A STUDY OF SHIGELLOSIS IN TASMANIA

by

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Chapter 1

INTRODUCTION

1. INTRODUCTION

In a recent publication, Tasmania was designated as an area of moderate or unknown status in regard to the overall problem of diarrhoea (DuPont and Pickering, 1980). This impression seems to be at least partly the fault of Tasmania itself. For example, between the years 1976 to 1983, only eleven cases of *Shigella* infection were reported, an average of 1.6 cases per year. This not only made it appear that Tasmania had a very low incidence of this infection in comparison with the rest of Australia, but also in fact, with most of the rest of the world.

Considering that Tasmania is no longer isolated from the mainland or the rest of the world, and also the fact that there is a large movement of people in both directions, including many tourists from countries with high incidence of diarrhoeal infections, it would seem very unusual to have so few cases of *Shigella* infections on record.

There could be a number of reasons for this situation, the main ones being:

(a) that dysentery (including *Shigella* infections) are not all being investigated and notified;

(b) that it is true there is a very low incidence which in itself is interesting, and should be investigated further in order to establish, if possible, the reasons for such a fortunate situation;

(c) infections do occur but for reasons unknown, are not detected, e.g. specimens are not being sent to the laboratories for investigation, or the organisms are not being isolated by the laboratories. This may involve the culture media presently available or those being used by diagnostic laboratories not

always recovering or isolating the organisms.

(d) The distance between the areas of collecting and the diagnostic laboratory may cause delay in the investigation. This could be significant as it is a well known fact that the shigellae need to be plated onto culture media almost immediately; even a short delay can reduce considerably the chance of isolating the organism.

Finally, there is a need to examine the possible introduction of a more rapid method to overcome the problem of delay in isolating *Shigella* infections combined with a more reliable method of detecting positive cases than the recovery of the organisms on culture media.

It was decided to combine the investigation of the alleged lack of *Shigella* infections in Tasmania with an overall research project as follows:

1. Research Protocol

To investigate the accuracy of presently available culture media to detect *Shigella* organisms using known strains and stock cultures of local strains to determine to what extent they can be recovered on available culture media, both directly and in dilution.

2. Due to the close relationship between *Escherichia coli* (A - D) (*Alkalescens dispar*) and *Shigella*, this former group of organisms will also be tested against the media as for *Shigella*.

3. To establish if *Shigella* infections are being missed, due to the fact that patients with diarrhoea are not being investigated by the laboratories, the cooperation of local doctors was obtained to submit specimens from any diarrhoeal patient, even if only having mild symptoms, and provided it was suspected the diarrhoea was due to infection.

Further, the co-operation of laboratories was obtained to submit diarrhoeal specimens they had found to be either positive or negative for microorganisms known to cause diarrhoea.

4. Having isolated suspected *Shigella* colonies, to establish which biochemical methods are most satisfactory for their rapid confirmation.

5. Due to the close relationship between *Escherichia coli* and *Shigella,* to investigate the frequency of toxigenic *E. coli* and invasive *E. coli* by gene probe analysis in those specimens from patients with diarrhoea in which no other possible pathogens had been isolated. This has never been investigated in Tasmania.

6. To investigate the possibility of developing a more rapid and accurate technique for the identification of *Shigella* infections in stool specimens, e.g. E.L.I.S.A. technique.

Chapter 2

LITERATURE REVIEW

2.1 HISTORICAL BACKGROUND

2.2 SOURCE AND SPREAD OF *Shigella*

2.3 RECENT ADVANCES IN THE UNDERSTANDING OF DIARRHOEAL DISEASES

2.4 PATHOGENESIS OF SHIGELLOSIS

2.5 CYCLIC PATTERN

2.6 DIAGNOSTIC PROBLEMS

2.7 LACK OF INTEREST

2.8 RESEARCH PROSPECTS AND NEEDS

2.9 COMPARATIVE STATISTICS

LITERATURE REVIEW

2.1 HISTORICAL BACKGROUND

The term 'dysentery' was used by Hippocrates to indicate a condition involving a list of symptoms including the frequent passage of stools containing blood and mucus, accompanied by straining and painful defecation. It was not until the end of the last century when the causes of amoebiasis and bacillary dysentery were determined, that the two major forms of dysentery could be accurately separated.

The different epidemiologic settings of the diseases were described by Osler (1890), followed by Councilman and Lafleur (1891). Shiga (1906) conclusively demonstrated that specific bacteria were present in the stools of many patients with dysentery, and agglutinins could be demonstrated in the serum of infected patients. Two years later, Flexner (1908) found a similar but serologically different organism in the stools of patients who acquired dysentery in the Philippines.

Shigellae have been a perennial problem. This has been especially so under conditions of overcrowding, for example wars and other conditions favouring the spread of the organisms from the human reservoir. More men died of diarrhoeal disease during the American Civil War than were killed in battle, and shigellosis continued to be a serious epidemic disease in the two World Wars (Freeman, 1985).

2.2 SOURCE AND SPREAD OF SHIGELLA

Bacillary dysentery occurs predominantly in areas of low sanitation and poor nutrition such as exists in developing

countries. However, shigellosis continues to be a localised problem also in developed countries in closely-housed populations, e.g. prisons, nursery schools, mental institutions or 'native' reservations (Kopecko et al., 1985).

It is interesting to note the comments of Cruickshank, et al. (1965), that "the lack of modern sanitation has been invoked to explain the frequency of *Shigella* epidemics in certain countries of the world. By contrast, in Britain we have seen the almost unrelenting increase in bacillary dysentery in the last thirty years, during which time environmental hygiene has been well above the standard obtained in materially less favoured communities".

At the time of Cruickshank's comments in 1965, there had been an increase of bacillary dysentery in Britain from just over 700 cases in 1933 to 32 000 in 1963 (Public Health Laboratory Service Statistics).

Since that time there has been a steady and remarkable change in the relative frequency of different *Shigella* species. In Britain and other European countries, not only has there been a fall in the number of cases, but today in Britain, most cases of shigellosis are caused by *S. sonnei* and are relatively mild. Only in the very young and old or debilitated are typical dysenteries sometimes seen (DuPont, 1980).

There are still many unexplained areas. If, as it has been said, the prevalence of dysentery reflects the hygienic standard of a country of community, this does not explain the increases reported in Britain during the period 1933 to 1963, with a peak of 44 000 in 1956. Everything from toilet seats to flies has been blamed for the spread of *Shigella* infections

(Cruickshank et al., 1965; Mackie and McCartney, 1978). The spread of organisms by flies from faeces to food, although a potential danger, seems of little importance in Britain where even during peak years, the highest incidence of infection took place during months of the year when house flies are uncommon (Cruickshank et al., 1965).

Australia, in certain parts of which the number of flies is very high and a constant irritant, does not seem to have a higher incidence of *Shigella* infections than Britain except in one area - the Northern Territory (Commonwealth Department of Health).

A comparative study by Hutchinson (1956) revealed that contamination was heaviest in the immediate vicinity of the toilet pedestal, that organisms passed through toilet paper onto fingers and that they could be recovered from fingers at least three hours after contamination, suggesting that this mode of contamination is of major significance in transmission. A natural ecological niche for the causative bacteria is unknown. *Shigella* strains have been found to remain viable in water for periods of up to six months and in rare cases, previously diseased individuals have been observed to excrete *Shigella* for periods of greater than one year (Levine et al., 1973 b , DuPont and Pickering, 1980).

The shigellae are unique among enteric pathogens in that as few as ten to one hundred viable organisms have been shown to cause dysentery in adults (Hornick, 1978). It would seem that this low number of bacteria required to initiate infection, combined with direct contact and virulence are the main

contributors to serious outbreaks (Kopecko et al., 1985).

2.3 RECENT ADVANCES IN THE UNDERSTANDING OF THE MECHANISMS OF DIARRHOEAL DISEASES

To date there are four distinct mechanisms recognised by which bacteria cause acute gastrointestinal (G.I.) illness.

2.3.1 Intoxication. This occurs by bacterial secretion of an exotoxin, usually in food before digestion, e.g. staphylococcal food poisoning. These enterotoxins generally cause excessive fluid and electrolyte secretion from the bowel. They do not cause histopathological changes in the intestine. In contrast the remaining three mechanisms require living and multiplying disease agents in the intestine.

2.3.2 Enterotoxigenic. Ingested bacteria adhere to and multiply on the surface of the small intestine, usually in the jejunum and duodenum but they cause no apparent mucosal damage. These organisms produce enterotoxins that stimulate the excessive fluid/electrolyte efflux resulting in profuse diarrhoea. *Vibrio cholera* and enterotoxigenic strains of *Escherichia coli* (E.T.E.C.) serve as typical examples. E.T.E.C. was first reported by DuPont et al (1971). Later it was shown two enterotoxins, one heat labile (LT) and the other heat stable (ST) were responsible for the disease.

2.3.3 Enteropathogenic. Characterised by strains of Enteropathogenic *E. coli* (E.P.E.C.) These agents do not make detectable levels of heat - stable or heat labile enterotoxins like the E.T.E.C., but do cause diarrhoea in the newborn and young children. Once ingested, these organisms cause

local disruption of the intestinal microvilli, they do not penetrate but attach to and colonise the underlying epithelial cell membrane throughout the intestine, and appear to produce a mucosal inflammation (Reviewed by Formal et al, 1983).

The precise cause of intestinal fluid loss is not known, but could involve the *Shigella* -like enterotoxins recently found to be produced by these strains (Wade et al., 1979; O'Brien et al., 1982).

2.3.4 Invasive. These strains penetrate the epithelial mucosa of the distal small intestine (i.e. ileum) or large intestine. These bacteria locally disrupt the microvilli, like the E.P.E.C. strains, and then proceed to invade the intestinal epithelial cells with ensuing intracellular multiplication. In some cases like *Salmonella*, the organisms disseminate throughout the host by septicaemia. This type of invasive mechanism, classically typified by *Shigella* and *Salmonella* is now thought to be used by invasive strains of *E. coli, Yersina, Vibrio parahaemolyticus, Campylobacter* as well as *Aeromonas hydrophila* and *A. (Plesiomonas) shigelloides*. Beyond invasive ability these bacteria may encode for cholera - like enterotoxins, shigella-like enterotoxins, haemolysins or other potential virulence factors.

Unlike other invasive bacterial disease, e.g. typhoid fever, *Shigella* infections are normally confined to the intestinal mucosa, and do not disseminate to any great extent.

2.4 PATHOGENESIS OF SHIGELLOSIS

Shigella must first invade epithelial cells of the colonic mucosa and must be able to multiply intracellularly (La Brec et al, 1964 a: Ogawa et al, 1967; Chiodini, 1988).

Ultrastructural studies of infected intestine have revealed that the first visible alteration in host colonic epithelium is: (a) localised disruption of the microvilli.

(b) The invading bacteria are then engulfed by an endocytic process (Formal et al., 1976, 1983).

(c) The intracellular bacteria are at first contained within the endocytic vacuole and later are found free in the cytoplasm.

(d) The microvilli are re-established and intracellular bacterial multiplication occurs. The intracellular bacteria disseminate laterally to adjacent epithelial cells and then to the lamina propria. *Shigella* rarely invade beyond the mucosa or enter the circulatory system.

(e) Intracellular multiplication of shigellae leads to epithelial cell necrosis, possibly due to the *Shigella* cytotoxin/enterotoxin.

(f) This process results in an acute inflammatory response involving an outpouring of polymorphonuclear leucocytes from the lamina propria. The neutrophils appear to limit the infection to the superficial layers of the colon, but dissemination of the bacteria and cell death can lead to focal ulceration of the epithelium.

(g) The infection is usually self-limiting and lasts from one to two weeks with normal mucosal cell replacement and effective inflammatory and immune response terminating the infection.

In recent years, with the advent of more advanced methods of identification, the situation has been made more comp-

licated by the recognition that certain groups of *E. coli* can be responsible for similar outbreaks of dysentery-type infections, (Gorbach et al., 1975; Rosenberg et al., 1977; Sack et al., 1977; Doyle et al., 1983 and Rowe and Gross, 1983).

In the last few years it has also become apparent that *Shigella* and some strains of *E. coli* are very closely related even to the extent that in an evolutionary sense they could be considered to be the same species (Formal et al., 1971; Silva et al., 1980; Brenner, 1981; O'Brien et al., 1982; Formal et al., 1983; and Kopecko et al., 1985.) Evidence accumulated over the past ten years (Kopecko et al., 1985) indicates that some strains of *E. coli* exhibit many of the key diagnostic biochemical traits of the shigellae (Silva et al., 1980), the *Shigella*-like enterotoxin (O'Brien et al., 1982) as well as the O-antigen structure of some *Shigella*.

It would appear that those *E. coli* encode the same chromosomal and plasmid virulence traits as the shigellae with perhaps one exception. In contrast to *Shigella*, dysenteric *E. coli* strains may require a large infective dose (10^8 cell) to cause disease (Formal et al., 1971 a).

2.5 CYCLIC PATTERN

Shigellosis has long been known to cause sudden explosive outbreaks. Pandemics are still occurring all over the world. Thus in 1970 a pandemic in Central America killed 20 000 people (Smith, 1981).

Since the description of bacteriological isolation procedures, cyclic epidemics of bacillary dysentery have been described,

each lasting twenty to thirty years (Kostrzwski et al., 1968).

In Europe during the first twenty-five years of this century, dysentery was generally caused by *S. dysenteriae 1* and the mortality was higher than was seen subsequently when other serotypes became prevalent. The cyclic pattern of serotype-specific shigellosis suggests that it takes a certain number of years for herd immunity to reach a critical level in a population.

After this critical level has been reached, one species of *Shigella* will disappear, to be replaced by another species. It would seem the disease attacks each generation, rendering the survivors immune, and must then wait for the occurrence of a non-immune offspring (Davison, 1922).

2.6 DIAGNOSTIC PROBLEMS

On the diagnostic side there are very serious technical problems regarding the accurate isolation and identification of shigellae. It has been recognised for many years that the possibility of isolating *Shigella* is decreased with the delay between collecting the specimen for examination and its reaching the laboratory (Baily et al., 1953; Kassur et al., 1960).

The only reasonably sure way of recovering the organisms is direct plating at the bedside from a rectal swab, or the immediate plating of a fresh specimen. This not only involves immense problems in developing countries where the outbreak of *Shigella* may be in an area some great distance from a laboratory, but even in the more developed areas where

there can be considerable delay from the time the patient is examined by a medical practitioner, and a specimen reaching the laboratory. This technical difficulty is further aggravated by the fact that few, if any, culture media yet available can be said to be reliable in recovering all strains of *Shigella* (Morris et al., 1970; Bhat et al., 1975 and Goyal et al., 1981).

2.7 LACK OF INTEREST

The whole situation is further complicated by the fact that in the last few years there has been a declining interest in *Shigella* infections in the developed countries. This seems mainly due to lower incidence and virulence of the *Shigella* strains found in these countries. Yet in vast areas of the world, mainly underdeveloped or developing countries, *Shigella* infections remain a significant cause of morbidity and mortality, particularly in pre-school children. This was confirmed at an international meeting held in Bangladesh in 1981. Thus Smith (1981) states that "Infectious diarrhoea in infants represents an extremely serious cause of death for underdeveloped areas of the world. Some five to eight million infants succumb to it each year. Half the deaths are due to Shigella".

2.8 RESEARCH PROSPECTS AND NEEDS

There is no doubt that because of the suffering and mortality *Shigella* infections still cause, combined with recent neglect of the subject and despite a better understanding of the disease itself, further research and re-evaluation of the

subject is needed. This is especially required in the areas where there are still serious problems in the isolation and identification of the organisms. Further studies are also required in Tasmania where it is widely held by local medical and laboratory staff that this State has a consistently low occurrence of *Shigella* infections (0 to 4 cases per year from a total population of 440,000) which seems to be supported by the statistics set out below in Tables 1 and 2.

In view of the low number of cases reported and a recent publication (DuPont and Pickering, 1980) in which Tasmania was designated an area of moderate or unknown risk for developing infectious diarrhoea, it was decided Tasmania would be an excellent venue to carry out further research.

Table 1. Shows a ten-year report of *Shigella* cases in the States of Australia (Australian Department of Health)

Year	N.S.W.	Vic.	Qld.	S.A.	W.A.	Tas.	N.T.	A.C.T.	Total No.
1976	*	-	42	85	-	1	268	-	396
1977	*	-	98	76	-	-	181	7	362
1978	*	-	66	47	-	1	277	3	394
1979	*	10	54	101	163	-	326	4	648
1980	*	20	134	100	87	2	184	18	545
1981	*	44	94	38	74	4	162	8	424
1982	40	23	87	30	87	3	165	2	437
1983	65	-	74	72	89	8§	257	2	567
1984	115	20	64	38	55	2	125	1	420
1985	149	30	135	84	82	1	252	1	734

* Not notifiable

\- Nil reported

§ The eight reported cases in Tasmania during 1983 included four cases detected by the University Medical Microbiology Laboratory during the first stages of the present research, and these four cases of *Shigella* had not been isolated by the other laboratories investigating the specimens. From the above statistics we can calculate the number of cases per 10 000 of the population in each Australian State; see Table 2.

Table 2 CASES REPORTED PER 10 000 FOR THE YEARS 1981 and 1985

State	Total population (in round figures)	Cases per 10 000 1981	1985
N.S.W.	5 500 000	N.R.	0.27
Victoria	4 100 000	0.10	0.08
Queensland	2 560 000	0.37	0.52
S.A.	1 360 000	0.28	0.61
W.A.	1 400 000	0.50	0.59
Tasmania	440 000	0.08	0.02
N.T.	150 000	10.80	17.00
A.C.T.	258 000	0.31	0.04

(Statistics from Department of Health, Canberra)

2.9.1 Comparative Statistics

It will be noted that until 1985 (Tables 1 and 2), Tasmania had a consistently lower number of *Shigella* cases than other States in Australia, with the exception of Victoria in 1985 and the A.C.T. which has shown a constant fall in cases reported over the last five years. It will also be noted that despite the low incidence in these three States, there has in fact been an overall increase in the number of *Shigella* cases over the last ten years. This is true even if we take into consideration the recent addition of N.S.W. cases (not reported until 1982). If we take the overall number of cases reported in 1981 (424) and 1985 (734), it will be seen there has been an average increase from 0.28 per 10 000 in 1981 to 0.48 per 10,000 in 1985 for the whole of Australia.

Table 3 shows the number of cases reported in England and Wales during the same period (1976-1985).

Year	Notifications	Cases per 10 000
1976	6217	1.27
1977	6208	1.26
1978	4332	Not calculated
1979	2787	" "
1980	2709	0.55
1981	3401	Not calculated
1982	2850	" "
1983	5004	" "
1984	6844	" "
1985	6059	1.23

(Statistics from Public Health Laboratory Services, Collindale, U.K.)

It will be noted that, with the exception of the years 1979-1982, the number of cases per 10,000 notified in England and Wales was more than twice the average of Australia. Individual results per 10,000 were not calculated for each year. It can be seen from the above table this was not necessary.

Table 4 shows the very high number of cases reported prior to 1975 in England and Wales. There are no comparative statistics for Australia, but in 1969 only 181 cases were notified for the whole of Australia. The figures for England and Wales during that year were 22,117. (Australian Commonwealth Department of Health, and Public Health Laboratory Service, U.K. statistics.)

Table 4 Shigellas

Laboratory Reports to C.D.S.C. England and Wales 1946-1982

Year	Shigella sonnei		Shigella flexneri		Shigella dysenteriae		Shigella boydii	
	No.	% Total	No.	% Total	No.	% Total	No.	% Total
1946	2364	87.0	316	11.6				
1947	508	57.3	335	26.4				
1948	1590	73.4	432	20.0				
1949	2135	88.1	255	10.5				
1950	13497	96.5	493	3.5				
1951	20549	98.1	395	1.9				
1952	11141	95.3	553	4.7				
1953	16067	95.9	683	4.1	Figures not			
1954	28282	96.8	928	3.2	available			
1955	34063	98.2	633	1.8				
1956	43726	98.6	613	1.4				
1957	25618	96.9	823	3.1				
1958	34563	98.2	621	1.8				
1959	31899	98.8	385	1.2				
1960	38169	99.0	379	1.0				
1961	18411	97.9	386	2.1				
1962	31054	98.4	469	1.5				
1963	32024	98.1	586	1.8				
1964	20666	96.8	663	3.1				
1965	26790	97.6	641	2.3				
1966	24336	97.8	513	2.1				
1967	22299	97.5	543	2.4	20	0.1	10	0.1
1968	21715	97.8	464	2.1	9	0.1	12	0.1
1969	23091	97.5	554	2.3	14	0.1	17	0.1
1970	9331	95.6	394	4.0	18	0.2	15	0.2
1971	9315	94.7	468	4.8	18	0.2	32	0.3
1972	6400	90.5	594	8.4	43	0.6	36	0.5
1973	5923	91.7	464	9.2	32	0.5	43	0.7
1974	6261	92.2	451	6.6	26	0.4	53	0.8
1975	5872	90.2	538	8.3	35	0.5	63	1.0
1976	4526	88.5	512	10.0	29	0.6	45	0.9
1977	6302	90.0	606	8.7	37	0.5	54	0.8
1978	3751	83.5	666	14.8	29	0.6	46	1.0
1979	1858	70.4	707	26.8	28	1.1	47	1.8
1980	1911	68.7	773	27.8	43	1.5	56	2.0
1981	3260	79.4	743	18.1	42	1.0	60	1.5
1982	2010	73.3	633	23.1	45	1.6	54	2.0

Note: 254 "Other" Shigellas also reported 1946-1949 and included in total

Table 5 Shows the incidence of *Shigella* infections per 10 000 of the population in a number of other countries of the world. These were obtained from the World Health Statistics Report for 1977, 1978 and 1979, and from Australian and British statistics.

Country	1977	1978	1979
Australia	0.24	0.26	0.43
Bahamas	0.25	3.70	5.0
Bangladesh	74.00	23.60	46.00
Canada	0.60	0.46	0.52
England/Wales	1.26	1.0	0.53
Guatemala	2.76	5.4	6.20
Hong Kong	0.60	0.76	0.80
India	50.00	7.50	86.40
Malawi	0.90	0.73	22.00
Channel Is	1.18	0.20	*N.R.
Tasmania	Nil	0.02	Nil

* Not Recorded

The above statistics have been set out to produce a comparison between Tasmania, Australia as a whole and other world countries. While it will be noted that in 1979 England and Wales had lower than their average number of cases, Tasmania still stands out with its extremely low numbers of reported cases.

2.9.2 Discussion

It must be accepted that there will be periodic fluctuations in the number of any infectious disease. This is shown clearly in Table 5. Such fluctuations will depend on a number of factors, not least of which are the notification, recording and confirmation of the infections, some or all of which are often neglected. For example, a study carried out in Vaucluse, France (Foulon, 1980), and set out again

by Velimirovic (1984), lists eight common diseases that were investigated and found to be 10% to 100% higher in frequency than was notified. In the case of intestinal infections there were 300 to 1000 cases, yet only twelve had been notified.

Bryan (1980) estimated that for each case of *Salmonella* reported in the U.S.A., twenty-nine remain unidentified. In the Netherlands it was estimated that the cases reported are only 1% to 5% of the real incidence (Velimirovic, 1984).

Such reports leave much room for scepticism in regard to statistics on infections such as those showing that Tasmania has a reported incidence of only 0.02 cases of shigellosis per 10 000 of the population, or that in rural Egypt, *Shigella* species is present in only 2% of diarrhoeal cases (Cook, 1988).

Due to modern techniques in the treatment of diarrhoeal infections (including *Shigella*) which is mainly oral rehydration (ORT) (Stanton et al., 1987), it would seem many cases of diarrhoea are not investigated microbiologically, and thus in many cases, a false impression of the prevalence of any specific microorganism responsible for the infection is created. From a clinical point of view this may not seem very important, especially in the majority of cases in the more developed countries where the patients will recover. But from a public health point of view in obtaining a true picture of the frequency, the detection of new or virulent strains, and antibiotic resistance is made very difficult. Burdon (1988) reported that strains of *Shigella* from many parts of the world are

resistant to commonly used antibiotics. In India it was found that 81% of *Shigella* isolates showed increasing multiple resistance.

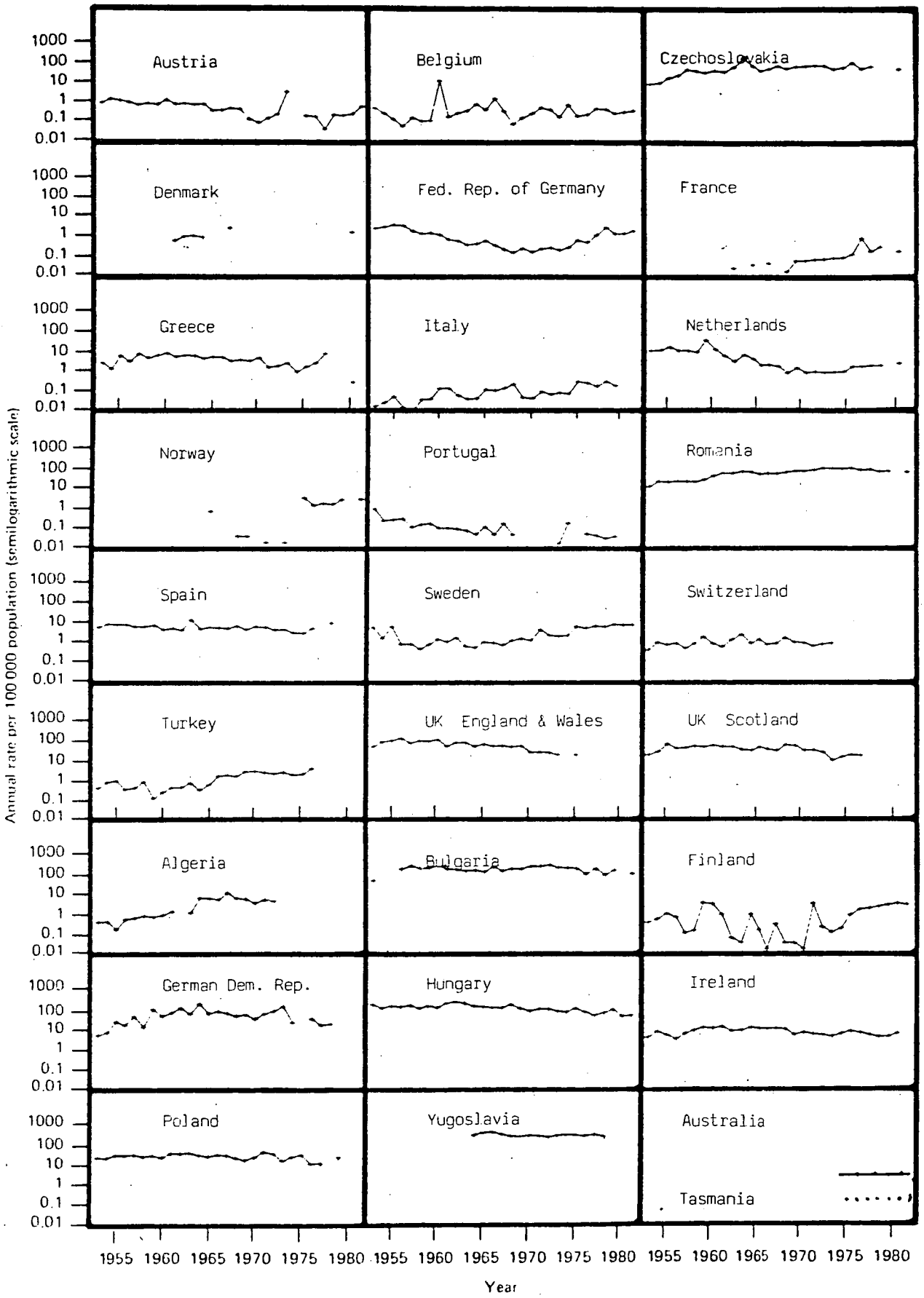
The need for travellers to be better informed about the dangers in high risk areas of contracting Travellers' Diarrhoea, including shigellosis, has been emphasised in a number of recent papers (Burdon, 1988; Chiodini, 1988). In these days of rapid air travel, there seems no reason why a traveller spending just a few hours in a high risk country could not be infected by a virulent or antibiotic-resistant strain, or both, of *Shigella*, and then only show symptoms of the infection on return to his home country, which may have a low endemic incidence of mild *Shigella*. If, to compound this, he worked in the food or catering industry, this could result in a serious epidemic with long term consequences. Transfer of *Shigella* virulence genes and antibiotic resistant plasmids (Kopecko et al., 1980, 1981, 1985; Silva, 1982; Sansonetti et al., 1982 b, 1983) to local strains would be possible. Therefore the detection, isolation, confirmation and accurate statistical records are of great importance.

It will be noted that 'statistically' Australia as a whole appears to have a lower incidence of *Shigella* infections than most countries, especially if one allows for the isolated high incidence in the Northern Territory (N.T.) with its low population density and high native Aboriginal component.

Table 6 shows the incidence of *Shigella* in European countries between 1952 and 1981 per 100,000 of population.

Table 6

Reported cases of bacillary dysentery[a] in some European countries, 1953-1981



[a] The numbers of cases reported in England & Wales and Scotland include amoebiasis as well as bacillary dysentery, while those reported in Algeria, Bulgaria, Finland, German Democratic Republic, Hungary, Ireland, Poland and Yugoslavia include all forms of

It will be noted that if one transfers Australian cases reported in Table 5 (average 0.3 per 10 000) this will be three cases per 100 000. This is much lower than most European countries with the exception of Finland, Norway and Italy which have recorded lower numbers. Tasmania, with an average number of cases of 0.2 per 100 000 since 1975 is very low and an exception to the majority of countries.

To summarise, it seems that studies on *Shigella* in Tasmania are warranted, bearing in mind published information available on shigellosis both on the mainland of Australia and overseas. There appeared to be an urgent need to establish to role of *Shigella* and certain related entero-bacteriaceae as causes of diarrhoea on the island. Also to establish whether or not the widely held belief in the low frequency of shigellosis in Tasmania was in fact true or not. Further, there seems a world wide need to develop a sensitive, specific and rapid test for identifying *Shigella* in stool specimens.

Chapter 3

CULTURE MEDIA

3.1 INTRODUCTION

3.2 PRELIMINARY STUDIES

3.3 MATERIALS AND METHODS

3.4 RESULTS

3.5 DISCUSSION

3.6 CONCLUSION

3. CULTURE MEDIA

3.1 INTRODUCTION

Over the years many media have been evaluated for their ability to isolate shigellae (Croft and Miller, 1956; Taylor, 1965; Taylor and Schelhart, 1968; Rollender, 1969; Dunn and Martin, 1971; Goo et al, 1973, and Goyal et al. 1981).

One of the problems facing all those attempting to carry out these evaluations is that the shigellae are not the only micro-organisms to cause gastrointestinal illness (G.I.). Therefore culture media used to investigate G.I. must of necessity be able to isolate as many possible pathogens as is scientifically feasible.

Obviously this sets limits on the isolation of individual species. In a comparative evaluation of selective media (Goyal et al, 1981) it was found the X.L.D. agar (Xylose, Lysine, Desoxycholate agar) isolated nine shigellae. In comparison, S.S. (Salmonella - Shigella) and D.C.A. (Desoxycholate Citrate agar) isolated only five shigellae in the same number of parallel specimens.

Although highly selective for the isolation of the salmonellae, Bismuth Sulphate agar (Dunn and Martin, 1971) is unsuitable for the isolation of the shigellae because except for some *Shigella flexneri* and *Shigella sonnei* strains, this medium inhibits the *dysenteriae* group of organisms.

It would seem that the media used to isolate the organisms causing G.I. in any individual laboratory will to some extent depend on personal preference, experience and the cost of the media. Despite the many papers produced over the years in which the overriding opinion of these investigators and others (e.g. McCarthy, 1966,

Taylor and Schelhart, 1967-1968; King and Metzager, 1968.I and II, and Bhat and Rajan, 1975) was that X.L.D. and Hektoen agar have a great advantage over other media for the isolation of the shigellae, it would seem few laboratories in Australia use them in combination.

3.2 PRELIMINARY STUDIES

It was decided to carry out a preliminary survey on the use of the media listed in 3.1. A questionnaire was compiled (see Appendix 1) and sent to thirty laboratories on the Mainland of Australia. A copy of the questionnaire was also sent to the seven laboratories in Tasmania. Half of the total laboratories were large central establishments in capital cities, while the rest were smaller country town laboratories.

An analysis of the response to this questionnaire is shown in Table 7.

RESULTS OF QUESTIONNAIRE

Table 7

	Mainland Australia		Tasmania	
	Used	Not Used	Used	Not Used
X.L.D.	20	6	2	5
Hektoen	0	26	0	7
S.S.	10	16	2	5
D.C.A.	12	14	7	0
MacConkey	15	11	7	0

Only two other media were used. One laboratory included Selenite F and the other Rappaport medium, neither of which are recommended for the isolation of the shigellae. Four laboratories did not reply.

In view of the past evaluation and the above survey it was decided to carry out a controlled investigation on the above listed culture media to ascertain and bring up to date the merits or otherwise of

the individual culture media's ability to isolate shigellae.

3.3 MATERIALS AND METHODS

X.L.D., Hektoen, D.C.A., S.S., MacConkey and Blood Agar Base were obtained from a number of firms including Oxoid, Difco and Institute Pasteur. All media were reconstituted and prepared according to the manufacturers' instructions. Known *Shigella* cultures were obtained from reference laboratories or from the University of Tasmania's own collection of known and confirmed isolates. When possible, these were fresh from patients (local or interstate) but some, e.g. *S. dysenteriae* are uncommon in Australia. Stool specimens were obtained from volunteer medical students and used as fresh normal controls.

Known pure cultures of *E. coli* (A - D) and salmonellae were obtained from reference laboratories (Microbiology Departments of the University of Queensland and the University of Melbourne). The investigations on the culture media were divided into two sections - (A) direct plating of known cultures, and (B) the plating of normal stool specimens that had been inoculated with known *Shigella* strains. All the *Shigella* strains used were in the S-phase and had been tested for purity, biochemically and serologically.

(A) Direct plating of known pure cultures

Standard eighteen-hour cultures ($\pm 10^9$ cfu/ml) of known *Shigella* strains grown in peptone water were diluted in tenfold steps commencing at 10^{-1} and continuing to a dilution of 10^{-8}. These dilutions were made in sterile Kahn tubes using peptone water as the diluent. From each of these dilutions, 0.5 ml was transferred to the individual culture media under investigation. The culture media had previously been prepared in petrie dishes in the normal manner. The 0.5 ml of the culture was then spread over the media with the

aid of sterile L-shaped glass rods, thus allowing good distribution of the colonies formed after incubation for eighteen to twenty-four hours at 37°C. This allowed the colonies to be counted accurately, especially in the higher dilutions.

These tests were carried out in duplicate for each of the media on five occasions covering a period of four weeks and with seven strains of shigellae.

(B) Plating of normal stool inoculated with *Shigella*

Normal faeces were liquefied in the proportion of 1 gram of faeces to 4 ml of physiological saline, resulting in a simulated diarrhoeal specimen. To this specimen was added increasing numbers of the *Shigella* organisms, commencing with 0.1 ml of an 18-hour peptone water culture, followed by additions equal to 0.2 ml, 0.5 ml, 1.0 ml and 2.0 ml of the pure culture. After mixing with the aid of a vortex mixer, specimens of the stools were plated out on different media in the normal procedure used for obtaining isolated colonies on solid media in petrie dishes.

Two sets of controls were also set up. (i) Pure 18-hour peptone water cultures were plated out onto the same duplicate media. (ii) The stool specimens were sterilised at 120°C for 15 minutes and after cooling to room temperature, the known *Shigella* culture in peptone water was added in the same proportion as before to the sterilised faecal specimens. These were plated out again as described.

It had been noted that in 1986 Mujibur Rahaman et al reported the use of a new medium, T.E.A. (Tekney Enteric Agar) which is composed of MacConkey Agar to which Potassium Tellurite (1µg/ml) is added. Although this was not commercially available in Australia, this medium was made in the laboratory for

limited testing and for comparison with the other accepted and documented media.

3.4 RESULTS

The results of the direct plating of pure cultures are given as a histogram in Fig. 1. It will be seen from Fig. 1 that the medium of choice for the isolation of the shigellae is X.L.D. in conjunction with Hektoen and a less selective medium such as MacConkey Agar. Blood agar was incorporated for control purposes only.

Despite strict adherence to the model method (B) a number of problems were encountered :

a) Recovery of *Shigella* after mixing with fresh stool was rare.

b) Of twelve normal stools from healthy medical students mixed with the same shigellae as used previously for the histogram, in only two cases could *Shigella* be recovered. In both cases the colonies were scanty and in no way representative of the number of organisms added to the stool specimen.

The overall results were erratic in regard to the culture media on which recovery was made, but in these two cases X.L.D. gave the best recovery rate.

The sterilised stool specimen controls gave the same results as the peptone water controls and recovery was excellent. The results indicated that the shigellae were being inhibited when added to fresh normal stools. To investigate this possible explanation, a further experiment was set up. From pure 18-hour peptone water cultures of *E. coli*, *Streptococcus feacalis* and individual *Shigella*, mixtures of these three organisms were prepared in varying amounts. These were then seeded onto MacConkey, X.L.D. and Hektoen culture media and results recorded (in Table 8).

MIXED CULTURE RESULTS

Table 8

	E. coli	*Strep Feacalis*	*Shigella*	Results of Culture
Mixture 1	0.05	0.05	0.05	Occasional *Shigella*
" 2	0.10	0.10	0.05	No *Shigella*
" 3	0.20	0.20	0.05	No *Shigella*
" 4	0.10	0.10	0.20	Occasional *Shigella*
" 5	0.10	0.10	0.50	Many *Shigella*
" 6	0.05	0.05	0.20	Small number of *Shigella*

The results indicated that the shigellae were being inhibited when added to normal stools. This would seem to depend on the number of normal flora present. Limited tests on T.E.A. were far from encouraging. While it was confirmed as previously reported (Mujibur Rahaman et al, 1986) that the *E. coli* were suppressed markedly, it was also found that the shigellae were also reduced in numbers and colony size. This was especially so with some strains of *S. sonnei* and *S. boydii* and the media on the whole seemed inferior to X.L.D. There was a further complication. The presence of potassium tellurite in this media also interfered with the results subsequently obtained with diagnostic kits, such as the A.P.I. and Microbact. False results were obtained with known pure cultures of *Shigella*, e.g. one *Shigella* was recorded as *Y. enterocolitica* and another as *Yersina pestis*. Without further and more extensive investigations it is difficult to accept that this medium has any advantages over others already in use.

3.5 DISCUSSION

There seems to be no advantage in attempting to simulate an artificial *Shigella* infection using normal stool specimens. In a previous survey Rollender et al (1969) stated: "The isolation

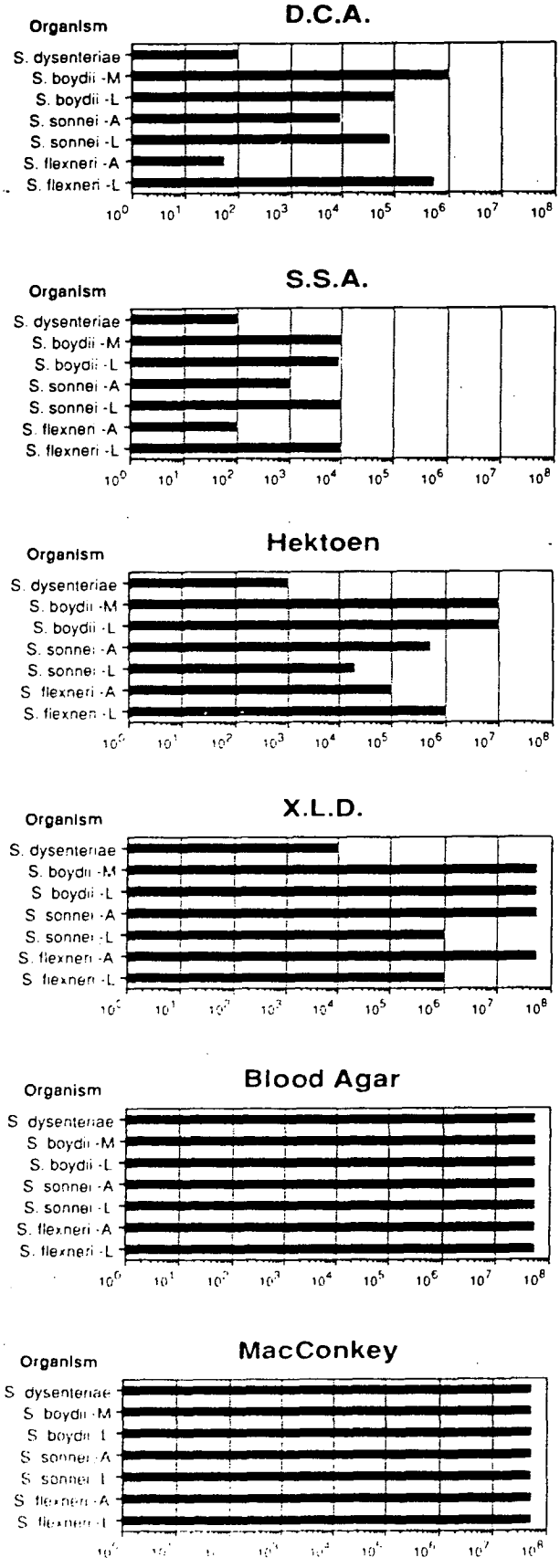
of *Salmonella* or *Shigella* organisms from clinical specimens in which microbial population is usually quite varied becomes complex. The efficiency of an isolation media may be judged by its success in recovering specific organisms from clinical material" (Izenberg et al, 1969).

Lennette et al (1980) also pointed out that "*Shigella* in particular are sensitive to acidic changes that occur in stools during storage." Table 8 adds support to Rollender's statement, and in fact may explain a number of other anomalies including the non-isolation of *Shigella* from a patient later to be found to have *Shigella* infections. A severe case would be more likely to result in good recovery of the organisms provided it was collected and plated in the accepted correct manner whereas a mild case may have many normal flora organisms present which would hinder or prevent the recovery of the shigellae. Therefore it would seem we must continue to rely on pure cultures to assess the merits of individual culture media.

From Fig. 1, it can be seen that the media of choice for the isolation of shigellae is X.L.D. in conjunction with Hektoen, and a less selective media such as MacConkey Agar.

While MacConkey and Blood Agar give the highest recovery rate, this is of course misleading when using pure cultures. In practice, one is often faced with specimens containing mixed flora, many of which are *E. coli*, and except in severe cases where a large number of *Shigella* are present, this will make it difficult to obtain a reasonable number of colonies for further identification, even if they are recovered at all.

Figure 1. Evaluation of Culture Media



Code.

L.= Local
M.= Melbourne
A.= Alice Springs.
The S.dysenteriae A.T.C.

3.6 CONCLUSION

X.L.D. combines the advantage of a good selective medium by containing a concentration of Sodium desoxycholate which allows the inhibition of coliforms without decreasing the ability of the media to support the shigellae.

D.C.A. and S.S. Agar gave very erratic recovery of shigellae, there being great variation of recovery between *Shigella* types. T.E.A. seems quite incompatible. Therefore for the purpose of this research, X.L.D., Hektoen and MacConkey media were used to isolate all suspected pathogens from cases of diarrhoea.

Chapter 4

TRANSPORT MEDIA

4.1 INTRODUCTION

4.2 MATERIALS AND METHODS

4.3 RESULTS

4.4 DISCUSSION

4.5 CONCLUSION

4. TRANSPORT MEDIA

4.1 INTRODUCTION

It has been an accepted fact for many years that the most accurate processing of faecal specimens for the isolation of *Shigella* is to carry out direct plating of faeces at the bedside from a freshly produced specimen or a rectal swab (Croft, 1956; Taylor et al., 1967; Bottone et al., 1986).

Such an ideal situation is often not possible even in a developed country like Australia, let alone in developing countries where the patients may be hundreds of kilometres from the laboratory. It was decided therefore in conjunction with the above investigation into the media of choice for the isolation of *Shigella*, to investigate transport media available for sending specimens over long distances, and the effect this delay may cause before laboratory investigations can be carried out.

For this work we had the full cooperation of the Microbiology Department at Alice Springs General Hospital where many investigations are required for the Northern Territory, an area of Australia with a high occurrence of *Shigella* infections.

4.2 MATERIALS AND METHODS

Four commonly used media were investigated, Buffered Glycerol Saline, Cary Blair, G.N. Broth (Hajna) and Buffered Peptone Water. The media used was obtained from either Difco or Oxoid.

Five of each of these media were sent to Alice Springs Hospital on a number of occasions covering a period of six months. In all, enough media was sent to investigate twenty-five known cases of shigellosis. Each of the four media was inoculated with a swab of

the patients' faeces so that a relatively standard technique was achieved.

This resulted in the receipt of four specimens from each patient in order that the recovery from each medium could be compared. The specimens were transported by air at room temperature and normally took twenty-four hours to reach Hobart.

On arrival they were inoculated onto X.L.D., MacConkey Agar and Hektoen Enteric Agar. The transport media were then kept at room temperature for periods of up to eight days, and reseeded every two days onto the above media again. Results are set out in Table 9.

4.3 RESULTS

EVALUATION OF TRANSPORT MEDIA

Table 9

Media	Days media held before plating	No. of Shigella isolated	% Recovery from 25 specimens
Buffered Glycerol Saline	1 - 2	15	60
	3 - 6	7	28
	7 - 8	4	16
Cary Blair	1 - 2	8	32
	3 - 6	4	16
	7 - 8	3	12
G.N.	1 - 2	5	20
	3 - 6	2	8
	7 - 8	0	0
Buffered Peptone	1 - 2	20	80
	3 - 6	8	32
	7 - 8	2	8

In an earlier evaluation of Cary-Blair medium, carried out by Wells et al., (1981), it had been found that if the specimens were transported in the medium at four degrees rather than at room temperature, the recovery of the *Shigella* organisms was markedly increased. It was thus decided to run a special test on this aspect.

Two sets of Cary-Blair medium were inoculated with known *Shigella*. One set of five was left at room temperature for twenty-four hours, and the other set kept at 4°C. In the first group, two of the *Shigella* could be recovered and in the refrigerated group, four *Shigella* were recovered.

4.4 DISCUSSION

A number of papers have been published over the years on the efficiency of the above media. Buffered glycerol-saline was recommended by Sachs (1939) and Colman (1945) for the preservation and transport of faecal specimens. In an investigation on B.G.S. (Morris et al, 1970), it was found that this solution was superior to Cary-Blair or G.N. broth for *Shigella*. This is supported by the findings above.

G.N. (Hajna) was found to be disappointing, only five *Shigella* being recovered from a total of twenty-five known positives. Morris et al (1970) also encountered this problem, recovering only eight from a total of sixty-eight specimens on direct plating. This was in contrast to the good recovery obtained by Taylor et al (1967) when the medium was used purely as an enrichment medium, inoculated and incubated at 37°C for eighteen hours and then plated onto X.L.D. etc. The latter workers found a 53% increase in the number of *Shigella* isolated over direct plating. Later they commented "Evidence that the choice of media has been demonstrated in previous studies" (Taylor, 1965).

It would seem that just as in direct plating, the choice of the transport media and the way in which it is used will affect the rate of isolation of *Shigella*.

Another aspect to these findings is the possibility that different strains of *Shigella* and even those with the same serotype, will react differently to their growth in culture media depending on their sensitivity to the chemical contents of that specific medium.

It is interesting to note that in Table 9 the best results were obtained with Buffered glycerol saline and Buffered peptone which contain no reagents known to inhibit *Shigella*. The Buffered peptone was found to be the best transport medium if subcultured onto solid media from two to three days. Longer periods tended to increase the number of *E. coli* in the transport medium to such an extent that the *Shigella* could no longer compete.

4.5 CONCLUSIONS

In view of the above results, for the purpose of this research, Buffered peptone water or Buffered glycerol saline were used where transport media was required.

In view of the findings that G.N. (Hajna) medium was a good enrichment medium (Taylor et al, 1967), and the other media being unsuitable in this respect, it was incorporated as an enrichment medium and inoculated after eighteen hours at 37°C onto a duplicate set of solid media as used for the direct cultures.

Chapter 5

SURVEY OF *SHIGELLA* INFECTIONS IN TASMANIA

5.1 INTRODUCTION

5.2 MATERIALS AND METHODS

5.3 RESULTS

5.4 DISCUSSION

5.5 CONCLUSION

5. SURVEY OF *Shigella* INFECTIONS IN TASMANIA

5.1 INTRODUCTION

It is a widely held concept by local medical and laboratory staff that Tasmania has a consistently low occurrence of *Shigella* infections. Since 1980 these have averaged about 1.6 cases per year from a total population of 440 000. This view is not helped by a recent publication (DuPont and Pickering, 1980) in which Tasmania was designated an area of moderate or unknown risk for developing infectious diarrhoea (Fig. 2). In fact the whole spectrum of diarrhoeal infections in Tasmania, until recently, has been a very neglected subject. Since 1980 a number of investigations have been made in this Department into specific areas (Goldsmid, 1980; Goldsmid et al, 1984; Kirov et al, 1984; Goldsmid, 1985; Kirov et al, 1986; Hunt et al in press), but until now no work has been carried out on the shigellae, the enterotoxigenic *E. coli*, enteroinvasive *E. coli* or the *E. coli (A-D) Alkalescenes Dispar* group. These pathogenic *E. coli*, be they E.T.E.C. E.I.E.C. or the *E. coli (A-D)* are seldom investigated or isolated in most countries, mainly due to the fact that the technical problems involved for a routine clinical laboratory plus the time factor tend to deter their investigation, excepting when there is an obvious outbreak of diarrhoea in which a pathogenic *E. coli* is suspect. At the same time with the advent of gene probes and commercial kits in this field of diagnosis, the testing procedures may become feasible in most routine diagnostic laboratories in the not-too-distant future. In the meantime, especially in areas like Tasmania, it is very valuable from a public health point of view to obtain statistics showing the true frequency of these microorganisms.

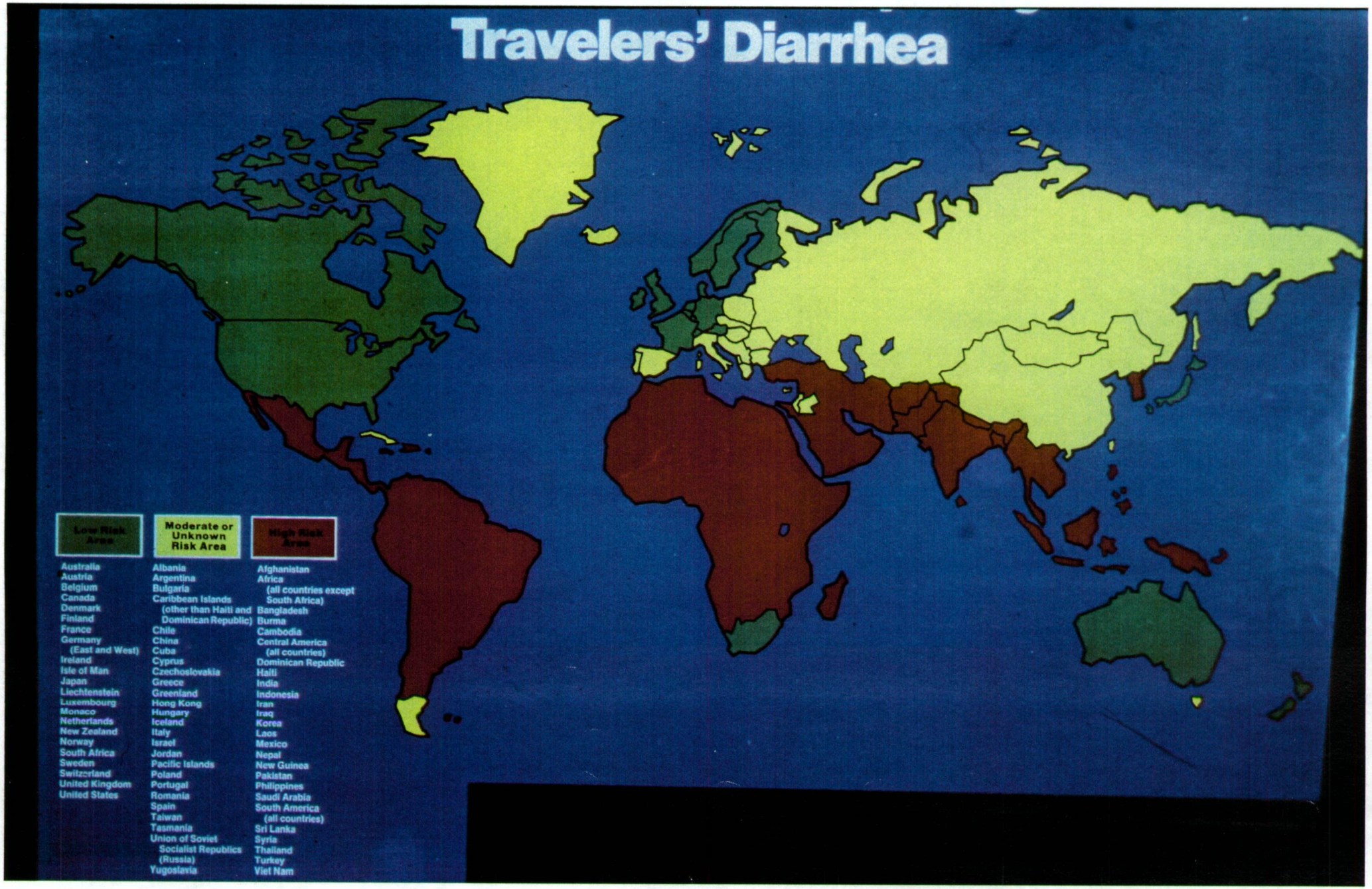


FIG. 2. MAP SHOWING TASMANIA AS A MODERATE OR UNKNOWN RISK AREA FOR DIARRHOEAL INFECTIONS

The shigellae on the other hand are a constant world wide problem, especially in developing or underdeveloped countries, for example Bangladesh (Gross et al, 1982) where *Shigella* was reported to be the second ranking cause of bacterial gastroenteritis in individuals over one year of age.

In the U.S.A., 70% of the *Shigella* isolates received by the Center for Disease Control are identified as *Shigella sonnei* (Blaser et al, 1983), currently the most prevalent species isolated in the developed world.

While it is true there has been an overall decrease in *Shigella* infections in the Western world, such a low incidence as found in Tasmania is almost unique. Even more surprising in these days of rapid international travel, and an increase in tourists especially from Asian countries where there is a high incidence of *Shigella*.

In developed Asian countries such as Japan, there has been a constant fall in shigellosis since a peak in 1952, now it remains at a constant of 700 to 1500 cases per year. At the same time it has been noted there has been an increase in imported cases. For example serotypes that have not been seen for several years in Japan (Yoshifumi Takeda, 1981) include *S. dysenteriae 6* and *7*, *Shigella flexneri 6*, and *S. boydii 2, 3, 5, 10* and *12*. The survey of the Research Association for Infectious Enteritis showed the imported strains of *Shigella* were mainly from the Philippines, Thailand, Korea, India and Taiwan. It would seem remarkable then if Australia in general, including Tasmania, now also enjoying a tourist boom with many visitors from Asian countries, should continually have an extremely low incidence of shigellosis. Having determined the best media and

methods available (Chap. 3 and 4) to isolate *Shigella*, a survey was conducted to determine the true incidence of *Shigella* infections in Tasmania. This was investigated in two parts. (1) The first involved the investigation of enteric pathogens in hospitalised mentally handicapped patients (Hunt et al, *in press*), and (2) a wider survey of Tasmania in general, over a two year period.

5.2 MATERIALS AND METHODS

The first major step to be taken was to obtain the aid and cooperation of Government and private medical laboratories in Tasmania and of local medical practitioners. No problems were encountered with the laboratories and we obtained their full cooperation, which consisted of sending us part of the specimens they had been requested to investigate for infectious diarrhoea.

It was not practical or possible to obtain the cooperation of every medical practitioner in Tasmania. Therefore a selected number in different areas cooperated by sending us stool specimens from patients with suspected infectious diarrhoea. As would be expected, most of these were obtained from local practitioners and private laboratories as most of the patients with such a clinical picture would be unlikely to be admitted to hospital.

The other area of investigation was the survey carried out at the hospital for the mentally handicapped where a detailed virological, bacteriological and parasitic study was carried out, the hospital having an ongoing problem with sporadic and epidemic diarrhoea. This study was carried out on mentally retarded and in many cases, physically handicapped, patients at

the Willow Court Centre, New Norfolk, Tasmania.

Stool specimens for enteric pathogens included 50 for parasitological studies, 71 for bacterial studies, 48 for rotavirus investigations and 116 anal tapes for enterobiasis.

For the parasite investigations, routine stools were collected and examined as Gomori-stained faecal smears and as centrifuged deposits after formalin-ethyl acetate concentration using the Evergreen faecal concentration system*. In addition, 22 direct faecal smears were stained using a modified Ziehl-Neelsen method for *Cryptosporidium*. The anal tapes were collected first thing in the morning and from most patients a single tape only was received. Testing for rotavirus was performed using the Rotalex Test.

In both surveys the following procedure was followed in regard to stool specimens:

All stool specimens were sent with the minimum of delay, and if sent any distance they were sent in transport media discussed under Chapters 4.4 and 4.5.

All specimens were plated directly onto X.L.D., Hektoen and MacConkey Agar for isolated colonies and incubated at 37°C for 18 hours. Further to this a pea-sized specimen of the stool was placed in 10 ml of G.N. (Hajna) medium used as an enrichment as advised by Taylor et al (in 1967), and after 18 hours this was then plated out on X.L.D. and the other media again.

All the plates were inspected for the presence of non lactose fermenting colonies. If present, individual colonies were

* Evergreen Scientific Los Angeles, U.S.A.

removed and placed into peptone water for four hours, and then examined for motility under a phase contrast microscope, plated out on X.L.D. and MacConkey media for purity check, and then inoculated onto a Microbact 24E for the identification of the Enterobacteriaceae (Disposable Products Pty Ltd, Adelaide, South Australia) following the manufacturer's instructions. The suspect colonies were further tested using Enterotube II (Roche Diagnostics, Mentone, Victoria, Australia) and again following the manufacturer's instructions. A.P.I. 20 E diagnostic kits (distrubuted by Carter Wallace [Aust] Pty Ltd, Frenchs Forest, Sydney, Australia) were occasionally used to aid in the identification of a difficult organism, but in general the other systems were used routinely. All the *Shigella* isolated were sent to reference laboratories for further comfirmatory checking.

A blood agar plate was incorporated into the routine also as an indicator for the presence of *Aeromonas*, and *Aeromonas* Agar (Gibco Laboratories, U.S.A.) used for isolation and further tests with the Microbact and A.P.I. systems. *Campylobacter* Growth Supplement (Oxoid Australia Pty Ltd, West Heidelberg, Victoria) was used for the isolation of the organism and suspected colonies were sent to a specialist laboratory for confirmation. Those plates which had no non-lactose fermenting organisms isolated despite the diarrhoeal symptoms were treated as follows. At least four colonies of the lactose fermenting organisms were tested from each plate using the Microbact and Enterotube II (Roche) systems to confirm they were *E. coli*.

These cultures were later tested by DNA probe techniques to ascertain if they were toxigenic or invasive *E. coli*. The methods used were the Non-Radioactive Kits for the detection of

Enterotoxigenic *Escherichia coli* and the P32 Radioactive test using the Nick Translation Kit, both of which were supplied by BRESA (Biotechnology Research Enterprise Pty Ltd, Adelaide, South Australia). These methods were used in preference to the use of suckling mouse assay (Dean et al, 1972) and the Sereny Test which require animal facilities and can be expensive and subject to variability if not carried out in a reference laboratory. (See Appendix 2.)

5.3 RESULTS

The results of the survey on the hospitalised mentally handicapped patients is given in Table 10. The wider survey on the frequency of *Shigella* infections in Tasmania is given in Table 11.

Table 10

Enteric pathogens isolated from mentally handicapped patients at Willow Court Centre.

SPECIMEN	+	%+	TOTAL
Stool (parasitology):			50
Giardia lamblia	1	2.0	
Dientamoeba fragilis	1	2.0	
Entamoeba coli	11	22.0	
Ent. histolytica	5	10.0	
Endolimax nana	6	12.0	
Blastocystis hominis	5	10.0	
Trichuris trichiura	4	8.0	
Anal tapes:			116
Enterobius vermicularis	22	19.0	
ZN stains			22
Cryptosporidium sp.	0	0	
Stool (Rotalex):			48
Rotavirus	0	0	
Stool (bacteriology):			71
NLF : *Proteus* spp	3	4.2	
Hafnia alvei	2	2.8	
Shigella flexneri	1	1.4	
Salmonella typhimurium	1	1.4	
Providencia sp	1	1.4	
Arizona sp	2	2.8	

LFC :	*Escherichia coli* (non-toxigenic)	71	100.0
	Esch. coli (toxigenic)	0	0
	Esch. coli (enteroinvasive)	1	1.4
	Esch. coli A.D. (non-toxigenic)	5	7.0
	Esch. coli A.D. (toxigenic)	0	0
	Esch. coli A.D. (enteroinvasive)	5	7.0
Miscellaneous:	*Yersinia enterocolitica*	1	1.4

Table 11

Organisms isolated from 201 stool specimens submitted for investigation by laboratories and local practitioners in Tasmania.

ORGANISMS	NUMBERS	% OF TOTAL COUNTS
Salmonella	6*	3.0
Arizona sp	8§	4.0
Aeromonas hydrophila	3¶	1.5
Campylobacter	4	2.0
Plesiomonas shigelloides	3	1.5
E. coli A-D) Alkalescens Dispar	6	3.0
Enterotoxigenic *E. coli*	3	1.5
Enteroinvasive *E. coli*	9	4.5
Shigella	8**	4.0
Total	50	25

* Of the 6 Salmonella, 5 were *Salmonella mississippi*, a common *Salmonella* in Tasmania; the other was *Salmonella typhimurium*.

§ Arizona is often missed due to the fact that 60% of strains utilise lactose within 24-to-48 hours, and therefore may not be selected for further testing.

¶ Of the 3 *Aeromonas*, 2 were found to be Toxigenic (confirmed by Gastroenterology & Nutrition Research Unit, Princess Margaret Hospital, Perth, Western Australia).

** The 8 *Shigella* consisted of the following: 1 *S. sonnei*, 1 *S. flexneri* 2b, 2 *S. boydii* 2, and 4 *S. boydii* 13.

5.4 DISCUSSION

As a general rule one might expect a high prevalence of enteric pathogens amongst institutionalised mentally handicapped patients. One might further expect that the species composition of the pathogens within such institutions would be more or less representative of the situation found in the general community of that area, albeit at a higher level. What was surprising in the present study however, was the high prevalence of intestinal

protozoa, both pathogenic and commensal, recovered - a finding at variance with the situation usually recorded in the general population in Tasmania. In fact, the results recorded in the present study were in general rather similar to those recorded in Queensland by Karik et al (1971), despite the very different geographic areas involved.

With the exception of six patients suffering from an obvious diarrhoea, all stool specimens received were formed. Of those with diarrhoea, one was diagnosed as having *Shigella flexneri,* one with *Salmonella typhimurium,* one had *Giardia lamblia,* one had enteroinvasive *Escherichia coli* and two had enteroinvasive *Escherichia coli* A.D. The finding of the *Shigella* was of particular significance as shigellae have not been commonly reported from Tasmania and there seems to be a common misconception in the State that shigellae are not of much significance here and are therefore often not looked for.

The common recovery of intestinal protozoa was surprising for Tasmania where these organisms are not commonly encountered in the community (Goldsmid, 1981). However, the prevalence of *Giardia* was lower than expected (probably due to the fact that repeat specimens were not generally available) as this species, together with *Blastocystis hominis* is one that does occur not uncommonly in this State (Goldsmid, 1980, 1984, 1985). There was an unexpectedly high prevalence of *Entamoeba histolytica* but these were probably non-invasive zymodemes as defined by Sargeaunt (1985), as no cases of invasive amoebiasis have been diagnosed over past years. An interesting finding was the identification of trophozoites of *Dientamoeba fragilis*, a species which can be associated with abdominal pain and diarrhoea, as

this species has not previously been recorded from Tasmania. It is interesting to speculate that this high prevalence of *D. fragilis*, is related to the high prevalence of threadworm, *Enterobius vermicularis*, which was recorded in 19% of the patients based on, mostly, single anal tapes. This protozoan has no cyst stage and is believed to be transmitted in the egg of the *Enterobius* (Pawlowsky, 1987). The only other helminth recorded was *Trichuris trichiura*, a species commonly recorded in overseas travellers and S.E. Asian refugees to Tasmania (Goldsmid, 1980, 1981, 1984), but not frequently encountered in the general community.

Karik et al (1971) in Queensland were aware of this after their study, commenting that the spread of infection was "an indication of the inherent difficulties which are encountered in these institutions and points to the important role of institutionalised prophylaxis which may need more thought and attention in future".

In this regard, the appointment of an infection control sister must be regarded as an essential in such institutions - as has been done at the Royal Derwent Hospital and Willow Court Centre. This appointment was a major factor in the mounting of this investigation. Further, any new patient or patients on return from visits outside should and must be screened on return. Once new pathogens have been introduced, they can spread rapidly and widely in these environments - not only to other patients but also to staff.

It will be seen (in Table 11) that the number of *Shigella* detected in the 201 cases of diarrhoea investigated are far in excess of the average one or two cases reported each year from a total population of 440 000. One of these cases was hospitalised, and

the *S. sonnei* was also isolated by the hospital laboratory.

Of the remaining seven cases, one was not detected by the laboratory investigating the case, and five had not been sent to a laboratory for investigation. One of the eight cases was an accidental laboratory infection from one of the patients suffering from *Shigella* infection.

It is interesting to also note how the number of *Salmonella* cases can give a clearer picture of the true situation regarding diarrhoeal infections.

It is a known fact (Commonwealth Health Statistics) that the average number of *Salmonella* cases isolated in Tasmania each year is 36. In the group of 201 in this study, six *Salmonella* were isolated or three for each year. It would seem then that only 1/12 of the number of cases have been investigated, 3X12=36.

On requesting the laboratories to inform us of the total number of patients that they would investigate for diarrhoea due to infection during a year, we were informed this would be about 2 400 or 201x12=2,412. On this basis then, one would expect with an average 4.0 cases of *Shigella* per year in this research, there would be about 48 cases per year, or about ten per 100,000, which it will be noted is in line with *Shigella* reported in other similar countries, and not the exceedingly low numbers associated with Tasmania.

It is also very interesting to note the number of Enterotoxigenic and Invasive *E. coli* which are not normally investigated in routine laboratories due to the many technical problems involved.

5.5 CONCLUSION

Although DuPont and Pickering (1980) designated Tasmania an area of moderate or unknown risk for developing infectious diarrhoea, local experience would not support a moderate risk situation. It is true certain areas of Tasmania do carry a higher risk of acquiring infectious diarrhoea (the Cradle Mountain-Lake St Clair walking track) and although this is probably due to the lack of toilet facilities and the area is liable to flooding, thus spreading feacal material (Ball, Personal Communication), the incidence of infection over the State as a whole is still low.

At the same time, recent studies, including the present investigation, have continued to elucidate the microorganisms associated with gastric infections.

While these studies have extended the list of endemic organisms known to be associated with diarrhoeal infections in Tasmania and have resulted in a better assessment of their prevalence, there is no evidence that they are significantly more common in Tasmania than in other similar geographical areas of the Western World.

At the same time, keeping in mind the experience of Japan and some European countries which have a large number of tourists each year, Tasmania must change its attitude that it is under no threat from these diseases, or that it is a unique area of the world with few or none of these infections.

Chapter 6

THE ENTEROTOXIGENIC AND ENTEROINVASIVE *E. COLI*

6.1 INTRODUCTION

6.2 MATERIALS AND METHODS

6.3 RESULTS

6.4 DISCUSSION

6.5 CONCLUSION

6. ENTEROTOXIGENIC AND ENTEROINVASIVE *E. coli*

6.1 INTRODUCTION

Escherichia coli was long regarded as a harmless commensal of the bowel and an indicator of faecal pollution. In recent years however, this organism has undergone intensive investigation since the recognition of its role in diarrhoeal disease. Much of this recognition was an out-growth of cholera research (Carpenter, 1982).

Dating back to the 1940s, enteropathogenic *E. coli* (E.P.E.C.) strains were found to be associated with diarrhoeal disease in infants. More recently *E. coli* strains of serotype O157:H7 have been incriminated in acute haemorrhagic colitis (Johnson et al, 1983; Rily et al, 1983).

Although research activities (Silva et al, 1980; Sansonetti et al, 1981; Boileau et al, 1984) have brought about a better understanding of the molecular mechanisms and virulence factors in regard to the enteropathogenicity of *E. coli*, little correlation exists between the presence of virulence markers such as enterotoxin production and biochemical behaviour (Lee et al, 1983).

Biological systems exist for assessing both heat-labile (LT) and heat-stable (ST) toxin, yet no single selective medium or biochemical marker distinguishes these strains from non-pathologic *E. coli*. The *Shigella* - like enteroinvasive *E. coli* (E.I.E.C.) may be distinguished by its failure to produce lysine decarboxylase, and in most instances by its lack of mobility, delayed lactose fermentation and anaerogenic fermentation of glucose (Silva et al, 1980). In general, however, both the E.T.E.C. and the E.I.E.C. strains of *E. coli* are very difficult if not impossible to

diagnose in a routine medical microbiology department. Therefore there is no doubt that many of these infections are overlooked, unless they are causing very severe symptoms or are present in large numbers. We are then left with little knowledge of their frequency in many countries, including Australia.

6.2 MATERIALS AND METHODS

As previously stated (Chapter 5.2), DNA Probe techniques were used for the detection of E.T.E.C. and E.I.E.C., and techniques are set out in full under Appendix 1 and 2.

These DNA Probe techniques were used in preference to suckling mouse assay (Dean et al, 1972) and the testing for keratoconjunctivitis in guinea pigs (Sereny Test) due to our limited animal facilities. The DNA Probe testing for the detection of E.T.E.C. was carried out using the BRESA non-radioactive kits. Each kit contained specific, synthethetic oligonucleotides probes, terminally labelled with alkaline phosphatase. These probes hybridize to the toxin genes in the enterotoxigenic *Escherichia coli* and are detected by a simple colour reaction. These kits consisted of Kit ETC4 LT Human, and Kit ETCO2 STI - b (STH) Human.

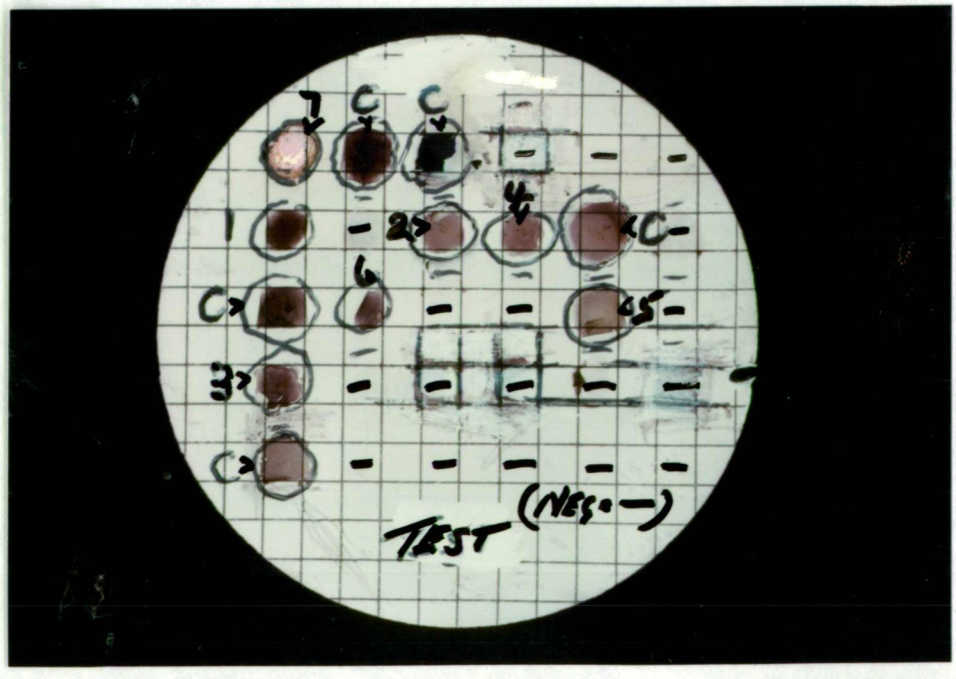
In the case of DNA probe testing for E.I.E.C., an *E. coli* agar stab containing the probe for the E.I.E.C. was sent to us by the courtesy of Dr Roy Robins-Browne from the University of Melbourne. The probe itself was a 17.kb EcorR1 fragment cloned from *Shigella flexneri* serotype 5 and ligated into pBR332. The resultant plasmid, pMR17 carries ampicillin and tetracycline resistance markers. (Boileau et al, 1984; Sethabutr et al, 1985). This probe was isolated from an unamplified culture of *E. coli* using an adaptation of the method of Birnboim and Doly (1979). This was performed

in the Department of Medicine molecular biology laboratory through the co-operation of Dr Greg Woods. The Nick Translation Kit (BRESA) and 32P was used for the radioactive method, in the detection of E.I.E.C.

From the 201 stools investigated 82 *E. coli* were isolated. These 82 *E. coli* were tested for purity by biochemical techniques using the Microbact 24 E, Roche Enterotube II and occassionally A.P.I. 20.E. systems. Only the Enterotube II (Roche) system specifically listed *E. coli* A - D *(Alkalescens dispar)*, the other systems list this organism under *E. coli* or *E. coli* inactive. The 6 *E. coli* A - D detected were all identified by the Roche method and serologically confirmed using Wellcome specific antisera (Wellcome Diagnostics, Concord, New South Wales). The 6 *E. coli* A - D were then incorporated into the DNA probe testing with the other 76 *E. coli*.

6.3 RESULTS

The resultant colour change for the DNA probe for the E.T.E.C. (BRESA) is set out in Fig. 3 below.



The colour reaction is a purple/red for a positive reaction and colourless to a slight yellow for a negative reaction. The cultures in the example (Fig. 2) have been inoculated in the form of a square 5 x 5. It will be seen the positive controls have been labelled C and the unknown positive tests labelled with a number, 1 to 6. With the majority of negatives the colour reaction is absent and therefore clear cut. Occassionally, the reaction can be doubtful (7). None of the *E. coli* A - D were positive.

The six possible positive results for E.T.E.C. were sent to a reference laboratory for confirmation (Princess Margaret Children's Research Foundation, Perth, Western Australia). They confirmed that three of the specimens were positive using the suckling mouse assay and an E.L.I.S.A. assay. In view of the fact that all our known positive controls worked, and we had 76 negative results, it seemed that the plasmids in our strains were probably unstable. But for the purpose of this survey we only included the three confirmed cases (Table 11).

The radioactive tests for E.I.E.C. were placed on a piece of photographic film in a cassette and left overnight. An example photograph of the result of this exposure is given in Fig. 4. It will be noted the positives are obvious, an example of a doubtful result (which should be repeated before a final report is made) is given, together with three negative results.

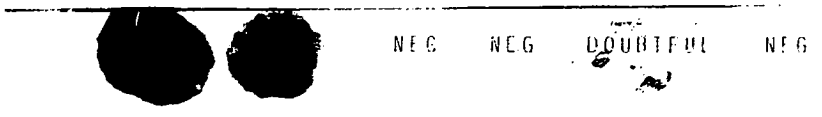


Figure 4 shows example of radioactive result for E.I.E.C.

These results were not sent for Sereny test confirmation as Wood et al (1986) have pointed out that the radioactive probe for E.I.E.C. was identical in accuracy with other tests.

From the 82 *E. coli* investigated nine unknown *E. coli* were found to be positive, all 6 *E. coli* A - D were found to be positive, the other 67 *E. coli* gave a negative result for E.I.E.C.

6.4 DISCUSSION

This was the first time a survey had been carried out in Tasmania on the incidence of the toxigenic and invasive *E. coli*. It was not initially expected that the incidence would be high, but in fact the frequency of the invasive *E. coli* was much higher than expected. Using the previous calculation (Chap. 5.4) we have a potential of 4.5 x12 or 54 cases of enteroinvasive *E. coli* per year 3 x 12 or 36 cases of *E. coli* A - D and 18 cases of enterotoxigenic *E. coli*, which explains a fair number of the diarrhoeas in which no pathogens are found or not investigated.

6.5 CONCLUSION

It has been shown that the incidence of pathogenetic *E. coli* in patients suffering from infective diarrhoea in Tasmania accounts for a fair number of cases remaining undiagnosed each year, and that the possibility that these organisms are the cause of diarrhoea should be kept in mind, especially when it can be seen we have a possible, theoretical incidence overall of 108 pathogenic *E. coli* cases per 100,000 each year.

Chapter 7

RAPID TEST FOR THE DETECTION OF *Shigella* INFECTIONS

7.1 INTRODUCTION

7.2 PRELIMINARY STUDIES

7.3 MATERIALS AND METHODS

7.3.1 METHODS

7.4 RESULTS

7.5 CONCLUSION

7. RAPID TEST FOR THE DETECTION OF *Shigella* INFECTIONS

7.1 INTRODUCTION

One of the important technical problems in the diagnosis of *Shigella* infections is the difficulty encountered in isolating the organism due to the time factor between receiving a specimen and obtaining a laboratory result. Complicating this situation is the delay between the inoculating of the media and growth. This can take from 18 to 48 hours. Further, the suspected pathogens have then to be taken from the plate, tested for purity and subsequently tested serologically with specific *Shigella* antisera. In some cases, it may be four to five days before a result is obtained.

These delays can have far-reaching consequences for patients suffering from severe dysentery, especially in Third World countries where the diagnosis may have to be made on purely clinical symptoms. In the pandemic which occurred in Central America in 1970, and in which 20 000 people died (Smith, A.L., 1981), many cases were first diagnosed as amoebic dysentery until later culture showed the infection to be due to *Shigella* organisms.

Some years ago, fluorescent antibody techniques were introduced and by 1960 many factors had increased interest in rapid identification procedures, including increased international travel resulting in increased spread of disease.

By 1969, over one thousand scientific papers had been published on aspects of fluorescent antibody techniques, (e.g. Beutner, 1961; Brown, 1963; Ayoub and Wannaker, 1964; Cherry and Moody, 1965; Bisset et al, 1969), but despite

this and although fluorescent antibody techniques have been available as diagnostic tools for nearly thirty years, their acceptance as a routine bacteriological procedure in clinical laboratories is disappointing.

It is true, there have been problems with fluorescent techniques in regard to the shigellae, but this is a problem not uncommon when producing antisera for *Shigella* identification. The cross reaction of *Shigella* antisera with other strains of the organism and with certain strains of *E. coli* has been a constant problem for many years (LaBrec et al., 1959; Thomason et al., 1965; Oaks et al., 1986).

Yet, although some excellent individual *Shigella* fluorescent antisera were produced and reported by Akiyoshi Kawamura (1969, 1983), which showed little or no cross reactions, making it possible to carry out direct tests even on feacal smears, such methods have not been accepted for routine use. With the decrease in the number of cases and severity of *Shigella* infections in the world's advanced nations, eventually the production of such sera became uneconomic, and it was no longer available commercially.

A more serious problem in the past, and this remains so for developing countries, is the cost of the necessary equipment (e.g. fluorescent microscope), and the staff with experience of the technique. With the advent of monoclonal antibody production (Kohler and Milstein, 1975) and spectacular advances in this branch of immunology with the production of antisera of exquisite specificity, there is no doubt that as this technology improves and reagents of higher quality become

available, fluorescent techniques may solve many of the earlier problems faced in the detection and identification of *Shigella* infections.

This is supported by Akiyoshi Kawamura and Yuzo Aoyama (1983) who, in their review of fluorescent techniques for the Enterobacteriaceae and other areas of application state:

> *The recent development with the use of monoclonal antibodies for making specific conjugates ... seems to guarantee that, for many years ahead, the immunofluorescence technique will remain an outstanding laboratory diagnostic tool in microbiology.*

In the meantime it is obvious that a rapid and reliable technique is required to solve the problems faced by those working in developing countries. This would need to take the form of a simple field kit if possible, where a faecal specimen could be tested on the spot. Even if this kit method only detected that the organisms were specifically *Shigella*, it would be sufficient. In most epidemics the strain is the same, therefore a diagnosis could be made on the spot and appropriate measures taken for treatment, and selected specimens sent to base or reference laboratories for specific group identification.

7.2 PRELIMINARY STUDIES

For some time now Microplate Methods of Enzyme-Linked Immunosorbent Assay pioneered by Engvall and Colleagues, and Van Weemen and Schuurs (Engvall et al., 1971 and 1972; Van Weemen and Schuurs, 1971) have been used in the detection of human, plant and other viruses (Feldmann et al., 1976; Voller et al., 1976; Clark and Adams, 1977).

Immunosorbent Assay Techniques have been used experimentally for the identification of enteroinvasive *Escherichia coli* and virulent *Shigella* strains (Tibor Pal et al., 1985).

They used a technique of coating the microtitre wells with the bacteria and then adding a conjugated anti-rabbit immunoglobulin G, labelled with horse radish peroxidase, and reported excellent results in detecting VMA (virulence marker antigen).

There are a number of assay type methods. These include the competitive, double antibody sandwich, indirect and solid phase methods. The double antibody sandwich technique seemed to be the method of choice for *Shigella*.

It was decided to carry out experiments with this method using *Shigella* antibody and antigen in substitution for viral reagents.

7.3 MATERIALS AND METHODS

The experiments for this part of the research had to be carried out in two parts, but for the sake of continuity, all the materials and reagents are listed here, and in Appendix 3.

Buffers: coating buffer 0.05 M - sodium carbonate at pH 9.6; substrate buffer: 10% diethanolamine adjusted to pH 9.8 with HCl; phosphate - buffered saline (PBS) - 0.02 M - phosphate plus 0.15 M - NaCl at pH 7.4; PBS with 0.05% Tween 20 (PBS - Tween).

Reagents: The enzyme was alkaline phosphatase (P 5521 Sigma)

and the substrate was 2 - nitrophenyl phosphate (Sigma, St Louis, U.S.A.) at 0.67 or 1.0 mg/ml in substrate buffer.

Shigella antisera was obtained from Difco (Detroit, Michigan, U.S.A. or Wellcome, Australia) or produced in rabbits at the Department of Agriculture Research Laboratories (New Town, Tasmania) under the direction of Dr Paul Guy, Research Fellow, who also contributed his expert advice on ELISA techniques. The method of producing the antisera in rabbits was that set out by Edwards and Ewing (8th Edition, 1970). A local strain of *Shigella flexneri 2b* (confirmed by Melbourne University Microbiology Department) was used for the antisera production and in the ELISA tests.

Equipment: Polystyrene microtitre system (Flow, Ayrshire, Scotland) plates, pipettes, etc.
Incubator 37°C, and Photometer for measuring optical density of paranitrophenol (hydrolysed substrate) at 405 nm.

7.3.1 METHODS

In the first instance commercial *Shigella* antisera was used, this was obtained from Difco (P.O. Box 1058, Detroit, MI 48232 U.S.A.) and Wellcome (Wellcome Diagnostics, P.O. Box 12, Concord, N.S.W., Australia).

This antisera was treated for purification of the IgG as follows:

1. To 1 ml of antisera was added 9 ml distilled water on a magnetic stirrer.
2. Ten ml of saturated ammonium sulphate was added drop by drop.

3. It was then left stirring for 45 minutes at room temperature.
4. Next, this was centrifuged to collect precipitate (3000 rpm).
5. The precipitate was dissolved in 2 ml of half-strength PBS.
6. Dialyzed three times against 500 ml half-strength PBS in the cold room.
7. The DE23 cellulose was equilibrated in half PBS overnight.
8. The gammaglobulin was washed through a DE23 column with half PBS.
9. The effluent was monitored at 280 nm and the gammaglobulin collected (first protein fraction to elute) in siliconised tubes.
10. The OD at 280 nm was adjusted to 1.4. This is equal to 1 mg/ml and stored at 4°C.

Conjugation of Alkaline Phosphatase with gammaglobulin

The method set out by Clark and Adams (1977) was used. Two mg alkaline phosphatase were dissolved in 1.0 ml of the purified gammaglobulin preparation and dialysed extensively against PBS at 4°C. Glutaraldehyde was added to 0.05% final concentration (Avrameas, 1969) and the mixture incubated at 22°C for four hours. Glutaraldehyde was then removed by dialysis against several changes of PBS and the conjugate stored with approximately 1% bovine serum albumin at 4°C.

Due to volume changes and possibly gammaglobulin losses during the conjugation procedure, all references to the use of conjugates are in terms of dilutions of the conjugate rather than absolute concentrations.

Coating of Plates

The purified gammaglobulin was diluted with coating buffer so that 200 µl contained approximately 2 µg. It was found on testing that 150 µl gave the best colour reaction.

Method

150 µl purified gamma globulin in coating buffer added to each well

↓

wash

↓

150 µl test sample in PBS - Tween added. *

↓

Incubated overnight at 6°C

↓

wash

↓

150 µl enzyme - labelled gammaglobulin added in PBS=Tween.

Incubated 3-to-4 hours at 37°C.

↓

wash

↓

300 µl p-nitrophenyl phosphate substrate in diethanolamine buffer added.

Incubated 0.5 to 2 hours at room temperature.

↓

Reaction stopped with 50 µl 3.0 m - NaOH.

Reaction yellow colour

↙ Visual assessment

↘ Photometric measurement at 405 nm

* This was a heat killed suspension of *Shigella flexneri 2b* isolated in Tasmania. The suspension was diluted to contain approximately 15,000 organisms per 150 µl. Unfortunately it was found the incubation times had to be increased, up to six hours with the labelled gammaglobulin and up to eight hours at the colour development stage.

Fig. 7 shows the colour reactions. The negative results are clear, and positive results are varying degrees of yellow. Table 12 records the plate set up and results.

It will be noted that the yellow colour reaction in these initial experiments was poor. This was not unexpected due to the fact we were using commercially produced antibody which was intended for direct agglutination tests and therefore the content of gamma G was not high.

Irrespective of the quality of the sera, it can be seen that Row 8 which contains the *Shigella flexneri* antibody and antigen gives the strongest reaction overall, being positive at dilution of the conjugate at 1:500 and 1:1000 in Row 8 B, C and D, and at 1:500 in E to F after pre-freezing the *Shigella* to break down the cell structure, and in G and H after boiling the *Shigella* to break down the cell structure, and in G and H after boiling the *Shigella* for a second time.

It will be observed that there are some fainter cross reactions, for example in Row 7 containing *Shigella boydii*, except for 7C which gives a reaction as strong as the reactions in Row 8. The known *Shigella sonnei* tended to give hardly discernible colour changes in Row 9. Fainter colour changes in some of the wells in Rows 10, 11 and 12 containing enteroinvasive strains of *E. coli* are noted.

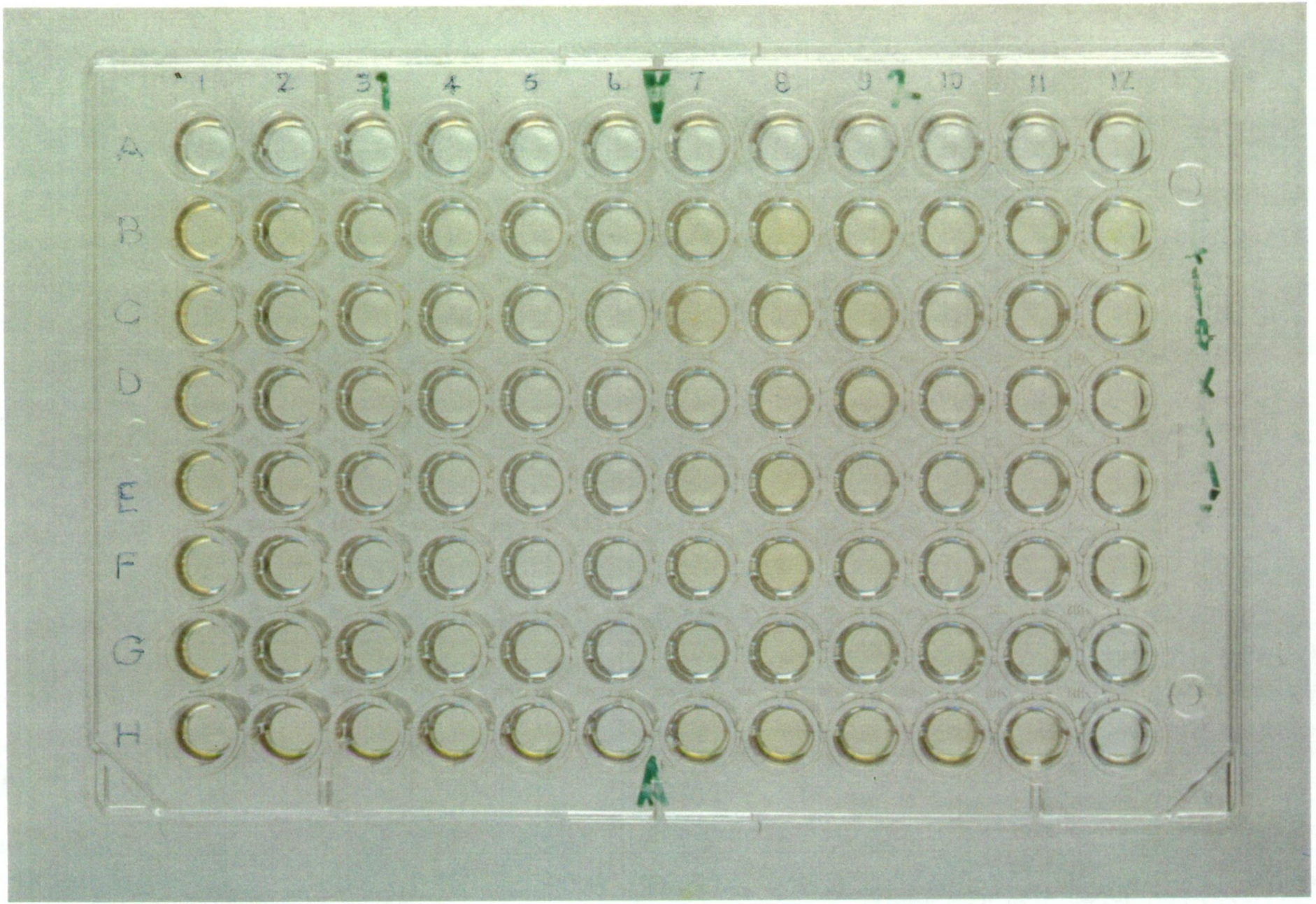


FIG. 7. INITIAL E.L.I.S.A. TEST USING COMMERCIALLY PRODUCED ANTISERUM
NOTE: FAINT COLOUR REACTION ONLY **(SEE TABLE 12 FOR KEY)**

TABLE 12

TEST: Shigella DESCRIPTION: _____ DATE: 16/12/88

COATING AG : S.flexner 2b (Anti) CONC: 1 x 2 ug/ml TIME: _____ TEMP: 5 37

TEST AG : S.flexner. E.coli + Shigella x 2 CONC: _____ TIME: _____ TEMP: 5 37

CONJ'D IgG : $^{1}/_{500} \times {}^{1}/_{1000}$ CONC: _____ TIME: _____ TEMP: 5 37

SUBSTRATE: p - nitrophenol phosphate CONC: 0.6 mg/ml TIME: _____ TEMP: Room ✓

NOTES: Shigella organisms frozen to break up cells to observe effect and also boiled to observe effect

1µg/Well (150µl containing 1µg) RESULTS 2µg/Well (150µl containing 2µg)

	boydii 1 13	flex 2 2b	Sonnei 3	E.coli 4 (AD)	E.coli 5 (ENT)	E.coli 6 (ENT)	boydii 7 13	flex 8 2b	Sonnei 9	E.coli 10 (AD)	E.coli 11 (ENT)	E.coli 12 (ENT)	
A	---	---	---	---	--- NEG	--- CONTROLES	---	---	---	---	---	---	A
B						--	D	++	D	-	-	D	B
C		COLOUR	REACTION				+	+	±	-	-	-	C
D		TOO UNSTABLE TO READ					-	+	±	-	-	-	D
E							D	++	-	-	-	-	E
F							D	++	-	-	-	-	F
G							-	+	D	D	-	-	G
H						---	D	+	D	D	-	-	H

(ENT)=ENTEROINVASIVE (D)=DOUBTFULL (++) = GOOD POS (+) = POS (±) = WEAK (-) = NEG

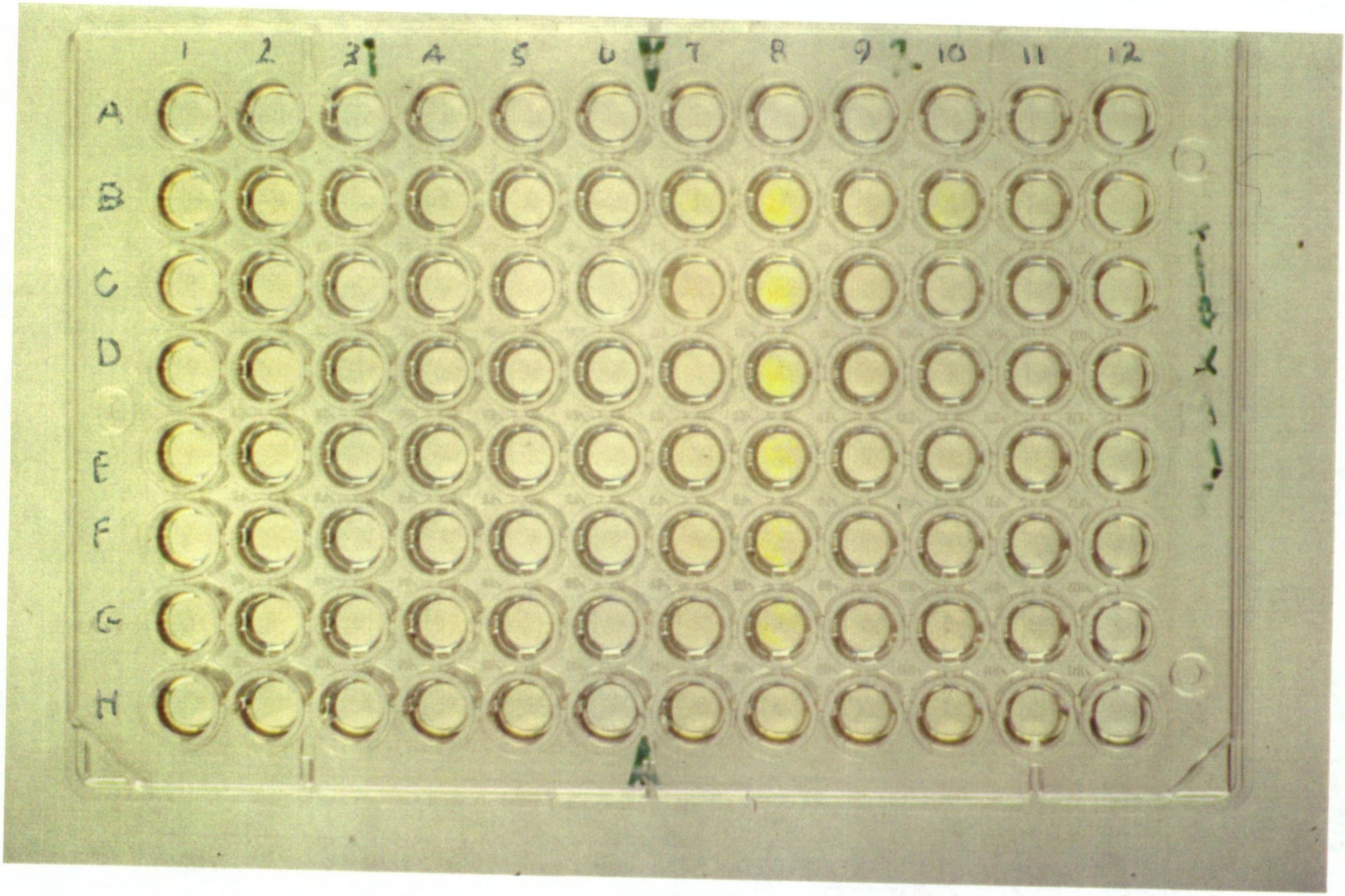


FIG. 8. INDIRECT E.L.I.S.A. TEST, USED FOR THE PRELIMINARY EXAMINATION OF THE *SHIGELLA* ANTISERUM PRODUCED IN A NEW ZEALAND WHITE RABBIT. **NOTE:** GOOD COLOUR REACTION AND SPECIFICITY.(SEE TABLE 13 FOR KEY)

Row A, wells 1 to 12, are negative controls with coating buffer. The best results for *Shigella flexneri* were in wells B8, E8 and F8 where the organisms had been frozen overnight before testing.

The wells on the left half of the plate A to H 1 to 6, to which 1 µg/ml of anti-*Shigella flexner* gammaglobulin had attached to the plate was ignored due to the lack of colour development.

Although the above technique showed promise the results were disappointing, which, as already stated, was not unexpected. In view of these results it was decided to produce our own *Shigella flexner* antisera in the hope of obtaining a high titre antibody mainly in the gammaglobulin fraction.

The culture of *Shigella* used for the immunisation of the rabbit was selected for specificity, smoothness and agglutination ability. The method of Edwards and Ewing (1970) was used for the immunisaiton of a New Zealand White rabbit. Their method of heating only the antigen was used in preference to a formalised culture. The rabbit was inoculated at five-day intervals over a period of 25 days, and then bled seven days later and tested for the production of *Shigella* antibody.

It was found that there was a much higher gammaglobulin antibody content than in the original commercially produced agglutinating antisera, and in fact it was approximately twelve times as much.

Preliminary checking for the suitability of the antisera, for use in the E.L.I.S.A. technique, was carried out using the indirect E.L.I.S.A. method. In this method, the wells were

first coated with the antigen (*S. flexneri 2b*), and then the antiserum from the rabbit tested at dilutions from 1:500 to 1:3000 at intervals of 500.

It will be seen from Fig. 8 and Table 13 that the colour reactions are more pronounced, giving a very good positive result up to well G. 8 (1:3000).

There are some faint cross reactions with *S. boydii* in well B. 7 at a 1:500 dilution, and with one of the *E. coli* wells, B. 10, at a 1:500 dilution.

In view of the promising results, it was decided to repeat the original double antibody sandwich technique using the new antiserum to coat the wells. Due to the previous good reactions by the indirect technique, the incubation times were run at varying intervals.

7.4 RESULTS

It was found with the new antiserum that the incubation time with the sample could be cut to six hours at 37°C or still left overnight at 6°C. The incubation time with the enzyme labelled gammaglobulin worked in two-to-three hours, and the colour development took place within fifteen minutes and was complete at forty minutes.

The results are set out in Fig. 9 and Table 14. It will be noted the results are excellent and specific for *S. flexneri 2b*. No cross reactions occurred against *S. boydii, S. sonnei, E. coli, S. flexneri 6* or reagent controls.

The ideal amount of the antibody gammaglobulin for coating the wells was found to be 1 µg per well. At 2 µg per well, a

TABLE 13

TEST: _____ DESCRIPTION: _____ DATE: _____

COATING AS AG: flexner 2b (Antigen) CONC: _____ TIME: _____ TEMP: 5 37

TEST AG AS: _____ CONC: _____ TIME: _____ TEMP: 5 37

CONJ'D IgG SpA: 1/500 - 1/300 CONC: 1 ug/as well TIME: _____ TEMP: 5 37

SUBSTRATE: p - netrophenol phosphate CONC: _____ TIME: _____ TEMP: Room ✓

NOTES: S.flex 2b used to coat well 8. B - G

Controls S. boydii 6 Wells 7. (B - G), S. sonnei Wells 9 (B - G), E. coli Wells 10. 11 & 12 (B - G)

RESULTS

	1	2	3	4	5	6	boydii 7	flex 2b 8	sonnei 9	E. coli 10	E. coli 11	E. coli 12	
A	-	-	-	-	- ←NEG. CONTROLS→	-	-	-	-	-	-	-	A
B				dilution of antisera	1/500		±	++	-	+	-	-	B
C					1/1000		±	++	-	-	-	-	C
D			N.A.		1/1500		-	++	-	-	-	-	D
E					1/2000		-	++	-	-	-	-	E
F					1/2500		-	+	-	-	-	-	F
G					1/3000		-	+	-	-	-	-	G
H				←COATING BUFFER CONTROL→									H

(D)=DOUBTFUL (+++)EXCELLENT (++) = GOOD POS (+) = POS (±) = WEAK (-) = NEG

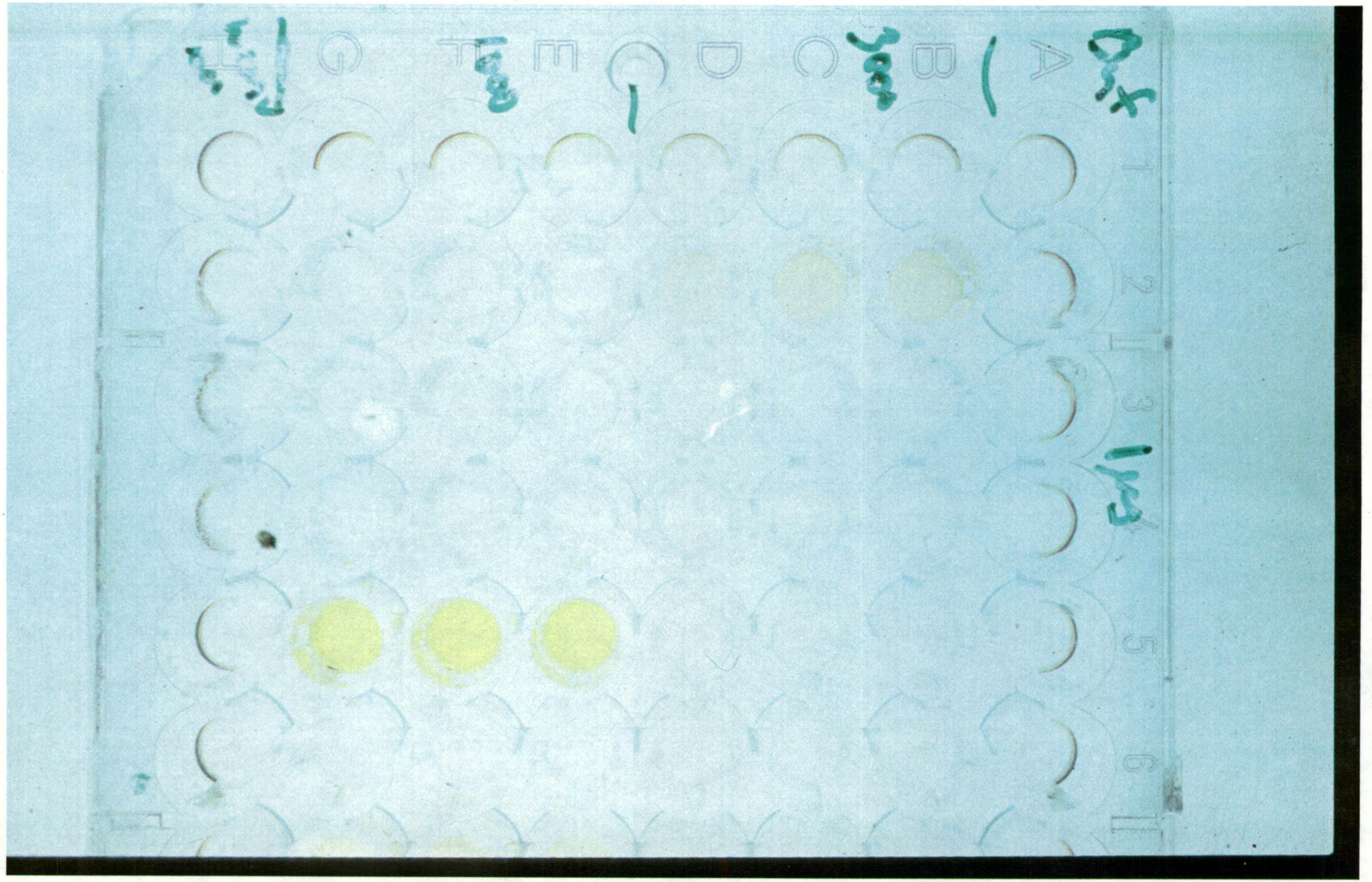


Fig 9. Shows excellent colour reaction and specific results for the newly adapted E.L.I.S.A. test. (see Table 14 for key) Notel better reaction at 1:1000 dilution.

able 14.

TEST: Shigella DESCRIPTION: _____ DATE: _____

COATING AS flexner 2b (Anti) CONC: _____ TIME: _____ TEMP: 5 x 37

TEST AG Shigella & E.coli CONC: _____ TIME: _____ TEMP: 5x 37x

CONJ'D IgG 1/500 - 1/3000 CONC: _____ TIME: _____ TEMP: 5 37x

SUBSTRATE: p - nitrophenol phosphate CONC: _____ TIME: _____ TEMP: Room x

NOTES: final coating 1µg AS PER WELL

Known Shigella organisms tested against anti flexner 2b

RESULTS

	S.boydii	S.flex 2b	S.sonnie	E.coli	S.flex 2b	E.coli	S.flex 6						
	1	2	3	4	5	6	7	8	9	10	11	12	
A	–	Buffer	Controle	–	–	–	–						A
B	–	+/–	–	–	NU	–	–						B
C	–	+/– 1/3000	–	–	NU	–	–						C
D	–	+/– 1/	–	–	NU	–	–						D
E	–	NU	–	–	+++	–	–						E
F	–	NU	–	–	+++ 1/1000	–	–						F
G	–	NU	–	–	+++ 1/	–	–						G
H	–	COATING BUFFER CONTROLES			–	–	–						H

ell not used (NU), Excellent Positive Reaction (+++), Strong Positive (++), Positive (+), Doubtful (±), Negative (–)

yellow colour developed with the coating buffer. Although this was weak in comparison with the strong known positive tests, it could cause confusion and result in it being read as a positive result.

7.5 CONCLUSION

In view of the promising results, in which it will be noted, an identification could be madein ten hours or less for the presence of *Shigella* organisms, it has been decided to investigate this rapid method of identifying *Shigella* under a separate research project.

It will be obvious that a number of aspects still need to be investigated. Will the same good results be obtained with a polyvalant antiserum, and will the results be specific for all the *Shigella* strains? Can the test be refined further, (which work to date indicates can be done) to produce, in kit form, a rapid one-well method which could be used in developing countries for the identification of *Shigella* in faecal specimens?

It will be realised that such investigations cannot be covered in this present research. Time, special equipment and specimens from patients suffering from *Shigella* infection are some of the essentials required before such an investigation can be carried out.

Chapter 8

GENERAL DISCUSSION AND CONCLUSIONS

8. GENERAL DISCUSSION AND CONCLUSIONS

This research has covered a wide spectrum of problems concerned with the detection, isolation and identification of *Shigella* and *E. coli* strains causing bacillary dysentery in Tasmania.

Although the objectives set out were in themselves important, it was felt that other important and perhaps wider problems concerning bacillary dysentery could also be examined at the same time. These included the identification and recording of other infective agents in the specimens to be investigated. To examine the subject in a wider context than for Tasmania alone, in the hope of being able to contribute constructive suggestions and methods in the diagnosis of dysentery, not only in Tasmania but in the developing world where these infections are a continuing scourge resulting in millions of deaths each year (Smith, 1981), especially among young children, and further complicated in some areas by primitive laboratory facilities.

It is obvious that in such situations, a simple and rapid method for the identification of the infection would be of invaluable help and the feasibility of achieving such a method was incorporated in the research.

It will be appreciated that in view of the extent of this research, involving many techniques, some new and not normally carried out in the average medical laboratory, plus the time involved including the co-operation needed from many other people, resulted in a very involved and taxing regimen.

Therefore by necessity, in retaining continuity, the investigations in this research have until now been set out under individual headings.

Before any other part of the research could commence, it had to be established which of the present available media and techniques would result in the most accurate isolation and identification of *Shigella* and the pathogenetic *E. coli*. This was essential in avoiding false statistics when one of the main aims was to establish the true incidence of *Shigella* infections in Tasmania. This aspect of the research was both enlightening and rewarding.

It has been established that presently used culture media leave a considerable amount to be desired in their reliability to result in the growth and isolation of the bacillary dysentery group of organisms (Chapter 3 and Fig. 1), confirming the findings of other studies, e.g. Morris et al., 1971; Bhat et al., 1975, and Goyal et al, 1981. Despite the many papers published over the years (Croft and Miller, 1956; Taylor, 1965; Taylor and Schelhart, 1968; Rollender, 1969; Dunn and Martin, 1971; Goo et al., 1973, and Goyal et al., 1981) pointing out the shortcomings of many culture media intended for the isolation of *Shigella*, and specific papers, e.g. McCarthy, 1966; King and Metzager, 1968, and Bhat and Rajan, 1975, stating the advantages of X.L.D. and Hektoen medium for the isolation of *Shigella*, it is more than surprising that many laboratories today still do not use these media, either individually or together.

Some laboratories continue to use media that was marketed over thirty years ago; a few use media that is quite unsuitable for the recovery of *Shigella*. It would seem that the culture medium used will to some extent depend on personal preference, cost and occasionally experience (Chapter 3 and Table 7).

It has been confirmed from this research that X.L.D. is the medium of choice, used in conjunction with Hektoen or some other

selective culture medium, and a less selective medium such as MacConkey.

Even though such a combination of culture medium is more likely to result in a higher recovery of *Shigella* organisms, at the same time it must be accepted that not even X.L.D. is one hundred per cent on its recovery rate, and it should be used in conjunction with an enrichment media which in this research, G.N. (Hajna) was found to result in the best recovery. One other aspect which causes poor recovery is the number of *Shigella* organisms present in the specimen. It has been shown that when there are small numbers, or few *Shigella* organisms present in a specimen containing moderate or large numbers of normal intestinal flora, (Chapter 3, Table 8) then recovery is markedly diminished on direct plating. This problem can be partly overcome by the use of an enrichment medium, but not always.

This would explain why in areas of the world where severe outbreaks of *Shigella* take place, recovery can be made on most enteric culture media, even if only a small number of colonies are isolated (provided the specimen is collected correctly). This is due to the fact that the patient is excreting large numbers of *Shigella* and little else.

On the other hand if we are faced with a moderate or mild infection, and investigating carrier states, then the recovery and isolation of the *Shigella* is made more difficult or impossible unless X.L.D. and an enrichment broth are used.

This all emphasises the point that there is still need for more research into producing better culture media and faster methods for detecting *Shigella* infections.

Having confirmed that X.L.D. in conjunction with an enrichment broth and Hektoen and MacConkey were the culture media of choice, it has now been established that the low number of *Shigella* cases previously reported in Tasmania (0.2 per 100 000 of population) is not a true reflection of the state of affairs here. The true number of cases found to be occurring in Tasmania is, in fact, in the region of ten cases per 100 000 each year.

The results show that if and when diarrhoeal infections are sent for laboratory investigation in Tasmania, then *Shigella* is found to be present in the patients' faeces much in the same numbers as in other similar countries, e.g. Britain, Ireland, Sweden, etc.

In regard to the reasons for the previous low numbers reported, the evidence points to the following reasons in order of importance.

1. The non-laboratory investigation of large numbers of patients suffering from diarrhoea.
2. Some laboratories not using the right combination of culture media, even when specimens are sent for investigation.
3. Specimens being collected incorrectly or being sent over long distances without being placed in transport media, e.g. a patient being requested to send his own specimen to a laboratory. If the specimen was collected in the morning and arrived at the laboratory in the afternoon, then the chance of recovering the *Shigella* is reduced by fifty per cent.

During the research it became clear that the main reason for the misleadingly low reported incidence of *Shigella* in Tasmania was due to the majority of infections being clinically moderate-to-mild in nature. In this case the majority of medical practitioners tended to treat the symptom conservatively, making no

requests for laboratory culture unless the symptoms worsened or continued for a prolonged period.

Whilst this would seem the correct practical procedure to follow, it contributes little to the area of public health, or to reflect the true state of affairs as to the real causes of diarrhoeal infections, be they bacteriological, virological or parasitological in nature. As stated by Velimirovic (1984), 'This is perhaps of little importance from the individual point of view, but for epidemiological purposes accurate aetiological diagnosis is essential'.

The above evidence is supported by the two surveys carried out and detailed in Tables 10 and 11. When the co-operation of medical practitioners was obtained to submit specimens that would not normally have been sent to a laboratory, not only was *Shigella* isolated, but many pathogens known to cause diarrhoea, e.g. *Salmonella, Aeromonas,* enterotoxigenic and enteroinvasive *E. coli, Giardia lambia,* etc.

In the Willow Court investigations one of the surprising results was the prevalence of intestinal protozoa, which was similar to those recorded in Queensland by Karik et al., (1971) rather than those normally reported in Tasmania.

Another interesting finding was the trophozoites of *Dientamoeba fragilis*, not previously recorded in Tasmania, and which can cause abdominal pain and diarrhoea. A pointer to the scrutiny that should be kept on imported infections is the finding of *Trichuris trichiura,* a species commonly recorded in overseas travellers and S.E. Asian refugees in Tasmania but not in the general community. This could happen with *Shigella* infections in Tasmania, such as those already reported in Japan (Yoshifumi Tekeda, 1981).

It is also very interesting to note that out of the eight shigellae detected, six were *Shigella boydii*. In most Western countries, over the years *Shigella sonnei* has become the predominant infection followed by *S. flexneri, S. dysenteriae* and with *S. boydii* showing the lowest incidence (about ten per cent of total numbers). The percentage in this investigation of *Shigella* in Tasmania showed that 75% of the cases were *S. boydii*.

This subgroup distribution pattern was exemplified by the frequency of the subgroups in travellers returning to the United Kingdom (1972-1978). About 70% of the *Shigella boydii* infections occurred in persons who had recently returned from developing countries (Velimirovic, 1984).

Again, although *Shigella boydii* is the lowest incidence strain in the United States, in an investigation carried out on water in Los Angeles County (Servillo et al., 1988) in which a number of people became infected at a recreational swimming site, 16% of the *Shigella* isolated were *S. boydii*.

Not one of the Tasmanian cases was related to any other, thus we are left with some intriguing questions.

1. Due to the fact that we are in the Asian region, is *S. boydii* an indigenous infection in Tasmania like *Shigella sonnei* is in the U.K.?
2. Is the infection brought here from Asia? None of those investigated had left Tasmaina in the last twelve months.
3. In view of the fact that humans are the common reservoir of *Shigella,* has *S. boydii* been here for many years, perhaps centuries, but not detected often, due to the non-laboratory investigation of most diarrhoeal cases?

Having established the **situation in regard to the incidence** of the shigellae in Tasmania, it came as no surprise that cases of diarrhoeal infections were caused by some strains of *E. coli*. What was surprising was the high number detected (9 EIEC, 3 ETEC and 6 *E. coli* A-D (Alkalescens - Dispar), a total of 12 at least, even if for the moment we ignore the debatable *E. coli* (A-D).

Enterotoxigenic *E. coli* (ETEC) is a major cause of diarrhoeal illness of children in developing countries, and is by far the most common cause of travellers' diarrhoea (70% - Velimirovic, 1984), often producing severe dysentery-like, or cholera-like, disease (Cvjetanovic, 1988; Cartwright, 1988).

Enteroinvasive *E. coli* (EIEC) have been isolated from older children and adults with dysentery-like disease. The EIEC have been responsible for extensive epidemics, e.g. 152 cases at a holiday camp in the German Democratic Republic in 1976, and a large outbreak in thirteen States of the U.S.A. from imported French cheese (Velimirovic, 1984). Unfortunately the detection of these *E. coli* infections is scientifically difficult, particularly in laboratories with limited facilities.

What is interesting is their detection in such numbers. In the 201 cases investigated, all suffered diarrhoeal illness disease but none had a history of travelling in the previous twelve months or had any mutual contact.

Therefore, it can only be assumed that in Tasmania at least, these two organisms are not an infrequent cause of diarrhoeal illness. The reasons for this are open to further investigation, but it is known ETEC have been isolated in many foods suspected of being the cause of diarrhoeal illness in Europe, e.g. shrimps,

mushroom and potato salad, hot dogs, bamboo shoots, cooked chicken, and cold beef etc. Poor sewage disposal systems, and cheese (as already stated) have been responsible for infections due to EIEC. In view of the fact that Australia is a country of high fast food consumption, and recently it is becoming obvious that sewage disposal in Australian States, including Tasmania, leaves much to be desired, then these factors may explain the high incidence of these infections in a small population for no obvious cause.

The *E. coli* (A-D) are a difficult group to hold responsible for diarrhoeal infections. Cruikshank et al., (1975) state rightly 'In their biochemical reactions they are easy to confuse with *Shigella*', but continue, 'They resemble *E. coli* in occurring in the intestine of healthy persons and in some cases of urinary tract infections'. As already stated previously many diagnostic aid procedures, e.g. Microbact 24 E, A.P.I., and reference laboratories no longer differentiate or report this group of organisms. Others like the Roche Enterotube 11 do.

Wellcome and Difco still produce diagnostic Alkalescens-Dispar antiserum. It would seem there is still some confusion concerning this group of organisms, but most laboratories identify them occasionally purely to exclude *Shigella* with which they can be confused, or in urinary tract infections in which the biochemical results are not those of an obvious *E. coli*.

It is interesting to note that these *E. coli* (A-D) isolated in Tasmania, all of which had been checked by Roche Enterotube 11, other biochemical tests, and with Wellcome and Difco antiserum, also gave a positive radioactive test result for enteroinvasiveness.

The microorganisms in the Alkalescens-Dispar (A-D) group are anaerogenic, nonmotile biotypes of *E. coli*. Edwards and Ewing (1970) state, 'Admittedly, the group is an artificial one and is retained simply as a matter of expediency'. In the early years many papers investigated Alkalescens-Dispar (A-D), (Braun, 1953; DuPont, 1955; Ewing et al., 1956) and the background and history of this subject is well covered in Edwards and Ewing (1970).

No recent papers or comments dealing with this aspect of the subject can be found, (confirmed by Dr John Murray, Flinders University, personal communication), and this again raises questions for further investigation.

1. In view of the fact that few laboratories now specifically identify *E. coli* (A-D - Alkalescens-Dispar), is it possible that some of the enteroinvasive *E. coli* are those which used to be labelled Alkalescens-Dispar?
2. Is a small number of the EIEC giving false reactions with the Enterotube 11 and specific antiserum for Alkalescens-Dispar?
3. Are these reactions peculiar to Tasmanian strains?

It will be noted under 6.2 that 82 *E. coli* were investigated, and of these, 67 gave negative results for enteroinvasiveness as did all the known negative controls, and positive controls gave the correct results. It would be extremely difficult then, to visualise a situation where only the *E. coli* (A-D) gave false results.

Even if we ignore the six cases of *E. coli* (A-D), the incidence of EIEC and ETEC is still higher than would be expected on the number of microorganisms causing diarrhoeal infections. No

E. coli O157:H7 were detected during the research. The media used for the isolation of *Shigella* was also found to be satisfactory for the isolation of the pathological *E. coli* provided a MacConkey plate was also incorporated to detect those few pathological *E. coli* which are adverse to some selective media, even XLD.

Present methods for detecting EIEC and ETEC cannot be recommended except for research or in reference laboratories. Present methods are expensive, time consuming, and call for experience and expertise, and seem quite unsuitable for use in a routine clinical laboratory.

Having achieved most of the main aims of this research, investigations were then commenced into the possibility of developing a more rapid technique for the identification of *Shigella*, which would be an immense asset to most laboratories, but especially so to those in the developing countries where equipment, staff and money are at a premium.
It was decided to examine the possibility of producing an ELISA test. This did not need to be quantitative - a single colour reaction would be ideal for small laboratories or clinics.

In the first instance the method set out by Clark and Adams (1976) was followed in co-operation with Dr P. Guy (Research Officer, Ministry of Agriculture Laboratories, Hobart) who has had extensive experience with ELISA methods in the detection of plant viruses.

As can be seen from the report of techniques in Chapter 7, at first our experiments were a little disappointing, which was not unexpected due to the fact that we were using commercially

produced antiserum which was intended for slide and tube agglutination tests. Added to this was the problem of cross reactions, especially with some of the *E. coli*, when using *Shigella* antisera.

Despite these problems, it was felt further research should be continued, especially in view of the fact that E.L.I.S.A. methods have been proved successful for the investigation of plant viruses (Clark and Adams, 1977) and in enhanced chemiluminescent enzyme immunoassays (Kricka and Thorpe, 1986) for parasites.

After producing a good *Shigella* antibody, excellent results were obtained with the E.L.I.S.A. method which had been adapted for the identification of *Shigella* organisms in the faeces of patients suffering from diarrhoeal infections, and further research is contemplated.

SUMMARY

Culture media, currently available for the isolation of *Shigella* have been evaluated and X.L.D., in conjunction with an enrichment medium (G.N. Hajna), were found to give the best recovery of the shigellae.

It has been shown that *Shigella* infections occur in Tasmania at a much higher level than was previously accepted. At the same time, it was shown that the true incidence of *Shigella* in Tasmania is no higher than many of its European counterparts.

The enterotoxin and enteroinvasive *E. coli* have also been shown to play a significant role in diarrhoeal infections in

Tasmania, and *E. coli* (A-D) is seen to be worthy of further investigation as a possible cause of diarrhoea.

A rapid method of detecting *Shigella* infections has been evolved, which shows promise and seems deserving of further research.

APPENDIX I

APPENDIX 1

App 1.1 INTRODUCTION

Certain strains of *E. coli* carry genes for enterotoxin production that may cause a diarrhoeal disease in humans and in the young of some farm animals, e.g. calves, lambs and piglets (Smith et al, 1970).

At least four types of *E. coli* enterotoxins have been characterised and the mechanism of their action described (Burgess et al, 1978). The heat labile toxin (LT) is similar to cholera toxin and is detected by visualising cytopathic effects on cultured mouse adrenal cell lines (Donta et al, 1974). The heat stable enterotoxin ST I (StI -a; Porcine, StI - b; Human) is detected by the use of suckling mouse assay (Dean et al, 1972) whereas the ST II toxin is assayed in ligated jejunial loops of pigs.

The genes encoding these enterotoxins are present on transmissible plasmids. They have been cloned by recombinant DNA techniques and their nucleotide sequences determined (So and McCarthy, 1980; Moseley et al, 1983; Leong et al, 1984; Yamamoto et al, 1984). DNA colony hybridization, originally developed as a screening method to detect recombinant DNA in bacterial isolates (Grunstein and Hogness, 1975) using cloned DNA or synthetic oligonucleotides as probes, is a reliable replacement for current tedious and expensive assays to detect enterotoxigenic *E. coli* (Moseley et al, 1980).

App 1.2 MATERIALS AND METHODS

The following reagents were supplied by BRESA in their test kits.

a) Membrane/Filter Sterile Pack, containing five cellulose acetate membranes marked with grids;

b) Whatman Filter Papers, 10 cm x 12 cm;

c) Polythene Heat Sealable Plastic;

d) Lysozyme, one vial containing 25 mg Lysozyme as a lyophilised powder, to be resuspended in 500 µl sterile distilled water;

e) Proteinase K. One vial containing 2 mg of Proteinase K as a lyophilised powder, and to be resuspended in 500 µl of sterile distilled water;

f) Denhardt's Solution. One vial containing 1.2 ml of sterilised 250 x Denhardt's solution;

g) Enzyme - Labelled Oligonucleotide Probe. One vial containing 1 µ g oligonucleotide coupled to alkaline phosphatase in a lyophilised form. This is to be dissolved in 4 ml of sterile water and add 16 µl of 5M NaCl and mix;

h) Nitro Blue Tetrazolium (NBT). One vial containing 250 µl of stock solution of 75 mg/ml NBT in 70% dimethylformide;

i) 5 - Bromo - 4 - Chloro - 3 - Indolyl Phosphate (BCIP). One vial containing 250 µl of stock solution of 50 mg/ml BCIP in dimethylformide.

SOLUTIONS REQUIRED

1. 5M NaCl (100 ml):	Dissolve 29.2 g of NaCl in 80 mL of distilled water. Adjust volume to 100 ml and sterilise by autoclaving.
2. 20 x SSC (1 litre):	3M NaCl, 0.3M Sodium Citrate
3. Lysing solution (200 ml):	0.5M NaOH, 1.5M NaCl
4. Neutralisation solution (200 ml):	1M Tris-Cl pH 7.0, 3M NaCl

5. Solution A (200 ml): 50mM Tris-Cl pH 8.0, 125mM NaCl, 10mM EDTA pH 8.0, 0.5% SDS.

6. Formaldehyde solution (50 ml): Mix 5 mL of 37% formaldehyde w/w with 45 ml of 20 x SSC. Store at room temperature (maximum 2 weeks).

7. Ethanol (redistilled)

8. Prehybridization solution: To make 2 ml , add the following:

Reagent	Vol. (ml)
20 x SSC	0.6
250 x Denhardt's Solution (Supplied BRESA)	0.2
20% SDS	0.05
Distilled H_2O	1.15

9. Hybridization solution: To make 2 ml , add the following:

Reagent	Vol. (ml)
20 x SSC	0.6
250 x Denhardt's Solution (supplied BRESA)	0.01
20% SDS	0.05
Distilled H_2O	1.34

10. Washing solution I (1 litre): 2 x SSC, 0.5% SDS, 0.5% Tween 20

11. Washing Solution II (1 litre): 0.1M Tris-Cl pH 9.5, 1M NaCl, 5mM $MgCl_2$

12. Solution B (100 ml): 0.1M Tris-Cl pH 9.5, 0.1M NaCl, 5mM $MgCl_2$

13. Termination solution (100 ml): 10mM Tris-Cl pH 8.0, 1.0mM EDTA pH 8.0

14. Sterile distilled water.

Although faeces may be inoculated directly onto the acetate membrane, in this survey the suspected *E. coli* were first isolated on X.L.D. media and individual colonies removed from the X.L.D. plate and placed into peptone water for four hours at 37°C.

The membrane was placed grid side up on MacConkey agar and then

inoculated by dotting 3 µl from each peptone water containing the individual suspected *E. coli* onto the individual grids. Each specimen was confined to one grid and a one grid space left between each specimen. In this way some forty specimens could be dotted on each membrane. A notch was made in one section of the membrane to identify the top line of inoculation, and a duplicate record kept by numbering the grids on a photocopy (Fig. 5).

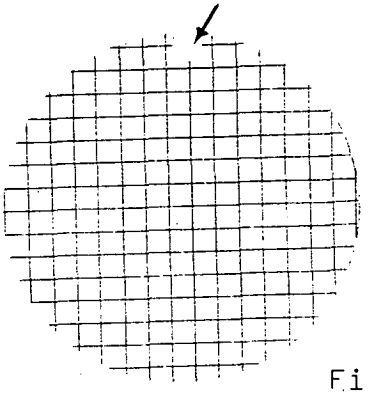


Fig. 5

Membrane Processing

1. After incubation of the membrane at 37°C for 18 hours on a MacConkey agar plate, the membrane was removed from the agar plate and placed on a Whatman filter paper (3MM Chr) well saturated with lysing solution and left at room temperature for twenty minutes.

2. The membrane was then transferred to a new filter paper saturated with neutralisation solution and left at room temperature for ten minutes.

3. The membrane was then transferred to a new filter paper saturated with solution A. It was left at room temperature for ten minutes.

4. The membrane was then transferred to a petrie dish containing 1 ml of solution A plus 100 µl of lysozyme stock solution, layer-

ing the membrane on the surface and being careful not to immerse it. The membrane was left in this solution for fifteen minutes.

5. To ensure DNA immobilisation the membrane was then placed on a filter paper saturated with formaldehyde solution and left at room temperature for six minutes.

6. The membrane was then transferred to a dry Whatman filter paper and left to dry by placing it 10 cm beneath a strong white light, i.e. Phillips 250 w infra red, made by Phillips Holland.

7. The membrane was then gently immersed in 10 mL of solution A (in a petrie dish) and left for one minute. Once wet the membrane regains its original flexibility.

8. A pouch was made from the polythene (supplied BRESA), and the membrane placed in the pouch. To 2 ml of fresh solution A was added 100 µl of Proteinase K stock solution. This was mixed and added to the pouch containing the membrane and sealed. It was then incubated at 50°C for thirty minutes in a shaking water bath. During this period all visible cell debris "peel off" the membrane.

9. The membrane was then removed from the pouch and immersed in 50 ml of ethanol for two minutes and then air dried. At this juncture the membrane can be stored at room temperature between filter circles.

Hybridization

10. The membrane was placed again into a polythene heat sealable pouch. Two ml of prehybridization solution was added, the pouch sealed and incubated at 50°C for forty-five minutes in a shaking water bath.

11. The membrane was removed and placed into a new polythene pouch. Two ml of hybridization solution was added plus 4 µl of probe stock solution, mixed, the pouch sealed and incubated at 50°C for forty-five minutes in the shaking water bath.

12. After hybridization, the membrane was removed from the pouch and placed in a wash tray containing 150 ml of washing solution I. Washing was carried out at room temperature with agitation for fifteen minutes. This washing was repeated once and then a final wash at room temperature for fifteen minutes was carried out.

13. Finally, the membrane was washed in at least 150 ml of washing solution II at room temperature twice for fifteen minutes each. This washing is important to free the membrane of SDS in order to avoid precipitation of substrate in Step 14.

Detection

14. The washed membrane was transferred into a petrie dish containing 5 ml of substrate solution. The substrate solution is made up as follows: add 20 µl NBT solution to 5 ml of solution B and gently mix. To this mixture add 20 mL of BCIP solution and mix. The substrate solution is made freshly before use.

15. The colour development is stopped by immersing the membrane in at least 10 ml of termination solution for five minutes at room temperature.

See Fig. 3 for colour reaction.

App 1.3 DISCUSSION

It will be seen that this method is, as stated by Moseley et al (1983), less tedious and expensive than other current assays for

enterotoxigenic *E. coli*. At the same time, although it was an excellent tool for this survey and research, it is still a time-consuming technique and requires the gaining of experience in this area of work if mistakes are to be avoided.

It would be difficult to recommend any of the current assays to any routine laboratory for the diagnosis of E.T.E.C. infections. The method described here would be of value if it was decided to set up a reference laboratory for such infections. A large number of tests could be carried out in a day and a half, large stocks of reagents could be made economically and the BRESA kits easily obtained at moderate cost. But for a routine clinical laboratory with perhaps only the occasional case of diarrhoea due to suspect *E. coli* infection, it has no advantage. It would seem then, that for the most part E.T.E.C. infections will go on being undiagnosed until perhaps such laboratories are able to obtain latex test methods such as the Oxoid Latex Test for *E. coli* O157:H7 implicated in haemorrhagic colitis.

App 1.4 CONCLUSION

The DNA probe (BRESA) method was found to be excellent for this survey and research project, but could not be recommended as a routine technique in the average clinical diagnostic laboratory without having large numbers of diarrhoea cases to investigate.

APPENDIX II

APPENDIX II

App 2.1 INTRODUCTION

Enteroinvasive *E. coli* (E.I.E.C.) are capable of producing bacillary dysentery in humans of all age groups. One of the first cases to be recognised was in Italy during 1944 and 1945 (Ewing and Gravatti, 1947). Since that time, E.I.E.C. have been found in many parts of the world. (Ewing et al, 1963; Gross, 1983). One of the best known outbreaks was in the United States between October 30 and December 10, 1971 (Marier et al, 1973; Tulloch et al, 1973). The vehicle of transmission being imported soft cheese. There were at least 387 cases in thirteen States and the District of Columbia. No reports of work on the isolation or frequency of E.I.E.C. can be found for Tasmania. Therefore it was decided to investigate the frequency using radioactive DNA probe technique.

APP 2.2 MATERIALS AND METHODS

The radioactive method (using ^{32}P) was the technique used. The DNA probe was sent to us by courtesy of Dr Roy Robins-Browne from the University of Melbourne. The probe itself was a 17.kb EcoR1 fragment cloned from *Shigella flexneri* serotype 5 and ligated into pBR332. The resultant plasmid, pMR17 carries antibiotic resistance markers. This probe was isolated from an unamplified culture of the *E. coli* using an adaptation method of Birnboim and Doly (1979) and the Nick Translation Kit was obtained from BRESA (Adelaide, South Australia). This work was performed in the Department of Medicine molecular biology laboratory with the co-operation and direction of Dr Greg Woods.

Preparation of Cellulose Acetate Membrane

A. A membrane was aseptically transferred onto a MacConkey Agar plate.

B. Using sterile toothpicks, suspect *E. coli* colonies were inoculated onto individual grids of the membrane, Fig. 6.

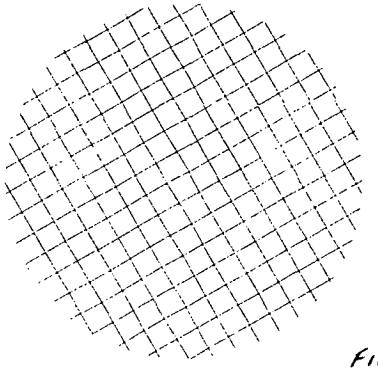


Fig 6.

The following reagents had been prepared.

Denaturing solution	0.05M NaOH 1.5 M NaCl
Neutralising solution	1.5 M NaCl 0.5 M Tris Cl pH 8.0

2 x SSPE prepare 20 x SSPE and dilute accordingly.

20 x SSPE	174.0 grams NaCl 27.6 grams $NaH_2PO_4 . H_2O$ 7.4 grams EDTA

Made to 1 litre and pH adjusted to 7.4 with ION NaOH.

C. Pieces of Whatman 3 MM paper were cut to fit neatly into the bottom of four petrie dishes. The paper in the first dish was flooded with SDS and any excess poured off. Using a blunt ended forceps the membrane was removed from the MacConkey plate and placed colony-side up, on the SDS impregnated paper.

D. The membrane was then transferred to the paper in the second petrie dish which had been saturated with denaturing solution, and left for five minutes.

E. Membrane transferred to third petrie dish in which paper was saturated with Neutralizing solution for 5 mins.

F. The membrane was then transferred to the fourth petrie dish in which the paper had been saturated with 2 x SSPE and left for five minutes.

G. The membrane was then removed, placed colony side up, on a dry 3MM paper and allowed to dry at room temperature. The membrane was then placed in 'Glad Wrap' and exposed to UV light for two-to-five minutes. The membranes can then be stored at 4°C.

Hybridization

The following solutions were made in advance.

20 x SSC

175.3 g NaCl
88.2 Sodium citrate

made to one litre and pH adjusted to 7.0 with NaOH.

Prewashing solution

50 mM Tris Cl 8pH 8.0
1 M NaCl
1 mM EDTA
0.1% SDS

Prehybridization solution

50% formamide
5 x Denhardts
5 x SSPE
0.1% DSD
100 µg/ml denatured salmon sperm DNA

Denhardts solution (50 x)

5 grams Ficoll Type 400
5 grams Polyvinylpyrrolidone
5 grams (BSA Pentax Fraction V made up to 500 mL)

Denatured salmon sperm DNA.

The DNA (Sigma type III sodium salt) was dissolved in water at a concentration of 10 mg/ml and stirred for two-to-four hours at room temperature to dissolve. The DNA was sheared by passing

it several times through a 21 gauge needle. The DNA was boiled for ten minutes and stored at -20°C. Just before use, the DNA was heated for five minutes in a boiling water bath then chilled quickly on ice.

Hybridization technique

a) The membranes were floated in a tray containing 6 x SSC until they had become thoroughly wetted from beneath. They were then submerged for five minutes.

b) The membrane was then immersed in 300 prewashing solution for one-to-two hours at 42°C.

c) The prewashing solution was removed and the membranes (if more than one) placed back to back in plastic bags which had been heat sealed at two edges. Twelve ml of prehybridization solution was added to the bags, and the bags sealed. The bags containing the membranes were then incubated at 42°C for four-to-six hours.

d) The ^{32}P probe was denatured by heating for five minutes at 100°C, then chilled briefly on ice. The denatured probe was then added to the hybridization solution (formula as for prehybridizaton) and placed in a plastic bag as for (c). The membranes were then transferred from prehybridization solution to the hybridization solution, the bag sealed and incubated at 42°C for 24 hours.

Post Hybridization

a) A cut was made in one corner of the plastic bag and using a pasteur pipette, the hybridization mixture was removed from the

bag. This was placed in an appropriate discard container for radioactive material.

b) The membranes were removed from the plastic bags, placed in a container and washed as follows.

1. 400 ml 2 x SSC, 0.1% SDS. Ten minutes at room temperature.
2. Repeat.
3. 400 ml 0.16 x SSC, 0.1% SDS. Twenty minutes at 65°C.
4. Repeat 3.
5. Repeat 3.
6. 400 ml 2 x SSC. Five minutes at room temperature.

c) The membranes were then placed on a piece of 3MM paper and left to dry for one hour at 37°C.

d) The membranes were placed on fresh 3 MM paper and a piece of photographic film placed over the membrane and left overnight. The next day the film was developed and read. See Fig. 4.

App. 2.3 DISCUSSION AND CONCLUSION

The radioactive DNA probe for enteroinvasive *E. coli*, although an excellent and accurate technique, is expensive, time consuming and requires a laboratory constructed to carry out radioactive tests including the safe disposal of radioactive waste. It is also essential to have staff with training and experience to carry out the tests or oversee and train others.

Again, this is an excellent research method, or for use in specialised laboratories, e.g. reference laboratory.

APPENDIX III

APPENDIX III

Buffers

Coating buffer (pH 9.6)

1.59 g Na_2CO_3

2.93 g $NaHCO_3$

0.2 g NaN_3

in litre H_2O

PBS (pH 7.4)

8.0 g NaCl

0.2 g KH_2PO_4

2.9 g Na_2HPO_4 $12H_2O$

0.2 g KCl

02 g NaN_3

in 1 litre H_2O

PBS Tween

999.5 ml PBS Plus

0.5 ml Tween 20

Sample buffer (0.1 M phosphate pH 7.4 - 0.33 M NaCl)

81 ml of 0.2 M NA_2H PO_4 in 0.33 M NaCl

19 ml of 0.2 M NaH_2 PO_4 in 0.33 M NaCl

100 ml of 0.33 M NaCl

Enzyme gammaglobuline buffer (PBS-Tween-PVP-ovalbumine)

PBS-Tween containing 2% polyvinyl-pyrrolidone (PVP; BDH Chemicals, MW44,000); and 0.2% ovalbumin (BDH Chemicals).

Substrate buffer

97 ml Diethanolamine

800 ml H_2O

0.2 g NaN_3

Add HCl to give pH 9.8

Make up to 1 litre with H_2O

Reagents

Purified gammaglobulin, diluted in coating buffer to approximately 200 µl of coating buffer to contain 2 µg gammaglobulin

Alkaline phosphatase labelled gammaglobulin, diluted in enzyme gammablobuline buffer

Enzyme substrate

0.6 mg/ml p-nitrophenylphosphate (Sigma Chemicals) in substrate buffer.

3 M NaOH solution.

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