0.75	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
1.00	YG (7.5)	YG	YG	YG	YG	YG	YG	YG	YG	YG (7.5)
1.25	YG	G	G	G	G	G	G	G	G	G
1.50	YG	G	G	G1	G1	G1	G1	G1	G1	G1
1.75	YG	G1	G1	G1	G1	G1	G1	G1	G1	G1
2.00	YG (7.5)	G2	G2	G2	G2	G2	G2	G2	G2	G2 (7.5)
2.25	YG	G2	G2	G2	G2	G2	G3	G3	G3	G3
2.50	YG	G2	G2	G2	G2	G3	G3	G3	G3	G3
2.75	YG	G2	G2	G2	G3	G3	G3	G3	G3	G3
3.00	YG (7.5)	G2	G2	G3	G3	G3	G3	G3	G3	G3 (7.5)

Y = ; YG = ; G = ; G1 = ; G2 = ; G3 =

Plates 16-17. Colonial characteristics of Y.enterocolitica on deoxycholate citrate sucrose agar plus 0.004% w/v malachite green

Plate 16. Serotype 0:3

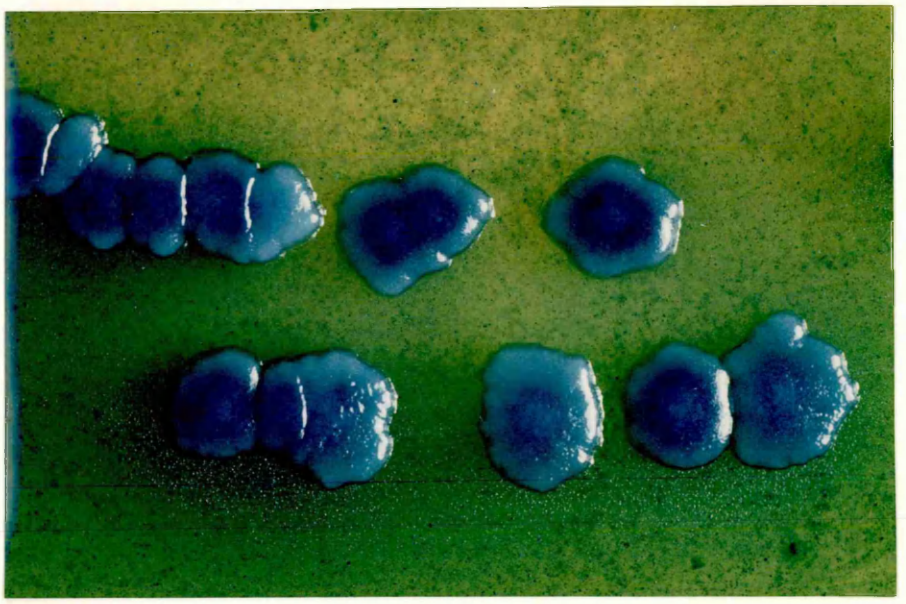
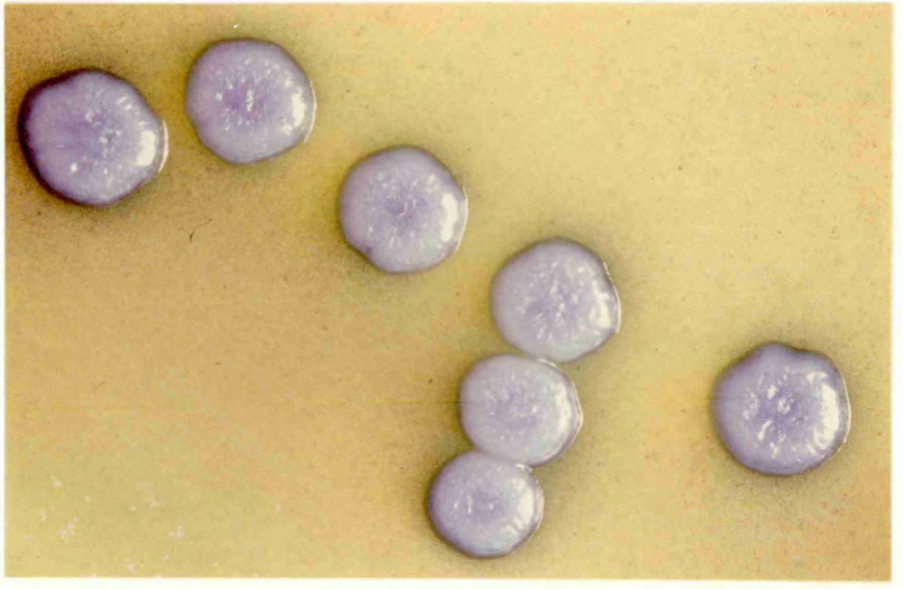


Plate 17. Serotype 0:9



Discussion

The isolation of any pathogen depends on either the colonial differentiation from other organisms which are also able to grow on a particular medium or on specific selectivity of the wanted organism. None of the agar media examined proved to be the "ideal" medium for the isolation of Y. enterocolitica. Many of the agars grew several other bacteria which had very similar colonial characteristics to Y. enterocolitica and others failed to grow Yersinia spp. Barclay (1981) reported finding similar problems in his recent study of some of the common enteric agars. However, to be able to make use of a medium which is already in use for other enteric pathogens, despite its shortcomings is somewhat preferable to adding yet another medium to the already large variety which has to be prepared by busy laboratories.

Agar media. Bismuth sulphite agar which was formulated for the isolation of Salmonellae has been recommended for the isolation of Y. enterocolitica by Hanna et al. (1977a), but the results of the present study showed this medium to produce very poor unreliable growth of Y. enterocolitica whichever brand was used (Table 20).

This study also showed that there was considerable strain variation of Y. enterocolitica on some of the other agars. It was particularly noticeable on DCA, MAC, LEE and SS agars, all of which contain lactose (Plates 2, 3, 5, 6, 7). Some strains of Y. enterocolitica were lactose positive and the colonies of these were generally much more opaque than those of the non-lactose fermenting strains. There was also some difference in colony size both between strains and within the same cultures.

Although size variation occurred on lactose sucrose urea agar (LSU) (Plate 4) the colonial appearance of Y. enterocolitica on this medium was less variable than on many of the other agars, and after a little experience was seen to be truly characteristic only of Y. enterocolitica. The most useful characteristic of the growth of Y. enterocolitica on LSU agar was the pungent smell which was quite unlike that produced by any other organism. The disadvantages of LSU were the large number of ingredients (17) and the essential requirements of a specific brand of dyes (Merck) and oxygen-free water for its preparation. However the method described simplified the preparation, and the disadvantages were

outweighed by the usefulness of the medium.

LEE agar (Lee 1977) showed some of the worst strain variations of any of the media studied (Plate 7). Lee recognized that other organisms do produce lipase positive colonies on his agar and that some strains of Y. enterocolitica are lipase negative, but he failed to report colonial variation between strains of Y. enterocolitica.

Wauters (1973) recommended the use of Salmonella-Shigella plus deoxycholate agar but emphasized that its use in conjunction with Wauters' broth was for the isolation of serotypes 0:3 and 0:9 only. This study (Table 24) showed that other serotypes of Y. enterocolitica and the other enterobacteria grew quite well on the agar but the colonies of serotypes 0:3 and 0:9 were small and there was little differentiation between them and the other organisms. A considerable disadvantage was the need to use a microscope with oblique light to identify the blurred edge and granular centre which Wauters described as characteristic of Y. enterocolitica serotypes 0:3 and 0:9. A stereo microscope as suggested by Wauters was not available and the use of an ordinary plate microscope was extremely fatiguing and time consuming.

The agars studied for colonial characteristics were not all examined quantitatively because they were made from similar base agars with only additional non-selective ingredients which would not be expected to affect the quantitative growth of the strains. The quantitative study (Table 25) confirmed the finding of the qualitative study, that most of the enterobacteria grow as readily as Y. enterocolitica on most of the agars in general use and which with few exceptions inhibited the Gram-positive organisms.

Both pectin and sodium oxalate agars were studied only briefly. The pectin agar was particularly difficult to make, requiring personal attention and production in very small batches. Many of the different types of organisms found in food enrichment cultures were able to grow on both pectin and oxalate agars making observation of any distinguishing features very difficult. For these reasons neither medium was very suited to the isolation of Y. enterocolitica from foods.

Broth media. The methods so far applied to the isolation of Y. enterocolitica have in the main involved the incubation of the broth media at 4°C for anything from 6-9 weeks. Such lengthy periods of incubation not only make the isolation of Y. enterocolitica inconvenient due to the large amount of refrigerator space required but, in the event of a

possible food posioning outbreak makes the confirmation of such an occurrence extremely protracted, and the possibility of any realistic follow-up work virtually out of the question. The effect which different incubation temperatures have on the growth of the reference organisms in the media was carried out with the intention of trying to speed up the isolation time by using a higher incubation temperature.

In this study it was shown that at least two strains of Y. enterocolitica were not able to grow in either selenite cystine (Figures 15 and 16) or selenite F broths (Figures 17 and 18). The remaining Y. enterocolitica strains grew rather more slowly in selenite cystine than the other enterobacteria and could quickly become overgrown in a mixed culture. Selenite F broth was a little more encouraging in so far that at 30°C the surviving strains of Y. enterocolitica actually grew faster than the other organisms but at 4°C this medium was lethal to both strains tested.

Gram-negative (GN) broth (Figures 12 and 13) was slightly inhibitory to all the test strains and when incubated at 30°C Y enterocolitica could be outgrown by any other bacteria present. However, when GN broth was incubated at 4°C both the Yersinia strains grew almost as strongly as at 30°C and as many other enterobacteria are not able to grow at this temperature selection by low temperature incubation would probably be the most suitable method for the use of this medium.

The growth of bacteria in phosphate buffered saline is dependant upon sufficient nutrients being supplied by the specimen under examination. The present study showed that even without any nutrient being present Y. enterocolitica can survive, although not grow, without significant loss of numbers for up to 2 weeks (Figure 14).

Many of the results presented in this study are similar to those recently reported by Barclay (1981). He also found that some strains of Y. enterocolitica could not be recovered from selenite F broth. He confirmed experimentally that when mixed with other organisms it was difficult to isolate Y. enterocolitica from GN broth incubated at 35°C although isolation was quite possible from the same medium incubated at 4°C. Barclay incubated his broth media at 35°C even though it is widely recognized that Y. enterocolitica grows better at 30°C or lower and this may have effected the recovery of the organism. Wauters' broth has been recommended for incubation at 22°C but the results in this study indicate that this may not be very good. The growth of

Y. enterocolitica was markedly slower at 22°C (Figure 20) than at 30°C (Figure 19) where they grew slightly more quickly even than the other enterobacteria. It was noted that some of the other enterobacteria did not grow in this medium. Incubation of Wauters' broth at 4°C (Figure 20) could not be recommended as neither strains of Y. enterocolitica grew during the period of the experiment.

Some surprising results were obtained from supplemented (sorbitol and bile salts) phosphate buffer (Figure 27). In this medium Y. enterocolitica 0:3 grew very poorly and died out rapidly at 30°C but this did not occur at the lower incubation temperatures. Mehlman et al. (1978) had reported this medium to be more sensitive than phosphate buffered saline and capable of enriching Y. enterocolitica when less than 1000 cells/ml were present in naturally contaminated pork, and when approximately 1000 cells/ml of a clinical isolate were present in an artificially inoculated pork sample, that strain could also be recovered.

As was expected growth in buffered peptone (Figure 21) was very similar to that in BHI (Figures 10 and 11).

The utilization of media which are already employed for the isolation of other organisms has certain advantages besides keeping down the work load of the laboratory. All too frequently media, especially enrichment broths, can remain unused on laboratory shelves for months. This is particularly true if a procedure is carried out only intermittently. During the storage period deterioration can occur and batch variation is liable to occur even with dehydrated products but a medium prepared from raw ingredients in a laboratory, whether it be by one laboratory assistant in the corner of the room or in a specially equipped media department, is potentially more prone to problems than commercial manufacture. The results of the study on batch variations and effects of prolonged storage emphasize one or two important points. Brain Heart Infusion (Figure 23) remained stable during prolonged storage but freshly made batches of GN broth (Figure 24) were found to be slightly more inhibitory than the stored batches. This was presumably because the sodium deoxycholate had deteriorated during storage. One batch of this particular medium had been stored for 22 months and neither strain of Y. enterocolitica survived even 6 h incubation. Whether this was caused by deterioration during the extended storage time or that the batch of dehydrated powder was unsatisfactory could not be determined as none of the powder remained. However, it does emphasize the importance of checking new batches of

powder before use. This of course means having a large enough stock of powder of any one batch to make testing practicable yet not having so much that it sits on the laboratory shelves so long that it also has time to deteriorate.

Buffered peptone water (Figure 25) was made from raw ingredients and it was not possible in this study to determine whether batch A, in which Y. enterocolitica grew very slowly, had deteriorated during the 10 months storage or was unsuitable from the time of production because earlier colony counts had not been carried out. This again emphasizes the need for careful checking at the time of preparation of such media.

New medium. A microbiologist's dream of the "ideal" agar medium is one which is capable of selecting, without any inhibition, the wanted pathogen whilst totally inhibiting all other organisms. There are few media which come near to matching up to this description and certainly when this project was begun even fewer media actually formulated with Y. enterocolitica in mind. Because of this lack of a really suitable medium for the isolation of Y. enterocolitica investigations were begun with the aim of either modifying an existing medium or formulating a new one.

The selectivity of a medium is usually attained by incorporating dyes, chemotherapeutic agents or other chemical agents in to a base medium. There were no reports in the literature of any studies of the effects of dyes or other chemicals on the growth of Y. enterocolitica but before proceeding with such an investigation it seemed relevant to examine all the reference strains for antagonism. Early in 1975 it had been reported (Thomas, personal communication) that a strain of S. flexneri 4a had been inhibited by a strain of E. coli. These two organisms had been mixed together in a simulated faecal specimen and sent out nationwide by the PHLS Microbiological Quality Control Laboratory. This was followed a few months later by the finding of a strain of S. sonnei being inhibited by S. hadar. The E. coli 21043/75 and S. sonnei were included in the reference strains for this project. The inhibition of enteric pathogens either by other enteric pathogens or indeed other members of the Enterobacteriaceae is not unknown, a few reports have appeared in the literature between 1947 and 1969 but there were none relating to Y. enterocolitica, probably because it was barely considered to be a pathogen at that time.

Although in the live cultures examined in this study on nutrient

media antagonism was not evident when young cultures were streaked across the original growth line of killed organisms some of the strains were inhibited. This antagonism was much more defined on selective agar especially DCSA (Table 31) where there was strong inhibition of all the Y. enterocolitica strains by E. coli, K. aerogenes, RM bacterium (Y. ruckeri) and S. typhimurium. All these strains including Y. enterocolitica also inhibited themselves. The cause of this inhibition was not clear but it could not be attributed to either acid production from the fermentation of lactose and/or sucrose because neither RM bacterium nor S. typhimurium fermented these sugars, or to the presence of bacteriophage as these were not seen in the experiments with live cultures. Other work (not reported in this thesis) carried out with further strains of Shigella, showed that those infected with phage produced plaques on the lawns of growth of certain strains.

A further possibility in the search for an explanation of antagonism is the production of bacteriocins. However, although the use and production of bacteriocins is well documented there appears to be no reports of bacteria being antagonistic against themselves. None of the early workers recorded such effects (Gratia & Fredericq 1946; Fredericq et al. 1946; Gardner 1950; Abbott & Shannon 1958) nor did those concerned primarily with the concept of antagonism (Wynne & Norman 1953; Hentges 1967, 1969; Halbert 1948).

Hentges (1969) suggested that volatile acids formed by E. coli in a mixed culture were bacteriocidal to Shigella but that the acids from fermentation of lactose and sucrose did not effect that organism.

The possibility of antagonism must be borne in mind when considering a new agar medium. Pathogens may be present on the medium in small numbers, especially when sub-cultured by the direct plating method, and even sometimes from enrichment media, and the loss of even one colony could cause a mis-diagnosis.

The range of dyes and chemicals available was unfortunately limited and the reference organisms were resistant to the majority of the agents examined. However there was a small number which showed variable inhibition of the test strains and of these malachite green incorporated into DCSA agar was the only agent to which Y. enterocolitica was resistant whilst many of the other reference organisms were sensitive (Table 34).

Malachite green is used in a few media currently in use, the best

known is probably Rappaport's broth for the isolation of Salmonella and of which Wauters' broth for the isolation of Y. enterocolitica is a modification. There are many early reports of the use of malachite green incorporated into agar media for the isolation of S. typhi (Padlewsky 1908; Peabody & Pratt 1908; Schindler 1909; Gaehtgens & Brückner 1910; Barth 1928). It was shown that at a neutral pH an optimum concentration of malachite green could be attained where S. typhi was able to grow freely but E. coli was inhibited.

The zoning of the inhibitory effect of malachite green in DCSA (Table 35) and the subsequent discovery of its relationship to sodium deoxycholate (Table 36) as shown in this present study was significant and of particular interest. A search of the literature revealed that Leifson (1935) carried out an extensive study of the effects of sodium deoxycholate during his researches to formulate a new medium for the isolation of salmonellae, the forerunner of the DCA agar still in use today. He made vague references to the fact that sodium deoxycholate will combine with chemicals which have one or more methyl groups and is also able to combine with various dyes and indicators such as methylene blue, gentian violet, fuchsin, neutral red and bromo thymol blue. Leifson made no mention of malachite green or any attempt to investigate the use of this phenomenon for the isolation of pathogens.

In summary, no one medium either agar or broth, old or new, stood out as being particularly suitable for the isolation of Y. enterocolitica from foods. However, lactose sucrose urea agar was seen to have some potential because of the distinct characteristics of Y. enterocolitica on this medium.

Investigations to formulate a new medium revealed that when malachite green and sodium deoxycholate was combined in certain concentrations an agent was formed which was inhibitory to many enterobacteria but not to Y. enterocolitica.

D. Isolation and identification of Yersinia enterocolitica from foods and other materials

Introduction

Isolation methods

The three basic methods which have been used for the isolation of enteric pathogens from foods and other materials are: direct plating, direct enrichment, and pre-enrichment.

Direct plating method. Generally the numbers of enteric pathogens expected to be found in food specimens, is at least several orders of magnitude less than in specimens from acute clinical cases of human infection and therefore the direct plating technique is of little value. Kapperud & Jonsson (1976) and Seelye & Yearbury (1979) did recover Y. enterocolitica by this method from trout and beef respectively. The latter workers did not, however, recover the organism until approximately 10,000 organisms/g were present which was only after 4 weeks storage of the beef.

Direct enrichment method. The direct enrichment method is generally employed for samples likely to contain large numbers of non-pathogenic contaminating organisms and has been used successfully for the isolation of Y. enterocolitica for which a wide range of broth media, incubation times and temperatures have been recommended. Phosphate buffered saline, however, has invariably been incubated at 4°C for anything from 3 weeks to 1 year (Botzler et al. 1976; Fukushima et al. 1979; Alonso et al. 1979; Peixotto et al. 1979). Occasionally other media such as cooked meat, yeast extract-casein cystine broth (Fukushima et al. 1979), peptone water and mannitol broth have also been employed at 4°C, whereas various selenite broths and Wauters' broth have been found more successful when incubated at 22°-25°C.

In comparisons of the direct plating and direct enrichment methods Leistner et al. (1975) recovered 50 strains of Y. enterocolitica from 215 samples of raw meat through direct enrichment but none by direct plating. In contrast Saari & Jansen (1979) recovered 89 isolates of Y. enterocolitica from unconcentrated river water by direct plating and a further 51 strains through enrichment.

Pre-enrichment method. Conflicting results have also been obtained from comparisons of direct enrichment and pre-enrichment techniques.

Schiemann (1980a) made 54 isolations of Y. enterocolitica from 63 samples of raw pork through direct enrichment but in an earlier study of raw milk his maximum recovery was obtained from Wauters' broth after pre-enrichment in phosphate buffered saline (Schiemann & Toma 1978).

Characterization of Y. enterocolitica

The characterization of Y. enterocolitica includes: biochemical classification; antibiotic susceptibility patterns; and serological identification.

Biochemical classification. The biochemical classification has been very confused because taxonomists have been undecided as to the identification and differentiation of true Y. enterocolitica and the Y. enterocolitica-like organisms. Recently the position was somewhat clarified by an international group of workers who have clearly defined Y. enterocolitica sensu stricto (Bercovier et al. 1980a), and also named three new species, Y. kristensenii (Bercovier et al. 1980b), Y. intermedia (Brenner et al. 1980) and Y. frederiksenii (Ursing et al. 1980). Unfortunately this classification has not yet been officially accepted by the International Committee on Systematic Bacteriology. Four schemes have also been described for the biotyping of Y. enterocolitica (Nilehn 1969b; Wauters 1970; Knapp & Thal 1973; Bercovier et al. 1980a) and are based on such tests as lecithinase (lipase), indole, and fermentation of xylose, aesculin and salicin. These schemes are also the subject of much disagreement.

Antibiotic susceptibility patterns. It has been suggested that there might be a correlation between antibiotic susceptibility patterns and specific biochemical characteristics but there is no real evidence to support this. Conflicting patterns have been reported by different workers (Nilehn 1967; Chester & Stotzky 1976; Raevuori et al. 1978). There is evidence, however, to suggest that some temperature related variations do occur (Kouwatli et al. 1979).

Serological identification. At the present time 57 Y. enterocolitica "O" antigens have been recognized. The relationship, if any, between serological type and pathogenicity in humans is as confused as the biochemical classification. Although serotypes O:3 (Wauters' biotypes 3 or 4) and O:9 (Wauters' biotype 2) are predominantly associated with human disease other serotypes including O:5,27, O:6,30 and O:8 which may

be Wauters' biotypes 1, 2 or 3 have also been implicated with increasing frequency.

Incidence of Y. enterocolitica in foods

The isolation of Y. enterocolitica from foods and other materials has been reported from many parts of the world. By far the largest number of isolations have been from raw meats including poultry, pork, beef and lamb (Leistner et al. 1975; Guthertz et al. 1976; Hanna et al. 1976; Schiemann 1980a; Norberg 1981). Yersinia enterocolitica has also been isolated with increasing frequency from dairy products including raw and pasteurized cow's milk, goat's milk, cheese and ice cream (Aldová et al. 1975; Schiemann & Toma 1978; Schiemann 1978a; Hughes 1979, 1980). Occasional isolations have been made from seafoods such as mussels, brown trout, oysters, crabs and shrimps (Spadaro & Infortuna 1968; Kapperud & Jonsson 1976; Peixotto et al. 1979). Mollaret et al. (1979) also reports isolations from vegetables and salad ingredients.

Although serotype 0:3 has been isolated from pork products other serotypes more commonly found in foods include 0:5; 0:5,27; 0:6,30; 0:8; 010K1 and 0:14.

Methods

1. **Comparisons of methods and media for the isolation of Yersinia enterocolitica from artificially and naturally contaminated foods and other materials**

a. Naturally and artificially contaminated animal feed - Study 1

The methods studied were:-

Enrichment

Pre-enrichment

The broth media studied were:-

Brain Heart Infusion broth (BHI)

Buffered peptone water (BP)

GN broth (GN)

Phosphate buffered saline (PS)

Wauters' broth (WB)

The incubation temperatures studied were:-

4°, 22°, 30°C.

Preparation of animal feed

Ten samples of animal feed (meat and bone meals), which had been received routinely into the Food Hygiene Laboratory, were mixed together thoroughly to give one uniform sample (F1).

i. Examination of naturally contaminated animal feed (F1)

The agar media studied were:-

Deoxycholate citrate agar (DCA)
Deoxycholate citrate sucrose agar (DCSA)
Lee agar (LEE)
Salmonella-Shigella agar (SS)

Method

Twenty-five g of the animal feed (F1) was placed into each of the enrichment broths and incubated as shown in Table 40.

Table 40

Broth media for the isolation of Y. enterocolitica from naturally and artificially contaminated animal feed

Medium	Number of jars	Quantity ml per jar	Incubation temperatures °C
Buffered peptone	3	225	4, 22, 30
Brain Heart Infusion	3	225	4, 22, 30
GN broth	3	100	4, 22, 30
	3	225	4, 22, 30
Phosphate buffered saline	1	100	4
Wauters' broth	2	100	22, 30
	3	225	4, 22, 30

The broth cultures incubated at 4°C were retained for up to 42 days and sub-cultured at weekly intervals. Those at 22°C were retained for up to 15 days and sub-cultured after 1, 3, 4 and 15 days and those at 30°C were retained for up to 4 days and sub-cultured after 1 and 4 days. The 225 ml broth cultures (with the exception of the Wauters' broth) were used as pre-enrichment media and after 3 days incubation 10 ml of each was transferred to 100 ml Wauters' broth incubated at 22°C for 15

days and sub-cultured after 1 and 15 days. The broths incubated at 22° and 30°C were initially sub-cultured to: one DCA plate incubated at 4°C for 14 days; two LEE agar plates incubated at 4° and 22°C for 14 days and 2 days respectively; and one SS agar plate incubated at 30°C for 2 days. After the first sub-culture DCSA plates were substituted for the DCA plate at 4°C and the SS agar plate at 30°C.

The broth cultures at 4°C were also sub-cultured to agar plates as described above and in addition a further DCSA plate was included and incubated at 22°C for 2 days.

Identification of Y. enterocolitica

Colonies suspected of being Y. enterocolitica were further sub-cultured as described on page 220.

ii. Examination of artificially contaminated animal feed (F2)

The reference strains studied were:-

Y. enterocolitica 0:3 Y. enterocolitica 0:6,30

The agar media studied were:-

Deoxycholate citrate sucrose agar (DCSA) Lee agar (LEE)

Inoculation of animal feed

Overnight broth cultures of the reference strains were diluted (see page 82) to give approximate concentrations of 1.5×10^6 Y. enterocolitica 0:3/ml and 5.75×10^5 Y. enterocolitica 0:6,30/ml.

A single 10 g sample of the homogenized animal feed (F1) was inoculated with 0.06 ml of both of the test strains, mixed very thoroughly with a sterile spatula and then added to another 100 g of animal feed (F1). This in turn was thoroughly mixed and 10 g of this homogenate was added to a further 100 g of the animal feed. This was again mixed well and the procedure repeated once more to obtain approximately 9 cells of Y. enterocolitica 0:3/g and 3.4 cells of Y. enterocolitica 0:6,30/g (F2).

Method

Twentyfive grams of the artificially contaminated animal feed (F2) was placed into each of the enrichment broths and incubated as shown in Table 40. The broth cultures at 4°C were retained for up to 42 days (6 weeks) and sub-cultured at weekly intervals, those at 22°C were

retained for 7 days and sub-cultured after 1, 3 and 7 days and those at 30°C were retained for 3 days and sub-cultured after 1 and 3 days.

In addition the 225 ml broth cultures (with the exception of Wauters' broth) were used as pre-enrichment media and after 3 days at 22° and 30°C and 7 days at 4°C 10 ml of each was transferred to 100 ml Wauters' broth, incubated at 22°C for up to 42 days and sub-cultured after 1, 3 and 7 days, and then at weekly intervals.

Each of the broth cultures was sub-cultured to three deoxycholate citrate sucrose agar plates and two LEE agar plates for incubation at 4°C for 14 days and 22° and 30°C (DCSA only) for 2 days.

Identification of Y. enterocolitica

Colonies suspected of being Y. enterocolitica were sub-cultured as described on page 220.

As outside facilities were not available at the time of this study for the complete serological identification, slide agglutination was carried out using sera supplied by the Yersinia Reference Laboratory (serotypes 0:3 and 0:6,30).

b. Comparison of media for the isolation of Y. enterocolitica from 194 foods and other materials - Study 2

The method studied was:-

The direct enrichment technique

The broth media studied were:-

Brain Heart Infusion broth (BHI) incubated at 30°C for 14 days
Buffered peptone water (BP) incubated at 4°C for 6 weeks
GN broth (GN) incubated at 30°C for 14 days
Wauters' broth incubated at 30°C for 14 days.

The agar media studied were:-

Deoxycholate citrate sucrose agar (DCSA) incubated at 30°C for 2 days
Lee agar (LEE) incubated at 22°C for 2 days

The 194 samples examined were:-

Animal feed - meat and bone meal (41)
Cheese, Halloumi type (3)
Coconut, desiccated (20)
Prawns, frozen cooked (20)
Swabs from cow, pig and lamb carcasses (96)
Swabs from slaughterhouse sewers (14)

Sampling

The animal feed, cheese, prawns and coconut had been received into the laboratory for routine examination for salmonella and other organisms. The animal carcass swabs were sampled at a local slaughterhouse for the express purpose of examining for the presence of Y. enterocolitica. As each carcass was washed some of the water draining from the neckend was absorbed onto a cotton wool throat swab which was then returned to the laboratory and examined within 2-3 h.

The sewer swabs were taken from the outside drains of the same slaughterhouse for a period of 4 weeks during November and December 1978. Triple pads of gauze were suspended for 7 days in the drains serving the loading bay, the slaughterhall, the outside holding corrals, the entrance ramp into the lairage, the exit of the save-all tank and the foul sewer from the slaughtermen's toilets.

Laboratory examination

Animal feed batch A, 25 g quantities of 15 animal feeds were incubated at 30°C in 225 ml BHI and GN broths.

Animal feeds batch B (26), cheese (3) and frozen cooked prawns (20), 25 g were incubated at 4°C for 6 weeks in 225 ml BP and at 30°C for 14 days in BHI and WB broths.

Coconut (20), 25 g were incubated at 4°C for 6 weeks in 225 ml BP.

Carcass swabs. The swabs were broken off and each incubated at 4°C in 10 ml BP.

Sewer swabs. The string holding the three layers of gauze was removed and each layer cut into two portions, each of the portions weighed approximately 25 g when soaked with water and debris. The portions of gauze were incubated at 4°C for 6 weeks in 225 ml BP and at 30°C for 14 days in BHI and WB broths.

Sub-culture. Each of the 4°C enrichments was sub-cultured to DCSA and LEE agar plates after 1, 3 and 6 weeks and the 30°C enrichments were sub-cultured to DCSA and LEE agar after 1, 2, 7 and 14 days.

Identification of Y. enterocolitica

Colonies suspected of being Y. enterocolitica were further sub-cultured and identified as described on page 220.

c. Comparison of media for the isolation of Y. enterocolitica from raw meats - Study 3

The method studied was:-

Direct enrichment technique

The broth media studied were:-

Brain Heart Infusion broth (BHI) incubated at 30°C for 2 days
Buffered peptone water (BP) incubated at 4°C for 6 weeks

The agar media studied were:-

Deoxycholate citrate sucrose (DCSA) agar incubated at 30°C for 2 days
Lactose sucrose urea (LSU) agar incubated at 30°C for 2 days
Lee agar (LEE) incubated at 22°C for 2 days

The 272 samples examined were:-

Beef mince (86)
Beef, various cuts including stewing steak, blade, topside, skirt, chuck steak and shin (33)
Pork mince (4)
Pork, various cuts including chops and belly (32)
Sausages, mainly pork but some meat unknown (16)
Pork, mechanically deboned (100)
Lamb, mince (1)

Sampling

Except for the mechanically deboned pork the samples were received as part of a survey of local butchers meat and primarily examined for the presence of Campylobacter spp.

The mechanically deboned pork samples were received primarily for the examination of general bacteriological quality and were collected from four processing plants in different parts of the country.

Laboratory examination

Beef mince (69), beef cuts (21), pork mince (4), pork cuts (28), sausages (14) and lamb mince (1), 25 g of each were incubated at 4°C for 6 weeks in 225 ml BP and sub-cultured to DCSA and LEE agars after 1, 3 and 6 weeks.

Beef mince (11), beef cuts (6), pork cuts (2) and sausage (1), 25 g of each were incubated at 4°C for 6 weeks in 225 ml BP and sub-cultured to

LSU and LEE agars after 1, 3 and 6 weeks.

Beef mince (6), beef cuts (6), pork cuts (2) and sausage (1), 25 g of each were incubated at 30°C for 2 days in 225 ml BHI and sub-cultured to LSU and LEE agars after 1 and 2 days.

Mechanically deboned pork (100), 100 g of each were homogenized in a Stomacher (Seward Ltd) with 100 ml of quarter-strength Ringer solution (RS). The bulk of each homogenate was required for other examinations so only 1 ml of each was incubated at 4°C for 6 weeks in 10 ml BP and sub-cultured to DCSA and LEE agars after 1, 3 and 6 weeks.

Identification of Y. enterocolitica

Colonies suspected of being Y. enterocolitica were further sub-cultured and identified as described on page 220.

d. Comparison of methods and media for the isolation of Y. enterocolitica from bulked mechanically deboned pork and miscellaneous vegetables - Study 4

The methods studied were:-

Direct enrichment Pre-enrichment

The broth media studied were:-

Buffered peptone water (BP) incubated at 4°C for 6 weeks
Phosphate buffered saline (PS) incubated at 4°C for 6 weeks
Wauters' broth (WB) pre-enriched from BP and PS and incubated at 22°C for 5 days

The agar media studied were:-

Deoxycholate citrate sucrose agar (DCSA)
Deoxycholate citrate sucrose malachite green agar (D4)
Lactose sucrose urea agar (LSU)

Each of these agars was incubated at 30°C for 48 h.

Lee agar (LEE) incubated at 22°C for 48 h.

Sampling

The unhomogenized remains of the 100 mechanically deboned pork samples (Study 3) which had been stored frozen for 20 weeks were bulked and homogenized to form 23 larger samples.

The 13 vegetables were received as part of a special survey on the general bacteriological quality of fresh vegetables.

Laboratory examination

Twenty-five gram of each sample were incubated at 4°C for 6 weeks in 225 ml BP and 225 ml PS and sub-cultured after 1, 3 and 6 weeks on to DCSA, D4, LSU and LEE agars.

After 2 weeks incubation 10 ml of each of the broth cultures were transferred to 100 ml WB, incubated at 22°C for 5 days and sub-cultured to DCSA, D4, LSU and LEE agars after 5 days.

Identification of Y. enterocolitica

Colonies suspected of being Y. enterocolitica were further sub-cultured as described on page 220.

e. Comparison of broth media for the isolation of Y. enterocolitica from 503 miscellaneous foods and other materials - Study 5

The broth media studied were:-

Brain Heart Infusion broth (BHI) incubated at 30°C for 2 days
Buffered peptone water (BP) incubated at 4°C for 3 weeks and also at 30°C for 2 days
GN broth (GN) incubated at 30°C for 2 days
Phosphate buffered saline (PS) incubated at 4°C for 3 weeks and also at 30°C for 2 days
Supplemented phosphate buffer (S) incubated at 4°C for 3 weeks

The agar medium studied was:-

Lactose sucrose urea agar (LSU)

The foods examined were:-

Cream, ultra heat treated (4)
Milk, pasteurized (67)
Yoghurt (1)
Eggs, clay (1)
Eggs, dried (15)
Sausages (26)
Chickens, raw frozen (101) (Salmonella survey)
Turkey (2)
Cockles , raw (49) (Survey, bacteriological quality)
Prawns, frozen cooked (30)

Shrimps, dried (7)
Animal feed, meat and bone meal (98)
Coconut, desiccated and frozen (16)
Ham, cooked (1)
Mineral waters (5)
Spices (20) (Survey, bacteriological quality)
Surface waters (4)
Vegetables (56) (Survey, bacteriological quality)

Sampling

Except for those shown as otherwise in the above list the samples were received for routine bacteriological examination.

Laboratory examination

All samples except cooked ham and spice, 25 g were incubated in duplicate 225 ml BP and PS (replicates at 4°C for 3 weeks and 30°C for 2 days), single 100 ml GN and BHI (at 30°C for 2 days) and in the later stages of the study also in single 225 ml S (at 4°C for 3 weeks).

Ham, 25 g was incubated only in BP at 4°C.

Spice, 10 g were incubated in duplicate 100 ml BP and PS (replicates at 4°C for 3 weeks and 30°C for 2 days) and single 40 ml GN and BHI broths incubated at 30°C for 2 days.

Sub-culture

All enrichment cultures were sub-cultured to LSU agar, those incubated at 4°C after 1, 2 and 3 weeks and those at 30°C after 1 and 2 days.

Identification of Y. enterocolitica

Colonies suspected of being Y. enterocolitica were sub-cultured and identified as described on page 220.

f. Additional laboratory examinations of pasteurized milk received for Study 5

i. Phosphatase test

In addition to the examination for Y. enterocolitica, 31 of the 67 pasteurized milks were also tested for the phosphatase reaction. The milks bottled by three dairies, London Co-operative Society (10), Express Dairy (11) and Unigate Dairies (10), were delivered to three

members of the laboratory staff and sampled daily between 22.7.80 and 30.7.80. The milk was kept overnight in the refrigerator before examination, except at the weekend when it was stored for up to 3 days. On arrival at the laboratory 20 ml were removed asceptically from each bottle and used for the phosphatase test.

The test (Great Britain 1963). The milk was allowed to warm to room temperature and 5 ml buffer-substrate (see page 77) was transferred to a test tube, the tube stoppered and the substrate warmed to 37°C (water bath). One ml of milk was added to the buffer substrate and the tube incubated at 37°C for 2 h. A blank was prepared using boiled milk. After incubation the tubes were mixed well and the colour compared with Lovibond comparator disc APTW.

Interpretation of test. A reading of 10 µg or less p-nitrophenyl/ml of milk was deemed satisfactory and indicated that the milk had been sufficiently pasteurized.

ii. Most probable number (MPN) Y. enterocolitica

Two of the 31 milk samples (examined for phosphatase reaction) were also examined for the most probable number (MPN) of Y. enterocolitica per g. The method was adapted from the American technique for the quantitative estimation of Salmonella in eggs.

Buffered peptone water (BP) was distributed into screw capped jars and milk added as follows:- Series A = 3 jars each containing 90 ml BP plus 10 ml milk; Series B = 3 jars each containing 9 ml BP plus 1 ml milk; Series C = 3 jars each containing 9 ml BP plus 0.1 ml milk.

The jars were incubated at 4°C for 3 weeks and sub-cultured to lactose sucrose urea agar after 1, 2 and 3 weeks.

The MPN Y. enterocolitica per ml was calculated according to the table adapted from Hoskins (1934) (see Appendix I).

2. Characterization of isolates from naturally contaminated foods and other materials

a. Biochemical characterization

i. Screening of suspicious colonies

All colonies suspected of being Y. enterocolitica were sub-cultured to the following media:-

Glucose peptone water sugar (Gluc PWS)
MacConkey agar (MAC)
Phenylalanine agar slope (PPA)
Urea slope (Urea)

The media were incubated at 30°C for 72 h.

ii. Full biochemical identification

Using a straight wire the presumptive Y. enterocolitica isolates were inoculated into the following media:-

Peptone water sugars containing; adonitol, arabinose, cellobiose, dulcitol, glycerol inositol, inulin, lactose, maltose, mannitol, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose (acid production), nutrient agar (catalase), Simmon's citrate (utilization of citrate), arginine, lysine and ornithine decarboxylase, gelatin, peptone water (indole), LEE agar (lipase), malonate broth, motility medium (37° and 22°C), glucose phosphate broth (MR and Voges-Proskauer), nitrate broth (nitrate reduction), ONPG broth and Hugh and Leifson medium (oxidation/fermentation).

All the media were incubated at 22°C for up to 7 days (gelatin 14 days) unless otherwise stated.

b. Antibiotic sensitivity patterns

Using the method described on page 86 the sensitivity patterns of 137 Yersinia isolates (126 Y. enterocolitica, 9 Y. intermedia and 2 Y. frederiksenii) were determined against Oxoid Multodiscs U4 and 725E (see page 77) and single sensitivity discs furazolidine 100 µg, neomycin 30 µg and sulphatriad 300 µg. The plates were incubated overnight at 30°C. One plate was also seeded with the antibiotic control E. coli NCTC 10418 and also incubated at 30°C.

c. Serological identification

The isolates suspected of being Y. enterocolitica were sent to the Yersinia Reference Laboratory (84 isolates) and later to the Division of Enteric Pathogens (156 isolates) for confirmation and serological typing.

i. A comparison of the serological typing results obtained from two laboratories using different methods

Eighty-four isolates were examined by both the PHLS Yersinia Reference Laboratory, which carried out serological typing using unsteamed antigen suspensions, and the Division of Enteric Pathogens which used antigen suspensions steamed for $\frac{1}{2}$ h.

ii. Serology of 30 non-typable (unsteamed) isolates

The non-typable (unsteamed) strains examined were:-

Y. enterocolitica Y1; Y29; Y42; Y44; Y45; Y46; Y47; Y48; Y49; Y50; Y51; Y52; Y53; Y54; Y55; Y56; Y57; Y58; Y59; Y60; Y62; Y68; Y73; Y74; Y75; Y77; Y78; Y79; Y94; Y99.

Sera were prepared in rabbits using the following non-typable isolates which were chosen because of their varied biochemical characteristics:-

Y. enterocolitica Y1
Y. enterocolitica Y29
Y. enterocolitica Y47
Y. enterocolitica Y73

Preparation of sera

Each of the four strains was grown in Hartley Digest broth at 22°C for 48 h, centrifuged and resuspended in 10 ml normal saline. The suspensions were then steamed for $2\frac{1}{2}$ h, washed three times in sterile saline and finally resuspended in 12 ml normal saline. Each rabbit received five inoculations during a 4 week period as follows:-

0.5 ml, 6 day interval 1 ml, 5 day interval 1 ml, 6 day interval 2 ml, 5 day interval 2 ml.

The rabbits were rested for 5 days then 40 ml of blood collected from each one from the ear vein. This was repeated 5 days later and then after a further 5 days they were bled out. On each occasion the blood was allowed to clot and the serum removed and pooled after the final bleed out.

Preparation of agglutinating suspensions (antigen)

Each strain was inoculated into 50 ml Hartley Digest broth, incubated at 22°C overnight and then steamed for $\frac{1}{2}$ h.

Method of agglutination

Doubling dilutions of the test sera (1/10-1/2500) were made in normal saline, a clean sterile pipette was used for each dilution.

In a WHO agglutination tray 0.5 ml volumes of sera and antigen were mixed giving dilutions of 1/20-1/5000, and incubated at 50°C overnight. Agglutination was observed using transmitted light.

Agglutination reactions of the non-typable strains against the new sera

Each of the new sera were tested for agglutination against the homologous antigens and also those of the other non-typable strains.

3. Incidence of Y. enterocolitica in foods and other materials

The results from Studies 1-5 on the comparisons of media and methods were collated to provide information on the incidence of Y. enterocolitica in foods and other materials obtained in the UK between 1978-1982.

The Division of Enteric Pathogens confirmed and serotyped the 156 strains isolated in studies 1-5. This information has been collated to show the source/serotype distribution of the strains.

Results

1. Comparisons of methods and media for the isolation of Y. enterocolitica from both artificially and naturally contaminated foods and other materials

a. Naturally and artificially contaminated animal feed - Study 1

i. Naturally contaminated animal feed (F1)

The results of the comparison of broth media and methods for the isolation of Y. enterocolitica are shown in Table 41. Yersinia enterocolitica was isolated from buffered peptone water (BP), GN broth (GN) 100 ml, GN broth 225 ml and phosphate buffered saline (PS) by direct enrichment after incubation for 35 days at 4°C but was not isolated from the Wauters' broth (WB) pre-enriched in the same broth cultures. In contrast the organism was isolated from WB pre-enriched in BP incubated at 22°C but not from the BP direct enrichment. Yersinia enterocolitica

Table 41

Comparison of the effects of combinations of broth media, methods and temperature on the time of isolation of Y. enterocolitica from naturally contaminated animal feed (F1)

Medium		Number of days incubation before Y. enterocolitica was isolated					
		Direct enrichment incubated at °C			Wauters' broth at 22°C pre-enrichment from °C		
		4	22	30	4	22	30
Buffered peptone water	225 ml	35	NF	NF	NF	15	NF
Brain Heart Infusion	225 ml	NF	NF	NF	NF	NF	NF
GN broth	100 ml	35	NF	NF	NT	NT	NT
	225 ml	35	NF	NF	NF	NF	NF
Phosphate buffered saline	100 ml	35	NT	NT	NT	NT	NT
Wauters' broth	100 ml	NT	NF	NF	NT	NT	NT
	225 ml	NF	15	4	NT	NT	NT

NF = not found; NT = not tested.

was also isolated by direct enrichment in WB incubated at 22° and 30°C after 15 and 4 days respectively but not from that incubated at 4°C.

The results of a comparison of agar media and incubation temperatures are shown in Table 42. Yersinia enterocolitica was isolated eleven times on agar media incubated at 22° and 30°C, all of which had been sub-cultured from broth cultures incubated at 4°C, but no isolations were made from broth cultures incubated at either 22° or 30°C. In contrast Y. enterocolitica was isolated three times on agar media incubated at 4°C and sub-cultured from broth cultures incubated at 22° and 30°C, and once only from those incubated at 4°C. A total of 21 of 482 suspicious colonies sub-cultured for identification proved to be Y. enterocolitica. The serotypes and isolation details of the 21 strains is shown in Table 43. Four different serotypes were identified, those from 4°C enrichments were predominantly serotype 0:8 (10 strains), followed by 0:7 (2) and one of each 0:5 and 0:6,30. One strain each of serotypes 0:5 and 0:8 were isolated from broth cultures incubated at 22°C whilst from those incubated at 30°C serotype 0:6,30 was the sole type isolated. All the strains isolated were biochemically Y. enterocolitica sensu stricto.

ii. Artificially contaminated animal feed (F2)

The results of the comparison of broth media and methods for the isolation of Y. enterocolitica from artificially contaminated animal feed is shown in Table 44. Yersinia enterocolitica was isolated from all the direct enrichment cultures incubated at 4°C except for WB 225 ml. At 22°C the organism was not found in BP, BHI or GN 225 ml but was recovered from all the direct enrichments incubated at 30°C. Yersinia enterocolitica was also isolated from WB which had been pre-enriched in BHI 225 ml and GN 225 ml incubated at 22°C but the organism was not isolated directly from these broth cultures. However, Y. enterocolitica was not isolated from WB which had been pre-enriched in GN 225 ml at 30°C and from which the organism had been isolated directly.

A comparison of agar media and incubation temperatures for the isolation of Y. enterocolitica from artificially contaminated animal feed (F2) is shown in Table 45. The pattern of isolations was similar to that which occurred with the naturally contaminated animal feed (F1). There were no isolations of Y. enterocolitica made from broth cultures incubated at 4°C when they were sub-cultured to agar media incubated at

Table 42

Comparison of agar media and incubation temperatures for the isolation of Y. enterocolitica from naturally contaminated animal feed (F1)

Agar medium	Agar incubation temperature °C	No. of occasions+ on which Y. enterocolitica was isolated from all enrichment cultures incubated at °C		
		4	22	30
Deoxycholate citrate sucrose	30	6	0	0
Salmonella-Shigella*	30	0	0	0
Deoxycholate citrate sucrose	22	5	0	0
Lee	22	0	0	0
Deoxycholate citrate*	4	0	0	0
Deoxycholate citrate sucrose	4	1	0	0
Lee	4	0	2	1

* This medium used for first sub-culture only.

+ NB: For the purposes of this table an occasion is the equivalent of each sub-culture to agar media.

Table 43

Serotypes and isolation details of 21 strains of Y. enterocolitica isolated from naturally contaminated animal feed (F1)

Enrichment medium	Incubation temperature °C	Incubation time (days)	Strain No.	Isolation agar(s)	Incubation temperature °C	Serotype "O"
Buffered peptone water	4	35	916A	DCSA	30	7
		35	917B	DCSA	22	7
GN 100 ml	4	35	912B	DCSA	30	8
			912C			8
		35	913B	DCSA	30	8
			913C			8
	4	42	712A	DCSA	30	8
		42	713A	DCSA	22	8
GN 225 ml	4	35	914B	DCSA	30	6,30
	4	42	715A	DCSA	22	8
		42	716A	DCSA	30	5
Phosphate buffered saline	4	35	910A	DCSA	30	8
			910B			8
		35	911	DCSA	22	8
Wauters' broth 225 ml	22	15	842A	LEE	4	5

Continued.....2/

Table 43 (Continued)

Enrichment medium	Incubation temperature °C	Incubation time (days)	Strain No.	Isolation agar(s)	Incubation temperature °C	Serotype "O"
Wauters' broth 225 ml	30	4	22	LEE	4	6,30
			900	DCSA	4	6,30
			902			6,30
			903			6,30
		4	904	LEE	22	6,30
Wauters' broth pre-enriched in buffered peptone water	22	15	843	LEE	4	8

DCSA = deoxycholate citrate sucrose agar.

Table 44

Comparison of the effects of combinations of broth media, methods and temperature on the time of isolation of Y. enterocolitica from artificially contaminated animal feed (F2)

Medium		Number of days incubation when isolation first made					
		Direct enrichment incubated at °C			Wauters' broth at 22°C pre-enriched from °C		
		4	22	30	4	22	30
Buffered peptone water	225 ml	15	NF	2	15	NF	2
Brain Heart Infusion	225 ml	15	NF	1	15	15	2
GN broth	100 ml	28	3	1	NT	NT	NT
	225 ml	21	NF	1	9	21	NF
Phosphate buffered saline	100 ml	21	NT	NT	NT	NT	NT
Wauters' broth	100 ml	NT	7	1	NT	NT	NT
	225 ml	NF	7	3	NT	NT	NT

NF = not found; NT = not tested.

Table 45

Comparison of the effects of combinations of agar media and incubation temperatures on the incidence of isolation of Y. enterocolitica from artificially contaminated animal feed (F2)

Agar medium	Incubation temperature °C	No. of occasions+ on which Y. enterocolitica was isolated from all enrichment cultures incubated at °C		
		4	22	30
Deoxycholate citrate sucrose	30	22	3	5
Deoxycholate citrate sucrose	22	8	3	1
Lee	22	6	0	3
Deoxycholate citrate sucrose	4	0	1	5
Lee	4	0	2	12

+ NB: For the purposes of this table an occasion is the equivalent of each subculture to agar media.

4°C but the largest number of isolations (34) was made when the same cultures were sub-cultured to agar media incubated at 22° and 30°C. In comparison there was also notably more isolations of Y. enterocolitica from broth cultures incubated at 30°C when they were sub-cultured to agar media incubated at 4°C than when they were sub-cultured to agar media incubated at 22° or 30°C.

A total of 347 colonies were sub-cultured for identification, of these 89 proved to be Y. enterocolitica. At the time that this work was carried out facilities were not available for the serological identification of these particular strains. Slide agglutination was unreliable and serotype 0:3 was not detected. Eventually, when the 89 strains could be further investigated, eight had either died or dried up but a summary of the serotypes and isolation details of the remaining 81 are shown in Table 46. Six of eight strains of serotype 0:3 were isolated from direct enrichment in WB incubated at 30° (5 strains) and 22°C (1 strain). The seventh strain was isolated from WB pre-enriched in buffered peptone water incubated at 4°C and the eighth strain from direct enrichment in phosphate buffered saline at 4°C.

Sixty-nine strains of serotype 0:6,30 were isolated readily from all the broth media examined with the exception of WB, only one strain was isolated from this medium.

In addition several natural contaminants were isolated. Serotype 0:5 was isolated twice from direct enrichment in BP at 4°C and GN at 22°C. One strain of serotype 0:6,31 was recovered from WB by direct enrichment and one non-typable strain was also found by direct enrichment in GN 100 ml at 30°C. These strains are not included in any of the later comparisons of biochemical or serological results.

b. Comparison of media for the isolation of Y. enterocolitica from 194 foods and other materials - Study 2

A comparison of media for the isolation of Y. enterocolitica from 194 foods and other materials is shown in Table 47. Yersinia enterocolitica was not isolated from any of the 41 animal feed, 3 cheese, 20 coconut, 20 prawns or the 96 carcass swabs.

Four of 14 sewer swabs yielded Y. enterocolitica. Three isolates were obtained by direct enrichment in BHI broth incubated at 30°C and all were serotype 0:5. Only one strain, serotype 0:5,27 was isolated from BP at 4°C. The four positive swabs were sampled from the

Table 46

Summary of serotypes and isolation media for 81 strains of Y. enterocolitica recovered from artificially contaminated animal feed (F2)

Incubation temperature °C	Method	Total no. strains	No. strains recovered					
			Serotype 0:3	Broth medium (no).	Serotype 0:6,30	Broth medium (no).	Other types	Broth medium (no).
4	Direct enrichment	26	1	PB (1)	24	BP (10) BHI (1) GN100 (3) GN225 (3) PB (7)	0:5 (1)	BP (1)
4	Pre-enrichment	13	1	Ex BP (1)	12	BHI (3) BP (2) GN225 (7)	0	
22	Direct enrichment	10	1	WB 100 (1)	8	WB100 (1) GN100 (7)	0:6,31 (1)	WB225 (1)
22	Pre-enrichment	1	0		0		0:5 (1)	Ex BHI225
30	Direct enrichment	14	5	WB100 (1) WB225 (4)	8	BP (2) GN100 (2) GN225 (1) BHI (3)	Not typable (1)	GN100 (1)
30	Pre-enrichment	17	0		17	Ex BP (4) Ex BHI (13)	0	

BHI = Brain Heart Infusion broth; BP = buffered peptone water; GN = Gram-negative broth; PB = phosphate buffered saline; WB = Wauters' broth; Ex = pre-enriched from.

Table 47

A comparison of media for the isolation of Y. enterocolitica from 194 foods and other materials - Study 2

Sample	No. examined	No. positive for Y. enterocolitica through Buffered peptone at 4°C		Brain Heart Infusion at 30°C		Wauters' broth at 30°C	
		LEE	DCSA	LEE	DCSA	LEE	DCSA
Animal feeds, Batch A	15*	NT	NT	0	0	NT	NT
Batch B	26	0	0	0	0	0	0
Cheese	3	0	0	0	0	0	0
Coconut, desiccated	20	0	0	NT	NT	NT	NT
Prawns, frozen cooked	20	0	0	0	0	0	0
Swabs from Cow carcass	21	0	0	NT	NT	NT	NT
Swabs from Lamb carcass	35	0	0	NT	NT	NT	NT
Swabs from Pig carcass	40	0	0	NT	NT	NT	NT
Swabs from Sewer	14	1	0	2	1	0	0
TOTALS	194	1	0	2	1	0	0
% positive		0.6	0	2.6	1.3	0	0

NT = not tested; LEE = Lee agar; DCSA = deoxycholate citrate sucrose agar; * = these samples were also examined in GN broth - Y. enterocolitica was not found.

slaughterhall drain, corral drain, the entrance to the lairage and the foul sewer from the slaughtermen's toilets. All the strains were biochemically Y. enterocolitica sensu stricto.

c. Comparison of media for the isolation of Y. enterocolitica from raw meats - Study 3

The comparison of media for the isolation of Y. enterocolitica from 272 raw meats is shown in Table 48.

Yersinia enterocolitica was isolated from 9 of 237 beef, pork, lamb and sausage samples incubated in BP at 4°C. The nine positive isolations were all identified on LEE agar and none were found on DCSA.

Two samples of the 35 incubated in BP at 4°C and BHI at 30°C yielded Y. enterocolitica. These strains were identified on LSU but not on LEE agar.

The isolates were predominantly serotype 0:5 followed by 0:6,30. Two strains isolated from sausages were biochemically Y. frederiksenii, one of these was serotype 0:16,29 and the other was non-typable.

d. Comparison of methods and media for the isolation of Y. enterocolitica from bulked mechanically deboned pork samples and miscellaneous vegetables - Study 4

Yersinia enterocolitica was not isolated from the 13 vegetables examined. The results of the comparison of isolation from mechanically deboned pork is shown in Table 49. The results compare direct enrichment through BP and PS and pre-enrichment through these media to WB for the isolation of Y. enterocolitica from bulked mechanically deboned pork. Yersinia enterocolitica was isolated from 16/23 bulked samples (Table 49) after 20 weeks storage at -20°C and contrasted markedly with the 1/100 isolated in Study 3 (Table 48). Thirteen of the 16 were obtained by direct enrichment with or without pre-enrichment and the remaining three by pre-enrichment only. Five of the 13 samples were positive by the direct enrichment method only.

By direct enrichment two samples yielded Y. enterocolitica after 1 weeks incubation, seven only after 3 weeks and four only after 6 weeks. Both BP and PS yielded the same number of isolations at 3 and 6 weeks.

All the isolations of Y. enterocolitica made from direct enrichment in BP and PS were on LSU except for one which was on DCSA + 0.004%

Table 48

A comparison of media for the isolation of Y. enterocolitica from 272 raw meats - Study 3

Food	Broth medium: Buffered peptone at 4°C			Broth medium: Buffered peptone at 4°C			Broth medium: Brain Heart Infusion at 30°C		
	No. examined	Sub-cultured to		No. examined	Sub-cultured to		No. examined	Sub-cultured to	
		LEE	DCSA		LEE	LSU		LEE	LSU
Beef, minced	69	5	0	11	0	1	6	0	0
Beef, cuts	21	0	0	6	0	0	6	0	1
Pork, minced	4	1	0	0	0	0	0	0	0
Pork, cuts	28	0	0	2	0	0	2	0	0
Sausage	14	2	0	1	0	0	1	0	0
Pork, MD	100	1	0	0	0	0	0	0	0
Lamb, minced	1	0	0	0	0	0	0	0	0
TOTALS	237	9	0	20	0	1	15	0	1
% positive		4	0		0	5		0	6.6

DCSA = deoxycholate citrate sucrose agar; LSU = lactose sucrose urea agar; MD = mechanically deboned.
LEE = Lee agar.

Table 49

A comparison of methods and media for the isolation of Y. enterocolitica from 23 bulked mechanically deboned pork samples - Study 4

Broth medium from which Y. enterocolitica was recovered (total no. of isolates/no. of isolates unique to the medium or method)			Agar medium on which Y. enterocolitica was recovered (total no. of isolates/no. of isolates unique to medium)			
			lactose sucrose urea	Lee	deoxycholate citrate sucrose	D4
Phosphate buffered saline		9/3	9/9	0/0	0/0	0/0
Buffered peptone water		10/4	10/10	0/0	0/0	0/0
Direct enrichment method		13/5	13/13	0/0	0/0	1/1
Total after incubation for	1 week	2/0	2/2	0/0	0/0	0/0
	3 weeks	9/2	9/9	0/0	0/0	0/0
	6 weeks	9/4	9/9	0/0	0/0	0/0
Wauters' broth pre-enriched through	Phosphate buffered saline	4/3	3/1	3/1	0/0	0/0
	Buffered peptone water	8/7	5/5	2/2	1/1	0/0
Pre-enrichment method		11/3	6/4	5/3	1/1	0/0

D4 = deoxycholate citrate sucrose agar plus 0.004% malachite green.

malachite green (D4) agar. Following pre-enrichment Y. enterocolitica was isolated on LSU (7/11), LEE agar (5/11) and DCSA and D4 agars (1/11 each). The total number of isolations by all enrichment procedures on LSU, LEE , DCSA and D4 agars were 32, 5, 1 and 1 respectively.

Nine serotypes were identified which included 0:5 (15); 0:5,27 (3); 0:6,30 (2); 0:6,31 (2); 0:7 (4); 0:8 (2); 10K1 (2); 0:12,25 (1) and 0:34 (5). Thirteen non-typable strains were also found, of these eight were biochemically Y. intermedia and one Y. frederiksenii.

e. Comparison of broth media for the isolation of Y. enterocolitica from 503 miscellaneous foods and other materials - Study 5

The results of a comparison of enrichment media for the isolation of Y. enterocolitica from 503 foods and other materials are shown in Table 50. Yersinia enterocolitica was isolated from 25 of the 503 samples examined (5.0%). Significantly the 4°C isolation rates were much higher at 1.4-3.3% as contrasted with 30°C which was 0.2-0.8%. The isolation rate for the 503 samples through S (11/331 - 3.3%) was slightly higher than those through BP, 14 (2.8%), and PS, 7 (1.4%) incubated at 4°C. The isolation rates of these same three media when compared only for the 331 samples which were common to all were S, 11 (3.3%); BP, 9 (2.7%) and PS, 4 (1.2%).

The serotypes included 0:5; 0:5,27; 0:6,30; 0:7; 0:8; 0:16,29; 0:18 and non-typable strains. One strain only, from chicken, was identified biochemically as Y. intermedia.

Summaries of the overall performance. The broth and agar media used in the studies are shown in Tables 51 and 52. The results only include those where 25 g of sample was incubated in 225 ml of broth medium.

The isolation rates from BP, PS and S incubated at 4°C were 4.5%, 3.3% and 3.3% respectively. At 30°C the recovery rates from BP and PS were only 0.4% and 0.2% respectively. Two other media also incubated at 30°C, BHI and GN, gave slightly higher rates of 1.0% and 0.8% respectively, and a third medium WB showed a 1.6% isolation rate but relatively few samples were examined in this latter medium.

The pre-enrichment method was investigated in one study involving 36 samples. The recovery rate from WB pre-enrichment from BP and PS was 30%. However, the recovery rates from the same BP and PS directly

Table 50

A comparison of methods and broth media for the isolation of Y. enterocolitica from 503 miscellaneous foods and other materials - Study 5

Samples	No. examined	No. samples positive	No. positive for Y. enterocolitica through						
			Buffered peptone water	Phosphate buffered saline	Supplemented phosphate buffer*	Buffered peptone water	Phosphate buffered saline	Brain Heart infusion	GN Broth
			at 4°C			at 30°C			
Cream, UHT	4	0	0	0	NT	0	0	0	0
Milk, pasteurized	67	13	7	5	4/52	2	1	2	4
Yoghurt	1	0	0	0	0/1	0	0	0	0
Egg, clay	1	0	0	0	NT	0	0	0	0
Egg, dried	15	0	0	0	0/15	0	0	0	0
Sausages	26	5	3	2	3/20	0	0	0	0
Chickens, frozen raw	101	5	2	0	4/50	0	0	0	0
Turkey	2	0	0	0	0/2	0	0	0	0
Cockles, raw	49	0	0	0	0/49	0	0	0	0
Prawns, frozen cooked	30	0	0	0	0/15	0	0	0	0
Shrimps, dried	7	0	0	0	0/7	0	0	0	0
Animal feed	98	1	1	0	0/61	0	0	0	0
Coconut	16	0	0	0	0/11	0	0	0	0
Ham, cooked	1	1	1	NT	NT	NT	NT	NT	NT
Mineral waters	5	0	0	0	NT	0	0	0	0
Spices	20	0	0	0	NT	0	0	0	0
Surface waters	4	0	0	0	NT	0	0	0	0
Vegetables	56	0	0	0	0/48	0	0	0	0
TOTALS	503	25	14	7	11/331	2	1	2	4
% positive		5.0	2.8	1.4	3.3	0.4	0.2	0.4	0.8

NT = not tested; * = supplemented phosphate buffer was only included in the later stages of this study and therefore not all samples were examined in this medium.

Table 51

Summary of the overall performance of the broth media used for the isolation of Y. enterocolitica from foods and other materials

Medium (direct enrichment 25 g sample/225 ml broth)	Temperature of incubation °C	No. samples examined	No. positive (%)
Buffered peptone water	4	760	34 (4.5)
Phosphate buffered saline	4	518	17 (3.3)
Supplemented phosphate buffer	4	331	11 (3.3)
Brain Heart Infusion	30	576	6 (1.0)
Buffered peptone water	30	482	2 (0.4)
GN broth	30	498	4 (0.8)
Phosphate buffered saline	30	482	1 (0.2)
Wauters' broth	30	64	1 (1.6)
Wauters' broth pre-enrichment	22	36*	11 (30.0)

* The isolation rates for the same samples in the buffered peptone water and phosphate buffered saline from which the Wauters' broth were pre-enriched were 30% and 25% respectively.

Table 52

Summary of the overall performance of the agar media used for the isolation of Y. enterocolitica from foods and other materials

Medium (sub-cultured from 225 ml enrichments containing 25 g sample)	No. samples examined	No. positive (%)
Deoxycholate citrate sucrose	252	4 (1.6)
D4	36*	1 (2.8)
Lactose sucrose urea	554	43 (7.8)
Lee	306	16 (5.2)

* These samples were also sub-cultured to deoxycholate citrate sucrose, lactose sucrose urea and Lee agars and the isolation rates were 2.8, 47.0 and 14.0% respectively.

D4 = deoxycholate citrate sucrose agar plus 0.004% malachite green.

were 30% and 25% respectively.

The three agar media most frequently used throughout the five studies were DCSA, LSU and LEE. The overall isolation rates on these agars was 1.6%, 7.8% and 5.2% respectively. The deoxycholate citrate sucrose agar plus 0.004% malachite green (D4) received only a limited trial but the recovery rate for the 36 samples was 2.8%. In the same study the recovery rates on DCSA, LSU and LEE agars was 2.8%, 47.0% and 14.0% respectively.

f. Additional laboratory examinations of pasteurized milk received for Study 5

i. Phosphatase test

The isolation of Y. enterocolitica from milk in relation to ug-nitrophenyl/ml (phosphatase reaction) is shown in Table 53. All the 31 milks examined contained 10 µg or less p-nitrophenyl/ml and therefore satisfied the phosphatase test indicating that they had been adequately pasteurized. Yersinia enterocolitica was isolated from 3/31 samples. Both the Unigate samples 1 and 3, from which Y. enterocolitica was isolated, were marked with the same code although they were delivered by the dairy two days apart.

ii. Most probable number (MPN) of Y. enterocolitica

Unigate samples 2 and 3 (Table 53) were both examined by the MPN test. Yersinia enterocolitica was not isolated from sample 2 either by the normal enrichment procedures of Study 5 or from the BP used for MPN. However, an MPN of 0.03 organisms/ml was obtained from sample 3 (Series A tubes 0/3 positive; Series B tubes 1/3 positive; Series C tubes 0/3 positive) - see Appendix 1.

Isolation of Y. enterocolitica from milk in relation to μg para-nitrophenyl/ml (phosphatase reaction)

Brand	Sample No.	Date sampled (July)	Cap code dating	μg p-nitro-phenyl per ml**	Y. entero-colitica
London Co-operative Society	1	20	6	0	NF
	2	21	7	0	NF
	3	22	7	0	NF
	4	23	3	<6	NF
	5	24	3	<6	NF
	6	28	5	<6	NF
	7	28	6	0	NF
	8	28	1	6	NF
	9	29	1	6	NF
	10	30	6	<6	NF
Express Dairy	1	21	20G	10	NF
	2	21	21G	6	Present
	3	22	23G	6	NF
	4	23	23G	0	NF
	5	24	25G	0	NF
	6	25	25G	0	NF
	7	28	27G	6	NF
	8	28	27G	<6	NF
	9	28	29G	<6	NF
	10	29	30G	6	NF
	11	30	30G	0	NF
Unigate Dairies	1	20	22	0	Present
	2	21	-	0	NF
	3	22	22	0	Present*
	4	23	25	0	NF
	5	24	25	0	NF
	6	28	26	0	NF
	7	28	27	0	NF
	8	28	30	0	NF
	9	29	30	6	NF
	10	30	31	0	NF

NF = not found; * = most probable number ≡ 3 organisms per 100 ml sample; - = code unknown; ** = results of 10 μg or less are considered to have been satisfactorily pasteurized.

2. Characterization of isolates from naturally contaminated foods and other materials

a. Biochemical characterization

i. Screening of suspicious colonies (from Studies 1i, 2, 3, 4, 5)

From a total of 782 suspicious colonies sub-cultured, 156 gave the results as shown in Table 54.

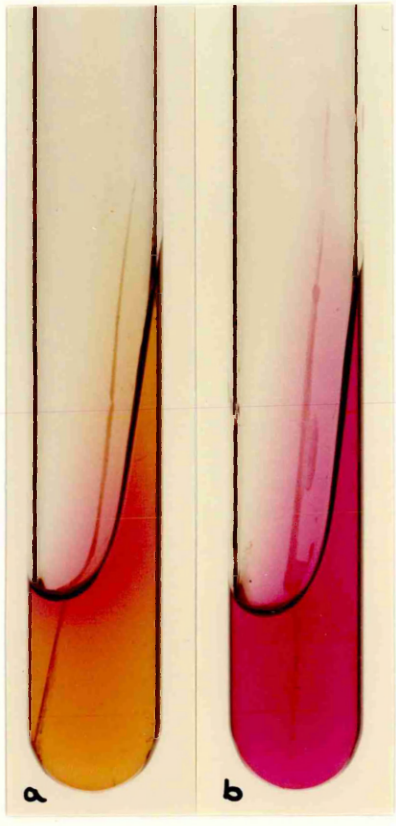
Table 54

Characteristics of 156 presumptive Y. enterocolitica isolates

Medium	Test result
Glucose peptone water sugar	Acid with or without small amount of gas. Lightly turbid, translucent growth. No pellicle. No deposit.
Urea slope	Urease activity, positive, the violet colour developed slowly over 48-72 h beginning at the point where the inoculation wire entered the stab at the base of the slope. The colour eventually spread through the entire medium (Plate 18).
Phenylalanine agar	Phenylalanine deamination negative.
MacConkey agar	Pure (no contaminant colonies). Non-lactose or lactose fermenting. Translucent or semi-translucent colonies.

The slow urease activity was characteristic of every presumptive Y. enterocolitica isolate (Table 54). With some strains the colour had begun to develop within 24-30 h. Strains of Proteus were characterized by a rapid and complete colour change occurring within 18-24 h incubation. Klebsiella strains produced a slow reaction which began at the top of the slope and gradually spread downwards, and although was usually complete

Plate 18. Urease activity by presumptive Y.enterocolitica



a = slow development of violet colour due to urease activity at the point where the inoculum wire entered the agar ; b = completed colour change.

by 72 h the colour change was frequently paler.

ii. Full biochemical identification of presumptive Y. enterocolitica isolates

A summary of the reactions of all the 156 isolates is shown in Table 55, and the biochemical classification according to Bercovier et al. (1980a) and sources of the isolates are shown in Table 56.

One hundred and fourty-four of the 156 isolates gave the typical reactions of Y. enterocolitica sensu stricto but the remaining 12 strains showed variable reactions in rhamnose, melibiose, raffinose and Simmon's citrate. Nine of these 12 isolates produced positive reactions in all four tests and were characteristic of Y. intermedia. The other three isolates were rhamnose positive, melibiose and raffinose negative and gave varied reactions in Simmon's citrate and were characteristic of Y. frederiksenii.

All (35/35) the Y. enterocolitica sensu stricto strains isolated from milk were lactose positive whereas only 9 of the 109 strains isolated from other sources had this characteristic.

b. Antibiotic sensitivity patterns

The antibiotic sensitivity patterns of 126 strains of Y. enterocolitica are summarized in Table 57.

The majority (greater than 74%) of the strains were resistant to ampicillin 25 μg, carbenicillin 100 μg, cephaloridine 5 μg, erythromycin 10 μg, furazolidine 100 μg, kanamycin 5 μg, penicillin G 1.5 units and streptomycin 10 μg and sensitive to colistin sulphate 10 μg, gentamycin 10 μg, neomycin 30 μg, sulphafurazole 500 μg, sulphamethoxazole/trimethoprim 25 μg and tetracycline 50 μg. Only 58% of the strains were resistant to cephaloridine 25 μg and 52 and 56% respectively were sensitive to sulphonamides 300 μg and chloramphenicol 10 μg.

The nine strains of Y. intermedia and two strains of Y. frederiksenii showed similar patterns to the Y. enterocolitica strains.

Table 52

The biochemical reactions of 156 presumptive *Y. enterocolitica* isolates

Test or substrate	Proposed neotype strain 161 (Bercovier *et al.* 1980a)	Isolates % + (total)	Isolates % + (delayed* reaction)
Adonitol	-	0	
L-Arabinose	+	100	
D-Cellobiose	+	100	1
Dulcitol	-	0	
D-Glucose	+	100	
Glycerol	+	100	4
i-Inositol	+	93	74
Inulin	-	0	
Lactose	-	34	10
Maltose	+	100	1
D-Mannitol	+	100	
Melibiose	-	6	
D-Raffinose	-	28	3
L-Rhamnose	-	10	
Salicin	+	99	
D-Sorbitol	+	100	
Sucrose	+	100	
Trehalose	+	100	
D-Xylose	+	100	
Catalase	+	100	
Citrate (Simmons')	-	10	
Decarboxylase arginine	-	0	
lysine	-	0	
ornithine	+	100	
Gelatin	-	0	
Indole	+	96	
Lipase	+	99	
Malonate	-	0	
Motility 22°C	+ (28°C)[a]	100	
37°C	-	0	
Methyl red 22°C	- (28°C)[a]	68	
37°C	+	100	
Nitrate reduction	+	100	
β-Galactosidase (ONPG)	+	100	
Oxidation/fermentation	F	100	
Phenylalanine deaminase	-	0	
Urease activity	+	100	
Voges-Proskauer 22°C	+ (28°C)[a]	97	
37°C	-	0	

* = reaction delayed 3 days or more; a = temperature used by Bercovier *et al.* (1980a).

Table 56

Biochemical classification according to the scheme of Bercovier et al. (1980a) of 156 Yersinia spp. isolated from foods and other materials

Samples	Y. enterocolitica sensu stricto rham - mel - raff - cit - lac -	Y. enterocolitica sensu stricto rham - mel - raff - cit - lac +	Y. frederiksenii rham + mel - raff - cit d	Y. intermedia rham + mel + raff + cit +	Total no. isolates
Milk, pasteurized	0	35	0	0	35
Beef, raw mince	6	0	0	0	6
Beef, raw cuts	1	0	0	0	1
Pork, raw mince	2	0	0	0	2
Pork, mechanically deboned	43	0	1	8	52
Sausages, raw	8	2	2	0	12
Chicken, raw frozen	5	0	0	1	6
Sewer swabs	5	1	0	0	6
Animal feed	30	4	0	0	34
Ham, cooked	0	2	0	0	2
Totals	100	44	3	9	156

d = variable; rham = rhamnose; mel = melibiose; raff = raffinose; cit = Simmons' citrate.

Table 57

Antibiotic sensitivity of 126 Y. enterocolitica strains isolated from food and other materials

Antibiotic/concentration		Sign	%	(% MR)
Ampicillin	2 µg	R	100	(0)
Ampicillin	25 µg	R	89	(10)
Carbenicillin	100 µg	R	90	(10)
Cephaloradine	5 µg	R	95	(0)
Cephaloradine	25 µg	R	58	(33)
Chloramphenicol	10 µg	S	56	(8)
Colistin sulphate	10 µg	S	79	(17)
Erythromycin	10 µg	R	100	(0)
Furazolidone	100 µg	R	74	(21)
Gentamycin	10 µg	S	97	(3)
Kanamycin	5 µg	R	84	(9)
Neomycin	30 µg	S	99	(0)
Penicillin G	1.5 units	R	100	(0)
Streptomycin	10 µg	R	85	(10)
Sulphafurazole	500 µg	S	94	(2)
Sulphonamides	300 µg	S	52	(40)
Sulphamethoxazole/ Trimethoprim	25 µg	S	96	(4)
Tetracycline	10 µg	S	100	(0)
Tetracycline	50 µg	S	100	(0)

R = resistant; MR = moderately resistant; S = sensitive.

c. Serological identification

i. Comparison of serological typing results obtained from two laboratories using different methods

A total of 84 cultures were examined by both laboratories and the results obtained from 78 of the 84 are compared in Table 58. Thirty-five of the 78 strains examined were found to be non-typable by the Yersinia Reference Laboratory using unsteamed antigen suspensions. In contrast only 20/78 could not be serologically identified by the Division of Enteric Pathogens using steamed suspensions. From a further 8 of the 78 cultures the two laboratories identified different serotypes.

Three of the six cultures not included in Table 58 were found not to be Y. enterocolitica by one laboratory but were identified serologically and biochemically as Y. enterocolitica by the other laboratory. The remaining three cultures were found by one or other of the laboratories to contain multiple serotypes.

ii. Serology of 30 of the non-typable (unsteamed) isolates

This study was carried out because of the large number of non-typable results returned from the Yersinia Reference Laboratory and at the time the facilities of the Division of Enteric Pathogens were not available.

The results of this study are shown in Table 59. The homologous titres of the four new sera were 2,500 or more. Serum No. 1 was agglutinated by four strains, nos. 1, 44, 56 and 60 to titres of 2,500 1280, 640 and 80 respectively. The first three strains were later identified as serotype 0:7 by the Division of Enteric Pathogens (DEP). They were biochemically Y. enterocolitica sensu stricto. The fourth strain, no. 60 was still non-typable when steamed and was biochemically Y. intermedia. The homologous antigens only, produced significant titres against sera nos. 29 and 73. Both these strains remained non-typable with steamed suspensions (DEP) and were biochemically Y. frederiksenii.

Serum 47 was agglutinated by five strains, nos. 48, 68, 74, 75 and 78 to titres of 160 or more. All these strains were also non-typable with steamed antigens (DEP) and were biochemically Y. intermedia.

Table 58

Comparison of steamed and unsteamed antigen suspensions for the serological typing of 78 food isolates

	No. of strains identified by "O" serotype													
Antigen	4,32	5	5,27	6,30	6,31	7	7,8	8	10K1	14	15	34	rough	not typable
Unsteamed*	0	26	0	7	0	0	1	0	0	4	1	0	0	35
Steamed**	2	25	3	4	2	4	0	2	1	3	1	5	2	20

* = PHLS Yersinia Reference Laboratory; ** = Division of Enteric Pathogens.

Table 59

Agglutination of 30 non-typable isolates with four new sera prepared from non-typable isolates

Strain No.	Agglutination titres against serum prepared from strain no. 1	29	47	73	"O" serotype using steamed antigen
1	**2500**	+	-	-	7
29	+	**5000**	-	-	NT
42	+	+	-	-	5
44	1280	+	+	-	7
45	-	-	-	-	NT
46	+	-	-	-	5,27
47	-	-	**2500**	-	NT
48	-	-	160	-	NT
49	+	+	-	-	NT
50	+	-	-	-	34
51	+	-	-	-	34
52	+	-	-	-	34
53	+	-	-	-	34
54	+	-	-	-	34
55	+	+	-	-	5,27
56	640	+	-	-	7
57	+	+	-	-	5,27
58	+	+	-	-	NT
59	+	+	-	-	NT
60	80	+	-	-	NT
62	-	-	-	-	10K1
68	-	-	640	-	NT
73	+	-	-	**2500**	NT
74	+	-	1280	-	NT
75	-	-	2500	-	NT
77	+	+	+	+	rough
78	-	-	2500	-	NT
79	+	+	+	-	rough
94	+	-	-	-	NT
99	+	+	-	-	NT

+ = minor reactions, titre <40; - = no reaction; NT = not typable;

[ ] = homologous antigen/sera.

3. Incidence of Y. enterocolitica in foods and other materials

A summary of the isolation of Y. enterocolitica from 1004 foods and other materials examined between 1978-1982 is given in Table 60. Yersinia enterocolitica sensu stricto was isolated from 53 (5.3%) samples which included raw beef, pork, sausages and chicken, sewer swabs, meat and bone meal, pasteurized milk and cooked ham. In addition organisms which were biochemically Y. intermedia were isolated from one chicken and six mechanically deboned pork samples and strains biochemically Y. frederiksenii were isolated from one mechanically deboned pork and two sausage samples.

One hundred and fifty-six isolates were sent to the Division of Enteric Pathogens for serotyping. Two cultures were found to contain two different serotypes bringing the total to 158 isolates. The serotype/source distribution of the 158 isolates is shown in Table 61.

A total of 158 isolates from 57 samples were confirmed and serotyped as Y. enterocolitica. Three of these isolates were later identified biochemically as Y. frederiksenii and nine as Y. intermedia. Some samples were found to contain multiple serotypes, the highest number from any one sample being the five serotypes, 0:5, 0:10K1, 0:14, 0:7 and one non-typable strain which were recovered from a single sample of pasteurized milk (Table 62). Fourteen different serotypes (125 strains) were identified, six strains were rough and therefore could not be serotyped and 27 were not typable with the available sera. The most commonly found serotype was 0:5 and this was present in many of the samples examined, including pasteurized milk, beef, pork, sausages, sewer swabs and animal feed. Much less commonly recovered were serotypes 0:5,27 and 0:6,30, both of which were isolated from beef, pork, sausages and sewer swabs and serotype 0:8 which was isolated from pasteurized milk, pork, sausages and animal feed.

Table 60

Isolations of Y. enterocolitica from foods and other materials obtained in the UK between 1978 and 1982

Sample	Countries of origin	Number Examined	Number Y. enterocolitica isolated
DAIRY PRODUCTS			
Cheese	Cyprus	3	0
Cream UHT	Denmark	4	0
Milk, pasteurized	UK	67	13
Yoghurt	Greece	1	0
EGGS			
Clay	China	1	0
Dried	West Germany, USA	15	0
RAW MEAT AND POULTRY			
Beef, minced	Unknown	86	6
Beef cuts	Unknown	33	1
Lamb, minced	Unknown	1	0
Pork, minced	Unknown	4	1
Pork cuts	Unknown	32	0
Pork MD* individual	UK	100	1
Pork MD* bulked	UK	23	14 (16)
Sausages	UK	40	6 (7)
Chicken, frozen raw	UK (100), Holland (1)	101	4 (UK) (5)
Turkey	USA	2	0
SEAFOODS			
Cockles, raw	UK	49	0
Prawns, frozen, cooked	Thailand, Malaysia	50	0
Shrimps, dried	Thailand, Malaysia	7	0
SLAUGHTERHOUSE			
Swabs from Cow carcass	UK	21	0
Swabs from Lamb carcass	UK	35	0
Swabs from Pig carcass	UK	40	0
Swabs from Sewer	UK	14	4
ANIMAL FEED			
Meat and bone meal	Unknown	140	2
MISCELLANEOUS			
Coconut	Phillipines, Sri Lanka	36	0
Ham	Unknown	1	1
Mineral waters	France	5	0
Spices	Unknown	20	0
Surface water	UK	4	0
Vegetables, raw	Unknown	69	0
TOTALS		1004	53 [5.3%] (57 = 5.7%)

*MD = mechanically deboned; ( ) total samples positive if Y. frederiksenii and Y. intermedia included.

Table 61

Source distribution of 158 Y. enterocolitica serotypes isolated 1978-1982

Samples	No. samples positive Y. enterocolitica sensu stricto		No. of strains identified by "O" serotype														
			4,32	5	5,27	6,30	6,31	7	8	10K1	12,25	14	15	16,29	18	34	Rough
Milk, pasteurized	13			16				1	1	3		4	1				3
Beef, raw mince	6			3	1	1											
Beef, raw cuts	1			1													
Pork, raw mince	1					2											
Pork, mecahnically deboned	15	(16)		15	3	2	2	4	2	2	1					5	3
Sausages, raw	6	(7)		2	1	1		1	3					1[a]	1		
Chicken, raw frozen	4	(5)	2				1	2									
Sewer swabs	4			5	1												
Animal feed	2			3		7		3	20	1							
Ham, oooked	1																
TOTAL	53	(57)	2	45	6	13	3	11	26	6	1	4	1	1	1	5	6

a = 1 strain from each biochemically Y. frederiksenii; b = 1 strain from raw chicken and 8 strains from mechanically deboned pork biochemically Y. intermedia; ( ) = no. of samples positive, including Y. frederiksenii and Y. intermedia.

Table 62

Isolation of multiple serotypes of Y. enterocolitica from individual samples (excluding all bulked materials)

Sample	No.	"O" serotypes isolated
Milk, pasteurized	1	5; 14
Milk, pasteurized	2	5; non-typable
Milk, pasteurized	3	5; 10K1; 14; 7; non-typable
Milk, pasteurized	4	15; rough
Sausages, raw	5	8; non-typable*
Sausages, raw	6	8; 6,30
Sausages, raw	7	5; 5,27; 7
Sewer swabs	8	5; 5,27

* = biochemically Y. frederiksenii.

Discussion

Comparisons of methods and media

Two of the basic isolation methods, namely, direct enrichment and pre-enrichment were compared. Because of the low likelihood of there being sufficient numbers of *Y. enterocolitica* present in most foods, direct plating was not included in these comparisons. The varied nature of the samples examined and the irregularity of their arrival into the laboratory caused one or two of the studies to be somewhat incomplete. Except for the sewer and carcass swabs and the milks the samples were received either as part of the normal routine commitment or as part of special surveys where other examinations took priority and often there was very little sample left for *Y. enterocolitica* investigations.

Naturally and artificially contaminated animal feed - Study 1. Both the naturally and the artificially contaminated animal feeds were examined simultaneously and therefore the level and identity of the naturally occurring *Y. enterocolitica* was unknown until after completion of both investigations.

Yersinia enterocolitica was isolated more frequently and after a shorter incubation time from the artificially inoculated sample (F2) than from the naturally contaminated sample (F1). This was presumably because of the increased level of contamination in F2. Both studies showed a higher recovery rate through direct enrichment incubated at 4°C than through direct enrichment incubated at either 22° or 30°C. *Yersinia enterocolitica* was not recovered through Wauters' broth (WB) incubated at 4°C in either study but was recovered from 22° and 30°C. Wauters (1973) recommends the incubation of his broth (WB) at 29°C or 22-29°C although he does not state that it should not be incubated at a lower temperature. The results of both this study and the evaluation of broth media in the previous section (see page) indicate that *Y. enterocolitica* will not grow in WB incubated at 4°C.

In neither part of this study did pre-enrichment show any significant advantage over direct enrichment and in one instance *Y. enterocolitica* was not isolated through pre-enrichment in WB when it had been recovered through the direct enrichment broth from which the WB had been inoculated. This finding was somewhat surprising as the pre-enrichment

technique is highly recommended for the isolation of other pathogens from processed (dried or frozen) foods.

There are no published reports of the utilization of variable temperatures, other than 22° and 30°C, for the incubation of agar media for the isolation of Y. enterocolitica. The results of both parts of Study 1 indicated that there might be some advantage in incubating the agar plate at the opposite temperature to that which the broth had been incubated (broth at 4°C, agar at 22° or 30°C and vice versa). This in theory could reduce the waiting time (6-7 weeks) for results if Y. enterocolitica was recoverable from broths incubated at 30°C for 2 days followed by sub-culture to agars incubated at 4°C for 14 days. However, the isolation rate was lower from broths incubated at 30°C than from those incubated at 4°C due most probably to rapid overgrowth of Y. enterocolitica by other organisms at this temperature. So to adopt the 30°C broth plus 4°C agar would result in a rather large proportion of false negative results, which would be most undesirable.

An attempt was also made to correlate media, temperature of incubation and serotype selection. None could be demonstrated on the agar media. However, in the broth media serotype 0:8 was isolated only by direct enrichment at 4°C (3) and pre-enrichment at 22°C (1) whereas serotype 0:6,30 was recovered by direct enrichment at 4°C (1) and 30°C (1). It is difficult with the low contamination rate of the naturally infected sample to attribute this result to anything but random sampling rather than to specific selection.

The picture with the artificially contaminated feed (serotypes 0:3 and 0:9 added, F2) was slightly different. Wauters (1973) stressed that the use of WB applied specifically to the isolation of serotypes 0:3, 0:9 and possibly serotypes 0:1 and 0:8. In this examination of artificially contaminated feed there was a significant selection of serotype 0:3 by Wauters' broth. This serotype was recovered seven times from WB (four after pre-enrichment in BP) and only once from phosphate buffer. Serotype 0:6,30 was recovered readily from most of the other media.

Comparison of media for the isolation of Y. enterocolitica from 194 foods - Study 2. As Y. enterocolitica had been recovered through buffered peptone water incubated at 4°C and Brain Heart Infusion broth and Wauters' broth incubated at 30°C in Study 1 these media were chosen for Study 2.

Unfortunately there was frequently insufficient material available so many of these samples were not examined in all three broth media.

The sewer swabs which had been sampled at a local slaughterhouse gave the most interesting results. All the swabs were heavily contaminated with debris from the sewers, which contained a considerable amount of straw and faecal matter. The build up of debris was partly caused by the swabs having to be left in situ for 7 days. Yersinia enterocolitica was isolated from three of the four positive samples through highly nutrient Brain Heart Infusion broth incubated at 30°C. This might be attributed to these particular swabs being taken at the end of a 4 week period during November to December 1978 when there was frequently a ground frost and which was especially hard during the fourth week. It may be postulated that cold temperature selection occurred in the sewers prior to collection. The psychrotrophs and organisms such as Y. enterocolitica were not only able to survive but also able to grow at the expense of the strict mesophilic organisms so that when enriched in BHI incubated at 30°C the Yersinia, being a mesophile and a facultative psychrotroph, was able to compete more readily.

Comparison of media for the isolation of Y. enterocolitica from 272 raw meats - Study 3. Very small quantities of these meats were received and an examination for Campylobacter spp. took priority. Only one broth medium could be chosen for this study but it was possible to compare the agar media.

Deoxycholate citrate sucrose agar faired very poorly, many problems beset this medium which were first manifested by the loss of the characteristic appearance of Y. enterocolitica. Further investigation showed that the appearance and growth of Salmonella spp. was affected also. Despite a full investigation and correction of a series of errors the medium has never regained the original characteristics (Kendall 1982b).

The apparent success of LEE agar in the first 237 samples and failure in the remaining 35 samples may have been due at least in part to Proteus swarming and masking the diagnostic features of Y. enterocolitica. This problem recurred from time to time throughout the studies and was caused by variation in the inhibitory quality of the bile salts in the MacConkey base medium. These variations, according to the manufacturer, were unavoidable (Kendall 1982b).

No opinion could be formed on the performance of lactose sucrose

urea agar as it had not been used for sufficient samples.

Comparison of methods and media for the isolation of Y. enterocolitica from bulked mechanically deboned pork and vegetables - Study 4. The problems encountered with LEE and DCSA agars as well as the size (1 g) of the samples examined may account for the isolation of Y. enterocolitica from only 1/100 samples of mechanically deboned pork in the original examination (Study 3). In this study 25 g quantities of the bulked mechanically deboned pork were examined.

The difficulties already experienced with LEE and DCSA agars were also repeated in this study. As the base for D4 agar is DCSA it must be assumed that this also was affected.

Yersinia enterocolitica was not isolated from the bulked samples of mechanically deboned pork in direct enrichment broths and sub-cultured on to LEE agar but there was no appreciable difference between the isolations made on LEE and lactose sucrose urea agars inoculated from the pre-enrichment broth. This difference in the isolation rates on these agars sub-cultured from the direct and pre-enrichment broths was probably due to the greater selectivity of the Wauters' broth resulting in a reduced background flora on the LEE agar sub-cultured from this medium.

There was no appreciable difference between the two broth media, buffered peptone and phosphate buffer, used for direct enrichment. Yersinia enterocolitica was recovered from the largest number of samples by direct enrichment. The organism was isolated from five samples by the direct enrichment technique only and from a further three samples by pre-enrichment only. This compares favourably with Schiemann's (1980a) study where he reported a greater number of isolations of Y. enterocolitica from raw pork through direct enrichment in Wauters' broth than through the same broth pre-enriched in phosphate buffered saline. In this study the majority of the isolations were made within 3 weeks incubation and both PS and BP yielded the same number of isolations after 3 and 6 weeks incubation.

The pre-enrichment technique and incubation for 6 weeks were not at this stage considered fruitful enough to continue their use in future studies and so to facilitate the examination of a larger number of samples in a greater range of broth media and incubation temperatures these techniques were abandoned. The continuing problems with LEE and DCSA agars rendered them unreliable and so these too were abandoned for

the time being. Following the success of lactose sucrose urea agar this was the only agar medium retained for Study 5.

Comparison of methods and media for the isolation of Y. enterocolitica from 503 miscellaneous foods and other materials - Study 5. Very significant differences were found in this study. Firstly, isolation rates from broth media incubated at 4°C were considerably higher than those from broth media incubated at 30°C. Secondly, isolation rates from buffered peptone water and supplemented phosphate buffer incubated at 4°C, both of which contain nutrients for growth, were at least twice that from phosphate buffer incubated at 4°C which relies on the nutrients present in the food to be sufficient for growth of the organisms. There was no significant difference between the recovery rates from buffered peptone water and supplemented phosphate buffer.

The summary of the overall performance of the broth media (Table 51) confirms quite clearly that 4°C was by far the most successful temperature for incubation of the broth media and that there was very little difference between buffered peptone, phosphate buffered saline and supplemented phosphate buffer. An insufficient number of samples were examined in Wauters' broth incubated at 30°C and by pre-enrichment in Wauters' broth at 22°C to form any clear opinion about this medium. However, the isolation rates from 36 samples which were examined in Wauters' broth pre-enriched from buffered peptone water and phosphate buffered saline and by direct enrichment in the latter two media were similar.

On the basis of these results there seemed little value in the additional time, work and media involved in transferring the primary broth culture to the secondary medium as in the pre-enrichment technique.

When Schiemann & Toma (1978) examined raw milk and compared direct enrichment in both Wauters' broth (modified Rappaport broth) and phosphate buffered saline with pre-enrichment from cooked meat medium and phosphate buffered saline to Wauters' broth they had a considerably higher recovery of Y. enterocolitica through the PB/WB combination than through any of the other media. More recent studies (Schiemann 1980a) have shown a complete reversal of these results. When examining raw pork the highest isolation rate was by direct enrichment in WB.

It is reasonable, therefore, to assume that as with Salmonella

isolation the media and method for the isolation of Y. enterocolitica must be tailored to suit the type of food under examination.

The summary of the overall performance of the agar media (Table 52) leaves no doubt that of the media compared in these studies LSU was the most successful for the isolation of Y. enterocolitica. This medium was used for the examination of more samples than the others but when compared directly with the other agars, DCSA, D4 and LEE, as it was in Study 4, the recovery rate on LSU was 47% compared to only 2.8% for DCSA and D4 and 14% for LEE. The advantages of LSU are that all strains of Y. enterocolitica have a characteristic quality and smell whatever their biochemical variations. The main disadvantages to its use are the large number of ingredients (17), the very specific demand for Merck dyes and the requirement for oxygen free water for preparation. Nevertheless these disadvantages are far outweighed by the success of this medium.

Despite the problems which beset LEE agar, from time to time, the overall performance of this medium was good, partly because of the very distinct characteristics of Y. enterocolitica, and also because all the strains isolated from the foods were lipase positive. However, it must be remembered that many strains of Y. enterocolitica do not produce the Tween reaction (lipase) and could therefore be easily missed on this agar; Lee (1977) was aware of this when he formulated the medium. He reported that this agar was more successful than Salmonella-Shigella agar for the recovery of "native isolates" of Y. enterocolitica from artificially inoculated meat and oyster samples and goes on to state that this medium was particularly useful for the differentiation of Y. enterocolitica from other non-lactose fermenting bacteria. This statement could no doubt lead to many strains of the organisms being ignored as lactose fermenting Y. enterocolitica occur quite frequently.

Additional laboratory examinations of pasteurized milk received for Study 5. Yersinia enterocolitica was isolated from 3/31 milks all of which had passed the phosphatase test which indicated that they had been adequately pasteurized. It is therefore reasonable to assume that post-pasteurization contamination had taken place particularly as Hanna et al. (1977d) demonstrated that the organism was destroyed in skimmed milk after heating at 60°C (140°F) for 3 min. Milk is "deemed

pasteurized when it has been retained either at a temperature of not less than 145°F (62.8°C) and not more than 150°F (65.6°C) for at least 30 min or at not less than 161°F (71.7°C) for at least 15 sec".

Isolation, identification and incidence

Biochemical characterization. One of the most helpful tests in the screening media was Christensen's urea slope. In general this medium is inoculated by streaking the test organism over the entire surface of the slope. However, during this study the agar was inoculated by a single streak with a straight wire from the bottom of the butt to the top of the slope. This proved to be of real diagnostic value, for without fail the Yersinia strains first turned the agar mauve at the entry point into the butt and then only later the remainder of the slope went mauve. This effect has not been reported by any other workers.

The clear cut division of the Y. enterocolitica and Y. enterocolitica-like group of organisms into four new species, Y. enterocolitica sensu stricto, Y. frederiksenii, Y. intermedia and Y. kristensenii is a recent development (Bercovier et al. 1980a; 1980b; Brenner et al. 1980; Ursing et al.1980). In applying this classification to the strains isolated in this study they could be identified easily. The majority of the 156 strains were Y.enterocolitica sensu stricto(144), with a few strains of Y.frederiksenii (3) and Y.intermedia (9), but Y.kristensenii was not found.

Antibiotic sensitivity patterns. Although there were only a few strains of Y. frederiksenii and Y. intermedia isolated in this study, and with which to compare Y. enterocolitica sensu stricto there were no obvious major differences between the species in their resistance/sensitivity to the antibiotics tested. Because of the varied methods by which antibiotic sensitivity patterns are determined direct comparison with particular reports is difficult. In general, however, Y. enterocolitica has been found to be sensitive to tetracycline, chloramphenicol, colistin, neomycin and sulphonamides, slightly sensitive to ampicillin and resistant to erythromycin and benzyl penicillin. The results of the present study were very similar.

Variations of the 'normal' pattern have been observed and Chester & Stotzky (1976) and Raevuori et al. (1978) attempted to correlate biochemical characteristics with antibiotic patterns whilst Kouwatli et al. (1979) have shown that incubation temperature may have some influence on results. Cornelis (1981), however, has correlated serotype and

production of β-lactamases with antibiotic patterns.

It has been shown that Y. enterocolitica can act as a recipient of R plasmids and so like many other enterobacteria it is possible for the antibiotic patterns of this organism to change if it has been associating with another organism carrying a resistance plasmid. It is known that S. typhi plasmids transfer better at 28°C than at higher temperatures. It is possible that plasmids are responsible for some of the variable factors which occur with Y. enterocolitica especially as temperatures below 30°C are frequently used for incubation of enrichment cultures where there are large populations of other enterobacteria which may be carrying plasmids.

Serological identification

Comparison of results obtained using different methods. It was reported by Wauters (1981) that Y. enterocolitica may change from a smooth (S) form to a rough (R) form more frequently than other enterobacteria and that this phenomenon is enhanced by growth at 37°C. Wauters maintains that in intermediate R forms apart from the specific 'O' antigen an R antigen is responsible for non-specific cross reactions between strains of unrelated 'O' groups and even with other Gram-negative bacteria. This especially occurs with heated bacteria while non-heated antigens give specific agglutination in the 'O' serum. R agglutinins can be removed by absorption with a rough unrelated Y. enterocolitica strain or even by an R form of E. coli.

Although Wauters prepares his sera in rabbits using heated antigens his agglutinations are carried out using unheated suspensions.

The PHLS Yersinia Reference Laboratory (YRL) follows the methods of Wauters whereas the PHLS Division of Enteric Pathogens (DEP) prepares its own sera and carries out agglutination using heated suspensions and pure absorbed sera, as they do for any other enteric pathogens with 'O' antigens. The purpose of the steaming is to destroy any 'K' antigen which might mask the 'O' antigen.

Fifteen of 35 previously untypable (unsteamed) strains which had been isolated in these studies could be identified without any cross reactions occurring using steamed suspensions and only two strains became rough during steaming. This increase in the number of strains which could be typed lends considerable support to the case for the use of steamed antigens for Y. enterocolitica agglutinations. The more

strains which can be fully identified the better the epidemiological picture which may be obtained.

Serology of 30 non-typable isolates. At the time that this study was carried out a large number of the food isolates had been reported as non-typable by YRL and the facilities of the DEP were not yet available. The four sera were prepared from non-typable strains with variable biochemical characteristics and antigenic links were demonstrated between some of these strains using steamed antigens. These links were later confirmed by the DEP who also identified the serotypes of 13 of the 30 strains.

Incidence

In general, the isolations of Y. enterocolitica reported in this study are from similar sources to those which have been identified in other parts of the world, with the possible exception of animal feeding meal, from which there are no other reported isolations.

Dairy products. The isolation of Y. enterocolitica from milk and dairy products has been reported from Australia, Canada, Czechoslovakia and France (Hughes 1979, 1980; Schiemann 1978a; Schiemann & Toma 1978; Aldová et al. 1975; Vidon & Delmas 1981) but by far the largest number of isolations have been made from the raw product. However, Schiemann (1978a) and Hughes (1979) both surveyed pasteurized dairy products, most of which were taken from the processing plants. Schiemann's study revealed an incidence of only 0.4% Y. enterocolitica in samples which included homogenized milk, skim milk, cream, milk shakes and chocolate milk. The only strain found, which came from homogenized milk was identified as serotype 0:34 and possessed atypical VP negative, citrate positive biochemical reactions. Hughes (1979) isolated a much larger number of strains from an indeterminate number of samples of pasteurized milk and cream from two factories. The majority of the strains were reported to be typical Y. enterocolitica but two isolates were said to be rhamnose, raffinose and citrate positive which if compared to the classification of Bercovier et al. (1980a) were probably Y. intermedia. Only two serotypes were identified, 0:5 and 0:5,27.

The isolation rate of 1% from the pasteurized milk examined in the present study was certainly considerably higher than that obtained in Schiemann's study, but it is difficult to compare with Hughes' results as

she failed to report the total number of samples examined. This higher isolation rate in the present study may in part be due to the milk having been sampled at the customer end of the processing chain and therefore any organisms present had more time to grow.

In contrast to the Canadian and Australian isolates the strains found in this present study were all biochemically Y. enterocolitica sensu stricto and represented a wide variety of serotypes. As in Hughes' study the most common serotype identified was 0:5 but of the other five types found a single isolation of serotype 0:8 was the most interesting as it was this serotype which was responsible for the chocolate milk outbreak in the USA (Black et al. 1978). These results also revealed that individual samples of pasteurized milk may contain numerous different serotypes.

It is reasonable to assume that the contamination of the pasteurized milk by Y. enterocolitica occurred after processing because, firstly, 31 of the samples were examined for and passed the phosphatase test which confirmed that they had been through the pasteurization process. Despite this Y. enterocolitica was isolated from 3 of the 31 samples. Secondly, Hanna et al. (1977d) were able to demonstrate that destruction of Y. enterocolitica occurred after heating at 60°C (140°F) for 3 min. The legal temperatures for pasteurization of milk in the UK is 145°F (62.8°C) for 30 min or 161°F (71.7°C) for 15 sec which, based on Hanna's figures, should be sufficient to destroy any Y. enterocolitica present.

Raw meat and poultry. Beef, pork and poultry have all been reported as sources of Y. enterocolitica and of these meats, pork has usually yielded the highest isolation rates.

Schiemann (1980a) obtained a 49% isolation rate of Y. enterocolitica from raw pork products but only 7% from processed meats. Forty per cent of his isolates were typable and many were identified as serotype 0:3, the majority of which came from fresh pork tongues. Serotype 0:5 was the next most frequently isolated, with one or two isolates only of five other serotypes, one of which was 0:8. Although it is obvious from Schiemann's paper that not all the strains were Y. enterocolitica sensu stricto it was not possible to identify the exact biochemical types because of insufficient information. Leistner et al. (1975) also reported a high isolation rate of Y. enterocolitica from raw pork (35%). This survey also included raw beef and poultry from which they isolated 11% and 29% respectively. The literal translation of the identity of

the organisms isolated was "Y. enterocolitica in the narrow sense" but as some of these strains fermented rhamnose, according to Bercovier et al. (1980a) at least a few of these isolates were probably Y. frederiksenii. In addition Y. enterocolitica-like organisms were found in beef (16%), pork (35%) and poultry (46%). Serotypes 0:5A, 0:6 and 0:7 were the most frequently found and 48% of the strains of Y. enterocolitica were non-typable.

Norberg's (1981) 24.5% isolation of Y. enterocolitica from frozen chicken was not very different to Leistner's results. Only 7 of 22 isolates were typable with the ten sera available and these belonged to 0:6, 0:4, 0:5b and 0:8. Biochemical results were not reported.

In contrast to the foregoing results those obtained in the present studies are somewhat low, only 5% isolations from beef, 21% from pork (including sausages) and 4% from chicken. These figures include only those foods where at least 25 g was examined and are for Y. enterocolitica sensu stricto only and not the other species. However, even the inclusion of these other species would not increase the isolation rates to anything like those obtained by Schiemann (1980a), Leistner et al. (1975) or Norberg (1981). In the present study the most common serotype was again 0:5 with 0:5,27, 0:6,30 and 0:8 much in evidence. It was also demonstrated that multiple serotypes may be present especially in sausages. Not altogether surprising considering the many sources, and the variety of the meats which are minced together to make the British sausage. Those produced by individual butchers on their own premises probably containing the greatest variety.

Other samples. The purpose of examining slaughterhouse sewer swabs had been to obtain some indication of the presence of Y. enterocolitica in domestic cattle, sheep and pigs in this country before investigating individual species. In the event the first isolations of Y. enterocolitica were not made until the end of the trial period and because of bad weather and other commitments both in the laboratory and at the slaughterhouse it was not possible to arrange further visits and carry out a more intensive survey. However, some carcass swabs were obtained during the early visits but Y. enterocolitica was not isolated. The reasons for the lack of isolates was most likely to be the nature and size of the samples. A throat swab cannot absorb very much fluid and any Y. enterocolitica present on the carcass would have been well

diluted by the wash water, but in the circumstances it was not possible to sample by any other means.

One of the four sewer swabs from which Y. enterocolitica was recovered was taken from the foul sewer leading from the slaughtermen's toilets. This suggests very strongly that the slaughtermen were infected from the animals. The practices of a slaughterhouse lend themselves to the men very easily becoming infected with whatever pathogens might be present on the carcasses, hand washing was very infrequent and for the most part pointless and in this particular establishment the men removed only their leather aprons before going to the canteen for their mealbreaks.

All the strains isolated from the swabs were Y. enterocolitica sensu stricto and not surprisingly one swab contained two serotypes. Serotype 0:5 was common to all.

The presence of Y. enterocolitica in animal feed (meat and bone meal) is to be expected considering the presence of the organism in raw meat. One of the two samples from which Y. enterocolitica was isolated was the bulked sample examined in Study 1 and from which several serotypes were obtained. The individual samples were not examined before bulking and therefore it was not possible to say whether there was one very contaminated feed or several containing single serotypes. However, what is surprising is that Y. enterocolitica has been isolated from only 1 of 139 samples examined since that study.

Cooked ham was the only other food from which Y. enterocolitica was isolated. There are few reports with which to compare this result but Schiemann (1980a) obtained 7% isolation of the organism from processed pork products but he did not examine ham as such and some of his samples were cured rather than cooked. Ham is a cooked (pasteurized) and cured product and Y. enterocolitica should have been destroyed by the processing. However this particular sample was a slice from a commercial (6-7 lb) vacuum packed block which had already been opened. Therefore contamination might have occurred either after processing and during the handling necessary to vacuum pack or after opening by the customer.

The overall incidence of the different serotypes isolated in the various studies was similar to those found by other workers. In the present studies where bulked samples were inoculated into multiple broth media and sub-cultured to multiple agars the number of isolates of

particular serotypes are artificially boosted. In particular the exceptionally high isolation rate of serotype 0:8 from the animal feed in Study 1 was mainly due to the use of multiple media although without it some of the serotypes present in smaller numbers might well have been missed.

In summary, the highest recovery rates of Y.enterocolitica were on lactose sucrose urea agar incubated at 30°C and from buffered peptone water incubated at 4°C; Yersinia spp. were isolated from 57/1004 samples including pasteurized milk, raw meats, sewer swabs, animal feeds and cooked ham; 144 isolates were biochemically Y.enterocolitica sensu stricto, 9 Y.intermedia and 3 Y.frederiksenii, Y.kristensenii was not found; 14 serotypes were identified including 0:5,27, 0:6,30, and 0:8, 17% of the isolates were non-typable.

GENERAL DISCUSSION

Yersinia enterocolitica, recognized as a cause of human infection for nearly 50 years has only been regarded seriously as a potential food poisoning agent since 1973, when several large community outbreaks were reported from Japan. The vehicles of infection of these outbreaks were never proven for although Y. enterocolitica was isolated from many patients, the common sources which were implicated epidemiologically were not identified bacteriologically.

Up to 1976, 34 serotypes of Y. enterocolitica were recognized, and the few workers who were interested in the organism had for many years considered serotypes 0:3 and 0:9 to be the only ones of medical importance. However, with the wider interest caused by the outbreaks more workers began investigating and many of the other serotypes were isolated from humans as well as from a variety of animal and food sources throughout the world.

Various media and methods were described for the isolation of Y. enterocolitica but many of these were for the isolation from faeces only. Little thought had been given to the difference in background flora of faeces and foods and the need to develop more suitable media and methods for isolation from foods. Although the ability of the organism to grow at 4°C had been widely exploited, this entailed prolonged incubation of up to 6-9 weeks which is impractical in the investigation of either food or faeces, especially from outbreaks.

Despite the increasing isolation of Y. enterocolitica from foods and the organism's implicated role in outbreaks, little information was available as to the ability of the organism to survive or grow in foods under varying conditions.

In the UK up to 1976 few clinical cases had been recognized in spite of the growing worldwide interest, and there were no published reports to show incidence of the organism in foods.

The investigations described in this work consist of; the first reported study of the growth and survival of Y. enterocolitica in foods stored under varying conditions including a study of the effect of pH and sodium chloride; the first comprehensive study of the growth, survival and isolation of the organism through various broth media and on agar media, including an examination of the effect of dyes and other agents which might be used to improve or formulate new media with particular reference to isolation from foods; the first intensive study of the incidence of Y. enterocolitica in foods and other materials

obtained in the UK.

It was shown that two test strains of Y. enterocolitica serotypes 0:3 and 0:9 were able to survive in buffered Brain Heart Infusion broth at pH 4.2 and were able to grow slowly at pH 4.4. These results were in contrast to those obtained by Hanna et al. (1977b) who reported little or no growth of Y. enterocolitica at pH 5.0 but in a later study Stern et al. (1980a) confirmed the ability of the organism to grow at values as low as pH 4.6. However he showed that at pH 4.4 there were some bacteriocidal or bacteriostatic influences. The results obtained in this study for Y. enterocolitica are similar to those reported for some of the other enteric pathogens. The minimum values at which the growth of S. typhi is initiated is pH 4.0-4.5 and that of S. paratyphi and S. schottmuelleri pH 4.5 (Corlett & Brown 1980).

The ability of Y. enterocolitica to survive at such low pH values does create the possibility that prolonged storage of even an acidic food might be hazardous. This potential hazard is much greater with "home made" products than with those commercially produced. The commercial manufacturer has to ensure a reasonable shelf life for his product and is therefore likely to keep the pH value as low as possible without making it organoleptically unacceptable. The types of acid food which would normally be thought to be safe and unlikely to cause illness from enteric pathogens includes such products as fermented vegetables, pickles, mayonnaise sauce and salad dressings. However, Y. enterocolitica has been isolated from egg mayonnaise although some authors appear to have translated this as tartar sauce (Aldová et al. 1975), and the related organism Y. pseudotuberculosis has been found in pickled products (Kuznetsov et al. 1975). Unfortunately the pH of neither of these products is known. Although the normal pH range of commercial mayonnaise sauces and salad dressings would be expected to be between pH 3.0-4.0, there are many on the market today which must be kept in the refrigerator after opening and presumably these are not so acidic. In this study it was shown that "home made" mayonnaise sauce may vary from pH 2.9-4.8 according to the recipe. However, despite the ability of the test strains to grow at pH 4.4 in the buffered Brain Heart Infusion broth, neither strain was able to survive for more than 18 h in sauce at pH 4.8. Both strains had died out much earlier in those with lower pH values. Whether this was entirely due to pH or other inhibitory factors is not known. What was significant was that when a portion of the mayonnaise sauce pH 4.15 was mixed with potato at

pH 6.0 the acidity of the mayonnaise sauce was neutralized and the finished potato salad was pH 5.8, and well within the range suitable for rapid growth of Y. enterocolitica. The home production of foods such as potato salad, egg mayonnaise and coleslaw is becoming more frequent, both in large scale catering institutions as well as for private parties. Outbreaks of food poisoning where this type of product has been the vehicle of infection are not unknown. In the USA a very large Shigella outbreak was traced to home made salad dressing (Weissman et al. 1974) and the potential hazard must not be ignored in this country. In one of the three Y. enterocolitica outbreaks recorded in the UK since 1979 coleslaw prepared in a hospital kitchen was implicated, although not bacteriologically proven, as the vehicle of infection (CDSC, unpublished data).

There is no doubt that factors other than pH are able to affect the growth of microorganisms in foods, one of these being sodium chloride content. This study showed that the growth of Y. enterocolitica incubated at 30°C is inhibited by increasing concentrations of sodium chloride and in 5% the test organisms were able to grow but much more slowly than in 3 or 4%. Similar results were later obtained by Stern et al. (1980a) who incubated their strains of Y. enterocolitica at 3° and 25°C. The combined effects of pH values and various sodium chloride concentrations were not investigated in this work.

The investigations presented in this thesis on the growth and survival of Y. enterocolitica in foods stored at a range of temperatures showed this organism to be exceedingly versatile and its presence in foods to be a cause for concern.

The types of food examined were chosen because of their association with food poisoning outbreaks mainly caused by other enteric pathogens, and because they frequently receive a considerable amount of handling after cooking, particularly in the preparation of buffet type meals. Early preparation of such meals could also mean that they are retained for prolonged periods in a refrigerator. The Y. enterocolitica test strains were able to grow to large numbers (1×10^8 organisms/g) within 7 days at 4°C in all the foods tested and similar numbers were attained within 30 h in the foods stored at 12°-36°C. The results later obtained by Stern et al. (1980b) from pasteurized milk incubated at 25°C were similar, but their results from incubation at 3°C showed a slightly slower rate of increase than those shown at 4°C in this present study.

None of the other common food poisoning bacteria are able to grow at such low temperatures and so the refrigerator which has hitherto been regarded as a safe place to store food could in the right, or wrong circumstances depending upon how one sees it, increase the chances of infection by Y. enterocolitica which would be able to outgrow many of the other bacteria present. The test strains did not affect either the appearance or smell of the foods in which they grew, and therefore the only clue to a particular food being unsuitable for consumption would be the smell or discolouration produced by spoilage organisms which may or may not be present.

The higher the freezing temperatures the more lethal they are to microorganisms; many more organisms are killed or injured between -2° to $-10^{\circ}C$ than at $-15^{\circ}C$ or more. Unfortunately, because high freezing temperatures tend to cause maximum harm to the foods themselves their use to destroy organisms is limited, and commercially frozen foods are usually stored at $-18^{\circ}C$ or less. When inoculated into foods (approximately 10^{3} organisms/ml) and stored at $-20^{\circ}C$ Y. enterocolitica survived without any reduction in numbers for at least 9 weeks. Once more the results reported by Hanna et al. (1977b) were at variance to those obtained in this study. They found that their strains inoculated into beef (10^{5}-10^{9} organisms/ml) declined rapidly and could not be detected after 4 weeks at $-20^{\circ}C$. Leistner et al. (1975) inoculated Y. enterocolitica into chickens and noted that after 90 days at $-18^{\circ}C$ there was only a slight decline in numbers. The results obtained by different workers cannot be reliably compared, varying inoculum levels (often not shown) together with strain variation may account for these considerable differences. That Y. enterocolitica can survive for some considerable time in the frozen state is without doubt. In the later studies naturally occurring strains of Y. enterocolitica were recovered from both commercially frozen raw chicken which had been stored for an indefinite time and from mechanically deboned pork which had been informally frozen in the laboratory and stored at $-20^{\circ}C$ for 20 weeks (140 days). It might be said that the presence of Y. enterocolitica or any other pathogen in such foods is of little consequence because they have to be cooked before consumption. However, frozen raw meat must be thawed prior to cooking and this process usually results in a quantity of fluid exuding from the food together with any bacteria present. The danger is the dissemination of these organisms by the

handler into the kitchen environment and onto other foods, which may already have been cooked, and where they may have time to multiply before ingestion. This is a proven method of spread of Salmonella spp. but add to this the ability of Y. enterocolitica to grow at 4°C where these contaminated cooked foods may be stored for 'safety' and a compound danger arises.

Although all the known food poisoning bacteria grow in the food vehicle before ingestion the mode of illness varies, but generally it is either caused by toxin pre-formed in the food during growth (Staph. aureus and some B. cereus), toxin formed during sporulation in the gut (C. perfringens), or by large dose infection of the intestine (Salmonella). The incubation period of Staph. aureus, C. perfringens and B. cereus are relatively short (2-6 h, 8-22 h and 1-16 h respectively) and it is a fact that in most of the outbreaks reported samples of the implicated food are available for laboratory examination. The causative organisms are present in countable numbers and are very easily isolated (Hobbs & Gilbert 1974). However, the food vehicles are seldom available in salmonellosis outbreaks. Most of the investigations have to be carried out in retrospect on similar foods, possibly from the same batch, in which the organisms may or may not have been given the opportunity to grow to countable numbers. Because the Salmonella spp. may only be present in very low numbers, 1-2 or less per 100 g of sample, effective enrichment techniques have to be used for isolation.

The incubation period of Y. enterocolitica infection is 3-7 days, which is even longer than that of Salmonella spp and it must be assumed that in the majority of cases the food vehicle will have been disposed of by the time the illness has manifested itself, let alone been recognized, and therefore any similar foods examined for epidemiological purposes will also contain only low levels of the pathogen.

The conventional methods for isolating enteric pathogens by incubating enrichment cultures at 37°C for 1-2 days had been found to be unsuitable even for the isolation of Y. enterocolitica from faeces, and incubation in phosphate buffered saline at 4°C for 6-9 weeks was the most frequently recommended procedure. It had been hoped in this work, that methods of isolation from foods could be found to speed up the examination time by perhaps using a higher incubation temperature in combination with different media.

No single broth stood out as superior to the others examined in these studies. The growth of the test strains in some of the true

enrichment broths (those containing selective agents) such as selenite F, selenite cystine and Wauters' broth was very variable, and whilst some of the Y. enterocolitica strains failed to grow, even in pure culture, many of the undesired enterobacteria grew freely. Growth of all the Y. enterocolitica test strains and the other enterobacteria was very good in both the non-selective nutrient type broths (Brain Heart Infusion, buffered peptone water) and in GN broth with no selection occurring, even in the latter medium.

It became obvious during the five studies on the isolation of Y. enterocolitica from foods that in practice the higher incubation temperatures were of limited value and that whichever broth medium was employed for enrichment, incubation at 4°C was virtually obligatory. However, it was possible to reduce the incubation period to 3 weeks instead of 6-9 weeks. Mehlman et al. (1978) suggested that using a supplemented phosphate buffer containing a source of nutrient (sorbitol) and a selective agent (bile salts) that Y. enterocolitica could be isolated earlier than through phosphate buffered saline. No evidence of this could be found in these studies as all the broth media gave similar results after each incubation period. The shortened incubation time meant the loss of a few isolations but was more acceptable both from the point of speed of reporting and from the amount of refrigerator space required to hold an increasing accumulation of enrichment cultures.

This latter point was endorsed when the size of the sample to be examined was considered. When looking for small numbers of Salmonella spp. in foods it is widely accepted that at least 25 g of food is required and that the ideal ratio of sample to broth medium is 1:10. Therefore 25 g of sample plus 225 ml of broth medium was used where possible. The wisdom of this was confirmed by the results obtained with mechanically deboned pork in studies 3 and 4. In study 3 one gram of each sample was incubated in 10 ml buffered peptone and Y. enterocolitica was isolated from 1 of the 100 samples examined. In study 4 the same samples were bulked to make 23 large samples and 25 g of each was incubated in 225 ml buffered peptone; Y. enterocolitica was isolated from 10 of the 23 samples.

The results reported by Schiemann & Toma (1978) and Schiemann (1980a) indicated that different media and methods may be required for the successful recovery of Y. enterocolitica from different foods. There were no obvious indications of any medium or method favouring isolation from particular foods examined in this present work.

However, the results did show that occasionally media may favour certain serotypes, for example serotype 0:3 inoculated into animal feed (Study 1) was recovered more often through Wauters' broth than through any of the others used. There was little evidence to show that any of the other serotypes isolated were favoured by any particular medium.

It became obvious during the course of the evaluation of media that most of the agar media currently in use such as deoxycholate citrate sucrose (DCSA), deoxycholate citrate (DCA), MacConkey (MAC), Hektoen enteric (HEK) and XLD agar together with the few which had been specially formulated for Y. enterocolitica isolation, such as Salmonella-Shigella plus deoxycholate (SS + D) and LEE agars grew too many of the unwanted organisms, and that there was very little colonial differentiation between Y. enterocolitica and these other organisms. Most of the agar media also displayed considerable variation in the colonial characteristics of different strains of Y. enterocolitica. Lactose sucrose urea (LSU) agar was no exception in supporting the growth of many unwanted species but exhibited less colonial variations between strains of Y. enterocolitica. In addition all the Y. enterocolitica strains grown on this medium produced a pungent characteristic smell which aided differentiation. Generally Y. enterocolitica required 48 h incubation at 30° or 22°C on all the agar media examined before the colonies were large enough for characteristics to be recognized. These findings were similar to those described by Nilehn (1969a) in her study of LSU, DCA and SS agars, when she also found differentiation of Y. enterocolitica somewhat easier on LSU agar. Only three agar media, namely, DCSA, LSU and LEE agars were compared comprehensively in the five studies of isolation from food samples reported in this work. Yersinia enterocolitica was identified more frequently on LSU agar than on the other agars. The characteristic smell of the organism on this medium being of considerable diagnostic value.

It was unfortunate that the base medium of DCSA failed during the studies and some months elapsed before a suitable agar could be produced on which Y. enterocolitica would grow satisfactorily. However, the diffential characteristics which had proved of some diagnostic value during the first study were no longer reproducible which may account for the poor isolation rate on this medium in later studies.

Deoxycholate citrate sucrose malachite green (D4) agar was the result of investigations to formulate a new medium or improve an old one.

The problems with the DCSA based medium also affected the D4 agar and studies had to be curtailed. However, before the problems occurred it had been shown that Y. enterocolitica was unaffected by malachite green incorporated into DCSA agar whilst other enterobacteria including Proteus were inhibited. Sodium deoxycholate was identified as the factor which combined with malachite green to cause the inhibition and it was shown that this effect only occurred when the two chemicals were in a certain ratio to one another. There are no published reports of such an effect occurring in relation to Y. enterocolitica, but Leifson (1935) did report that dyes such as methylene blue, safranin and neutral red when combined together with sodium deoxycholate and sodium citrate exhibited extremely toxic effects. Leifson was interested in finding an improved medium for the isolation of Salmonella spp. but he did not describe any investigations into the possible practical use of the sodium deoxycholate/dye combination and malachite green was not mentioned in his studies. Other workers may have discovered this effect and either never followed it up or decided that it was not worth reporting. Midgley (1966) did report in an obscure East European publication that the genus Pasteurella, which at that time included the organisms now known as Y. enterocolitica, Y. pestis and Y. pseudotuberculosis, could be divided into two groups on the basis of sensitivity to various dyes including malachite green. Organisms of the genus Yersinia were able to grow well on serum agar containing 1:50,000 concentration of this dye whereas the organisms of the genus Pasteurella were unable to do so. However, further studies should be carried out to investigate the full potential of this phenomena.

Overall the evaluation and isolation studies showed that a basic method of incubating 25 g of sample at 4°C for 3 weeks in 225 ml buffered peptone and sub-culturing after 1, 2 and 3 weeks onto lactose sucrose urea agar will provide reasonably reliable results with the minimum of effort. This does not purport to be the ideal method for the isolation of Y. enterocolitica but a technique with which to compare others as research progresses. Good methods and media for the isolation of Salmonella spp. from foods were not achieved overnight but only after many carefully controlled studies over many years and there is no reason to suppose that Y. enterocolitica will be any different.

The biochemical identification of the isolates from the food samples was facilitated by the recent publication of a series of papers

by an international group of workers (Bercovier et al. 1980a, 1980b; Brenner et al. 1980; Ursing et al. 1980). The majority of the strains isolated were Y. enterocolitica sensu stricto with a few strains only of Y. frederiksenii and Y. intermedia. Yersinia kristensenii was not found. It is difficult to compare these isolates with those reported earlier by many other workers because all too often biochemical reactions were not fully described or strains were referred to simply as "environmental" or Y. enterocolitica-like organisms. Yersinia enterocolitica is frequently referred to as a non-lactose fermenting organism and it must be noted that all the strains isolated from the pasteurized milk and some from the sausages, sewer swabs, animal feed and ham did ferment lactose. To the unwary the use of an agar medium containing only lactose would result in a significant number of strains being overlooked.

The epidemiological investigation of food poisoning outbreaks relies heavily on methods of differentiation other than biochemical identification. Serological typing is one of the most common means of further differentiating many species of enterobacteria and its value lies in the ability to be able to type the maximum number of isolates. In the present studies there was a significant difference between the results obtained with Wauters' widely accepted method using unsteamed antigens and those with the Division of Enteric Pathogens method which employed steamed antigen suspensions. The use of steamed suspensions is a standard procedure for the identification of the "O" antigens of Shigella spp., Salmonella spp. and E. coli. Two of the arguments put forward against the use of steamed suspensions for the serotyping of Y. enterocolitica are that heating causes roughness (R forms), and also after heating many cross-reactions occur (Wauters 1981). Only 6 of the 158 strains isolated during these studies were found to be rough after steaming, 5 of these 6 R forms were isolated through phosphate buffered saline or supplemented phosphate buffer. Phosphate buffered saline has frequently been recommended and used for the isolation of Y. enterocolitica and it is conceivable that the enrichment substrate also plays a part in the production of the R forms. The cross-reaction argument has no substance as these can be eliminated by the preparation of pure absorbed sera.

These studies showed that not only Y. enterocolitica sensu stricto was able to be serotyped with the existing sera. The "O" sera 1-34 are presumed to have been prepared from Y. enterocolitica sensu stricto but

one strain of Y. frederiksenii was identified as 0:16,29. However, it is known that many of the type strains used in the preparation of the "O" groups which have been added to the serotyping scheme in recent years, that is types 0:35 onwards, are not all Y. enterocolitica sensu stricto but include Y. intermedia, Y. frederiksenii and possibly Y. kristensenii. If and when the new biochemical classification is accepted internationally then it is possible that separate serotyping schemes might have to be considered for each of the new species.

These studies have demonstrated that Y. enterocolitica is present in foods obtained in the UK and that these foods which include dairy products, raw meat and poultry and cooked ham are similar to those from which the organism has been isolated in many other parts of the world.

How much of a real hazard this organism presents by its presence in these products is difficult to ascertain. Neither of the so-called medically important serotypes 0:3 or 0:9 were isolated from any of the 1004 samples examined. However, serotypes 0:5,27, 0:6,30 and 0:8 which have all been associated with human illness were found. In particular, one strain of serotype 0:8 was recovered from pasteurized milk. Although serotype 0:8 has not been recognized as a cause of illness in Europe it was the proven cause of a large scale outbreak of food poisoning in the USA in which the vehicle of infection was found to be chocolate milk (Black et al. 1978). This serotype is also commonly associated with human infection throughout the States.

Even as this thesis is being typed two more milkborne outbreaks of Y. enterocolitica infection have been reported from the USA. One, for which no details are available, is said to have occurred last year but the second, more recent outbreak, reported involvement of 172 cases and to have been caused by serotype 0:16,18 (Lofgren et al. 1982).

Despite the report by Hanna et al. (1977d) which showed that Y. enterocolitica is destroyed by heating at 60°C for 3 min there was a very high incidence (19%) of the organism in the pasteurized milk examined in these studies and several samples contained multiple serotypes. The organism was even isolated from milks proven to have been pasteurized by the phosphatase reaction. In contrast Schiemann (1978a) reported only 0.4% incidence in pasteurized dairy products.

There are two possible reasons for these results, either some strains of Y. enterocolitica are resistant to pasteurization temperatures, or the cleanliness in the bottling plants is not as good as it should be and post-pasteurization contamination had occurred. Hughes' (1980)

investigations in dairy plants in Australia showed that the latter reason was responsible for contamination there. As with other pathogens, Y. enterocolitica has a prelidiction for the young and during a recent winter a $3\frac{1}{2}$ year old girl in the UK was taken ill with abdominal pain and diarrhoea which lasted for 2 weeks and was followed by a relapse 3-4 weeks later. The child had no known contact with any animal source of Y. enterocolitica but serotype 0:8 was isolated from faeces (Chessum, personal communication). Children drink large quantities of milk, Y. enterocolitica enjoys cold temperatures and milk keeps better in the winter months. Could it be that Y. enterocolitica could grow to sufficient numbers to cause diease before spoilage organisms were able to make the milk unpalatable ? The growth studies carried out during the present investigations indicate that this is possible. On the other hand serotype 0:8 is known to be invasive as indicated by the Sereny test and therefore only small numbers of the organism would be needed to cause illness.

The number of cells of Y. enterocolitica required in a food before illness can occur and indeed the exact mode of pathogenicity is still debatable. Although in a human volunteer test 3.5×10^9 organisms taken orally were followed by typical illness (Szita et al. 1973), it has been suggested by Une (1977a) that Y. enterocolitica is invasive and therefore similar in action to Shigella spp. and some Salmonella spp. In this case as already mentioned, only low numbers of the organism would be required to cause illness. However, even if a pathogen does not possess either the ability to produce toxin or is of the invasive type it is still possible for small numbers to cause illness. A factor often overlooked is the nature of the vehicle of infection, the ingredients of which may form protective layers round the organism and enable it it travel through the stomach and into the intestine where it can multiply. A very recent (July 1982) outbreak of salmonellosis in the UK was caused by the consumption of chocolate bars containing less than 10 Salmonella per 100 g.

Recently Kapperud (1982) has reported that some strains of Y. enterocolitica sensu stricto are able to form heat stable toxin at $4^{\circ}C$. Although most of the strains which were capable of doing so were human isolates a few (4%) of the environmental strains tested showed a similar property and Y. kristensenii showed the highest incidence of toxin production at both 4° (47%) and $37^{\circ}C$ (49%). Even more recently

Olsvik & Kapperud (1982) have shown that five strains of Y. enterocolitica although growing readily in whole and skimmed milk at 4°C were unable to produce detectable toxin. It has also been shown that the invasiveness as indicated by the Serény test may be plasmid associated (Zink et al. 1978; Gemski et al. 1980; Portnoy et al. 1981). Presumably such a plasmid can be lost at any time and this therefore could account for some of the variable results obtained by different workers. It is also possible that just as the plasmids associated with S. typhi are more freely transferred at temperatures below 30°C that during the low temperature enrichment required for isolation of Y. enterocolitica that plasmids of this organism might also be lost (or gained) ?

Despite the relatively high incidence (5.3%) of Y. enterocolitica sensu stricto found in the foods and other materials examined in these studies there are few cases of human infection reported annually in the UK. However, there are significant numbers of outbreaks of gastroenteritis of unknown aetiology (GUA) (Appleton et al. 1981) which epidemiologically have a common food source. Laboratories examining clinical material from suspected food poisoning outbreaks readily investigate for the well known pathogens such as Salmonella spp., C. perfringens, Staph. aureus, B. cereus and even Vibrio parahaemolyticus, but Y. enterocolitica is either quietly ignored or looked for half heartedly.

When no bacterial pathogens are found thoughts turn to viruses and in some cases these have proved to be the causative agent but in many more the agent has remained unidentified. The faecal specimens from many of these GUA outbreaks are finally sent to the Virus Reference Laboratory in Colindale and the samples from one or two have been examined recently by the Food Hygiene Laboratory. Unfortunately the faeces are usually at least 4-5 weeks old before arrival but in one of these outbreaks in which no other pathogen (bacterial or viral) was isolated, Y. enterocolitica was recovered from 4 of 26 patients examined. Serotype O:5 was identified in 3 of the 4 patients, one of whom also had a non-typable strain. The fourth patient was infected only with a non-typable strain.

The patients concerned were related but no further details are available. The significance of these findings is difficult to assess at this stage partly because the normal carriage of Y. enterocolitica in healthy humans in the UK is unknown, but further more careful and earlier investigations of GUA outbreaks are indicated.

The more one investigates Y. enterocolitica the more questions remain to be answered. This study set out to investigate some of the growth parameters of Y. enterocolitica in foods, to evaluate media and methods already in use and to improve upon them if possible and to determine the presence, if any, of the organism in foods and other materials obtainable in the UK. As work progressed further areas of study were indicated in order to obtain the true significance of the presence of Y. enterocolitica in foods. These areas include investigations into production and detection of toxin in foods, modes of infection/intoxication, heat resistance especially in relation to Y. enterocolitica isolated from pasteurized milk and earlier and more detailed investigations of outbreaks of gastroenteritis of unknown aetiology. The latter study can only be done if laboratories are educated and encouraged to look for Y. enterocolitica themselves at the onset of the outbreak rather than leave it to other reference laboratories to search when the specimens are dried up or overgrown with moulds. A knowledge of the normal carriage rate in healthy humans in the UK would also be valuable in deciding the significance of this potential pathogen in this country.

REFERENCES

ABBOTT J.D. & SHANNON R. 1958. A method for typing Shigella sonnei using colicine production as a marker. Journal of Clinical Pathology 11, 7-77.

AHVONEN P & ROSSI T. 1970. Familial occurrence of Yersinia enterocolitica infection and acute arthritis. Acta paediatrica Scandinavica 206, (Supplement), 121-122.

ALDOVÁ E, ČERNÁ J, JANEČKOVÁ M & PEGRÍMKOVÁ J. 1975. [Yersinia enterocolitica and its demonstration in the food.] Československá hygiena 20, 395-404.

ALONSO J.M., BERCOVIER H., SERVAN J. & MOLLARET H.H. 1979. Contribution to the study of the ecology of Yersinia enterocolitica in France. Contributions to Microbiology and Immunology 5, 132-143.

ANDRADE E. 1906. Influence of glycerin in differentiating certain bacteria. Journal of Medical Research 14, 551-556.

ANJORIN F.I., STURROCK R.D., SUBBUSWAMY S.G., LAWAMNDE R.V. & FAKUNLE Y.M. 1979. Yersinia enterocolitica infection from West Africa - a case report. Transactions of the Royal Society of Tropical Medicine and Hygiene 73, 634-635.

APPLETON H, PALMER S.R. & GILBERT R.J. 1981. Foodborne gastroenteritis of unknown aetiology: a virus infection ? British Medical Journal 282, 1801-1802.

ASAKAWA Y., AKAHANE S., KAGATA N., NOGUCHI M., SAKAZAKI R. & TAMURA K. 1973. Two community outbreaks of human infection with Yersinia enterocolitica. Journal of Hygiene 71, 715-723.

ASAKAWA Y., AKAHANE S., SHIOZAWA K. & HONMA T. 1979. Investigations of source and route of Yersinia enterocolitica infection. Contributions to Microbiology and Immunology 5, 115-121.

AULISIO C.C.G., MEHLMAN I.J. & SANDERS A.C. 1980. Alkali method for rapid recovery of Yersinia enterocolitica and Yersinia pseudotuberculosis from foods. Applied and Environmental Microbiology 39, 135-140.

BARCLAY G. 1981. The laboratory isolation and identification of Yersinia enterocolitica and Yersinia pseudotuberculosis. Canadian Journal of Medical Technology 43, 34-41.

BARTH E. 1928. Die Anreicherung von Typhus-und Paratyphusbacillen auf Farbstoffnährböden. Zeitschrift für Hygiene und Infektionskrankheiten 109, 611-615.

BARTLETT C.L.R., HOOPER W.L., MAIR N.S., FISHER J., ANDERSON M. & FOX E. 1982. Yersiniosis in a boarding school. In preparation.

BERCOVIER H., BRENNER D.J., URSING J., STEIGERWALT A.G., FANNING G.R., ALONSO J.M., CARTER G.P. & MOLLARET H.H. 1980a. Characterization of Yersinia enterocolitica sensu stricto. Current Microbiology 4, 201-206.

BERCOVIER H., URSING J., BRENNER D.J., STEIGERWALT A.G., FANNING G.R., CARTER G.P. & MOLLARET H.H. 1980b. Yersinia kristensenii: a new species of Enterobacteriaceae composed of sucrose-negative strains (formerly called atypical Yersinia enterocolitica or Yersinia enterocolitica-like). Current Microbiology 4, 219-224.

BLACK R.E., JACKSON R.J., TSAI T., MEDVESKY M., SHAYEGANI M., FEELEY J.C., MACLEOD K.I.E. & WAKELEE A.M. 1978. Epidemic Yersinia enterocolitica infection due to contaminated chocolate milk. New England Journal of Medicine 298, 76-79

BOTTONE E.J. 1977. Yersinia enterocolitica - a panoramic view of a charismatic microorganism. CRC Critical Reviews in Microbiology 5, 211-241.

BOTZLER R.G., WETZLER F.T., COWAN A.B. & QUAN T.J. 1976. Yersiniae in pond water and snails. Journal of Wildlife Diseases 12, 492-496.

BOWEN J.H. & KOMINOS S.D. 1979. Evaluation of a pectin agar medium for isolation of Yersinia enterocolitica within 48 hours. American Journal of Clinical Pathologists 72, 586-590.

BOYCE J.M., EVANS D.J., EVANS D.G. & DuPONT H.L. 1979. Production of heat-stable, methanol-soluble enterotoxin by Yersinia enterocolitica. Infection and Immunity 25, 532-537.

BOYD W.C. 1956. Fundamentals of Immunology 3rd ed. New York: Interscience Publishers Inc.

BRENNER D.J., BERCOVIER H., URSING J., ALONSO J.M., STEIGERWALT A.G., FANNING G.R., CARTER G.P. & MOLLARET H.H. 1980. Yersinia intermedia: a new species of Enterobacteriaceae composed of rhamnose-positive, melibiose-positive, raffinose-positive strains (formerly called Yersinia enterocolitica or Yersinia enterocolitica-like). Current Microbiology 4, 207-212.

BUCKERIDGE S.A., SEAMAN A. & WOODBINE M. 1980. Effects of temperature and carbohydrate on the growth and survival of Yersinia enterocolitica, In: Microbial Growth and Survival in Extremes of Environment ed. Gould G.W. & Corry J.E.L. p 206-214. Society for Applied Bacteriology Technical Series No 15. London: Academic Press.

CARTER P.B. 1975. Pathogenicity of Yersinia enterocolitica for mice. Infection and Immunity 11, 164-170.

CHESTER B. & STOTZKY G. 1976. Temperature-dependent cultural and biochemical characteristics of rhamnose positive Yersinia enterocolitica. Journal of Clinical Microbiology 3, 119-127.

CHIN G.N. & NOBLE R.C. 1977. Ocular involvement in Yersinia enterocolitica infection presenting as Parinaud's oculoglandular syndrome. American Journal of Ophthalmology 83, 19-23.

CHRISTENSEN S.G. 1979. Isolation of Yersinia enterocolitica 0:3 from a well suspected as the source of yersiniosis in a baby. Acta Veterinaria Scandinavica 20, 154-156.

CHRISTENSEN W.B. 1946. Urea decomposition as a means of differentiating Proteus and paracolon cultures from each other and from Salmonella and Shigella types. Journal of Bacteriology 52, 461-466.

CHURCHMAN J.W. 1912. The selective bactericidal action of Gentian violet. Journal of Experimental Medicine 16, 221-245.

COLE S.W. & ONSLOW H. 1916. On a substitute for peptone and a standard nutrient medium for bacteriological purposes. Lancet ii, 9-13.

CORLETT D.A. & BROWN M.H. 1980. pH and acidity. In: Microbial Ecology of Foods. 1. Factors affecting life and death of microorganisms, by the International Commission on Microbiological Specifications for Foods, p 92-111. New York: Academic Press.

CORNELIS G. 1981. Antibiotic resistance in Y. enterocolitica. In: Yersinia enterocolitica. ed. Bottone E.J. Boca Raton: CRC Press.

COWAN S.T. & STEEL K. 1974. Manual for the Identification of Medical Bacteria 2nd edn. London: Cambridge University Press.

CRICHTON E.P. 1978. Suppurative conjunctivitis caused by Yersinia enterocolitica. Canadian Medical Association Journal 118, 22-23.

DRIGALSKI V. & CONRADI H. 1902. Ueber ein Verfahren zum Nachweis der Typhusbacillen. Zeitschrift für Hygiene 39, 283-300.

DUDLEY M.V. & SHOTTS E.B. 1979. Medium for isolation of Yersinia enterocolitica. Journal of Clinical Microbiology 10, 180-183.

EDEL W. & KAMPELMACHER E.H. 1973. Comparative studies on the isolation of "sublethally injured" salmonellae in nine European laboratories. Bulletin of the World Health Organization 48, 167-174.

EDEN K.V., ROSENBERG M.L., STOOPLER M., WOOD B.T., HIGHSMITH A.K., SKALIY P., WELLS J.G. & FEELEY J.C. 1977. Waterborne gastroenteritis illness at a ski resort - isolation of Yersinia enterocolitica from drinking water. Public Health Reports 92, 245-250.

EISENBERG P. 1919. Untersuchungen über spezifische Desinfektionsvorgänge II. Mitteilung: Ueber die Wirkung von Salzen und Ionen auf Bakterien. Centralblatt für Bakteriologie, Parasitenkunde, und Infektionskrankheiten I. Abteilung: Originale 82, 69-208.

EISS J. 1975. Selective culturing of Yersinia enterocolitica at a low temperature. Scandinavian Journal of Infectious Disease 7, 249-251.

EL-ZAWAHRY Y.A. & ROWLEY D.B. 1979. Radiation resistance and injury of Yersinia enterocolitica. Applied and Environmental Microbiology 37, 50-54.

ESSEVELD H. & GOUDZWAARD C. 1973. On the epidemiology of Yersinia enterocolitica infections: pig as the source of infections in man. Contributions to Microbiology and Immunology 2, 99-101.

EWING W.H., DAVIS B.R. & REAVIS R.W. 1957. Phenylalanine and malonate media and their use in enteric bacteriology. Public Health Laboratory 15, 153-161.

EWING W.H., ROSS A.J., BRENNER D.J. & FANNING G.R. 1978. Yersinia ruckeri sp. nov., the Redmouth (RM) Bacterium. International Journal of Systematic Bacteriology 28, 37-44.

FALCAO D.P., EWING W.H. & DOWELL V.R. 1979. Cultural characteristics of Yersinia enterocolitica and Yersinia pseudotuberculosis on differential media. Contributions to Microbiology and Immunology 5, 88-94.

FEELEY J.C., LEE W.H. & MORRIS G.K. 1976. Yersinia enterocolitica. In: Compendium of Methods for the Microbiological Examination of Foods ed. Speck M.L. p 351-357. Washington: American Public Health Association.

FERMI C. 1898. Die Mineral - und organischen Säuren, die Alkali, die Alkaloide, das Jodkali und das arsensaure Kali zur Differenzierung der Mikroorganismen. Centralblatt für Bakteriologie, Parasitenkunde Infektionskrankheiten. I. Abteilung: Originale 23, 208-217 & 266-273.

FLEMING A. 1929. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. British Journal of Experimental Pathology, 10, 226-236.

FRANCIS D.W., SPAULDING P.L. & LOVETT J. 1980. Enterotoxin production and thermal resistance of Yersinia enterocolitica in milk. Applied and Environmental Microbiology 40, 174-176.

FREDERICQ P., THIEBAULT J. & GRATIA A. 1946. Recherches sur les propriétés antibiotiques des germes du genre Bacillus. Compte Rendu de la Societé de Biologie 140, 1035-1037.

FREDERIKSEN W. 1964. A study of some Yersinia pseudotuberculosis-like bacteria (Bacterium enterocoliticum and Pasteurella X). Proceedings of the 14th Scandinavian Congress of Pathology and Microbiology, Oslo, p 103-104. Oslo: Norwegian University Press.

FUKUSHIMA H., ITO Y., SAITO K., TSUBOKURA M. & OTSUKI K. 1979. Role of the fly in the transport of Yersinia enterocolitica. Applied and Environmental Microbiology 38, 1009-1010.

FUNG D.Y.C. & MILLER R.D. 1973. Effect of dyes on bacterial growth. Applied Microbiology 25, 793-799.

GAEHTGENS W. & BRÜCKNER G. 1910. Vergleichende Untersuchungen über einige neuere Typhusnährböden und Erfahrungen über den Wert der Agglutination, Blutkultur und Stuhlzüchtung für die Diagnose des Abdominaltyphus. Centralblatt für Bakteriologie, Parasitenkunde unde Infektionskrankheiten.I.Abteilung Originale 53, 559-576.

GALTON M.M., LOWERY W.D. & HARDY A.V. 1954. Salmonella in fresh and smoked pork sausage. Journal of Infectious Diseases 95, 232-235.

GARDNER J.F. 1950. Some antibiotics formed by Bacterium coli. British Journal of Experimental Pathology 31, 102-111.

GARROD L.P. 1933. Inhibitory selective culture media. In: Saint Bartholomew's Hospital Reports 66, p 203-252. London: John Murray.

GARROD L.P., LAMBERT H.P. & O'GRADY F. 1973. Antibiotic and Chemotherapy. 4th edn. Edinburgh and London: Churchill Livingstone.

GEMSKI P., LAZERE J.R. & CASEY T. 1980. Plasmid associated with pathogenicity and calcium dependency of Yersinia enterocolitica. Infection and Immunity 27, 682-685.

GEIGY SCIENTIFIC TABLES 1962. ed Diem K. p. 314-315. Manchester: Geigy Pharmaceuticals.

GILES N. & CARTER M.J. 1980. Yersinia enterocolitica in budgerigars. Veterinary Record 107, 362-363.

GINI G.A. & TORRES M.F. 1979. Primeros aislamientos de Yersinia enterocolitica en Central América y revisión de la literatura. Revista Latinoamericana de Microbiologia 21, 107-113.

GRACE de M., LAURIN M-F., BÉLANGER C., ROLLAND P.E., BLAIS R., BRETON J.P. & MARTINEAU G. 1976. Yersinia enterocolitica gastroenteritis outbreak - Montreal. Canada Diseases Weekly Report 2, 41-44 & 73-74.

GRATIA A. & FREDERICQ P. 1946. Diversité des souches antibiotiques de B. coli et étendue variable de leur champ d'action. Compte Rendu de la Societé de Biologie 140, 1032-1033.

GREAT BRITAIN 1963. Food and Drugs, Milk and Dairies; The Milk (Special Designation) Regulations 1963. Statutory Instrument 1963 No. 1571. London: H.M.S.O.

GREENSTEIN A.J. & DREILING D.A. 1974. Postoperative combined undermining infection of abdominal wound due to Yersinia enterocolitica. Mount Sinai Journal of Medicine 41, 665-666.

GUTHERTZ L.S. FRUIN J.T., SPICER D. & FOWLER J.L. 1976. Microbiology of fresh comminuted turkey meat. Journal of Milk and Food Technology 39, 823-829.

GUTMAN L.T., OTTESEN E.A., QUAN T.J., NOCE P.S. & KATZ S.L. 1973. An inter-familial outbreak of Yersinia enterocolitica enteritis. New England Journal of Medicine 288, 1372-1377.

HAJNA A.A. 1955. A new enrichment broth medium for Gram-negative organisms of the intestinal group. Public Health Laboratory 13, 83-90.

HALBERT S.P. 1948. The antagonism of coliform bacteria against Shigellae. Journal of Immunology 58, 153-167.

HANNA M.O., ZINK D.L., CARPENTER Z.L. & VANDERZANT C. 1976. Yersinia enterocolitica-like organisms from vacuum-packaged beef and lamb. Journal of Food Science 41, 1254-1256.

HANNA M.O., STEWART J.C., CARPENTER Z.L. & VANDERZANT C. 1977a. Development of Yersinia enterocolitica-like organisms in pure and mixed cultures on different bismuth sulphite agars. Journal of Food Protection 40, 676-677.

HANNA M.O., STEWART J.C., CARPENTER Z.L. & VANDERZANT C. 1977b. Effect of heating, freezing and pH on Yersinia enterocolitica-like organisms from meat. Journal of Food Protection 40, 689-692.

HANNA M.O., STEWART J.C., CARPENTER Z.L. & VANDERZANT C. 1977c. Effect of packaging methods on the development of Yersinia enterocolitica on beef steaks. Journal of Food Safety 1, 29-37.

HANNA M.O., STEWART J.C., CARPENTER Z.L. & VANDERZANT C. 1977d. Heat resistance of Yersinia enterocolitica in skim milk. Journal of Food Science 42, 1134 & 1136.

HANNA M.O., STEWART J.C., ZINK D.L., CARPENTER Z.L. & VANDERZANT C. 1977e. Development of Yersinia enterocolitica on raw and cooked beef and pork at different temperatures. Journal of Food Science 42, 1180-1184.

HARTLEY P. 1922. The value of Douglas's medium for the production of diphtheria toxin. Journal of Pathology and Bacteriology 25, 479-486.

HARVEY R.W.S. & PRICE T.H. 1968. Elevated temperature incubation of enrichment media for the isolation of salmonellas from heavily contaminated materials. Journal of Hygiene 66, 377-381.

HENTGES D.J. 1967. Inhibition of Shigella flexneri by the normal intestinal flora. 1. Mechanisms of inhibition by Klebsiella. Journal of Bacteriology 93, 1369-1373.

HENTGES D.J. 1969. Inhibition of Shigella flexneri by the normal intestinal flora. II. Mechanisms of inhibition by coliform organisms. Journal of Bacteriology 97, 513-517.

HENTGES D.J. & FULTON M. 1964. Ecological factors influencing the relationships between Klebsiella and Shigella in mixed cultures. Journal of Bacteriology 87, 527-535.

HIGHSMITH A.K., FEELEY J.C., SKALIY P., WELLS J.G. & WOOD B.T. 1977. Isolation of Yersinia enterocolitica from well water and growth in distilled water. Applied and Environmental Microbiology 34, 745-750.

HOBBS B.C. & GILBERT R.J. 1974. Microbiological counts in relation to food poisoning. Proceedings of the 4th International Congress of Food Science and Technology 3, 159-169.

HOSKINS J.K. 1934. Most probable numbers for evaluation of coli-aerogenes tests by fermentation tube method. Public Health Report 49, 393-405.

HUGH R. & LEIFSON E. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. Journal of Bacteriology 66, 24-26.

HUGHES D. 1979. Isolation of Yersinia enterocolitica from milk and a dairy farm in Australia. Journal of Applied Bacteriology 46, 125-130.

HUGHES D. 1980. Repeated isolation of Yersinia enterocolitica from pasteurized milk in a holding vat at a dairy factory. Journal of Applied Bacteriology 48, 383-385.

HUGHES D. & JENSEN N. 1981. Yersinia enterocolitica in raw goats' milk. Applied and Environmental Microbiology 41, 309-310.

INOUE M. & KUROSE M. 1975. Isolation of Yersinia enterocolitica from cow's intestinal contents and beef meat. Japanese Journal of Veterinary Science 37, 91-93.

JAMESON J.E. & EMBERLEY N.W. 1956. A substitute for bile salts in culture media. Journal of General Microbiology 15, 198-204.

JAYNE-WILLIAMS D.J. 1963. Report of a discussion on the effect of the diluent on the recovery of bacteria. Journal of Applied Bacteriology 26, 398-404.

JUHLIN I. & ERICSON C. 1961. A new medium for the bacteriologic examination of stools (LSU-agar). Acta Pathologica et Microbiologica Scandinavica 52, 185-200.

KAPPERUD G. 1980a. Studies on the pathogenicity of Yersinia enterocolitica and Yersinia enterocolitica-like bacteria. 1. Enterotoxin production at 22°C and 37°C by environmental and human isolates from Scandinavia. Acta Pathologica et Microbiologica Scandinavica Section B 88, 287-291.

KAPPERUD G. 1980b. Studies on the pathogenicity of Yersinia enterocolitica and Yersinia enterocolitica-like bacteria. 2. Interaction with HeLa cells among environmental and human isolates from Scandinavia. Acta Pathologica et Microbiologica Scandinavica Section B 88, 293-297.

KAPPERUD G. 1980c. Yersinia enterocolitica and Yersinia-enterocolitica-like bacteria isolated from healthy humans in Norway. Acta Pathologica et Microbiologica Scandinavica Section B 88, 303-306.

KAPPERUD G. 1982. Enterotoxin production at 4°, 22° and 37°C among Yersinia enterocolitica and Yersinia enterocolitica-like bacteria. Acta Pathologica et Microbiologica Scandinavica Section B 90, 185-189.

KAPPERUD G. & JONSSON B. 1976. Yersinia enterocolitica in brown trout (Salmo trutta L.) from Norway. Acta Pathologica et Microbiologica Scandinavica Section B 84, 66-68.

KAPPERUD G. & LANGELAND G. 1981. Enterotoxin production at refrigeration temperature by Yersinia enterocolitica and Yersinia enterocolitica-like bacteria. Current Microbiology 5, 119-122.

KENDALL M. 1982a. Bismuth sulphite agar for the isolation of Salmonellae. In: Quality Assurance and Quality Control of Microbiological Culture Media. Proceedings of the Symposium held on 6th and 7th September 1979, Calas de Mallorca, ed Corry J.E.L., p 133-139. Darmstadt: G.I.T. Verlag Ernst Giebeler.

KENDALL M. 1982b. Quality assurance of culture media: invited comments. In: Quality Assurance and Quality Control of Microbiological Culture Media. Proceedings of the Symposium held on 6th and 7th September 1979, Calas de Mallorca, ed Corry J.E.L., p. 39-45. Darmstadt: G.I.T. Verlag Ernst Giebeler.

KILLIAN H. 1924. Brillantgrün, seine elektiv-bactericide Wirkung und seine Verwendung zur Typhus - und Paratyphusdiagnose. Zeitschrift für Hygiene und Infecktionskrankheiten 103, 193-203.

KITASATO S. 1888. Ueber das Verhalten der Typhus - und Cholerabacillen zu säure - oder alkalihaltigen Nährböden. Zeitschrfit für Hygiene 3, 404-426.

KNAPP W. & THAL E. 1973. Die biochemische Charakterisierung von Yersinia enterocolitica (syn. "Pasteurella X") als Grundlage eines vereinfachten O-Antigenschemas. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene I. Abteilung: Originale. Reihe A 223, 88-105.

KOUWATLI K., BEJAR V., RUIZ-BRAVO A. & RAMOS-CORMENZANA A. 1979. Sensitivity of Yersinia enterocolitica to several antimicrobial agents. Microbios Letters 11, 137-142.

KRUMWEIDE C. & PRATT J.S. 1914. Further observations on the growth of bacteria on media containing various anilin dyes, with special reference to an enrichment method for typhoid and paratyphoid bacilli. Journal of Experimental Medicine 18, 501-512.

KUZNETSOV V.G., RAKOVSKY V.I., GREBENSHCHIKOV L.A., LOSKUTOV A.N., DAGAYEVA A.R. & SOMOV G.P. 1975. [Study of the epidemiology of the far-eastern scarlatina-like fever in the Primorsk region. Report 1. Contamination of vegetables, roots and pickled provisions with Yersinia pseudotuberculosis in the foci of scarlatina-like fever]. Zhurnal Microbiologii, Epidemiologii I Immunobiologii 52 No 10, 34-38.

LEE W.H. 1977. Two plating media modified with Tween 80 for isolating Yersinia enterocolitica. Applied and Environmental Microbiology 33, 215-216.

LEE W.H., HARRIS M.E., McCLAIN D., SMITH R.E. & JOHNSTON R.W. 1980. Two modified selenite media for the recovery of Yersinia enterocolitica from meats. Applied and Environmental Microbiology 39, 205-209.

LEE W.H., McGRATH P.P., CARTER P.H. & EIDE E.L. 1977. The ability of some Yersinia enterocolitica strains to invade HeLa cells. Canadian Journal of Microbiology 23, 1714-1722.

LEIFSON E. 1935. New culture media based on sodium deoxycholate for the isolation of intestinal pathogens and for the enumeration of colon bacilli in milk and water. Journal of Pathology and Bacteriology 40, 581-599.

LEISTNER L., HECHELMANN H., KASHIWAZAKI M. & ALBERTZ R. 1975. Nachweis von Yersinia enterocolitica in Faeces und Fleisch von Schweinen, Rindern und Geflügel. Fleischwirtschaft 55, 1599-1602.

LOFGREN J.P., KONIGSBERG C., RENDTORFF R., ZEE V., HUTCHESON R.H., RAUSA A., BOWER D. & RIECKEN W.E. 1982. Multi-state outbreak of yersiniosis. Morbidity and Mortality Weekly Report 31, 505-506.

LOWE G.H. 1962. The rapid detection of lactose fermentation in paracolon organisms by the demonstration of β-D-galactosidase. Journal of Medical Laboratory Technology 19, 21-25.

MacCONKEY A.T. 1908. Bile salt media and their advantages in some bacteriological examinations. Journal of Hygiene 8, 322-334.

MAIR N.S. 1977. Yersinia infections. In: Topics in Gastroenterology, No 5, ed. Truelove S.C. & Lee E. p. 325-338. Oxford: Blackwell.

MAIR N.S., WHITE G.D., SCHUBERT F.K. & HARBOURNE J.F. 1970. Yersinia enterocolitica infection in the Bush-baby (Galago). Veterinary Record 86, 69-71.

MALLMAN W.L. & DARBY C.W. 1941. Uses of a lauryl sulfate tryptose broth for the detection of coliform organisms. American Journal of Public Health 31, 127-134.

MARTIN S.E., FLOWERS R.S. & ORDAL Z.J. 1976. Catalase: its effect on microbial enumeration. Applied and Environmental Microbiology 32, 731-734.

MEHLMAN I.J., AULISIO C.C.G. & SANDERS A.C. 1978. Problems in the recovery and identification of Yersinia from food. Journal of the Association of Official Analytical Chemists 61, 761-771.

MIDGLEY J. 1966. The sensitivity to basic dyes of Pasteurella and some other Gram-negative genera. Publications de la Faculté des Sciences de l'Université J.E. Purkyně, Brno Series K38, 282-288.

MOLLARET H.H., BERCOVIER H. & ALONSO J.M. 1979. Summary of the data received at the WHO Reference Centre for Yersinia enterocolitica. Contributions to Microbiology and Immunology 5, 174-184.

MOLLARET H.H. & NICOLLE P. 1965. Sur la fréquence de la lysogénie dans l'espèce nouvelle Yersinia enterocolitica. Compte Rendu de l'Académie des Sciences, Paris 260, 1027-1029.

MORS V. & PAI C.H. 1980. Pathogenic properties of Yersinia enterocolitica. Infection and Immunity 28, 292-294.

MUNGER S.F., WETZLER T.F, HEYWARD A.A. & SWARTZ R.G. 1980. Isolation of Yersinia enterocolitica from Saxidomus giganteus harvested from Seattle beaches. Abstracts of the Annual Meeting of the American Society for Microbiology, p.206.

NICOLLE P., MOLLARET H., HAMON Y. & VIEU J.F. 1967. Étude lysogénique, bactériocinogénique et lysotypique de l'espèce Yersinia enterocolitica. Annales de l'Institut Pasteur 112, 86-92.

NILÉHN B. 1967. Studies on Yersinia enterocolitica. Acta Pathologica et Microbiologica Scandinavica 69, 83-91.

NILÉHN B. 1969a. Studies on Yersinia enterocolitica. Growth on various solid media at 37°C and 25°C. Acta Pathologica et Microbiologica Scandinavica 77, 685-697.

NILÉHN B. 1969b. Studies on Yersinia enterocolitica with special reference to bacterial diagnosis and occurrence in human acute enteric disease. Acta Pathologica et Microbiologica Scandinavica Supplement 206.

NILÉHN B. 1973. Host range, temperature characteristics and serologic relationships among Yersinia phages. Contributions to Microbiology and Immunology 2, 59-67.

NILÉHN B. & ERICSON C. 1969. Studies on Yersinia enterocolitica. Bacteriophages liberated from chloroform treated cultures. Acta Pathologica et Microbiologica Scandinavica 75, 177-187.

NORBERG P. 1981. Enteropathogenic bacteria in frozen chicken. Applied and Environmental Microbiology 42, 32-34.

OKAMOTO K., INOUE T., ICHIKAWA H., KAWAMOTO Y., HARA S. & MIYAMA A. 1980. Adherence of Yersinia enterocolitica to mammalian epithelial cell lines. Microbiology and Immunology 24, 1013-1022.

OLŠOVSKÝ Z., OLŠÁKOVÁ V., CHOBOT S. & SVIRIDOV V. 1975. Mass occurrence of Yersinia enterocolitica in two establishments of collective care of children. Journal of Hygiene, Epidemiology, Microbiology and Immunology 19, 22-29.

OLSVIK Ø. & KAPPERUD G. 1982. Enterotoxin production in milk at 22°C and 4°C by Escherichia coli and Yersinia enterocolitica. Applied and Environmental Microbiology 43, 997-1000.

OYE van E. 1978. Sur la survie de Yersinia enterocolitica dans le milieu exterieur. Revue d'Epidémiologie et de la Santé Publique 26, 131-136.

PADLEWSKY L. 1908. Eine neue Anwendungsmethode des Malachitgrünagars zum Nachweis von Bacillen der Typhusgruppe. Centralblatt für Bakteriologie Parasitenkunde und Infektionskrankheiten I Abteilung Originale 47, 540-544.

PAI C.H. & MORS V. 1978. Production of enterotoxin by Yersinia enterocolitica. Infection and Immunity 19, 908-911.

PAI C.H., MORS V. & TOMA S. 1978. Prevalence of enterotoxigenicity in human and non-human isolates of Yersinia enterocolitica. Infection and Immunity 22, 334-338.

PAI C.H., SORGER S., LAFLEUR L., LACKMAN L. & MARKS M.I. 1979. Efficacy of cold enrichment techniques for recovery of Yersinia enterocolitica from human stools. Journal of Clinical Microbiology 9, 712-715.

PEABODY F. & PRATT J. 1908. Ueber den Wert von Malachitgrünnährböden zur Differenzierung von Typhus - und Colonbacillen. Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten I Abteilung: Originale 45, 550-558.

PEDERSEN K.B., WINBLAD S. & BITSCH V. 1979. Studies on the interaction between different O-serotypes of Yersinia enterocolitica and HeLa cells. Acta Pathologica et Microbiologica Scandinavica Section B 87, 141-145.

PEIXOTTO S.S., FINNE G., HANNA M.O. & VANDERZANT C. 1979. Presence, growth and survival of Yersinia enterocolitica in oysters, shrimp and crab. Journal of Food Protection 42, 974-981.

POKORNÝ J. 1974. [On the question of survival of Yersinia enterocolitica in water environment]. Československá Hygiena 19, 414-418.

PORTNOY D.A., MOSELEY S.L. & FALKOW S. 1981. Characterization of plasmids and plasmid-associated determinants of Yersinia enterocolitica pathogenesis. Infection and Immunity 31, 775-782.

PRUITT D.V. & JOHNSON M.G. 1978. Recovery of tolerance to sodium chloride or bile salts No 3 by heat-stressed cells of Yersinia enterocolitica 107 exposed to 5°C or an overlay. Abstracts of the Annual Meeting of the American Society for Microbiology, p.187.

RABSON A.R. & KOORNHOF H.J. 1973. Yersinia enterocolitica infections in South Africa. Contributions to Microbiology and Immunology 2, 102-105.

RAEVUORI M., HARVEY S.M., PICKETT M.J. & MARTIN W.J. 1978. Yersinia enterocolitica: In vitro antimicrobial susceptibility. Antimicrobial Agents and Chemotherapy 13, 888-890.

RAPPAPORT F., KONFORTI N. & NAVON B. 1956. A new enrichment medium for certain salmonellae. Journal of Clinical Pathology 9, 261-266.

RESTAINO L., JETER W.S. & HILL W.M. 1980. Thermal injury of Yersinia enterocolitica. Applied and Environmental Microbiology 40, 939-949.

ROBINS-BROWEN R.M., JACOBS M.R., KOORNHOF H.J. & MAUFF A.C. 1979. Yersinia enterocolitica biotype 1 in South Africa. South African Medical Journal 55, 1057-1059.

SAARI T.N. & JANSEN G.P. 1979. Waterborne Yersinia enterocolitica in the Midwest United States. Contributions to Microbiology and Immunology 5, 185-196

SARTORIUS F. 1928. Neue Farbstoffnährböden zur Elektivzüchtung von Typhus - und Paratyphusbacillen. Zeitschrift für Hygiene und Infektionskrankheiten 108, 637-647.

SCHIEMANN D.A. 1978a. Association of Yersinia enterocolitica with the manufacture of cheese and occurrence in pasteurized milk. Applied and Environmental Microbiology 36, 274-277.

SCHIEMANN D.A. 1978b. Isolation of Yersinia enterocolitica from surface and well waters in Ontario. Canadian Journal of Microbiology 24, 1048-1052.

SCHIEMANN D.A. 1979a. Effect of dyes on the quantitative recovery of Yersinia enterocolitica. Applied and Environmental Microbiology 38, 205-211.

SCHIEMANN D.A. 1979b. Synthesis of a selective agar medium for Yersinia enterocolitica. Canadian Journal of Microbiology 25, 1298-1304.

SCHIEMANN D.A. 1980a. Isolation of toxigenic Yersinia enterocolitica from retail pork products. Journal of Food Protection 43, 360-365.

SCHIEMANN D.A. 1980b. Yersinia enterocolitica: observations on some growth characteristics and response to selective agents. Canadian Journal of Microbiology 26, 1232-1240.

SCHIEMANN D.A. & TOMA S. 1978. Isolation of Yersinia enterocolitica from raw milk. Applied and Environmental Microbiology 35, 54-58.

SCHINDLER H. 1909. Über Malachitgrünnährböden. Zeitschrift für Hygiene und Infektionskrankheiten 63, 91-110.

SCHILLINGER J.E. & McFETERS G.A. 1978. Survival of Escherichia coli and Yersinia enterocolitica in stream and tap waters. Abstracts of the Annual Meeting of the American Society for Microbiology, p. 175.

SEBES J.I., MABRY E.H. & RABINOWITZ J.G. 1976. Lung abscess and osteomyelitis of rib due to Yersinia enterocolitica. Chest 69, 546-547.

SEELYE R.J. & YEARBURY B.J. 1979. Isolation of Yersinia enterocolitica-resembling organisms and Alteromonas putrefaciens from vacuum-packed chilled beef cuts. Journal of Applied Bacteriology 46, 493-499.

SIMON C.E. & WOOD M.A. 1914. The inhibitory action of certain aniline dyes upon bacterial development. American Journal of the Medical Sciences 147, 247-260 & 524-540.

SIMMONS J.S. 1926. A culture medium for differentiating organisms of typhoid-colon aerogenes groups and for isolation of certain fungi. Journal of Infectious Diseases 39, 209-214.

SKERMAN V.B.D., McGOWAN B. & SNEATH P.H.A. 1980. Approved lists of bacterial names. International Journal of Systematic Bacteriology 30, 225-420.

SOLTÉSZ L.V., SCHALÉN C. & MÅRDH P. 1980. An effective, selective medium for Yersinia enterocolitica containing sodium oxalate. Acta Pathologica et Microbiologica Scandinavica Section B 88, 11-16.

SONNENWIRTH A.C. 1976. Isolation and characterization of Yersinia enterocolitica. Mount Sinai Journal of Medicine 43, 736-745.

SPADARO M. & INFORTUNA M. 1968. Isolamento di Yersinia enterocolitica in Mitilus galloprovincialis lamk. Bollettino della Societa Italiana di Biologia Sperimentale 44, 1896-1897.

STANEK G., ROTTER M. & KOLLER W. 1979. Yersinia enterocolitica - ein in Österreich noch selten isolierter Infektionserreger. Wiener Klinische Wochenschrift 91, 221-226.

STARR M.P., CHATTERJEE A.K., STARR P.B. & BUCHANAN G.E. 1977. Enzymatic degradation of polygalacturonic acid by Yersinia and Klebsiella species in relation to clinical laboratory procedures. Journal of Clinical Microbiology 6, 379-386.

STEARN E.W. & STEARN A.E. 1926. Conditions and reactions defining dye bacteriostasis. Journal of Bacteriology 11, 345-357.

STERN N.J. 1981. Isolation of potentially virulent Yersinia enterocolitica from variety meats. Journal of Food Science 46, 41-42 & 51.

STERN N.J., PIERSON M.D. & KOTULA A.W. 1980a. Effects of pH and sodium chloride on Yersinia enterocolitica growth at room and refrigeration temperatures. Journal of Food Science 45, 64-67.

STERN N.J., PIERSON M.D. & KOTULA A.W. 1980b. Growth and competitive nature of Yersinia enterocolitica in whole milk. Journal of Food Science 45, 972-974.

SZITA J., KÁLI M. & RÉDEY B. 1973. Incidence of Yersinia enterocolitica infection in Hungary. Contributions to Microbiology and Immunology 2, 106-110.

THATCHER F.S. & CLARK D.S. eds. 1968. Microorganisms in Foods: Their Significance and Methods of Enumeration. Toronto: University of Toronto Press.

TODD, E.W. & HEWITT L.F. 1932. A new culture medium for the production of antigenic streptococcal haemolysin. Journal of Pathology and Bacteriology 35, 973-974.

TOIVANEN P., TOIVANEN A., OLKKONEN L. & AANTAA S. 1973. Hospital outbreak of Yersinia enterocolitica infection. Lancet i, 801-803.

TOMA S. & LAFLEUR L. 1974. Survey on the incidence of Yersinia enterocolitica infection in Canada. Applied Microbiology 28, 469-473.

TSUBOKURA M., OTSUKI K. & ITAGAKI K. 1973. Studies on Yersinia enterocolitica. 1. Isolation of Yersinia enterocolitica from swine. Japanese Journal of Veterinary Science 35, 419-424.

UNE T. 1977a. Studies on the pathogenicity of Yersinia enterocolitica. 1. Experimental infection in rabbits. Microbiology and Immunology 21, 349-363.

UNE T. 1977b. Studies on the pathogenicity of Yersinia enterocolitica. II. Interaction with cultured cells in vitro. Microbiology and Immunology 21, 365-377.

URSING J., BRENNER D.J., BERCOVIER H., FANNING G.R., STEIGERWALT A.G., BRAULT J. & MOLLARET H.H. 1980. Yersinia frederiksenii: a new species of Enterobacteriaceae composed of rhamnose-positive strains (formerly called atypical Yersinia enterocolitica or Yersinia enterocolitica-like). Current Microbiology 4, 213-217.

VIDON D.J.M. & DELMAS C.L. 1981. Incidence of Yersinia enterocolitica in raw milk in Eastern France. Applied and Environmental Microbiology 41, 355-359.

WAUTERS G. 1970. Contribution à l'étude de Yersinia enterocolitica. Thesis. University of Louvain.

WAUTERS G. 1973. Improved methods for the isolation and recognition of Yersinia enterocolitica. Contributions to Microbiology and Immunology 2, 68-70.

WAUTERS G. 1981. Antigens of Yersinia enterocolitica. In: Yersinia enterocolitica, ed. Bottone E.J. Boca Raton: CRC Press.

WAUTERS G., LE MINOR L. & CHALON A.M. 1971. Antigènes somatiques et flagellaires des Yersinia enterocolitica. Annales de l'Institut Pasteur 120, 631-642.

WAUTERS G., LE MINOR L., CHALON A.M. & LASSEN J. 1972. Supplément au schéma antigénique de Yersinia enterocolitica. Annales de l'Institut Pasteur 122, 951-956.

WEISSFELD A.S. & SONNENWIRTH A.C. 1980. Yersinia enterocolitica in adults with gastrointestinal disturbances: need for cold enrichment. Journal of Clinical Microbiology 11, 196-197.

WEISSMAN J.B., WILLIAMS S.V., HINHAN A.R., HAUGHIE G.R. & GANGAROSA E.J. 1974. Foodborne shigellosis at a country fair. American Journal of Epidemiology 100, 178-185.

WILSON H.D., McCORMICK J.B. & FEELEY J.C. 1976. Yersinia enterocolitica infection in a 4 mouth old infant associated with infection in household dogs. Journal of Pediatrics 89, 767-769.

WINBLAD S. 1968. Studies on O-antigen factors of Yersinia enterocolitica. Symposium Series in Immunobiological Standardization 9, 337-342.

WRIGHT H.D. 1933. The importance of adequate reduction of peptone in the preparation of media for pneumococcus and other organisms. Journal of Pathology and Bacteriology 37, 257-282.

WYNNE E.S. 1947. Antagonism by Aerobacter strains. Journal of Bacteriology 53, 469-478.

WYNNE E.S. & NORMAN J.O. 1953. On the concept of "direct antagonism" in bacteria. Journal of Infectious Diseases 93, 243-246.

ZEN-YOJI H. 1978. Enteropathogenicity of Escherichia coli and Yersinia enterocolitica. In: Gastrointestinal Infections in South East Asia III. Proceedings of 5th SEAMIC Seminar Tokyo 1978. Ed. Fukumi H., Ohashi M., Murakami D.U.M., Kudoh Y. & Nakamura A. p. 82-84. Tokyo: South East Asian Medical Information Centre.

ZEN-YOJI H., MARUYAMA T., SAKAI S., KIMURA S., MIZUNO T. & MAMOSE T. 1973. An outbreak of enteritis due to Yersinia enterocolitica occurring at a Junior High School. Japanese Journal of Microbiology 17, 220-22.

ZEN-YOJI H., SAKAI S., MARUYAMA T. & YANAGAWA Y. 1974. Isolation of Yersinia enterocolitica and Yersinia pseudotuberculosis from swine, cattle, and rats at an abattoir. Japanese Journal of Microbiology 18, 103-105.

ZINK D.L., FEELEY J.C., WELLS J.G., VANDERZANT C., VICKERY J.C. & O'DONOVAN G.A. 1978. Possible plasmid-mediated virulence in Yersinia enterocolitica. Texas Journal of Science Special publication No 4 (Trans Gulf Coast Molecular Biology Conference) III, 155-163.

APPENDIX I

Most probable numbers per gram/ml of sample*

No. of tubes positive in Series A 10 ml	Series B 1 ml	Series C 0.1 ml	MPN	No. of tubes positive in Series A 10 ml	Series B 1 ml	Series C 0.1 ml	MPN
0	0	0	LT 0.03	2	0	0	0.091
0	0	1	0.03	2	0	1	0.14
0	0	2	0.06	2	0	2	0.20
0	0	3	0.09	2	0	3	0.26
0	1	0	0.03	2	1	0	0.15
0	1	1	0.061	2	1	1	0.20
0	1	2	0.092	2	1	2	0.27
0	1	3	0.12	2	1	3	0.34
0	2	0	0.062	2	2	0	0.21
0	2	1	0.093	2	2	1	0.28
0	2	2	0.12	2	2	2	0.35
0	2	3	0.16	2	2	3	0.42
0	3	0	0.094	2	3	0	0.29
0	3	1	0.13	2	3	1	0.36
0	3	2	0.16	2	3	2	0.44
0	3	3	0.19	2	3	3	0.53
1	0	0	0.036	3	0	0	0.23
1	0	1	0.072	3	0	1	0.39
1	0	2	0.11	3	0	2	0.64
1	0	3	0.15	3	0	3	0.95
1	1	0	0.073	3	1	0	0.43
1	1	1	0.11	3	1	1	0.75
1	1	2	0.15	3	1	2	1.20
1	1	3	0.19	3	1	3	1.60
1	2	0	0.11	3	2	0	0.93
1	2	1	0.15	3	2	1	1.50
1	2	2	0.20	3	2	2	2.10
1	2	3	0.24	3	2	3	2.90
1	3	0	0.16	3	3	0	2.40
1	3	1	0.20	3	3	1	4.60
1	3	2	0.24	3	3	2	11.00
1	3	3	0.29	3	3	3	GT 11.00

MPN = mean probable number; LT = less than; GT = greater than.

* = adapted from MPN table of Hoskins (1934).

KENDALL, M. and GILBERT, R.J. 1980 Survival and Growth of Yersinia enterocolitica In: Microbial growth and survival in extremes of environment ed. Gould, G.W. & Corry, J.E.L. pp 215-226. Society for Applied Bacteriology Technical Series No. 15. Academic Press:London

Survival and Growth of *Yersinia enterocolitica* in Broth Media and in Food

MARGARET KENDALL AND R. J. GILBERT

Food Hygiene Laboratory, Central Public Health Laboratory, London, UK

Sporadic cases of human gastrointestinal infection due to *Yersinia enterocolitica* have been recognized and reported with increasing frequency since 1966 (Highsmith *et al.* 1977). Workers in various countries throughout the world have recorded the occurrence of this organism (Rabson & Koornhof 1972; Zen-Yoji & Maruyama 1972; Hinderaker *et al.* 1973; Rakovský *et al.* 1973; Vandepitte *et al.* 1973; Kohl *et al.* 1976; Szita & Svidró 1976). Useful reviews of a general nature have also been published (Mair 1975; Morris & Feeley 1976; Highsmith *et al.* 1977; Bottone 1977).

At least five outbreaks in which a common food or water source was suspected are known to have occurred in the period 1973–1978. Three of these were explosive community outbreaks in Japan involving junior and primary schools. Zen-Yoji and co-workers described an incident in which 198 of 1086 pupils suffered typical symptoms of abdominal pain (76%), fever (61%), headache (60%), diarrhoea (36%), malaise (33% and vomiting (12%). Half of the patients experienced severe pain in the umbilical region or lower right quadrant and three cases underwent appendectomy. One hundred and thirty two isolates of *Y. enterocolitica* were serotype 0:3, two were serotype 0:5 and six serotype 0:9: one patient carried both types 0:3 and 0:9 (Zen-Yoji *et al.* 1973). The other two incidents in Japan (Asakawa *et al.* 1973) were similar to the one described above and affected a total of 733 children and teachers. All the strains isolated were *Y. enterocolitica* type 0:3. The fourth outbreak occurred in Czechoslovakia (Olšovský *et al.* 1975) and centred on two establishments for the collective care of children, both of which received their food from a single kitchen. In none of the outbreaks described above was the vehicle of infection identified.

The fifth outbreak (Black *et al.* 1978) occurred in a village in the USA

where 37 of 119 people reporting ill with abdominal pain and fever were found to be infected with *Y. enterocolitica* type 0:8. The same serotype was also isolated from 1 of 60 people reporting diarrhoea without abdominal pain and fever. Twenty three of the culture positive patients, 9 of 40 culture negative and one symptomless child had a raised antibody level (>128). Sixteen patients underwent appendectomy. The common food source of the organism was identified as a chocolate milk drink. This was purchased from a small dairy which was the exclusive supplier of milk to the local schools. The chocolate milk was prepared by adding chocolate syrup to previously pasteurized milk in an open vat and mixing by hand with a perforated metal stirring rod. *Yersinia enterocolitica* type 0:8 was isolated from 1 of 4 unopened cartons of milk obtained from the school cafeteria.

Yersinia enterocolitica has been isolated from many sources including mussels (Spadaro & Infortuna 1968), drinking water (Lassen 1972), oysters (Toma 1973), poultry meat, pork and beef (Leistner *et al.* 1975) and also from milk (Schiemann & Toma 1978). Pigs, dogs, hares and chinchillas are known hosts of the organism.

The biochemical classification and differentiation of *Y. enterocolitica* and *Yersinia*-like organisms is still under debate but detailed schemes for characterization of these bacteria have been described by many workers (Wauters 1970; Darland *et al.* 1974; Sonnenwirth 1976; Mehlman *et al.* 1978).

Two biotyping schemes which sub-divide *Y. enterocolitica* into five biochemical groups have also been described (Niléhn 1969; Wauters 1970) and further sub-division of the species is possible using a serotyping scheme based on the somatic antigens (Wauters 1970) and by phage typing (Niléhn & Ericson 1969). There are 34 serotypes of which types 0:3, 0:8 and 0:9 have been most commonly associated with human illness. In a correlation study of serological and phage types it was found that most of the human serotypes were phage types II or III (Niléhn 1973).

This report is part of a larger study on the growth, survival and isolation of *Y. enterocolitica* in various foods.

Materials and Methods

Organisms

Two human isolates of *Y. enterocolitica* serotypes 0:3 and 0:9 were supplied by the Yersinia Reference Laboratory, Public Health Laboratory, Leicester.

Media

All dehydrated media were prepared and sterilized according to the manufacturers' instructions.

Brain Heart Infusion broth (*BHI*): Dehydrated Difco Bacto No. B37.

Brain Heart Infusion agar plates (*BHI+dye*): BHI with the addition of (% w/v) powdered agar 1·2; lactose 1; 1% solution neutral red 0·5.

Buffered Brain Heart Infusion broth (*BBHI*): Sterile double strength BHI mixed with an equal volume of sterile 0·68 M McIlvaine's citric acid phosphate buffer solution of the required pH (Giegy Scientific Tables 1962).

GN broth (Hajna 1955) (*GN*): Dehydrated Difco Bacto No. 0486.

Phosphate buffered saline (*PB*): 0·067 M potassium dihydrogen phosphate/disodium hydrogen phosphate (pH 7·6) in 0·85% (w/v) sodium chloride.

Quarter strength Ringer's solution (*RS*): Oxoid tablets No. BR 52.

Selenite broth (*SB*): (% w/v) sodium hydrogen selenite 0·4; peptone 0·5; mannitol 0·4; disodium hydrogen phosphate 0·75; sodium dihydrogen phosphate 0·53 in distilled water. Sterilized by filtration.

Selenite cystine broth (*SC*): Dehydrated Difco Bacto No. 0687.

Wauters' broth (Wauters 1973) (*WB*): Sterile 1% Bacto tryptone No. B123 620 ml; sterile M/15 disodium hydrogen phosphate 160 ml; sterile 40% magnesium chloride 208 ml; 0·2% (w/v) malachite green 6·4 ml; 1000 μg ml^{-1} carbenicillin 1·2 ml. The medium was stored at 4°C until required.

Foods

Hard-boiled egg, boiled fish, rice, potato, and roast chicken were prepared and cooked by normal domestic methods. After cooking, the bones and other non-edible parts were removed and where necessary the foods were chopped or finely homogenized. These foods were chosen primarily because of their previous implication in other types of food-borne outbreaks. Studies on other foods with a higher natural contaminating flora have still to be completed.

Growth studies

Preparation of inocula. Overnight BHI broth cultures incubated at 30°C were diluted with RS to give the required concentration for each experiment.

Surface colony counts. 10-fold dilutions were prepared in RS and counts made by a modified surface drop method (Thatcher & Clark 1968) on BHI plus dye plates.

Growth at different pH values: The two test strains were inoculated separately into 20 ml volumes of BBHI broth at pH 2·4, 3·6, 4·2, 4·4, 4·6, 4·8, 5·4, 6·6 and 7·8 to give approx. 10^3 colony forming units (cfu) ml^{-1} and incubated at 22°C for 72 h. Surface colony counts were carried out after 0, 8, 14, 18, 22, 26, 30, 38, 48, 62 and 72 h and the plates incubated at 30°C for 48 h.

Growth in various media. Both test strains were inoculated separately into each of 3×100 ml volumes of BHI, WB, SB, SC, GN and PB to give approx. 100 cfu ml^{-1}. The broths were incubated at 4° and 22°C for 13 d and at 30°C for 72 h. Surface colony counts were carried out after 1, 2, 5, 9 and 13 days at 4°C and 0, 6, 24 and 48 h at 22° and 30°C; plates were incubated at 30°C for 48 h.

Growth in foods. 10 gram quantities of each homogenized food were distributed in sterile 1 lb screw-capped jars. 0·06 ml volumes of the two test strains were inoculated separately on to the surface of each of 20×10 g samples to give approx. 10^3 cfu g^{-1}. Five inoculated jars of food were incubated at 30°C, six at 4°C and seven at −20°C. One jar was retained and a surface colony count carried out immediately. The counts were carried out by homogenizing a 10 g sample with 90 ml RS to give a 1/10 dilution, from which further dilutions were prepared.

Surface colony counts were carried out on the foods incubated at 30°C after 5, 8, 14, 24 and 30 h, from those stored at 4°C after 1, 2, 3, 7, 10 and 21 d and at −20°C after 1, 2, 3, 21, 35, 49 and 63 d. All the plates were incubated at 30°C for 48 h.

Where it was suspected that counts would be <100 g^{-1}, and therefore beyond the lower limits of the counting method, 90 ml volumes of BHI broth were added to the 10 g portions of food instead of RS and these suspensions incubated at 30°C for 48 h as enrichment cultures after being used as 1/10 dilutions for the surface colony counts. Sub-cultures were made on BHI + dye plates and incubated at 30°C for 48 h. The presence or absence of *Y. enterocolitica* was recorded.

Results

Effect of pH

Figure 1 shows the growth curves of *Y. enterocolitica* serotype 0:3 at various pH values in BBHI; similar results were obtained with serotype 0:9. The lower the pH value the more prolonged was the lag period. At pH 3·6 and below there was a rapid decrease in the number of *Y. enterocolitica* and within 48 h the number of both strains had diminished to below the limits of the counting method. At pH 4·2 both strains sur-

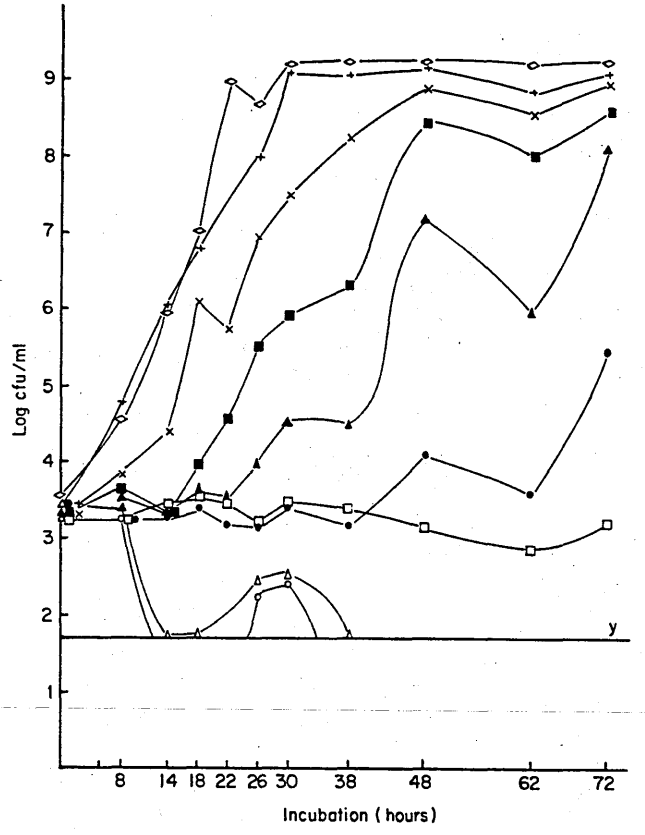


Fig. 1. Growth of *Y. enterocolitica* serotype 0:3 in BBHI at 22°C. ○, pH 2·4; △, pH 3·6; □, pH 4·2; ●, pH 4·4; ▲, pH 4·6; ■, pH 4·8, x, pH 5·4; +, pH 6·6; ◇, pH 7·8; y, lower limit of counting method.

vived for 72 h with little or no variation from the original count. pH values of 4·2–4·4 appeared to be the threshold of inhibition/growth and although at pH 4·4 the lag period was prolonged, growth did occur, particularly after 38 h, and finally reached a level of $>2{\cdot}75\times10^5$ cfu ml^{-1} at 72 h. Growth of both strains occurred more readily at pH 4·8 and all values above this. For example at pH 6·6 and 7·8 levels of $>1\times10^9$ cfu ml^{-1} were attained within 30 h.

Growth in enrichment media

In BHI both strains of *Y. enterocolitica* grew to levels greater than 5×10^8 cfu ml^{-1} within 48 h at 22° and 30°C. At 4°C the count rose more steadily and finally reached $>5\times10^8$ cfu ml^{-1} after 9 d. BHI was used

as a standard against which the growth in all the other media was compared. The growth patterns of the two test strains in GN were almost identical to those in BHI, the counts attained being 5×10^8 cfu ml^{-1} after 10–11 d at 4°C.

Although PBS does not provide any organic nutrients for growth both strains survived for 13 d at 4°C with no decrease in count. At 22°C, the number of organisms fell below the lower counting limit within 2–5 d.

The two selenite broths SB and SC demonstrated very different characteristics. Figure 2a shows the growth of both serotypes in SC at 4°, 22° and 30°C. By comparison with BHI there was inhibition of *Y. enterocolitica* as would be expected in a selective medium. At 4°C growth increased steadily to $2{\cdot}75\times10^6$ and 5×10^5 cfu ml^{-1} respectively after 13 d. The experiment was terminated before the upper limit of bacterial growth was attained. Nevertheless, at 30°C counts of 2×10^6 and 2×10^7 and at 22°C $6{\cdot}2\times10^4$ and 8×10^5 cfu ml^{-1} were obtained within 48–72 h.

SB failed to support the growth of serotype 0:3 at any temperature and this strain was undetectable by the methods used after the initial count at 0 h. Serotype 0:9 was also undetectable at 4°C after the initial counts but at 22° and 30°C numbers of this serotype rose to $1{\cdot}2\times10^5$ and $1{\cdot}5\times10^8$ cfu ml^{-1} respectively within 2 d.

In WB only one strain, serotype 0:9, appeared to survive at 4°C with evidence of some growth after 9 d; serotype 0:3 was not isolated after 1 d. Both strains grew well at 22° and 30°C to $>3\times10^7$ cfu ml^{-1} in 2 d (Fig. 2b).

Growth in foods

Growth patterns of *Y. enterocolitica* in hard boiled egg and boiled fish are representative of the results from all five foods (Fig. 3a & b). At 30°C the colony counts rose rapidly to $>1\times10^8$ cfu g^{-1} within 30 h. There was a steady increase in count at 4°C over a period of 10 d by which time the number of *Y. enterocolitica* had reached the same level as at 30°C. Both strains of *Y. enterocolitica* survived for at least 8–9 weeks after storage at −20°C, the number of viable cells remaining at the same level as the initial count.

Boiled potato was the only food tested which became discoloured by the growth of the *Y. enterocolitica.*

Discussion

The results obtained in this study for growth of *Y. enterocolitica* are comparable in many respects with those of Hanna *et al.* (1977*a*). These workers adjusted the pH of BHI with sodium hydroxide or hydrochloric

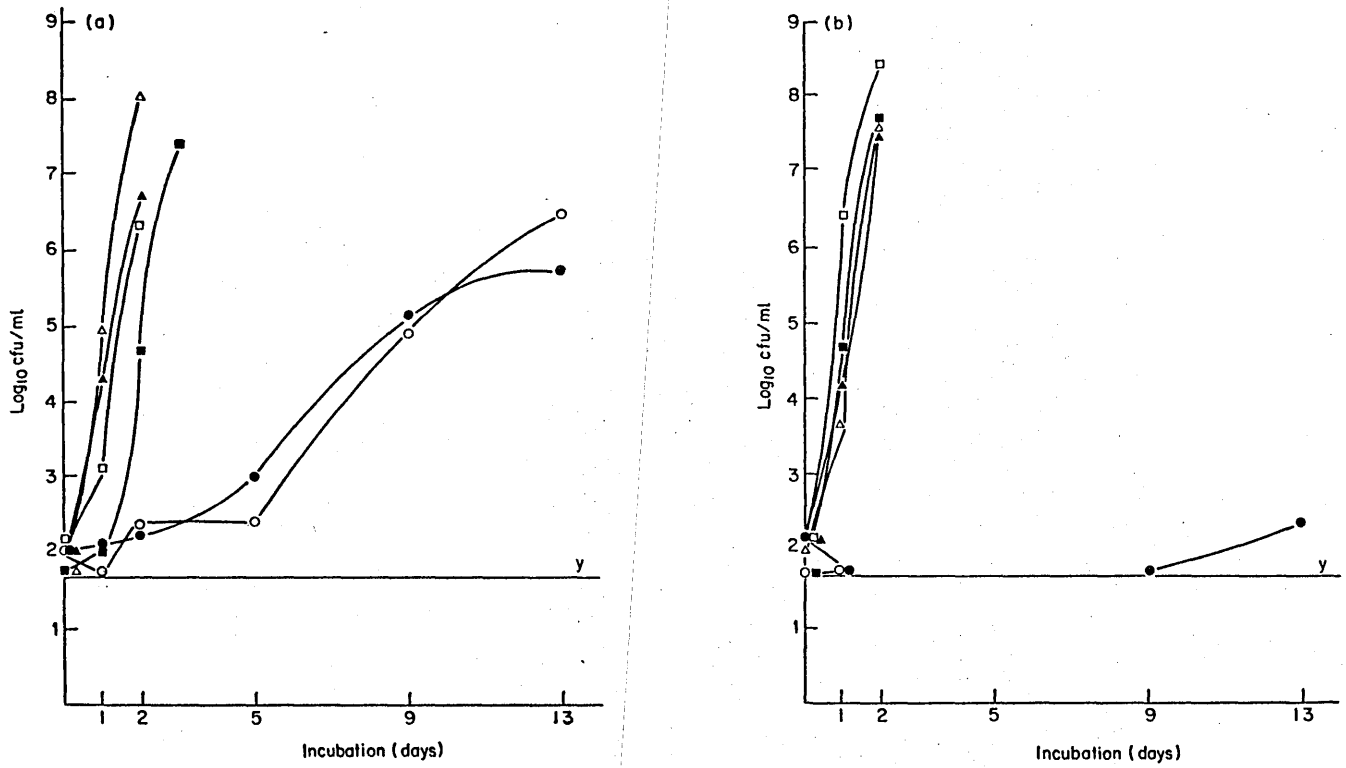


Fig. 2. Growth of *Y. enterocolitica* in enrichment media: (a) selenite cystine, (b) Wauters broth. Serotype 0:3 open symbols; serotype 0:9 solid symbols; ○ ●, 4°C; △ ▲, 22°C; □ ■, 30°C; y, lower limit of counting method.

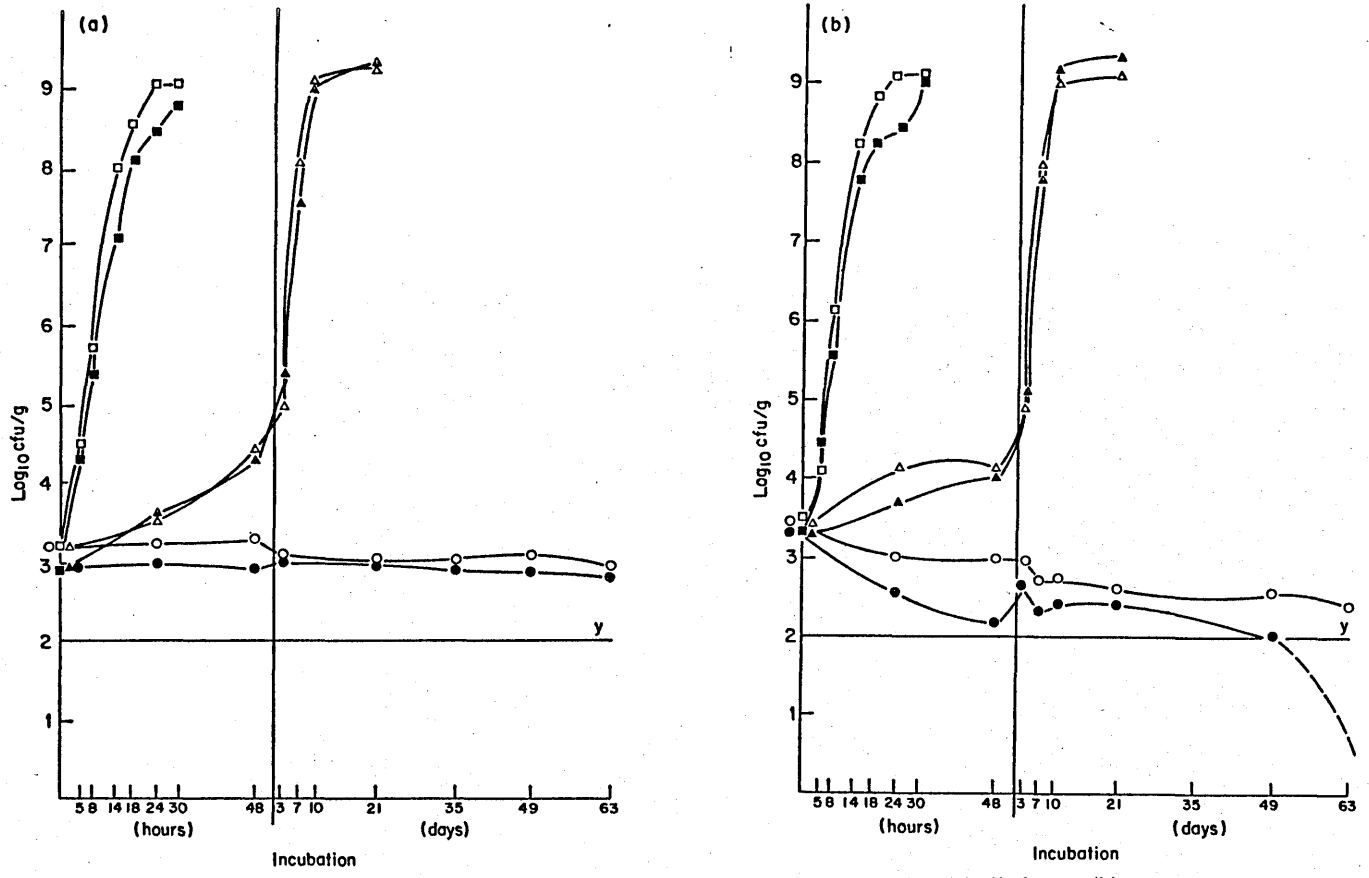


Fig. 3. Growth and survival of *Y. enterocolitica* in foods: (a) hard boiled egg, (b) boiled fish. Serotype 0:3 open symbols; serotype 0:9 solid symbols; ○, ● —20°C; △ ▲, 4°C; □ ■, 30°C; y, lower limit of counting method; (- - -), *Y. enterocolitica* present $< 100/g^{-1}$.

acid prior to sterilization and only recorded the growth of the organisms over 24 h. The only difference between our results and those of Hanna and co-workers is that they found no increase in growth over 24 h at pH 5·0 whereas our results show a definite increase in numbers within that period. Our findings indicate that *Y. enterocolitica* can grow to high and therefore presumably hazardous levels in certain foods, even with a relatively low pH, when stored for prolonged periods.

Various methods have been recommended for the isolation of *Y. enterocolitica* but there appears to be little information in the literature relating to the performance of specific media. Toma & Diedrick (1975) used phosphate buffered saline pH 7·6 incubated at 4°C for the isolation of *Yersinia* from the caecal contents of pigs. Inoue & Kurose (1975) used both phosphate buffered saline and selenite broth with novobiocin when investigating cow's intestinal contents and beef meat. Of the 24 isolations obtained only one was made from selenite—novobiocin. Phosphate buffered saline was also used successfully in the investigation of the chocolate milk outbreak (Black *et al.* 1978). Schiemann & Toma (1978) compared four methods of isolating *Yersinia* from milk in which they included phosphate buffer and a modified Rappaport broth. The modified Rappaport broth was identical in formula to that of the Wauters broth used in this study. One millilitre of sample was inoculated into 10 ml of enrichment media as follows: (1) modified Rappaport broth incubated at room temperature for 5 d; (2) Butterfields phosphate buffer pH 7·2 incubated at 4°C for 14 d; (3) modified Rappaport broth inoculated with 1 ml of phosphate buffer culture (incubated at 4°C for 14 d) and incubated at 23°C for 5 d; and (4) modified Rappaport broth inoculated with 1 ml from a cooked meat culture (incubated at 23°C for 28 d and incubated at 23°C for 5 d. The four methods gave 6, 8, 24 and 3 isolations respectively.

Extended incubation at 4°C is widely used as an enrichment technique for *Y. enterocolitica* but not all media which support growth at 22° and 30°C are suitable for use in low temperature procedures. This appears to be true for Wauters broth as shown by the results presented in this paper. However when Schiemann & Toma (1978) used this medium at 22°C in combination with PB at 4°C (Method 3) isolations were substantially increased. Our results show that *Y. enterocolitica* survives in PB at 4°C but does not multiply without the addition of nutrients. The increased recovery of this organism by Schiemann & Toma's method 3 could have been due to *Y. enterocolitica* surviving better in the PB than the competing flora due to the presence of milk in the sample. Selection might have been further enhanced when some of the culture was transferred to modified Rappaport broth and incubated at 22°C for 5 d. The

results obtained in this study with SB and SC show the former medium to be unsuitable as an enrichment medium for *Y. enterocolitica*. The main difference between the two media apart from the fact that SB contains mannitol and SC lactose is that SC also contains 0·001 % *l*-cystine.

There are few published reports of studies on the growth and survival of *Y. enterocolitica* in foods. Our results are similar to those obtained by Hanna *et al.* (1977b) for raw and cooked beef and pork. At 7°C the main increase in numbers occurred between 3 and 7 d storage. Hanna *et al.* (1977*a*) also examined the effect of freezing on the survival of *Y. enterocolitica* in cooked beef. They used two inoculum levels: 1×10^3 to 1×10^4 and 1×10^6 to 1×10^7 cfu g^{-1} and storage was at −20°C. One strain of *Y. enterocolitica* could not be detected after two weeks and two other strains could not be detected after four weeks. These results are quite different to those presented in this paper where very small numbers of *Y. enterocolitica* could still be demonstrated even after 9 weeks at −20°C.

In conclusion, the results presented in this paper indicate that *Y. enterocolitica* can grow to high and therefore presumably hazardous levels in foodstuffs stored at domestic refrigerator temperature (4°C). In a warm atmosphere (22°C) even those foodstuffs with the relatively low pH of 4·8, if kept for a prolonged period (48 h), could be a hazard.

Further investigations are needed to find the ideal technique for isolation of *Y. enterocolitica* and the enrichment broth/incubation temperature combination needs to be carefully selected.

We are grateful to Dr Diane Roberts for her helpful advice and criticism in the preparation of this paper.

References

Asakawa, Y., Akahane, S., Kagata, N., Noguchi, M., Sakazaki, R. & Tamura, K. 1973 Two community outbreaks of human infection with *Yersinia enterocolitica*. *Journal of Hygiene, Cambridge* **71**, 715–723.

Black, R. E., Jackson, R. J., Tsai, T., Medvesky, M., Shayegani, M., Feeley, J. C., Macleod, K. I. E. & Wakelee, A. M. 1978 Epidemic *Yersinia enterocolitica* infection due to contaminated chocolate milk. *New England Journal of Medicine* **298**, 76–79.

Bottone, E. J. 1977 *Yersinia enterocolitica* a panoramic view of a charismatic micro-organism. *CRC Critical Reviews in Microbiology* **5**, 211–241.

Darland, G., Ewing, W. H. & Davis, B. R. 1974 The biochemical characteristics of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. *US Department of Health, Education and Welfare* No. (CDC) 75–8294.

Geigy Scientific Tables 1962 ed. Diem, K. pp. 314–315, Manchester: Geigy Pharmaceuticals.

Hajna, A. A. 1955 A new enrichment broth medium for gram-negative organisms of the intestinal group. *Public Health Laboratory* **13**, 83–90.

HANNA, M. O., STEWART, J. C., CARPENTER, Z. L. & VANDERZANT, C. 1977*a* Effect of heating, freezing and pH on *Yersinia enterocolitica*-like organisms from meat. *Journal of Food Protection* **40,** 689–692.

HANNA, M. O., STEWART, J. C., ZINK, D. L., CARPENTER, Z. L. & VANDERZANT, C. 1977*b* Development of *Yersinia enterocolitica* on raw and cooked beef and pork at different temperatures. *Journal of Food Science* **42,** 1180–1184.

HIGHSMITH, A. K., FEELEY, J. C. & MORRIS, G. K. 1977 *Yersinia enterocolitica:* A review of the bacterium and recommended laboratory methodology. *Health Laboratory Science* **14,** 253–260.

HINDERAKER, S., LIAVAAG, I. & LASSEN, J. 1973 *Yersinia enterocolitica* infection. *Lancet II,* 322–323.

INOUE, M. & KUROSE, M. 1975 Isolation of *Yersinia enterocolitica* from cow's intestinal contents and beef meat. *Japanese Journal of Veterinary Science* **37,** 91–93.

KOHL, S., JACOBSON, J. A. & NAHMIAS, A. 1976 *Yersinia enterocolitica* infections in children. *Journal of Pediatrics* **89,** 77–79.

LASSEN, J. 1972 *Yersinia enterocolitica* in drinking-water. *Scandinavian Journal of Infectious Diseases* **4,** 125–127.

LEISTNER, L., HECHELMANN, H., KASHIWAZAKI, M. & ALBERTZ, R. 1975 Nachweis von *Yersinia enterocolitica* in faeces und fleisch von Schweinen, Rindern und Geflügel. *Fleischwirtschaft* **55,** 1599–1602.

MAIR, N. S. 1975 Yersiniosis (Infections due to *Yersinia pseudotuberculosis* and *Yersinia enterocolitica.*) In *Diseases Transmitted from Animals to Man.* eds Hubbert, W. T., McCulloch, W. F. & Schnurrenberger, P. R. Ch. 13, pp. 174–185, Illinois: Thomas.

MEHLMAN, I. J., AULISIO, C. C. G. & SANDERS, A. C. 1978 Problems in the recovery and identification of *Yersinia* from food. *Journal of the Association of Analytical Chemists* **61,** 761–771.

MORRIS, G. K. & FEELEY, J. C. 1976 *Yersinia enterocolitica*: a review of its role in food hygiene. *Bulletin of the World Health Organisation* **54,** 79–85.

NILÉHN, B. 1969 Studies on *Yersinia enterocolitica* with special reference to bacterial diagnosis and occurrence in human enteric disease. *Acta Pathologica et Microbiologica Scandinavica* Supplement 206.

NILÉHN, B. 1973 Host range, temperature characteristics and serologic relationships among *Yersinia* phages. In *Contributions to Microbiology and Immunology,* Yersinia, Pasteurella *and* Francisella ed. Winblad, S. Vol. 2, pp. 59–67, Basel: Karger.

NILÉHN, B. & ERICSON, C. 1969 Studies on *Yersinia enterocolitica.* Bacteriophages liberated from chloroform treated cultures. *Acta Pathologica et Microbiologica Scandinavica* **75,** 117–187.

OLŠOVSKÝ, Z., OLŠÁKOVÁ, V., CHOBOT, S. & SVIRIDOV, V. 1975 Mass occurence of *Yersinia enterocolitica* in two establishments of collective care of children. *Journal of Hygiene, Epidemiology, Microbiology and Immunology* **19,** 22–29.

RABSON, A. R. & KOORNHOF, H. J. 1972 *Yersinia enterocolitica* infections in South Africa. *South African Medical Journal* **46,** 798–803.

RAKOVSKÝ, J., PAUČKOVÁ, V. & ALDOVÁ, E. 1973 Human *Yersinia enterocolitica* infections in Czechoslovakia. In *Contributions to Microbiology and Immunology,* Yersinia, Pasteurella *and* Francisella ed. Winblad, S. Vol. 2, pp. 93–98, Basel: Karger.

SCHIEMANN, D. A. & TOMA, S. 1978 Isolation of *Yersinia enterocolitica* from raw milk. *Applied and Environmental Microbiology* **35,** 54–58.

SONNENWIRTH, A. C. 1976 Isolation and characterization of *Yersinia enterocolitica. Mount Sinai Journal of Medicine* **43,** 736–745.

SPADARO, M. & INFORTUNA, M. 1968 Isolamento di *Yersinia enterocolitica* in *Mitilus galloprovincialis* lamk. *Bollettino della Società Italiana di Biologia Sperimentale* **44,** 1896–1897.

SZITA, J. & SVIDRÓ, A. 1976 A five year study of human *Yersinia enterocolitica* infections in Hungary. *Acta Microbiologica Academiae Scientiarum Hungaricae* **23,** 191–203.

THATCHER, F. S. & CLARK, D. S. 1968 *Microorganisms in Foods. Their Significance and Methods of Enumeration.* Toronto: University of Toronto Press.

TOMA, S. 1973 Survey on the incidence of *Yersinia enterocolitica* in the province of Ontario. *Canadian Journal of Public Health* **64,** 477–487.

TOMA, S. & DIEDRICK, V. R. 1975 Isolation of *Yersinia enterocolitica* from swine. *Journal of Clinical Microbiology* **2,** 478–481.

VANDEPITTE, J., WAUTERS, G. & ISEBAERT, A. 1973 Epidemiology of *Yersinia enterocolitica* infections in Belgium. In *Contributions to Microbiology and Immunology,* Yersinia, Pasteurella *and* Francisella ed. Winblad, S. Vol. 2, pp. 111–119, Basel: Karger.

WAUTERS, G. 1970 *Contribution a l'étude de* Yersinia enterocolitica. *Thesis,* University of Louvain.

WAUTERS, G. 1973 Improved methods for the isolation and recognition of *Yersinia enterocolitica.* In *Contribution to Microbiology and Immunology,* Yersinia, Pasteurella *and* Francisella ed. Winblad, S. Vol. 2, pp. 68–70, Basel: Karger.

ZEN-YOJI, H. & MARUYAMA, T. 1972 The first successful isolations and identification of *Yersinia enterocolitica* from human cases in Japan. *Japanese Journal of Microbiology* **16,** 493–500.

ZEN-YOJI, H., MARUYAMA, T., SAKAI, S., KIMURA, S., MIZUNO, T. & MAMOSE, T. 1973 An outbreak of enteritis due to *Yersinia enterocolitica* occurring at a Junior High School. *Japanese Journal of Microbiology* **17,** 220–222.

KENDALL, M. 1982 Quality assurance of culture media: Invited comments. In: Quality Assurance and Quality Control of Microbiological Culture Media. Proceedings of the Symposium held on 6th and 7th September 1979, Calas de Mallorca, ed. Corry, J.E.L. pp39-45. G.I.T. Verlag : Ernest Giebeler.

Quality Assurance of Culture Media: Invited Comments

MARGARET KENDALL

Food Hygiene Laboratory,
Central Public Health Laboratory,
Colindale Avenue, London, NW9 5HT, England

Summary

The eight constituent laboratories of the Central Public Health Laboratory are serviced by a media department with an average daily production of 100 litres of bulk media, 1500 agar plates and 4000 filled tubes and bottles. Some 160 items are held in stock and at least a further 200 special items are produced on request. Quality control procedures are carried out on a 5% random sample of each batch of medium and include sterility, pH, accuracy of volume, sodium chloride concentration, amino-nitrogen titrations and growth checks of control organisms.

Communication between the manufacturers of basic ingredients and dehydrated media, the ready for use media department and the user is a vital factor in successful quality control of media. Alterations in ingredients which appear to be minor can cause major disasters in the laboratory. The use of control organisms and careful observation of test media by experienced workers is essential.

Two case histories are reported which illustrate some problems which have arisen and the manner in which they were overcome.

Introduction

These comments are divided into I) background information about the Media Production Department at the Central Public Health Laboratory, Colindale; II) general comments about quality control and quality assurance; and III) two case histories illustrating specific problems relating to media production. The opinions expressed are based on personal experience together with points raised in discussions with colleagues and with commercial contacts.

Media Production Department, Colindale

This department supplies most of the media requirements of eight constituent laboratories at the Central Public Health Laboratory and also a small number of items to associated clinical laboratories. However, it is not unusual for individual laboratories to prepare batches of experimental media or those required for a specific research project within their own departments.

The workforce of the Media Department (MD) is equivalent to 20 full time staff. The average daily production is 100 litres of bulk media, 1500 agar plates and 4000 tubes and small bottles of media. Most agar plates are poured by machine and the tubes and bottles are filled either mechanically, or manually if an aseptic technique is required. Some 160 items held in stock are available imme-

diately and a further 200 or so special items are prepared on request either on a regular (60 items) or on an occasional basis (140 items).

For most of the last 21 years in which the author has used the MD, quality control of the media produced has been dependent upon the user alerting the MD when something has been observed to be wrong. In the early years the MD was run by a technician with no formal qualifications but who had some bench experience, but there was no quality controller. This arrangement was followed by several years of a trained manager with media production experience together with several quality controllers with varying bench experience. In the 18 months prior to this meeting the MD manager has been a qualified technician who has been backed up by a quality controller who is similarly qualified; both of these staff have considerable microbiological bench experience. Having a keen experienced and qualified quality controller means that besides reliable basic routine testing some research can now be carried out in conjunction with the reference laboratories.

Quality Control Testing

The present quality control procedures are carried out on a 5% random sample from each batch of medium except where stated.

1. *Sterility.* A 5% random sample is taken from media autoclaved after distribution and the entire batch of aseptically filled media. The samples are incubated overnight and inspected for contamination before issue and the aseptic fills are incubated for 48h. All samples must be sterile.
2. *pH.* This is checked on all media and must be within the variation ±0.2 unless a more critical pH is required, as with citrate media.
3. *Volume.* A volume variation is allowed within ±0.2 ml in 10 ml. Tightness of the caps during autoclaving is important to reduce volume loss.
4. *Salt concentration.* Determination of salt concentration is essential only when it is a critical ingredient in a medium e. g. salt broths for vibrios.
5. *Amino nitrogen titrations.* These are used to standardize media produced from raw meat.
6. *Growth.* Tests are carried out using the organisms for which the medium was developed and also those relevant to the user laboratory. Where indicators are included in the formula, the colour (pH) of the uninoculated medium is checked. Growth and/or inhibition of the relevant organisms and any colour change produced is noted.

No general use is made of the surface colony count technique and agar gel strength is not tested. The single quality control technique in regular use which uses the surface colony count is the standardization of sodium deoxycholate powder for use in LEIFSON agar (HYNES 1942) and LEIFSON sucrose agar (modified from HYNES 1942). LEIFSON agar plates containing varying amounts of sodium deoxycholate are prepared and a surface colony count carried out using a broth culture of a standard strain of *Escherichia coli* diluted in broth cultures of *Salmonella cubana* and *Shigella sonnei.* The number of colonies of each organism and their size and appearance in each dilution is taken into account in deciding on the correct concentration of the sodium deoxycholate required to give optimal conditions for the isolation of the enteric pathogens.

This procedure has been developed by and used mainly in one of the Colindale reference laboratories (J. DE MELLO, unpublished). Every batch of sodium deoxycholate is checked prior to its distribution within the Public Health Laboratory Service.

General Comments

The manufacturers of various media ingredients and complete dehydrated media, the ready-for-use media production department and the user all share some responsibility for the production of good media. Adequate communication is probably the most important single factor influencing quality assurance and it is essential that this takes place between all levels from manufacturer to user (Table 1).

The Manufacturer

It is often difficult to obtain a full specification for raw materials, and this view is confirmed by contacts involved in manufacturing dehydrated media. Alterations to an apparently minor specification of a single raw ingredient can cause quite dramatic problems for the user and can render a medium completely useless. A new process may have been introduced because of increased speed, greater convenience, unavailability of a particular crude product or more often for economy, but no new specification is published to warn the user. The resulting aggravation and wastage of time and money could be avoided or at least reduced by better communication between suppliers and users. The user might at least be able to carry out some tests before purchasing large quantities of stock.

A recent example is the case of a well known brand of peptone. The type of meat used in its production was changed due to unavailability of the original meat. Media made from the 'new' peptone began to fail and suspicion finally fell on this product. Enquiries revealed the nature of the change and after being told the problem the manufacturers modified the process to suit the new meat being used. In the meantime other peptones had been found to replace this particular brand in many of our media.

Another example is specifications or lack of them for dyes. The formula for lactose sucrose urea agar (LSU) (JUHLIN & ERICSON 1961) specifies the use of MERCK dyes and it seems from discussions with other users that the use of this brand of dye is important. Various firms produce the same products much more cheaply and more readily available in the UK but when confronted these producers can give no specification as to the purity of their products.

These comments about communication or lack of it apply equally to the dehydrated media manufacturer.

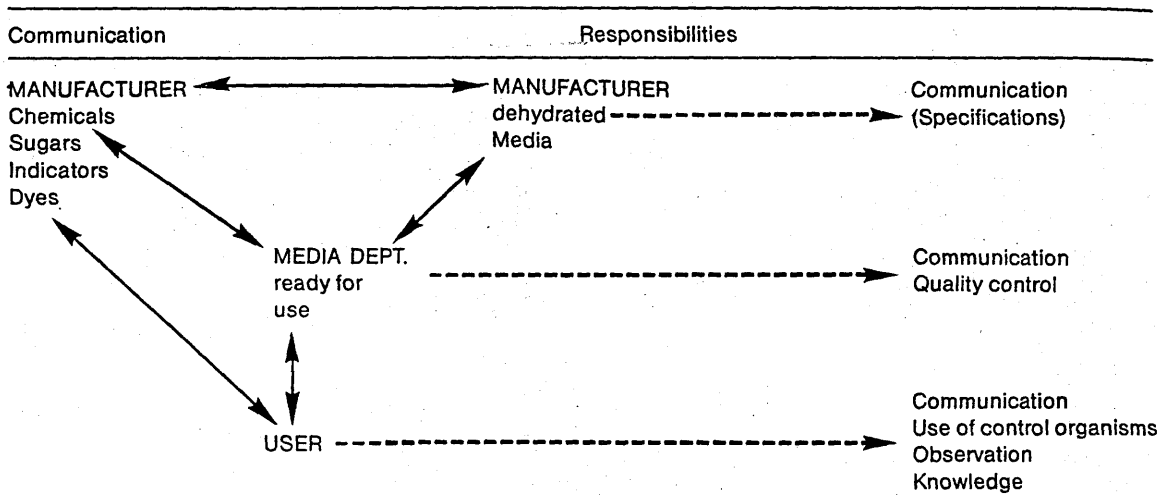
The Ready-for-Use Media Department

Hopefully, one could expect less problems with a local private media kitchen but it is all too easy in a large institute for laboratories to work in isolation.

Communication

This is vital between the ready-for-use department and the various manufacturers and users and also within the department's own organisation (Table 1).

Table 1 *Communication between and responsibilities of manufacturers and users*



Careful and adequate training of all staff and good supervision by qualified personnel contributes a great deal to the production of high quality media. Training should not be just to show how to do a specific job, but include explanations and demonstrations of the appearance and use of the end products and the consequences to the user if they are not made correctly. The greater the knowledge and understanding of any worker the more care and interest will be taken in the job. Faults may even be observed earlier in the production chain which could save hours of unnecessary work and expense.

Qualified supervision is vital if training as described is to take place and be effective. Interest needs to be shown not just in the end product but in the person taking part in its preparation. The use of old, inaccurate and inadequate machines will inspire only boredom and carelessness. Equipment should be regularly checked and upgraded where necessary.

Quality Control

Well trained production staff and good supervision should enable the Quality Control Section to keep routine tests to a minimum. A few simple tests might be used on each small distribution batch with more comprehensive testing on bulk media. This should result in more time being available for research quality control such as testing the effects of different peptones and investigating discrepancies between similar types of media e. g. blood agar. This adds a more interesting dimension to the Quality Controller's job.

The User

The media kitchen should be informed not only which medium is required but also its purpose and its origin otherwise once more a lack of interest and understanding can contribute to the production of poor product.

The use of control organisms for comparison with test results should be the norm and not the exception as is all too often the case. Hoping that errors and variations will be spotted during the use

of a medium may be disastrous. The inclusion of control organisms does not of course preclude constant observation. Exceptions to the rule do occur and atypical strains are not unknown.

STOKES (1975) recommends the use of surface viable count techniques in the control of both liquid and solid media. This is because it shows not only the quantity of growth but also the size of colonies. She later describes (STOKES 1979) a national quality control service organized in the UK for clinical specimens. This involves distributing simulated specimens to interested laboratories. A similar system could be valuable for monitoring media in food microbiology laboratories.

Knowledge

Occasionally amongst research workers one finds that knowledge concerning the medium under discussion is minimal, often because preparation, use and even observation of results were carried out by other members of staff. A working knowledge of any medium being used is essential if reliable results are to be obtained.

In the author's own laboratory it has been the practice for research staff at least initially, to prepare their own experimental media. Difficulties have often been foreseen and overcome by this action.

Case Histories

Case A. LEIFSON Sucrose agar (LS)

In July 1978 batches of this agar, which had been in daily use in the author's laboratory for many years, were observed to be pink instead of the usual brown colour and less inhibitory than previously. Also, the colonial appearance of *Yersinia enterocolitica* with which the author was working had changed dramatically.

The ingredients, quantities used and method of production were investigated and the following discoveries were made. 1. Yeastrel had at some time been substituted for the Lab-Lemco designated in the original formula, and more important, the yeastrel product was an impure and very va-

Table 2 *Haemolysis on horse blood agar prepared with different nutrient bases (SAUNDERS & BALL 1980)*

Organisms	Number of strains	Nutrient base	
		Group 1*	Group 2**
Strep. salivarius	6	-***	+****
Strep. salivarius	7	-	-
Lancefield Group A streptococci	7	+	+
Various greening and non haemolytic streptococci	15	-	-

*Group 1 Colindale Hartley Digest; Gibco Blood Agar Base; Oxoid Blood Agar Base No. 1; Oxoid Nutrient Broth No. 2; Bacto Blood Agar Base; Bacto Blood Agar Base No. 2; Bacto Heart Infusion; Bacto Nutrient Broth.

**Group 2 Columbia Blood Agar Base by Oxoid, Bacto, Mast and Gibco; Oxoid Blood Agar Base No. 2.

*** - Either greening or no change in blood about colony.

**** + Haemolysis about colony.

riable commercial catering brand. 2. The lactose and sucrose were of a cheap, impure quality and not Analytical Reagent grade; 3. The original formula had been simplified by rounding up the original water volume of 900 ml to 1000 ml but no corresponding adjustment had been made to the quantities of the other ingredients; and 4. The method of standardization of the sodium deoxycholate had undergone a slight change and this had resulted in a second but slight variation to the water volume.
During the preparation of several earlier batches of this medium three faults occurred which resulted in variations in the growth of *Salmonella* and *Yersinia* test strains: 1. A batch of medium received mild overheating and non-lactose fermenting organisms appeared as lactose fermenters – it was significant that this batch had been made up with the cheap impure sugars; 2. The quality of the growth of the test organisms varied with the thickness of the agar which was irregular due to poor service of the distributing machine; and 3. Ferric citrate was added instead of ferric ammonium citrate in solution A resulting in a low pH, opalescence which was probably due to precipitation of the deoxycholate, and a reduction in inhibiton.

The following steps were taken to eliminate or reduce the problem: 1. The variable yeastrel was replaced by Oxoid yeast extract powder; 2. Impure sugars were replaced by Analytical Reagent grade; 3. The water volumes were adjusted to those stated in the original formula; 4. Allowance was made for the variation in the volume of the sodium deoxycholate solution; 5. Checks and careful adjustment were carried out on the plate pouring machines to ensure only minimal variation in the thickness of agar; and 6. Checks and adjustment of pH were instituted not only after the autoclaving of the LS base but also after the addition of solutions A and B.

Despite the alterations and procedures implemented it has not been possible to revert to all the original characteristics of this agar.

Case B (SAUNDERS & BALL 1980) Blood agar bases.

This history does not illustrate a QC problem so much as a need for users to be more explicit in publications and indicates a possible area for future investigations.

Frequently in recent months organisms have been received for serotyping at Colindale as "Group A haemolytic streptococci, penicillin-resistant". These strains had all been isolated on Columbia blood agar and were serologically tested using a co-agglutination test kit which showed them to be Group A. The Reference Laboratory found them to be non-haemolytic on COLINDALE HARTLEY digest blood agar, non groupable by extraction methods and were identified as penicillin-resistant *Streptococcus salivarius.*

The results obtained by Media Department Quality Controller are shown in Table 2. Blood agar plates prepared from Columbia agar produced by MAST, Oxoid, Difco and Gibco as well as with Oxoid blood agar base No. 2 grew strains of *Strep. salivarius* with marked haemolysis. SAUNDERS and BALL have shown that the age and type of blood used can affect the haemolysis. The intensity of haemolytic reaction increased the older the horse blood used and the longer the plates were stored. When grown on Oxoid Columbia base and Oxoid blood agar base No. 2 supplemented with defribrinated sheep or human blood all strains gave reactions typical for *Strep. salivarius* and were non-haemolytic.

This case emphasizes the need to be specific in stipulating the type and brand of base agar and blood being used. A question which might be asked is are any other organisms affected e. g. clostridia?

In conclusion we can use all the sophisticated tests in the world but if staff are untrained, poorly supervised, and there is a lack of communication at all levels from supplier to user our media production will cause problems. As users we can be more specific and detailed in stating our requirements in publications.

Acknowledgement

The author wishes to thank Dr D. ROBERTS and Mrs. K. SAUNDERS for their advice in the preparation of this paper.

References

HYNES, M. 1942 The isolation of intestinal pathogens by selective media. *Journal of Pathology and Bacteriology* 54, 193–207.

JUHLIN, I. & ERICSON, C. 1961 A new medium for the bacteriologic examination of stools (LSU-agar). *Acta Pathologica et Microbiologica Scandinavica* Supplement 206.

SAUNDERS, K. & BALL, L. C. 1980 The influence of the composition of blood agar on beta haemolysis by *Streptococcus salivarius. Journal of Medical Laboratory Sciences 37,* 341–345.

STOKES, E. J. 1975 *Clinicical Bacteriology* 4th edn, pp. 343–383, London: Edward Arnold.

STOKES, E. J. 1979 Quality control in microbiology. In *Recent Advances in Infection* vol. 1 eds Reeves, D. & Geddes, A., pp. 193–204, London: Livingstone.

KENDALL, M. 1982 Bismuth Sulphite agar for the isolation of salmonellae. In: Quality Assurance and Quality Control of Microbiological Culture Media. Proceedings of the Symposium held on 6th and 7th September 1979, Calas de Mallorca, ed. Corry, J.E.L. pp133-139. G.I.T. Verlag : Ernest Giebeler.

Bismuth Sulphite Agar for the Isolation of Salmonellae

MARGARET KENDALL

Food Hygiene Laboratory, Central Public Health Laboratory, Colindale Avenue, London, NW9 5HT, England

Summary

A study was carried out to compare the growth of salmonellae, including *Salmonella typhi,* on varying depths of bismuth sulphite agar in relation to (a) pre-use storage time (b) artificial drying time and (c) brand and batch variation.

Results of tests with Oxoid bismuth sulphite agar and salmonellae other than *Salm. typhi* showed that the length of time that the medium could be stored at 4°C before use increased with increased depth of agar. However, for the isolation of *Salm. typhi,* even the thickest plates (9 mm deep) needed to be used within 4 days of preparation. Typical colonies of the other salmonella serotypes tested could be recognized after up to 19 days pre-use storage. Variation in the artificial drying times produced no significant effects on the growth characteristics of any of the salmonella strains tested.

Variations between different batches of a particular brand of the medium and between different brands were apparent.

Introduction

WILSON (1923) showed that many organisms of the *Salmonella* group are able to reduce sulphites to sulphides in the presence of a fermentable sugar. Together with BLAIR he developed a glucose bismuth sulphite iron medium for the isolation of *Salmonella typhi* (WILSON & BLAIR 1926, 1927). Several modifications of this WILSON & BLAIR medium (WB) have been produced (TABET 1938; WILSON 1938; DE LOUREIRO 1942). That of WILSON (1938) has been found suitable for the isolation of most types of salmonellas (McCOY 1962).

Irregularities in appearance and growth of *Salm. typhi* on various modifications of the medium have been recorded and some of these were found to be due to variations in the peptone (WILSON 1938), impurity of the ferric salt or inaccurate adjustment of pH (POT 1943). COOK (1952) showed that it was necessary to store WB plates for 4 days at 4°C in order to obtain typical results with *Salm. typhimurium.*

In the years preceding the work reported here there were many discussions with colleagues as to whether WB should or should not be stored at 4°C before use. Also, many requests were received from the Media Department asking for specifications on the quantity of agar required in each plate so that mechanical distribution methods could be used. There was also an increasing awareness that the varying depths of the agar, which were the normal result of manual distribution of the medium, were producing irregular results both in quality of growth and colonial appearance.

Dehydrated WB was in regular routine use in the Food Hygiene Laboratory and a study of the literature produced by some of the commercial firms showed that there was a considerable variety of opinion on (a) the quantity of agar required in each Petri dish and (b) the length of pre-use storage time, if any, of the poured plates. None of the manufacturers mentioned the size of the Petri dish

Table 1 *Summary of instructions by commercial media production companies for the preparation of bismuth sulphite agar*

Brand	Volume of agar in each Petri dish	Petri dish size	Pre-use storage instructions for poured plates
Oxoid	25 ml	Not specified	3 days at 4°C (for all salmonellae except *Salm. typhi.*)
Difco	-	Not specified	Cold moist atmosphere if necessary
BBL	20 ml approx.	Not specified	Use same day

which should be used (Table 1). In more recent literature OXOID Ltd recommended dispensing 20 ml into a 10 cm diameter Petri dish (OXOID 1976) and later 25 ml per plate (OXOID 1979).

Standard 90 mm disposable plastic Petri dishes are more commonly used at the present time than they were when this work was carried out, 10–12 years ago. It was then not uncommon, for the sake of economy, to divide the all glass dishes to give two bases, one of which was 89 mm diameter and the other 98 mm diameter. Metal lids were fitted to complete the two "new" dishes. The significance of the variation in size can be appreciated when a volume of medium is stated but the plate size is unspecified.

The following is a report of a study carried out to compare the growth of salmonellae, including *Salm. typhi,* on varying depths of medium inrelation to 1) the pre-use storage time, 2) artificial drying times, 3) batch variation and 4) brand variation.

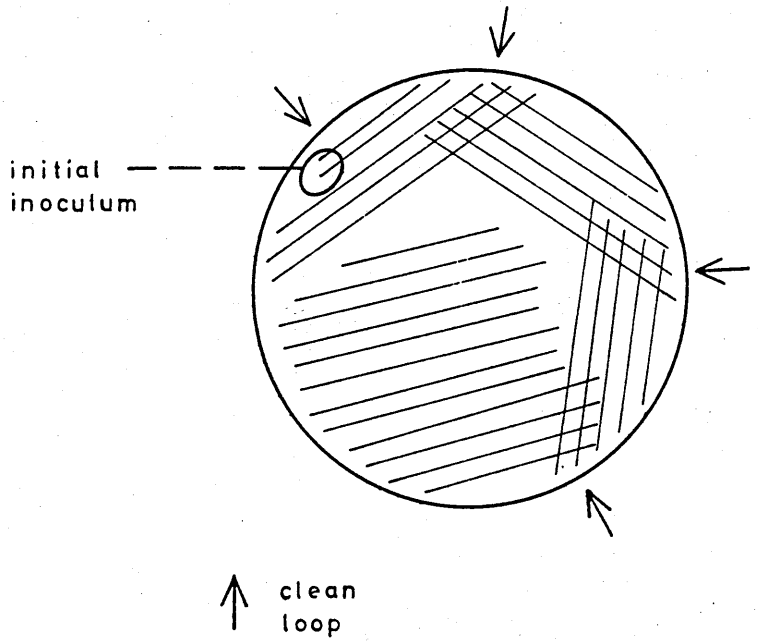


Figure 1. Method used for the inoculation of bismuth sulphite agar plates.

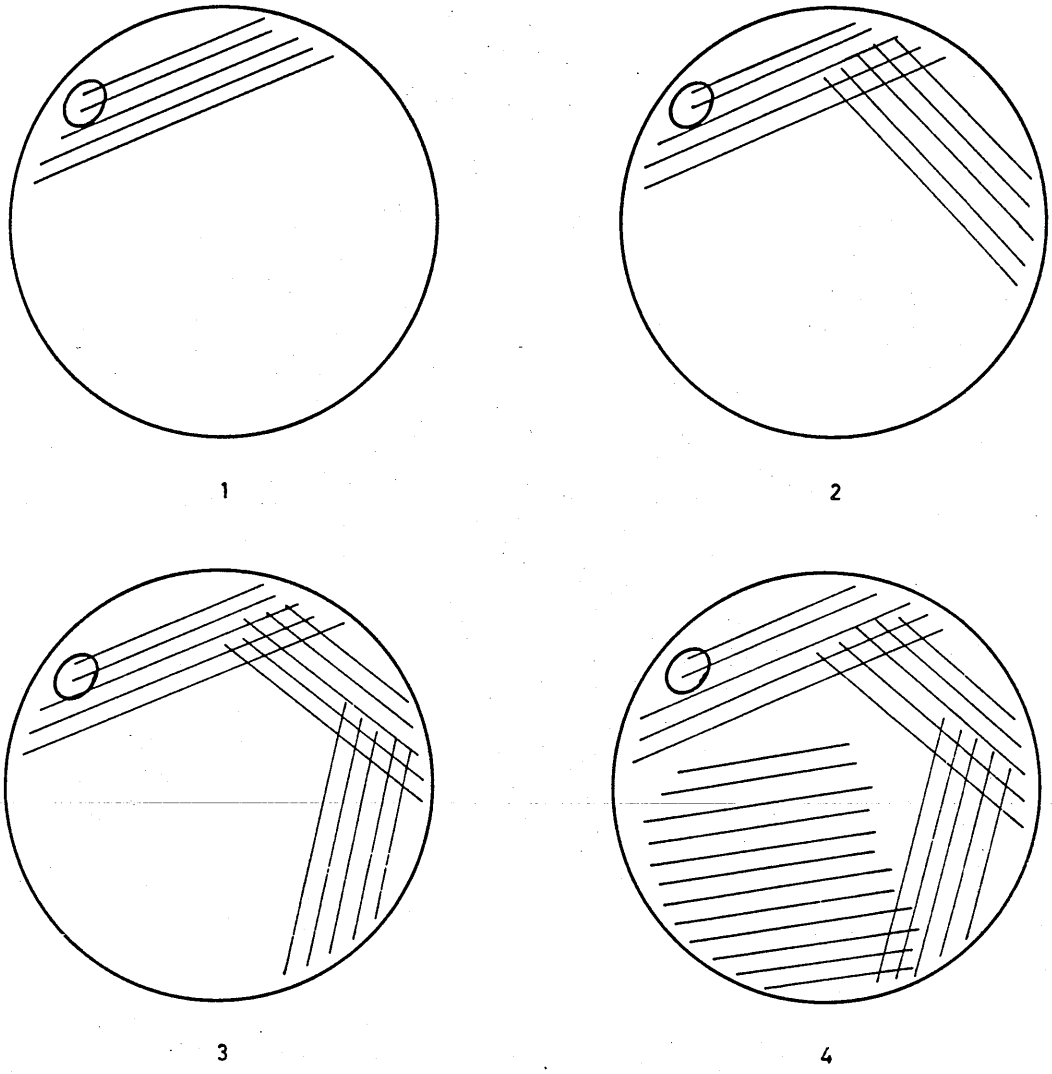


Figure 2. Grading of growth on bismuth sulphite agar plates.

Methods

Oxoid and Difco bismuth sulphite agars (WB) were prepared according to the manufacturers, instructions and dispensed in 15 ml, 20 ml, 25 ml, 30 ml, and 40 ml volumes into 90 mm plastic Petri dishes using sterile bulb pipettes, to give approximately 4, 5, 6, 7 and 9 mm depth of agar respectively. The agar was allowed to set and, except where the effects of different drying times were being investigated, dried with the dishes open at 37°C for 2 hours and then either used immediately or stored at 4°C for up to 19 days.

The organisms used were *Salm. typhi* NCTC 8394; *Salm. paratyphi A* NCTC 8002; *Salm. paratyphi B* F7064/67; *Salm. cholerae-suis* NCTC 5735; *Salm. typhimurium* F4212/67; *Salm. typhimurium* F3684/60; *Salm. worthington* F3717/67; *Salm. dublin* F6951/68; *Salm. anatum* F2210/67; *Salm. pullorum* NCTC 5776; *Proteus vulgaris* NCTC 4175; *Providence* sp. NCTC 6345 and *Escherichia coli* NCTC 9001.

A standard method of inoculation was maintained by streaking one loopful of an overnight nutrient broth culture onto the surface of each plate using the method shown in Fig. 1. All plates were incubated at 37°C for 48 hours and the quality of growth recorded as illustrated in Fig. 2. Colony types were recorded as 1) typical; sharp black centre, clear periphery, blackening of agar and sheen around colony, 2) atypical; pale green colony, or 3) mixed; mixture of both typical and atypical colony types.

Results & Discussion

The effect of age and thickness of WB agar on the growth of salmonellae was studied and the results obtained with *Salm. typhi* and *Salm. typhimurium* are shown in Table 2. *Salm. typhi* was more easily recognizable as a typical salmonella on plates stored at 4°C for 4 days or less. The thicker the agar the longer the plate could be stored before inoculation and still show characteristic colonies and therefore remain potentially usable. *Salm. typhimurium* could be easily recognized on plates stored for more than 2 days; the 15, 20, 25, 30 and 40 ml plates showed typical colonies after 2, 6, 10, 16 and 17 days storage respectively. A similar pattern was seen in the results of *Salm. paratyphi A, Salm. worthington, Salm. dublin,* a second strain of *Salm. typhimurium, Salm. anatum,* and *Salm. paratyphi B.* The number of days which the plates could be stored and still produce recognizable salmonella colonies varied between those shown for *Salm. typhi* and *Salm. typhimurium* (Table 2). *Salm. cholerae-suis* and *Salm. pullorum* typically did not produce black colonies with a metallic sheen and the results were graded only according to the quality of growth. Increased thickness of agar and length of storage time resulted in heavier growth on the plates. The batch of agar used for this trial was also used on a further three occasions over a period of 2½ years and each time exhibited similar characteristics.

Table 2 *Growth of salmonellae on bismuth sulphite agar (Oxoid batch A) of varying thickness in relation to the pre-use storage time*

Organism	Volume of agar ml per plate	Number of days pre-use storage at 4°C																			
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Salm. typhi NCTC 834	15	1A	1A	1A	1A	1A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	20	4T	2A	1M	1A	1A	1A	1A	0	0	0	0	0	0	0	0	0	0	0	0	0
	25	4T	3M	3T	2M	1A	1A	1A	0	0	0	0	0	0	0	0	0	0	0	0	0
	30	4T	3T	3T	3T	2A	1A	1A	1A	1A	0	0	0	0	0	0	0	0	0	0	0
	40	4T	4T	4T	4T	4T	2A	2M	2A	2A	1A	1A	1A	1A	0	0	0	0	0	0	0
Salm. typhimurium F4212/67	15	4T	2A	3T	3A	3M	2M	3M	2M	2M	2M	2M	3M	2M	2M	2M	2M	2M	–	2M	2M
	20	4T	4T	3T	3T	3T	2M	3T	3M	3T	2M	2M	2M	3M	2M	2M	2M	3M	2M	2M	2M
	25	4T	4T	4T	4T	3T	3T	3T	3T	3T	3T	3T	3M	3M	3M	3M	3M	3M	3M	3M	3M
	30	4M	4T	4M	4T	4M	4T	3T	4T	4T	4T	3T	3M	3M	3T	3T	3T	3T	3M	2M	3M
	40	4A	4M	4T	4T	4M	4T	4T	4T	4T	4T	4T	4T	4T	4T	3T	4T	4T	4T	3M	3M

A = atypical colonies
M = mixed atypical and typical colonies
T = typical colonies
0–4 = amount of growth expressed as values between 0 and 4 (see Fig. 2)

Table 3 *Growth of salmonellae on bismuth sulphite agar (Oxoid batch B) of varying thicknesses in relation to pre-use storage times and artifical drying times*

Organism	Quantity of agar ml per plate	Number of days pre-use storage at 4°C														
		0					4					9				
		Artificial drying time in hours														
		0	½	1	1½	2	0	½	1	1½	2	0	½	1	1½	2
Salm. typhi NCTC 8394	15	3M	2A	2M	2M	3A	1A	1A	0	1A	0	0	0	0	0	0
	20	4T	3M	3M	3M	3M	2A	2A	2A	1A	0	0	0	0	0	0
	25	4M	4T	4M	4T	4T	3A	2A	2M	2M	3M	0	0	0	0	0
	30	4M	4T	4M	4T	4M	3M	4M	3M	2A	3M	0	1A	1A	0	1A
	40	4T	4T	4M	4T	4T	4M	4T	4T	4T	4T	2A	4M	4M	1A	2A
Salm. typhimurium F3684/60	15	3M	3M	3M	3M	3M	3A	3A	3M	3M	3M	4A	4A	4A	3M	3A
	20	4M	3M	3M	3T	3T	3M	3A	3T	3M	3T	4A	4A	4A	3M	3A
	25	3M	3T	3T	3T	3T	3M	3M	3M	3T	3M	4A	4A	4A	3M	3A
	30	3T	4T	3T	3T	3T	3M	3T	4M	3T	4M	4M	4M	4A	4M	4M
	40	4T	4T	4T	3T	3T	4T	4M	4M	3T	4M	4M	4T	4M	4M	4T

A = atypical colonies
M = mixed atypical and typical colonies
T = typical colonies
0–4 = amount of growth expressed as values between 0 and 4 (see Fig. 2)

The time allowed for pre-use drying of agar plates varies from laboratory to laboratory. Therefore, a study was made of the growth of four strains of *Salmonella* on WB in relation to thickness of agar, pre-use storage time at 4°C and drying time; a summary of the results obtained with *Salm. typhi* and *Salm. typhimurium* are shown (Table 3). Although a different batch of OXOID WB was used to that in the previous trial, the general characteristic was similar, the thicker the agar the longer the plate could be stored at 4°C prior to use. The variation in drying times had little effect on the

Table 4 *Growth of salmonellae,* **Escherichia coli** *and* **Proteus vulgaris** *on different batches of bismuth sulphite agar**

Organism	Oxoid batch	Number of days pre-use storage at 4°C						Organism	Oxoid batch	Number of days pre-use storage at 4°C					
		1	2	3	4	5	6			1	2	3	4	5	6
Salm. typhi NCTC 8394	C	1A	1A	0	0	0	0	*Pr. vulgaris* NCTC 4175	C	3	3	1	0	0	0
	D	3M	3A	1A	0	0	0		D	4	3	2	4	1	1
	E	4T	3M	4M	3M	4M	3M		E	4	4	4	4	4	4
Salm. typhimurium F4212/67	C	4T	4T	3T	4T	3T	3M	*Esch. coli* NCTC 9001	C	0	0	0	0	0	0
	D	4T	4T	3T	4T	4T	4T		D	0	0	0	0	0	0
	E	4A	4M	4A	4M	4M	4M		E	1	1	1	2	2	2

A = atypical colonies
M = mixed atypical and typical colonies
T = typical colonies
0–4 = amount of growth expressed as values between 0 and 4 (see Fig. 2)

*all the plates contained 30 ml of medium

Table 5 *Growth of salmonellae,* **Escherichia coli** *and* **Proteus vulgaris** *on two different brands of bismuth sulphite agar*

Organism	Brand batch	Volume of agar (ml per plate)	Number of days pre-use storage at 4°C				
			0	1	2	3	4
Salm. typhi NCTC 8394	Oxoid A	20	4M	1A	1A	2A	1A
		40	4M	4T	4T	4T	4M
	Difco A	20	3A	3A	3A	3A	3A
		40	3A	3A	3A	3A	3A
Salm. typhimurium F4212/67	Oxoid A	20	4M	4T	4T	4T	4T
		40	2M	4M	4M	4T	4T
	Difco A	20	1M	2M	4M	4M	4A
		40	1M	2M	2M	2T	2T
Pr. vulgaris NCTC 4175	Oxoid A	20	3	2	1	1	1
		40	1	2	2	3	3
	Difco A	20	3	3	2	2	1
		40	3	3	3	4	4
Esch. coli NCTC 9007	Oxoid A	20	1	1	1	1	0
		40	1	2	2	2	2
	Difco A	20	1	1	1	1	1
		40	0	0	1	1	1

A = atypical colonies
M = mixed atypical and typical colonies
T = typical colonies
0–4 = amount of growth expressed as values between 0 and 4 (see Fig. 2)

appearance or growth of the colonies of the test strains. Of the other two strains tested, *Salm. dublin* showed similar growth characteristics to *Salm. typhi* and *Salm. typhimurium* but on this particular batch of medium, *Salm. paratyphi A* failed to produce any typical colonies.

Three different batches of OXOID WB were compared directly using a standard quantity of agar (30 ml) in each plate with pre-use storage times ranging from 1–6 days. Five strains of salmonella and three strains of common Enterobacteriacae were used. The results obtained with *Salm. typhi, Salm. typhimurium, Proteus vulgaris* and *Escherichia coli* are shown in Table 4. Considerable variation was observed between the different batches of WB. On batches C and D *Salm. typhi* produced few typical colonies after 1 day storage at 4°C only whereas on batch E a mixture of typical and atypical colonies were observed after up to 6 days storage. *Salm. typhimurium* grew well and produced typical or mixed colonies on all three batches. The other serotypes tested, *Salm. anatum, Salm. paratyphi* and a second strain of *Salm. typhimurium,* all produced typical or mixed colonies on batches C and D and atypical colonies on batch E. *Providence* sp. showed similar characteristics to *Pr. vulgaris* on all three batches of WB. In general, batch E was less inhibitory than batches C and D.

A comparison between OXOID batch A and DIFCO batch A was carried out using the same strains as the previous experiment. The results obtained for *Salm. typhi, Salm. typhimurium, Pr. vulgaris* and *Esch. coli* are shown in Table 5. *Salm. typhi* did not produce any typical colonies on DIFCO batch A and growth was less heavy than that on OXOID batch A. *Salm. typhimurium* also grew less

well on the DIFCO than on the OXOID agar, but a few typical colonies were observed on the 40 ml plates which had been stored at 4°C for 3 days or more before use. Similar results were observed with *Salm. anatum, Salm. paratyphi B* and a second strain of *Salm. typhimurium.* The DIFCO medium was less inhibitory to *Proteus* than the OXOID medium but the converse was observed with *Esch. coli* and *Providence* sp.

The work reported here was carried out over a period of 2½ years and because of the relatively small quantities of dehydrated medium available from most of the batches it was not possible to carry out more comprehensive trials. These results show that for OXOID WB agar the pre-use storage time at 4°C can be extended when the thickness of the agar is increased. When required for the isolation of *Salm. typhi,* plates should be used within 4 days. This confirms the instructions issued by OXOID (Table 1).

However, there is a need to stipulate thickness of agar required per plate rather than the plate size. The exact quantity of agar could then be ascertained for any size of Petri dish. The results obtained with OXOID batch A agar indicate that the dehydrated medium may be stable for 2–3 years, but as a general rule control strains should be used for media control testing because considerable variations have been demonstrated between batches and between brands.

The variations obtained with WB agar are not unique. Other authors have reported similar findings with brilliant green agar (READ & REYES 1968).

The author wishes to thank Dr. D. ROBERTS for her advice in the preparation of this paper.

References

COOK, G. T. 1952 Comparison of two modifications of bismuth sulphite agar for the isolation and growth of *Salmonella typhi* and *Salmonella typhimurium. Journal of Pathology and Bacteriology 64,* 559–566.

LOUREIRO, J. A. de 1942 A modification of WILSON and BLAIR'S bismuth sulphite agar (stabilized stock solutions). *Journal of Hygiene, Cambridge 42,* 224–226.

McCOY, J. H. 1962 The isolation of salmonellae. *Journal of Applied Bacteriology 25,* 213–224.

POT, A. W. 1943 The isolation of *Bacterium typhosum. Journal of Pathology and Bacteriology 55,* 100–103.

READ, R. B. & REYES, A. L. 1968 Variation in plating efficiency of salmonellae on eight lots of brilliant green agar. *Applied Microbiology 16,* 746–748.

TABET, F. 1938 A modification of WILSON and BLAIR'S bismuth medium suitable for both typhoid and paratyphoid bacilli. *Journal of Pathology and Bacteriology 46,* 181–193.

WILSON, W. J. 1923 Reduction of sulphites by certain bacteria in media containing a fermentable carbohydrate and metallic salts. *Journal of Hygiene, Cambridge 21,* 392–398.

WILSON, W. J. 1938 Isolation of *Bacterium typhosum* by means of bismuth sulphite medium in water and milk-borne epidemics. *Journal of Hygiene, Cambridge 38,* 507–519.

WILSON, W. J. & BLAIR, E. M. McV. 1926 A combination of bismuth and sodium sulphite affording an enrichment and selective medium for the typhoid-paratyphoid groups of bacteria. *Journal of Pathology and Bacteriology 29,* 310–311.

WILSON, W. J. & BLAIR, E. M. McV. 1927 Use of a glucose bismuth sulphite iron medium for the isolation of *B. typhosus* and *B. proteus. Journal of Hygiene, Cambridge 26,* 374–391.