การผลิตโมโนโคลนอลแอนติบอดีต่อ *Yersinia enterocolitica*

นางสาววิลสา คำจริง

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PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST *Yersinia enterocolitica*

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วิลสา คำจริง : การผลิตโมโนโคลนอลแอนติบอดีต่อ *Yersinia enterocolitica*. (Production of monoclonal antibodies against *Yersinia enterocolitica*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.ศิริรัตน์ เร่งพิพัฒน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร.นันทิกา คงเจริญพร, 99 หน้า.

ผลิตและศึกษาลักษณะสมบัติของโมโนโคลนอลแอนติบอดีต่อ *Yersinia enterocolitica* ซึ่งเป็นแบคทีเรียในอาหารที่ก่อให้เกิดโรคเยอซินิโอซิส ทำให้เกิดภาวะของกระเพาะอาหารและลำไส้อักเสบ รวมทั้งโรคแทรกซ้อนอื่นๆ เซลล์ไฮบริโดมาผลิตขึ้นโดยการหลอมรวมเซลล์มัยอีโลมา NS-1 กับเซลล์ม้ามของหนูทดลองสายพันธุ์ ICR ที่ปลูกภูมิคุ้มกันด้วย *Y. enterocolitica* สายพันธุ์ ATCC 27729 เพียงอย่างเดียวหรือผสมรวมกับสายพันธุ์ MU ทั้งในรูปแบบคงสภาพและเสียสภาพ คัดเลือกเซลล์ไฮบริโดมาที่สร้างแอนติบอดีต่อ *Y. enterocolitica* โดยวิธี dot blotting จากการหลอมรวมเซลล์ทั้งหมด 5 ครั้ง ได้โมโนโคลนอลแอนติบอดีทั้งหมด 25 โคลน แบ่งออกเป็น 9 กลุ่ม ตามความจำเพาะต่อแบคทีเรียที่ใช้ทดสอบซึ่งตรวจสอบโดยวิธี dot blotting โมโนโคลนอลแอนติบอดี 5 กลุ่มแรกมีความจำเพาะต่อ *Y. enterocolitica* เพียงอย่างเดียวเท่านั้น แต่ไม่สามารถจับแบคทีเรียดังกล่าวได้ทุกสายพันธุ์ที่นำมาทดสอบ โมโนโคลนอลแอนติบอดีกลุ่มที่ 6 และ 7 มีความจำเพาะต่อ *Y. enterocolitica* ทุกสายพันธุ์ที่นำมาทดสอบ แต่แสดงปฏิกิริยาข้ามกับแบคทีเรียในสกุล *Yersinia* บางสายพันธุ์ รวมทั้งแสดงปฏิกิริยาข้ามกับ *Edwardsiella tarda* เฉพาะแอนติบอดีกลุ่มที่ 7 ในขณะที่โมโนโคลนอลแอนติบอดีกลุ่มที่ 8 และ 9 มีความจำเพาะต่อ *Y. enterocolitica* และแบคทีเรียสกุล *Yersinia* ทุกสายพันธุ์ที่นำมาทดสอบ รวมทั้งแสดงปฏิกิริยาข้ามกับแบคทีเรียแกรมลบที่อยู่ในวงศ์ Enterobacteriaceae แอนติบอดีทั้งหมดนี้แสดงความจำเพาะต่อแถบโปรตีนแอนติเจนที่มีน้ำหนักโมเลกุลอยู่ในช่วง 10 ถึง 43 กิโลดาลตัน เมื่อตรวจสอบด้วยวิธี Western blotting และสามารถตรวจหา *Y. enterocolitica* ในช่วงพิสัย 10^6 ถึง 10^8 CFU ml^{-1} ด้วยวิธี dot blotting ทั้งนี้ขึ้นอยู่กับแอนติบอดีในแต่ละกลุ่ม เมื่อตรวจสอบไอโซไทป์ของโมโนโคลนอลแอนติบอดีพบว่า แอนติบอดีส่วนใหญ่มีไอโซไทป์ IgM ส่วนที่เหลือถูกจัดจำแนกอยู่ในแต่ละสับไอโซไทป์ของไอโซไทป์ IgG โมโนโคลนอลแอนติบอดีบางโคลนที่ผลิตได้จากการศึกษาในครั้งนี้ สามารถนำไปใช้ประโยชน์ในเทคนิคทางวิทยาภูมิคุ้มกัน เพื่อตรวจหาการปนเปื้อนของ *Y. enterocolitica* ในตัวอย่างอาหารหรือตัวอย่างทางคลินิกได้ต่อไป

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WILSA KHAMJING : PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST *Yersinia enterocolitica*. ADVISOR : ASSOC. PROF. SIRIRAT RENGPIPAT, Ph.D., CO-ADVISOR : NANTHIKA KHONGCHAREONPORN, Ph.D., 99 pp.

Monoclonal antibodies (MAbs) against *Yersinia enterocolitica*, a foodborne pathogen causing gastroenteritis and other complications known as yersiniosis, were produced and characterized. Hybridomas were produced by fusion NS-1 mouse myeloma cells with spleen cells of ICR mouse immunized with heat-killed and SDS-mercaptoethanol treated form of *Y. enterocolitica* ATCC 27729 or mixed with MU isolate. Screening of hybridomas producing antibody against *Y. enterocolitica* was performed by dot blotting. Twenty-five MAbs were obtained from five fusions and can be divided into 9 groups according to their specificities to several bacterial strains as determined by dot blotting. The first five groups of MAbs were specific only to *Y. enterocolitica*, but not recognized all isolates tested. MAbs in group 6 and group 7 reacted with all isolates of *Y. enterocolitica* tested and also showed cross-reaction with some *Yersinia* spp. and *Edwardsiella tarda*, especially for group 7. MAbs in group 8 and group 9 reacted with all isolates of *Y. enterocolitica* and *Yersinia* spp. as well as other Gram-negative bacteria which belong to Enterobacteriaceae. These MAbs recognized *Y. entericolitica* at molecular weight ranging from 10-43 kDa by Western blotting and could detect *Y. enterocolitica* within the range from approximately 10^6-10^8 CFU ml^{-1} by dot blotting depending on the group of MAbs. The major isotype of the MAbs was IgM and the remaining MAbs were determined as various groups of IgG isotypes. Some of MAbs produced in this study would be used as a tool in immunological-based methods for specific detection of *Y. enterocolitica* in food or clinical samples.

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LIST OF ABBREVIATIONS

%	=	percent
~	=	about
<	=	less than
>	=	more than
µg	=	microgram
µl	=	microlitre
µm	=	micrometre
CFU	=	colony forming unit
cont.	=	continued
g	=	gram
h	=	hour
kb	=	kilobase
kDa	=	kilo Dalton
lb/in^2	=	pounds/square inch
M	=	molar
mA	=	milliampère
MDa	=	megadalton
mg	=	milligram
min	=	minute
ml	=	millilitre
mm	=	millimetre
N	=	normal
nm	=	nanometre
no.	=	number
^{o}C	=	degree Celcius
rpm	=	revolution per minutes
sec	=	second
V	=	voltage
v/v	=	volume by volume
w/v	=	weight by volume

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CHAPTER I

INTRODUCTION

Food safety is the first important issue which should be realized by the food manufacturers and food exporters. Unsafe food is caused by many factors including microbiological hazards, chemical hazards and physical hazards. Microbial agents are involved in the incidence of foodborne diseases and affect the health of consumers. Furthermore, they also have great economic impacts on every country.

Yersinia enterocolitica is one of the most important foodborne pathogen that causes acute gastroenteritis known as yersiniosis. The main symptoms of infections are diarrhea, abdominal pain and fever. However, in a small proportion of cases, complications such as appendicitis-like syndromes, reactive arthritis, erythryma nodosum and septicemia can be observed (Cover and Aber, 1989). *Y. enterocolitica* is a Gram-negative, rod-shaped bacterium which is a member of the Family Enterobacteriaceae. It is widely distributed in both terrestrial and aquatic ecosystems and also in animal reservoirs, with pig serving as a major reservoir for human pathogenic strains (Bottone, 1997). This pathogenic bacterium has increased the opportunities for transmission through foods due to poor sanitation, improper sterilization techniques by food handlers and improper storage of foods. Human infections are caused by the consumption of contaminated food, especially pork or water (Doyle and Cliver, 1990; Bottone, 1997). Of special significance in food hygiene is the ability of *Y. enterocolitica* to grow in foods under refrigeration temperatures with no apparent signs of spoilage (Hudson and Mott, 1993). Therefore, the detection of *Y. enterocolitica* in contaminated food is necessary to ensure food quality and safety of consumers.

Culture methods based on enrichment, isolation and identification of isolated bacterial colonies in foods are most commonly used for microbial detection. These methods are effective, inexpensive and can give both qualitative and quantitative information on the number of microorganism. However, culture methods are labor-intensive using a variety of selective media and time-consuming. They require 4-21 days to obtain results (Nielsen et al., 1996). This delay is an obvious inconvenience in many industrial applications, particularly in the foods sector which is inadequate for making timely assessments on the microbiological safety of foods. Molecular

methods such as polymerase chain reaction (PCR) for detecting *Y. enterocolitica* have been reported (Wren and Tabaqchali, 1990; Nakajima et al., 1992). They are the most specific and sensitive methods but require trained technicians, special equipment and expensive cost to perform the assay. Furthermore, the length of time to obtain results at least 48 h due to extensive sample pretreatment including enrichment and extraction (Shriver-Lake et al., 2007) and high sensitivity can result in false-positive reactions (Lazcka et al., 2007). Alternative methods to detect bacteria and solve these problems are needed. Immunological methods involving the specific binding of an antibody to an antigen are one of the most suitable to detect bacteria because they provide detection specificity and sensitivity with cost-effective, rapid detection and simple to use by untrained personnel (Mackie, 1996). However, an essential for these methods is antibodies. Polyclonal antibodies obtain inadequate specificity and cause cross-reaction with related antigens in other bacteria, while monoclonal antibodies are superior in terms of their specificity and capability to produce unlimited quantities which would be the appropriate immunological tools for bacterial detection.

Therefore, the objectives of this study were to produce and characterize monoclonal antibodies against *Yersinia enterocolitica*.

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CHAPTER II

LITERATURE REVIEWS

2.1 History

The genus *Yersinia* belongs to family Enterobacteriaceae and composes of 11 species, which includes three well-recognized human pathogens namely *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*. *Y. pestis* is a bacterium that causes bubonic or pneumonic plague which is one of the oldest infectious diseases (Nesbakken, 2006; Bhunia, 2008). *Y. pseudotuberculosis* and *Y. enterocolitica* were established as foodborne bacterial pathogens causing gastrointestinal diseases. However, most cases of human illness which were frequently reported were caused by *Y. enterocolitica* (Delmas and Vidon, 1985; EFSA, 2006). All three species carry a 70-kb virulence plasmid (pVY) and target to lymphoid tissues, which are importance for infection and to overcome host defence mechanism (Wren, 2003; Bhunia, 2008). The remaining eight species—*Y. frederiksenii, Y. intermedia, Y. kristensenii, Y. bercovieri, Y. mollaretii, Y. rohdei, Y. ruckeri, and Y. aldovae*—are referred to as *Y. enterocolitica*-like bacteria. They are considered to be nonpathogenic bacteria because they are absent from classical *Yersinia* virulence markers (Sulakvelidze, 2000).

The historical aspects of *Yersinia* spp. have been described since Mclver and Pike isolated a small Gram-negative coccobacillus from facial abscesses of a 53-year-old farm worker in 1934. They, later were identified it as *Flavobacterium pseudomallei*. In 1939, during working at the New York State, Department of Health, Schleifstein and Coleman studies *F. pseudomallei* isolate and other four isolates from facial lesions and intestines of patients with symptoms of enteritis which resembled *Actinobacillus lignier* and *Pasteurella pseudotuberculosis*. However, these five isolates were biochemically differed from those two species and three of the five isolates were from enteric contents, so they concluded these isolates were new species and proposed the name as *Bacterium enterocoliticum* (Bottone, 1997, 1999). In 1944, the genus *Yersinia* was established by Van Longhem to honor the French bacteriologist Alexander Yersin, who first isolated the plague bacillus *Y. pestis*. Twenty years later, Wilhelm Frederiksen claimed *B. enterocoliticum* to be genus *Yersinia* and later changed to *Y. enterocolitica*.

The taxonomy of *Yersinia* was revised in 1976 by Brenner et al., who applied classical biochemical tests especially fermentation of sucrose, rhamnose, raffinose and melibiose and the utilization of citrate (Table 2.1) and DNA-DNA hybridization to classify relatedness group among *Y. enterocolitica* and *Y. enterocolitica*-like bacteria. As a result of these studies, three groups of *Y. enterocolitica*-like bacteria were separated from *Y. enterocolitica* and four *Yersinia* species—*Y. enterocolitica, Y. intermedia, Y. frederiksenii, Y. kristensenii*—were established in 1980.

In 1984, Bercovier et al. proposed the name *Y. aldovae* for isolates which had been recovered from aquatic ecosystems, and in 1987 Aleksic et al. proposed *Y. rohdei* for isolates from human and dog feces. Furthermore, *Y. bercovieri* and *Y. mollaretii* were proposed by Wauters in 1988 for isolates formerly classified as *Y. enterocolitica* biotypes 3A and 3B, respectively. *Y. ruckeri,* which is the oldest *Y. enterocolitica*-like species, was proposed the species designation in 1978 by Ewing et al.; however, several researchers have questioned about the validity of this classification and believed that this bacterium actually may be distinct, perhaps not even *Yersinia* species (Sulakvelidze, 2000).

2.2 *Yersinia enterocolitica*

2.2.1 Characteristics of *Y. enterocolitica*

Y. enterocolitica is a Gram-negative, rod-shape bacterium and a tendency toward pleomorphism ranging from small coccobacilli with rounded ends and bipolar staining to more elongated bacilli in occasionally (Bottone, 1999). The bacterium has 0.5-0.8 x 1-3 μm in sizes, which does not form a capsule or spores (Bercovier and Mollaret, 1984). Bacterium is facultative anaerobe and grows in a wide range of temperature with optimum growth at 25-29 ^{o}C. Moreover, it is a psychrotroph means that it can grow in refrigeration temperature. It can survive in 5% NaCl and a pH range of 4-10. It is non-motile at 35-37 ^{o}C, but motile at 22-25 ^{o}C with peritrichous flagella (Palumbo, 1986; Lechowich, 1988; Sutherlane and Varnam, 2003). For culturing, *Y. enterocolitica* grows slowly on sheep blood agar, MacConkey agar , Hektoen-Enteric (HE) agar producing pinpoint colonies approximately 0.5-2 mm diameter after 24 h of incubation (Swaminathan et al., 1982; Bottone, 1999). In addition, selective media, cefsulodin-irgasan-

novobiocin or CIN agar is used for selective isolation which provides small colonies having deep red center with sharp border surrounded by clear colorless zone with entire edge (Weagant and Feng, 2007).

2.2.2 Taxonomy

Based on variation in biochemical reactions, *Y. enterocolitica* is divided into six biotypes which differentiates between pathogenic (biotype 1B, 2, 3, 4, 5) and nonpathogenic strains (biotype 1A) as shown in Table 2.2. The main pathogenic strains for human are biotype 2, 3 and 4 (Khan et al., 2009).

Furthermore, *Y. enterocolitica* is also classified into approximately 60 serotypes using heat-stable "O" (somatic) antigens. Of these 60 serotypes, only 11 serotypes are associated with human infections. Serotypes which predominate in human infections are O:3, O:8, O:9, and O:5,27 (Schiemann, 1989; Wannet et al., 2001; Bhunia, 2008). The correlation between human pathogenicity, biotypes, the O-antigens, ecologic and geography distributions were presented in Table 2.3.

2.2.3 Sources

Y. enterocolitica is ubiquitous which widely distributed in the environment, animals and a variety of foods. Pig is the major reservoir for *Y. enterocolitica* which cause infections in humans, in particular strains of serotype O:3 and O:9 (Schiemann, 1989; Bottone, 1997). The bacteria are most present in oral cavity, especially the tongue and tonsils (Nesbakken et al., 2003). However, other animal species including cattle, sheep, goats, deer, poultry, rodents, dogs and cats may also carry pathogenic strains (Bottone, 1999, Nesbakken, 2006; Bhunia, 2008). Environmental isolates are generally nonpathogenic strains which belong to the biotype 1A (Bhunia, 2008).

Table 2.1 Biochemical characteristics of *Yersinia* species[a]

Reaction	*Yersinia* species[b]										
	Y. pestis	***Y. pseudotuberculosis***	***Y. enterocolitica***	***Y. intermedia***	***Y. frederiksenii***	***Y. kristensenii***	***Y. mollaretii***	***Y. bercovieri***	***Y. aldovae***	***Y. rhodei***	***Y. ruckeri***
Glucose	+	+	+	+	+	+	+	+	+	+	+
Sucrose	0	0	+	+	+	0	+	+	0	+	0
Rhamnose	0	+	0	+	+	0	0	0	+	0	0
Raffinose	0	0	0	+	0	0	0	0	0	+	v
Melibiose	v	+	0	+	0	0	0	0	0	v	0
Cellobiose	0	0	+	+	+	+	+	+	0	+	0
Sorbose	0	0	v	+	+	+	+	0	0	ND	ND
Ornithine decarboxylase	0	0	+	+	+	+	+	+	+	+	+
Voges-Proskauer (25 °C)	0	0	+	+	+	0	0	0	+	0	0
Indole	0	0	v	+	+	v	0	0	0	0	0
Urease production	0	+	+	+	+	+	+	+	+	+	+
Citrate	0	0	0	v	v	0	0	0	0	+	+
Motility (25 °C)	0	+	+	+	+	+	+	+	+	+	+

[a] Adapted from Bottone, 1997

[b] +, positive; 0, negative; v, variable; ND, not determined

[c] Biotype 5 strains may vary in some reactions.

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Table 2.2 Biochemical reaction for classification biotypes of *Y. enterocolitica*[a] (Bottone, 1997, 1999)

Test	Biotype reaction[b]					
	1A	**1B**[c]	**2**	**3**	**4**	**5**
Lipase activity	+	+	0	0	0	0
Salicin (acid production in 24 h)	+	0	0	0	0	0
Esculin hydrolysis (24 h)	+/0	0	0	0	0	0
Xylose (acid production)	+	+	+	+	0	v
Trehalose (acid production)	+	+	+	+	+	0
Indole production	+	+	v	0	0	0
Ornithine decarboxylase	+	+	+	+	+	+(+)
Voges-Proskauer test	+	+	+	+	+	+(+)
Pyrazinamidase activity	+	0	0	0	0	0
Sorbose (acid production)	+	+	+	+	+	0
Inositol (acid production)	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	0

[a] Modified from Wauters et al., 1987

[b] +, positive; 0, negative; (+), delayed positive; v, variable

[c] Biotype 1B is mainly comprised of strains isolated in the United States.

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Table 2.3 Virulence of *Y. enterocolitica* associated with biotype, serotype, ecologic and geographic distribution (Bottone, 1997, 1999)

Associated with human infections	Biotype	Serotype(s)	Ecologic/geographic distribution
Yes	1B	O:8; O:4; O13a,13b; O:18; O:20; O:21	Environment, pig (O:8), United States, Japan, Europe, The Netherlands (O:8-like)
	2	O:9; O:5,27	Pig, Europe (O:9), United States (O:5,27), Japan (O:5,27), Sweden, The Netherlands
	3	O:1,2,3; O:5,27	Chinchilla (O:1,2,3), pig (O:5,27)
	4	O:3	Pig, Europe, United States, Japan, South Africa, Scandinavia, Canada, The Netherlands
	5	O:2,3	Hare, Europe
No[a]	1A	O:5; O:6,30; O:7,8; O:18; O:46, nontypeable	Environment, pig, food, water, animal and human feces, global

[a] May be opportunist pathogens in patients with underlying disorders.

2.3 Pathogenicity

2.3.1 Virulence factors

Some strains of *Y. enterocolitica* are invasive bacterial pathogens which can invade and colonize in intestinal epithelial cells as well as lymph nodes resulting in clinical diseases. Human pathogenic strains carry a 70-kb virulence plasmid (pVY) or about 48 MDa in size which are absent in nonpathogenic strains (Sutherland and Varnam, 2003). However, the presence of the plasmid alone is not sufficient for infection in human host. The chromosomal-encoded virulence genes are also necessary for full expression of virulence in the bacterium (Table 2.4) (Nesbakken, 2006). These virulence factors are expressed depending on temperature which occurs mostly at

37 °C. Furthermore, *Y. enterocolitica* can produce enterotoxin (Yst) when it is cultured at 20-30 °C (Pai et al., 1978). The structure and function of Yst is very similar to the heat-stable enterotoxin (ST) of *E. coli* (Boyce et al., 1979). Yst is heat-stable and remains active at pH range of 1-11 at 37 °C for 4 h, which is involved in diarrhea. Although the toxin production could not be found in vivo, foodborne illness following consumption of food containing toxin has been suggested (Roberts and Tompkin, 1996). This incidence is based on the fact that Yst is able to resist gastric acidity and enzymatic degradation as well as temperatures used in food processing and storage operations, without losing activity (Boyce et al., 1979; Okamoto et al., 1981). Therefore, disease caused by *Y. enterocolitica* does not only to be foodborne infection, but it may also be foodborne intoxication.

2.3.2 Mechanism

Following consumption of food or water contaminated with *Y. enterocolitica*, bacteria translocate to terminal ileum and proximal colon where the primary site of infection is. Bacteria can invade the cell using strategy as shown in Figure 2.1. Initially, *Y. enterocolitica* attach to mucus membrane and cross the epithelial cells of the intestine through the M cells by using chromosomal-encoded determinant, Inv which interacts with the β1-integrin receptor located on the M cells of the follicle-associated epithelium of intestinal Peyer's patch. When bacteria ingested from cold food or water adapt to host temperature at 37 °C, the expression of plasmid- and chromosomal-encoded determinants, YadA and Ail are induced to assist in attachment and invasion. After that, bacteria inject Yersinia outer membrane proteins, YOPs into macrophages through type III secretion system (TTSS) resulting in paralysis of the actin cytoskeleton, thereby blocking phagocytosis process. Bacteria induce apoptosis of macrophage; therefore, bacteria are then released and able to reinvade the epithelial cells. Moreover, bacteria can multiply within the lymphoid follicle causing necrosis and abscess in Payer's patch. Bacteria also spread to mesenteric lymph nodes which cause characteristic lymphadenitis, as well as disseminating to liver, spleen and lungs (Sansonetti, 2004; Bhunia, 2008).

Table 2.4 *Y. enterocolitica* chromosomal and plasmid-encoded virulence determinants effective in gastrointestinal infection (Bottone, 1999; Bhunia, 2008)

Genomic origin	Determinant	Functions	Expressed temperature
Chromosome *inv locus*	Invasin	Attachment/invasion	28 °C
ail locus	AiL	Attachment/invasion; serum resistance	37 °C
yst locus	Yst (enterotoxin)	Fluid secretion in intestine	28 °C
hem	Hem R and other proteins	Heme receptor – removes iron bound to heme proteins	37 °C
irp2	HMWP 1 and 2	Synthesized under iron starvation by high-pathogenicity strain involved in iron or siderophore uptake	37 °C
Plasmid *yad*	YadA	Attachment/invasion	37 °C
	YopH	Resistance to phagocytosis by macrophages. Dephosphorylation of host cell proteins	37 °C
	YopB	Suppresses tumor necrosis factor alpha release from macrophages. Evasion of immune and inflammatory responses	37 °C
	YopE	Translocated into target cell at zone of contact between *Y. enterocolitica* and eukaryotic cell; leads to disrupt actin cytoskeleton and prevent phagocytosis	37 °C
	YopP	Macrophage apoptosis; alters the expression of cytokines	37 °C
	YopT	Interfere with actin cytoskeleton formation	37 °C

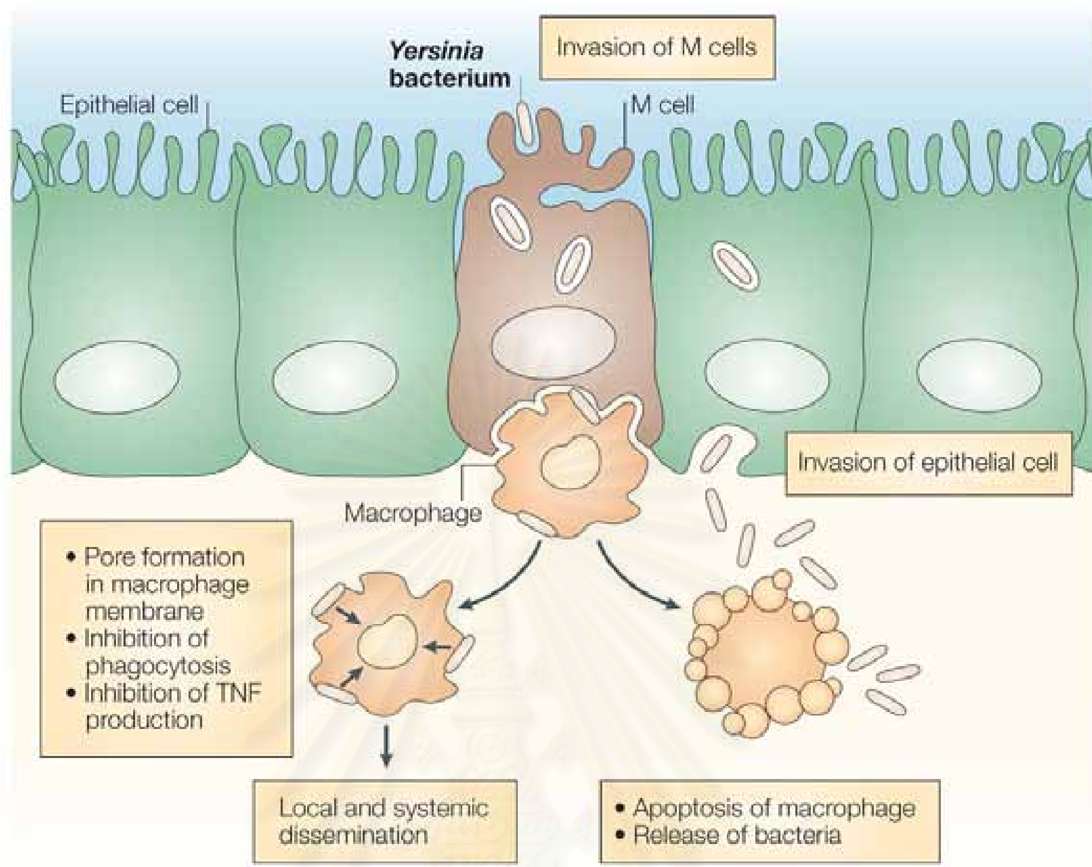

Figure 2.1 Steps of *Y. enterocolitica* translocation through the intestinal epithelial barrier and development of infectious process, *Y. enterocolitica* is transported from intestinal lumen to mucosal lymphoid tissues via M cells. Bacteria survive inside macrophage by blocking phagocytosis and also induce macrophage apoptosis. This process allows them to invade the epithelial cells basolaterally, disseminate locally and systemically, and spread to mesenteric lymph nodes (Sansonetti, 2004).

2.4 Yersiniosis

2.4.1 Clinical diseases

The infection disease caused by *Y. enterocolitica* is called yersiniosis which causes a variety of symptoms depending on the strain of *Y. enterocolitica* (biotype and serotype), the dose, genetic factors, the age and physical condition of the host (Bottone 1977; Larson 1979) (Table 2.5). Gastroenteritis is the most common clinical syndrome of *Y. enterocolitica* infection in

humans (Mollaret et al., 1979; Cover and Aber, 1989). Infants in the first year of life are the most susceptible to *Y. enterocolitica* infections (Vandepitte and Wauters, 1979). In young children, acute enterocolitis with fever and diarrhea which may be bloody is a common presentation. In older children and adults, acute terminal ileitis and mesenteric lymphadenitis are the predominant symptoms. These manifestations result in abdominal pain at the right lower quadrant of the abdomen which is often confused with appendicitis (Bottone 1999; Sutherland and Varnam, 2003). Moreover, nausea and vomiting can occur with lower frequency (Schiemann, 1989; Nesbakken, 2006). The event is usually self-limiting, although long-term illnesses have been reported (Saebo, 1983). The infection dose of *Y. enterocolitica* which is required for the disease is approximately 10^7-10^9 CFU depending on the immunological status of the host and the natural infectivity of the organism. Symptoms generally appear within 24-30 h after consumption of contaminated food and the illness lasts for 3-28 days for infant and 1-2 weeks for adults (Bhunia, 2008). *Y. enterocolitica* is also associated with various extraintestinal symptoms, which may not be involved in gastroenteritis before, such as septicemia, pharyngitis and meningitis. In addition, *Y. enterocolitica* infection can cause postinfection sequelae such as erythema nodosum and reactive arthritis.

2.4.2 Transmission

Y. enterocolitica is spread by fecal-oral route which is transmitted to human via many routes as shown in Figure 2.2. Infection with *Y. enterocolitica* is most often acquired by consumption of contaminated food or water. Poor sanitation, improper sterilization techniques by food handlers and improper storage of food can contribute the bacteria for contamination. As say in the beginning that pig is a major reservoir for the human pathogenic serotypes. The association between pig carriage and human infections has been established through epidemiological investigations. Schiemann and Fleming (1981) reported that serotype O:3 was common in both pig and humans in eastern Canada. In western Canada, serotype O:8 and O:5,27 were most common in humans, but only serotype O:5,27 could be found in the throat of slaughter-age pigs (Toma and Lafleur, 1981). It seemed reasonable that *Y. enterocolitica* which is frequently found in the oral cavity of pigs at the age of slaughter should be spread to pork products during slaughter and processing and then to the customer. But there was only one consumer pork product

that has consistently shown the presence of pathogenic *Y. enterocolitica*. This incidence might be described that the enrichment and isolation methods normally applied were not suitable to detect low numbers of pathogenic strains among high populations of other bacteria which were common in samples (Schiemann, 1989).

Other animals also appeared to be a reservoir of *Y. enterocolitica*, but with few exceptions, they were not recognized as pathogen (Schiemann, 1989). A few human cases of yersiniosis have been related directly to contact with infected animals (Ahvonen et al., 1973; Wilson et al., 1976). Moreover, there was a fascinating hypothesis that rodents might be reservoirs for the highly pathogenic serotypes O:8 and O:21 found in North America, and the bacteria might be transmitted by rodent fleas, as in *Y. pestis* (Schiemann, 1989).

Y. enterocolitica which is found common in the environment including soil, sewage and water could be recognized as nonpathogenic strains and no clinical evidence of yersiniosis (Eden et al., 1977). However, cases of yersiniosis were reported by consumption of water from a mountain stream (Keet, 1974). This phenomenon might be explained by contamination of water with the bacteria. Water was susceptible to contaminate with feces from animals by surface run-off from rain or snowmelt, or by leakage from septic tanks or open restrooms in the surrounding areas (Nesbakken, 2006). Therefore, water would appear to be a potentially important vehicle for transmission of *Y. enterocolitica*.

In addition, human yersiniosis can be an important nosocomial disease. Person-to-person transmission has been reported under certain circumstances, for example in hospitals (Toivanen et al. 1973) or among family members (Marks et al., 1980; Lee et al., 1990).

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Table 2.5 Clinical spectrum of *Y. enterocolitica* infections (Bottone, 1999)

Type of infection	Symptom/population
Gastrointestinal	Enterocolitis, predominantly in young children; concomitant bacteremia may also be present in infants Pseudoappendicitis syndrome (children older than 5 years; adults) Acute mesenteric lymphadenitis Terminal ileitis
Septicemia	Especially in immunosuppressed individuals and those in iron overload or being treated with deferrioxamine Transfusion related (usually leads to septic shock syndrome)
Metastatic	Focal abscesses: liver, kidney, spleen, lung Cutaneous manifestations: cellulitis, pyomyositis, pustules and bullous lesions Pneumonia, cavitary pneumonia Meningitis Panophthalmitis Endocarditis, infected mycotic aneurysm Osteomyelitis
Postinfection sequelae	Arthritis Myocarditis Glomerulonephritis Erythema nodosum
Pharyngitis	Inflammation

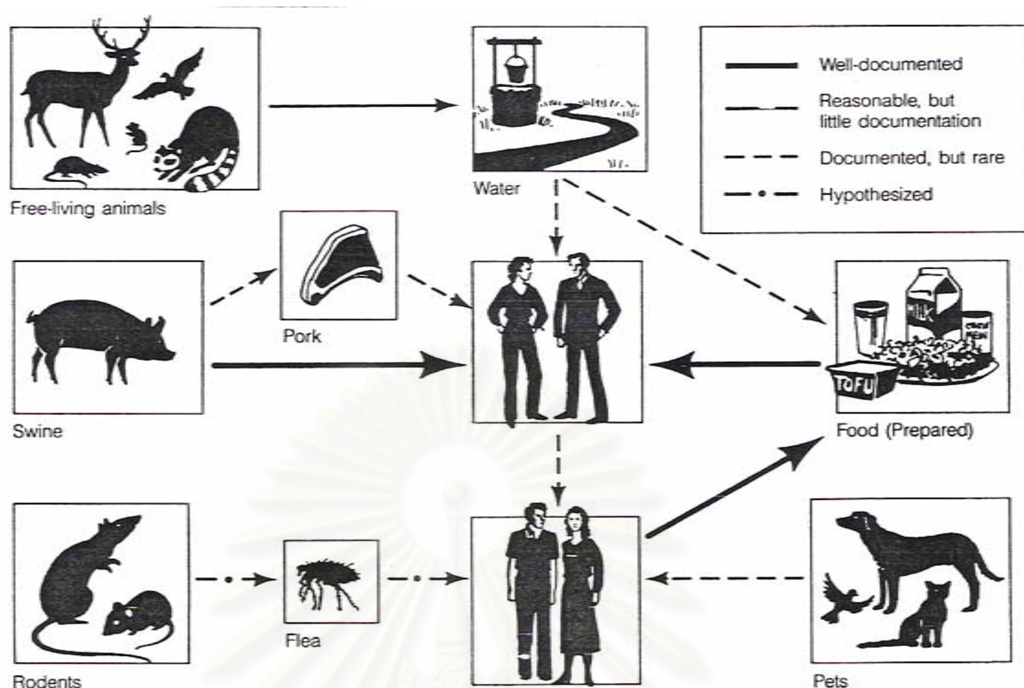

Figure 2.2 Pathways showing transmission of *Y. enterocolitica* to humans. Illustrations of reservoirs, vehicles and the relative degree of documentation available for each route of transmission (Schiemann, 1989)

2.4.3 Epidemiology

Most cases of yersiniosis are sporadic. *Y. enterocolitica* has been isolated from humans in many countries, but it seems to be found most frequently in cooler climates and infections appear to peak in autumn and winter months (Mollaret et al., 1979; Tauxe, 2002). The widespread nature of *Y. enterocolitica* has been well-documented; by the mid-1970s, Mollaret et al. (1979) had collected reports of isolates from 35 countries on six continents (Nesbakken, 2006). The outbreak was most occurred by consumption of contaminated food especially milk, pork, tofu and water (Sutherland and Varnam, 2003). The first foodborne outbreak of *Y. enterocolitica* occurred in New York state in 1976 affecting 222 children due to consumption of chocolate milk contaminated with serotype O:8 (Bhunia, 2008).

Human yersiniosis was the third most frequently reported zoonosis in Europe after campylobacteriosis and salmonellosis, respectively. In 2005, a total of 9,630 recorded cases of yersiniosis were reported in Europe. Most of cases (89%) were caused by *Y. enterocolitica*, but a

few cases caused by *Y. pseudotuberculosis* were also reported. However, the number of reported yersiniosis cases in human had been decreased slightly since 2002. *Y. enterocolitica* was occasionally found from various types of food, including pork, beef, cow milk and vegetables. The highest incidences were reported in Lithuania, Finland, Sweden and Germany and most reported cases occurred in the age groups 0-4 and 5-14 years. Furthermore, the European Food Safety Agency reported about foodborne outbreaks that there were 9 outbreaks caused by *Y. enterocolitica* (0.2% of all outbreaks). This was 82% decrease compared to 51 outbreaks reported in 2004. Outbreaks with *Y. enterocolitica* affected 22 people and 32% were hospitalized (EFSA, 2006).

Although yersiniosis did not occur frequently, the Center for Diseases Control and Prevention (CDC) estimated that about 17,000 cases of human diseases occurred due to *Y. enterocolitica* infection annually in the USA, which was a far more common disease in Northern Europe, Scandinavia, and Japan (Walderhaug, 1991). Furthermore, a recent US foodborne outbreak survey report indicated that there were 8 outbreaks linked to *Y. enterocolitica* with 87 cases between 1998 and 2002. The incidence of this bacterium decreased by 49% from 1996-1998 to 2005 (Bhunia, 2008).

2.5 Treatment

In more severe or complicated cases, antibiotic treatment is necessary for *Y. enterocolitica* infections. Antibiotic susceptibility patterns of *Y. enterocolitica* are serotype specific and governed in part by the production of β-lactamase. *Y. enterocolitica* produces two types of β-lactamases (enzymes that hydrolyse the β-lactam ring of the β-lactam antibiotics): β-lactamase A and β-lactamase B. β-lactamase A hydrolyzes a variety of penicillins and cephalosporins, whereas β-lactamase B exhibits strong cephalosporinase activity.

These β-lactamases account for resistance to penicillin and many penicillin derivatives including ampicillin, cephalothin and carbenicillin (Cornelis, 1973, 1975). However, newer β-lactam antibiotics such as ceftriaxone, ceftazidime, cefoxitin, moxalactam and cefamandole are found to be effective (Scribner et al., 1982; Soriano and Vega, 1982). Two types of β-lactamase are produced in serotype O:3 and O:9, whereas only a type B β-lactamase is

produced in serotype O:5,27 (Matthew et al., 1977). Unlike the other serotypes, serotype O:8 is succeptible to ampicillin but is variably resistant to carbenicillin and cephalothin (Bottone, 1997).

Y. enterocolitica is usually susceptible to the aminoglycosides (gentamicin, kanamycin, streptomycin), polymycin, chloramphenicol, trimethoprim-sulfamethoxazole, ciprofloxacin and tetracycline (Scribner et al., 1982; Bottone, 1999). Furthermore, *Y. enterocolitica* is also sensitive to imioenem and aztreonam antibiotics (Bhunia, 2008). Broad-spectrum cephalosporins, often in combination with an aminoglycoside, are effective for patients with extraintestinal *Y. enterocolitica* infection (Gayraud et al., 1993).

2.6 Prevention and control procedures

To prevent or reduce the incidence of *Y. enterocolitica* infections and the outbreak of yersiniosis, good sanitation from farm level through food processing and personal hygiene must be performed as following ways:

2.6.1 Minimizing contact between infected and non-infected herds in order to reduce the herd prevalence of *Y. enterocolitica* (Nesbakken, 2006).

2.6.2 Because of the high prevalence of *Y. enteocolitica* in pig herds, strict slaughter hygiene will remain an important means to reduce carcass contamination with *Y. enterocolitica* as well as other pathogenic microorganisms (Skjerve et al., 1998).

2.6.3 In order to prevent cross-contamination, raw meats should be separated from other foods as well as knives, cutting board and other equipment.

2.6.4 Knives, equipment and machines used to cut or process raw meat products must be cleaned and disinfected with appropriate and effective agents before being used for handling other foods.

2.6.5 *Y. enterocolitica* is a psychrotroph, which is able to multiply at low temperatures; therefore, refrigeration of food does not prevent their growth. However, the rate at which this takes place will be reduced (Nesbakken, 2006).

2.6.6 Avoid eating raw or undercooked meats, as well as unpasteurized milk or milk products.

2.6.7 Do not drink untreated surface water such as stream, pond or river. It must be assumed that this water is contaminated with animal feces. So, water from these sources should be boiled or disinfected before using in food manufacturing or drinking.

2.6.8 To ensure that there is adequate heat for cooking of meats because inadequate heat-treatment of meat is a risk for human yersiniosis (Ostroff et al., 1994). The minimum internal temperature for poultry and other meats are 74 °C and 71 °C, respectively (BCCDC, 2006).

2.6.9 Wash hands before eating and preparing food, after contact with animals, and after handling raw meats.

2.6.10 Dispose of animal feces in a sanitary manner.

2.7 Diagnosis methods

Although *Y. enterocolitica* is found ubiquitously in the environment, it is a concern mostly because of its role as a foodborne pathogen. So, detection of the bacteria is necessary to ensure food quality and safety of consumer. Various methods for detection of *Y. enterocolitica* have been described.

2.7.1 Culture methods

Culture methods are most commonly used for isolation of *Y. enterocolitica* from foods, clinical specimens and environmental samples. The procedure is based on enrichment in liquid media, isolation of pure cultures on selective media and identification of isolates colonies by biochemical and serological tests (Swaminathan et al., 1982). Furthermore, isolated strains can be further characterized by biotyping, serotyping, antimicrobial susceptibility and testing for virulence properties. As the numbers of *Y. enterocolitica* in foods are usually low and there is often a great variety of background flora, enrichment in selective medium such as irgasan-

ticarcillin-potassium chlorate (ITC) or bile-oxalate-sorbitol (BOS) broth is necessary to increase numbers of the bacteria. Because of the property of *Y. enterocolitica* which is able to grow at 4 ^{o}C, enrichment at this temperature has been used. Furthermore, the bacterium is more tolerant of alkaline conditions, so post-enrichment alkali treatment with potassium hydroxide (KOH) often results in higher isolation rates (Aulisio et al., 1980). Selective media, cefsulodin irgasan novobiocin (CIN) agar and Salmonella-Shigella deoxycholate calcium chloride (SSDC) agar are frequently used and found to be the most effective for isolation (Sutherland and Varnam, 2003).

Culture methods are the oldest bacterial detection technique and remain the standard detection method. The advantages of the methods are effective, inexpensive and can give both qualitative and quantitative information on the number of microorganism. However, the procedure is labor-intensive which uses a variety of culture media and time-consuming due to enrichment step and biochemical characterization. These methods take at least 4 days for a positive result and 21 days for a negative result (Nielsen et al., 1996).

2.7.2 Molecular detection methods

Polymerase chain reaction (PCR) is one of molecular detection method which is widely used for bacterial detection. The method is based on isolation, amplification and detection of the amplicons by agarose gel electrophoresis. There are many reports for detection of *Y. enterocolitica* in naturally contaminated samples and for the identification of pathogenic isolates using PCR method (Wren and Tabaqchali, 1990; Nakajima et al., 1992; Fredriksson-Ahomaa and Korkeala, 2003). This method often uses primers targeting genes in the virulence plasmid (PVY) including *virF* (Thisted-Lambertz et al., 1996; Weynants et al., 1996), the *yadA* (Kapperud et al., 1993), *IcrE* (Viitanen et al., 1991) and *yopT* (Arnold et al., 2001). Because virulence plasmid of *Y. enterocolitica* may lose during culture, subculture or storage (Blais and Philippe, 1995), PCR method targeting chromosomal virulence genes, often *ail* gene, have also been designed. Furthermore, a combination of genes from the virulence plasmid and the chromosome, namely *virF* and *ail* genes are used in multiplex PCR (Kanebo et al., 1995; Nilsson et al., 1998).

PCR has the advantages of being rapid, sensitive and specificity. However, PCR might also be too sensitive which cause false-positive results due to dead cells present in relatively high numbers, or to the presence of partly homologous target sequences in nonpathogenic *Yersinia* or in other bacterial species (EFSA, 2007; Lazcka et al., 2007). Furthermore, false-negative results may occur due to the presence of inhibitory substances in clinical, food and environmental samples, which reduce or even block the amplification capacity of PCR (Lantz et al., 2000). To overcome these problems, an enrichment step prior to PCR is essential to concentrate the bacteria, to decrease the risk of false-positive results due to detection of dead cells and to dilute out the inhibitors in the samples. Moreover, positive results should be confirmed with culture methods (Swaminathan and Feng, 1994; EFSA, 2007). Generally, PCR is a lot less time-consuming and the results were obtained within one working day. When enrichment step was included in PCR method, the entire procedure may take 2-3 days to complete.

Although PCR method is easy to perform, it requires a high cost instrument which must be carried out by trained technicians and there is expensive cost to perform the assay. Furthermore, the electrophoretic detection of amplification products including agarose gel electrophoresis, ethidium bromide staining and visual examination of the gel under ultraviolet light, is not optimal for high volume screening of food samples in quality-control laboratories (Swaminathan and Feng, 1994).

2.7.3 Imunological methods

Immunological methods involving the specific binding of an antibody to an antigen were used as alternative method to reduce the test time and cost for detection. Among these methods, the enzyme-linked immunosorbent assay (ELISA) is the most widely used for bacterial detection and sandwich ELISA is appropriate for detection of bacteria in foods (Swaminathan and Feng, 1994). This method gives high accuracy, high specificity and high sensitivity. It is cost-effective, rapid detection and easy to perform by untrained personnel (Mackie, 1996). However, the requirement for immunological methods is antibodies which specific to interesting antigens such as cell-surface, cytoplasmic antigen or bacterial toxin.

2.8 Antibodies

Antibodies (also known as immunoglobulins, or Igs) are powerful immunological tools for bacterial detection. They are a group of glycoproteins secreted by plasma cells that circulate in the bloodstream. They are found in mammalian serum and other bodily fluids which form part of the immune system and protect the body against pathogens. All antibodies have the same general structure made up of four polypeptide chains linked covalently by disulfide bonds. The four chain structure is composed of two small (light, L) and two large (heavy, H) polypeptide chains arranged in a Y-shaped heterodimeric molecule (Figure 2.3). The light chain contains identical sequence of about 200 amino acids which common to all antibodies (isotypes) and are of two distinct forms, kappa (κ-type) and lambda (λ-type). Each antibody contains either identical kappa or lambda chains. The heavy chain contains about 400-500 identical amino acid residues. There are five types of heavy chains namely γ, α, μ, δ and ε based on structural differences in carboxy-terminal portion of heavy chains. Antibodies are divided into five classes or isotypes depending on the type of H-chain (Figure 2.4). They differ in size, charge, structure, amino acid composition and carbohydrate content (Roitt et al., 1985). The physical and biological properties of the individual isotypes of antibody were summarized in Table 2.6.

Sequence analysis of antibodies reveals that both light and heavy chains are composed of two segments, namely the constant region and the variable region. The constant region (C) is the carboxy-terminal of chains which shows the same or constant in amino acid sequence among antibodies of the same class, except minor inherited differences. The constant domains help to stabilize the antibody structure. By contrast, the variable region (V) is the amino-terminal half of chains which shows a wide variation in amino acid sequence. The variables regions of heavy (V_H) and light (V_L) chains sequences are folded to form a cup-like site for binding the antigen.

The molecule of antibodies can be cleaved by proteolytic enzymes pepsin and papain into specific fragments with specific biological properties, leading to know that antibodies are bifunctional. They comprise of two regions including Fab and Fc region. Fab (fragment, antigen binding) region is concerned with antigen binding, while Fc (fragment, crystallizable) region is

responsible for the biological activity of the antibody and plays an important role in the immune response such as binding complement, binding to cell receptors on macrophages and monocytes.

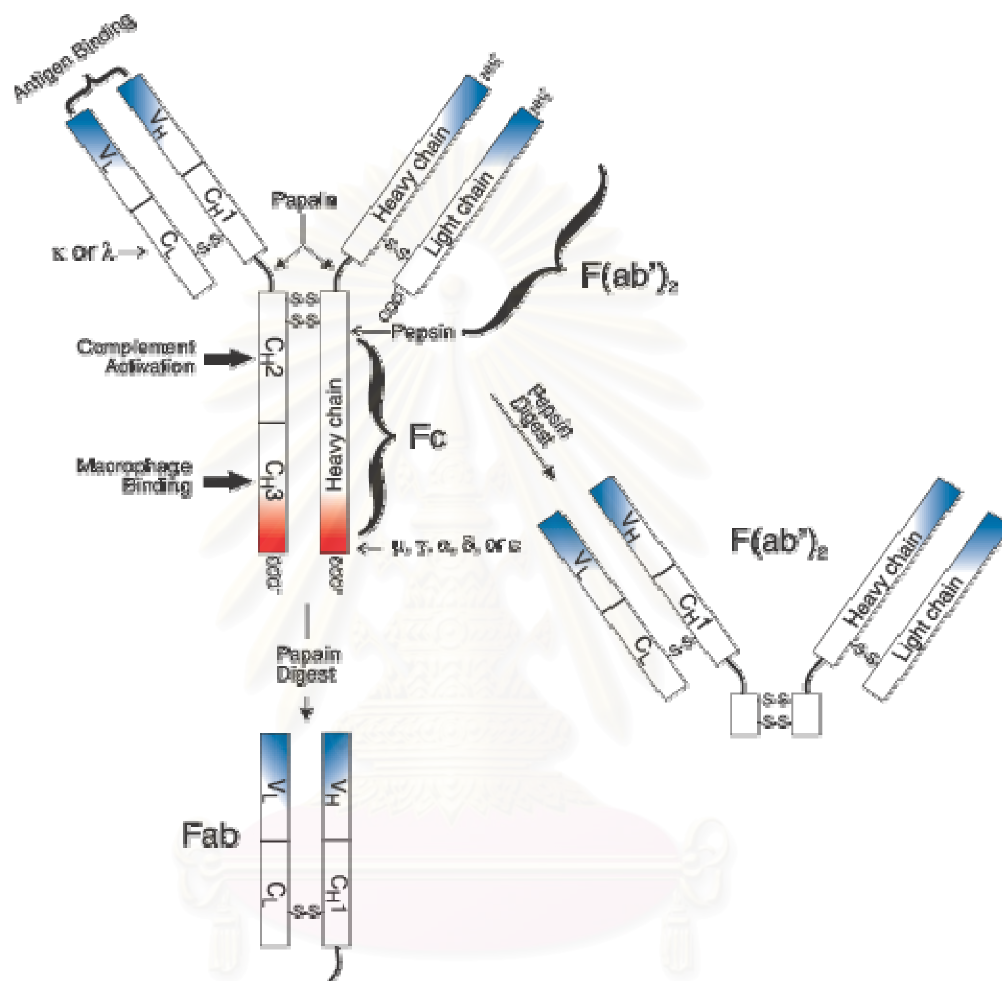

Figure 2.3 Schematic representation of an antibody molecule

Available from: http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/Technical-Notes-and-Product-Highlights/Antibody-Structure-and-Classification.html [2009, April 8]

Table 2.6 The physical and biological properties of human and mouse antibody isotypes and subisotypes[a]

Antibody	Structure	Light chain	Heavy chain	Function(s)
IgM	Pentamer	κ หรือ λ	μ	First response antibody. Expressed on the surface of B cells and in a secreted form with very high avidity. Eliminates pathogens in the early stages of B cell mediated immunity before there is sufficient IgG.
IgG	Monomer	κ หรือ λ	γ_{1}, γ_{2}, γ_{3}, or γ_{4} (in human) γ_{1}, γ_{2a}, γ_{2b}, or γ_{3} (in mouse)	Major Ig in serum. Provides the majority of antibody based immunity against invading pathogens. The only antibody capable of crossing the placenta to give passive immunity to fetus.
IgA	Dimer	κ หรือ λ	α_{1} or α_{2}	Found in mucosal areas, such as the gut, respiratory and urogenital tract, and prevents colonization by pathogens. Resistant to digestion and is secreted in milk
IgE	Monomer	κ หรือ λ	ε	Binds to allergens and triggers histamine release from mast cells and is involved in allergy. Also protects against parasitic worms
IgD	Monomer	κ หรือ λ	δ	Function unclear, but mainly as an antigen receptor on B cells that have not been exposed to antigens.

[a] Available from:
http://www.abcam.com/index.html?pageconfig=resource&rid=11258&pid=11287[2009, April 9]

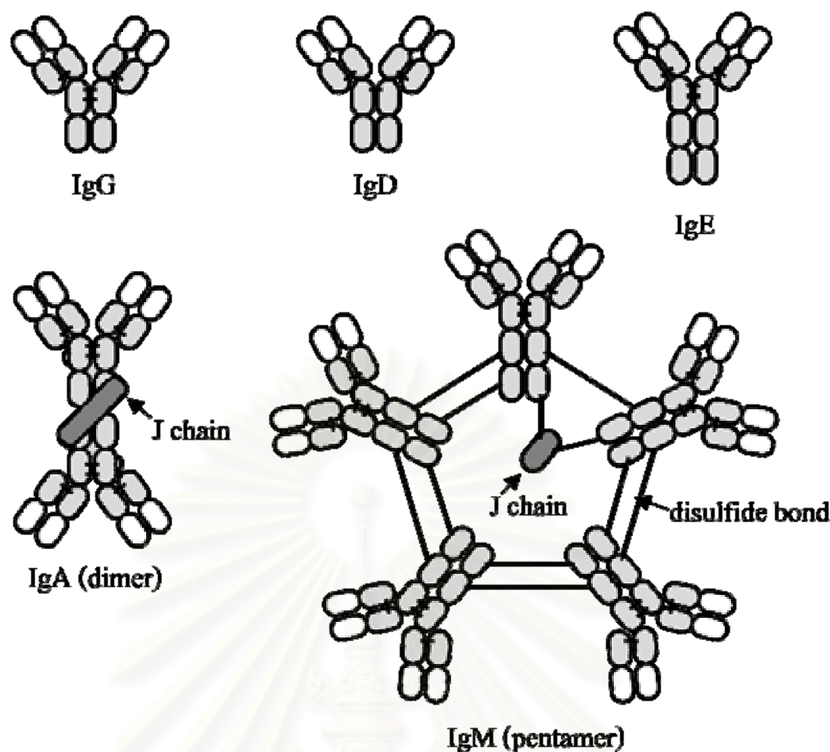

Figure 2.4 Structure of individual isotypes of antibody (adapted from Goldsby et al., 2000)

The properties of immunological assays are indicated by the quality of the antibody preparation. Different antibody preparations vary greatly in the affinity and specificity of their interaction with antigen. There are two types of antibodies, polyclonal and monoclonal antibodies. Both antibodies can be used in the immunological methods. The method uses polyclonal antibodies offer many advantages, for example they recognize multiple epitopes of the antigens, making them more tolerant of small changes in the nature of antigen, like polymerization or slight denaturation and they are a preferred choice for detection of denaturated proteins. However, they also present limitations such as variable affinity and limited production. Furthermore, the inadequate specificity of the polyclonal antibodies leading to cross-reaction with related antigens in other bacteria may yield false-positive results (Swaminathan and Feng, 1994). Therefore, the development of hybridoma technology and the ability to produce monoclonal antibodies with high specificity can lead to significant improvements in the specificity of the assays.

2.8.1 Monoclonal antibodies

Monoclonal antibodies (MAbs) are antibodies produced by a single clone of B lymphocyte (B cell) which were first produced by Köhler and Milstein in 1975. They advised hybridoma technology for generating MAbs by fusion B cells of an immunized animal (usually a mouse or rat) with a cell line that grows continuously (called myelomas) in culture, so that the fused cells (hybridomas) will grow continuously and secrete one kind of antibody molecule. An outline of the experimental protocol for production of MAbs was shown in Figure 2.5. Each MAb interacts with a particular small region on the antigen called an epitope. Therefore, Mabs are highly specific and overcome some of the problems of unwanted cross-reactions that occur with polyclonal antibodies. MAbs are important tools which used in a variety of immunological research, in diagnosis of disease and in treatment of such disease as infections and cancer. Characteristics of MAbs as well as their advantages and disadvantages were shown in Table 2.7.

Table 2.7 Comparison between polyclonal and monoclonal antibody (Liddell, 2001)

Properties	Polyclonal antibody	Monoclonal antibody
Production time	Minimum six weeks	Minimum four months
Immunogen purity	Essential	Not essential
Cost to produce	Inexpensive	Expensive
Technology required	Low	High
Skilled required	Low	High
Concentration	About 1 mg/ml	Culture supernatant: 5-10 μg/ml static and 100-200 μg/ml fermenter Ascites: 2-10 mg/ml
Quantity	About 100 ml serum/rabbit and 1 ml serum/mouse	Limitless
Specificity	Recognizes multiple epitopes on any one antigen	Recognizes only one epitope on an antigen
Affinity	Heterogeneous	Homogeneous
Quality	Batch to batch variability	Constant and renewable source and all batches will be identical

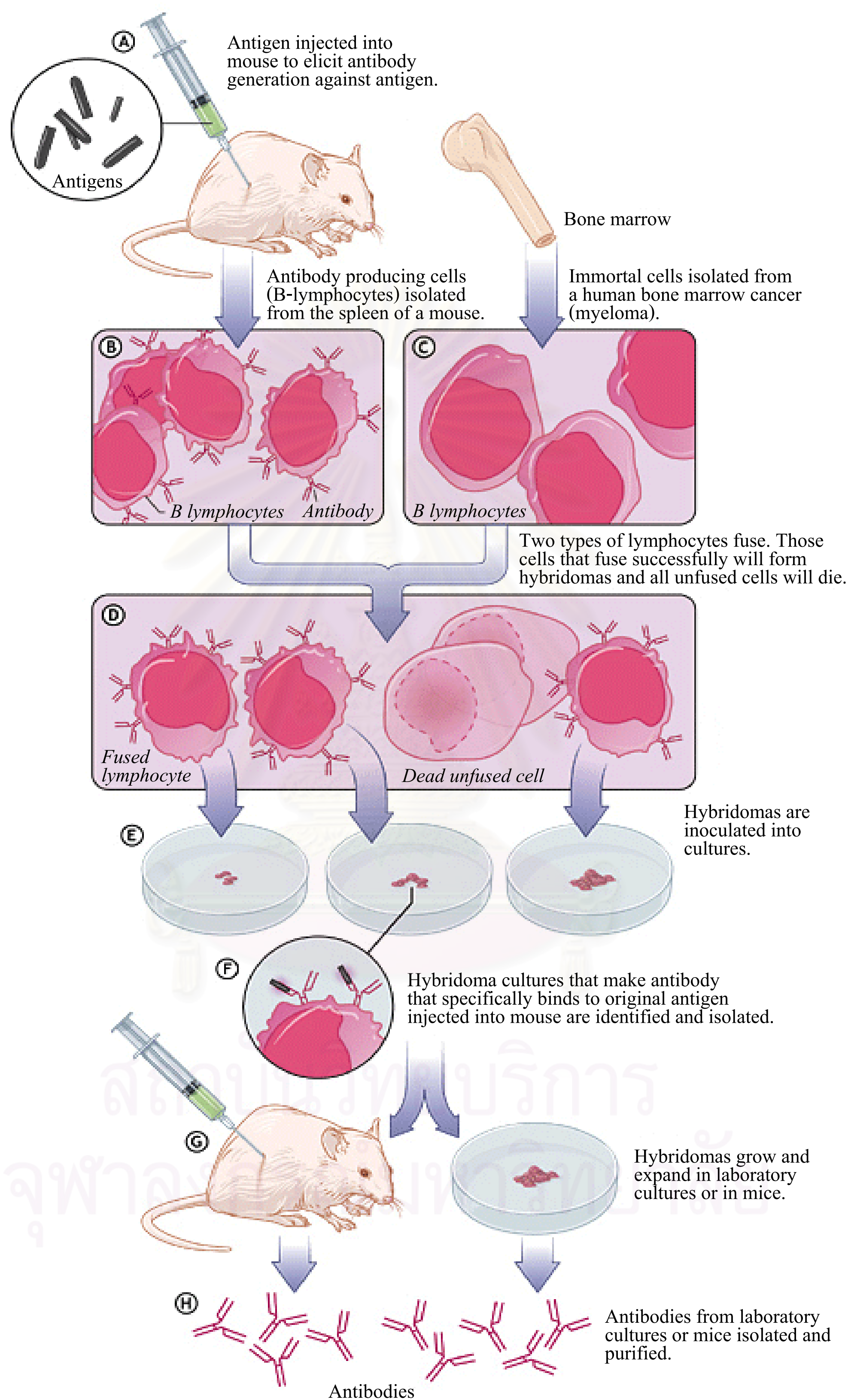

Figure 2.5 Monoclonal antibodies production

Available from: http://www.cancerresearch.org/Resources.aspx?id=600[2009, April 9]

2.8.1.1 Principle of monoclonal antibody production

The first step in production of monoclonal antibodies is to inject the mouse with antigen which the desired antibody will react to. This antigen stimulates an immune response in the mouse, and its B lymphocytes begin producing antibodies that will recognize the antigen. Next, the spleen of the mouse which contains a concentrated source of B cells is removed, and the B cells are then fused with myelomas to produce hybridomas by using polyethylene glycol (PEG) as fusogen. When cells are treated with PEG, their membranes fuse and multinucleated cells called heterokaryons are formed (Ringertz and Savage, 1976; Abbott and Povey, 1995). However, the fusion events are poorly controlled. In addition to the fused B cells and myeloma cells or hybridomas, myeloma: myeloma-fused cells and B cell: B-cell-fused cells as well as unfused cells may be occurred. Therefore, a selection procedure is required.

Littlefield (1964) developed a selection method for ensuring that all other cells would die in the media except the fused hybridomas. This method was based on biosynthesis of nucleotides. Cells have two ways of producing nucleic acid, including *de novo* pathway and salvage pathway. The main biosynthetic pathway, *de novo* pathway can be blocked by the folic acid antagonist, aminoperin. However, the blockade can be overcome by alternative salvage pathway, which utilizes the bases and nucleosides produced by degradation of nucleic acid via the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK) (Figure 2.6).

HAT medium, a medium containing hypoxanthine, aminopterin and thymidine, is a selective medium for hybridomas. Aminopterin selects against myeloma cells by blocking the main biosynthetic *de novo* pathway. The myeloma cells which are defective in the enzyme HGPRT ($HGPRT^-$) cannot multiply and then die in the HAT medium because they cannot produce nucleotides by *de novo* and salvage pathway. The B cells cannot survive for more than a few days because they have a short life span. Therefore, only hybridomas can survive in the medium because the myelomas provide the ability to grow continuously in tissue culture and B cells contributed the functional HGPRT enzyme necessary to overcome the aminopterin block (Figure 2.7).

After fusion, the viable hybridomas are screened for their antibodies specific to the antigen. There are many screening assays such as ELISA, dot blotting, Western blotting or immunohistochemistry. These assays will vary according to the desired antibody specificity and the intended use of the product. Hybridomas producing the desired antibodies are cloned by limiting dilution method to ensure that a hybridoma is stable and single-cell cloned. The stable clones are further expanded to high volume in culture medium or injected in the peritoneal cavity of mice which called mouse ascites method.

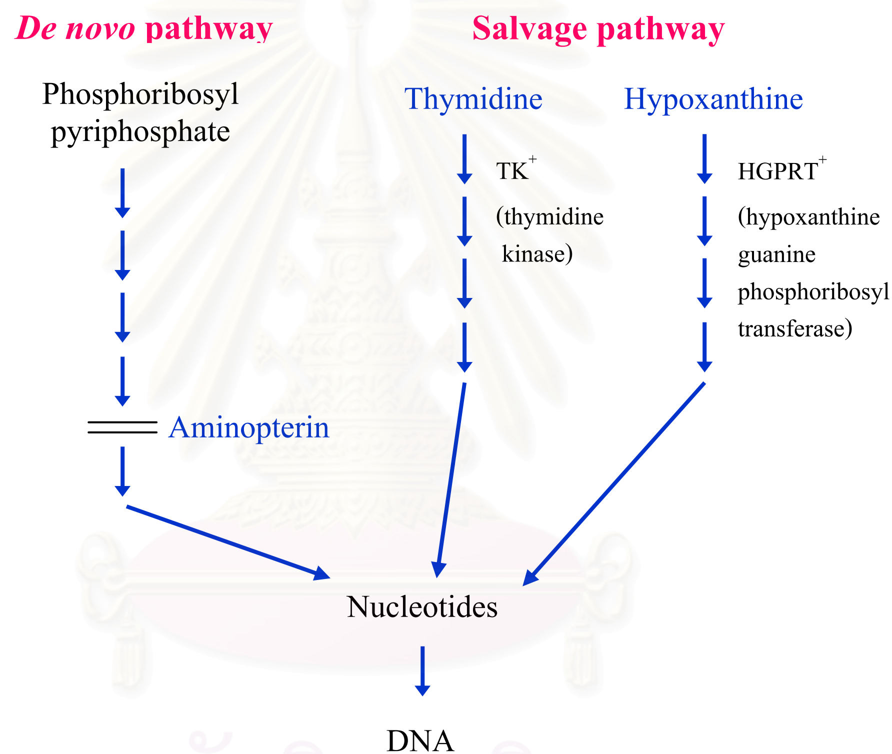

Figure 2.6 Nucleotide syntheses by *de novo* pathway and salvage pathway which related to hybridoma selection in HAT medium. When the main biosynthetic *de novo* pathway is blocked by aminopterin, cells can synthesize nucleotides using the salvage pathway if hypoxanthine and thymidine are provided (Abbas et al., 1991).

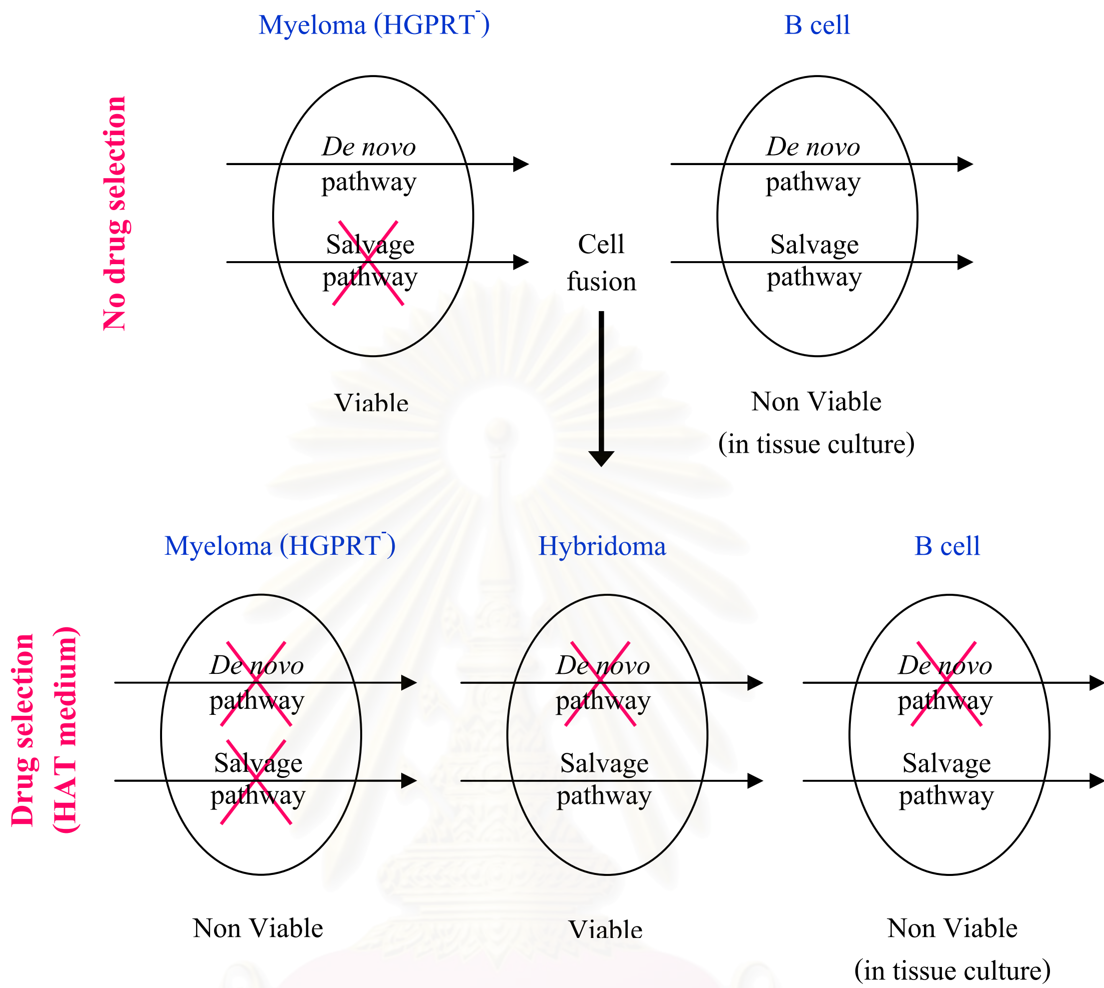

Figure 2.7 Selection for viable hybridomas in HAT medium (Harlow and Lane, 1988)

2.9 Researches on monoclonal antibodies against bacterial pathogens

There are many monoclonal antibodies which were produced against other foodborne pathogens including *S.* Typhimurium (Jaradat and Zawistowski, 1996; Schneid et al., 2005), *E. coli* (Padhye and Doyle, 1991; Zhao and Liu, 2005), *Vibrio* spp. (Qadri et al., 1994), *B. cereus* (Quinlan and Foegeding, 1997; Charni et al., 2000) and *L. monocytogenes* (Lin et al., 2006; Heo et al., 2007), whereas there is a few report on *Y. enterocolitica*. Examples of research on monoclonal antibodies against *Y. enterocolitica* were described as follow:

Heesemann et al. (1986) prepared MAbs against different released proteins of *Y. enterocolitica* serotype O:9. These MAbs used as probes in immunoblotting permitted identification of cross-reactive proteins from different *Yersinia* spp. and serotypes. The immunoblot analysis showed that Mab9–200 recognized the 46-kDa released protein of *Y. enterocolitica* of serotypes O:3, O:9 and O:5,27, and shared a common epitope with the 58-kDa protein of *Y. enterocolitica* of serotype O:8 and the 67-kDa protein of *Y. pseudotuberculosis* of serotypes I and III. Mab9–15 reacted with the 36-kDa protein of *Y. enterocolitica* of serotypes O:9, O:3 and O:8, and the 34-kDa protein of *Y. enterocolitica* of serotype O:5,27 and *Y. pseudotuberculosis* of serotypes I and III. Conversely, MAb-128 recognized an epitope present only on the 25-kDa proteins of *Y. enterocolitica* strain, but not on those of *Y. pseudotuberculosis*. The researchers suggested that this epitope is to be specific-specific which could not be achieved by mouse polyclonal antibodies.

Pekkola-Heino et al. (1987) produced MAbs against core and O-polysaccharide of *Y. enterocolitica* O:3 polysaccharide. Mice were immunized with whole bacterial cells and outer membrane protein. MAb 2B5 reacted with purified core-lipid A complex by enzyme immunoassay suggesting that the target determinant is in the outer core. This antibody recognized all *Y. enterocolitica* O:3 tested strains and also reacted with many other Gram-negative bacteria. MAb 2B5 which reacted to core-lipid A complex was visualized both with *Y. enterocolitica*, *Brucella abortus* and Haemophilus influenza by immunoblotting; however there is the only positive reaction was seen with *Y. enterocolitica* by immunofluorescence assay. Furthermore, MAb A6 reacted with purified O-polysaccharide chains in enzyme immunoassay, recognized all *Y. enterocolitica* O:3 tested strains, and showed no cross-reactions with other bacteria. A typical ladder pattern was not seen in the immunoblotting analysis with A6 suggesting that the O-chain of *Y. enterocolitica* O:3 may be different from those in other Gram-negative bacteria.

Schmidt and Sethi (1987) produced 7 MAbs specific to *Y. enterocolitica* serotype O:3, O:8 and O:9 and then characterized by indirect fluorescence assay (IFA) and agglutination reactions. MAb 2D8 which isotyped as IgG3 showed specific reactivity in both assay with all 70 strains of *Y. enterocolitica* serotype O:3 tested. Three MAbs designated 8E9, 10G11 and 11G2 isotyped IgG3 showed definite positive reactions in both assay only with all the strains representing serogroup O:9. MAb 1G2 reacted specially in the IFA test with all the strains

representing serogroup O:3 and O:9 indicating that this antibody recognizes an immunodeterminant shared by serogroups O:3 and O:9. The remaining MAbs, 4C2 and 6G5, showed reactivity in both assays with "esculin negative" biogroups 1 (pathogenic American strains) strains of serogroup O:8, and also exhibited narrow cross reactivity with other serotypes. Of these MAbs, MAbs 2D8 and 8E9 were purified and/or conjugated with fluorescein isothiocyanate (FITC) which developed by Acris Antibodies GmbH for probing the presence of pathogenic *Y. enterocolitica* organism in clinical and food materials.

Levasseur et al. (1992) produced MAb to detect rapidly all Enterobacteriaceae by fusion NS1 myeloma cells with splenocytes from mice immunized with heat-killed whole cell of E. coli O14:K7. Of these 6 obtained MAbs, MAb, designated CX9/15 is able to specifically recognize all Enterobacteriaceae as well as *Y. enterocolitica*, except *Erwinia chrysanthemi* when assay by indirect immunofluorescence. However, this MAb showed cross-reaction to non-enterobacteria including *Plesiomonas shigelloides*, *Aeromonas hydrophila* and *Aeromonas sobria*. This recognition spectrum strongly suggested that CX9/15 recognized the enterobacterial common antigen which presented a band of 20 kDa on the crude extract of an enterobacterium by Western blotting.

Li et al. (1992) produced monoclonal antibodies for detection of *Y. enterocolitica* serotype O:3 in pig feces by colony immunoblotting method. Heat-killed whole cells of *Y. enterocolitica* O:3 were used as antigens for immunization in BALB/C mice. MAb namely Yab-2 reacted specifically with strain of *Y. enterocolitica* O:3 when determined by ELISA without any cross-reactivity to other serotypes and Gram-negative bacteria. The MAb could detect single colonies of the bacteria in the presence of calculated 3.1×10^{8} heterologous organisms in pig feces. The result of Western blotting showed that the MAb reacted strongly with the lipopolysaccharide (LPS) O-antigens of *Y. enterocolitica* serotype O:3.

In addition to MAbs, Hochel and Škvor (2007) prepare rabbit IgG antibodies against whole cells of *Y. enterocolitica* O:3, O:9 and a group of pathogenic *Y. enterocolitica* strain (O:3, O:5,27, O:8 and O:9). The limiting titers of sera were within range 1: 9.5×10^{4}-1: 7.5×10^{5} CFU ml^{-1}. The Western blotting of *Yersinia* lipopolysaccharides showed that IgG against the single serotype O:3 interacted with high-molar-mass LPS of O:3, whereas other antibodies were

bound to low-molar-mass LPS of serotypes O:3, O:5,27, O:9 and strain *Y. enterocolitica* (CNCTC Y 2/68). IgG against the group of pathogenic serotypes also weakly interacted with low-molar-mass LPS of serotypes O:5, O:6,30, and O:10. The cross-reactivity of the antibodies with *Y. pseudotuberculosis* Ia and/or *Y. rohdei* b, d, e, f, i, which was observed by means of dot blotting using the whole bacterial cells as an antigen, but was not shown by LPS of these bacteria. The detection limits of these antibodies were $3x10^6$–$7x10^6$ CFU ml^{-1} determined by indirect competitive ELISA method.

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CHAPTER III

MATERIALS AND METHODS

3.1 Microorganisms

Table 3.1 List of bacterial strains and sources used in this study

Bacteria	Source
Yersinia enterocolitica	
Yersinia enterocolitica ATCC 27729	Department of Medical Science, Ministry of Public Health, Nonthaburi, Thailand
Yersinia enterocolitica MU	Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand
Yersinia enterocolitica CWC-01-0079	Department of Enteric Disease, Armed Forces Research Institute of Medical Science, Bangkok, Thailand
Yersinia enterocolitica CWC-01-0045	
Yersinia enterocolitica CWC-01-0275	
Yersinia enterocolitica CW-01-0199	
Yersinia enterocolitica SM-4	
***Yersinia* spp.**	
Yersinia pseudotuberculosis MU	Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand
Yersinia frederiksenii CWC-01-0149	Department of Enteric Disease, Armed Forces Research Institute of Medical Science, Bangkok, Thailand
Yersinia frederiksenii CWC-01-0093	
Yersinia frederiksenii DMST 19212	Department of Medical Science, Ministry of Public Health, Nonthaburi, Thailand
Yersinia kristensenii DMST 19209	
Yersinia ruckeri B04023	Department of Aquatic Science, Burapha University, Chonburi, Thailand

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Table 3.1 (cont.)

Bacteria	Source
Other Gram-negative bacteria	
Escherichia coli ATCC 25922	Department of Medical Science, Ministry of Public Health, Nonthaburi, Thailand
Vibrio vulnificus ATCC 27562	
Vibrio mimicus ATCC 33653	
Aeromonas hydrophila DMST 2798	
Salmonella Typhimurium ATCC 13311	
Enterobacter cloacae	Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand
Klebsiella pneumoniae	
Citrobacter freundii	
Serratia marcescens	
Proteus mirabilis	
Providencia rettgeri	
Edwardsiella tarda	
Shigella flexneri	
Vibrio cholera Eltor	
Pseudomonas aeruginosa	
Acinetobacter baumanii	
Morganella morganii	Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand
Salmonella Typhi	
Vibrio parahaemolyticus ATCC 17802	
Other Gram-positive bacteria	
Listeria monocytogenes DMST 17303	Department of Medical Science, Ministry of Public Health, Nonthaburi, Thailand
Staphylococcus aureus ATCC 25923	
Bacillus cereus ATCC 1729	Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand
Enterococus faecalis ATCC 7080	

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3.2 Animals and Cell lines

- ICR mice (outbred strain) female 6-8 weeks old were purchased from the National Laboratory Animal Centre, Mahidol University, Nakhon Pathom, Thailand.
- Myeloma cells P3/NSI/1-Ag4-1 (NS-1) were obtained from the American Type Culture Collection (ATCC) No: TIB-18

3.3 Chemicals, Antibodies and Kits

• 2-mercaptoethanol	Sigma-Aldrich, USA
• 3,3’ Diaminobensidine tetrahydrochloride (DAB)	Sigma-Aldrich, USA
• 30% Hydrogen peroxide (H_2O_2)	Merck, Germany
• 40% Acrylamide and Bis-acrylamide solution	Bio-Rad, USA
• Absolute ethanol	Merck, Germany
• Absolute methanol	Merck, Germany
• Acetic acid	Merck, Germany
• Aminopterin	Sigma-Aldrich, USA
• Ammonium persulfate ($(NH_4)_2S_2O_8$; APS)	Bio Basic Inc., Canada
• API 20E	BioMérieux, France
• Bacto Agar	Difco Laboratories, USA
• BCA^{TM} Protein Assay Kit	Pierce, USA
• Bovine serum albumin (BSA)	Sigma-Aldrich, USA
• Bromphenol blue	Sigma-Aldrich, USA
• Cefsulodin-irgasan-novobiocin agar (CIN)	Difco Laboratories, USA
• Citric acid	Merck, Germany
• Cobalt (III) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$)	Carlo Erba Reagenti, Italy
• Coomassie brilliant blue R-250	USB Corporation, USA
• Crystal violet	Sigma-Aldrich, USA
• D-glucose	Sigma-Aldrich, USA
• Diethyl ether	Merck, Germany
• Dimethylsulfoxide (DMSO)	Fluka, Switzerland
• di-Sodium hydrogen phosphate (Na_2HPO_4)	Merck, Germany

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• Fetal calf serum (FCS)	Invitromax, USA
• Freund's complete adjuvant (FCA)	Sigma-Aldrich, USA
• Freund's incomplete adjuvant (FIA)	Sigma-Aldrich, USA
• Gentamicin	T.P. drug laboratories (1969) Co., Ltd., Thailand
• Glycerol	Merck, Germany
• Glycine	Sigma-Aldrich, USA
• Horseradish peroxidase-conjugates goat anti mouse IgG (GAM-HRP)	Jackson Immuno, USA
• Hydrochloric acid (HCl)	Merck, Germany
• Hypoxanthine	Sigma-Aldrich, USA
• Iodine	Merck, Germany
• L-glutamine	Sigma-Aldrich, USA
• Mouse Monoclonal Antibody Isotyping Kit	Sigma-Aldrich, USA
• N, N, N', N'-Tetramethy ethylenediamine (TEMED)	Bio Basic Inc., Canada
• *o*-Phenylenediamine (OPD)	Abkem Iberia L.S., Spain
• Penicillin G	Sigma-Aldrich, USA
• Peroxidase labeled Goat Anti-Mouse IgG (Fab Specific)	Sigma-Aldrich, USA
• Polyethylene glycol (PEG)	Sigma-Aldrich, USA
• Ponceau S	Sigma-Aldrich, USA
• Potassium chloride (KCl)	Merck, Germany
• Potassium dihydrogen phosphate (KH_2PO_4)	Merck, Germany
• Prestained moleular weight markers	Fermentas, Canada
• RPMI 1640 medium	Biochrom AG, Germany
• Salfanin O	Merck, Germany
• Skim milk	Anline, Thailand
• Sodium chloride (NaCl)	Merck, Germany
• Sodium dodecyl sulfate (SDS)	Amersham Biosciences, England
• Sodium hydroxide (NaOH)	Merck, Germany
• Sodium pyruvate ($C_3H_3O_3Na$)	Sigma-Aldrich, USA

- Streptomycin — Sigma-Aldrich, USA
- Sulfuric acid (H_2SO_4) — Merck, Germany
- Thimerosal — Sigma-Aldrich, USA
- Thymidine — Sigma-Aldrich, USA
- Tris [hydroxymethyl] aminomethane (Trisma base) — Sigma-Aldrich, USA
- Tryptic Soy Broth (TSB) — Difco Laboratories, USA
- Tween 20 — Riedel-de Haën, UK

3.4 Equipments

- -20 ^{o}C and -70 ^{o}C Freezer — Sanyo, Japan
- 30 ^{o}C and 37 ^{o}C Incubater — Memmert, Germany
- 4 ^{o}C Refrigerator — Mitsubishi Electric, Japan
- 5% CO_2 Incubator (model 311) — Thermo Electron Corporation, USA
- 96-well EIA/RIA plate — Nunc, Denmark
- Autoclave (model ES-315) — Tomy, Japan
- Cell culture dish, 60 and 90 mm — Bibby Sterilin Ltd., UK
- Centrifuge tube 15 and 50 ml — CLP, USA
- Cryotube — Nunc, Denmark
- Filter paper — Whatman, Germany
- Heat block : Thermomixer Compact — Eppendorf, Germany
- High Speed Refrigerated Centrifuge (model J2-21) — Beckman, USA
- Hot air oven (model D06063) — Memmert, Germany
- Hot plate stirrer (model C-MAG HS 10) — Becthai, Thailand
- Hypodermic needle 18G and 21G — Nipro, Japan
- Inverted microscope — Nikon, Japan
- Laminar flow (‘clean’ model V6) — Lab Survice LTD part, Thailand
- Liquid Nitrogen Tank 34 HC Taylor Wharton Cryogenic — Harsco Corporation, USA
- Microcentrifuge tube 1.5 ml — Axygen Scientific, USA

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Item	Manufacturer
• Micropipette P2, P20, P100, P200, P1000 and P5000	Gilson, France
• Microplate reader	Titertek multiskan, Finland
• Microscope (model CH 30RF200)	Olympus, USA
• Nitrocellulose transfer membrane	Whatman, Germany
• Orbital shaker	Bioblock Sciencetific, France
• Petri Dish	Hycon, Germany
• pH meter (model S20-K)	Mettler Toledo, USA
• Pipettes	HBG, Germany
• Precision weighting balance (model AG 204 and PG 4002-5)	Mettler Toledo, Switzerland
• Protein III System for SDS-PAGE	Bio-Rad, USA
• Refrigerated incubater shaker (Innova™ 4330)	New Brunswick Scientific, USA
• Refrigerated Microcentrifuge 6500	Hettich Zentrifugen, Germany
• Semi-dry Electrophoretic Transfer Cell : Trans-Blot® SD	Bio-Rad, USA
• Spectrophotometer (Genesys 20 model 4001/4)	ThermoSpectronic, USA
• Syringe 1 and 5 ml	Nipro, Japan
• Tip 0.01, 0.2 and 1 ml	Axygen Scientific, USA
• Tissue cell culture plate 24-, 48- and 96-well	Corning Incorporation, USA
• Ultra-Pure Water Purification System	Elga, England
• Ultrasonic disruptor (model UD-201)	Tomy, Japan
• Vacuum pump	Iwaki, Japan
• Vortex mixer (model G560E)	Scientific Industries, Inc., USA
• Water bath	Becthai, Thailand

3.5 Experimental procedures

Flow diagram of all experiments was shown in Figure 3.1.

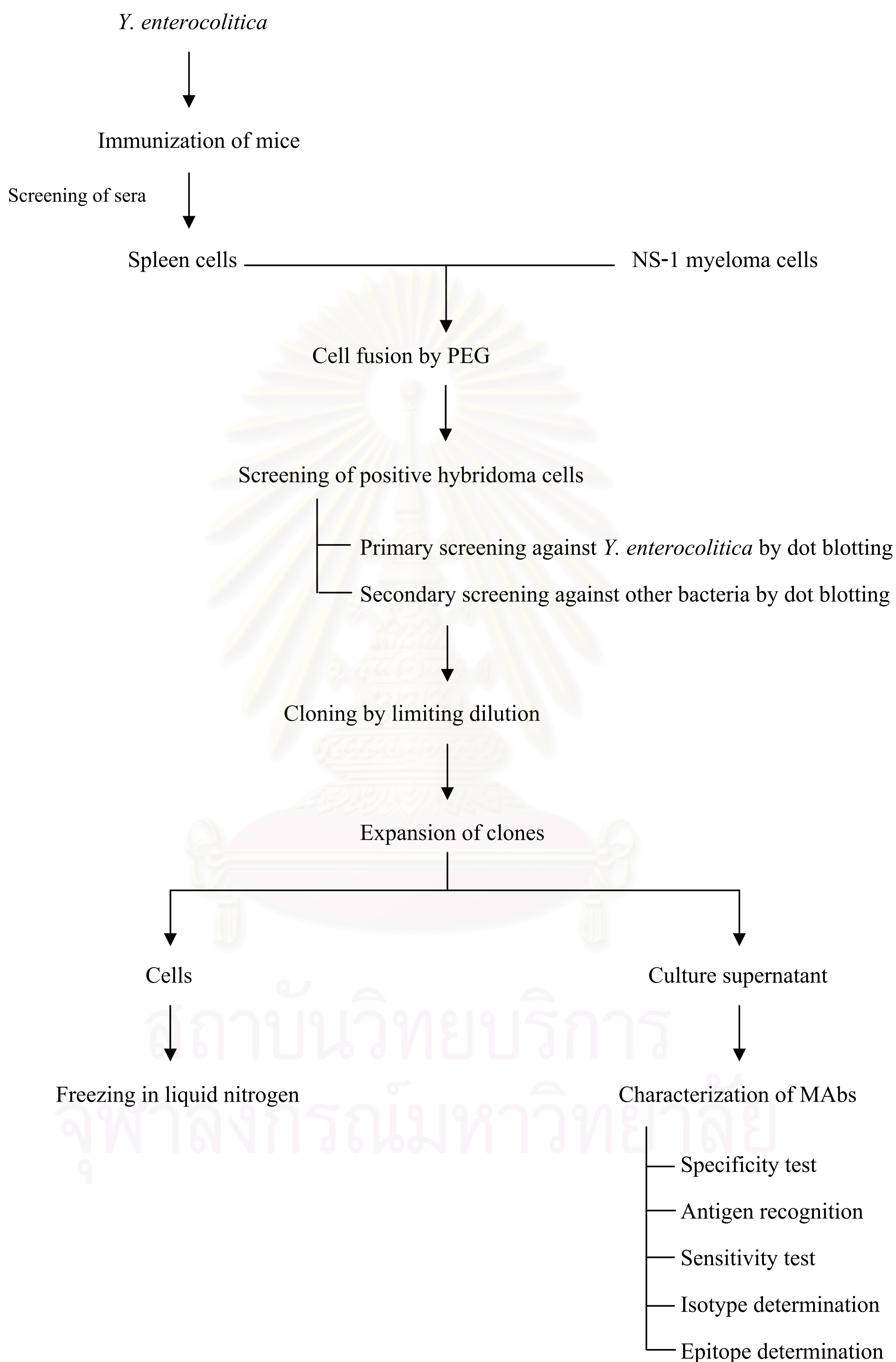

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Figure 3.1 Flow diagram of an experiment

3.5.1 Bacterial preparation

Yersinia enterocolitica and other bacteria in this study (Table 3.1) were stored at -70 °C in 20% glycerol in order to keep as stock bacterial cultures until use. To prepare a sufficient of bacterial cells, stock cultures of *Yersinia* spp. were inoculated in tryptic soy broth (TSB) and cultured at 30 °C for 24 h with agitation. Other bacteria were cultured at 37 °C for 24 h with agitation in either TSB or TSB supplemented with 2% (w/v) NaCl for the growth of *Vibrio* species. The bacteria were harvested by centrifugation at 8,000 rpm for 10 min at 4 °C and washed twice with 0.15 M phosphate buffered saline (PBS) pH 7.2. The pellets were resuspended in PBS, and then the absorbance of the final bacterial suspensions was adjusted to the optical density (OD) of 1.0 at 660 nm. The numbers of viable cells were determined as CFU by spread plates on tryptic soy agar (TSA). This was equivalent to approximately 10^9 CFU ml^{-1}. Moreover, identification of bacteria was confirmed by API 20E strips according to the manufacturer's instruction. The bacteria were identified using the database API LAB Plus provided by the manufacturer.

3.5.2 Antigen preparation

Y. enterocolitica ATCC 27729 and *Y. enterocolitica* MU were prepared as antigen for immunization in two forms, heat-killed and SDS-mercaptoethanol treated forms. Each of bacterial suspension was heat-killed at 60 °C for 60 min to inactivate the bacteria, and then cooled to 4 °C. To treat with SDS-mercaptoethanol, the heat-killed bacteria were mixed with 2x treatment buffer (Appendix B) at a ratio 1:1 (v/v), and then boiled for 90 sec. After that, the mixture was dialyzed against three changes of PBS at 12 h intervals. For screening and characterization of monoclonal antibodies, other bacterial suspension was heat-treated as the same procedure as mentioned above. Both of heat-killed and SDS-mercaptoethanol treated forms of antigens were divided into several aliquots and stored at -20 °C for later use (Phianphak et al., 2005).

3.5.3 Immunization

The mixture of heat-killed and SDS-mercaptoethanol treated forms of *Y. enterocolitica* at ratio 1:1 were used as antigens to produce MAbs (Table 3.2). Five ICR mice were immunized by intraperitoneal injection (i.p.) with 50 µl of the antigen, emulsified in equal volumes of Freund's complete adjuvant. Thereafter, mice were boosted with different times in 2 weeks (details shown in Table 4.2) using the same amount of antigen emulsified in equal volumes of Freund's incomplete adjuvant. One week later, blood was taken from each mouse by tail bleeding, and then centrifuged at 10,000 rpm for 5 min at 4 ^{o}C for collecting the serum. The antibody response was tested against *Y. enterocolitica* by dot blotting and Western blotting as described below. Three day before fusion, the mouse showing the highest titer was final booster with the same antigen without adjuvant to use as spleen donor for hybridoma production. Serum samples were obtained before first immunization to be used as negative control.

Table 3.2 Antigen(s) for immunization in each mouse

Mouse number	Antigen(s)
1	*Y. enterocolitica* ATCC 27729
2	*Y. enterocolitica* ATCC 27729
3	*Y. enterocolitica* ATCC 27729
4	*Y. enterocolitica* ATCC 27729 and *Y. enterocolitica* MU
5	*Y. enterocolitica* ATCC 27729 and *Y. enterocolitica* MU

3.5.4 Hybridoma production

Hybridomas were produced according to the method developed by Köhler and Milstein (1976) with modifications described by Mosmann et al. (1979). NS-1 myeloma cells were fused with spleen cells of the selected mouse using 50% polyethylene glycol (PEG) as a fusogen. All media and reagents for hybridoma production were prepared as described in the Appendix C.

3.5.4.1 Myeloma cell lines

Myeloma partners used for fusion were NS-1. They were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) in a humidified 5% CO_2 incubator at 37 ^oC. The myelomas were maintained in exponential phased growth by subculturing for one week prior to fusion. Approximately 10^7 cells of NS-1 myeloma were added into a 50 ml of a polypropylene tube and then centrifuged at 1,500 rpm for 5 min. After the supernatant was discarded, RPMI 1640 medium supplemented with 0.2 mg ml^{-1} gentamicin was added and then placed in a humidified incubator to use in the fusion step.

3.5.4.2 Spleen cells

Y. enterocolitica immunized mice was anesthetized with diethyl ether before blood drawn by cardiac puncture, and their serum were pooled and stored at -20 ^oC to be used as positive control in the immunological assay. The spleen was collected aseptically from euthanized mice and gentle washed in sterile Petri dishes containing RPMI 1640 medium supplemented with 0.2 mg ml^{-1} gentamicin to minimize the chance of contamination. Then the connective tissues were carefully removed as much as possible during extensive soak in the medium. A single cell suspension was prepared by cutting the spleen into small pieces and then crushing by use a 10 ml syringe plunger through a sterile grid into the medium. Spleen cells were harvested by centrifugation at 1,500 rpm for 5 min and resuspended in 5 ml of the medium for fusion with myeloma cells.

3.5.4.3 Fusion procedure

Myeloma cells (3.5.4.1) were added into a 50 ml of a polypropylene tube containing the spleen suspension (3.5.4.2) at a 3:1 ratio. It was centrifuged at 1,500 rpm for 5 min and all of the supernatant was discarded as completely as possible. After mixing the cells by tapping the tube, 1 ml of pre-warmed (37 ^oC) 50% (w/v) polyethylene glycol (PEG) was added dropwise to the cell suspension using a Pasteur pipette and simultaneously the tube was slightly agitated. This step was performed exactly for 1 min. After that, 30 ml of RPMI 1640 medium supplemented with 0.2 mg ml^{-1} gentamicin was added gently into the suspension, and mixed with the pipette.

Pellet the cells by centrifugation and resuspended with the medium again in order to wash PEG from the cells. The fused cells were suspended in hypoxanthine-aminopterine-thymidine (HAT) selective medium supplemented with 20% FCS. The suspension was dispensed (200 µl $well^{-1}$) into twelve 96-well culture plates. All plates were placed in a humidified 5% CO_2 incubator at 37 ^{o}C.

Half medium of the wells was replaced by fresh HAT medium on day 7 post-fusion. Cell growth in the bottom of the wells was examined using inverted microscope. When cells grow about two-thirds of the wells (10 to 12 days after fusion), the hybridoma culture supernatant in each well was screened for antibody activity against *Y. enterocolitica* as described below. Cells were grown in HAT medium for 2 weeks and then HAT was substituted by HT medium (HAT medium without aminopterine).

3.5.5 Hybridoma screening

3.5.5.1 Primary screening against *Y. enterocolitica* by dot blotting

Heat-killed form of *Y. enterocolitica* approximately 10^9 CFU ml^{-1} used as antigen in immunization step was used for primary screening by dot blotting. Bacterial samples were applied to a nitrocellulose membrane as 1 µl $spot^{-1}$. Positive and negative controls, sera from immunized and nonimmunized mouse, respectively were also dotted onto the membranes. The membranes were dried at 37 ^{o}C for 30 min and blocked in 5% skim milk at room temperature (RT) for 30 min to prevent nonspecific binding of the antibodies. After washed with PBS, the membrane was dried and stored at -20°C to use subsequently.

The membranes were incubated in hybridoma culture supernatant diluted 1:2 in PBS from each culture for 3 h and then washed 4 times for 5 min each with 0.05% Tween-20 in PBS (PBST). After that, horseradish peroxidase-conjugates goat anti mouse IgG (GAM-HRP) diluted 1:2000 in PBS was added and incubated for 2 h. After washing as above, the membranes were treated with DAB substrate solution (Appendix E) for 5 min and the color reaction was stopped by washing thoroughly in deionized water. All steps were performed at room temperature with continuous rocking. The positive wells which appeared as a dark spot were further screened by dot blotting against *Y. enterocolitica* and other bacteria.

3.5.5.2 Secondary screening against *Y. enterocolitica* and other bacteria by dot blotting

The positive wells in primary screening were tested for cross-reactivity by dot blotting against *Y. enterocolitica* ATCC, *Y. enterocolitica* MU, and seven other bacteria including *Y. pseudotuberculosis* MU, *Y. frederiksenii* DMST 19212, *Y. kristensenii* DMST 19209, *Y. ruckeri*, *E. coli*, *S.* Typhimurium, and *V. cholera*. The bacteria approximately 10^9 CFU ml^{-1} in heat-killed form were spotted onto nitrocellulose membrane as 1 µl $spot^{-1}$ in each block of 3x3 mm. The membranes were dried and blocked in 5% skim milk in PBS. After washed in PBS, the membranes were incubated in hybridoma culture supernatant which gave positive results in primary screening (1:2 dilution in PBS) at RT for 3 h and then processed as described above in primary screening section. Hybridomas from the wells showing antibody patterns of interest with strong signals were selected for subsequent cloning by limiting dilution method.

3.5.6 Cloning of hybridoma cell by limiting dilution

Selected hybridomas were cloned by limiting dilution using HT medium supplemented with 20% FCS to ensure that a hybridoma is stable and single-cell cloned. From each positive well, 10 µl of the cell suspension was diluted with the medium to obtain about 80 viable cells and then mixed with 10 ml of HT medium. One hundred microlitres of the mixture was dispensed into 96-well plate containing 100 µl of HT medium. The plates were placed in a humidified 5% CO_2 incubator at 37 ^{o}C for 10 to 12 days. Wells containing a single colony were tested for antibody production by dot blotting. The selected hybridomas were cloned a minimum of 3 times or continue repeating the cloning until every well tested is positive in order to achieve stable single clones producing only a homogeneous antibody, and further stored in liquid nitrogen, while the supernatants were characterized as described below.

3.5.7 Cryopreservation

3.5.7.1 Cell freezing

Stable antibody-producing clones were expanded from wells to plates. The cell pellets were harvested by centrifugation at 1,500 rpm for 5 min and mixed with 1 ml of freezing

medium (Appendix C), which was kept cold at 4 ^{o}C. The suspension was transferred to labeled sterile cryotube using a Pasteur pipette. After closing the cap, the tube was placed into a foam box and put the box into a -70 ^{o}C freezer for 24 h. After that, the tube was transferred into a container of liquid nitrogen (-196 ^{o}C) for long-term storage.

3.5.7.2 Cell thawing

The cryotube from liquid nitrogen was swung at 37 ^{o}C in a water bath for a few minutes until the contents have thawed. The cells were transferred immediately into a tube containing 10 ml of RPMI 1640 medium and centrifuged at 1,500 rpm for 5 min. The cell pellets were resuspended in a new sterile Petri dish with 20 ml of RPMI 1640 medium supplemented with 20% FCS and placed in a humidified 5% CO_2 incubator at 37 ^{o}C.

3.5.8 Characterization of monoclonal antibodies

3.5.8.1 Specificity test

The specificity of the MAbs was determined for the reactivity of the antibody with *Y. enterocolitica* and other bacterial species by dot blotting. Heat-killed bacteria samples (10^9 CFU ml^{-1}) listed in table 3.1 were spotted onto nitrocellulose membranes as 1 µl spot^{-1}. The bacteria were spotted in each block of 4x4 mm. The membranes were dried at 37 ^{o}C for 30 min and blocked in 5% skim milk in PBS at RT for 30 min. Subsequently, the membranes were reacted with various MAbs diluted 1:100 (or serum diluted 1:20,000) in 5% skim milk in PBST at RT for 3 h. The membranes were washed 4 times for 5 min each with PBST and then incubated in GAM-HRP diluted 1:2000 in PBS at RT for 2 h. After washing, DAB substrate solution was added to the membranes and reacted at RT for 5 min. The color reaction was stopped by washing the membranes in deionized water. The intensity of the reaction with each MAb was compared by eye and scored relative to the reaction against antigen.

3.5.8.2 Antigen recognition by Western blotting

3.5.8.2.1 SDS-PAGE

SDS-PAGE was performed according to the method described by Laemmli (1970) with slight modification, using a 1.5 mm thick slab gels with 5% stacking gel, 12% separating gel and 15-laned comb for applying samples. All reagents were prepared as described in the Appendix D. First of all, total protein concentrations of each of heat-killed antigens were determined by BCA^{TM} Protein Assay Kit according to the manufacturer's instructions (Appendix F). Two micrograms of proteins from each sample was denatured by mixing with equal volume of SDS staining dye, boiled for 5 min and then loaded into each well of the gels. Protein markers with a molecular weight ranging from 19 to 117 kDa were used as standard. Samples were electrophoresed at a constant voltage of 100 V for 95 min in Western blot running buffer using protein III system. The separated bands of proteins in the gels were either visualized by immersion in staining solution or electroblotted onto nitrocellulose membrane.

3.5.8.2.2 Western blotting

Immediately after completion of the electrophoresis, the stacking gel was removed and size of the separating gel was measures. The gel was equilibrated in transfer buffer for 5 min. During gel equilibration, 6 pieces of Whatman filter paper and a nitrocellulose membrane were cut to the same size as the separating gel. The membrane was pre-wetted in deionized water and then immersed in transfer buffer prior to use. After that, the gel was placed onto the membrane, which spliced with the filter papers layered to form a sandwich in a semi-dry transfer apparatus. Any air bubbles stuck in the layer were eliminated by gentle rolling a glass test tube on the layer. The electroblotting was carried out for 2 h at a constant current 75 mA per one gel. After transfer, the membrane was stained with Ponceau S solution to ensure the presence of proteins and then marked the site of the protein lanes. The membrane was washed in PBS before blocking in 5% skim milk in PBS at RT for 30 min. Afterward, the membrane was cut into strips, incubated with various MAbs diluted 1:100 (or serum diluted 1:20,000) in 5% skim milk in PBST at RT for 3 h and then processed as described above in the specificity test section.

3.5.8.3 Sensitivity test

Five-fold serial dilutions of heat-killed *Y. enterocolitica* from 10^9 to 10^5 CFU ml^{-1} diluted in PBS were prepared. One microlitre of each dilution was spotted onto a nitrocellulose membrane and processed for dot blotting using various MAbs as described above in the specificity test section. The lowest bacterial cell concentration which gave positive reaction was observed as the detection limit of *Y. enterocolitica* using dot blotting technique.

3.5.8.4 Isotype determination

Isotype of mouse immunoglobulins secreted by the hybridomas were determined using Sigma's Mouse Monoclonal Antibody Isotyping Kit. The assay based on sandwich ELISA principle. The procedure was conducted according to the manufacturer's instruction with slight modification. Briefly, 96-well plate was coated with 100 µl of isotype specific antibodies (Goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA) diluted 1:1000 in PBS. The plate was incubated at 37 ^{o}C for 1 h and then washed 3 times with PBST (300 µl $well^{-1}$). Subsequently, 100 µl of hybridoma culture supernatants to be tested diluted 1:20 in PBS and a known IgG3 antibody at concentration 3 µg ml^{-1} used as positive control were added to each well, and incubated at 37 ^{o}C for 1 h. After washing with PBST, 100 µl of peroxidase labeled Goat anti-mouse IgG (Fab specific) diluted 1:2000 in PBST was added to the plate followed by incubate at RT for 30 min. The plate was final washed with PBST and then 150 µl of OPD substrate solution (Appendix E) was added. The plate was incubated in the dark at RT for 10 min. The reaction was stopped by adding 50 µl of 1 M H_2SO_4 to each well and the absorbance was measured at 492 nm using microplate reader.

3.5.8.5 Epitope determination

To determine whether the MAbs recognize the same epitope, the ELISA additivity test was performed on paired MAbs as described by Friguet et al. (1983) with some modifications. Cell suspension of *Y. enterocolitica* (3.5.1) was broken by ultrasonication for 5 min twice. Each of the antigens was adjusted to 10^7 CFU ml^{-1} using PBS to use as antigen for the assay. One hundred microlitres of the antigen was coated into the wells of 96-well plate at 4 ^{o}C

overnight. The plate was washed 3 times with PBST and then blocked in 5% skim milk in PBS (300 µl well^{-1}) at 37 °C for 1 h. After washing, 100 µl of each participating MAb at saturated concentrations to the coated antigen was added individually or in pairs into the plate and incubated at 37 °C for 2 h. The plate was washed as above followed by the addition of 100 µl of GAM-HRP diluted 1:10,000 in PBS and incubated at 37 °C for 1 h. After washing, 150 µl of OPD substrate solution (Appendix E) was added. The plate was incubated in the dark at RT for 10 min. The reaction was stopped by adding 50 µl of 2.5 M H_2SO_4 to each well and the absorbance was measured at 492 nm using microplate reader.

Absorbancy values resulting in the ELISA additivity test were expressed as additive index (AI) which calculated by the following formula:

$$AI = \left[\frac{2A_{1+2}}{(A_1 + A_2)} - 1 \right] \times 100$$

Where A_1, A_2, A_{1+2} are absorbance values with the first antibody alone, the second antibody alone and the mixture of two antibodies together, respectively. An AI of $> 30\%$ was interpreted as additive, meaning the MAbs recognized distinct epitopes, while AI of $< 30\%$ was interpreted as not additive, meaning they recognized the same epitope or epitopes of similar structures (Friguet et al., 1983).

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CHAPTER IV

RESULTS

4.1 Characteristics and biochemical test of *Y. enterocolitica* ATCC 27729 and MU

The characteristics of *Y. enterocolitica* ATCC 27729 and MU used as antigens for immunization including the morphology of the bacterial colonies on TSA and CIN agar plates, and Gram staining were determined. The results found that *Y. enterocolitica* ATCC 27729 was Gram-negative coccobacilli, while *Y. enterocolitica* MU was more elongated than the other one. The colonial morphology of these two isolates on TSA for 24 h of incubation at 30 ^{o}C appeared as small to medium colony (1-2 mm diameter) with cream color, circular, convex, entire margin, smooth, translucent and buttery. In addition, *Y. enterocolitica* growing on CIN agar, highly selective medium for *Y. enterocolitica*, produced deep red center with a transparent margin or bull's-eye colonies (Figure 4.1). The identification of the *Y. enterocolitica* ATCC 27729 and MU were confirmed using the API 20E strip which was based on the biochemical reactions shown in Table 4.1. Although, biochemical tests of two isolates of *Y. enterocolitica* differed in acetoin production (VP), the percentage of identification to *Y. enterocolitica* was 99.5% and 99.9% for ATCC 27729 and MU, respectively.

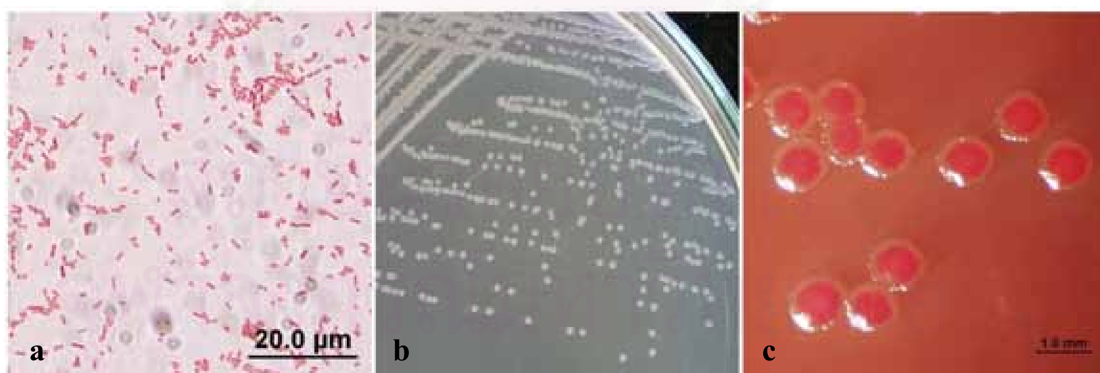

Figure 4.1 Light microscopic photographs of Gram staining of *Y. enterocolitica* ATCC 27729 (a) (magnification 1,000X). The morphology of *Y. enterocolitica* colonies growing on TSA (b) and CIN agar (c)

Table 4.1 Biochemical tests of *Y. enterocoliica* ATCC 27729 and MU by API 20E.

Biochemical tests	***Y. enterocolitica***	
	ATCC 27729	**MU**
β-galactosidase (ONPG)	-	-
Arginine dihydrolase (ADH)	-	-
Lysine decarboxylase (LDC)	-	-
Ornithine decarboxylase (ODC)	+	+
Citrate utilization (CIT)	-	-
H_2S production (H_2S)	-	-
Urease (URE)	+	+
Tryptophane deaminase (TDA)	-	-
Indole production (IND)	-	-
Acetoin production (VP)	+	-
Gelatinase (GEL)	-	-
Fermentation/Oxidation Glucose (GLU)	+	+
Mannitol (MAN)	+	+
Inositol (INO)	+	+
Sorbitol (SOR)	+	+
Rhamnose (RHA)	-	-
Saccharose (SAC)	+	+
Melibiose (MEL)	-	-
Amygdalin (AMY)	+	+
Arabinose (ARA)	+	+
Cytochrome-oxidase (OX)	-	-
API 20E code number	0115723	0114723
% identification to *Y. enterocolitica*	99.5%	99.9%

+ = positive, - = negative

4.2 Immunization of mice

One week after completion of the immunization, the sera from five ICR mice were collected to evaluate the *Y. enterocolitica*-specific antibody response against *Y. enterocolitica* ATCC 27729 compared to the serum from nonimmunized mouse by dot blotting. All antisera had a high titer of 1: 2.56×10^5 to 1: 1.02×10^6 with the antigen depending on each mouse, while the immunoreactivity of the nonimmunized mouse serum used as negative control to the antigen was not observed (Figure 4.2).

Furthermore, the sera were analyzed by Western blotting against *Y. enterocolitica* ATCC 27729 and *Y. enterocolitica* MU which were used for immunization, as well as *Y. frederiksenii* DMST 19212 and *E. coli* ATCC 25922 which were representatives of *Yersinia* sp. and other Gram-negative bacteria, respectively (Figure 4.3). The profiles of these proteins which were revealed by SDS-PAGE and stained with Coomassie blue showed similar bands in each species of *Yersinia* tested, but different in *E. coli*. In Western blotting, all antisera demonstrated a series of numerous immunoreactive bands not only to these two isolates of *Y. enterocolitica*, but also to *Y. frederiksenii*. The results showed major similar bands at about 45 kDa and 10-11 kDa. Another immunoreactive band at about 20 kDa was observed in antisera of immunized mouse number 3, 4 and 5. Furthermore, all antisera also showed some cross-reactivity to *E. coli*, while immunoreactive band against all separating antigens was not detected in nonimmunized mouse (Figure H1). However, antibodies responses of these immunized mice were raised enough to used as spleen donor for undertaking the hybridoma production.

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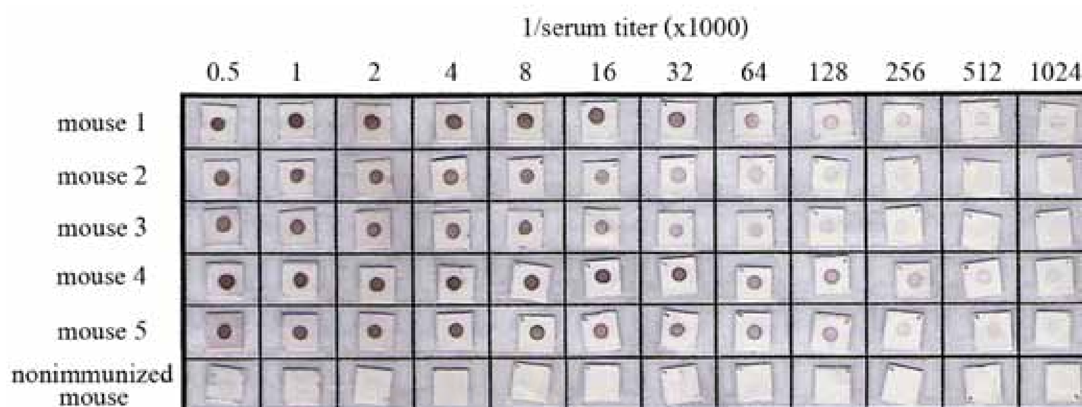

Figure 4.2 Dot blotting for specificity of mice antisera. The serum of immunized mouse number 1, 2, 3, 4, 5 and nonimmunized mouse were tested against heat-killed *Y. enterocolitica* ATCC 27729.

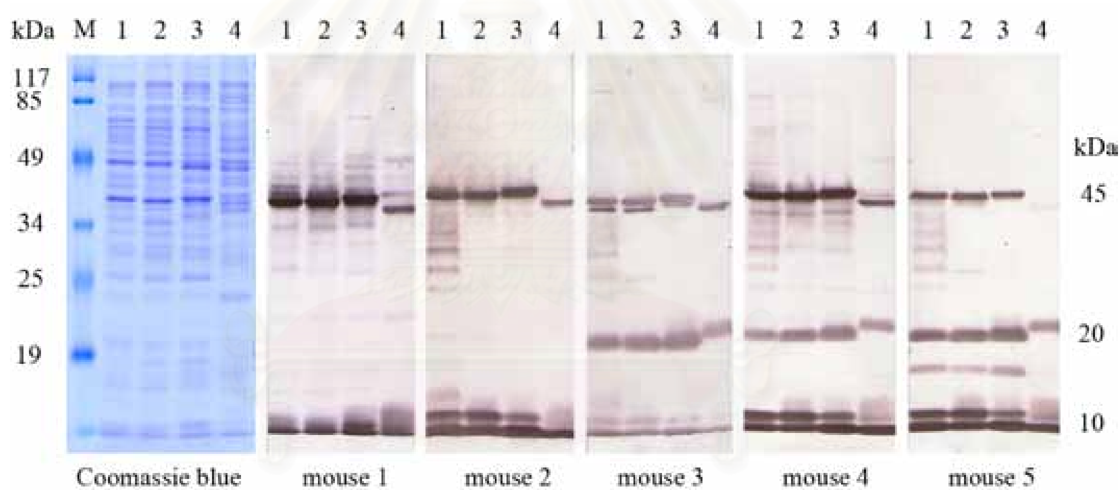

Figure 4.3 SDS-PAGE and Western blot analysis for specificity of mice antisera. The serum of the immunized mouse numbered 1, 2, 3, 4 and 5 at dilution 1:20,000 were tested against SDS-PAGE separated whole cell lysates of *Y. enterocolitica* ATCC 27729 (lane 1), *Y. enterocolitica* MU (lane 2), *Y. frederiksenii* DMST 19212 (lane 3), and *E. coli* ATCC 25922 (lane 4); M, molecular weight marker in kDa was shown on the left side.

4.3 Hybridoma production

Five fusions were performed after immunization with *Y. enterocolitica* ATCC 27729, or mixing with *Y. enterocolitica* MU (Table 3.2). The fusion products were dispensed into twelve 96-well cell culture plates (1152 wells) and hybridomas were observed. After primary and secondary screening by dot blotting, the hybridomas producing a strong immunoreactivity to the antigens used for immunization and showing different antibody pattern of interest were cloned. Finally, 25 monoclones were obtained and their respective MAbs subjected to further characterization. For each fusion, the percentage of the wells contained hybridoma clones, the wells which gave positive results after primary and secondary screening and the designation of monoclones were shown in Table 4.2.

Table 4.2 Hybridoma production for each fusion

Fusion no.	Mouse no.	Number of immunization	Hybridoma (%)	1st screen (wells)	2nd screen (wells)	Clone number
1	3	4	100	109	1	YE5
2	1	5	-[a]	-[a]	-[a]	-[a]
3	2	6	94.88	224	6	YE35, 37, 38, 42, 44, 51
4	4	3	92.71	124	10	YE59, 62, 64, 66, 67, 68, 69, 70, 75, 81
5	5	4	62.24	226	8	YE83, 86, 90, 96, 102, 105, 107, 108

[a] no clone was obtained due to bacterial contamination.

4.4 Characterization of monoclonal antibodies

4.4.1 Specificity test

The specificity of MAbs from all 25 monoclones was determined by dot blotting with various heat-killed bacterial strains listed in Table 3.1. These 25 MAbs can be divided into 9 groups according to their antigen specificities. The results of representative MAbs from each group namely YE108, YE86, YE44, YE90, YE38, YE5, YE42, YE105 and YE66 respectively, were shown in Figure 4.4. The reactivities of MAbs in each group were as follows:

MAbs in group 1 consisted of seven clone including YE67, YE70, YE81, YE83, YE96, YE107 and YE108. These MAbs reacted only with *Y. enterocolitica* MU without any cross-reactivity to the other isolates of *Y. enterocolitica* or other bacteria tested.

MAb in group 2 consisted of one clone, namely YE86 whose MAb reacted with *Y. enterocolitica* MU and showed weakly cross-reactivity to *Y. enterocolitica* ATCC 27729.

MAbs in group 3 consisted of nine clones including YE37, YE44, YE59, YE62, YE64, YE68, YE69, YE75 and YE102. These MAbs reacted with *Y. enterocolitica* ATCC 27729 and *Y. enterocolitica* CWC-01-0079 with slighter immunoreactivity.

MAb in group 4 consisted of one clone, namely YE90. The result of this MAb differed from MAb in group 2 is that it showed stronger reactivity to *Y. enterocolitica* ATCC 27729 than *Y. enterocolitica* MU.

MAbs in group 5 consisted of two clones including YE35 and YE38. These MAbs reacted with *Y. enterocolitica* ATCC 27729, *Y. enterocolitica* MU and *Y. enterocolitica* CWC-01-0079 isolates.

MAb in group 6 consisted of one clone, namely YE5. This MAb reacted with all isolates of *Y. enterocolitica*, and also cross-reacted with three isolates of *Yersinia* spp. including *Y. pseudotuberculosis* MU, *Y. frederiksenii* DMST 19212 and *Y. kristensenii* DMST 19209.

MAb in group 7 consisted of one clone, namely YE42. This MAb reacted with all isolated of *Y. enterocolitica* liked the previous group. Furthermore, it also cross-reacted with four isolates of *Yersinia* spp. including *Y. frederiksenii* DMST 19212, *Y. frederiksenii* CWC-01-0149, *Y. frederiksenii* CWC-01-0093 and *Y. kristensenii* DMST 19209 as well as one species of the other Gram-negative bacteria: *E. tarda*.

The two remaining groups of MAb were group 8 which consisted of one clone, YE 105 and group 9 which consisted of two clones, YE51 and YE66. These MAbs reacted prominently with all isolates of *Y. enterocolitica* and *Yersinia* spp. tested. Furthermore, the antibodies also showed cross-reactions with other Gram-negative bacteria including *E. coli*, *E. cloacae*, *K. pneumoniae*, *C. freundii*, *S. marcescens*, *M. morganii*, *S.* Typhimurium, *S.* Typhi and *S. flexneri*. Nevertheless, there was a distinction between these two groups is that MAb in group 8 showed cross-reactivity to *P. rettgeri*, whereas MAbs in group 9 showed cross-reactivity to *E. tarda*.

Unexpectedly, all produced MAbs and nonimmunized mouse antiserum (Figure H2) were found to react with *Staphylococcus aureus* on dot blotting. As MAbs in group 1 and group 2 showed strong reactivity to *Y. enterocolitica* MU, this bacterial strain was used as antigen for subsequently characterization. By contrast, *Y. enterocolitica* ATCC 27729 was used as antigen for characterization of MAbs in the other groups. The specificities of MAbs and results of the other tests were summarized in Table 4.6.

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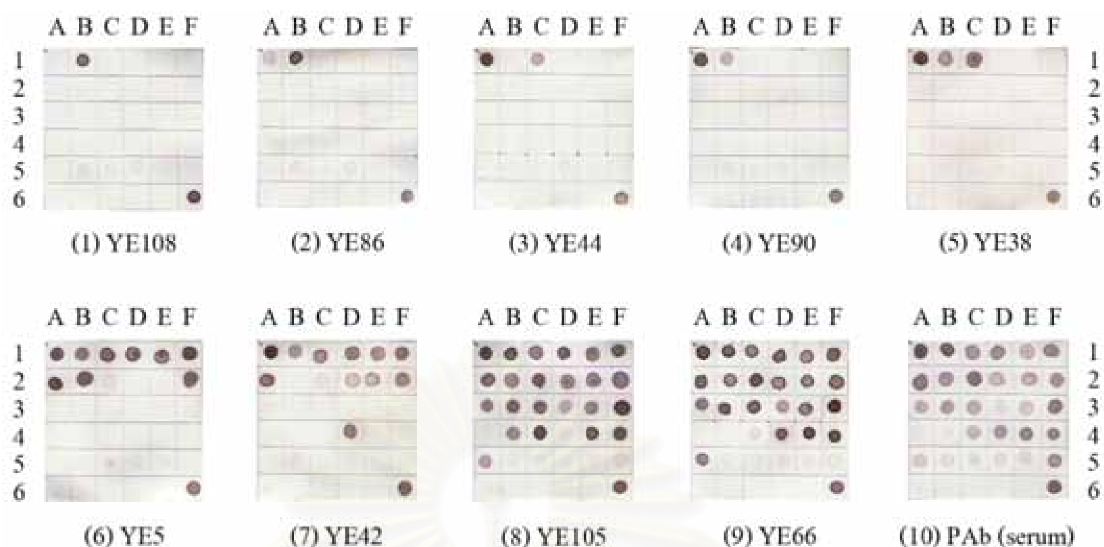

Figure 4.4 Specificity of MAbs assayed by dot blotting. Heat-killed bacteria (~10^9 CFU ml^{-1}) were spotted onto nitrocellulose membrane 1 µl $spot^{-1}$ and treated with various representative MAbs from each group (1-9) at dilution 1:100 or polyclonal antibody (10) at dilution 1:20,000. Each bacterium was spotted onto each block of the membrane as follows:

Row 1: (A) *Y. enterocolitica* ATCC 27729; (B) *Y. enterocolitica* MU; (C) *Y. enterocolitica* CWC-01-0079; (D) *Y. enterocolitica* CWC-01-0045; (E) *Y. enterocolitica* CWC-01-0275; (F) *Y. enterocolitica* CW-01-0199

Row 2: (A) *Y. enterocolitica* SM-4; (B) *Y. pseudotuberculosis* MU; (C) *Y. frederiksenii* DMST 19212; (D) *Y. frederiksenii* CWC-01-0149; (E) *Y. frederiksenii* CWC-01-0093; (F) *Y. kristensenii* DMST 19209

Row 3: (A) *Y. ruckeri* B04023; (B) *Escherichia coli*; (C) *Enterobacter cloacae*; (D) *Klebsiella pneuminoae*; (E) *Citrobacter freundii*; (F) *Serratia marcescens*

Row 4: (A) *Proteus mirabilis*; (B) *Providencia rettgeri*; (C) *Morganella morganii*; (D) *Edwardsiella tarda*; (E) *Salmonella* Typhimurium; (F) *Salmonella* Typhi

Row 5: (A) *Shigella flexneri*; (B) *Vibrio cholera*; (C) *Vibrio parahaemolyticus*; (D) *Vibrio vulnificus*; (E) *Vibrio mimicus*; (F) *Aeromonas hydrophila*

Row 6: (A) *Pseudomonas aeruginosa*; (B) *Acinetobacter baumanii*; (C) *Listeria monocytogenes*; (D) *Bacillus cereus*; (E) *Enterococcus faecalis*; (F) *Staphylococcus aureus*

4.4.2 Antigen recognition by Western blotting

The characteristics of *Y. enterocolitica* antigens recognized by produced MAbs were analyzed using SDS-PAGE followed by Western blotting (Figure 4.5). The results found that MAbs in group 1 reacted with a protein band of 10 kDa on *Y. enterocolitica* MU, while MAb in group 2 failed to react with any protein band on the blot. MAbs in group 3 reacted with 43, 39, 35, 32, 28 and 24 kDa proteins for *Y. enterocolitica* ATCC 27729. However, the antibodies from two MAbs, YE64 and YE75, reacted with protein bands at molecular weight similarities to the other clones in this group with weakly immunoreactivity. MAb in group 4 reacted weakly with protein bands of 32, 28 and 12 kDa. MAbs in group 5 recognized protein bands of 39, 35, 32, 28, 24, 15 and 12 kDa and also weakly bands of 21 and 18 kDa. MAb in group 6 reacted with a protein band of 10 kDa and weakly band of 24 kDa. MAb in group 7 reacted with a smear band ranging from 13 to 11 kDa. Finally, the two remaining groups of MAb, group 8 and group 9, recognized two protein bands at molecular weight of 11 and 10 kDa and also weak band at 14 kDa.

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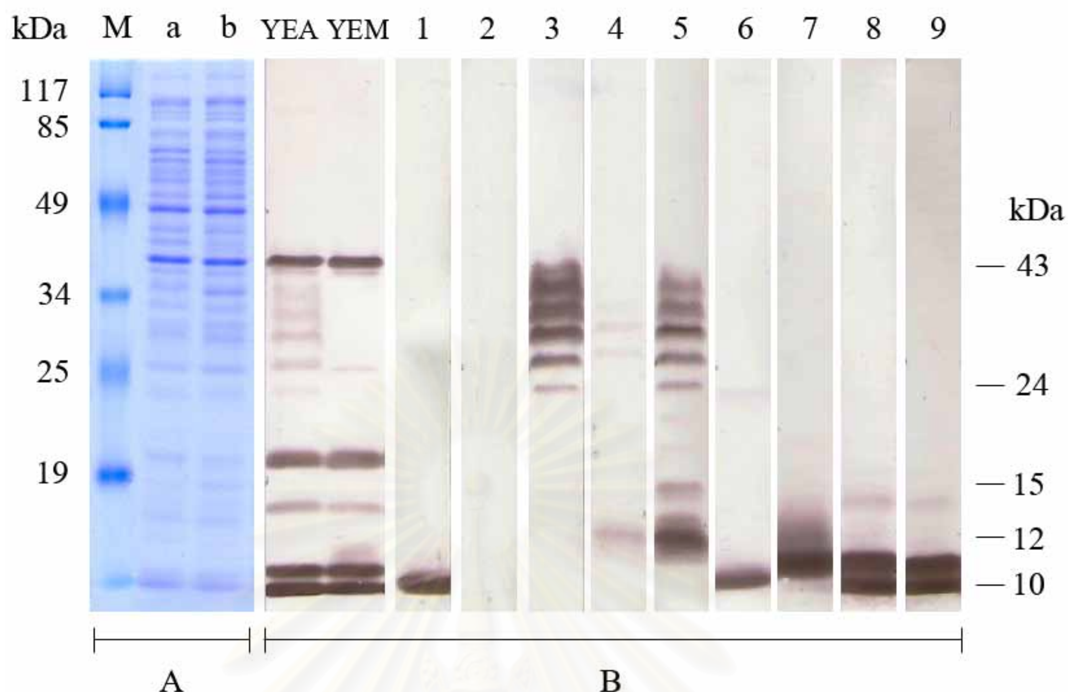

Figure 4.5 SDS-PAGE and Western blot analysis of *Y. enterocolitica* antigens recognized by the MAbs. Heat-killed *Y. enterocolitica* ATCC 27729 (lane a) and *Y. enterocolitica* MU (lane b) were electrophoresed and stained with Coomassie blue (A) or electroblotted onto nitrocellulose membrane and then treated with various representative MAbs from each group at dilution 1:100 (B). The separating *Y. enterocolitica* MU was treated with MAb YE108 (lane 1) and YE86 (lane 2), whereas the separating *Y. enterocolitica* ATCC 27729 was treated with the other MAb as follows: YE44 (lane 3), YE90 (lane 4), YE38 (lane 5), YE5 (lane 6), YE42 (lane 7), YE105 (lane 8), and YE66 (lane 9). In addition, a polyclonal antibody of immunized mouse number 5 at dilution 1:20,000 was tested against *Y. enterocolitica* ATCC 27729 (YEA) or *Y. enterocolitica* MU (YEM). M, molecular weight marker in kDa was shown on the left side.

4.4.3 Sensitivity test

The sensitivity of MAbs was determined by dot blotting with 5-fold serial dilution of heat-killed *Y. enterocolitica* ATCC 27729 or MU isolate depending on their specificity to the antigen. The results shown in Figure 4.6 indicated that the detection limit of MAbs in group 1 and group 2 was 1.4 x 10^8 CFU ml^{-1} of *Y. enterocolitica* MU. Furthermore, the sensitivity of MAbs in group 3, 4, 5, 8 and 9 was 2.4 x 10^7 CFU ml^{-1}, while MAb in group 6 was 1.2 x 10^8 CFU ml^{-1} of *Y. enterocolitica* ATCC 27729. MAb in group 7 showed the highest sensitivity which could detect *Y. enterocolitica* ATCC 27729 at 4.8 x 10^6 CFU ml^{-1}.

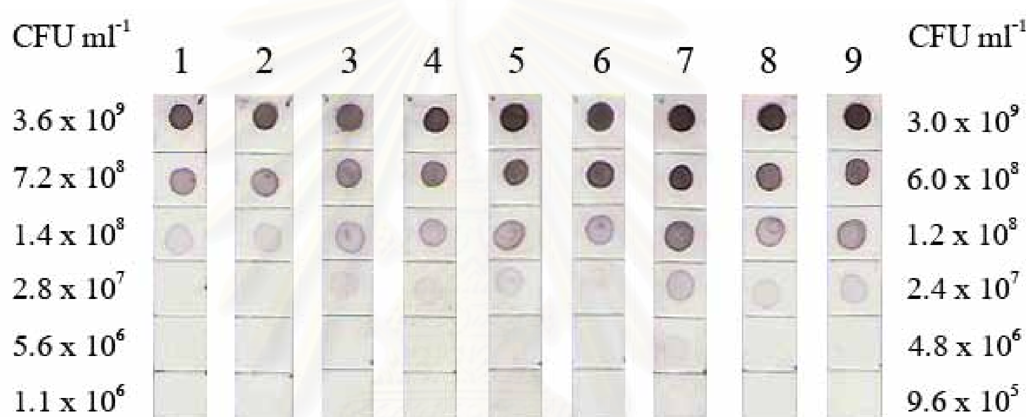

Figure 4.6 Sensitivity of the MAbs assayed by dot blotting. Five-fold serial dilution of heat-killed *Y. enterocolitica* MU (left) was spotted onto nitrocellulose membrane as 1 µl $spot^{-1}$ and then treated with MAb YE108 (1) and YE86 (2) at dilution 1:100, whereas that of *Y. enterocolitica* ATCC 27729 (right) was treated with the other MAbs from each group at the same dilution as follows: YE44 (3), YE90 (4), YE38 (5), YE5 (6), YE42 (7), YE105 (8), and YE66 (9).

4.4.4 Isotype determination

The isotype of MAbs was determined using Sigma's Mouse Monoclonal Antibody Isotyping Kit. The absorbance values of all MAbs for each fusion which was measured at 462 nm were shown in Table 4.3. The isotype of MAbs was indicated in bold number with gray shading. The results showed that thirteen of these MAbs were IgM, five were IgG2b, four were IgG2a, two were IgG3, and one was IgG1 isotype.

Table 4.3 The absorbance values of MAbs for isotype determination

Fusion	MAb	A_{462} [a]					
		IgG1	**IgG2a**	**IgG2b**	**IgG3**	**IgM**	**IgA**
1	YE5	0.118	0.100	**2.841**	0.711	0.266	0.113
3	YE35	0.073	0.065	**2.776**	0.166	0.192	0.060
	YE37	0.183	0.152	0.563	0.434	**2.703**	0.123
	YE38	0.078	0.064	**2.798**	0.156	0.185	0.059
	YE42	0.062	**1.245**	0.159	0.142	0.153	0.059
	YE44	0.134	0.109	0.210	0.689	**2.582**	0.104
	YE51	0.093	**1.077**	0.169	0.769	0.285	0.079
4	YE59	0.077	0.089	0.157	0.121	**2.597**	0.074
	YE62	0.064	0.068	0.118	0.110	**2.522**	0.109
	YE64	0.118	0.105	**2.522**	0.376	0.309	0.088
	YE66	0.059	**1.919**	0.120	1.232	0.178	0.056
	YE67	0.076	0.082	0.155	0.290	**2.566**	0.060
	YE68	0.070	0.060	0.237	0.171	**2.578**	0.061
	YE69	0.059	0.058	0.170	0.142	**2.535**	0.056
	YE70	0.107	0.108	0.182	0.226	**2.552**	0.073
	YE75	0.077	0.072	0.154	**2.317**	0.112	0.063
	YE81	0.072	0.070	0.140	0.255	**2.551**	0.062
5	YE83	0.151	0.206	0.324	0.350	**2.611**	0.135
	YE86	**1.466**	0.089	0.124	0.161	0.200	0.080
	YE90	0.070	0.081	**2.337**	0.254	0.197	0.064
	YE96	0.330	0.493	0.837	0.879	**2.601**	0.279
	YE102	0.147	0.214	0.301	0.293	**2.491**	0.118
	YE105	0.062	**0.527**	0.095	0.100	0.105	0.058
	YE107	0.268	0.350	0.484	0.533	**2.503**	0.118
	YE108	0.077	0.127	0.119	**1.140**	0.125	0.067
Control IgG3		0.265	0.424	0.677	**1.968**	0.470	0.173

[a] A_{462} absorbance at 462 nm. Values were based on duplicated experiments.

4.4.5 Epitope determination

All the representative MAbs from each group were tested by the ELISA additivity test to determine whether the MAbs recognize the same epitope. Table 4.4 showed the absorbance values of each MAb and pairs thereof at saturated concentration to *Y. enterocolitica* ATCC 27729 or MU isolate used as coating antigen. The results of the ELISA additivity test on pairs of the MAbs were calculated to the additive index (AI) as shown in Table 4.5. From the results, it was found that pairs of MAb YE42 with the other, except YE44, had AI values of more than 30% as well as pair of YE44 and YE5 as shown in bold number with gray shading, indicating that these antibodies recognized with distinct epitopes of *Y. enterocolitica* ATCC 27729. On the other hand, the other pairs of MAb had AI values of less than 30% indicating that these antibodies recognized the same epitope or epitopes of similar structures.

Table 4.4 Absorbance values of each MAb and pairs thereof at saturated concentration to the coating antigen[a]

Dilution	**A_{462}[b]**								
	1:20	**1:10**	**1:20**	**1:5**	**1:20**	**1:50**	**1:10**	**1:10**	**1:20**
MAb	**YE108**	**YE86**	**YE44**	**YE90**	**YE38**	**YE5**	**YE42**	**YE105**	**YE66**
YE108	0.663	0.842							
YE86	-	0.761							
YE44			0.457	0.685	1.161	2.268	0.750	0.950	1.345
YE90			-	1.478	1.665	2.194	1.590	1.678	1.852
YE38			-	-	1.797	2.250	1.851	1.862	1.977
YE5			-	-	-	2.301	2.294	2.259	2.301
YE42			-	-	-	-	0.923	1.399	1.743
YE105			-	-	-	-	-	1.178	1.635
YE66			-	-	-	-	-	-	1.713

[a] The sonicated *Y. enterocolitica* MU (10^7 CFU ml^{-1}) was used as coating antigen for clones YE108 and YE86, while the sonicated *Y. enterocolitica* ATCC 27729 (10^7 CFU ml^{-1}) was used as coating antigen for the remaining clones.

[b] A_{462} absorbance at 462 nm. Values were based on duplicated experiments.

Table 4.5 Additive index (AI) for the MAbs as assessed by indirect ELISA[a]

MAb	Additive index (%)[b]								
	YE108	YE86	YE44	YE90	YE38	YE5	YE42	YE105	YE66
YE108	-	18.26							
YE86	-	-							
YE44			-	0	3.02	**64.47**	8.70	16.21	23.96
YE90			-	-	1.68	16.12	**32.44**	26.36	16.08
YE38			-	-	-	9.81	**36.10**	25.18	12.65
YE5			-	-	-	-	**42.31**	29.86	14.65
YE42			-	-	-	-	-	**33.17**	**32.25**
YE105			-	-	-	-	-	-	13.11
YE66			-	-	-	-	-	-	-

[a] The sonicated *Y. enterocolitica* MU (10^7 CFU ml^{-1}) was used as coating antigen for clones YE108 and YE86, while the sonicated *Y. enterocolitica* ATCC 27729 (10^7 CFU ml^{-1}) was used as coating antigen for the remaining clones.

[b] An additivity reaction was considered positive when the additive index > 30%.

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Table 4.6 Grouping and characteristics of the MAbs[a]

Group	MAb(s)	Isotype	Sensitivity (CFU ml^{-1})	Antigen (kDa)	Specificity
1	YE67, YE70, YE81, YE83, YE96, YE107	IgM	1.4×10^{8}	10	YEM (+++)
	YE108	IgG3			
2	**YE86**	IgG1	1.4×10^{8}	ND	YEA (+), YEM (+++)
3	YE37, **YE44**, YE59, YE62, YE68, YE69, YE102	IgM	2.4×10^{7}	43, 39, 35, 32, 28, 24	YEA (+++), YE0079 (++)
	YE64	IgG2b			
	YE75	IgG3			
4	**YE90**	IgG2b	2.4×10^{7}	32, 28, 12	YEA (+++), YEM (++)
5	YE35, **YE38**	IgG2b	2.4×10^{7}	39, 35, 32, 28, 24, 21, 18, 15, 12	YEA (+++), YEM (++), YE0079 (++)
6	**YE5**	IgG2b	1.2×10^{8}	24, 10	*Y. enterocolitica* (+++), YP (+++), YF19212 (+), YK (+++)
7	**YE42**	IgG2a	4.8×10^{6}	13-11	*Y. enterocolitica* (+++), YF19212 (+), YF0149 (++), YF0093 (++), YK (+++), ET (+++)
8	**YE105**	IgG2a	2.4×10^{7}	14, 11, 10	*Yersinia* spp. (+++), 10 species of other Gram-negative bacteria
9	YE51, **YE66**	IgG2a	2.4×10^{7}	14, 11, 10	*Yersinia* spp. (+++), 10 species of other Gram-negative bacteria

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[a] Sensitivity and specificity were determined by dot blotting. The size of detected antigen in kDa was determined by Western blotting. YEA, *Y. enterocolitica* ATCC 27729; YEM, *Y. enterocolitica* MU; YE0079, *Y. enterocolitica* CWC-01-0079; YP, *Y. pseudotuberculosis* MU; YF19212, *Y. frederiksenii* DMST 19212; YF0149, *Y. frederiksenii* CWC-01-0149; YF0093, *Y. frederiksenii* CWC-01-0093; YK, *Y. kristensenii* DMST 19209; ET, *E. tarda*; ND, not determined. The intensity of staining for the specificity test was arbitrary scored as +++ = very intense staining, ++ = moderate staining, + = light staining. The bold numbers were representative MAbs used in various tests.

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CHAPTER V

DISCUSSION

Monoclonal antibodies against *Y. enterocolitica* were produced by fusion spleen cells from immunized mice with NS-1 myeloma cells. In this study, heat-killed and SDS-mercaptoethanol treated forms of *Y. enterocolitica* were used as antigens for immunization. Inactivated whole bacterial cells which are large molecule and complex are considered good antigen for stimulating immune system (Bhunia, 2008). Furthermore, whole cells of *Y. enterocolitica* were degraded by treating with SDS-mercaptoethanol to increase an immune response against proteins in the cells. Dot blotting was selected to screen the hybridomas producing antibodies against *Y. enterocolitica* as well as to characterize of the MAbs. This method used for detection an antigen in solution is rapid, simple and cost-effective, requires only 1 μl of sample and can easily be processed simultaneously with a large number of samples (Sulimenko and Dráber, 2004).

Profiles on SDS-PAGE of proteins from different *Y. enterocolitica* and *Y. frederiksenii* were similar. Moreover, the antiserum collected from each immunized mouse also showed similar immunoreactive bands to all two isolated of *Y. enterocolitica* ATCC and MU as well as *Y. frederikseneii* (Figure 4.3). The result showed a close relationship among these *Yersinia* species. However, these antisera showed reactivity to *E. coli*, a representative of Gram-negative bacteria. This occurrence may due to whole cells of *Y. enterocolitica* or their lysates composed of multiple epitopes. Different B cells were stimulated to synthesize antibodies which one of those capable of binding a distinct epitope. Thus, a complex mixture of antibodies synthesized which were found in serum showed cross-reactivity to related antigens in other bacteria.

Twenty-five MAbs were obtained and they can be divided into 9 groups according to their specificities as determined by dot blotting (Figure 4.4 and Table 4.6). MAbs in group 1 to group 5 were specific only to *Y. enterocolitica*; however, they did not recognize all isolated of *Y. enterocolitica* tested suggesting the heterogeneity among *Y. enterocolitica* strains. This suggestion was supported by its taxonomy which was classified into 60 serotypes according to their “O” somatic antigens. Therefore, these MAbs was suggested to recognize some serotypes of

Y. enterocolitica which may be used for strain-specific identification. The MAbs in group 6 and 7 showed reactivity to all isolated of *Y. enterocolitica* tested, but they showed cross-reactivity to other *Yersinia* spp. as well as *E. tarda*, especially for group 7. It could be suggested that these MAbs recognized common epitope of *Yersinia* spp. which possessed closely related structure in the cross-reaction species (Goding, 1996). Therefore, these two groups of MAbs can be used to differentiate *Yersinia* spp. from other Gram-negative and Gram-positive bacteria. The MAbs in group 8 and 9 showed reactivity to all isolated of *Y. enterocolitica* and *Yersinia* spp. and also reacted with other Gram-negative bacteria. It was noticeable that all Gram-negative bacteria reacted with these MAbs belonged to family Enterobacteriaceae. Therefore, these two groups of MAbs can be used for detection of enterobacteria. Furthermore, these two groups distinctly recognized the two identical bands at 11 and 10 kDa in Western blotting (Figure 4.5), believing that they were enterobacterial common antigens. However, the intensity of the reaction with individual MAb which was visually examined on the dot blotting, suggested that they were considerably variation in the level of antigen expression between the different species and in particular between the different isolates of *Y. enterocolitica*.

Unexpectedly, all produced MAbs and nonimmunized mouse antiserum reacted with *Staphylococcus aureus* in various degrees immunoreactivity on dot blotting (Figure 4.4 and Figure H2) which corresponded to previous reports (Okret et al., 1984; Bérubé et al., 1989; Prahkarnkaeo, 2005). One probable explanation might be due to protein A which is a surface protein found in the cell wall of *S. aureus*. It binds with the Fc region of immunoglobulins, especially IgG isotype (Boyle, 1987). Some variants of IgA and IgM can also bind to staphylococcal protein A (Chalon et al., 1979; Lindmark et al., 1983). Thus, all MAbs could bind to *S. aureus* with various affinities resulting in the positive results in dot blotting. However, the problem of nonspecific binding might be overcome by treating the membrane with a 0.1% solution of fetal bovine serum before reacting with antibodies because serum contains large amounts of IgG that will react with protein A of *S. aureus* and block nonspecific reactions (Bérubé et al., 1989). Another choice to dissolve this problem is that using selective media such as MacConkey agar, which inhibited Gram-positive bacteria as well as *S. aureus* in sample tested, coupled with immunological method.

Although the target antigens for MAbs in this study were not identified, band patterns in immunoblotted lysates of *Y. enterocolitica* reacted with the MAbs might suggested by comparing with the other reports. A ladder-like banding pattern having molecular weight higher than 15 kDa recognized for the MAbs in group 3 and 5 (Figure 4.5) were suggested to react with O-antigen of lipopolysaccharide (LPS). Furthermore, the broad band pattern recognized for the MAb in group 7 and the single band or two bands at low molecular weight of proteins running at dye front recognized for the other groups of MAbs as well as the MAbs in group 5 were suggested to react with core LPS (Westerman et al., 1997; Jung et al., 2001; Skurnik and Bengoechea, 2003; Hochel and Škvor, 2007). However, the immunoblot of SDS-PAGE separated LPS of *Y. enterocolitica* reacted with all of these MAbs should be performed in order to confirm the suggestions of these results.

It was surprising that only MAb YE86 in group 2 was not capable of detecting protein bands in Western blotting (Figure 4.5). This evidence could be explained that it possibly recognized conformational epitopes which may have been altered under the denaturing conditions used in SDS-PAGE, leading to no binding detected (Goding, 1996; Lin et al., 2006). This is one of the properties of MAbs that sometimes may be too specific to the pretreated antigens.

Although MAb YE86 in group 2 which distinctly reacted with *Y. enterocolitica* MU could not detect any protein bands in Western blotting, YE86 was assumed that it recognized the same epitope of bacteria as YE108 in group 1 did. This assumption is explained by the result of epitope determination showing that the absorbance values did not increase significantly when the antibodies of YE86 were combined with YE108 for testing on *Y. enterocolitica* MU and an additive index is not more than 30% (Table 4.4 and 4.5).

When the antibodies of YE42 in group 7 combined individual with these of YE90, YE38, YE5, YE105 or YE66 which were representative of the MAbs in group 4 to 6 and group 8 to 9 respectively, additive index were more than 30% (Table 4.5) indicating that these MAbs most likely recognized distinct epitopes of *Y. enterocolitica* ATCC 27729. These results corresponded with those of dot and Western blotting, which showed that they reacted differently to several bacterial strains and having different pattern of protein bands that be recognized though they were the same core LPS.

Furthermore, the highest additive index at 64.47% was calculated when the antibodies of YE44 in group 3 and YE5 in group 6 were combined which indicated that they recognized epitopes distinctly (Table 4.5). This indication corresponded with the result of Western blotting is suggested that YE44 reacted with O-antigen, while YE5 reacted with core LPS. However, the positive additivity reactions were not observed when YE44 were mixed with other antibodies of the MAbs that reacted with core LPS. The observe differences might be explained that these MAbs recognized the same epitope or epitopes of similar structures which were present in different parts of LPS.

The results of sensitivity test found that the MAbs could detect *Y. enterocolitica* by dot blotting with different sensitivities ranging from approximately 10^6-10^8 CFU ml^{-1} (~10^3-10^5 cells $spot^{-1}$) (Figure 4.6 and Table 4.6). However, these sensitivities could be increased by using other types of immunoassay such as sandwich ELISA or a cocktail mixture of MAbs which recognized different epitopes of the same antigen (Canfield et al., 1985; Ziai et al., 1987; Wang et al., 1994). Furthermore, some reagents such as fluorescent or chemiluminescent product was used instead of a colored product can increase the sensitivity of immunoassay approximately 10-fold (Swaminathan and Konger, 1986).

The MAbs produced in this study, especially group 1 to group 5 would be used as a tool in many immunological-based methods to detect or differentiate *Y. enterocolitica* from other *Yersinia* spp., other Gram-negative and Gram-positive bacteria. However, the detection of *Y. enterocolitica* in naturally contaminated or spiked food samples by these MAbs should be performed in order to continue for development of an immunoassay-based test kit for the most effective and rapid detection of *Y. enterocolitica* in the future.

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CHAPTER VI

CONCLUSION

1. Twenty-five monoclonal antibodies (MAbs) against *Y. enterocolitica* were obtained from five fusion NS-1 mouse myeloma cells with spleen cells of ICR mice immunized with heat-killed and SDS-mercaptoethanol treated forms of *Y. enterocolitica* ATCC 27729 or mixed with MU isolate and they can be divided into 9 groups according to their apparent specificities to several bacterial strains as determined by dot blotting.

2. Of these 9 groups, MAbs in group 1 to group 5 were specific only to *Y. enterocolitica* without any cross-reactivity to *Yersinia* spp. and other bacteria tested. However, they did not recognize all isolates of *Y. enterocolitica* tested.

3. MAbs in group 6 and group 7 reacted with all isolates of *Y. enterocolitica* tested, but showed cross-reactivity to *Yersinia* spp. and *E. tarda* especially for group 7.

4. MAbs in group 8 and group 9 reacted with all isolates of *Y. enterocolitica* and *Yersinia* spp., and also reacted with other Gram-negative bacteria which belong to Enterobacteriaceae.

5. By Western blotting, these MAbs recognized the protein of *Y. enterocolitica* with various molecular weights ranging from 10 to 43 kDa.

6. These MAbs could detect *Y. enterocolitica* with different sensitivities ranging from approximately 10^6-10^8 CFU ml^{-1} (~10^3-10^5 cells spot^{-1}) as determined by dot blotting. All of these, MAb YE 42 showed the highest sensitivity at 4.8 x 10^6 CFU ml^{-1} or 4,800 cells spot^{-1}.

7. Thirteen of these MAbs were determined as IgM, five were IgG2b, four were IgG2a, two were IgG3, and one was IgG1 isotype.

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APPENDICES

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APPENDIX A

Culture media for bacterial growth

1. Tryptic soy broth (TSB)

Tryptone	17	g
Soytone	3	g
Dextrose	2.5	g
NaCl	5	g
K_2HPO_4	2.5	g

Adjusted pH to 7.3 and bring total volume to 1000 ml using distilled water. The medium was then sterilized by autoclaving at 121 ^{o}C, 15 lb/in^{2} for 15 min.

2. Tryptic soy agar (TSA)

Tryptone	17	g
Soytone	3	g
Dextrose	2.5	g
NaCl	5	g
K_2HPO_4	2.5	g
Agar	15	g

Adjusted pH to 7.3 and bring total volume to 1000 ml using distilled water. The medium was then sterilized by autoclaving at 121 ^{o}C, 15 lb/in^{2} for 15 min.

3. Cefsulodin-irgasan-novobiocin agar (CIN)

Peptone	17	g
Proteose peptone	3	g
Yeast extract	2	g
Mannitol	20	g
Sodium pyruvate	2	g
NaCl	1	g

$MgSO_4 \cdot 7H_2O$	10	mg
Sodium deoxycholate	0.5	g
Sodium cholate	0.5	g
Irgasan®	4	mg
Agar	13.5	g
Crystal violet	1	mg
Neutral red	30	mg
Yersinia Antimicrobial Supplement (CN)		
Cefsulodin	4	mg
Novobiocin	2.5	mg

Adjusted pH to 7.4 and bring total volume to 1000 ml using distilled water. The medium was sterilized by autoclaving at 121 °C, 15 lb/in^2 for 15 min. After cool to 45-50 °C, 10 ml of rehydrated Yersinia Antimicrobial Supplement was aseptically added to the medium and mixed well.

APPENDIX B

Reagents

1. 2x treatment buffer (10 ml)

1 M Tris-Cl pH 6.8	1.25	ml
10% SDS	4	ml
Glycerol (87%)	2.29	ml
2-mercaptoethanol	1	ml
Distilled water adjusted volume to	10	ml

2. 1 N HCl

HCl (37%)	8.28	ml
Distilled water adjusted volume to	100	ml

3. 1 N NaOH

NaOH	4	g
Distilled water adjusted volume to	100	ml

4. 0.85% NaCl

NaCl	8.5	g
Distilled water adjusted volume to	1000	ml

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APPENDIX C

Media and reagents for hybridoma production

1. Stock 100x HT

Hypoxanthine	0.1360 g	in 20 ml distilled water
Thymidine	0.0388 g	in 20 ml distilled water

Dissolve the solutions above and total volume was adjusted to 100 ml using distilled water. Then, the solution was divided into aliquots and stored at -20 ^{o}C before use.

2. Stock 100x HAT

Hypoxanthine	0.1360 g	in 20 ml distilled water
Aminopterin	0.0018 g	in 20 ml distilled water
Thymidine	0.0388 g	in 20 ml distilled water

Dissolve the solutions above and total volume was adjusted to 100 ml using distilled water. Then, the solution was divided into aliquots and stored at -20 ^{o}C before use.

3. RPMI 1640 medium

RPMI 1640 (Roswell Park Memorial Institute)	10.43	g
$NaHCO_3$	2	g
L-glutamine	0.1	g
Glucose	2	g
Sodium pyruvate	0.11	g
Distrilled water	1000	ml

The solution of penicillin G and streptomycin were added to a final concentration of 100,000 units and 100 mg per litre, respectively. The medium was sterilized by Millipore membrane (pore size 0.22 µm) filtration and stored at 4 ^{o}C.

4. HT medium

RPMI 1640 medium	1000	ml
100x HT	10	ml

The medium was mixed well and then sterilized by Millipore membrane (pore size 0.22 µm) filtration and stored at 4 °C.

5. HAT medium

RPMI 1640 medium	1000	ml
100x HAT	10	ml

The medium was mixed well and then sterilized by Millipore membrane (pore size 0.22 µm) filtration and stored at 4 °C.

6. 50% (w/v) polyethylene glycol (PEG)

PEG was thawed in a 60 °C water bath. The solution was prepared by dissolving 2 g of PEG in 2 ml of RPMI 1640 medium. The solution was divided into aliquots with the volume of 1 ml and stored at 4 °C. The aliquot of PEG solution was placed in a humidified 5% CO_2 incubator at 37 °C before use.

7. Freezing medium (10% DMSO)

Dimethyl sulfoxide (DMSO)	10	ml
RPMI 1640 medium	90	ml

The medium was stored at 4 °C before use.

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APPENDIX D

Buffers and reagents for SDS-PAGE and Western blotting

1. 10% SDS

Sodium dodecyl sulfate (SDS)	10	g
Deionized water adjusted volume to	100	ml

2. 10% APS

Ammonium persulfate (APS)	1	g
Deionized water adjusted volume to	10	ml

3. 1 M Tris-HCl pH 6.8

Trisma base	12.11	g

The small volume of deionized water was added and pH was adjusted with 1 N HCl to 6.8, and then deionized water was added to reach 100 ml final volume.

4. 1.5 M Tris-HCl pH 8.8

Trisma base	18.17	g

The small volume of deionized water was added and pH was adjusted with 1 N HCl to 8.8, and then deionized water was added to reach 100 ml final volume.

5. 2x Laemmli buffer (SDS-dye) (10 ml)

1 M Tris-HCl pH 6.8	1	ml	(final concentration 100 mM)
10% SDS	4	ml	(4% v/v)
Glycerol (87%)	2.29	ml	(20% v/v)
Bromphenol blue	0.001	g	
HPLC water adjusted volume to	10	ml	

The solution was stored at -20 ^{o}C.

6. SDS staining dye

2x Laemmli buffer (SDS-dye)	900	µl
2-mercaptoethanol	100	µl

The solution was stored at -20 °C.

7. SDS-polyacrylamide gel preparation

7.1 12% separating gel (8 ml)

Sterile water	3.436	ml
40% Acrylamide and Bis-acrylamide solution	2.4	ml
1.5 M Tris-HCl pH 8.8	2	ml
10% SDS	0.08	ml
10% APS	0.08	ml
TEMED	0.004	ml

7.2 5% stacking gel (2 ml)

Sterile water	1.204	ml
40% Acrylamide and Bis-acrylamide solution	0.25	ml
1 M Tris-HCl pH 6.8	0.504	ml
10% SDS	0.02	ml
10% APS	0.02	ml
TEMED	0.002	ml

8. 5x running buffer for SDS-PAGE

Trisma base	15.1	g
Glycine	94	g
SDS	5	g
Deionized water adjust volume to	1000	ml

For working solution, 1x running buffer was prepared by diluting 100 ml of 5x running buffer to total volume 500 ml using deionized water.

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9. Transfer buffer for Western blotting

Trisma base	5.08	g
Glycine	2.9	g
SDS	0.37	g
Deionized water	800	ml
Absolute methanol	200	ml

The buffer was stored at 4 °C till use.

10. Staining and destaining solution

10.1 Staining solution

Coomassie brilliant blue R-250	2.5	g
Methanol	500	ml
Acetic acid	100	ml
Distilled water adjust volume to	1000	ml

The solution was stored in a dark bottle.

10.2 Destaining solution

10.2.1 Destain I (50% methanol, 10% acetic acid)

Methanol	500	ml
Acetic acid	100	ml
Distilled water adjusted volume to	1000	ml

10.2.2 Destain II (5% methanol, 7% acetic acid)

Methanol	50	ml
Acetic acid	70	ml
Distilled water adjusted volume to	1000	ml

Procedure of gel staining

After electrophoresis, the separating bands of proteins in the gel were visualized by immersion in staining solution (10.1) for 1-2 h. The gel was then washed in destain I (10.2.1) for 1 h with 1-2 changes and followed by destain II (10.2.2) until the gel was cleared.

11. Staining blot (Ponceau S solution)

Ponceau S	0.1	g
Acetic acid	5	ml
Deionized water adjusted volume to	1000	ml

The solution was stored in a dark bottle.

Procedure of membrane staining

After the proteins were transferred from the gel to the nitrocellulose membrane, the proteins on the membrane was visualized by immersion in Ponceau S solution for 5 min with continuous rocking. The membrane was then washed in deionized water until the membrane was cleared.

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APPENDIX E

Buffers and reagents for immunoassay

(Dot blotting, Western blotting and ELISA)

1. 0.15 M Phosphate buffer saline (PBS), pH 7.4

NaCl	8	g
KCl	0.2	g
KH_2PO_4	0.2	g
Na_2HPO_4	1.15	g
Distilled water adjusted volume to	1000	ml

Adjusted pH to 7.4

2. 0.05% Tween 20 in PBS (PBST)

Tween 20	500	µl
PBS	1000	ml

3. 5% skim milk

Skim milk	5	g
PBS	100	ml

4. 5% skim milk in PBST

Skim milk	5	g
PBST	100	ml

5. 1% $CoCl_2$

$CoCl_2 \cdot 6H_2O$	1.83	g
Distilled water adjusted volume to	100	ml

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6. 0.15 M Phosphate Citrate buffer, pH 5.0

Na_2HPO_4	9.5	g
Citric acid	7.3	g
Distilled water adjusted volume to	1000	ml

Adjusted pH to 5.0 and the buffer was stored at 4 ^{o}C in a dark bottle before use.

7. OPD substrate solution

o-Phenylenediamine (OPD)	40	mg
30% H_2O_2	0.04	ml
0.15 M Phosphate citrate buffer	100	ml

The substrate solution was freshly prepared in a dark bottle before use.

8. DAB substrate solution

3,3’ Diaminobensidine tetrahydrochloride (DAB)	15	mg
30% H_2O_2	0.05	ml
1% $CoCl_2$	0.125	ml
PBS (1)	50	ml

The substrate solution was freshly prepared in a dark bottle before use.

9. 1 M H_2SO_4 (stopping solution)

H_2SO_4 (96%)	55.53	ml
Distilled water adjusted volume to	1000	ml

10. 1% Thimerosal

Thimerosal	1	g
Distilled water adjusted volume to	100	ml

The solution was stored in a dark bottle and used in concentration of 0.01% as a preservative in immunoglobulins to prevent bacterial contamination.

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APPENDIX F

Determination of protein concentration by BCA^{TM} Protein Assay Kit

The BCA^{TM} Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitative determination of total protein using bovine serum albumin (BSA) as a protein standard.

1. Procedure

1.1 Pipette 25 µl of each standard or sample replicate into each well of 96-well plate.

1.2 Add 200 µl of the working reagent (BCA^{TM} reagent A: reagent B at the ratio of 50: 1) to each well and mix plate thoroughly on the plate shaker for 30 sec.

1.3 Cover the plate and incubate at 37 ^{o}C for 30 min.

1.4 Cool the plate to RT.

1.5 Measure the absorbance at 562 nm using microplate reader.

2. Preparation of a standard curve of BSA

BSA stock solution (1 mg ml^{-1}) was diluted in PBS to get the final BSA concentration of 0, 50, 100, 200, 400, 600, 800, 1000 µg ml^{-1} (Table F1). Each of concentrations was used as standard for determination of the protein concentration of samples. The protein standard curve was prepared by plotting the 562 nm absorbance value for each BSA standard versus its concentration in µg ml^{-1} (Figure F1).

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Table F1 Preparation of BSA standards and the 562 nm absorbance values

BSA stock 1 mg ml^{-1} (µl)	PBS (µl)	BSA concentration (µg ml^{-1})	A_{562}
0	200	0	0.000
10	190	50	0.107
20	180	100	0.167
40	160	200	0.337
80	120	400	0.576
120	80	600	0.983
160	40	800	1.197
200	0	1000	1.471

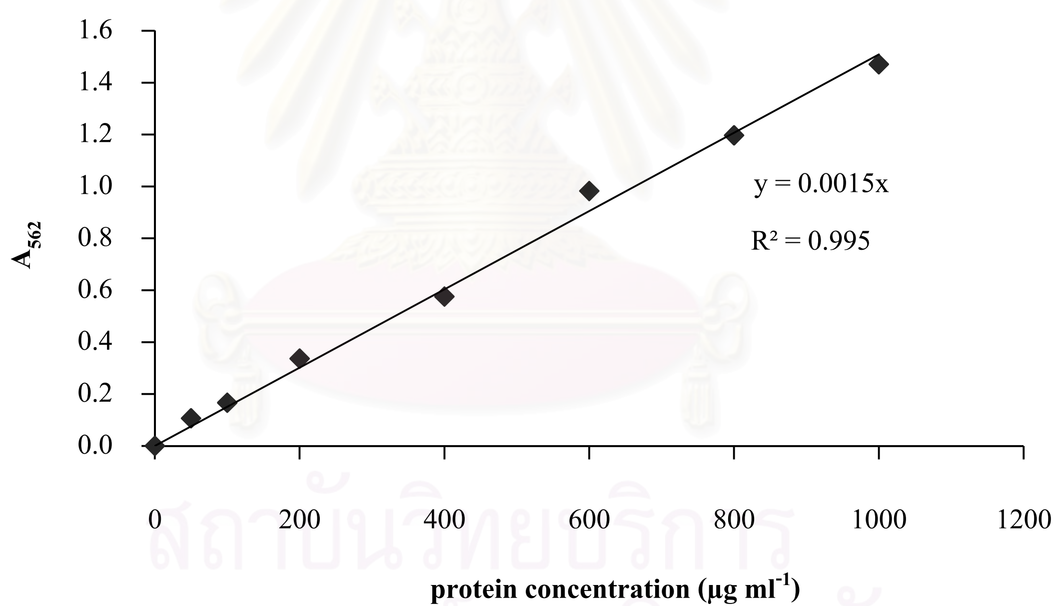

Figure F1 BSA standard curve by BCA™ Protein Assay Kit

3. Determination of protein concentration in a heat-killed bacteria

The protein concentration of the samples was determined using the slope of the BSA standard curve (Table F2).

Table F2 The 562 nm absorbance values and protein concentration of the samples

Sample	A_{562}	Protein concentration (µg ml^{-1})
Y. enterocolitica ATCC 27729	0.455	303.00
Y. enterocolitica MU	0.441	294.00
Y. frederiksenii DMST 19212	0.496	330.67
E. coli ATCC 25922	0.549	366.00

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APPENDIX G

Molecular weight determination

The molecular weight of an unknown protein could be determined directly from a semilog graph of the molecular weight of protein marker versus their mobility (Table G1 and Figure G1). The relative mobility (Rf) of the protein was calculated according to the following formula:

$$\text{Relative mobility } (Rf) = \frac{\text{Distance of the protein migrate}}{\text{Distance of the tracking dye migrate}}$$

Table G1 The molecular weight of protein marker and their relative mobility

Protein marker	Molecular weight (kDa)	Relative mobility (Rf)
β-galactosidase	117	0.10
Bovine serum albumin	85	0.15
Ovalbumin	49	0.27
Carbonic anhydrase	34	0.42
β-lactoglobulin	25	0.56
lysozyme	19	0.73

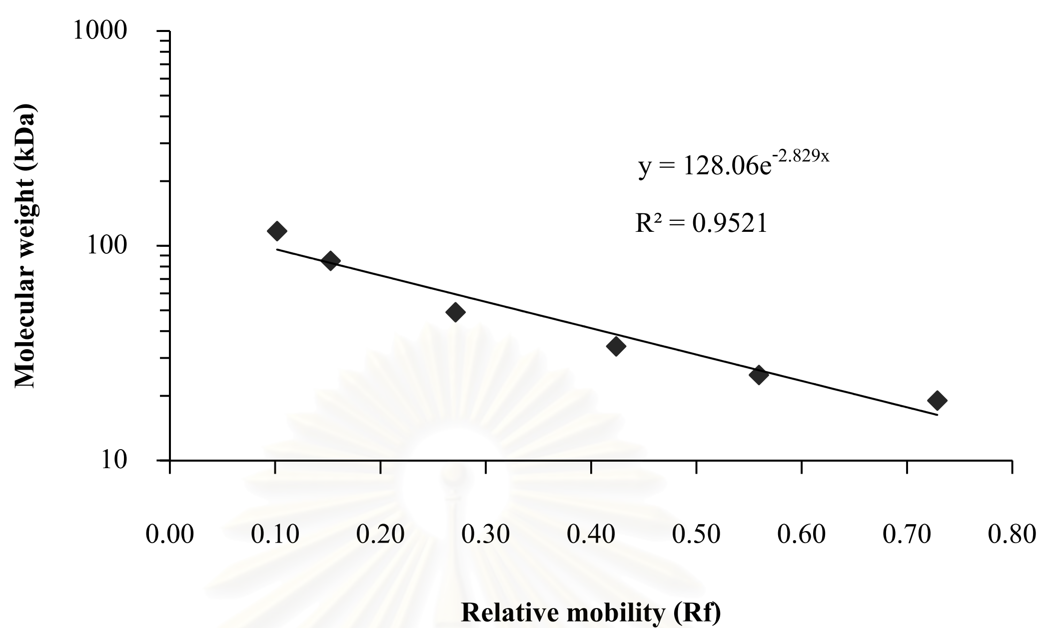

Figure G1 Standard curve of protein marker separated by SDS-PAGE

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APPENDIX H

Confirmation of negative control

1. Refer to Figure 4.3 (4.2 Immunization of mice in page 52), more figure (Figure H1) of negative control was added for confirmation.

Serum from nonimmunized mouse used as negative control in this experiment was tested for specificity by Western blotting. The result showed that immunoreactive band against separating antigens was not detected (Figure H1). This negative result confirmed that there was no antibody against *Y. enterocolitica* and other bacterial tested in mouse before immunization.

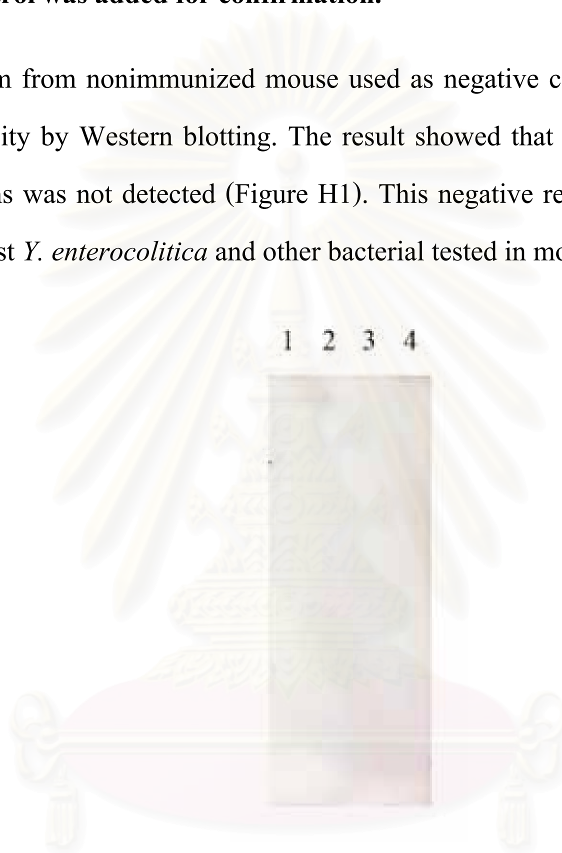

Figure H1 Western blot analysis for specificity of nonimmunized mouse antiserum. The serum at dilution 1:20,000 was tested against whole cell lysates of *Y. enterocolitica* ATCC 27729 (lane 1), *Y. enterocolitica* MU (lane 2), *Y. frederiksenii* DMST 19212 (lane 3), and *E. coli* ATCC 25922 (lane 4).

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2. Refer to Figure 4.4 (4.4 Characterization of monoclonal antibodies, a subtitle of 4.4.1 Specificity test in page 55), more figure (Figure H2) of negative control was added for confirmation.

Serum from nonimmunized mouse was tested for specificity against bacterial strains listed in Table 3.1 by dot blotting. The result did not show any immunoreactivity to bacterial strains by dot blotting except *S. aureus*.

Figure H2 Specificity of nonimmunized mouse antiserum assayed by dot blotting. Heat-killed bacteria (~10^9 CFU ml^{-1}) were spotted onto nitrocellulose membrane 1 µl $spot^{-1}$ and treated with the serum at dilution 1:20,000. Each bacterium was spotted onto each block of the membrane as follows:

Row 1: (A) *Y. enterocolitica* ATCC 27729; (B) *Y. enterocolitica* MU; (C) *Y. enterocolitica* CWC-01-0079; (D) *Y. enterocolitica* CWC-01-0045; (E) *Y. enterocolitica* CWC-01-0275; (F) *Y. enterocolitica* CW-01-0199

Row 2: (A) *Y. enterocolitica* SM-4; (B) *Y. pseudotuberculosis* MU; (C) *Y. frederiksenii* DMST 19212; (D) *Y. frederiksenii* CWC-01-0149; (E) *Y. frederiksenii* CWC-01-0093; (F) *Y. kristensenii* DMST 19209

Row 3: (A) *Y. ruckeri* B04023; (B) *Escherichia coli*; (C) *Enterobacter cloacae*; (D) *Klebsiella pneuminoae*; (E) *Citrobacter freundii*; (F) *Serratia marcescens*

Row 4: (A) *Proteus mirabilis*; (B) *Providencia rettgeri*; (C) *Morganella morganii*; (D) *Edwardsiella tarda*; (E) *Salmonella* Typhimurium; (F) *Salmonella* Typhi

Row 5: (A) *Shigella flexneri*; (B) *Vibrio cholera*; (C) *Vibrio parahaemolyticus*; (D) *Vibrio vulnificus*; (E) *Vibrio mimicus*; (F) *Aeromonas hydrophila*

Row 6: (A) *Pseudomonas aeruginosa*; (B) *Acinetobacter baumanii*; (C) *Listeria monocytogenes*; (D) *Bacillus cereus*; (E) *Enterococcus faecalis*; (F) *Staphylococcus aureus*

BIOGRAPHY

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Academic presentation:

Khamjing, W., Khongchareonporn, N. and Rengpipat, S. 2008. Production of monoclonal antibodies against *Yersinia enterocolitica*. Proceedings of the 9th National Grad Research Conference, p.120. Graduate School, Burapha University, Chonburi, Thailand. March 14-15, 2008. (Oral presentation) (Full text in CD-ROM)

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