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I am submitting herewith a dissertation written by Mohammad Kazem Amin entitled "Growth Characteristics of Yersinia enterocolitica in Selected Food Products." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Technology and Science.

F. Ann Draughon

F. Ann Draughon, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of the Graduate School

GROWTH CHARACTERISTICS OF *YERINIA ENTEROCOLITICA* IN SELECTED FOOD PRODUCTS

A Dissertation

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Degree

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Mohammed Kazem Amin

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DEDICATION

To my parents, whose love and support I have cherished and always will.

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ABSTRACT

Growth characteristics of five strains of Yersinia enterocolitica in pasteurized milk inoculated with either 10 or 1,000 CFU/ml Y. enterocolitica and stored at 4° C for up to 21 days was determined. All five strains of Y. enterocolitica competed very well with background microflora of pasteurized milk and reached level of log 5.0 to 8.0 CFU/ml after 14 days at 4° C. The initial level of inoculation had little or not effect on the total number of Y. enterocolitica after 14 or 21 days in pasteurized milk at 4° C.

An improved isolation technique based upon the alkalotolerance characteristics of Y. enterocolitica was developed and compared with conventional direct plating (DP) for recovery and enumeration of Y. enterocolitica from pasteurized milk. The pre-plating alkaline diluent (PPAD) technique couple with plating on Cefsulodin-Irgasan-Novobiocin (CIN) agar virtually eliminated all interference from background microflora and allowed easy isolation and enumeration of Y. enterocolitica. PPAD technique was shown to be an effective method for recovery and enumeration of Y. enterocolitica from pasteurized milk stored at 4° C, when Y. enterocolitica reached late log phase growth or stationary phase growth.

The ability of Y. enterocolitica to penetrate and invade shell eggs and broken-out liquid eggs was investigated. Y. enterocolitica was recovered from shell eggs after 3 to 7 days using both the temperature differential (TD) and the pressure differential (PD) method of inoculations. The rate and extent of infection was substantially increased by

supplementation of suspension medium with 20 ppm iron. Y. enterocolitica grew rapidly in egg yolks and whole eggs and survived and grew somewhat slower in egg whites at 4° C. Supplementation of egg whites with 10 ppm iron improved growth of Y. enterocolitica in egg whites. Additionally, it was shown that grow patterns of Y. enterocolitica in raw liquid eggs were not significantly different ($P<0.05$) from those of pasteurized liquid eggs.

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CHAPTER I

INTRODUCTION

Yersinia enterocolitica is emerging as an important foodborne and waterborne enteropathogen with the unique characteristic of being capable of growth at refrigeration temperatures. *Y. enterocolitica* has been recognized to be among the most frequent causes of human gasteroentritis (Marks et al., 1980; Weissfeld and Sonnenwirth, 1980; Shayegani et al., 1981; Hookamp-Korstanje et al., 1986). Marks et al. (1980) reported that gastroenteritis due to *Y. enterocolitica* was second only to that of *Salmonella*. Similarly, Weissfeld and Sonnenwirth (1980) found that the incidence of yersiniosis was equal to salmonellosis. Since early 1970, several outbreaks of yersiniosis have been reported from the United States, Canada, Japan and European countries (Asakawa et al., 1973; Olsovsky et al., 1975; Eden et al., 1977; Black et al., 1978; CDC, 1982). Epidemiologically, food and water were suspected as the vehicle of infections in virtually all cases.

Y. enterocolitica has been frequently isolated from a variety of foods such as raw and pasteurized milk, pork, beef and their products. In the last ten years, pasteurized milk has been implicated as a vehicle of infection in two of three well-known documented outbreaks of yersiniosis in the United States (Black et al., 1978; CDC, 1982; Aulisio et al., 1983).

Occurrence of *Y. enterocolitica* in poultry and poultry products has also been reported (Leistner et al., 1975; Gutertz et al., 1976;

Norberg, 1981; DeBoer et al., 1982; Greenwood and Hooper, 1985). DeBoer et al. (1982) reported the recovery of Y. enterocolitica from 68% of tested poultry parts. Leistner et al. (1975) and Norberg (1981) recovered Y. enterocolitica from more than 25% of fresh and frozen chicken meat samples. The occurrence of Y. enterocolitica in poultry meat and poultry products suggests there is a possibility of contamination of eggs and egg products.

Out of a vast number of research projects on Y. enterocolitica, only a few have been devoted to the mode of growth of Y. enterocolitica in food products under refrigeration conditions. Therefore, the objectives of this study were: (1) to determine the growth characteristics of Y. enterocolitica in pasteurized skim milk and liquid eggs stored at 4° C; (2) to develop an improved isolation techniques for recovery and enumeration of Y. enterocolitica from pasteurized milk, and (3) to investigate the ability of Y. enterocolitica to penetrate shell egg and invade egg contents during storage at 10° C.

CHAPTER II

REVIEW OF LITERATURE

A. *YERSINIA ENTEROCOLITICA*

Classification and Characteristics

The genus *Yersinia* was proposed by Van Loghem in 1944 to be named after the French bacteriologist A.J.E. Yersin to include those species of *Pasteurella*: *Yersinia* (*Pasteurella*) *pestis* and *Yersinia* (*Pasteurella*) *pseudotuberculosis* which differed from the hemorrhagic septicemic group. Thal (1954) proposed the inclusion of the genus *Yersinia* in the *Enterobacteriaceae* family. In 1964, Frederiksen reported that several previous isolates including *Bacterium enterocoliticum* of Schleifstein and Coleman (1939), *Pasteurella pseudotuberculosis rodentum* of Hassig et al. (1949) and *Pasteurella* X of Daniels and Goudzwaard (1963) had similar biochemical reactions and antibiotic susceptibility patterns. However their 0 antigens were different from those of *Y. pseudotuberculosis*. Thus the author proposed classification of these group of microorganisms into a new species names *Yersinia enterocolitica* in the genus *Yersinia*.

The species *Y. enterocolitica* is composed of gram-negative, facultatively anaerobic, coccobacilli or rod shaped bacteria with a marked tendency toward pleomorphism. Cell morphology is affected by factors such as growth temperature, culture age and growth medium composition (Bottone, 1977). *Y. enterocolitica* strains are found to be nonmotile at 37° C but motile when grown at 30° C or below by means of peritrichous flagella (Bercovier and Mollaret, 1984).

However, nonmotile strains have also been reported (D'amato and Tomfohrde, 1981; Bottone, 1977). Y. enterocolitica strains are a biochemically heterogenous group with some reactions being temperature dependent (Bottone, 1977; Bercovier et al., 1979). All strains of Y. enterocolitica are classified into two groups referred to as typical and atypical strains. The typical strains of Y. enterocolitica are able to ferment glucose, arabinose, cellobiose, galactose, maltose, manitol, sorbitol and trehalose but not lactose, adonitol, dulcitol, raffinose, rhamnose or melibiose (Bottone, 1977). Swaminathan et al. (1982) divided atypical strains of Y. enterocolitica into the four major groups: sucrose negative, metabolically inactive sucrose negative, rhamnose positive and metabolically more active rhamnose positive. Some investigators classify Y. enterocolitica on the basis of their biochemical reactions, into several groups so called "biotypes." The most widely used biotype classifications are those of Nilehn (1969), Wauters (1970) and Knapp and Thal (1973). Nilehn (1969) and Wauters (1970) divided Y. enterocolitica strains into 5 biotypes whereas Knapp and Thal (1973) assigned them into four different biotypes (Table 1). The considerable variation in the biochemical activities among Y. enterocolitica strains (regardless of the biotype scheme used) raised the question as to whether all strains truely belong to the same species. In this regard, Mehlman and Aulisio (1978) proposed the name Y. rhamnophilica for those strains which tested positive for rhamnose, raffinose, melibiose, trehalose, citrate, indole and Voges-Proskauer tests, while Y. enteritidis was

Table 1. Major Biotypes Classifications for Yersinia enterocolitica

Biochemical Reaction	Biotypes of Y. enterocolitica[a]													
	Nilehn (1969)					Wauters (1970)					Knapp & Thal (1973)			
	1	2	3	4	5	1	2	3	4	5	1	2	3	4
Lecithinase						+	-	-	-	-				
Salicin	+	-	-	-	-						-	-	+	V
Esculin (hydrolgsis)	+	-	-	-	-						-	-	+	V
Indole	+	+	-	-	-	+	(+)	-	-	-	-	+	+	V
D-xylose	+	+	+	-	-	+	+	+	-	-	V	+	+	V
Lactose (Of medium oxidative)	+	+	+	-	-	+	+	+	-	-				
Nitrate (reduction)	+	+	+	+	-	+	+	+	+	-				
Trehalose	+	+	+	+	-	+	+	+	+	-				
Ornithine (decarboxylase)	+	+	+	+	-	+	+	+	+	-				
β-Galactosidase	+	+	+	+	-	+	+	+	+	-				
Voges-Proskauer	+	+	+	+	-	+	+	+	+	-				
Sucrose	+	+	+	+	-									
Sorbose	+	+	+	+	-									
D-Sorbitol	+	+	+	+	-									

[a]Adapted from Knapp and Thal (1973)
+ = positive
(+) = delayed positive
- = negative
V = variable

used to identify indole-negative strains (Knapp and Thal, 1973). Brenner et al. (1976) characterized Y. enterocolitica strains on the basis of their deoxyribonucleic acid (DNA) hybridization and biochemical reactions and concluded that Y. enterocolitica strains form 3 to 4 distinct DNA relatedness groups which can be defined biochemically. The authors suggested that although all DNA relatedness groups are closely related and should remain in the genus Yersinia, only one group corresponds to the typical strains. The remaining atypical groups are either rhamnose positive; rhamnose, melibiose, α-methylglucoside and raffinose positive; or sucrose negative. In 1979, Brenner proposed the separation of atypical rhamnose positive and melibiose, α-methylglucoside and raffinose positive strains from Y. enterocolitica into new species named Y. frederiksenii and Y. intermedia respectively. Bercovier et al. (1980) suggested the name Y. kristensenii for the sucrose-negative strains. Ewing et al. (1978) recommended the inclusion of a new species into genus Yersinia for thecausative bacterium of red mouth disease of rainbow trout and some other fresh water fish. Bercovier and Mollaret (1984) in Bergey's manual of systematic bacteriology classified the genus Yersinia into seven separate species as follows: Y. pestis, Y. pseudotuberculosis, Y. enterocolitica, Y. intermedia, Y. frederiksenii, Y. kristensenii and Y. ruckeri.

B. EPIDEMIOLOGY

Incidence in Human

Reports of human Yersinia enterocolitica infections have been made from a number of countries on all five continents. Although recovery of Y. enterocolitica has been reported from various clinical specimens such as stool, urine, blood, mesenteric lymphnode, eye, pus, peritoneal fluid, spleen, bile, bowel, colon abscess, abdominal abscess, infected skin, excised appendix, resected pieces of ileum, throat, trachea and sputum (Zen-Yoji and Maruyama, 1972; Weaver and Jordon, 1973; Bottone et al., 1974; Marks et al., 1980; Van Noyen et al., 1980; Shayegani et al., 1981; Mollee and Tilse, 1985), the mode of transmission and subsequent infection of humans by Y. enterocolitica is not fully understood. Foodborne (Anonymous, 1976; Black et al., 1978; CDC, 1982; Shayegani et al., 1983), waterborne (Keet, 1974; Highsmith et al., 1977), person-to-person (Ahvonen and Rossi, 1970; Solem and Lassen, 1971), person-to-food (Olsovsky et al., 1975) and animal-to-person transmission (Ahvonen, 1972; Gutman et al., 1973) have been incriminated as possible modes of Y. enterocolitica infection to human.

Improvement in isolation and identification methodology and better understanding of Yersinia infection by physicians and clinical workers have increased the rate of isolation and the numbers of reported cases of human infection over the past years. Weaver and Jorden (1973) reported in the 6-year period from 1966 to 1972 only 29 isolates of Y. enterocolitica were received by Center for Disease

Control (CDC) from various clinical laboratories in the Unites States, whereas, Shayeganiet al. (1981) reported 120 human isolates identified as Y. enterocolitica by Department of Health Laboratories, New York State, during a 4-year period from 1976 to 1980. Van Noyen et al. (1980) conducted an 8-year study from 1970 to 1978 for isolation of Y. enterocolitica from fecal specimens of patients with gastroenteritis in Belgium. Their study consisted of three phases since improvements were made in isolation procedures as new techniques became available during the course of study. The authors reported a recovery rate of 1% for the first phase and 2.3% and 4.7% for phases 2 and 3, respectively. The increased rate of isolation during the study was assumed to be primarily due to improvements and adjustments in the isolation procedure from one phase to another (Van Noyen et al., 1980).

Seasonal variation in the incidence rate of Y. enterocolitica has been noted. Several reports have suggested a high frequency of incidence of yersiniosis is in the cold months (Esseveld and Goudzwaard, 1973; Mair, 1973; Rakovsky et al., 1973; Vandepitte et al., 1973; Szita and Svidro, 1976; Vandepitte and Wauters, 1979). However, this can not be substantiated by the finding of Marks et al. (1980) and Marriot et al. (1985) or the fact that most human yersiniosis outbreaks have occurred between April and September (Asakawa et al., 1973; CDC, 1982; Maruyama, 1982; Shayegani et al., 1983).

Geographic distribution of certain serotypes of Y. enterocolitica has been documented but is not exclusive. It is apparent that serotype 0:8 is the most predominant serotype in the

United States (Black et al., 1978; Shayegani et al., 1981) and serotype 0:3 has been more frequently isolated in Canada, European countries, Japan and Australia (Asakawa et al., 1973; Zen-Yoji et al., 1973; Toma and Lafleur, 1974; Olsovsky et al., 1975; Pai et al., 1979; Maruyama, 1982; Marriot et al., 1985; Mollee and Tilse, 1985; Hoogkamp-Korstanje et al., 1986).

The significance of human yersiniosis becomes apparent when comparing the incidence of Y. enterocolitica to the occurrence of other well-known enteropathogenic organisms. In general, Y. enterocolitica has been recognized to be among the most frequent causes of bacterial gastroenteritis. Marks et al. (1980) reported that the incidence rate of common enteropathogenic bacteria in Montreal children with gastroenteritis as being 4.4%, 2.8% and 1.1% for Salmonella, Y. enterocolitica and Shigella respectively. Weissfeld and Sonnenwirth (1980) reported that the frequency of yersiniosis in adults with enteric infection was equal to that of salmonellosis. Shayegani et al. (1981) showed Salmonella, Shigella and Y. enterocolitica were the most prevalent enteropathogens in individuals with gastroenteritis in New York State with isolation rates of 9.0%, 1.9% and 1.6% respectively. In 1986, HooKamp-Korstanje et al. reported the following recoveries: Salmonella, 7.8%; Campylobacter jejuni, 4.6%; Y. enterocolitica, 2.9%; and Shigella, 0.2% from patients with gastroenteritis.

Enteric infection due to double infection with Y. enterocolitica and other enteropathogenic bacteria have been also

shown. Cases of concomitant infection with Y. enterocolitica - Salmonella (Rabson and Koornhof, 1972; Vandepitte et al., 1973; Delorme et al., 1974; Marks et al., 1980), Y. enterocolitica - Shigella (Mollaret, 1972) and Y. enterocolitica - enteropathogenic E. coli (Rabson and Koornhof, 1972) have been reported.

Incidence in Animals

Recovery of Yersinia enterocolitica from various species of healthy and sick animals has been reported from many countries around the world. Y. enterocolitica has been isolated from farm animals (Mair, 1973; Tsuobokura et al., 1973; Zen-Yoji et al., 1974; Leistner et al., 1975; Inoue and Kurose, 1975; Pedersen, 1976; Pedersen and Winblad, 1979; Wauters, 1979; Wooley et al., 1980; Doyle et al., 1981; Shayegani et al., 1981), pet animals (Mair, 1973; Rakovsky et al., 1973; Tsuobokura et al., 1975; Kaneko et al., 1977; Vaschenok et al., 1977; Shayegani, 1981), zoo animals (Mair et al., 1970; Langford, 1972; Otsuki et al., 1973; Hacking and Sileo, 1974; Toma and Lafleur, 1974; Szita et al., 1980), wild mammals (Mair, 1973; Otsuki et al., 1973; Toma, 1973; Hacking and Sileo, 1974; Kapperud, 1977), commercial fur-bearers (Langford, 1972; Mair, 1973; Toma and Lefleur, 1974), birds (Hubbert, 1972; Toma, 1973; Hacking and Sileo, 1974; Kato et al., 1985), rodents (Zen-Yoji et al., 1974; Kapperud, 1975; Tsubokura et al., 1975; Pokorna and Aldova, 1977; Kaneko et al., 1978; Shayegani et al., 1981), and fish and shell fish (Rakovsky et al., 1973; Toma, 1973; Kapperud and Jonsson, 1976; Peixotto et al., 1979; Aulisio et al., 1980). Although most animal isolates differ biochemically and

serologically from those of humans, isolates belongingn to serotypes 0:3, 0:8 and 0:9 which are commonly involved in human infections have been isolated from various animals, particularly, swine (Ahvonen and Rossi, 1970; Esseveld and Goudzwaard, 1973; Zen-Yaji et al., 1974; Doyle et al., 1981; Hurvell, 1981; Shayegani, 1981).

Swine have been incriminated as a possible reservior of human pathogenic strains of Y. enterocolitica (Vandepitte et al., 1973; Esseveld and Goudzwaard, 1973; Hurvell et al., 1979). In 1981, Doyle et al. isolated Y. enterocolitica serotype 0:8 from pig tongues. This serotype is frequently involved in human infections in the United States. In this study the authors referred to the oral cavity as a natural reservior for Y. enterocolitica. Several investigators also reported a higher isolation of Y. enterocolitica from tonsils and tongues of swine than from fecal material (Wauters and Janssens, 1976; Christensen, 1979; Pedersen, 1979; Christensen, 1980). In addition to swine, dogs have been suspected to be a reservoir for Y. enterocolitica and implicated as a possible mode of transmission in an interfamilial outbreak of yersiniosis (Ahvonen et al., 1973; Toma, 1973; Gutman et al., 1973; Tsuobokura et al., 1975; Pedersen, 1976; Wilson et al., 1976; Yanagawa et al., 1978).

Various reports regarding the significance of animals in transmission of Y. enterocolitica to humans have been made. Few studies have reported the direct relationship between transmission of Y. enterocolitica from animal to human (Ahvonen, 1972; Gutman et al., 1973). Shayegani et al. (1981) conducted a 4-year study in the New York State area to determine the incidence of Y. enterocolitica in

human, animal, and environmental sources and to characterize the isolates. The authors reported some isolates with the same characteristics from various sources and concluded the possible transmission of Y. enterocolitica from environment or animal to human. Kato et al. (1985) found a high incidence rate of Y. enterocolitica in Japanese wild birds and reported human enteropathogenic Y. enterocolitica was not found among the isolates in the wildlife. It was concluded that although wildlife have been thought to play an important part in the maintenance and dissemination of Y. enterocolitica in nature, their significance as a reservior of human enteropathogenic Y. enterocolitica was not supported by this study.

Incidence in Food

Recovery of Yersinia enterocolitica from raw and processed food products has been reported by many investigators (Table 2). Additionally, isolation of Y. enterocolitica from drinking water (Lassen, 1972; Eden et al., 1977; Jansen and Saari, 1977; Caprioli et al., 1978; Christensen, 1981), well water (Highsmith et al., 1977; Schiemann, 1978b), stream water (Keet, 1974; Shayegani et al., 1981; Bartley et al., 1982; Meadows and Snudden, 1982; Weagant and Kaysner, 1983) and sea water (Harvey et al., 1976; Weagant and Kaysner, 1983) has been documented. Epidemiologically food and water have been incriminated or strongly suspected as vehicles of infections in virtually all known outbreaks of Y. enterocolitica infections (Asakawa et al., 1973, Eden et al., 1977; Black et al., 1978; CDC, 1982; Maruyama, 1982; Aulisio et al., 1983; Shayegani et al., 1983).

Table 2. Some Reported Incidences of Yersinia enterocolitica in Foods

Food Product	No. Sample Tested	Incidence Rate (%)	Serotype[a]	Location	Reference
Dairy Products					
Raw milk					
	2	100.0	NA[b]	Quebec, Canada	Caprioli et al. (1978)
	55	18.2	0:16,34; 0:16; 0:6,30	Ontario, Canada	Schiemann (1978a)
	131	22.0	0:5; 0:6, 30; 0:15; 0:14; 0:7,8; 0:4,33; 0:22	Ontario, Canada	Schiemann and Toma (1978)
	NA	NA	0:13,15; 0:5a; 0:23, 15	New South Wales, Australia	Hughes (1979)
	329	20.6	0:4; 0:5; 0:6	Ontario, Canada	Schiemann (1979b)
	251	10.0	NA	Denmark	Christensen (1981)
	81	4.9	NA	Finland	Hanninen and Raevuori (1981)
	100	13.0	0:5a; 0:6; 0:13; 0:25; 0:5b	Sweden	Norberg (1981)
	160	2.5	0:6,31; 0:8	New York, USA	Shayegani et al. (1981)
	75	81.4	0:5; 0:7,8; 0:27; 0:6; 0:4,32	Alsaca, France	Vidon and Delmas (1981)

Table 2. (Cont'd).

Food Product	No. Sample Tested	Incidence Rate (%)	Serotype[a]	Location	Reference
	233	54.5	0:5; 0:7; 0:6	France	Delmas (1983)
	100	13.0	NA	Wisconsin, USA	Moustafa et al. (1983)
	NA	21.0	NA,	Scotland	Roy (1983)
	374	3.2	0:13,7; 0:5,27; 0:6, 31; 0:7,8; 0:14; 0:22	Shimane, Japan	Fukushima et al. (1984)
	62	25.0	NA	Zaria, Nigeria	Umoh et al. (1984)
	30	13.3	NA	Italy	Franzin and Fantino (1985)
	40	25.0	NA	Wisconsin, USA	Vasavada et al. (1985)
	170	10.0	0:5,27; 0:34; 0:6, 30; 0:4; 0:7	Northern Ireland	Walker and Gilmour (1986b)
Raw goat milk					
	274	12.8	0:16; 0:15; 0:10; 0:55; 0:14; 0:15; 0:22; 0:52,53	New South Wales, Australia	Jensen and Hughes (1980) Hughes and Jensen (1981)
	50	18.0	0:6,30	Northern Ireland	Walker and Gilmour (1986c)

Table 2. (Cont'd).

Food Product	No. Sample Tested	Incidence Rate (%)	Serotype[a]	Location	Reference
Pasteurized milk					
	4	25.0	0:8	New York, USA	Black, et al. (1978)
	265	0.4	0:34	Ontario, Canada	Schiemann (1978a)
	20	5.0	0:5a	New South Wales,	Hughes (1980)
	100	1.0	NA	Australia	
	NA	NA	0:6,30; 0:5,27; 0:7 +7,8	England	Greenwood and Hooper (1985)
	150	6.7	0:34	Northern Ireland	Walker and Gilmour (1986b)
Pasteurized milk and cream					
	NA	NA	0:5a; 0:5b; 0:6; 0:13,15	New South Wales, Australia	Hughes (1979)
Reconstituted dried milk					
	NA	NA	0:8; 0:34	New York, USA	Shayegani et al. (1983)
Cheese					
	1	100.0	0:4,32	Quebec, Canada	Caprioli et al. (1978)
(curd)	76	9.2	0:16; 0:4	Ontario, Canada	Schiemann (1978a)
(fresh)	4	25.0			Luppi and Bucci (1983)
(cottage)	NA	7.0	NA	Scotland	Roy (1983)
(cheddar)	127	0.8	NA	Ontario, Canada	Brodsky (1984)

Table 2. (Cont'd).

Food Product	No. Sample Tested	Incidence Rate (%)	Serotype[a]	Location	Reference
Ice Cream					
	38	2.6	NA	Zaria, Nigeria	Umoh et al. (1984)
	100	18.0	0:39,41; 0:5; 0:7; 0:14; 0:30 47; 0:31,41 0:4; 0:6	France	Delmas and Vidon (1985)
	NA	NA	0:5,27; 0:7+	England	Greenwood and Hooper (1985)
Yogurt (plain and flavored)					
	77	3.8	NA	Zaria, Nigeria	Umoh et al. (1984)
Meat and Meat Products					
Raw beef					
	61	24.6	NA	Japan	Inoue and Kurose (1975)
	37	10.8	NA	West Germany	Leistner et al. (1975)
	1	100.0	0:4,32	Quebec, Canada	Caprioli et al. (1978)
	12	83.3	NA	Maryland, USA	Lee et al. (1980)
Vacuum packed beef					
	107	9.4	0:22; 0:17	Texas, USA	Hanna et al.(1976)
	87	11.5	NA	Texas, USA	Seideman et al. (1976)
(pH<6.0)	12	16.7	NA	New Zealand	Seelye and Yearbury (1979)

Table 2. (Cont'd).

Food Product	No. Sample Tested	Incidence Rate (%)	Serotype[a]	Location	Reference
(pH>6.0)	12	33.3	NA	New Zealand	Seelye and Yearbury (1979)
Vacuum packed lamb					
	30	6.7	0:22; 0:17	Texas, USA	Hanna et al. (1976)
Raw pork					
	29	34.5	NA	West Germany	Leistner et al. (1975)
	30	73.3	NA	Maryland, USA	Lee et al. (1980)
(different parts and cuts)					
	76	39.5	0:5; 0:16; 0:21; 0:13,7	Ontario, Canada	Schiemann (1980a)
(ground)					
	15	60.0	0:5; 0:8; 0:13m6	Ontario, Canada	Schiemann (1980a)
	10	100.0	0:5; 0:6,30; 0:41,42; 0:7, 13; 0:7,8; 0:21; 0:36	Ontario, Canada	Schiemann (1980a)
Pork tongue					
	142	52.8	0:3	Belgium	Wauters and Janssens (1976)
	37	65.0	0:3; 0:17		Schiemann (1980a)

Table 2. (Cont'd).

Food Product	No. Sample Tested	Incidence Rate (%)	Serotype[a]	Location	Reference
	31	42.0	0:8; 0:6,30; 0:3; 0:13,7; 0:18; 0:46		Doyle et al. (1981)
	23	8.7	0:19	Maryland, USA	Stern (1981)
Processed pork products					
	3	100.0	0:4,32; 0:6,31	Quebec, Canada	Caprioli et al. (1978)
	69	7.2	0:17; 0:13,7	Ontario, Canada	Schiemann (1980a)
	166	37.4	0:5; 9:7,8; 0:10,34,K1; 0:6; 0:27; 0:30; 0:X; 0:14; 0:25,35 0:35; 0:36; 0: 41,42,43; 0:K1	France	Delmas and Vidon (1985)
Chicken					
	121	28.9	NA	West Germany	Leistner et al. (1975)
(frozen)	82	24.5	0:6; 0:4; 0:5b; 0:8	Sweden	Norberg (1981)
(raw and processed)	108	68.0	0:5; 0:7,8: 0:25; 0:14; 0:5,27; 0:6,30: 0:10K,34; 0:4;	Netherlands	DeBoer et al. (1982)

Table 2. (Cont'd).

Food Product	No. Sample Tested	Incidence Rate (%)	Serotype[a]	Location	Reference
			0:7; 0:10; 0:10K 0:18; 0:11; 0:11, 23; 0:12,25; 0:16; 0:16,29		
Turkey					
	75	4.0	NA	California, USA	Gutertz et al. (1976)
Seafoods					
Fish (Brown trout)	20	15.0	NA	Norway	Kapperud and Jonsson (1976)
Oysters					
	4	100.0	0:6,30; 0:5; 0:7,13	Ontario, Canada	Toma and Lafleur (1974)
	45	13.3	NA	Texas USA	Peixotto et al. (1979)
Shrimp					
	50	4.0	NA	Texas USA	Peixotto et al. (1979)
Crab (blue)					
	58	20.7	NA	Texas USA	Peixotto et al. (1979)
Vegetables					
Raw					
	65	32.3	0:6; 0:5; 0:7,19; 0:4; 0:7; 0:7,8; 0:7,19; 0:7,8,	France	Delmas and Vidon (1985)

Table 2. (Cont'd).

Food Product	No. Sample Tested	Incidence Rate (%)	Serotype[a]	Location	Reference
			19; 0:10,34,K1; 0:14; 0:25,35; 0:30		
Other Foods					
Pancake					
	1	100.0	0:4,32	Quebec, Canada	Caprioli et al. (1978)
Cake with cream					
	171	28.7	0:39,41; 0: 10,34,K1; 0:5; 0:14; 0:6; 0:7,8; 0:10,K1; 9:10,34 0:8,19; 0:7,8, 13,19; 0:7,8, 13,19; 0:8,13; 0:25,35; 0:30; 0:35,36; 0:39,43	France	Delmas and Vidon (1985)
Cake without cream					
	90	17.8	0:5; 0:7,8,13, 19; 0:5,27; 0:6; 0:7,8,13; 0:7,8, 19; 0:10,34; 0: 14; 0:30; 0:30,47; 0:39,41; 0:39,41, 42,43	France	Delmas and Vidon (1985)

Table 2. (Cont'd).

Food Product	No. Sample Tested	Incidence Rate (%)	Serotype[a]	Location	Reference
Fresh cream cake					
	NA	NA	0:5,27; 0:34	England	Greenwood and Hooper (1985)
Liquid egg					
	NA	NA	0:34	England	Greenwood and Hooper (1985)

[a]Serotypes given not necessarily represents serotypes of all isolates recovered from a particular food.
[b]NA = Not available.

Isolates belonging to serotypes 0:5,27; 0:6,30; 0:5, 0:6,31 and 0:7,8 are the second most frequent human clinical isolates after 0:3, 0:9 and 0:8 (Pai et al., 1979; Marks et al., 1980; Shayegani et al., 1981). Such isolates have been recovered from raw milk (Schiemann and Toma, 1978; Shayegani et al., 1981; Walker and Gilmour, 1986b), meats and meat products (DeBoer et al., 1982), seafood (Toma and Lafleur, 1974), and vegetables (Delmas and Vidon, 1985).

Although many food isolates of Y. enterocolitica are biochemically and serologically different from most human pathogenic strains and generally believed to be atypical and nonpathogenic to humans, cases of human infection caused by such strains have been reported. The illness is usually mild and may have different manifestations than those caused by typical clinical strains (Bottone et al., 1974; Bottone, 1978). Even though some food isolates may belong to the same serotypes known to be pathogenic, Lee et al. (1981) stated that the pathogenicity of a food isolate which is not implicated in foodborne illness cannot be assumed regardless of serotype and biotype and should be assessed for their clinical significance.

The virulence potential of pathogenic Y. enterocolitica is assumed to be plasmid-mediated (Zink et al., 1980). The possibility that an atypical strain of Y. enterocolitica may acquire a virulence plasmid from pathogenic strains or from other enteropathogens has been suggested (Zink et al., 1980). While improvement in isolation and identification procedures in recent years have shown higher frequency in the rate of occurrence of Y. enterocolitica in various food

products, more studies are needed to determine whether those methods are useful in isolation of all Y. enterocolitica strains from foods, since not all food isolates may be pathogenic.

Outbreaks of Yersiniosis

Outbreaks of yersiniosis reported in the United States and other countries are given in Table 3. Although the mode of infec-tion in most of the yersiniosis outbreaks has not been identified, food or water has been suspected as a vehicle of transmission of Y. enterocolitica in virtually all the outbreaks (Asakawa et al., 1973; Zen-Yoji et al., 1973; Olsovsky et al., 1975; Black et al., 1978; Anonymous, 1976; Eden et al., 1977; CDC, 1982; Maruyama, 1982; Aulisio et al., 1983; Shayegani et al., 1983). One exception was reported in which the initial transmission to humans began with a infected dog and puppies and subsequent person to person contact resulted in the infection of 16 of 21 family members. This outbreak resulted in 2 appendectomies and two deaths (Gutman et al., 1973).

Raw milk was reported the most likely source of Y. enterocolitica in an outbreak which affected 137 elementary school children and one adult in Montreal Canada (Anonymous, 1976).However, serotype 0:6,30 which was isolated from raw milk differed from serotype 0:5,17 isolated from the ill children. Consumption of con-taminated pastuerized milk has been linked to several outbreaks of yersiniosis (Black et al., 1978; CDC, 1982; Maruyama, 1982). In 1982, the interstate outbreak of enteritis caused by Y. enterocolitica was

Table 3. Outbreaks of Yersiniosis

Date	Location	# of Cases	Major Symptoms	Source of Infection	# Hospitalized[a]	Serotype Involved	Reference
Sept. 1971	Karvina, Czechoslovakia	15	Afebrile with slight abdominal pain; anorexia with diarrhea	Person-to-Food	1(0)	0:3	Olsovsky et al. (1975)
31 Jan-26 Feb 1972	Shizuoka, Japan	189	Abdominal pain, fever, diarrhea, nausea, vomiting	Unknown	Unknown (Unknown)	0:3	Asakawa et al. (1973)
11-22 July 1972	Shizuoka, Japan	544	Abdominal pain, fever, diarrhea, nausea, vomiting	Unknown	None	0:3	Asakawa et al. (1973)
14-22 July 1972	Tochigi, Japan	198	Abdominal pain, fever, headache, diarrhea, malaise, vomiting, nausea	Unknown	Unknown(3)	0:3; 0:9; 0:5	Zen-Yoji et al. (1973)
April 1973	North Carolina, USA	16	Fever, diarrhea, severe abdominal pain, vomiting, pharyngitis, headache	Dog	5(2)[b]	NA	Gutman et al. (1973)
Dec. 1974-Jan. 1975	Montana, USA	750	Nausea, fatigue, diarrhea, abdominal cramps, vomiting, muscle aches, headache, fever	Well water	None	0:4,33[c]; 0:18,28; 0:3	Eden et al. (1977)

Table 3. (Cont'd)

Date	Location	# of Cases	Major Symptoms	Source of Infection	# Hospitalized[a]	Serotype Involved	Reference
April 1975	Montreal, Canada	138	Diarrhea, vomiting, lack of appetite, headache, shivering, fever	Raw milk	NA	0:6,30; (from raw milk) 0:5,27 (from patient)	Anonymous (1976)
Sept.-Oct. 1976	New York, USA	222	Fever, abdominal pain, diarrhea, rash	Chocolate milk	36(16)	0:8;	Black et al. (1978)
April,	Okinawa, Japan	1,051	NA	Pasteurized milk	NA	0:3	Maruyama (1982)
July, 1981	New York, USA	239	Abdominal pain, fever, nausea, vomiting, diarrhea	Reconsituted dried milk and turkey chow mein	7(5)	0:8; 0:34	Shayegani et al. (1983)
Dec. 1981-Feb. 1982	Washington, USA	87	Abdominal cramps, fever, headache, diarrhea, photo-sensitivity	Tofu	Unknown (Unknown)	0:8; 0:Tocoma; 0:11,24	Aulisio et al. (1983)
June 11-July 29, 1982	Memphis-inter-state area, USA	172	Abdominal cramps, fever, headache, diarrhea, photo-sensitivity	Pasteurized Milk	Unknown (Unknown)		CDC (1982)

[a]Number in parenthesis represents number of patients were underwent appendoctomy.
[b]Two deaths as result of this outbreak were also reported.
[c]Only isolated from non-chlorinated well water used for drinking.

assumed to be due to the consumption of contaminated pasteurized milk processed in a Memphis, Tennessee plant. Of 172 confirmed cases, 148 patients exhibited infections with diarrhea, and/or abdodminal pain and fever. In addition to the enteritis, 24 patients experienced infections of the throat, blood, urinary tract, central nervous system and wounds. A large majority of the victims were hospitalized and 17 underwent appendectomies. Epidemiological studies conducted by the Department of Health estimated the possible number of actual infections to be much higher than the 172 cases reported. Although pasteurized milk was suspected as the source of infection, milk from suspected lots was not available for culture and later isolation attempts from various lots failed to produce Y. enterocolitica. The same serotypes involved in the outbreak were later isolated from a crate of outdated milk from the same plant on a hog farm where the milk was fed to the hogs (CDC, 1982).

Two outbreaks of Y. enterocolitica due to water have been reported in the United States. Eden (1977) found that approximately 750 visitors and employees of a Montana ski resort contracted a gastrointestinal illness characterized by diarrhea between December 1974 and January 1975. The source of infection was assumed to be the nonchlorinated well water used for drinking, since Y. enterocolitica along with coliforms were isolated from the water samples. However, Y. enterocolitica could not be obtained from fecal specimens of infected individuals. In another outbreak described by Ausilio et al. (1983), 87 individuals were infected with Y. enterocolitica in

Seattle, Washington due to the consumption of contaminated tofu. The source of infection was suspected to be well water used in the processing of the tofu.

It is noteworthy that the majority of people with yersiniosis were children, 18 years of age and under, who experienced the major symptoms of diarrhea, abdominal pain, vomiting, headaches, fever and nausea (Asakawa et al., 1973; Zen-Yoji et al., 1973; Olsovsky et al., 1975; Black et al., 1978; Anonymous, 1976; Shayegani, et al., 1983).

C. ISOLATION METHODOLOGY

Since the recognition of Yersinia enterocolitica as an important enteropathogenic organism, many isolation methods have been recommended for the recovery and identification of this bacterium from foods, clinical specimens and environmental samples. Table 4 presents a number of selected isolation methods with the appropriate enrichment broths, plating media and incubation conditions under which effective recovery of Y. enterocotilica isolates from various inoculated and naturally infected food samples have been demonstrated.

Selective Enrichment

Initial isolation methods used for recovery of Y. enterocolitica from foods were similar to those developed for isolation of this bacterium from fecal and clinical specimens of which cold enrichment technique was the method of choice (Zen-Yoji et al., 1974; Eiss, 1975; Leistner et al., 1975; Feeley et al., 1976; Lee, 1979; Van Pee and Stragier, 1979). Cold enrichment with phosphate buffered saline (PBS)

Table 4. Selected Isolation Methods for Yersinia enterocolitica from Foods

Pre-enrichment/ Cold Enrichment	Selective Enrichment	Post-enrichment Alkali Treatment	Plating Medium	Reference
Butterfield, Phosphate buffer (PB) at 4° C for 14d	Modified rappaport broth (MRB) at 23° C for 5 d	----	MacConkey+Tween 80 (MT); modified deoxyribonuclease	Schiemann and Toma (1978)
Phosphate buffered saline (PBS) at 4° C for 7-10 d	----	0.5% KOH for 1 min	MacConkey	Aulisio et al. (1980)
----	Modified selenite broth at 22° C for 2-3 d	0.25% KOH for about 1 min	Bismuth sulfite (BS); deoxyribonuclease sorbital+Tween 80; MT	Lee et al. (1980)
PBS + 1% Sorbitol + 0.15% bile salts (PBSSB) at 4° C for 7-28 d	Selenite broth at 25° C for 2 d	----	MacConkey; xylose-lysin-desoxycholate	Norberg (1981)
----	Novobiocin-polymyxin B sulfate-crystal violet-tryptic soy (NPC) broth at 18° C for 4 d	0.25% KOH	MacConkey; cefsulodin-irgasan-novobiocin (CIN)	Park et al. (1981)
Cooked meat medium at 4° C for 21 d	------	----	Salmonella-Shigella; Endo; deoxycholate-citrate; deoxycholate-citrate + 0.5% sucrose	Shayegani et al. (1981)

Table 4. (Cont'd)

Pre-enrichment/ Cold Enrichment	Selective Enrichment	Post-enrichment Alkali Treatment	Plating Medium	Reference
PBSSB at 4° C for 14-28 d	----	0.5% KOH for few sec	MacConkey	DeBoer et al. (1982)
--------	PBS at 25° C for 1-3 d	0.25% KOH for 2 min 0.5% KOH for 15 sec	MacConkey	Doyle and Hugdahl (1983)
PBSSB at 4° C for 21 d	-----	0.5% KOH	CIN	Moustafa et al. (1983)
Peptone-yeast extract broth (PYB) at 15° C for 1-2d/ at 4° C for 7-9 d	Bile-oxalate-sorbose (BOS) broth at 22-25° C for 3-5 d	----	CIN	Schiemann (1984)
---------	Cellobiose-cephal-otine-ampicilin (CCA) broth at 15° C for 4 d	0.5% KOH for 15 sec	CCA	DeFelip et al. (1985)
PBSSB at 4° C for 9 d	BOS at 22° C for 5 d	0.25% KOH for 2 min	CIN	Delmas and Vidon, (1985)

was originally developed by Patterson and Cook (1963) for isolation of Y. pseudotuberculosis and was later adapted for recovery of Y. enterocolitica. The selectivity of cold enrichment in PBS and several other media is based on the ability of Y. enterocolitica to grow at refrigeration temperature. The usefulness of cold enrichment methods for the isolation of Y. eterocolitica has been well documented; however, a number of drawbacks including an incubation period of 7-28 days (Zen-Yoji et al., 1974; Greenwood et al., 1975; Norberg, 1981; Mustafa et al., 1983) and the inability to isolate clinically important serotypes (Pai et al., 1979; Van Noyen et al., 1980) have been discussed. Pai et al. (1979) evaluated the efficiency of cold enrichment in PBS for isolation of Y. enterocolitica from fecal specimens and reported that cold enrichment was an effective method for recovery of most serotypes of Y. enterocolitica, except 0:3. Serotype 0:3 is known to be of clinical importance in countries outside the United States (Bottone, 1977). Van Noyen et al. (1980) similarily indicated the inefficiency of cold enrichments techniques for recovery of serotypes 0:3 and 0:9 from fecal and clinical specimens. Lee (1979) evaluated the effectiveness of cold enrichment in PBS at 4° C for isolation of Y. enterocolitica from inoculated meats and oyster samples and concluded that this method was unsatisfactory for the isolation of Y. enterocolitica. Mehlman et al. (1978a) experimentally inoculated pork with Y. enterocolitica to observe the effectiveness of the cold enrichment technique on recovery. The authors noted the duration of recovery and initial

inoculum were directly related and also noted an improved recovery of Y. enterocolitica in PBS when samples were homogenized. Mehlman et al. (1978a) concluded that nutritional composition of enrichment was a critical factor for isolation of Y. enterocoliticia and reported supplementation of PBS with 1% sorbitol and 0.15% bile salts resulted in a higher recovery rate in a shorter incubation period.

In an effort to find a selective one step enrichment method for recovery of Y. enterocolitica several studies have been undertaken. Wauters (1973) tested selenite broth and Rappaport medium for their selectivity for Y. enterocolitica and observed a poor recovery rate. A modified rappaport medium was formulated which contained more magnesium chloride, less malachite green and the addition of a small amount of carbenicillin to the original formula. The modified rappaport medium termed magnesium chloride-malachite green-carbenicillin (MMC) enhanced rapid recovery of clinically important serotypes 0:3 and 0:9 (Wauters, 1973). Two modified selenite enrichment broths incubated at 22° C for 2-3 d were used for the recovery of Y. enterocolitica from meats (Lee et al., 1980). The modified selenite medium was made by the incorporation of malachite green, carbenicillin and sodium selenite to PBS. Lee et al. (1980) reported rapid recovery of Y. enterocolitica including clinical serotype 0:3 and 0:8 from inoculated meat samples using the modified selenite broths. The authors also noted that restricting the meat sample size improved the efficiency of these selective enrichment media. Contrary to these findings, Doyle et al. (1981) found that enrichment in modified rappaport broth or modified

selenite broth was ineffective in recovering both pathogenic and environmental strains from pork tongues. Other one step enrichment methods have been shown to be an effective means for the recovery of Y. enterocolitica from food and water. In 1981, Shayegani et al. successfully isolated Y. enterocolitica from raw milk and water using cooked meat medium at 4° C up to 21 days. Harvey et al. (1976) recovered Y. enterocolitica from water samples using glucose broth as an enrichment medium at room temperature for 96 h followed by anaerobic plating on MacConkey-Tween 80.

Due to the long incubation and/or difficulty in isolation of some pathogenic strains of Y. enterocolitica by one step enrichment methods, two step enrichment methods were devised. In a comparative study conducted by Schiemann and Toma (1978), four enrichment procedures were evaluated for their effectiveness in isolating Y. eterocolitica from raw milk. Enrichment in Butterfield's phosphate buffer incubated at 4° C for 14 days followed by incubation in modified rappaport broth at 23° C for 5 days was the most effective method for recovery as compared to modified rappaport at 23° C for 5 days, Butterfield's phosphate buffer at 4° C for 14 days and two step enrichment in cooked meat at 23° C for 28 days followed by modified rappaport broth at 23° C at 5 days. Norberg (1981) studied the occurrence of Y. enterocolitica in frozen chicken by using a two step enrichment procedure consisting of pre-enrichment in phosphate buffered saline containing 1% sorbitol and 0.5% bile salts (PBSSB) at 4° C for 7-28 days followed by selective enrich-selenite broth at 25° C for 48 h. A recovery rate of 25.4% was

concluded. Of several two step enrichment methods used for recovery of Y. enterocolitica from food (Schiemann, 1978a; Schiemann and Toma, 1978; Doyle et al., 1981; Norberg, 1981; Schiemann, 1982; Schiemann, 1983b), Schiemann (1984) claimed that a pre-enrichment in peptone-yeast extract broth at 4° C for 1-2 days and selective enrichment in bile-oxalate-sorbose (BOS) broth at 22°-25° C for 3-5 days was the most reliable method.

Aulisio et al. (1980) first described the relative resistance of Y. enterocolitica to short exposure to weak alkaline treatment as compared to other gram negative microorganisms. Through this finding Aulisio et al. (1980) developed a rapid recovery method by mixing a specific amount of cold enrichment cultures from naturally and artifically contaminated foods to a solution of 0.5% potassium hydroxide (KOH) in 0.5% sodium chloride for few seconds and then streaking for isolation on MacConkey agar. The authors reported post-enrichment alkali treatment increased both recovery rate and sensitivity and decreased the incubation time required as compared to cold enrichment methods alone. Doyle et al. (1981) examined several enrichment techniques with and without post enrichment alkali treatment for isolation of virulent Y. enterocolitica from pork tongues and concluded cold enrichment in PBS followed by alkali treatment was an effective method. Weissfeld and Sonnenwirth (1982) experimented with possible use of KOH treatment in the recovery of Y. enterocolitica from seeded fecal samples. All strains of Y. enterocolitica were recovered after 2 minutes exposure to 0.5% KOH solution. They

concluded alkaline treatment to be a good substitute for tedious conventional enrichment methods. A later comprehensive study by Schiemann (1983a) examined the parameters affecting the alkalotolerance of Y. enterocolitica in regard to selective isolation from foods. The alkali resistance of Y. enterocolitica was found to be affected by suspension medium, temperature, concentration of KOH and growth phase (Schiemann, 1983a). An attempt to standardize the KOH treatment for the recovery of Y. enterocolitica from food, treatment in peptone supplemented 0.5% KOH in 0.5% NaCl was followed by the addition of a phosphate buffer (pH 6.6). This was found to minimize destruction of Y. enterocolitica cells as compared with other gram-negative competitors. However, it was determined that alkali treatment was not adequate for the recovery of Y. enterocolitica from the enrichment of inoculated foods in comparison with selective enrichment in bile-oxalate-sorbose (BOS) broth (Schiemann, 1983a). In an effort to develop a rapid isolation method with a higher selectivity, and sensitivity for recovery of Y. enterocolitica from foods, Doyle and Hugdahl (1983) found, that alkali treatments, ranging from 0.5% KOH for 15 seconds to 0.25% KOH for 2 minutes could be used successfully. Despite apparent difference in alkalotolerence of various strains, it was concluded that all strains of Y. enterocolitica could be recovered from enrichment culture containing minimum of 10^4 viable Yersinia cells/ml after post-enrichment in KOH solution. Post-enrichment alkali treatment was also found to be useful for recovery of Y. enterocolitica from foods and environmental samples by

other investigators (Lee et al., 1980; Park et al., 1981; DeBoer et al., 1982; Schiemann, 1982; Moustafa et al., 1983; Weagant and Kaysner, 1983; DeFelip et al., 1985; Delmas and Vidon, 1985).

Selective Plating

Initial selective plating media used for the isolation of Y. enterocolitica were similar to those media normally utilized for the isolation of other enteropathogens. However, Y. enterocolitica strains vary in their ability to grow on such selective media (Bottone, 1977). Selective media such as bismuth sulphite, Endo, eosin methylene blue, MacConkey, desoxycholate-citrate, Salmonella-Shigella (SS), Hektoen enteric, and xylose-lysine-desoxycholate (XLD) agars have been shown to support the growth of Y. enterocolitica serotypes 0:3, 0:8 and 0:9 (Chester and Stotzky 1976; Bottone, 1977). Y. enterocolitica on such media as Endo, Salmonella-Shigella, desoxycholate-citrate, and MacConkey agar developed pinpoint (0.05mm) colonies after incubation at 22° C or 37° C for 24 h. In contrast Escherichia coli and Salmonella species formed substantially large colonies (4 to 5 mm) during the same period (Bottone, 1977). Due to the slow growth rate of Y. enterocolitica on commonly used enteric media, other microorganisms may outgrow Y. enterocolitica making its detection difficult. Therefore, Wauters (1973) and Toma (1973) suggested the use of a stereoscopic microscope at 13X magnification and oblique transillumination for identification of Y. enterocolitica colonies on the agar surface.

Wauters (1973) reported Y. enterocolitica to be extremely tolerant of deoxycholate at concentrations of 3% and greater. He suggested the addition of 2% sodium deoxycholate to Salmonella-Shigella agar improved its selectivity for Y. enterocolitica and allowed the rapid growth of serotypes 0:3 and 0:9. This medium was found to be superior for recovery of Y. enterocolitica over desoxycholate-citrate and Salmonella-Shigella agars (Wauters, 1973). In 1977, Lee modified two selective plating media in an attempt to ease the differentiation of Y. enterocolitica from other lactose-negative gram-negative microorganisms. MacConkey agar was modified by incorporating Tween 80 and calcium chloride. Lipolytic strains of Y. enterocolitica form flat wrinkled colonies surrounded by a centeric sheen after incubation at 25° C for 48 h (Lee, 1977b). The second medium was a modification of deoxyribonucleuse (DNase) agar described to be used in conjunction with cold enrichment method. The modified DNase was prepared by the addition of Tween 80, sorbitol, sodium lauryl sulphate and calcium chloride. Y. enterocolitica colonies are typically translucent, colorless or pink and lack lipolytic and nuclease reaction.

Van Noyen et al. (1980) reported that the selectivity of MacConkey agar for the recovery of Y. enterocolitica serotypes 0:3 and 0:9 from clinical specimens was inadequate. However, Salmonella-Shigella agar proved to be a sufficient direct plating medium for the recovery of Y. enterocolitica from clinical specimens. Similarly, Weissfeld and Sonnenwirth (1980) found a higher percentage of recovery of Y. enterocolitica isolates resulted after cold enrichment of fecal speciments using Salmonella-Shigella agar rather than MacConkey agar.

In 1979, Schiemann, after intensive studies which characterized optimal growth requirements of Y. enterocolitica and evaluated the selectivity of various chemical agents, dyes and antibiotics (Schiemann, 1979a; 1980b), formulated a new selective differential plating medium termed cefsulodin-irgasan-novobiocin (CIN) agar (Schiemann, 1979c). This medium was found to provide quantitative recoveries of 40 various strains of Y. enterocolitica in which Y. enterocolitica forms dark red-centered colonies that can be easily differentated from other microorganisms. This new medium was noted to be highly inhibitory to Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae and Proteus mirabilis. Recovery of Y. enterocolitica from fecal specimens on CIN agar was compared to Salmonella-Shigella and MacConkey agar (Schiemann, 1979c). Dudley and Shotts (1979) prepared a new medium, cellobiose-arginine-Lysine (CAL) agar for the recovery of Y. enterocolitica. The organism utilizes cellobiose and forms burgundy to red colonies due to the change in pH and the presence of neutral red in the agar. A problem with this medium (CAL) is that after 36 h of incubation at 25° C, the color of Y. enterocolitica colonies changes to white or orange (Dudley and Shotts, 1979). This is due to decarboxylation activity. Soltesz et al. (1980) described a new selective medium called 'Y' medium, for isolation of Y. enterocolitica from fecal specimens. The medium is composed of casein hydrolysate and peptone as carbon and energy sources and sodium oxalate, sodium desoxycholate and bile salts as selective agents. This medium effectively inhibited most gram-negative

bacteria, members of the Enterobacteriaceae, Pseudomonas spp. and Proteus, as well as gram-positive microorganisms (Soltesz et al., 1980).

A comparative study by Harmon et al. (1983) evaluated seven selective differential plating media for their effectiveness in the isolation of various serotypes of Y. enterocolitica from pure cultures and from inoculated ground pork. The authors reported CIN agar, in spite of its inhibitory effect on some serotypes, to be the most effective in the isolation of Y. enterocolitica, followed by bismuth sulfite, MacConkey and MacConkey-Tween-80 agars. The least effective media were found to be cellobiose-arginine-lysine, desoxycholate citrate and Salmonella-Shigella agars. Schiemann (1983b) compared the performance of five selective plating medium for recovery of Y. enterocolitica from beef stew inoculated with Y. enterocolitica as well as ten other gram-negative bacteria. Although none of the plating media were found to be superior, CIN agar gave the highest number of confirmatory Y. enterocolitica colonies (Schiemann, 1983b). The findings of Walker and Gilmour (1986a) also support earlier studies which showed that CIN was the most selective medium for the recovery of Yersinia spp.

The most recent isolation method for the recovery of Y. enterocolitica from food was suggested by DeFelip et al. (1985) who formulated an enrichment medium, cellobiose-cephalotine-ampicillin (CCA) broth, and the same medium with agar, CCA agar, for selective isolation of Y. enterocolitica. CCA broth was prepared by incorpo-

rating several nutrients and energy sources including cellobiose as well as two antibiotics, cephalotine and ampicillin, and was found to be highly selective for the isolation of Y. enterocolitica even when a number of other microorganisms were present. In a comparative study, the usefulness of an isolation method using CCA enrichment broth followed by selective plating on CCA agar was compared to several isolation methods. They concluded that the CCA broth and agar were superior to BOS and CIN for recovery of Y. enterocolitica from foods (DeFelip et al., 1985).

CHAPTER III

GROWTH CHARACTERISTICS OF *YERSINIA ENTEROCOLITICA* IN PASTEURIZED SKIM MILK

A. INTRODUCTION

Yersinia enterocolitica has been frequently isolated from raw and pasteurized milk (Schiemann, 1978a; Schiemann and Toma, 1978; Hughes, 1980; Norberg, 1981; Shayegani et al., 1981; Vidon and Delmas, 1981; Fukushima et al., 1984; Walker and Gilmour, 1986b). Pasteurized milk has been implicated as the vehicle of infection in two yersiniosis outbreaks in the United States (Black et al., 1978; CDC, 1982). However, the pasteurization process has shown to destroy *Y. enterocolitica* (Hanna et al., 1977a; Lovett et al., 1982). It is generally accepted that *Y. enterocolitica* enters milk as a post-pasteurization contaminant (Black et al., 1978; CDC, 1982). Since post-pasteurization contamination of milk with *Y. enterocolitica* is possible, the number of cells contaminating pasteurized milk and the rate at which the pathogen grows in the presence of the background microflora would determine how great the hazard might be from post-pasteurization contamination. Therefore, this study was undertaken to characterize the growth of *Y. enterocolitica* in refrigerated pasteurized milk at 4° C when inoculated at various levels with *Y. enterocolitica*.

B. MATERIALS AND METHODS

Organisms and Preparation of Inoculum

Five strains of *Y. enterocolitica* were used in this study: IP383 (Serotype 0:9), E657 (Serotype 0:5,27), WA20 (Serotype 0:8), Y7P (Serotype 0:8) and C122-76 (Serotype unknown). These strains were provided by Dr. M. P. Doyle (Food Research Institute, Madison, WI). Stock cultures were maintained on Tryptone Soya Agar (TSA, Oxoid, Basingstoke, Hants, England). The cultures were transferred at bi-monthly intervals, incubated at 25° C for 18 h and maintained at 4° C. *Escherichia coli* ATCC 25922 and *Citrobacter freundii* ATCC 8090 were purchased from the American Type Culture Collection. They were grown at 32° C and maintained as described above.

Inocula of *Y. enterocolitica* were prepared by transferring loopfuls of the actively growing culture into 250 ml screw-cap Erlenmeyer flasks containing 99 ml of Brain Heart Infusion Broth (BHI, Oxoid) and incubating at 25° C for 18 h. *E. coli* and *C. freundii* were grown at 32° C for 18 h.

Growth of Y. enterocolitica in Pasteurized Milk

Freshly pasteurized milk (HTST, 167° C, 15 sec) was obtained from the University of Tennessee Creamery. Pasteurized skim milk (99 ml) was aseptically transferred into 250 ml screw cap Erlenmeyer flasks. Milk was inoculated with either 10 or 1000 *Y. enterocolitica* CFU/ml. Serotypes IP383, E657, WA20, Y7P, and C122-76 were evaluated individually. The inoculated flasks were stored at 4° C. Flasks were sampled at 0, 3, 7, 14 and 21 days. Dilutions were prepared in 0.1%

peptone water and samples were spread plated in duplicate on MacConkey Agar, Cefsulodin- Irgansan-Novobiocin (CIN) and TSA. All culture media were purchased from Oxoid (Basingstoke, England). One set of plates was incubated at at 25° C for 48 h and the other set at 32° C for 24 h. Plates were enumerated for total aerobic plate count (TSA) and for Y. enterocolitica count (CIN and MacConkey agar). Representative colonies were selected from CIN and MacConkey agar to positively con-firm identification of Y. enterocolitica by biochemical testing and morphology. Three replications were performed. Generation times were calculated for each strain.

C. RESULTS AND DISCUSSION

The preliminary studies showed that when freshly pasteurized milk was co-inoculated with 10, 100 or 1000 cells of Y. enterocolitica and C. freundii or E. coli, Y. enterocolitica was easily distinguished from background microflora and coliforms on both MacConkey agar and CIN agar. E. coli did not grow on CIN. C. freundii grew on CIN but did not form a typical Y. enterocolitica colony.

Growth of Y. enterocolitica in Pasteurized Milk

Pasteurized milk not inoculated with Y. enterocolitica (controls) had aerobic plate counts (APC) which averaged approximately 1000 CFU/ml immediately after pasteurization (Figure 1). The APC for the same sample after one week of storage at 4° C reached log 7 CFU/ml and within 14 days, APC was greater than log 9.0 CFU/ml. Overall, the temperature of incubation of plates had no significant effect on recovery, however, there was occasional differences in recovery at 25

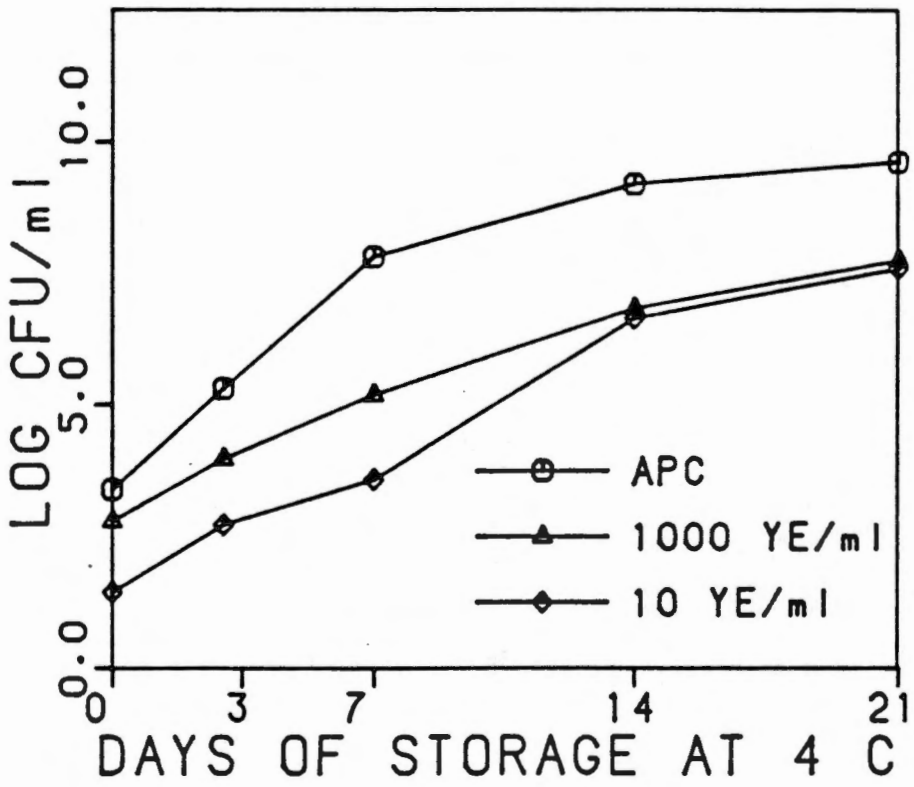

Figure 1. Growth of Yersinia enterocolitica in pasteurized skim milk inoculated with either 10 or 1000 CFU/ml in the present of naturally occurring competitive background microflora (APC = Aerobic Plate Count of control). Data points represent the mean of three replications for five strains, IP383, E657, WA20, Y7P and C122-76.

vs. 32° C. MacConkey agar was not suitable for enumeration of Y. enterocolitica from pasteurized milk stored at 4° C due to interferrence by the background psychrotrophic microflora. This finding was in contrast to the discovery in the preliminary experiments that Y. enterocolitica was easily distinguishable from other bacteria on MacConkey agar. Preliminary experiments were conducted using milk that had a low background microflora, thus there was little competition with Y. enterocolitica. This finding may help explain why some researchers have found MacConkey agar a suitable medium for isolation and enumeration of Y. enterocolitica and others have not (Mehlman et al., 1978a; Vidon and Delmas, 1981; Harmon and Swaminathan, 1983). Greater than 95% of the colonies typical of Y. enterocolitica on CIN were confirmed as Y. enterocolitica.

All strains of Y. enterocolitica competed favorably with background microflora whether inoculated with 10 cells/ml or 1000 cells/ml. All strains of Y. enterocolitica had somewhat similiar growth patterns in refrigerated pasteurized milk (Figure 1). Although levels of Y. enterocolitica at 14 days were not affected by size of inoculum, after 7 days the pasteurized milk inoculated with 1000 cells/ml contained log 1.4 to 2.0 CFU/ml more Y. enterocolitica than the treatments with the lower level of inoculation. It is interesting to note that even when the background microflora had reached approximately log 9.0 CFU/ml at 14 days of incubation, the Y. enterocolitica counts continued to increase through 21 days.

Generation times of Y. enterocolitica under various conditions of storage are shown in Table 5. Generation times for all strains

Table 5. Generation Times of Y. Enterocolitica in various substrates at 4° or 25° C

Strain	Serotype	Substrate	Temp C	Generation Time (hours)
Y7P	0:8	Sterile Skim Milk	25	1.43
Y7P	0:8	Sterile 10% NFDM	25	1.93
Y7P	0:8	BHI	25	1.28
IP383	0:9	BHI	25	1.03
E657	0:5,27	BHI	25	1.03
WA20	0:8	BHI	25	1.33
C122-76	Unknown	BHI	25	1.00
Y7P	0:8	Pasteurized Milk	4	25.90
IP383	0:9	Pasteurized Milk	4	17.70
E657	0:5,27	Pasteurized Milk	4	16.80
WA20	0:8	Pasteurized Milk	4	22.90
C122-76	Unknown	Pasteurized Milk	4	22.60

studied were generally shorter in BHI broth than in sterile skim milk or sterile 10% non-fat dried (NFD) milk at 25° C. This may be due to the destruction of some nutrients in the milks by the sterilization process. At 25° C, Y. enterocolitica did not compete favorably with the background microflora of pasteurized milk and ac-curate generation could not be obtained by using CIN as the plating medium. At 4° C in pasteurized milk, generation times ranged from 16.8 to 25.9 h which was an 8-fold to 18-fold increase over the generation times at 25° C for sterile skim milk or NFD milk. This translates into a log 0.9 to 1.4 increase in Y. enterocolitica each day at 4° C in pasteurized milk depending on the strain present.

Generation times reported in this study for Y. enterocolitica in pasteurized milk are quite similiar to those reported by Stern et al. (1980) for pooled strains in UHT milk at 3° C. This is another indication of the competitive nature of Y. enterocolitica in pasteurized milk at 4° C. There are frequent references to generation times of Y. enterocolitica in various culture medias (Schiemann, 1980b; Stern et al., 1980) and in UHT milk (Stern et al., 1980; Hellman and Heinrich, 1985). German scientists, Hellman and Heinrich (1985) studied the growth of Y. enterocolitica in pasteurized milk inoculated with mouse feces containing Y. enterocolitica and found that levels increased from <5 CFU/100 ml to log 4.0 CFU/ml after seven days. Counts leveled off and did not increase, possibly due to the competition of bacteria in the fecal material.

The milk used in this study was obtained freshly pasteurized and was code dated (sell by) for 14 days. The results indicate that Y. enterocolitica at initial levels as low as 10 cells/ml could grow to log 5.0 to 7.0/ml within two weeks (depending on strain). Szita and co-workers (1973) reported that a human volunteer consumed log 9.0 cells of Y. enterocolitica and developed yersiniosis within 48 hours. Carter and Collins (1974) reported that when mice consumed log 7.0 cells of Y. enterocolitica, all developed the infection. Therefore, within two weeks after contamination of pasteurized milk with 10 cell of Y. enterocolitica/ml, a person consuming only four ounces (120 ml) could develop yersiniosis since they would be receiving in excess of log 9.0 cells. Since a major portion of the population consuming pasteurized milk is children, the infective dose should probably be considered to be somewhat lower.

The results of this study concerning the competiveness of Y. enterocolitica differ from those of Stern et al. (1980) who stated that Y. enterocolitica did not compete favorably with common psychrotrophs of pasteurized milk and could not be detected after 10 days at 3° C. However, they did their study in sterile UHT milk inoculated with pure cultures of specific psychrotrophic organisms which had been previously isolated by Higoshi et al. (1975) from both raw and pasteurized milk. For enumeration of Y. enterocolitica, they used nutrient agar which is not a selective medium. Our results are supported by the recent report of Hellman and Heinrich (1985) which

showed rapid growth of Y. enterocolitica in pasteurized milk inoculated with mice feces containing low levels of Y. enterocolitica.

The use of two different temperatures for incubation of CIN plates was based on finding of Nilehn (1969) who reported higher recoveries of Y. enterocolitica on media containing bile salts when plates were incubated at room temperature vs. 37° C and Mehlman et al. (1978a) who stated that recovery of some strains of Y. enterocolitica was affected by incubation temperature. The findings of this study also showed that the recovery of Y. enterocolitica on CIN at 25° C vs. 32° C was somewhat strain dependent. However, if results are needed within 24 h instead of 48 h, the higher temperature (32° C) is acceptable with the understanding that Y. enterocolitica counts may be 0.5 to 1.0 log lower at the higher temperature.

The research literature shows that Y. enterocolitica is destroyed by pasteurization (Hanna et al., 1977a; Lovett et al., 1982) and it is generally accepted that Y. enterocolitica enters milk as a post-pasteurization contaminant (Black et al., 1978; CDC, 1982). Dogs are known reservoirs of Y. enterocolitica (Ahvonen et al., 1973; Pedersen, 1976) and outbreaks of yersiniosis have been traced to family pets (Gutman et al., 1973). Therefore, pasteurized milk can be contaminated in the home by the consumer. Swine and other members of the animal kingdom are also significant reservoirs of Y. enterocolitica (Vidon and Delmas, 1981; Swaminathan et al., 1982). The four strains of known serotype used in our study tested positive with WA-SAA, an antiseru48

that agglutinates mouse virulent, but not avirulent Y. enterocolitica (Doyle and Hugdahl, 1983). Therefore, it was assumed that strains found capable of growing in pasteurized milk are capable of producing yersiniosis. The findings that Y. enterocolitica grows rapidly and competes well in pasteurized milk emphasize the need for education of the general public and the industry concerning the growth of psychrotrophic pathogens in refrigerated milk after pasteurization.

CHAPTER IV

PRE-PLATING ALKALINE DILUENT (PPAD) TECHNIQUE FOR RECOVERY OF YERSINIA ENTEROCOLITICA FROM MILK

A. INTRODUCTION

Over the years, many selective isolation methods have been developed for recovery and enumeration of Y. enterocolitica from clinical specimens, food and environmental samples (Wauters, 1973; Leistner et al., 1975; Feeley et al., 1976; Schiemann and Toma, 1978; Norberg, 1981; Shayegani et al., 1981; Schiemann, 1984). However, a number of drawbacks including the requirement of long incubation time and the inefficiency in recovery of some clinically important strains have limited the value of such methods (Zen-Yoji et al., 1974; Pai et al., 1979; Van Noyen et al., 1980; Norberg, 1981; Mustafa et al., 1983). Aulisio et al. (1980) first described a rapid isolation method for recovery of Y. enterocolitica from foods by mixing a weak alkali solution with cold enrichment cultures for a short period and immediately streaking for isolation. The authors reported the alkali treatment increased both selectivity and sensitivity. Following their finding, several other researchers have reported the efficiency of alkali treatment for recovery of Y. enterocolitica from various sources (Doyle et al., 1981; Weissfeld and Sonnenwirth, 1982; Doyle and Hugdohl, 1983; Delmas and Vidon, 1985). Therefore, the objective of this study was to develop an improved isolation technique for

Y. enterocolitica from milk based upon its alkalotolerance and to examine growth parameters which affect recovery.

B. MATERIALS AND METHODS

Organisms and Preparation of Inoculum

Five strains of Y. enterocolitica were used in this study: IP383 (Serotype 0:9), E657 (Serotype 0:5,27), WA20 (Serotype 0:8), Y7P (Serotype 0:8) and C122-76 (Serotype unknown). These strains were provided by Dr. M. P. Doyle (Food Research Institute, Madison, WI). Stock cultures were maintained on Tryptone Soya Agar (TSA, Oxoid, Basingstoke, Hants, England). The cultures were transferred at bi-monthly intervals, incubated at 25° C for 18 h and maintained at 4° C. Escherichia coli ATCC 25922, Citrobacter freundii ATCC 8090 were purchased from the American Type Culture Collection and Pseudomonas fluorescens was obtained from departmental stocks, Department of Food Technology and Science, University of Tennessee, Knoxville. They were grown at 32° C on TSA and maintained at 4° C.

Inocula of Y. enterocolitica were prepared by transferring loopfuls of the actively growing cultures into 250 ml screw-cap Erlenmeyer flasks containing 99 ml of Brain Heart Infusion Broth (BHI, Oxiod) and incubating at 25° C for 18 h. E. coli, C. freundii and P. fluorescens were grown at 32° C for 18 h.

Inoculation of Pasteurized Milk Samples

Freshly pastuerized skim milk was obtained from the University of Tennessee Creamery. Pasteurized milk (99 ml) was aseptically

transferred into 250 ml screw-cap Erlenmeyer flasks and was inoculated at the day of pasteurization with either 10 or 1000 CFU/ml of Y. enterocolitica. Serotypes IP383, E657, WA20, Y7P and C122-76 were evaluated individually. The inoculated milk samples were stored at 4° C and sampled at 0, 3, 7, 14 and 21 days of storage.

Inoculation of UHT Milk and BHI Broth

UHT milk was obtained from a commercial producer and BHI broth was prepared according to manufacturer's instructions. UHT milk and BHI broth (99 ml) were aseptically transferred into 250 ml screw-cap Erlenmeyer flasks. The UHT milk and BHI broth were inoculated with approximately 10,000 CFU/ml Y. enterocolitica strains and incubated at 25° C for 20 h after which the plating was performed for enumeration of Y. enterocolitica. Additional UHT milk samples were also inoculated with approximately 10,000 CFU/ml E. coli, C. freundii and P. fluorescens and incubated at 32° C for 20 h after which the milk samples were plated. Each bacterial strain or species was tested separately.

Recovery and Enumeration

Enumeration of Y. enterocolitica from inoculated pasteurized milk, UHT milk and BHI samples were performed by using both conventional direct plating (DP) and pre-plating alkaline diluent (PPAD) techniques.

Conventional Direct Plating (DP) Technique. Appropriate dilution of milk samples were prepared in (0.01 M) phosphate-buffered

saline (PBS) pH 6.6 with 0.02% peptone and samples were spread plated on MacConkey Agar, Cefsulodin-Irgasan-Novobiocin (CIN) Agar and Tryptone Soya Agar (TSA). All culture media were purchased from Oxold (Basingstoke, England). The plates were incubated at 25° C for 36 h and enumerated for total aerobic plate count (APC) and Y. enterocolitica count.

Pre-plating Alkaline Diluent (PPAD) Technique. A modification of the alkali treatment of Aulisio et al. (1980) was used to prepare the stock solution by dissolving an appropriate amount of potassium hydroxide (KOH) in 0.5% NaCl to give the stock solution of 44% KOH. The stock KOH was filter sterilized and stored at 4° C. The alkaline base diluent (ABD) was prepared by mixing a solution of 0.5% NaCl and 0.06% peptone and autoclaving at 121° C for 15 min. To perform the alkaline treatment, 8.9 ml of ABD was aseptically added to a sterile test tube; an aliquot of 0.1 ml of the 44% stock KOH was added to give a final concentration of 0.44% KOH (pH approximately 12.3); 1 ml of milk sample was added. The tube was mixed vigorously by Vortex and a 1 ml aliquot was transferred to 9 ml of phosphate buffered saline (PBS, 0.01 M, pH 6.6) containing 0.02% peptone. To prevent excessive damage to Y. enterocolitica, the entire treatment from delivery of milk to the alkaline diluent to the first dilution in PBS was completed within 30 sec. Appropriate PBS dilutions were spread plated on TSA, CIN and MacConkey agar. Plates were incubated at 25° C for 36 h. Two replications were performed of all experiments.

C. RESULTS AND DISCUSSION

Recovery of Y. Enterocolitica from Pasteurized Milk

Y. enterocolitica increased rapidly in pasteurized milk inoculated with either 10 or 1000 CFU/ml at 4° C. By the seventh day, most strains except for the WA20 strain, exceeded log 5.50 CFU/ml in the milk samples inoculated with 1000 CFU/ml. By the 14th day *Y. enterocolitica* counts for the same samples were in log 5.20 to 8.52 CFU/ml range (Table 6). *Y. enterocolitica* counts for the milks with initial inoculum of 10 CFU/ml exceed log 6 CFU/ml after 14 days of storage (Table 7). An exception was the WA20 strain which yielded log 4.23 CFU/ml. On CIN plates from the non-alkaline treatment group, crowding by background microflora was severe and it was difficult to obtain accurate readings. After 14 days of storage at 4° C, pasteurized milk with initial inoculum of 1000 CFU/ml had *Y. enterocolitica* counts of log 8.08, 8.32, 8.52, 7.53 and 5.20 CFU/ml for the various *Y. enterocolitica* strains (Table 6) by using conventional spread plating. After PPAD treatment, the Y. enterocolitica counts were log 6.86, 8.05, 8.07, 7.06 and 4.10 CFU/ml, respectively for the same strains (Table 6). Therefore, the alkaline pre-plating treatment caused a reduction of log 0.27 to 1.47 *Yersinia* CFU/ml. This difference was particularly pronounced at the 10 CFU/ml inoculation level (Table 7). After storage of milk for 21 days at 4° C, the alkaline treatment had virtually no effect ($P<0.05$) on most strains. Although, *Y. enterocolitica* was detected at 0, 3, and 7 days by using the conventional direct plating

Table 6. Enumeration of Yersinia enterocolitica from Pasteurized Skim Milk Inoculated with 10^3 CFU/ml and Stored at 4° C up to 21 Days Using Direct Plating (DP) and Pre-plating Alkali Diluent (PPAD) Techniques on Tryptone Soya Agar (TSA), MacConkey (MAC) and Cefsulodin-Irgasan-Novobiocin (CIN) Agars

Strain	Initial Inoculum (CFU/ml)	14 d						21 d					
		TSA		MAC		CIN		TSA		MAC		CIN	
		DP	PPAD	DP	PPAD	DP	PPAD	DP	PPAD	DP	PPAD	DP	PPAD
		Log CFU/ml[a]											
E657	10^3	8.48^{b}	7.96^{b}	8.57^{b}	7.80^{b}	8.08^{b}	6.86^{b}	8.91^{b}	8.85^{b}	8.79^{b}	8.83^{b}	8.60^{c}	8.50^{c}
C122-76	10^3	8.47^{b}	8.15^{b}	8.41^{b}	8.09^{b}	8.32^{b}	8.05^{b}	8.81^{b}	8.44^{b}	8.78^{b}	8.38^{b}	8.52^{b}	8.33^{b}
IP383	10^3	8.47^{bc}	8.31^{cd}	8.46^{bcd}	8.28^{d}	8.52^{b}	8.07^{e}	8.75^{b}	8.63^{b}	8.76^{b}	8.69^{b}	8.57^{b}	8.62^{b}
Y7P	10^3	8.46^{b}	7.74^{cd}	8.23^{bc}	7.52^{de}	7.53^{de}	7.06^{e}	8.81^{b}	8.59^{b}	8.74^{b}	8.43^{b}	8.41^{b}	8.01^{b}
WA20	10^3	7.61^{b}	5.52^{c}	7.29^{b}	4.45^{cd}	5.20^{cd}	4.10^{d}	8.68^{b}	8.0^{bc}	8.45^{b}	7.60^{cd}	7.91^{bc}	6.94^{d}
Control	0	7.62^{b}	$<3^{cf}$	7.48^{b}	$<3^{cf}$	$<3^{cf}$	$<3^{cf}$	8.45^{b}	$<3^{cf}$	8.50^{b}	$<3^{cf}$	$<3^{cf}$	$<3^{cf}$

[a]Values represent the mean of two replications. No cells were recovered with PPAD prior to 14 days storage at 4° C.

[b-e]Means in the row within a given storage day followed by the same letters are not significantly different ($P<0.05$).

[f]For analysis of variance and mean separations, a value of $\log_{10}$ CFU/ml = 3.00 was used since no cells were detected at that level.

Table 7. Enumeration of Yersinia enterocolitica from Pasteurized Skim Milk Inoculated with 10^1 CFU/ml and Stored at 4° C up to 21 Days Using Direct Plating (DP) and Pre-plating Alkali Diluent (PPAD) Techniques and Plating on Tryptone Soya Agar (TSA), MacConkey (MAC) and Cefsulodin-Irgasan-Novobiocin (CIN) Agars

Strain	Initial Inoculum (CFU/ml)	14 d						21 d					
		TSA		MAC		CIN		TSA		MAC		CIN	
		DP	PPAD	DP	PPAD	DP	PPAD	DP	PPAD	DP	PPAD	DP	PPAD
		Log CFU/ml[a]											
E657	10^1	7.96^b	6.62^{cd}	8.22^b	5.87^d	7.06^c	4.40^e	8.82^b	8.44^b	8.88^b	8.42^b	8.40^c	7.74^c
C122-76	10^1	7.83^b	6.62^b	7.94^b	6.80^b	6.98^b	6.59^b	8.62^b	8.23^b	8.64^b	8.14^b	8.29^b	7.93^b
IP383	10^1	8.29^b	7.43^b	8.10^b	7.25^b	7.84^b	6.48^b	8.69^b	8.49^b	8.76^b	8.30^b	8.43^b	8.32^b
Y7P	10^1	7.48^b	5.21^{cd}	7.62^b	4.84^{cd}	6.38^{bc}	4.48^d	8.55^b	7.92^b	8.55^b	7.93^b	7.86^b	7.36^b
WA20	10^1	7.56^b	<3^{df}	7.45^b	<3^{df}	4.23^c	<3^{df}	8.56^b	6.09^{cd}	8.47^b	5.81^{cd}	6.98^c	4.93^d
Control	0	7.62^b	<3^{cf}	7.48^b	<3^{cf}	<3^{cf}	<3^{cf}	8.45^b	<3^{cf}	8.50^b	<3^{cf}	<3^{cf}	<3^{cf}

[a]Values represent the mean of two replications. No cells were recovered with PPAD prior to 14 days storage at 4° C.

[b-e]Means in the row within a given storage day followed by the same letters are not significantly different ($P<0.05$).

[f]For analysis of varience and mean separations, a value of Log_{10} CFU/ml = 3.00 was used since no cells were detected at that level.

(DP) technique on CIN agar, none of the strains could be detected until 14 days when the alkaline pre-plating technique (PPAD) was employed.

Recovery of Y. enterocolitica From UHT Milk and BHI Broth

In UHT milk and BHI broth inoculated with strains of Y. enterocolitica and incubated at 25° C for 18 h, all strains of Y. enterocolitica grew to over log 8.5 CFU/ml (Table 8). Y. enterocolitica enumerated on CIN using the pre-plating alkaline diluent (PPAD) technique were 8.63, 8.54, 8.44, 8.74 and 8.49 CFU/ml in UHT milk for the five strains which showed no significant differences ($P<0.05$) as compared to the conventional plating procedure on CIN agar. In BHI broth, when non-alkali plating was used, all strains grew to levels of log 8.86, 8.89, 8.97, 8.93 and 8.86 CFU/ml on CIN agar plates (Table 9), whereas the PPAD treatment gave log 8.52, 8.48, 8.63, 8.45 and 7.69 CFU/ml, respectively. E. coli counts were log 9.42 and 8.91 CFU/ml for TSA and MacConkey agar. However, E. coli subjected to the PPAD treatment were reduced below detectable numbers on TSA and MacConkey agar. C. freundii and P. fluorescens were also reduced from log 9.20 CFU/ml to non-detectable numbers by the PPAD treatment when plated on TSA or MacConkey agar (Table 8 and 9).

Four of the Y. enterocolitica strains grown in BHI broth for 18 h at 25° C showed little or no injury ($P<0.05$) when subjected to PPAD treatment. The WA20 strain was susceptible to PPAD injury and its counts were log 8.91 and 7.69 CFU/ml, respectively, for TSA and CIN (Table 9). UHT milk appeared to have some protective effect on Y.

Table 8. Comparison of Direct Plating (DP) and Pre-plating Alkaline Diluent (PPAD) Technique for Enumeration of Five Strains of Yersinia enterocolitica Grown in UHT Milk at 25° C for 20 h and Plated on Tryptone Soya Agar (TSA), MacConkey (MAC), Cefsulodin-Irgasan-Novobiocin (CIN) Agars

	Log_{10} CFU/ml[a]					
	TSA		MAC		CIN	
Organism	DP	PPAD	DP	PPAD	DP	PPAD
E657	8.78	8.72	8.80	8.59	8.51	8.63
C122-76	8.67	8.63	8.70	8.57	8.61	8.54
IP383	8.87	8.74	8.73	8.63	8.68	8.44
Y7P	8.69	8.74	8.79	8.69	8.79	8.74
WA20	8.78	8.72	8.78	8.75	8.74	8.49

[a]Values represent the mean of two replications and those in rows are not significantly different ($P<0.05$).

Table 9. Comparison of Direct Plating (DP) and Pre-plating Alkaline Diluent (PPAD) Technique for Enumeration of Five Strains of Yersinia enterocolitica and Three Other Gram-Negative Organisms Grown in Brain Heart Infusion Broth and Plated on Tryptone Soya Agar (TSA), MacConkey (MAC) and Cefsulodin-Irgasan-Novobiocin (CIN) Agars

	Log_{10} CFU/ml[a]					
	TSA		MAC		CIN	
Organism	DP	PPAD	DP	PPAD	DP	PPAD
Yersinia enterocolitica						
E657	8.94[b]	8.52[b]	8.94[b]	8.58[b]	8.86[b]	8.52[b]
C122-76	8.95[b]	8.57[b]	8.94[b]	8.40[b]	8.89[b]	8.48[b]
IP383	8.95[b]	8.59[b]	9.05[b]	8.64[b]	8.97[b]	8.63[b]
Y7P	8.96[b]	8.50[b]	8.97[b]	8.41[b]	8.93[b]	8.45[b]
WA20	8.91[b]	8.88[b]	8.80[b]	8.72[b]	8.86[b]	7.69[c]
Escherichia coli						
ATCC 25922	9.42[b]	<3[ce]	8.91[b]	<3[ce]	<3[ce]	<3[ce]
Citrobacter freundii						
ATCC 8090	9.20[b]	<3[ce]	9.14[b]	<3[ce]	9.20[b]	<3[ce]
Pseudomonas fluorescens						
FTS 20	9.10[b]	<3[de]	<7[cf]	<3[de]	<7[cf]	<3[de]

[a]Values represent the mean of two replications.

[b-d]Means in the same row followed by the same letters are not significantly different ($P<0.05$).

[ef]For analysis of variance and mean separations, values at Log_{10} CFU/ml = 3.00 and Log_{10} CFU/ml = 7.00 were used since no cells were detected at that dilution.

enterocolitica subjected to PPAD treatment since even strain WA20 showed no significant reduction ($P<0.05$) on CIN as compared to TSA. Such protective effect may be due to protein and/or fat content of milk. Aulisio and co-workers (1980) first described an alkali method for rapid recovery of Y. enterocolitica from food. Their procedure involved streaking directly to MacConkey agar from the alkali treatment after 3 to 5 min. They reported a 3 to 5 log reduction in Y. enterocolitica due to the alkali treatment. Their report was a breakthrough in Y. enterocolitica methodology. Schiemann (1983a) studied their procedure further and using the method of Aulisio et al. (1980) characterized the alkalotolerance of selected gram-negative organisms and studied the effect of temperature and level of KOH.

The major modifications which were made to adapt this procedure to milk and improve recovery and isolation of Y. enterocolitica are the lowering of the KOH concentration from 0.5% to 0.44% in the initial alkali treatment and the rapid transition from alkali treatment (30 sec vs. 5 min) to a pH 6.6 buffering diluent (PBS) to prevent unwanted cellular destruction before plating sample.

With PPAD method, E. coli, C. freundii, and P. fluorescens were reduced below detectable levels in BHI broth even when in stationary phase growth after 18 h at 32° C; whereas, four of the Y. enterocolitica strains were unaffected by the PPAD treatment. The WA20 strain, one of the most sensitive of all Y. enterocolitica to environmental stress, was reduced by only one log. In stationary phase, PPAD treatment did not cause significant injury of Y. enterocolitica.

Therefore, the CIN medium could be used successfully.

In pasteurized milk, the PPAD treatment was highly effective for selective isolation of Y. enterocolitica once the organisms had reached late-log or early stationary phase. The PPAD treatment was so highly selective for Y. enterocolitica that TSA and MacConkey agar gave counts very similiar to CIN for pasteurized milk samples.

There are numerous problems in the recovery and isolation of Y. enterocolitica from foods (Mehlman et al., 1978a). The combination of a selective medium such as CIN with the PPAD treatment virtually eliminated all background microflora and greatly facilitated the enumeration of Y. enterocolitica in food systems having high microbial numbers.

CHAPTER V

EXPERIMENTAL INFECTION OF SHELL EGGS WITH *YERSINIA ENTEROCOLITICA*

A. INTRODUCTION

The susceptibility of shell eggs to microbial invasion has been long recognized. A number of researchers have shown that several species of microorganisms including a few enteropathogens are able to penetrate and infect shell eggs (Lorenz et al., 1952; Stokes et al., 1956; Garibaldi and Bayne, 1960; Brant and Starr, 1962; Garibaldi and Bayne, 1962a,b; Paton and Ayres, 1964; Brown et al., 1966a,b; Rizk et al., 1966b; Clark and Bueshchkens, 1985). Although a high incidence of *Yersinia enterocolitica* in poultry and poultry products has been reported (Leistner et al., 1975; Norberg, 1981; DeBoer et al., 1982); the possible invasion of shell eggs by this bacterium has not been studied. Therefore, this investigation was undertaken to determine the ability of *Y. enterocolitica* to penetrate and infect shell eggs in the presence or absence of iron supplementation in bacterial suspension; to investigate the rate and extent of infection of shell eggs over extended storage (28 d) at 10° C; and to compare the effectiveness of the inoculation method used to infect the shell eggs.

B. MATERIALS AND METHODS

Source of Eggs

Nest clean, unwashed, newly laid eggs from one strain of Single Comb White Leghorns were obtained from The University of Tennessee poultry farm. Eggs were collected the day of lay and transported to the laboratory where they were stored at room temperature. The eggs were inoculated within 24 h of storage.

Organism

Two strains of *Y. enterocolitica* were used in this study: IP383 (serotype 0:9) and Y7P (serotype 0:8). These strains were provided by Dr. Michael P. Doyle (Food Research Institute, Madison, WI). Stock cultures were grown and maintained on Tryptone Soya Agar (TSA, Oxoid, Basingstoke, Hants, England). The cultures were transferred at bi-monthly intervals, incubated at 25° C for 24 h and maintained at 4° C.

Preparation of Inoculum

Prior to preparation of inocula, the cultures were subcultured for two consecutive days by transferring loopfulls of cultures into 250 ml screw-cap Erlenmeyer flasks containing 99 ml of Brain Heart Infusion broth (BHI, Oxoid) and incubated at 25° C for 18-20 h. Inocula of *Y. enterocolitica* were prepared by transferring 1 ml of actively growing cultures into the flasks containing 99 ml of BHI broth and incubating at 25° C for 18-20 h.

Preparation of Bacterial Suspension

An 18 to 20 h culture in BHI broth was centrifuged at 5000 rpm (3020 x g) for 15 min and washed twice with sterile distilled water and resuspended to volume in sterile distilled water. This was done to avoid nutrient carry over into the suspension. Serial dilutions were made in sterile distilled water to obtain bacterial suspensions with approximately 10^6 CFU/ml. Strains IP383 and Y7P were evaluated separately. The actual numbers of Y. enterocolitica of bacterial suspensions were determined by spread plating appropriate dilutions of the suspensions on Cefsulodin-Irgasan-Novobiocin agar (CIN, Oxoid) and TSA agar before and after inoculation of the eggs. This was done to determine the effects of centerifugation, suspension in distilled water and the various treatments on viability of Y. enterocolitica.

Preparation of Bacterial Suspension with Iron Supplement

An identical bacterial suspension was prepared to which 20 ppm iron in the form of a sterile aqueous solution of ferrous sulfate ($FeSO_4.7H_2O$) was added prior to inoculation of eggs. The actual number of Y. enterocolitica cells in the iron supplemented suspension was determined by spread plating appropriate dilutions of the suspension on TSA and CIN agars before and after inoculation of eggs.

Inoculation of Eggs

Inoculation of eggs was performed by using two experimental infection techniques.

Temperature Differential. The infection technique of Lorenz et al. (1952) was used to experimentally infect the eggs. The eggs were pre-warmed to 37° C for eight h prior to immersion. The pre-warmed

eggs were completely immersed in the 10° C bacterial suspension with and without iron supplement for exactly 5 min, after which they were drained and air dried for one h at room temperature and stored at 10° C. At selected intervals, the content of eggs were examined for total Y. enterocolitica counts.

Pressure Differential. A modification of the technique of Clark and Bueschkens (1985) was used for pressure differential inoculation. A pressure cooker was modified by replacing the gauge with a hose outlet and connecting the chamber to an air cylinder. The room tempered (20-22° C) eggs were placed in containers containing the same temperature bacterial suspension (20-22° C) with and without the iron supplement. The containers of eggs were then placed in the chamber of the pressure vessel. The pressure of the chambers was raised 1 atmosphere (14.7 psi) and held for one min. It then was released over 20 min. Upon withdrawal, the eggs were drained and air dried at room tempera-ture for one h prior to incubation at 10° C.

Sampling and Enumeration

At 0, 3, 7, 14, 21 and 28 days of storage 3 eggs from each treatment (4 eggs on day 7) were immersed in 70% alcohol for 5 min and then drained and flamed to sterilize the outer shells prior to removal of egg contents. The individual egg content was poured aseptically into sterile 4 oz. specimen container containing 10-15 sterile glass beads and shaken vigorously so that contents were mixed well. Serial dilutions of well-mixed samples were prepared in phosphate-buffered

saline (pH 7.2, 0.1 M, 0.85% NaCl) containing 0.02% peptone. Aliquots of 0.1 ml of appropriate dilutions were spread plated on CIN agar. Plates were inoculated at 25° C for up to 48 h before enumeration of Y. enterocolitica counts.

C. RESULTS AND DISCUSSION

Infection of Shell Egg by Y. enterocolitica

Temperature differential method. Examination of the shell egg contents, subjected to temperature differential inoculation with or without iron supplementation (Table 10), yielded no Y. enterocolitica CFU/ml immediately after inoculation (day 0) and on day 3 of storage at 10° C. An exception was limited growth in one egg within a non-iron treated group on day 3. After 7 days of storage, shell eggs treated with iron showed a marked increase in the rate and extent of infection by Y. enterocolitica. Infection rates were >69% as compared to ca 8.5% for non-iron treated groups. The overall infection rate reached 100% for iron supplemented groups after 14 days of storage and remained the same thereafter until the end of the storage period (28 days). After twoweeks of storage, Y. enterocolitica counts in iron treated eggs were greater than 10^5 CFU/ml of egg contents.

The temperature differential method of inoculation has been a commonly chosen technique for experimental infection of shell egg with common egg spoilage and pathogenic microorganisms by many investigators (Lorenz et al., 1952; Stokes et al., 1956; Garibaldi and Bayne, 1960;

Table 10. Infection of Shell Eggs by Yersinia Enterocolitica strains, Y7P and IP383, Using the Temperature Differential Inoculation Method and Storing the Eggs at 10° C for up to 28 days

Strain of YE	Inoculation Method	Incubation Time (day)	No. Eggs Examined	No. Eggs infected	Mean[a] Yersinia CFU/ml infected Eggs (log)	% Infected Eggs
Y7Ph	T.D.[b]	0	6	0	<1	00.0
		3	6	1	2.68	16.7
		7	8	0	<1	00.0
		14	6	2	1.50	33.3
		21	6	0	<1	00.0
		28	6	1	1.30	16.7
	T.D.+Fe	0	6	0	<1	00.0
		3	6	0	<1	00.0
		7	8	6	4.17	75.0
		14	6	6	5.32	100.0
		21	6	6	6.47	100.0
		28	6	6	6.51	100.0
IP383	T.D.	0	6	0	<1	00.0
		3	6	0	<1	00.0
		7	8	1	1.30	14.3
		14	6	4	1.86	66.7
		21	6	1	1.0	16.7
		28	6	1	1.85	16.7
	T.D.+Fe	0	6	0	<1	00.0
		3	6	0	<1	00.0
		7	8	5	4.52	71.4
		14	6	6	6.07	100.0
		21	6	6	6.75	100.0
		28	6	6	6.98	100.0

[a]Values represent the mean of two replications; three eggs were examined in each replication for a given storage time; exception was day 7 on which 4 eggs were examined.

[b]T.D. = Temperature differential inoculation.

Brant and Starr, 1962; Garibaldi and Bayne, 1962a, b; Paton and Ayres, 1964; Rizk et al., 1966b; Clark and Bueshchkans, 1985). Inoculation of eggs by this method is carried out by immersing pre-warmed shell eggs in a cold bacterial suspension. The immersion of a warmed egg into a cold suspension medium causes interior content of egg (yolk and white egg) to contract more than shell, therefore causing the microbial cells along with suspension medium to be pulled into the egg through the shell pores (as a result of suction) and deposited on the shell membranes (Haines and Moran, 1940; Board, 1966). Depending on the prevailing conditions, temperature and/or availability of iron, the microbial cells may colonize the membrane and subsequently invade the egg contents. Some pertinent factors indentified in playing an important role in the incidence of infection of shell eggs using temperature differential method of inoculation are: temperature differential between eggs and microbial suspension (Lorenz et al., 1952; Brant and Starr, 1962), number of microorganisms in suspension medium (Lorenz et al., 1952; Brant and Starr, 1962), duration of immersion of eggs in microbial suspension (Garibaldi and Bayne, 1960; Brant and Starr, 1962), quality and thickness of egg shell (Orel, 1959; Sauter and Petersen, 1974), treatment of shell before immersion (Brown et al., 1966b), concentration of iron in microbial suspension (Brant and Starr, 1962; Garibaldi and Bayne, 1962a), age of egg at the time of inoculation (Elliott, 1954) and temperature of storage after inoculation (Stokes et al., 1956; Rizk et al., 1966b). There is

reason to believe that similar factors may be involved in invasion and infection of shell eggs at the time of lay and during its subsequent handling, washing and storage (Solowey et al., 1946; Lorenz et al., 1952; Forsythe et al., 1953).

Pressure Differential Method. Examination of egg contents inoculated with Y. enterocolitica using the pressure differential method in presence or absence of iron (Table 11) yielded no Y. enterocolitica immediately after inoculation. On day 3 of storage at 10° C, Y. enterocolitica was recovered from only one treatment group which had iron supplementation. After 7 days of storage at 10°C, the infection rate of eggs subjected to iron supplementation reached 75%, whereas, that of non-iron treated groups was less than 19%. The overall infection rate after 14 days of storage reached 100% in the presence of iron with Yersinia counts over 10^5 CFU/ml of egg content. Both strains showed similar growth characteristics in the infection of shell eggs utilizing the pressure differential method of inoculation.

The pressure differential method has been used by other investigators to either artifically inoculate shell eggs with Campylobacter jejuni (Clark and Bueshchkens, 1985) or prevent spoilage by treating eggs with antibiotics (Alls et al., 1963). The results from the present study showed that the pressure differential method of inoculation was equally effective as the temperature differential method in infecting the shell eggs with Y. enterocolitica (Table 10 and 11). The experiment illustrates that with the pressure differential

Table 11. Infection of Shell Eggs by Yersinia enterocolitica Strains, Y7P and IP383, Using the Pressure Differential Inoculation Method and Storing the Eggs at 10° C for up to 28 Days

Strain of YE	Inoculation Method	Incubation Time (day)	No. Eggs Examined	No. Eggs infected	Mean[a] Yersinia CFU/ml Infected Egg (log)	% Infected Egg
Y7P	P.D.[b]	0	6	0	<1	00.0
		3	6	0	<1	00.0
		7	8	1	1.0	14.3
		14	6	1	1.50	16.7
		28	6	0	<1	00.0
	P.D.+Fe	0	6	0	<1	00.0
		3	6	0	<1	00.0
		7	8	5	1.63	71.4
		14	6	6	5.56	100.0
		21	6	6	6.66	100.0
		28	6	6	6.48	100.0
IP383	P.D.	0	6	0	<1	00.0
		3	6	0	<1	00.0
		7	8	2	1.0	28.6
		14	6	4	1.52	66.7
		21	6	4	1.88	66.7
		28	6	6	2.53	66.7
	P.D.+Fe	0	6	0	<1	00.0
		3	6	4	1.53	66.7
		7	8	7	3.55	87.5
		14	6	6	5.87	100.0
		21	6	6	6.62	100.0
		28	6	6	6.59	100.0

[a]Values represent the mean of two replications; three eggs were examined in each replication for a given storage time with the exception of day 7 on which 4 eggs were examined.

[b]P.D. = Pressure differential inoculation.

technique, bacterial cells are forced through the shell by external pressure, whereas in temperature differential technique, the vacuum created inside the eggs due to differences in temperature between egg content and outside, causes the bacterial cells to be pulled through the shell and deposited on the membranes.

Yersinia enterocolitica Count of Suspension Medium

Enumeration of Y. enterocolitica counts in the suspension medium before and after inoculation of the shell egggs is shown in Table 12. The viable cell counts were determined by spread plating the appropriate dilutions of bacterial suspension on CIN and TSA plates before and after each treatment. The counts on TSA as compared to that on CIN were used to determined whether centrifugation, suspension of Y. enterocolitica cells in distilled water or temperature and pressure differential methods of inoculations had caused any physical stress or damage to the Yersinia cells. The results show no significant differences between viable Y. enterocolitica counts before and after the treatments in the absence of iron. Supplementation of bacterial suspension with 20 ppm iron significantly reduced Yersinia counts and increased injury. Strain Y7P was somewhat more sensitive in the presence of iron immediately after iron supplementation, but the overall counts for both strains in the iron supplemented suspension medium were significantly reduced (Table 12).

The results indicate that the reduction in cell number of Y. enterocolitica observed after iron supplementation of bacterial

Table 12. Yersinia enterocolitica Count, Strain Y7P and IP383, in Bacterial Suspension Before and After Treatment of Egg Using Temperature and Pressure Differential With and Without Iron Supplementation

		Log CFU/ml			
		Before TMT		After TMT	
Strain	Treatment	TSA	CIN	TSA	CIN
Y7P	T.D.[d]	6.04[a]	6.01[a]	6.02[a]	5.84[a]
Y7P	T.D.+Fe	6.00[a]	5.10[b]	5.36[b]	3.88[c]
IP383	T.D.	6.02[a]	5.98[a]	5.97[a]	5.79[a]
IP383	T.D.+Fe	5.92[a]	5.76[a]	5.81[a]	4.48[b]
Y7P	P.D.[e]	5.95[a]	5.95[a]	6.00[a]	5.80[a]
Y7P	P.D.+Fe	5.95[a]	5.23[b]	4.94[bc]	3.58[c]
IP383	P.D.	5.99[a]	5.91[a]	6.00[a]	5.82[a]
IP383	P.D.+Fe	5.99[a]	5.74[a]	4.80[b]	3.87[c]

[a-c]Means in rows followed by different letters are significantly different ($P<0.05$).
[d]T.D. = Temperature differential treatment.
[e]P.D. = Pressure differential treatment.

suspension was due to the presence of iron. Physical stress such as centrifugation, temperature or pressure differential and suspension in distilled water had minimal or no effect on Y. enterocolitica since in non-iron treated groups, before and after treatments, no reduction was observed in Yersinia count. Garibaldi and Bayne (1962a) made a similar observation and reported that the addition of 10 ppm iron into bacterial suspensions resulted in approximately 2 log CFU/ml reduction in viable bacterial counts of tested organisms. It is well known that some metallic ions including iron are essential for growth of microorganisms and that concentrations over their growth requirements may result in toxicity effects (Salle, 1948; Weinberg, 1960).

Mode of Infection of Shell Egg by Y. enterocolitica

The exterior structure of shell egg including cuticle, shell and shell membranes are known to be effective as mechanical barriers against microorganisms (Garibaldi and Stokes, 1958; Hartung and Stadelman, 1962; Lifshitz et al., 1964; Vadehra and Baker, 1968). Nevertheless, under certain environmental and experimental conditions, gram-negative bacteria may penetrate these barriers and infect egg content. It is known that the cuticle plays an important role in preventing microbial penetration through shell (Vadehra and Baker, 1968). The protective effect of cuticle is known to diminish with the age of egg as well as physical and chemical treatments which eggs may be subjected to during handling, storage and washing (Brown et al., 1966b; Ball et al. 1975).

Penetration of the egg shell by various microorganisms has been the subject of many studies (Scott, 1930; Schalm, 1937; Haines and Moran, 1940; Stuart and McNally, 1943; Mallmann and Davidson, 1944; Soloway et al., 1946). Garibaldi and Stokes (1958) demonstrated that egg shell easily permits the passage of microorganism through pores when microbial suspensions were filtered through the shell. The finding of this study is in accord with the earlier studies. Therefore, Y. enterocolitica, like common egg spoilage microorganisms is capable of penetrating the shell and subsequently infecting the egg content under the designated experimental conditions. Microbial penetration of shell via pores is demonstrated, but little is known about the mode of penetration of microorganisms through shell membranes.

The importance of different exterior structures of shell eggs as mechanical barriers was the subject of study by Lifshitz et al. (1964) who reported that inner shell membrane is of prime importance, followed by shell and outer membrane as a barrier against microbial penetration. Although the shell membranes have been shown to act as microbial filters (Haines and Moran, 1940; Garibaldi and Stokes, 1958), their resistance to microbial penetration diminishes upon contact with microorganisms. Membranes are affected by concentration of microbial challenge and presence of iron (Hartung and Stadelman, 1962) and the age of egg (Hartung and Stadelman 1963). The authors illustrated this effect of age to be due to alkaline hydrolysis of

albuminous material caused by high pH of egg white. It has been shown that several common egg spoilage microorganisms are capable of growing on the shell membrane as well as in the suspension of comminuted shell membranes (Stuart and McNally, 1943; Stokes and Osborne, 1956; Elliott and Brant, 1957; Garibaldi and Stokes, 1958; Board, 1965). The structural compositionn of shell membrane was described by Simons and Wiertz (1963) as a network of keratin fibers with a polysaccharide mantle which are cemented by interstitial albuminous matrix. Walden et al. (1956) proposed that enzymatic digestion of albuminous material due to microbial growth on the shell membrane may be the means by which microorganisms penetrate the membranes. Their proposal was supported by works of Garibaldi and Stokes (1958) and Brown et al. (1965). In retrospect, Board (1965) cited that bacterial proteolysis of shell membrane is unlikely to play an important role in microbial penetra-tion of shell membranes. Later in 1966, Board suggested that an ap-parent resistance of shell membranes to microbial penetration is pri-marily due to the antimicrobial effects of egg white and shell mem-branes per se may not play an important role in restraining microbial invasion of egg content.

The lag period of 3 to 7 days (time from inoculation of egg with Y. enterocolitica to occurrence of the organism in egg content) observed in this study correlates with the findings of other investigators who cited the lag period of up to 20 days, depending on type of test organisms used as well as inoculation and storage

conditions employed (Gillespie and Scott, 1950; Orel, 1959; Fromm and Monroe, 1960; Garibaldi and Bayne, 1960; Board and Ayres, 1965). It has been suggested that the duration of lag period primarily reflects the antimicrobial properties of egg white and the invasion of egg content takes place only when microorganisms overcome such effects or when yolk makes contact with the shell membranes (Brooks, 1960; Board, 1964; Board and Ayres, 1965; Board, 1966). Brooks (1960) reported that suspending shell membrane pieces in egg white enhances the growth of Pseudomonas sp. Therefore it appears that shell membranes play dual rules by providing protection against antimicrobial properties of egg white and furnishing essential nutrients for the growth of organisms to colonize and eventually invade the egg content. During the course of colonization on the shell membranes, bacteria may excrete metabolic products which may diffuse into the egg white making this medium more favorable for growth either by changing the pH of the immediate micro-environment around the colonies and/or by releasing iron-binding compounds, siderophores, to obtain their iron needs (Brooks, 1960; Garibaldi and Bayne, 1960; Garibaldi and Bayne, 1962a).

The results of this study indicate that supplementation of bacterial suspension with iron substantially increased both the rate and extent of infection of shell eggs by Y. enterocolitica. Similar results have also been reported in studies which dealt with common egg spoilage bacteria (Garibaldi and Bayne, 1960; Brant and Starr, 1962; Garibaldi and Bayne, 1962a,b) and other pathogenic microorganisms

(Sauter and Petersen, 1974; Clark and Bueschkens, 1985). Garibaldi and Bayne (1962b) found the spoilage of shell eggs washed with well water having varying levels of soluble iron, was directly proportional to the iron content of the water. The authors concluded that the minute concentrations of iron which pentrated through the shell were sufficient to enhance the growth of organisms on the shell membrane and consequently enabled them to alter their immediate growth environment by rapid production of diffusable metabolic products. Since production of iron-binding compounds, siderosphores, by Y. enterocolitica has not been detected (Perry and Brubaker, 1979), this coupled with the ability of the bacterium to survive and have limited growth in egg white (as reported in Chapter VI of this study) may suggest that Y. enterocolitica is capable of utilizing a different mechanism to obtain its iron requirement. One such mechanism was reported by Perry and Brubaker (1979) to be a cell-bound iron transport system. Another possibility is the excretion of soluble micromolecules such as lipopolysaccharide, similar to that reported for Esherichia coli, which are able to bind or localize iron from iron-limited medium (Kochan et al., 1977).

The findings of this study indicate that Y. enterocolitica contaminates eggs in a manner analogous to common egg spoilage microorganisms and Salmonella spp. It is possible for Y. enterocolitica to penetrate egg shells and subsequently infect egg contents due to improper washing, storage or handling conditions. Contamination of wash water with a trace amount of soluble iron salts,

may significantly increase the incidence of Y. enterocolitica infection. Since no sign of infection can be recognized in Y. enterocolitica infected eggs during candling of eggs or inspection of broken-out egg contents, such eggs may find their way to egg breaking plants and contaminate a large volume of broken-out eggs or be used in the home or other food establishments and contaminate other foods. Generally, foods containing eggs are cooked or baked in such a way that Y. enterocolitica and other pathogens are destroyed. However, raw eggs and egg products may be used in foods such as egg nog, mayonnaise, and salad dressings where inhibition and destruction of pathogens is assumed to occur due to low pH or low refrigeration temperature. Since Y. enterocolitica survives at pH of 4.6 and grows fairly rapidly in the refrigerator, it is important that eggs and egg products consumed raw be free of Y. enterocolitica. Careful handling of eggs and milk and education of public concerning the hazards of psychrotrophic foodborne pathogens are essential to reduce the incidence of yersiniosis.

CHAPTER VI

GROWTH CHARACTERISTICS OF *YERSINIA ENTEROCOLITICA* IN RAW AND PASTEURIZED LIQUID EGGS

A. INTRODUCTION

The inability of a large number of microorganisms to invade eggs has been shown to be due to the bacteriostatic and bacteriocidal effects of egg white against such microorganisms (Schade and Caroline, 1944; Brooks, 1960; Garibaldi, 1960; Board, 1966). Studies have shown some species of microorganisms, including *Salmonella*, are able to survive and grow in liquid eggs and egg whites (Ayres and Taylor, 1956; Garibaldi, 1960; Rizk et al., 1966a,b; Garibaldi, 1970). However, *Salmonella* does not grow in refrigerated eggs due to its mesophilic nature (Ayres and Taylor, 1956; Rizk et al., 1966b). The growth of psychrotrophic pathogens in refrigerated eggs has not been investigated. Therefore studies regarding the safety of refrigerated eggs and egg products should be a priority. This investigation was designed to investigate the growth patterns of *Y. enterocolitica* in raw and pasteurized liquid egg whites, yolks and whole eggs at 4° C and study the effect of iron supplementation of egg whites on the growth of *Y. enterocolitica*.

B. MATERIALS AND METHODS

Source of Eggs

Nest clean, unwashed, newly laid eggs from one strain of Single Comb White Leghorns were obtained from The University of Tennessee poultry farm. Eggs were collected the day of lay and transported to the laboratory where they were stored at 10° C for one week prior to the experiment.

Organism

Two strains of Y. enterocolitica were used in this study: IP383 (serotype 0:9), and Y7P (serotype 0:8). These Y. enterocolitica strains were provided by Dr. Michael P. Doyle (Food Research Institute, Madison, WI). Stock cultures were grown and maintained on Tryptone Soya Agar (TSA, Oxiod, Basingstoke, Hants, England). The cultures were tranferred at bi-monthly intervals, incubated at 25° C for 24 h and maintained at 4° C.

Preparation of Inoculum

Prior to preparation of inocula, the cultures were subcultured for two consecutive days by transferring loopfulls of cultures into 250 ml screw-cap Erlenmeyer flasks containing 99 ml of Brain Heart Infution broth (BHI, Oxoid) and incubated at 25° C for 18-20 h. Inocula of Y. enterocolitica was prepared by transferring 1 ml of actively growing cultures into the flasks containing 99 ml of BHI broth and incubating at 25° C for 18-20 h.

Preparation of Samples

The eggs were immersed in 70% alcohol for 5 min then drained and flamed to destroy organisms in the shell. The sterile shell eggs were aseptically cracked and opened at one end to remove the content.

Egg whites, egg yolks and whole eggs were obtained in separate sterile Mason Jars containing glass beads and the contents were mixed well by shaking the jar vigorously. The well-mixed liquid eggs were transferred into two sets of 20x150 mm screw cap tubes. One set of the egg samples was batch pasteurized as follows: egg whites at 57° C for 4 min, egg yolks at 60° C for 6.5 min and whole eggs at 60° C for 4 min. The above pasteurization conditions met the regulations set forth by the U. S. Department of Agriculture (Section 59.570 paragraph (b), 7CFR, 1986). One flask of raw egg white and one flask of pasteurized egg whites were supplementated with a sterile solution of ferrous sulfate ($FeSO_4.7H_2O$) to give an iron concentration of 10 ppm (10 mg/L) of egg white.

All egg samples were inoculated with 10^3 cells/ml of Y. enterocolitica. Strains IP383 and Y7P were evaluated individually. The egg samples were stored at 4° C and sampled at 0, 3, 7, 14, 21, and 28 days. Appropriate dilutions of the egg samples were prepared in physiological saline and spread plated on Cefsulodin-Irgasan-Novobiocin (CIN, Oxoid) and TSA agars. The plates were incubated at 25° C for 36 h and enumerated for Y. enterocolitica counts. Representative colonies were selected from CIN to positively confirm identification of Y. enterocolitica.

C. RESULTS AND DISCUSSION

Growth of Y. enterocolitica in Egg White

The growth patterns of Y. enterocolitica in egg whites and egg whites supplemented with 10 ppm iron are shown in Figure 2. Y. enterocolitica counts increased significantly ($P<0.05$) in egg whites and iron supplemented egg whites; however, the rate and extent of growth was minimal. No significant differences ($P<0.05$) were found between the two test strains in regard to growth in egg whites with and without iron supplementation. There were also no significant differences ($P<0.05$) in the rate and extent of growth in raw egg white vs pasteurized egg whites.

Egg white has long been known to possess bacteriostatic and bactericidal effects against microorganisms (Brooks, 1960; Garibaldi, 1960). Such an effect was also noted by Schade and Caroline (1944) and stability of the effect was observed even at 60° C for one hour. This may explain the similarity in the growth patterns observed between raw and pasteurized egg whites in this study. Factors influencing the bactericidal and bacteriostatic effects include: lysozyme (Fleming, 1922; Ayers, 1958), conalbumin (Feeney and Nagy, 1952; Garibaldi, 1960; Board, 1964; Board, 1966), ovomucoid (Ayres, 1958), avidin (Eakin et al., 1940), high pH (Sharp and Whitaker, 1927), lack of free water (Nath and Baker, 1973), presence of only native protein (Romanoff and Romanoff, 1949) and physical properties of egg white (Board, 1964; Yadav and Vadehra, 1977). Among the factors cited, conalbumin plays a major role in inhibiting the

invasion of microbes into the egg whites by binding tightly with iron (Feeney and Garibaldi, 1948; Garibaldi, 1960). Weinberg (1978) stated the pres-ence of iron was essential for microbial growth. Johnston (1956) established 0.3 ppm of iron as being present in egg white. However Feeney and Garibaldi (1948) and Garibaldi (1960) indicated that due to the formation of conalbumin-Fe complex, iron is rendered unavailable for microbial growth. Newly laid egg whites have a pH of 7.6-7.8, but after a few days of storage the pH increases up to 9.1-9.2 (Sharp and Powell, 1931). Such a high pH enhances the coupling of conalbumin and iron (Board and Ayres, 1965; Garibaldi, 1960).

A number of microorganisms: Escherichia coli, Bacillus megaterium, Salmonella typhimurium, Enterobacter aerogenes, Enterobacter cloacae, Pseudomonas sp. and Actinomycetes sp. have the ability to absorb necessary iron from an iron deficient medium by utilizing complex iron transport mechanisms. Soluble low molecular weight compounds termed siderophores are biosynthesized and released into their immediate growth environment, which then binds with iron and reenters the cells (Garibaldi and Bayne, 1962a,b; Garibaldi, 1970; Garibaldi, 1971; Garialdi, 1972; Lankford, 1973; Perry and Brubaker, 1979). Although the production of siderophores by Yersinia species has not been reported, Perry and Brubaker (1979) found that Y. enterocolitica and Y. pseudotuberculosis possessed the ability to use siderophores produced by other microorganisms. These authors also indicated a cell-bound iron carrier to be the method of iron transport in Y. enterocolitica and Y. pseudotuberculosis from iron deficient medium. Egg whites do not support the growth of several pathogenic

bacteria under refrigeration temperature, although production of siderophores by Salmonella have been reported (Garibaldi, 1970; Garibaldi, 1972). Salmonella failed to grow in egg whites at 10° C and lower (Ayres and Taylor, 1956; Rizk et al., 1966b). Egg white has been found to have inhibitory effects on Shigella dysenteriae and Staphylococcus aureus. (Schade and Caroline 1944) and to have toxic effects on Campylobacter jejuni (Clark and Bueschkens, 1986).

Therefore, regardless of the type of iron transport system used by certain microorganisms, it is apparent that possession of such mechanisms enables these microorganisms to reverse the inhibitory effect of conalbumin (Garibaldi, 1970). The findings of this study suggest that Y. enterocolitica is the only known pathogen which is able to survive and grow in the adverse environmental condition of egg whites at refrigeration temperature.

The results showed that supplementation of egg whites with 10 ppm iron significantly improved ($P<0.05$) the growth of Y. enterocolitica (Figure 2). This concides with prior studies (Schade and Caroline, 1944; Garibaldi, 1960; Board and Ayres, 1965) which indicate that supplementation of egg white with 20 ppm iron saturates the conalbumin content completely and reverses the inhibitory effect of egg white on gram-negative microorganisms. Garibaldi (1960) reported that partial saturation of egg white conalbumin with 1 ppm or 7 ppm iron brings about differing responses from various organisms.

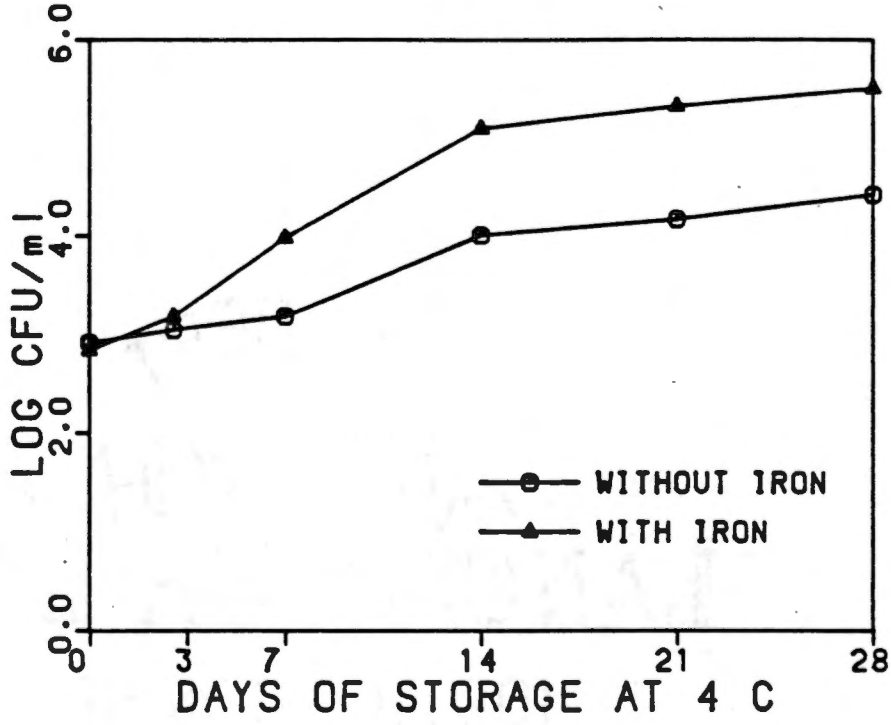

Figure 2. Growth of Yersinia enterocolitica in liquid egg whites with and without 10 PPM iron suplementation. Data points represent the mean of two replications for two strains (IP383 and Y7P) and two treatments (raw and pasteurized).

The results of this study indicate that the partial saturation of egg whites with 10 ppm iron significantly ($P<0.05$) improved the growth of Y. enterocolitica. At this level of supplementation (10 ppm), iron binds tightly with conalbumin thereby rendering the exogenous iron unavailable as a free iron source for Y. enterocolitica (Board and Ayres, 1965). Since Y. enterocolitica is able to grow in egg whites, it is obvious that it possesses a mechanism for obtaining its iron requirement. Additional research is needed to further characterized proposed mechanisms such as that proposed by Perry and Brubaker (1979).

Growth of Y. enterocolitica in Egg Yolks

Growth pattern of Y. enterocolitica in liquid yolks inoculated with approximately 10^3 CFU/ml and stored at 4° C are shown in Figure 3. Viable Yersinia count reached approximately log 7 CFU/ml after only 7 days of storage and up to log 8.5 CFU/ml after 14 days. The growth then leveled off and no significant changes were observed in Y. enterocolitica counts thereafter through the end of the experiment (28 days). Growth patterns of both test strains were similar and not effected by the plating medium used. There was no significant differences ($P<0.05$) between Y. enterocolitica growth in the raw yolk as compared to the pasteurized yolk samples.

The findings of this study indicate that the rate and extent of Y. enterocolitica growth in the yolks is substantially greater than those reported for pasteurized skim milk at 4° C (Amin and Draughon, 1987). Other pathogenic bacteria such as Salmonella and Campylobacter jejuni commonly associated with eggs and poultry do not

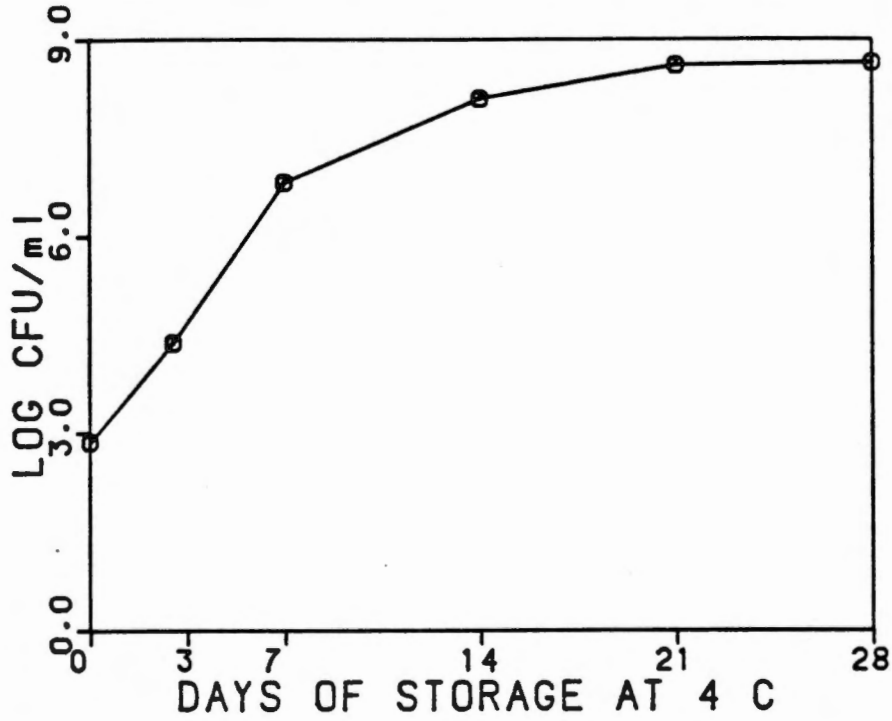

Figure 3, Growth of Yersinia enterocolitica in liquid egg yolks. Data points represent the mean of two replications for two strains (IP383 and Y7P) and two treatments (raw and pasteurized).

mimic the growth characteristics of Y. enterocolitica under similar prevailing condition (Rizk et al., 1966b; Clark and Bueschkens, 1986). Clark and Bueschkens (1986) reported the growth of Campylobacter jejuni in egg yolks at 42° C under controlled atmosphere and Rizk et al. (1966b) found that viable Salmonella pullorum counts decreased during storage at 2° C and increased only when temperature exceeded 10° C.

It is noteworthy that the number of Yersinia cells increased to over log 8.5 CFU/ml in the yolks, yet visual symptoms of infection were not observed in these samples. Therefore, it is imperative that proper handling and storage of yolks and yolk products be done to avoid public health hazard.

Growth of Y. enterocolitica in Whole Eggs

Growth patterns of Y. enterocolitica in whole eggs are shown in Figure 4. No significant differences ($P<0.05$) in the rate and extent of growth were found between the test strains. The plating medium had little or no effect on the recovery of Y. enterocolitica from whole eggs. The growth of this organism in raw whole eggs was not significantly different ($P<0.05$) to that in pasteurized liquid whole eggs. Y. enterocolitica reached approximately log 6 CFU/ml after 7 days of storage at 4° C and after 14 days, the viable counts increased to over log 7.5 CFU/ml. The rate and extent of Y. enterocolitica growth in whole eggs surpassed that reported for pasteurized skim milk under similar growth conditions (Amin and Draughon, 1987).

It is well known that several egg white proteins are biologically active and have antimicrobial properties (Ayres, 1958; Garibaldi, 1960; Brooks, 1960; Board, 1966). Such protective effects are virtually eliminated by substances present in egg yolks (Brooks, 1960; Board, 1964).

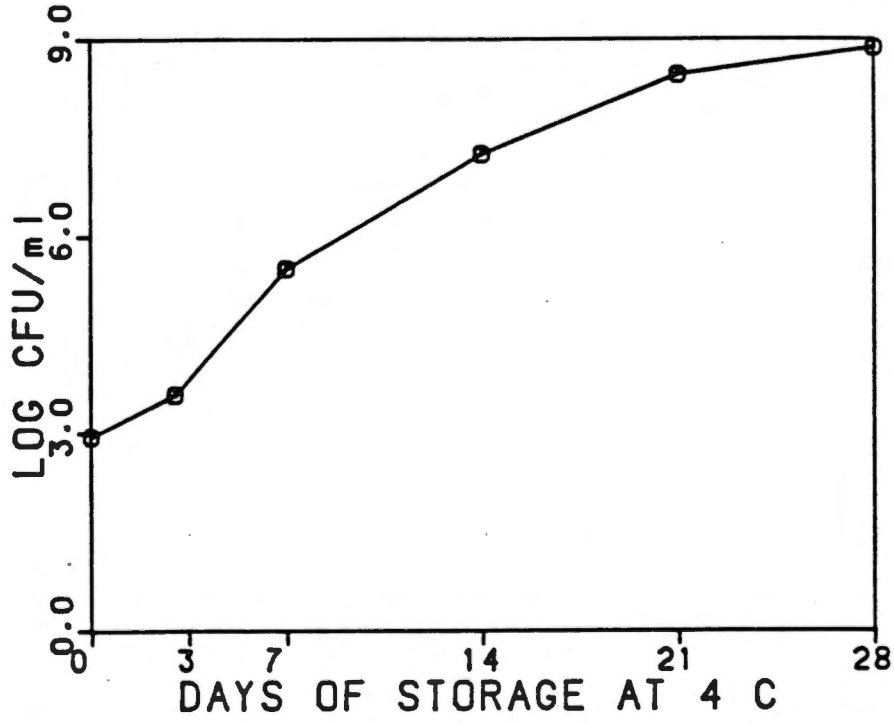

Figure 4. Growth of Yersinia enterocolitica in liquid whole eggs. Date points represent the mean of two replications for two strains (IP383 and Y7P) and two treatments (raw and pasteurized).

This may explain why Y. enterocolitica was able to grow rapidly in liquid whole eggs.

During the course of this study no physical sign of infection or microbial growth were observed in liquid whole eggs. Therefore inspection of liquid eggs merely by visual or olfactory signs is not appropriate since such products may be infected without obvious signs.

CHAPTER VII

SUMMARY

The overall objectives of this study were twofold; first, to determine the growth characteristics of Yersinia enterocolitica in pasteurized milk and to develop an improved isolation method for the recovery of this enteropathogen from milk. The second objective was to investigate, for the first time, the ability of Y. enterocolitica to infect and grow in shell eggs and broken-out liquid eggs under refrigeration temperatures.

The findings of the first part of this study indicated that all five test strains of Y. enterocolitica grew rapidly in pasteurized skim milk inoculated either with 10 or 1,000 CFU/ml Y. enterocolitica and stored at 4° C for up to 21 d. The final level of Y. enterocolitica in the milk samples was over log 5 CFU/ml and not affected by the initial level of inoculum after two weeks of storage at 4° C. Y. enterocolitica competed favorably with the background microflora of pasteurized milk. CIN agar was shown to be an effective selective-differential plating medium for recovery and enumeration of Y. enterocolitica. However, after the level of background microflora of milk samples surpassed that of Y. enterocolitica, the crowding of plates with such microflora made the enumeration of Y. enterocolitica difficult.

The pre-plating-alkaline diluent (PPAD) techniques was developed and found to be an effective technique for recovery of Y. enterocolitica from milk, but only when the Y. enterocolitica were in

late log phase growth or early stationary phase growth. PPAD method recovered Y. enterocolitica from milk samples inoculated with either 10 or 1,000 CFU/ml and stored at 4° C after 14 days of storage.

Both test strains of Y. enterocolitica were able to penetrate and infect shell eggs treated by temperature differential or pressure differential inoculation methods. In both techniques, the inoculation of shell eggs was carried out by immersing the eggs in a bacterial suspension under designated conditions. Supplementation of bacterial suspensions with 20 ppm iron increased both the rate and extent of Y. enterocolitica infection of shell eggs.

The findings of the liquid egg study showed that Y. enterocolitica grew in egg white. However, the rate and extent of growth was limited. Supplementation of egg white with 10 ppm iron improved the growth of Y. enterocolitica. Similar growth patterns were also observed in pasteurized egg white samples. Rapid growth of Y. enterocolitica was also shown in raw and pasteurized liquid whole eggs and yolks at 4° C. The growth of Y. enterocolitica in pasteurized milk and liquid eggs and its ability to invade and infect shell eggs under refrigeration temperature, emphasize the need for sanitation and proper handling of such products.

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