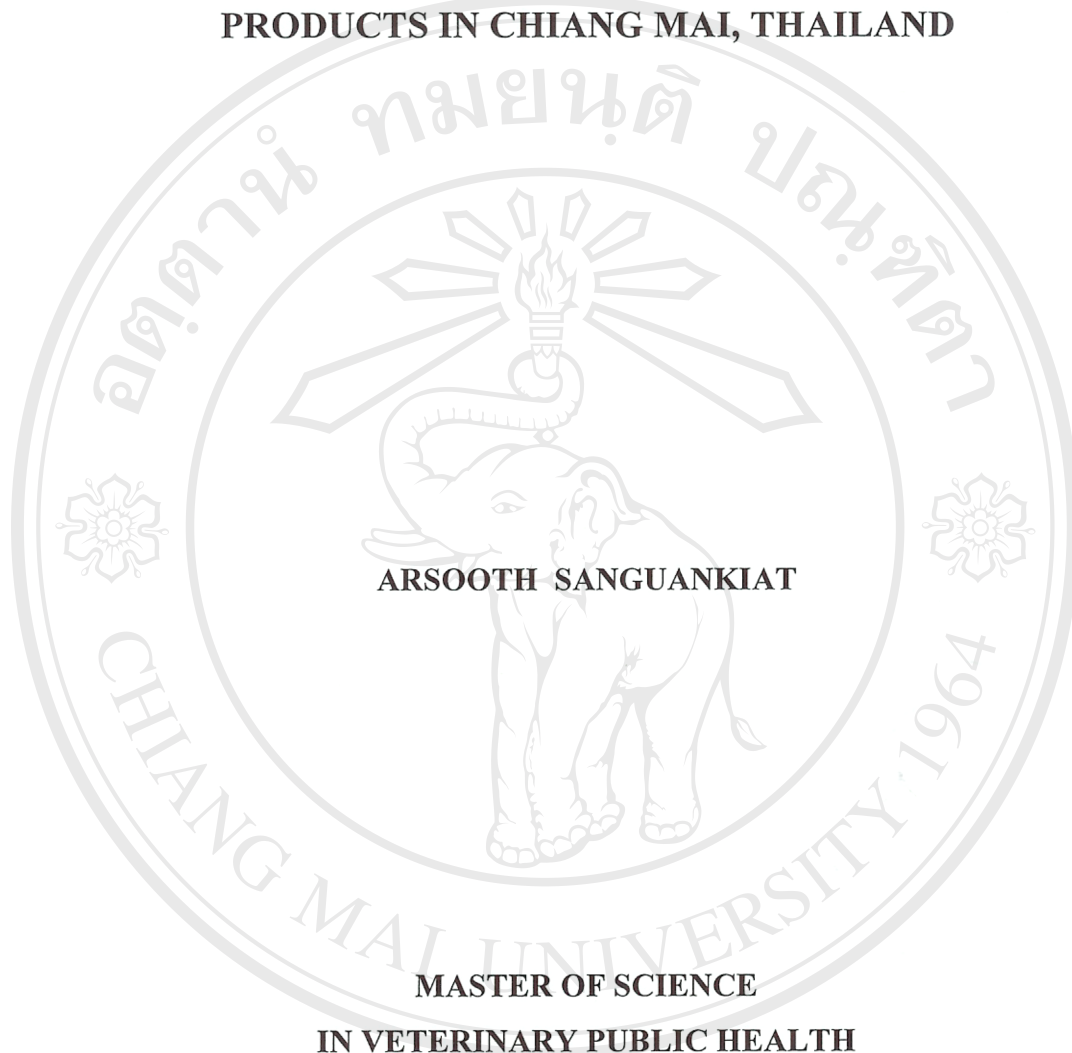
A CROSS-SECTIONAL STUDY OF SALMONELLA IN PORK PRODUCTS IN CHIANG MAI, THAILAND



ARSOOTH SANGUANKIAT

MASTER OF SCIENCE
IN VETERINARY PUBLIC HEALTH

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CHIANG MAI UNIVERSITY AND FREIE UNIVERSITÄT BERLIN

SEPTEMBER 2005

ISBN 974-9887-76-X

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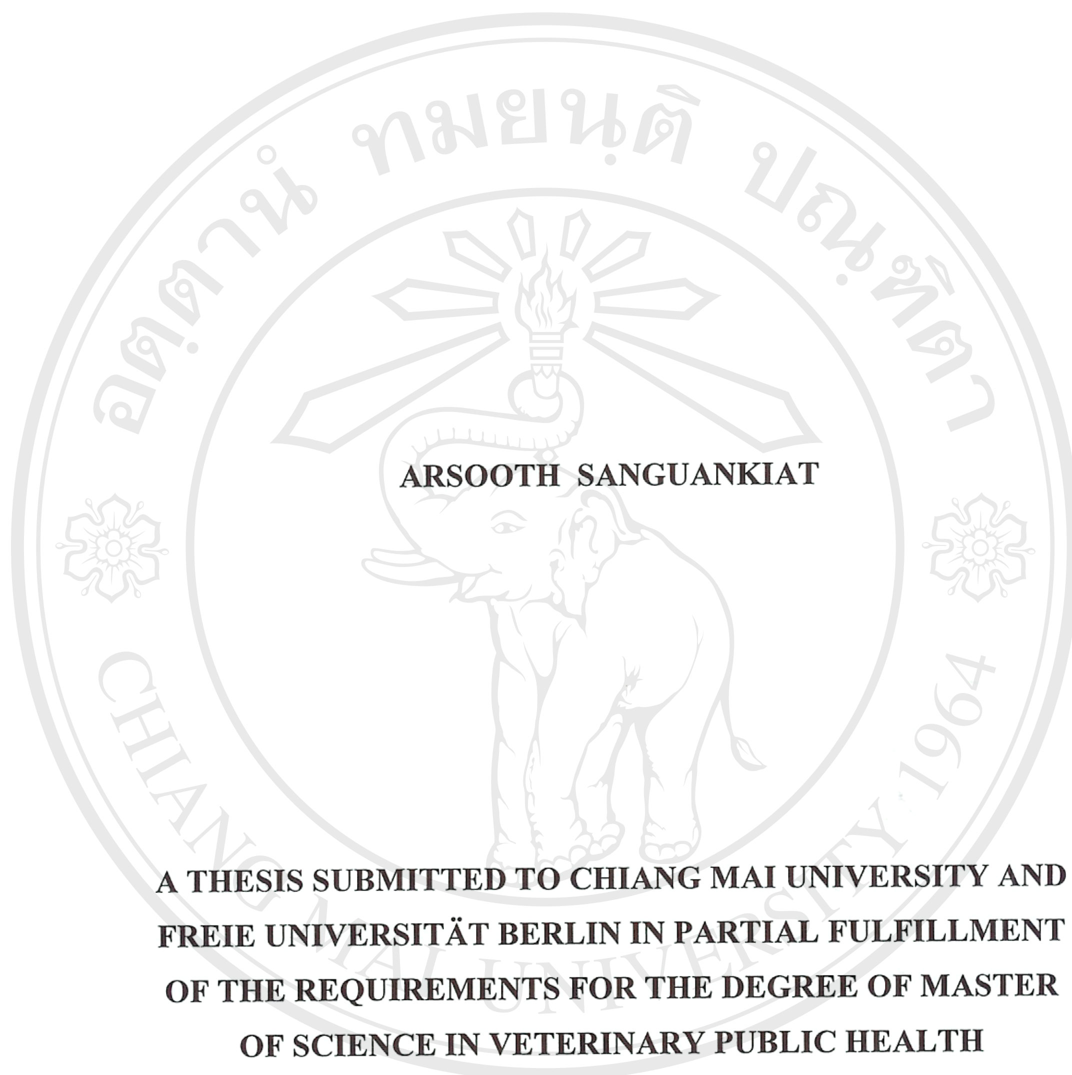

ARSOOTH SANGUANKIAT

A THESIS SUBMITTED TO CHIANG MAI UNIVERSITY AND FREIE UNIVERSITÄT BERLIN IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN VETERINARY PUBLIC HEALTH

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EXAMINING COMMITTEE

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Thesis title A Cross-Sectional Study of Salmonella in Pork Products in Chiang Mai, Thailand

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ABSTRACT

The occurrence of Salmonella in foods of animal origin in Chiang-Mai province was studied using the pork production chain as a model. This cross-sectional study investigated several phases of the pork production chain (cut, transported, and retail) and the environment in a slaughterhouse in a cutting unit. A total number of samples of 846 samples: 173 samples of cut pork, 173 samples of transported pork, 200 samples of retail pork (10 bones, 29 bellies, 9 ribs, 23 collars, 33 loins, 33 packs of ground pork, 13 shoulder meats, 21hams, and 29 fillets) and 300 samples from slaughterhouse environment were investigated for Salmonella. Salmonella was detected in cut, transported, and retail pork products with the following percentages: 54.63 % cut, 70.16 % transported and 34.50 % retail products. It was also found that the cut pork samples were significantly different from the transported pork samples (p=0.0346) and retail pork products (p=0.0034). The prevalence ratio (PR) of cut pork and transported pork in this study was 1.327 (95%CI: 0.971-1.814), indicating the transport process as a risk factor. In retail products, bone products had the highest 70% (7/10) and the collar the lowest positive samples, 17.4% (4/23) of Salmonella. Environmentally, the highest percentage of salmonellae positivity, found during the duration of cutting 25% (95%CI: 16.8-34.6%). The most frequent serogroup in pork and environmental samples was serogroup C. The five most prevalent serotypes isolated from pork and environment of the slaughterhouse were S. Rissen (45.3 %), S. Typhimurium (16.3%), S. Krefeld (10.6), S. Stanley (6.3%) and S. Lagos (6.0%). In ten occasions, salmonellae were isolated more than one serotype (50%) in environment and most of serotypes that found in environmental samples were isolated from pork. These results suggest that the quality of carcasses coming to cutting affected slaughterhouse environment and affect on the quality of pork in terms of bacterial contamination. Application of HACCP (Hazard Analysis Critical Control Point), GMP (Good Manufacturing Practice) system and strong staff educational programs would greatly improve the hygienic standards in pork product processing.

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ชื่อเรื่องวิทยานิพนธ์	การศึกษาแบบตัดขวางของเชื้อซัลโมเนลลาใน ผลิตภัณฑ์เนื้อสุกรในจังหวัดเชียงใหม่ ประเทศไทย
ผู้เขียน	นายอาสูตร สงวนเกียรติ
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คณะกรรมการที่ปรึกษาวิทยานิพนธ์	ศ.ดร. Reinhard Fries ประธานกรรมการ(FU-Berlin) รศ.ดร. เรณู ปิ่นทอง ประธานกรรมการ(CMU)

บทคัดย่อ

การศึกษาการปนเปื้อน Salmonella ในอาหารประเภทเนื้อ ในจังหวัดเชียงใหม่ โดยใช้ห่วงโซ่การผลิตเนื้อหมูเป็นแบบจำลอง ศึกษาส่วนหน้าคัด(Cross sectional study) ของกระบวนการผลิต มีจุดประสงค์เพื่อศึกษาแต่ละขั้นตอนในห่วงโซ่กระบวนการผลิต ตั้งแต่ขั้นตอนการตัดแต่ง ขนส่ง จนถึงตลาดขายปลีก รวมทั้งศึกษาสภาวะแวดล้อมภายในห้องตัดแต่งของโรงฆ่าสัตว์ ได้ทำการศึกษาทั้งหมด 846 ตัวอย่าง ศึกษาตัวอย่างจากห้องตัดแต่งซาก 173 ตัวอย่าง ตัวอย่างที่ได้ผ่านกระบวนการขนส่งตามปกติแล้ว 173 ตัวอย่าง อีก 200 ตัวอย่าง เป็นตัวอย่างจากตลาดขายปลีก ซึ่งประกอบด้วยส่วนกระดูก 10 ตัวอย่าง ส่วนท้อง 29 ตัวอย่าง ซี่โครง 9 ตัวอย่าง คอ 23 ตัวอย่าง สันนอก 33 ตัวอย่าง และหมูบด 33 ตัวอย่าง เนื้อส่วนไหล่ 13 ตัวอย่าง เนื้อส่วนตะโพก 21 ตัวอย่าง และส่วนสันใน 29 ตัวอย่าง และเป็นตัวอย่างจากสิ่งแวดล้อมภายในห้องตัดแต่งซากอีก 300 ตัวอย่าง ปรากฏว่าพบ Salmonella ในเนื้อหมูจากห้องตัดแต่ง (cut pork) เนื้อหมูผ่านการขนส่ง และเนื้อหมูจากตลาดขายปลีก เป็นจำนวน 54.63 ,70.16 และ 34.50 ตามลำดับ ปริมาณ Salmonella จากตัวอย่างหมูจากห้องตัดแต่งแตกต่างกับตัวอย่างที่ได้ผ่านการขนส่ง(p= 0.0346) และแตกต่างกับผลิตภัณฑ์ ในร้านขายปลีกอย่างมีนัยสำคัญยิ่งทางสถิต(p= 0.0034) อัตราส่วนการปนเปื้อน (prevalence ratio) ของตัวอย่างหมูจากห้องตัดแต่งกับตัวอย่างที่ได้ผ่านการขนส่งเท่ากับ 1.327 (95 %CI:0.971-1.814) แสดงว่าการขนส่งเป็นปัจจัยเสี่ยงต่อการปนเปื้อน การศึกษาในผลิตภัณฑ์ขายปลีกนี้พบว่า ผลิตภัณฑ์ส่วนกระดูก ปนเปื้อนมากที่สุด 70 % (7/10) และพบผลิตภัณฑ์ส่วนคอมีการปนเปื้อนน้อยที่สุด 17.40 % (4/23) พบการปนเปื้อน Salmonella มากที่สุดในสภาวะแวดล้อมของห้องตัดแต่ง 25 % (95 %CI:16.8-34.6%) ซึ่งเป็นกลุ่มซีโรวาซี (serogroup C) มากที่สุด พบชนิดซีโรวา(serotypes)ที่แยกได้จากตัวอย่างเนื้อหมูและสิ่งแวดล้อมในโรงฆ่าสัตว์มากที่สุด 5 ชนิด คือ *S.* Rissen (45.3%), *S.* Typhimurium (16.3%), *S.* Krefeld (10.6%) , *S.* Stanley(6.0%) และ *S.* Lagos(6.0%),พบชนิดซีโรวา(serotypes)ที่แยกได้จากตัวอย่างสิ่งแวดล้อมในโรงฆ่าสัตว์เป็นชนิดเดียวกับที่พบในเนื้อหมู จากผลการศึกษานี้แสดงว่าคุณภาพของซากสัตว์ที่นำเข้าไปตัดแต่งในห้องตัดแต่งเป็นผลต่อสิ่งแวดล้อมในโรงฆ่าสัตว์ และเป็นผลต่อคุณภาพการปนเปื้อนของเนื้อหมู การใช้ระบบเอชเอซีซีพี , จีเอ็มพี และการให้การศึกษาฝึกอบรมแก่พนักงานจะช่วยปรับปรุงมาตรฐานสุขอนามัยในกระบวนการผลิตผลิตภัณฑ์เนื้อหมูเป็นอย่างมาก

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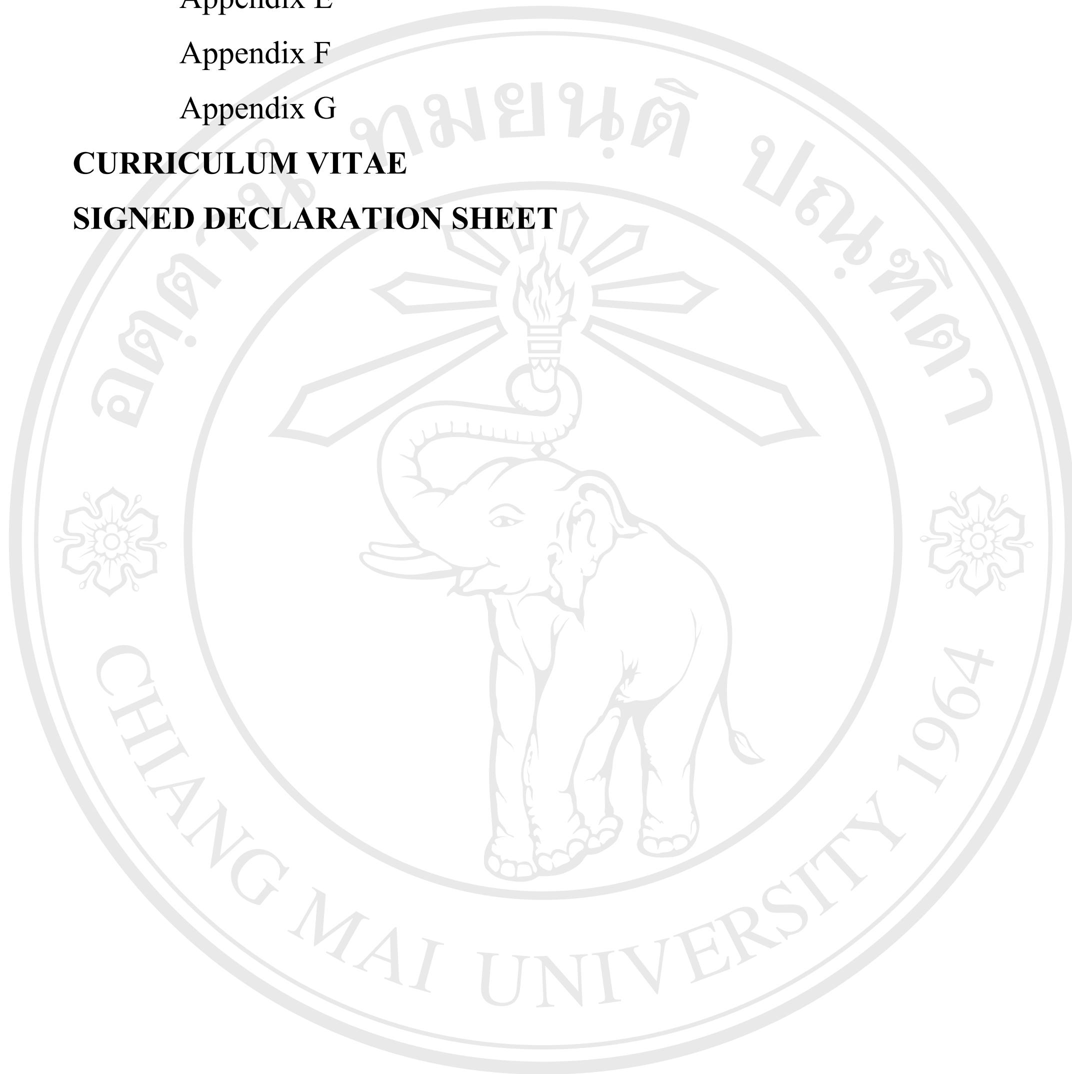

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ABBREVIATIONS AND SYMBOLS

BPLS	=	Brilliant green phenol red lactose sucrose
BPW	=	Buffer peptone water
CDC	=	Center of Disease Control
CI	=	Confidence Interval
CMU	=	Chiang Mai University
Countd.	=	Continued
df	=	Degree of freedom
DNA	=	Deoxynucleic Acid
e.g.	=	Example
etc.	=	Et cetera
EU	=	European Union
FU	=	Freie Universität Berlin
g	=	grams
GMP	=	Good Manufacturing Practice
h	=	hours
HACCP	=	Hazard Analysis Critical Control Point
H_2S	=	Hydrogen sulfide
ISO	=	International Organization for Standardization
LPS	=	Lipopolysaccharide
min	=	minute
MKTTn	=	Muller-Kauffmann tetrathionate novobiocin
ml	=	milliliters
mm	=	millimeters
No.	=	Number
NSSC	=	National *Salmonella* and *Shigella* Center
^{o}C	=	degree Celsius
p	=	Probability value
PR	=	Prevalence ratio

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RVS	=	Pappaport-Vassiliadis medium with soya
SD	=	Standard deviation
SE	=	Standard error
SOP	=	Standard Operation Prescriptions
TSI	=	Triple sugar iron
VP	=	Voges-Proskauer
WHO	=	World Health Organization
XLD	=	Xylose lysine deoxycholate
β	=	Beta
μm	=	Micromillimeters
χ^2	=	Chi-square

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1. INTRODUCTION

The genus *Salmonella* is one of the widely studied foodborne pathogens. It commonly found in a variety of food products especially in food of animal origin such as poultry, eggs, pork, beef and also dairy products (Escartin *et al*., 1995; Yan *et al*., 2003; Bangtrakulnonth *et al*., 2004). Pork is one of the most important sources of food-borne salmonellosis in humans (Van der Gaag *et al*., 2004).

Members of the genus *Salmonella* are Gram-negative bacilli belonging to the family Enterobacteriaceae. The latter the most is a most heterogeneous collection of medically important bacteria (Murray *et al*., 2003; Yan *et al*., 2003). Salmonellae are ubiquitous organisms found in soil, water, and vegetation worldwide, and are frequently part of the intestinal flora of most animals, including humans. Salmonellae are nonspore forming, mostly motile and facultative anaerobes (Yan *et al*., 2003). They can reduce nitrates to nitrites, ferment glucose, and oxidase negative. Generally, salmonellae are categorized into typhoidal and non-typhoidal group (Holt *et al*., 2003; Hane, 2003). *Salmonella* Typhi and *Salmonella* Paratyphi are members of the typhoidal group. They can cause typhoid disease and paratyphoid disease only in humans. These infections are generally transmitted from one person to another without animal involvement (Murray *et al*., 2003; Yan *et al*., 2003).

The genus *Salmonella*, named in 1885 after the veterinary pathologist, Danial E. Salmon, causes diseases worldwide (Escartin *et al*., 1995; Ayofo *et al*., 2002; Hald *et al*., 2003; Bangtrakulnonth *et al*., 2004). At present, there have been more than 2,500 serovars of Genus *Salmonella* identified (Popoff, 2001; Bangtrakulnonth *et al*., 2004). However, only a limited number of serovars are of public health importance (Bangtrakulnonth *et al*., 2004). Members of the species *Salmonella enterica* are the main causative agents of human gastroenteritis (Mead *et al*., 1999).

Although slaughterhouses and retail shops depend greatly on the quality of the raw materials and products received, they do also bear responsibility for the quality of the end products and for the prevention of contamination of the edible products (Lo

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Fo Wong *et al*., 2002). Some of the risk factors is that play great roles in the on carcass cross-contamination are handling, time and temperature (Lo Fo Wong *et al*., 2002; Alban *et al*., 2005).

Large quantities of raw meat from different origins are handled closely at the slaughterhouses and retail markets where cross-contaminations possibly occur. There may be contaminated carcasses and cuts from both single and different types of animals at the retail markets. Retail is probably the weakest point in the commercial cold chain for contamination to occur (James and Bailey, 1990). The appropriate time and temperature for *Salmonella* either a container or a room stimulate the multiplication of the bacteria that expose consumers to high risk of acquiring infection.

This study was concerned with *Salmonella* in pork products in Chiang-Mai province. This model consists of three research studies. The first one was collect samples of feces and blood of pigs at the farm. Samples from bellies, jowl, back and ham of carcass from the same pig were collected subsequently by the second investigator.

The third investigator, this study, was to determine the prevalence and the most common serovars of *Salmonella* that found in raw pork before packaging in slaughterhouse and after transportation and also meat at supermarkets. Total positive samples used for the prevalence of *Salmonella* determination were in supermarkets. Then, such results were used to detect the weak points of the commercial pork chain and to define the origin of *Salmonella* contamination in Chiang Mai province, Thailand.

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Objectives of this current study were; (1) to estimate *Salmonella* spp. contamination in pork (cut, transported, and retail); (2) to determine *Salmonella* spp. in environment in the slaughterhouse; (3) to assess the common serotype of *Salmonella* spp. in cut pork, transported pork, and retail pork products in the province

2. LITERATURE REVIEW

2.1 Genus *Salmonella*

Salmonellae, belonging to the family Enterobacteriaceae, are gram negative bacteria rods which ferment glucose and other sugars and are oxidase-negative. They are catalase-positive, non-spore forming facultative anaerobes. They can grow well on MacConkey agar and also can reduce nitrates to nitrites. These bacteria are facultative anaerobes that do not utilize lactose but usually produce H_2S. The genus *Salmonella* contains more than 2500 serotypes and consists of two species: (1) *Salmonella enterica* which is divided into six subspecies (S. *enterica* subsp. enterica; I, *S. enterica* subsp. salamae; II, *S. enterica* subsp. arizonae; IIIa, *S. enterica* subsp. diarizonae; IIIb, *S. enterica* subsp. houtenae; IV, and *S. enterica* subsp. indica; VI) and (2) *Salmonella bongori* with was formerly subspecies V (Popoff, 2001; Yan *et al*., 2003).

The antigenic formulae of *Salmonella* serotypes are defined and maintained by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France (WHO Collaborating Centre). The new serotypes are listed in annual updates of the Kauffmann-White scheme (Brenner *et al*., 2000).

2.2 Nomenclature for *Salmonella*

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Since 1885, when the veterinary scientist, Danial E. Salmon discovered the first *Salmonella* strain (Yan *et al*., 2003), scientists have used different systems for *Salmonella* nomenclature. However, uniformity in *Salmonella* nomenclature is necessary for communication between scientists, health officials and the public. Common usage often combines several nomenclatural systems that inconsistently divide the genus into species, subspecies, subgenera, groups, subgroups, and serotypes (serovars), which causes confusion (Brenner *et al*.,2000).

The nomenclature for the genus *Salmonella* has evolved from the initial one serotype-one species concept proposed by Kauffmann. Serotyping is based on the identification of somatic (O) and flagellar (H) antigens using specific anti-sera. Each serotype is considered a separate species (for example, *S. paratyphi A, S. galinarum, S. enteritidis*). If this concept were used, it would result in 2501 species of *Salmonella* (Popoff, 2001) (Table 1). Other taxonomic proposals have been based on the clinical role of a strain, on the biochemical characteristics that divide the serotypes into subgenera, and ultimately, on genomic relatedness (Brenner *et al*., 2000).

The central development in *Salmonella* taxonomy occurred in 1973 when Crosa *et al.* (1973) demonstrated, using DNA-DNA hybridization, that all serotypes and sub-genera I, II, and IV of *Salmonella* and all serotypes of "Arizona" were related at the species level. Thus, they belonged to a single species. The single exception, subsequently described later, is *S. bongori*, previously know as subspecies V. By DNA-DNA hybridization however, it is a distinct species. Since *S. choleraesuis*, causative agent of swine salmonellosis, appeared on the Approved List of Bacterial Names as the type species of *Salmonella*, it had priority as the species name. The name "choleraesuis", however, refers to both a species and a serotype, which causes confusion (Brenner *et al*., 2000). In addition, the serotype Choleraesuis is not reproductive of the majority of serotypes because it is biochemically distinct, being arabinose and trehalose negative.

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Table 1: Actual number of *Salmonella* species, subspecies, serotypes and their usual habitats

***Salmonella* species and subspecies**	**Usual habitat**	**Number of serotypes within subspecies**	
		1998	**2001**
Salmonella enterica subsp. enterica (I)	Warm-blooded animals	1454	1478
Salmonella enterica subsp. salamae (II)	Cold-blooded animals and the environment	489	498
Salmonella enterica subsp. arizonae (IIIa)	Cold-blooded animals and the environment	94	94
Salmonella enterica subsp. diarizonae (IIIb)	Cold-blooded animals and the environment	324	327
Salmonella enterica subsp. houtenae (IV)	Cold-blooded animals and the environment	70	71
Salmonella enterica subsp. indica (VI)	Cold-blooded animals and the environment	12	12
Salmonella bongori (V)	Cold-blooded animals and the environment	20	21
Total		**2463**	**2501**

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In 1986, the Subcommittee of Enterobacteriaceae of the International Committee on Systematic Bacteriology at the XIV International Congress of Microbiology unanimously recommended that the name of the species for *Salmonella* be changed to *S. enterica*, a name coined by Kauffmann and Edwards in 1952, because no serotype shares this name. In 1987, Le Minor and Popoff of the WHO Collaborating Centre formally made a proposal as a "Request for an Opinion" to the Judicial Commission of the International Committee of Systematic Bacteriology. In the same year, they also proposed that the seven subgenera of *Salmonella* be referred to as subspecies

(subspecies I, II, IIIa, IIIb, IV, V, and VI). Subgenus III was divided into IIIa and IIIb by genomic relatedness and biochemical reactions Subspecies IIIa (*S. enterica* subsp. *Arizonae*) includes the monophasic "*Arizona*" serotypes and subspecies IIIb (*S. enterica* subsp. *diarizonae*) contains the diphasic serotypes. All "*Arizona*" serotypes had been incorporated into the Kauffmann-White scheme by Rohde in 1979 (Brenner *et al*., 2000). The recommendation was adopted by CDC, by Ewing in 1986 in the 4th edition of Edward's and Ewing's Identification of Enterobactericeae, and by others.

Nonetheless, the Judicial Commission denied the request, to change the type species for *Salmonella*. Although the Judicial Commission was generally in favor of *S. enterica* as the type species of *Salmonella*, its members believed that the status of *Salmonella* Typhi, the causative agent of typhoid fever, was not adequately addressed in this request for an opinion. They were concerned that if *S. enterica* were adopted as the species, *Salmonella* serotype Typhi would be referred to as *Salmonella enterica* subsp. *enterica* serotype Typhi and might be missed or overlooked by physicians in the same way that *S. choleraesuis* subsp. *choleraesuis* serotype Typhi might be overlooked. From this perspective, nothing would be gained by changing the type species name. The Judicial Commission, therefore, ruled that *S. choleraesuis* be retained as the legitimate type species pending an amended request for an opinion. To comply with this ruling, in 1999 Euzéby made an amended request, which was, to adopt *S. enterica* as the type species of *Salmonella* while retaining the species "*S. typhi*" as an exception.

At present, the nomenclatural system is based on recommendations from the WHO Collaborating Centre (Brenner *et al*., 2000). The number of *Salmonella* species and *Salmonella* nomenclature currently seen in the literature is summarized in Tables 1 and 2, respectively.

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Table 2: Examples of *Salmonella* nomenclature currently seen in literature (Brenner *et al*., 2000)

Complete name	CDC designation	Other designations
S. enterica subsp. *enterica* ser. Typhi	*Salmonella* ser. Typhi	*Salmonella typhi*
S. enterica subsp. *enterica* ser. Typhimurium	*S.* ser. Typhimurium	*Salmonella typhimurium*
S. enterica subsp. *salamae* ser. Greenside	*S.* ser. Greenside	*S.* II 50:z:e,n,x, *S. greenside*
S. enterica subsp. *arizonae* ser. 18:z4,z23:2	*S.* IIIa 18:z4,z23:2	"*Arizona hinshawii*" ser. 7a,7b:1,2,5:2
S. enterica subsp. *diarizonae* ser. 60:k:z	*S.* IIIb 60:k:z	"*A. hinshawii*" ser. 24:29:31
S. enterica subsp. *houtenae* ser. Marina	*S.* ser. Marina	*S.* IV 48:g,z51:2, *S. marina*
S. bongori ser. Brookfield	*S.* ser. Brookfield	*S.* V 66:z41:2, *S. brookfield*
S. enterica subsp. *indica* ser. Srinagar	*S.* ser. Srinagar	*S.* VI 11:b:e,n,x, *S. srinagar*

2.3 Structure of a *Salmonella* bacterium

Morphologically, a *Salmonella* bacterium is a straight rod of 0.7-1.5 µm in width and 2-5 µm in length. There are three common compartments of salmonellae. The first compartment is cytosol, in which the processes of genetic replication and protein expression occur (McClane *et al*., 1999; Murray *et al*., 2002). The second compartment is a cell envelope, containing a cell wall and cytoplasmic membranes critical to the structure and function of the pathogen. The other compartments are surface structures that lie external to the cell envelope (e.g., capsules; O or K antigen, flagella; H antigen) (Figure 1).

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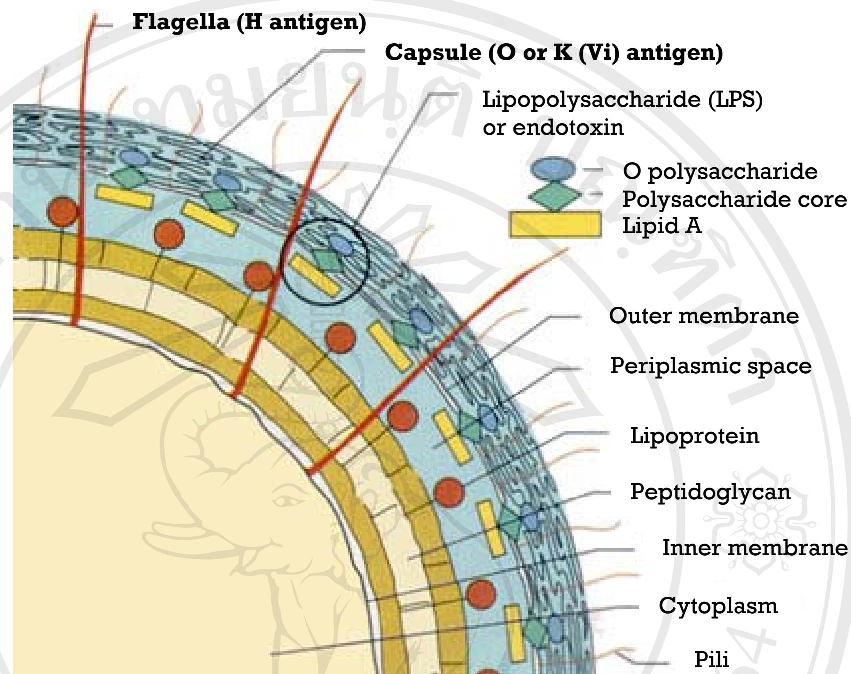

Source: Murray et al. (2002)

Figure 1: Antigenic structure of *Salmonella* and bacteria in family Enterobactericeae

2.4 *Salmonella* serotype antigens and designation

O antigen is a carbohydrate (also called a polysaccharide) that is the out-most component of lipopolysaccharide (LPS). It is a polymer of O subunits; each O subunit is composed of four to six sugars depending on the O antigen. Variations in O antigens result from the sugar components of the O subunit, the nature of the covalent bonds between the sugars of the subunit, and in the nature of the linkage between O subunits that form the O antigen polymer (Anonymous, 2004b).

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O antigens are designated by numbers and are divided into O serogroups, also called O groups. O groups are designated by the primary O factor(s) that are associated with the group. Many of the common O groups were originally designated by letters and are still commonly referred to by letters (e.g., *S.* Typhimurium belongs

to Group O:4 within Group B, *S*. Enteritidis belongs to group O:9 within Group D1; *S*. Paratyphi A belongs to Group O:2 within Group A).

Additional O factors are associated with some O groups and are often variably present or variably expressed. The *Salmonella* O groups and the additional O antigens may be present in serotypes of that group. When multiple O factors are present, they are listed sequentially and separated by commas.

H antigen is the filamentous portion of the bacterial flagella. It is made up of protein subunits called flagellin (Anonymous, 2004b). The ends of flagellin are conserved and give the filament its characteristic structure. The antigenically variable portion of flagellin is the middle region of the protein, which is surface-exposed. *Salmonella* is unique among the enteric bacteria in that it can express two different H antigens, which are encoded by two different genes. Expression of the two genes is coordinated so that only one flagellar antigen is expressed at a time in a single bacterial cell (MaClane *et al*., 1999). The two distinct flagellar antigens are referred to as Phase 1 and Phase 2. "**Monophasic**" isolates are those that express only a single flagellin type. These occur naturally in some serotypes (e.g., ***S.*** Enteritidis, *S*. Typhi, most subspecies IIIa and IV serotypes), or can occur through the inactivation or loss of the gene encoding the Phase1 or Phase 2 antigen. On the contrary, subspecies IIIb (*S. enterica* subsp. *diarizonae*) contains the **diphasic serotypes**. *Salmonella* Phase variation diagrams are shown in Figure 2.

Some antigens are composed of multiple factors, which are separated by commas; for example, the second phase antigen of *S.* Typhimurium is composed of factors 1 and 2. The H antigens composed of multiple factors are grouped into complexes.

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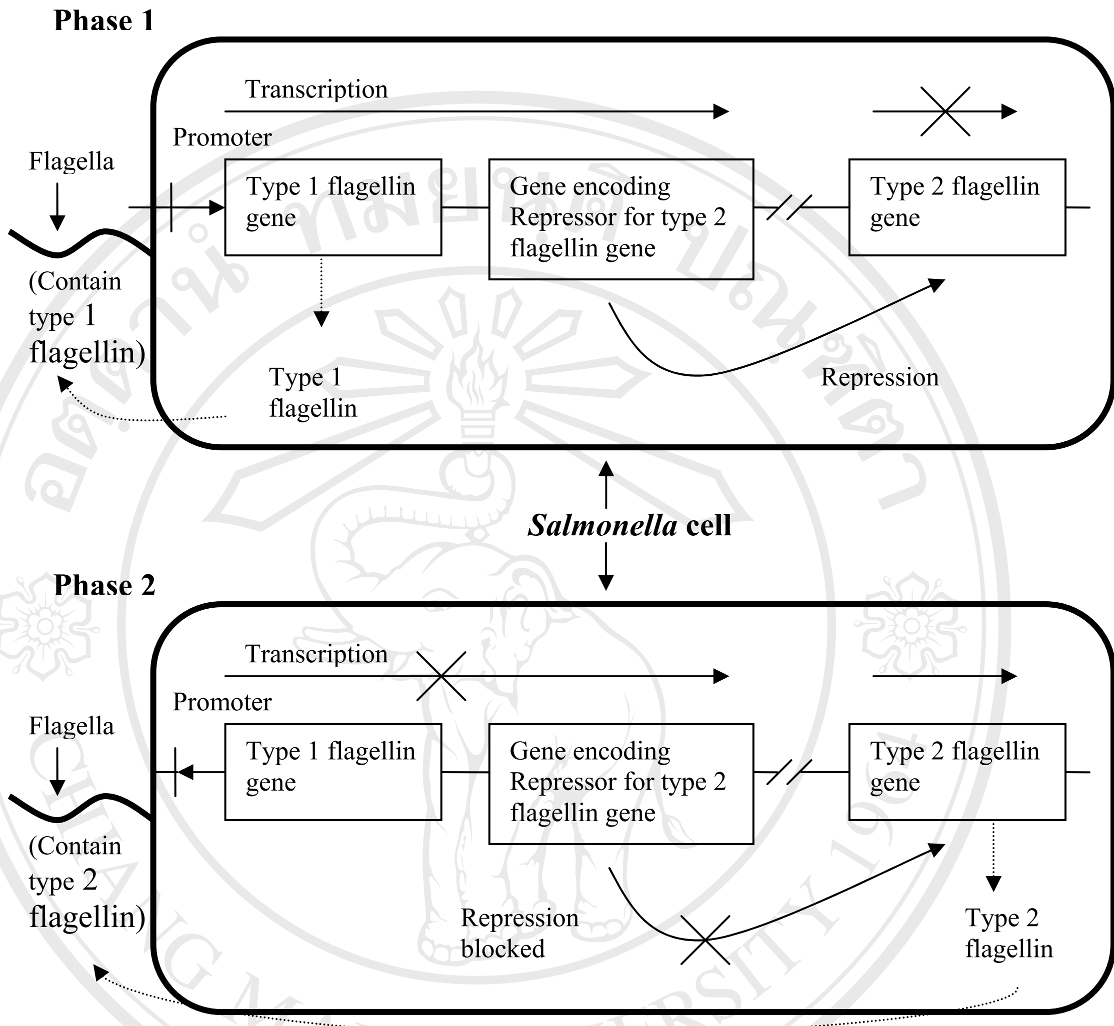

Source: MaClane and Mietzner (1999)

Figure 2: *Salmonella* Phase variation diagrams

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In the Kauffmann-White Scheme, all serotypes can be designated by a formula. In the first column of the scheme, the names of the serovar for serovars of *S. enterica* subsp. *enterica* are present (e.g., Kisangani, Paratyphi A, Enteritidis, Hadar, etc.). For other subspecies of *S. enterica*, the subspecies to which the serovar is indicated by a Roman numeral *(S. enterica* subsp. *salamae*; II, *S. enterica* subsp. *arizonae*; IIIa, *S.*

enterica subsp. *diarizonae*; IIIb, *S. enterica* subsp. *houtenae*; IV, and *S. enterica* subsp. *indica*; VI) and also the serovars of S. *bongori* ,the “V” was retained to avoid confusion with the serovar name of *S. enterica* subsp. *enterica* (Popoff, 2001).

The typical formats for a serotype formula (e.g., *S.* Typhimurium and *S.* Weltevreden) are shown in Figure 3.

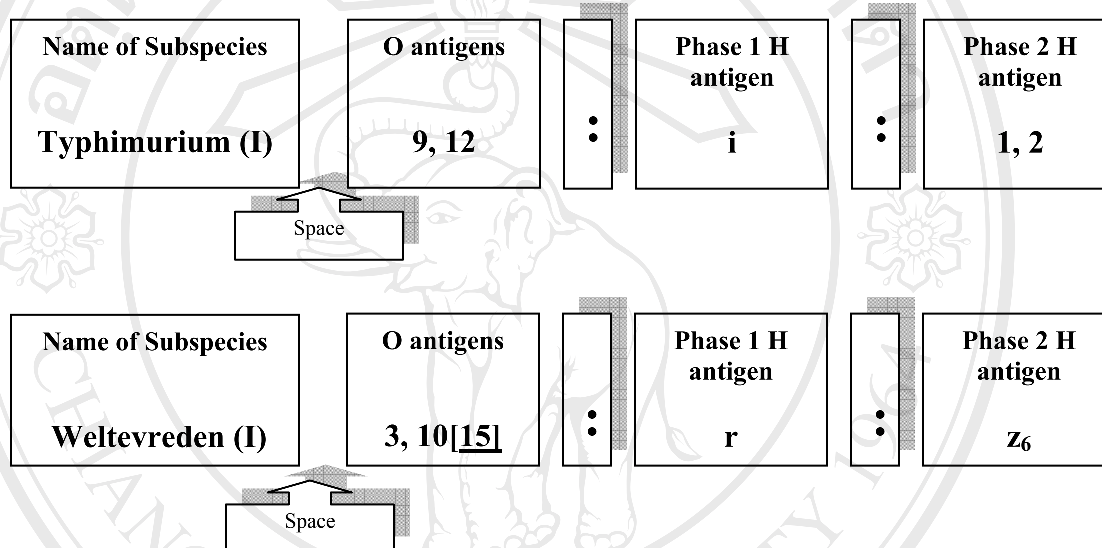

Figure 3: Diagram of typical format for the serotype formula of *S.* Typhimurium and *S.* Weltevreden

In some cases, O and H factors are variably present. This is indicated in the generic serotype formula by underlining when the factor is encoded on a bacteriphage (e.g., 1) or by square brackets (e.g., [5]) when the antigen is variably present. Weakly recognized antigens are indicated by parentheses e.g., (k).

The absence of an H antigen is indicated by a minus sign (“-”) for a particular phase (e.g., *S.* IV 48:g,z_{51}:-) (Figure 4).

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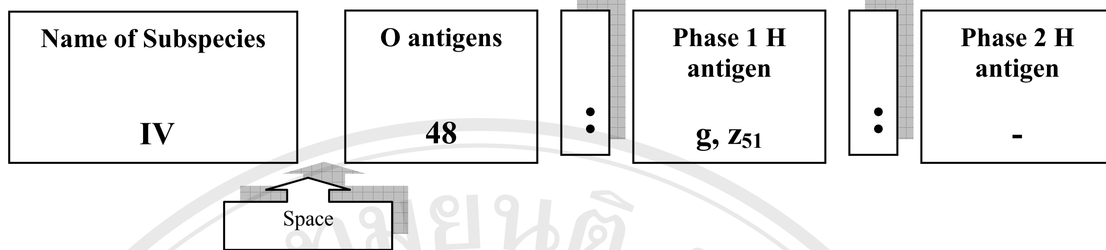

Figure 4: Diagram of typical format for the serotype formula of *S.* IV 48:g,z_{51}:-

Rarely, isolates express a third H antigen that is noted by a colon followed by the antigen after the phase 2 H antigen e.g., S. II 13,23:b:[1,5]:z_{42} .

2.5 Salmonellosis

2.5.1 Salmonellosis in humans

Salmonellosis is considered to be one of the most common human foodborne illnesses (Swanenburg *et al*., 2001; Murray *et al*., 2002; Lo Fo Wong *et al.,* 2002). *Salmonella* Enteritidis and *S.* Typhimurium have accounted for the majority of cases of human salmonellosis for over many years and, have consistently been the most commonly-implicated pathogens in outbreaks of foodborne disease (Hane, 2003; Hu *et al*., 2003).

Contributing risk factors include contacts with infected individuals, exposure to contaminated environments, or the ingestion of foods containing this microorganism. The clinical presentation of human salmonellosis correspond to either the enteric fever syndrome following infection with typhoid or paratyphoid strains or to the nontyphoid gastroenteritis/enterocolitis with positive progression to a more serious systemic infection (Hane, 2003; Hu *et al*., 2003).

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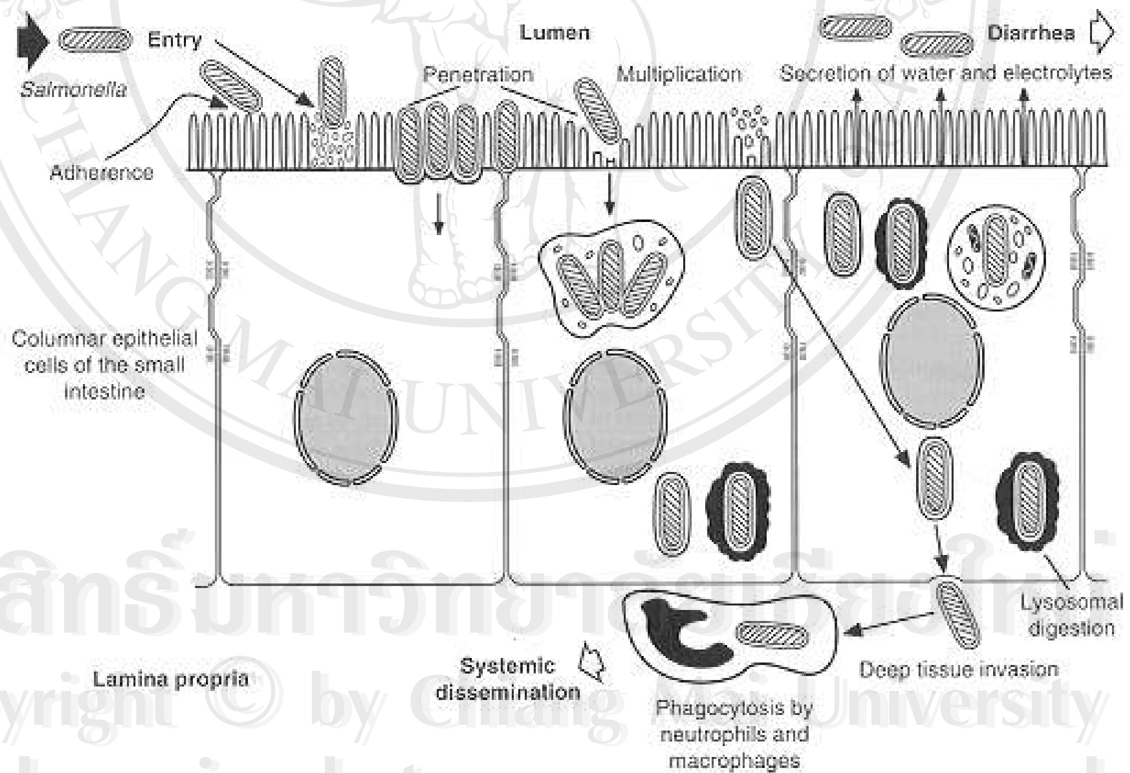
In most cases of enteric fever, the etiological agent is *S. enterica* serovar Typhi. This serovar causes typhoid fever (Hu *et al*., 2003). Other salmonellae, specifically serovars Paratyphi A, B, and C can also cause enteric fever but the symptoms are milder and the mortality is low (Hu *et al*., 2003).

The clinical symptoms of typhoid or paratyphoid salmonellosis may appear 7 to 28 hours after exposure to these serovars. Watery diarrhea or, infrequent constipation, persistent and spiking fever, abdominal pain (cramps), headache, nausea, prostration, and a rash of rose spots on shoulders, thorax, or abdomen may occur (Hu *et al*., 2003). Complications of enteric fever include intestinal bleeding from ileal ulcers or intestinal perforation resulting from hyperplasia of the epitheliolymphoid Peyer's patches (Hu *et al*., 2003).



Source:http://www.surrey.ac.uk/SBMS/ACADEMICS_homepage/mcfadden_johnjoe/img/salmonellaepithelial%20interactions.jpg

Figure 5: *Salmonella*-epithelial cell interactions

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The symptoms of non-typhoid salmonellosis may include nausea, abdominal cramps, diarrhea with watery and possibly mucous stool tinged with blood, fever of short (less than 48 hours) duration, and vomiting that appear 8 to 72 hours following exposure to the bacterium (Hane, 2003). The *Salmonella* – epithelial cell interactions are shown in Figure 5.

The National *Salmonella* and *Shigella* Center (NSSC) of the National Institute of Health, Department of Medical Science, Ministry of Public Health, Nonthaburi, Thailand is an important institute that has been working as the national reference laboratory of Thailand. Non-typhoidal salmonellosis is a major cause of food-borne illness in Thailand (Vaeteewootacharn *et al*., 2005). In the NSSC annual report 2003, a total of 141 serovars were identified among the 5005 isolations; 139 and 2 serovars from non typhoidal *Salmonella* and typhoidal *Salmonella*, respectively. The top five of the salmonellae serovars from human rectal swabs and stool were *S*. Weltevreden (13.1%), *S*. Stanley (9.6%), *S*. Anatum (7.4%), *S*. Rissen (7.2%), *S*. Enteritidis (5.2%). The top five Salmonellae serovars from 428 human blood samples were also reported ; *S*. Enteritidis (46.0%), *S*. Cholereraesuis (22.2%), *S*. I. ser. 4,12:i:- (5.8%), *S*. Typhimurium (3.9%), and *S*. I. ser. 4,5,12:i:- (3.5%) (Bangtrakulnonth *et al*., 2004).

According to the Center of Disease Control and Prevention (CDC), Atlanta, Georgia, USA, *S.* Typhimurium was highest (19.7%) reported in 2003. *S.* Enteritidis, *S.* Newport, *S.* Heidelberg, *S.* Javiana were reported in the top 5 most frequently isolated serovars in 2003 (14.5, 11.5, 5.4 and 4.9%, respectively) (Anonymous, 2004b).

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2.5.2 Salmonellosis in Animals

Salmonellosis commonly occurs in domestic animals, the consequences of infection range from sub-clinical carrier status to acute fatal septicemia. Some *Salmonella* serotypes such as *Salmonella* Pullorum in poultry, *Salmonella* Dublin in cattle and *Salmonella* Choleraesuis in pigs are relatively host-specific. In contrast,

Salmonella Typhimurium has a comparatively wide host range. The *Salmonella* serotypes of importance in domestic animals and the consequences of infection are shown in Table 3 and differential characters of *Salmonella* species and subspecies are shown in Table 4.

The NSSC annual report 2003 also reported *Salmonella* serovars isolated from animals. The top five salmonellae serovars were *S.* Enteritidis (55.5%), *S.* Stanley (4.7%), *S.* Rissen (4.7%), *S.* Weltevreden (3.9%), and *S.* Lexington (3.1%). In the zoonoses report 2003 of the Department of Environment Food and Rural Affairs, United Kingdom , the top five *Salmonella* serotypes in pig incidents were *S.* Typhimurium (70.1%), *S.* Derby (14.3%), *S.* Kedougou (3.0%), *S.* Reading (2.6%), and *S.* Montevidio (1.3%) (Anonymous, 2004a).

Table 3: *Salmonella* serotypes of human and animals clinical importance and the consequences of infection

Salmonella serotype	Host	Consequences of infection
Salmonella Typhimurium	Many animal species	Enterocolitis and septicemia
	Humans	Food poisoning
Salmonella Dublin	Cattle	Many disease conditions
	Sheep, horses, dogs	Enterocolitis and septicemia
Salmonella Choleraesuis	Pigs	Enterocolitis and septicemia
Salmonella Pullorum	Chicks	Pullorum disease (bacillary white diarrhoea)
Salmonella Gallinarum	Adult birds	Fowl typhoid
Salmonella Arizonae	Turkeys	Arizona or paracolon infection
Salmonella Enteritidis	Poultry	Often sub-clinical in poultry
	Many other species	Clinical disease in mammals
	Humans	Food poisoning
Salmonella Brandenburg	Sheep	Abortion

Source: Quinnand *et al., (2003)*

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Table 4: Differential characters of *Salmonella* species and subspecies

Species	***S. enterica***						***S. bongori***
Subspecies	*enterica*	*salamae*	*arizonae*	*diarizonae*	*houtenae*	*indica*	
Characters							
Dulcitol	+	+	-	-	-	d	+
ONPG (2h)	-	-	+	+	-	d	+
Malonate	-	+	+	+	-	-	-
Gelatinase	-	+	+	+	+	+	-
Sorbitol	+	+	+	+	+	-	+
Culture with KCN	-	-	-	-	+	-	+
L(+)-tartrate [(a)]	+	-	-	-	-	-	-
Galacturonate	-	+	-	+	+	-	+
γ-glutamyltransferase	+(*)	+	-	+	+	+	+
β-glucuroidase	d	d	-	+	-	+	-
Mucate	+	+	+	-(70%)	-	d	+
Salicine	-	-	-	-	+	-	-
Lactose	-	-	-(75%)	+(75%)	-	d	-
Lyse by phage O1	+	+	-	+	-	+	d

(a) = *d*-tartrate.

(*) = Typhimurium d, Dublin -

\+ = 90% or more positive reaction.

\- = 90% or more negative reaction

d = different reaction given by different serovars.

Source: Popoff (2001)

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3. MATERIAL AND METHODS

Three separate studies on the prevalence of *Salmonella* in the pork production chain were performed. Sampling was carried out from the same pigs studied at farm level, prior to slaughter, and during the slaughtering process.

3.1 Place of investigation and data collection

This study was performed at a slaughterhouse and retail markets (supermarkets) in the Chiang Mai Province, Thailand. The slaughterhouse handled approximately eighty pigs per day from farms located mainly in the Chiang Mai and Lamphun provinces in the northern part of Thailand.

3.2 Materials for *Salmonella* determination

Collection of samples started after the pork carcasses were handled removed from the slaughter chilling room into the cutting room. In this room, carcasses were cut by staff members and each respective carcass was termed "**cut pork**". Some portions from the same carcasses were sent to the packaging unit of the slaughterhouse, packed into containers and transported by a slaughterhouse truck to the laboratory. These were called "**transported pork**".

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Retail pork from the same batches of pork parts, such as bones, bellies, ribs, collars, loins, ground pork, shoulder meats, hams, and fillets were sent to meat departments in supermarkets. Some of these were sampled, bought and taken to the laboratory for analysis.

Environmental samples were taken in the slaughterhouse the same day as the pigs arrived in the slaughterhouse. The samples taken from surfaces of cutting boards, plastic curtains, knives, shackles, and staff hands were examined.

3.3 Collection of pork samples

3.3.1 Cut pork samples

Samples from each carcass, already individually identified at the farm were collected in the cutting room prior to packaging. Five samples were collected, i.e. belly, fillet, neck, shoulder and loin (Appendix D). These pieces called "cut pork" were combined in the same package for analysis. Sampling started at the beginning of the day (8.30-9.00 am.). The temperature of the carcasses and room temperatures were recorded (Figure 6).

3.3.2 Transported pork samples

After cutting processing, each part of the individually identified pork such as the neck of the identified carcass was packed in the same plastic bag, from the cutting unit, therefore, there were 5 packs of samples which were already sampled and packaged at the packaging unit of the slaughterhouse. They were kept in the chilling room for 1-2 days and they were transported to the faculty of Veterinary Medicine, Chiang Mai University by slaughterhouse truck, and called "transported pork". In the laboratory, 5 parts of each pig were recombined to be one sample to obtained (Figure 6).

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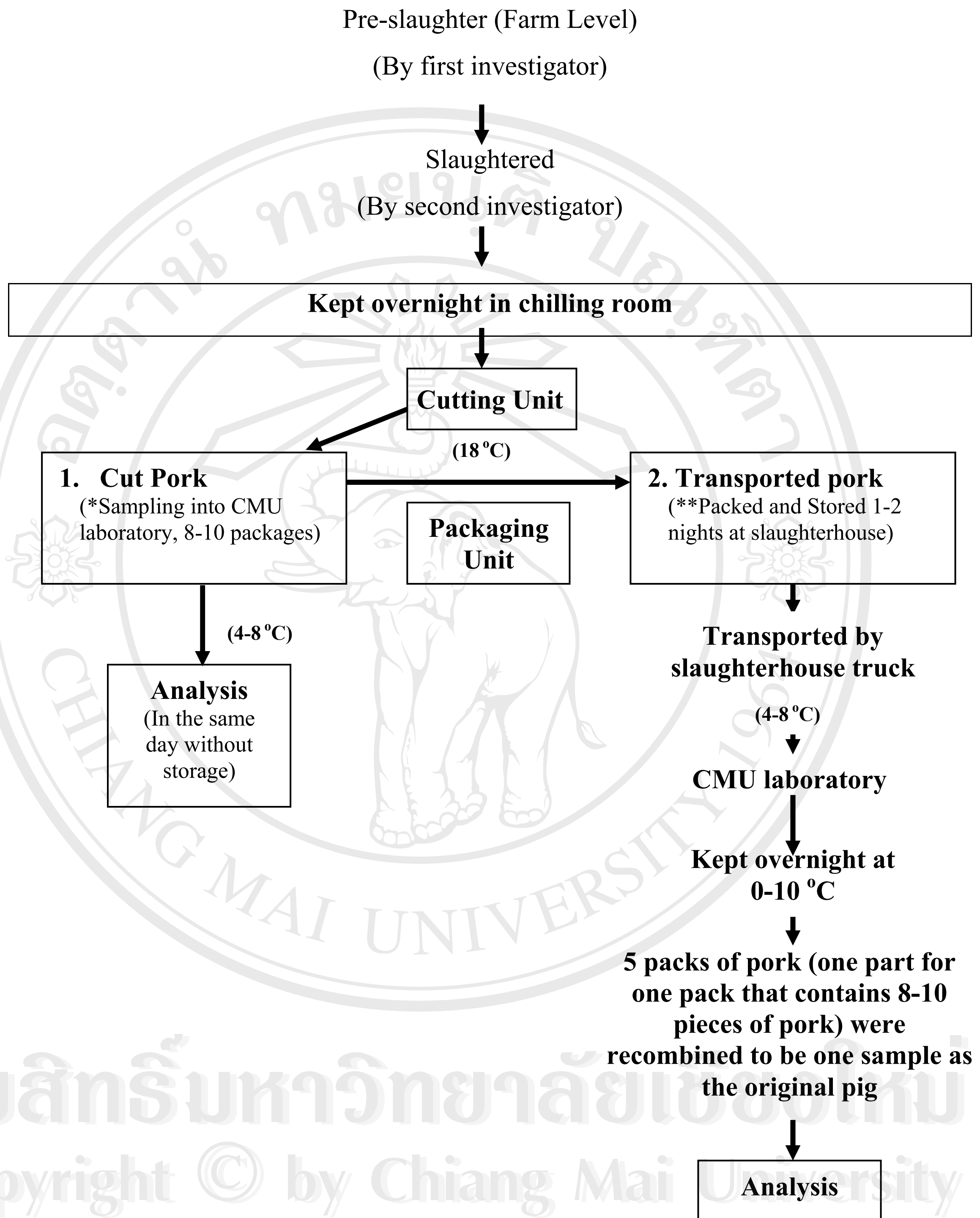

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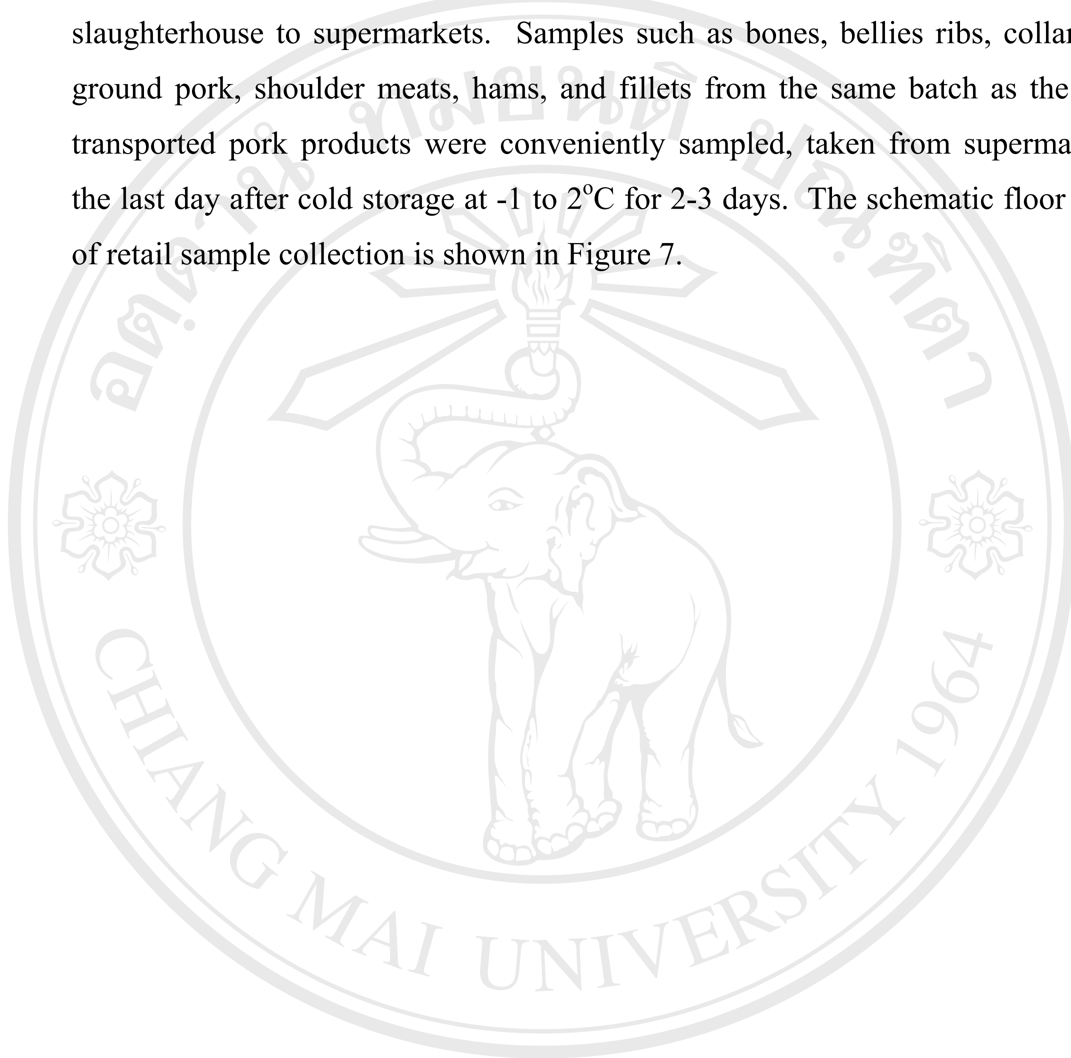
Figure 6: Schematic representation of "cut pork" and "transported pork" products

1)* 8-10 pigs = 8-10 pork samples, each pig collecting 5 parts: belly, loin, shoulder, fillet and neck. 5 parts of each pig combined in the same package

2)**The same 10 pigs as in 1)* ,each part of carcass such as neck of all 10 pigs were packed in the same plastic bag. Therefore, there were 5 packs of samples. Then, they were kept them in a chilling room 1-2 days and after that sent to the CMU laboratory. In the laboratory, 5 parts of each pig were combined to be one sample (10 samples from 10 pigs).

3.3.3 Retail pork samples

For retail products, identified packaged pork was transported from the slaughterhouse to supermarkets. Samples such as bones, bellies ribs, collars, loins, ground pork, shoulder meats, hams, and fillets from the same batch as the cut and transported pork products were conveniently sampled, taken from supermarkets on the last day after cold storage at -1 to 2°C for 2-3 days. The schematic floor diagram of retail sample collection is shown in Figure 7.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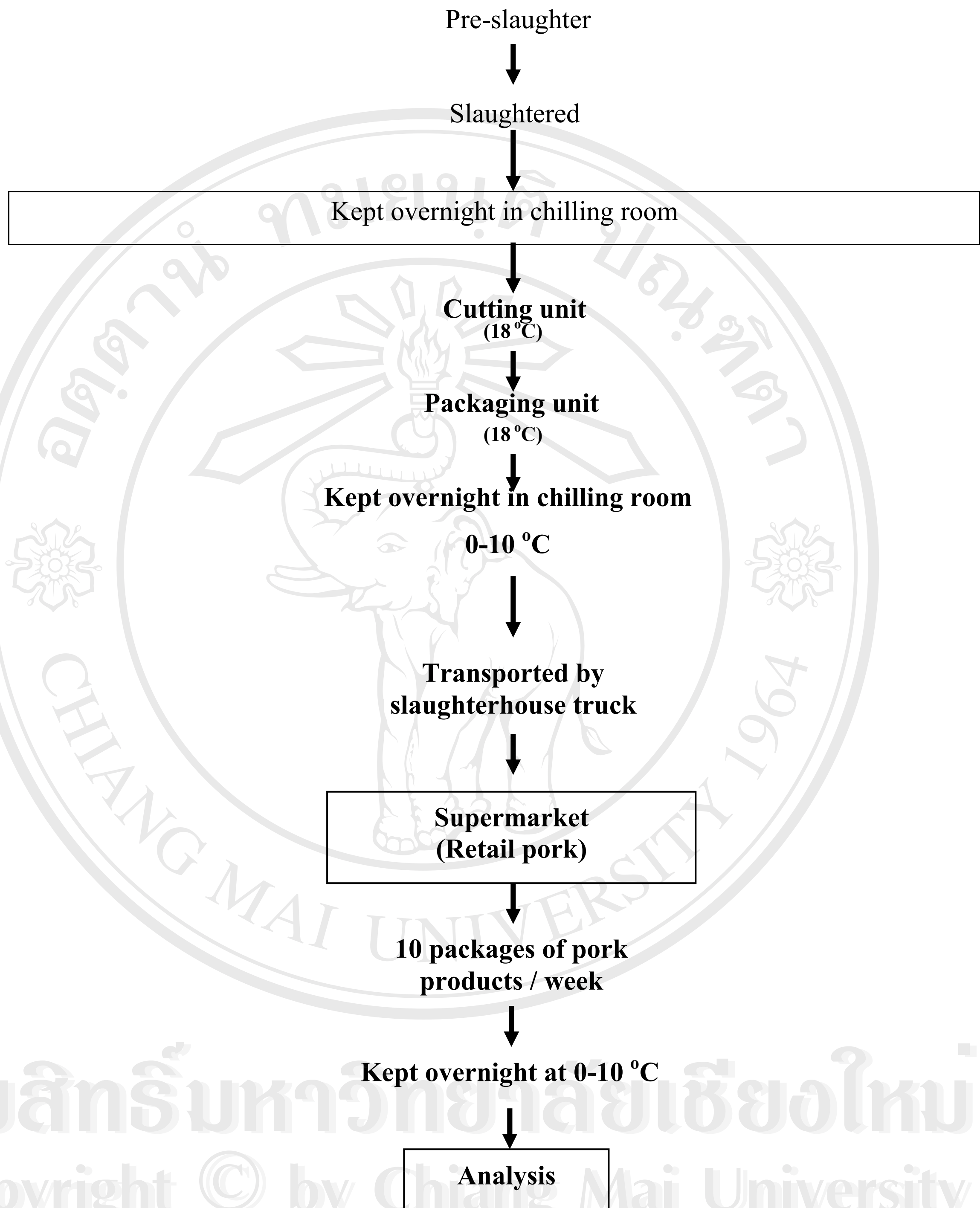

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Figure 7: Schematic flow diagrams of convenient sampling of retail pork products from supermarkets

3.4 Collection of Environmental samples

Samples from equipment (cutting boards, plastic curtains, knives, shackles, hands of staff) were collected by a non–destructive method (swab technique) as described in section 3.6. There were 3 time intervals for environmental sample collection in the slaughterhouse: (1) prior to cutting (8.00-8.30); the first swab environmental samples were collected from cutting boards, plastic curtain, knife, shackles and hands of staff, called "**before cutting operation**"; (2) During cutting (8.30-11.00), the second swab samples were collected from cutting boards, plastic curtain, knife shackle and hands of staff again, called "**during cutting operation**"; (3) the third swab samples at the same position were done after cleaning and disinfecting during a lunch break (11.00-12.00), called "**after disinfecting operation**". The distribution of environmental samples collected at 3 intervals in the cutting unit of the slaughterhouse per day is shown in Table 5.

Table 5: Collection of environmental samples at 3 intervals per day in the slaughterhouse

Sampling materials	Before cutting operation	During cutting operation	After disinfecting operation	Total
Swab technique		**Number of samples**		
Cutting board	1	1	1	3
Plastic curtain	1	1	1	3
Knife	1	1	1	3
Shackle	1	1	1	3
Hands of staff	1	1	1	3
Total				15[a]

[a] Total number of swabbing samples per day

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3.5 Sample size determination

The sample size was calculated by Win Episcope software (Win Episcope®, Version 2.0, 1998). Estimation of numbers of samples was based on an assumption of the prevalence of *Salmonella* infection in pre-slaughter pigs in the Chiang Mai province of 69.5% (ranging from 50-83%) (Patchanee, 2002), with a 95% confidence interval, and with an accepted error rate of 8%. All together 346 samples, from 20 farms, were collected from the slaughterhouse (173 samples from "cut pork" and 173 samples from "transported pork").

For retail, all samples were collected from one supermarket in the Chiang Mai province on one occasion. Ten samples of several types of products were collected from supermarkets. All sampling took place between January–May 2005. In total, 200 pieces of retail pork samples were collected.

A total of 300 environmental swabs in the slaughterhouse were collected.

3.6 Methods of sample preparation

3.6.1 Destructive method

From one pig, five pieces of tissues totaling 25 g were collected from five parts, pooled and put into sterile plastic bags (Stomacher). In the latter, 225 ml of sterile non-selective pre-enrichment medium was added (Buffered peptone water) at ambient temperature and sent to the laboratory unit in Chiang Mai University.

Wrappings from samples in packages from retail markets were removed carefully without touching the pork. Sterile forceps were used for putting 25g of samples into sterile plastic bags. Another 225 ml buffered peptone water was used.

3.6.2 Non-destructive method (swab technique)

The swab samples were taken from the surface of the environmental samples. The cotton swabs were wrapped in aluminum foil and were sterilized for 15 minutes

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at 121 °C before use. The swabs were held in sterile forceps and the surfaces were swabbed 10 times from top to bottom carefully applying firm pressure on every surface (Table 5) according to the EU decision 2001/471. Prior to sampling, the swab was moistened with normal saline solution. After swabbing the surfaces, the swabs were put into 50 ml of sterile buffered peptone water in a plastic bag and shaken by hand for 2 minutes. The fluid was kept in an ice box (4-5 °C). All samples were forwarded to the laboratory for analysis.

3.7 *Salmonella* isolation procedures

In this study, conventional methods for the detection of *Salmonella* were carried out, following ISO 6579:2002 (Microbiology of food and animal feeding stuffs-horizontal method for the detection of *Salmonella* spp.) The Diagram of the procedure for the detection of *Salmonella* spp. is given in Figure 8.

3.7.1 Non-selective pre-enrichment

Using the non-destructive technique, a swab sample (cotton swab) was put into 50 ml of buffer peptone water (BPW). With the destructive method, 25 g of pork was transferred into a stomacher bag with 225 ml BPW. They were shaken in a stomacher for 2 min. All samples were incubated at 37 °C ± 1 °C for 18 ± 2 h.

3.7.2 Selective enrichment

The pre-enrichment broth was mixed and 0.1 ml was transferred into a tube containing 10 ml of RVS broth (Rappaport-Vassiliadis medium with soya). For the 2nd environment, 1 ml of the pre-enrichment broth was transferred into a tube containing 10 ml of MKTTn broth (Muller-Kauffmann tetrathionate novobiocin broth).

The inoculated RVS broth was incubated at 41.5 °C±1 °C for 24 h ± 3 h and the inoculated MKTTn broth at 37 °C±1 °C for 24 h ± 3 h.

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3.7.3 Plating and identification

After incubation for 24 h ± 3 h, a loop of material from RVS broth and MKTTn was transferred and streaked separately onto the surface of XLD agar (Xylose lysine deoxycholate agar) and BPLS agar (Brilliant green Phenol Red Lactose Sucrose agar) separately. The plates were incubated in an inverted position at 37 ^{o}C±1 ^{o}C for 24 h ± 3 h. After incubation, the plate was checked for growth of typical *Salmonella* colonies.

Typical colonies of *Salmonella* grow on XLD agar with a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator (*Salmonella* H_2S negative variants (e.g. *S.* Paratyphi A). On XLD agar typical colonies are pink with a darker centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening).

Typical colonies of *Salmonella* grow on BPLS agar a have a reddish color and translucent colony.

3.7.4 Confirmation

Five typical colonies per plate grown on the XLD agar and BPLS agar were transferred and inoculated on triple sugar iron agar (TSI), incubated at 37 ^{o}C±1 ^{o}C for 24 h ± 3 h.

If fewer than five typical or suspected colonies per Petri dish were observed, all suspected colonies were streaked on the surface of pre-dried nutrient agar plates, in a manner which allowed well-isolated colonies to develop. The inoculated plates were incubated at 37 ^{o}C±1 ^{o}C for 24 h ± 3 h.

Pure cultures were used for biochemical and serological confirmation.

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3.7.4.1 Biochemical confirmation

3.7.4.1.1 TSI agar

Streak the agar slant surface and stab the butt. Incubate at 37 °C±1 °C for 24 h ± 3 h. Interpret the changes in medium as follows.

Table 6: Meaning of TSI agar for *Salmonella* spp.

Area of Reaction	Result	Meaning
Butt		
	Yellow	glucose positive (glucose used)
	Red (unchanged)	glucose negative (glucose not used)
	Black	formation of hydrogen sulfide
	Bubbles or cracks	gas formation from glucose
Slant surface		
	Yellow	lactose and/or sucrose positive
	Red(unchanged)	lactose and sucrose negative

Typical *Salmonella* cultures show alkaline (red) slant and acid (yellow) butts with gas formation (bubble) and (in about 90% of the cases) formation of hydrogen sulfide (blackening of the agar).

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3.7.4.1.2 Urea agar

Streak the agar slant surface. Incubate at 37 °C±1 °C for 24 h ± 3 h. and examine at intervals.

If the reaction is positive, splitting of urea liberates ammonia, which changes the colour of phenol red to rose-pink and later to deep cerise (a moderate red). The reaction is often apparent after 2 h to 4 h.

3.7.4.1.3 L-Lysine decarboxylation medium

Inoculate just below the surface of the liquid medium. Incubate at 37 ^{o}C±1 ^{o}C for 24 h ± 3 h.

Turbidity and a purple colour after incubation indicate a positive reaction. A yellow colour indicates a negative reaction.

3.7.4.1.4 Detection of β-galactosidase

Suspend a loopful of the suspected colony in a tube containing 0.25 ml of the saline solution.

Add one drop of toluene and shake the tube. Put the tube in the water bath set at 37 ^{o}C and leave for several minutes (approximately 5 min). Add 0.25 ml of the β-galactosidase reagent for detection of β-galactosidase and mix.

Replace the tube in the water bath set at 37 ^{o}C and leave for 24 h ± 3 h, examining the tube at intervals.

A yellow colour indicates a positive reaction. The reaction is often apparent after 20 min.

3.7.4.1.5 Medium for Voges-Proskaur (VP) reaction

Suspend a loopful of the suspected colony in a sterile tube containing 3 ml of the VP medium (7g of peptone, 5g of glucose and 5g of dipotassium hydrogen phosphate in 1000 ml water). Incubate at 37 ^{o}C±1 ^{o}C for 24 h ± 3 h.

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After incubation, add two drops of the creatine solution, three drops of ethanolic solution of 1-naphthol and then two drops of the potassium hydroxide solution; shake after the addition of each reagent.

The formation of a pink to bright red colour within 15 min indicates a positive reaction.

3.7.4.1.6 Medium for indole reaction

Inoculate a tube containing 5 ml of the tryptone/ tryptophan medium with the suspected colony. Incubate at 37 ^{o}C±1 ^{o}C for 24 h ± 3 h. After incubation, add 1ml of the Kovacs reagent. The formation of red ring indicates a positive reaction. A yellow-brown ring indicates a negative reaction.

The interpretation of biochemical test for detection of *Salmonella* spp. is shown in table 7, the whole procedure in Figure 8.

3.7.4.2 Serological confirmation and Serotyping

The presence of *Salmonella* O-, Vi- and H-antigens was tested using slide agglutination reaction with the appropriate anti-sera, from pure colonies and after elimination of auto-agglutinationable strains. For agglutination testing, the prescription of the manufacturer of the antiserum was used following SIFIN (Institut für Immunpräparate und Nährmedien GmbH) and the serotyping *Salmonella* procedure of the Institute of Meat Hygiene and Technology, Freie Universität Berlin, Germany, described in Appendix B.

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Table 7: Interpretation of biochemical test for salmonellae

Biochemical Test	Reaction
TSI acid from glucose (+gas formation)	Positive
TSI acid from lactose	Negative
TSI acid from sucrose	Negative
TSI hydrogen sulfide produced	Positive
Urea hydrolysis	Negative
Lysine decarboxylation	Positive
β-galactosidase reaction	Negative
Voges-Prokauer reaction	Negative
Production of indole	Negative

3.8 Data management and analysis

Program Excel version 2003 (Microsoft® Office Excel 2003, Microsoft Office Professional Edition, 2003) and NCSS statistical software (Hintze, 2001) were used for collection, management and analysis of the data. Descriptive statistics were used to describe the result for prevalence analysis. The prevalence ratio (PR) was used in this study to measure association of cut and transported pork by using the Win Episcope program (Win Episcope®, Version 2.0, 1998). The value of PR indicates; (1) PR equal to 1 means no association exists between occurrence of disease and exposure; (2) PR less than 1 means the exposure is positively associated to the disease (risk factor); (3) PR more than 1 means the exposure is negatively associated to the disease (preventive factor).

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		Result or Disease		
		+	-	
Factor	+	A	B	A+B
	-	C	D	C+D
		A+C	B+D	N

$$PR = \frac{\frac{A}{A+B}}{\frac{C}{C+D}} \quad ;$$

A = Number of result both were positive
B = Number of result in first step (cut) was positive and in second step (transported) was negative
C = Number of result in first step was negative and in second step was positive
D = Number of result both were negative

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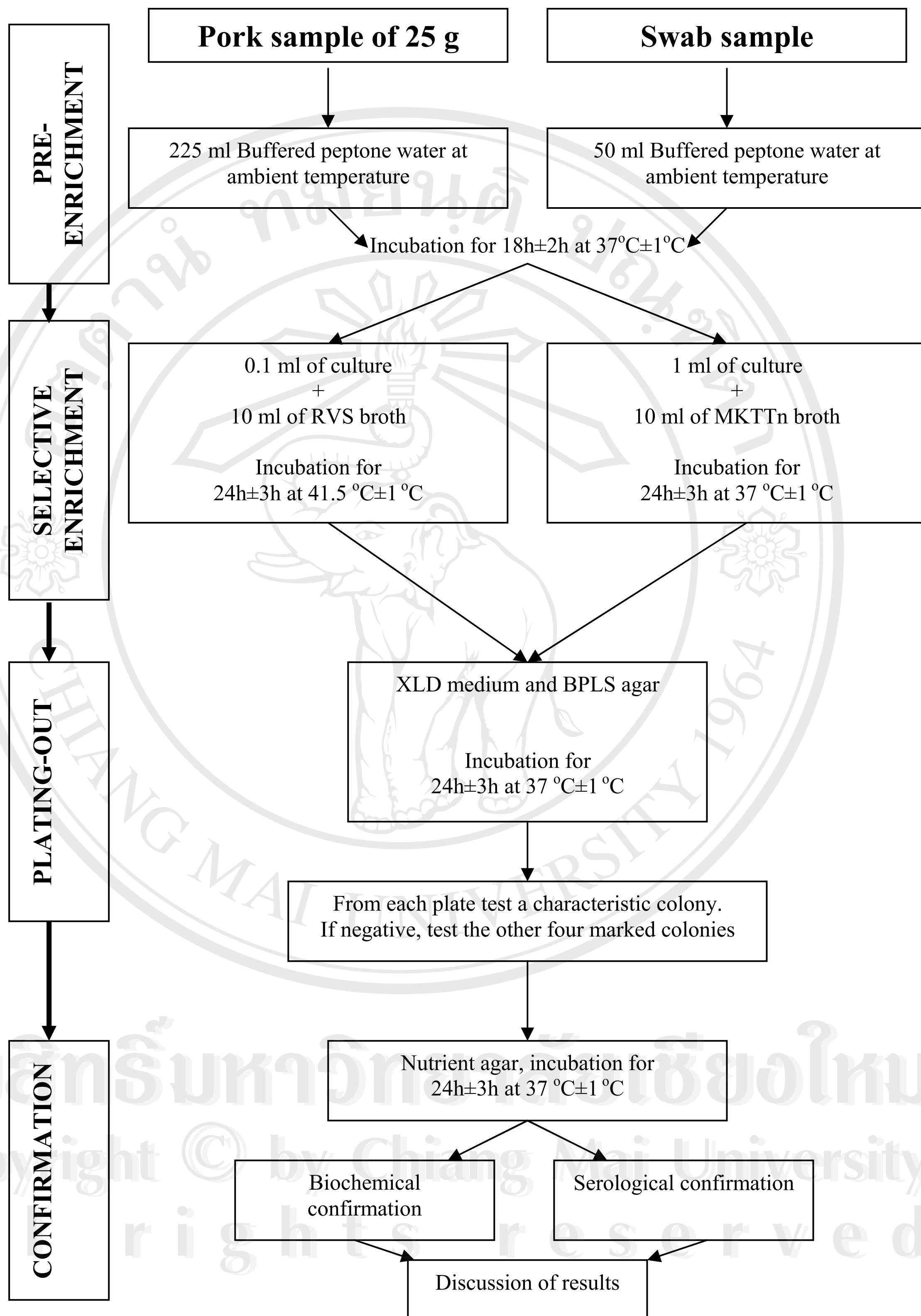

Figure 8: Diagram of procedure for detection of *Salmonella* spp.

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4. RESULTS

The total number of samples analyzed in this study was 846 samples. Four groups of samples were used for the interpretation of this study; (1) cut pork; (2) transported pork; (3) retail pork; (4) environmental samples of the slaughterhouse. 173 samples of “cut pork”, 173 samples of “transported pork”, 200 samples of retail pork (10 bones, 29 bellies, 9 ribs, 23 collars, 33 loins, 33 packs of ground pork, 13 shoulder meats, 21 hams, and 29 fillets) and 300 samples from the slaughterhouse environment (Table 8 and 9) were available.

Table 8: Number of samples of pork for salmonellae analysis

Sampling materials	Number of samples
Destructive method	
Cut pork	173
Transported pork	173
Retail pork	200
Total	546

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Table 9: Number of samples collected from slaughterhouse environment

Swab surfaces	Number of samples			Total
	Before cutting	**During cutting**	**After disinfecting**	
Cutting board	20	20	20	60
Plastic curtain	20	20	20	60
Knife	20	20	20	60
Shackle	20	20	20	60
Hands of staff	20	20	20	60
Total	***100***	***100***	***100***	***300***

4.1 Prevalence of *Salmonella* in pork meat

The prevalence of salmonellae on 173 pig carcasses and 200 pieces of pork during the period of the study, January – May 2005 is shown in Figure 9 within 20 farms, and the summary of descriptive statistics in 3 types of pork during 5 months is shown in Table 10 and Figure 11. Two farms of retail samples were 100 % positive and also were 100% positive in "cut pork" and "transported pork". Most of time during the study, *Salmonella* was presented (Figure 9).

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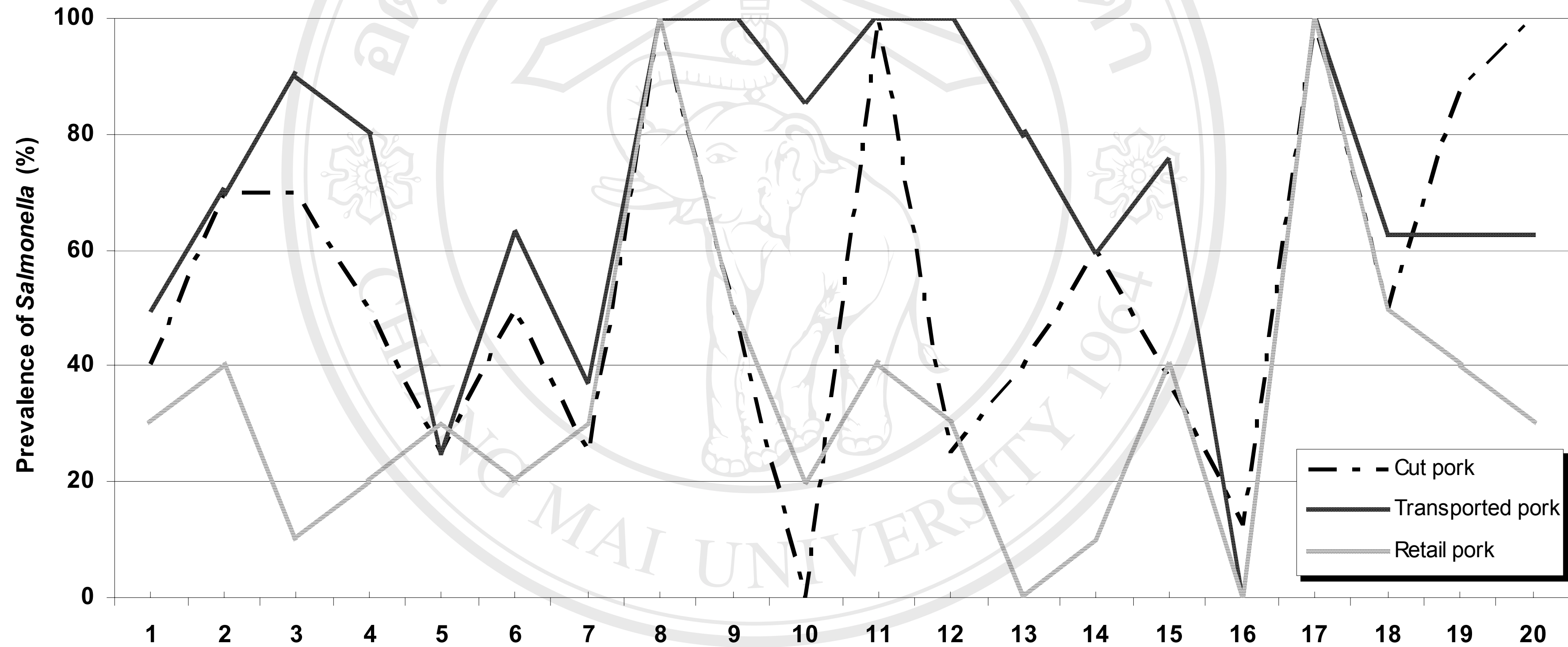

Figure 9: Prevalence of *Salmonella* in 3 types of pork sample in 20 farms

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Table 10: Summary of descriptive statistics in 3 types of pork products

Parameter / Type of pork	Count (n)	Mode	Median	Mean	95% Confidence Interval: Lower limit	95% Confidence Interval: Upper limit	Standard Deviation (SD)	Standard Error (SE)	Minimum	Maximum	χ^2	*p*-value	df
Cut	20	50,100	50	55.49	40.21344	69.03655	30.79297	6.88551	0	100	59.53	0.000005	19
Transported	20	100	72.5	70.52	57.42083	82.89916	27.2196	6.08648	0	100	55.96	0.000017	19
Retail	20	30	30	34.50	22.02706	46.97294	26.65076	5.95929	0	100	59.72	0.000004	19

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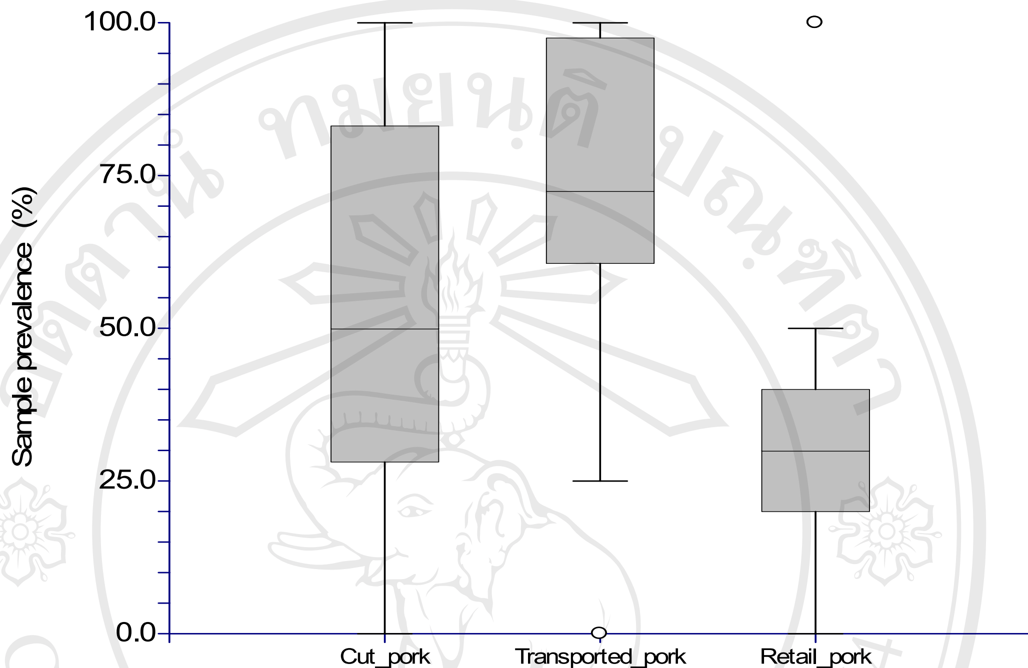

Figure 10: Box-and-whisker plots of sample prevalence of *Salmonella* positivity in three types of pork products

4.1.1 Prevalence of *Salmonella* in "cut pork"

Salmonellae were isolated from a total 55.49% (96/173) of "cut pork" samples (55.49%; 95% CI: 40.21-69.03%) ranging from 0 (1 farm) to 100% (4 farms). The prevalence of "cut pork" at least one farm had significantly different from others (χ^2=59.53; df=19; p=0.000005) (Table 10). Dates of sampling, number of samples examined, number of positive sample and sample prevalence in each farm are shown in Table 11.

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Table 11: Prevalence of *Salmonella* in "cut pork"

Farm ID	Date of Sampling	No. of Samples examined	No. of Positive samples	Sample prevalence (%)	95% Confidence Interval	
					Lower limit	Upper limit
1	8 Jan 2005	10	4	40.0	13.69	72.63
2	15 Jan 2005	10	7	70.0	35.37	91.91
3	27 Jan 2005	10	7	70.0	35.37	91.91
4	4 Feb 2005	10	5	50.0	20.14	79.86
5	6 Mar 2005	8	2	25.0	4.45	46.42
6	10 Mar 2005	8	4	50.0	17.45	82.55
7	11 Mar 2005	8	2	25.0	4.45	64.42
8	19 Mar 2005	8	8	100.0	59.77	98.84
9	20 Mar 2005	8	4	50.0	17.45	82.55
10	26 Mar 2005	7	0	0.0	1.32	43.91
11	30 Mar 2005	10	10	100.0	65.55	99.08
12	3 Apr 2005	8	2	25.0	4.45	64.42
13	6 Apr 2005	10	4	40.0	13.69	72.63
14	21 Apr 2005	10	6	60.0	27.37	86.31
15	25 Apr 2005	8	3	37.5	10.24	74.11
16	28 Apr 2005	8	1	12.5	0.66	53.32
17	2 May 2005	8	8	100.0	59.77	98.84
18	5 May 2005	8	4	50.0	17.45	82.55
19	7 May 2005	8	7	87.5	46.68	99.34
20	10 May 2005	8	8	100.0	59.77	98.84
	Total	***173***	***96***	***55.49***	***40.21***	***69.04***

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4.1.2 Prevalence of *Salmonella* in "transported pork"

Salmonellae were isolated from 122 of 173 "transported pork" samples (70.52%; 95% CI: 57.42-82.89%). The proportions ranged from 0 % (1 farm) to 100%. The prevalence of "transported pork" had at least one prevalence significantly different among others farm prevalences (χ^2=55.96; df=19; p=0.000017) (Table 10). Dates of

sampling, number of samples examined, number of positive sample and sample prevalence in each farm are shown in Table 12.

Table 12: Prevalence of *Salmonella* in "transported pork"

Farm ID	Date of Sampling	No. of Samples examined	No. of Positive samples	Sample prevalence (%)	95% Confidence Interval	
					Lower limit	Upper limit
1	10 Jan 2005	10	5	50.0	20.14	79.86
2	16 Jan 2005	10	7	70.0	35.37	91.91
3	28 Jan 2005	10	9	90.0	54.11	99.48
4	5 Feb 2005	10	8	80.0	44.22	96.46
5	7 Mar 2005	8	2	25.0	4.45	64.42
6	11 Mar 2005	8	5	62.5	25.89	89.76
7	13 Mar 2005	8	3	37.5	10.24	74.11
8	20 Mar 2005	8	8	100.0	59.77	98.84
9	21 Mar 2005	8	8	100.0	59.77	98.84
10	28 Mar 2005	7	6	85.7	42.01	99.25
11	30 Mar 2005	10	10	100.0	65.55	99.08
12	5 Apr 2005	8	8	100.0	59.77	98.84
13	8 Apr 2005	10	8	80.0	44.22	96.46
14	24 Apr 2005	10	6	60.0	27.37	86.31
15	27 Apr 2005	8	6	75.0	35.58	95.55
16	30 Apr 2005	8	0	0.0	1.16	40.23
17	4 May 2005	8	8	100.0	59.77	98.84
18	7 May 2005	8	5	62.5	25.89	89.76
19	10 May 2005	8	5	62.5	25.89	89.76
20	12 May 2005	8	5	62.5	25.89	89.76
	Total	***173***	***122***	***70.52***	***57.42***	***82.89***

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4.1.3 Prevalence of *Salmonella* in "cut pork" and "transported pork"

There was a significant (p=0.0346) difference between prevalence of "cut pork" and "transported pork". The prevalence ratio (PR) of "cut pork" and "transported pork" in this study was 1.195 (95% CI: 0.981-1.455). This indicated an association between "cut pork" and "transported pork" (Table 13). But, the prevalence increased from 55.49 % (96/173) in "cut pork" to 70.52 % (122/173) in "transported pork".

Table 13: 2x2 table of association between *Salmonella* at "cut pork" and *Salmonella* at "transported pork" and the prevalence ratio of their association

		Salmonella at "transported pork"		
		Yes	No	*Total*
Salmonella at "cut pork"	Yes	73	23	*96*
	No	49	28	*77*
	Total	*122*	*51*	*173*

		Logarithmic approximation		χ^2 Approximation	
		Lower Limit	Upper Limit	Lower Limit	Upper limit
Prevalence ratio (PR)	1.195	0.976	1.464	0.981	1.455

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4.1.4 Prevalence of *Salmonella* in retail pork

In retail products, 69 out of 200 samples were positive for *Salmonella* testing (34.50%; 95% CI: 22.02-46.97%). In two farms 100% positively and the most frequently occurring is 30%. At least one of the prevalences of retail pork also was

significantly different among other farm (χ^2=59.72; df=19; p=0.000004) (Table 10). Dates of sampling, number of samples examined, number of positive samples and sample prevalence in each farm are shown in Table 14.

The results of salmonellae positive in retail products were found in variable percentages. 70% (7/10) of positive sample was found in bone product and the lowest positive sample was 17.4% (4/23) in collar samples as showed in Figure11

Table 14: Prevalence of *Salmonella* in retail pork

Farm ID	Date of Sampling	No. of Samples examined	No. of Positive samples	Sample prevalence (%)	95% Confidence Interval	
					Lower limit	Upper limit
1	10 Jan 2005	10	3	30.0	8.09	64.63
2	16 Jan 2005	10	4	40.0	13.69	72.62
3	28 Jan 2005	10	1	10.0	0.52	45.89
4	5 Feb 2005	10	2	20.0	3.54	55.78
5	7 Mar 2005	10	3	30.0	8.09	64.63
6	11 Mar 2005	10	2	20.0	3.54	55.78
7	13 Mar 2005	10	3	30.0	8.09	64.63
8	20 Mar 2005	10	10	100.0	65.55	99.08
9	21 Mar 2005	10	5	50.0	20.14	79.86
10	28 Mar 2005	10	2	20.0	3.54	55.78
11	30 Mar 2005	10	4	40.0	13.69	72.62
12	5 Apr 2005	10	3	30.0	8.09	64.63
13	8 Apr 2005	10	0	0.0	0.92	34.45
14	24 Apr 2005	10	1	10.0	0.52	45.89
15	27 Apr 2005	10	4	40.0	13.69	72.62
16	30 Apr 2005	10	0	0.0	0.92	34.45
17	4 May 2005	10	10	100.0	65.55	99.08
18	7 May 2005	10	5	50.0	20.14	79.86
19	10 May 2005	10	4	40.0	13.69	72.62
20	12 May 2005	10	3	30.0	8.09	64.63
	Total	***200***	***69***	***34.50***	***22.02***	***46.97***

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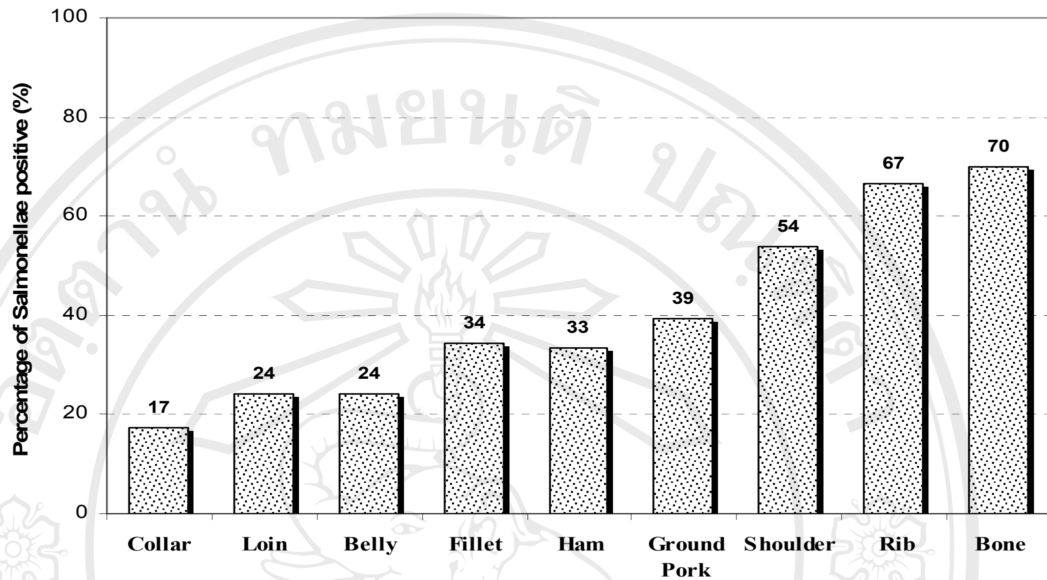

Figure 11: Percent distributions of salmonellae positive retail products

4.2 *Salmonella* in the environment of the slaughterhouse

Salmonellae positive results from the environment are shown in Table 15 and Figure 12. The highest percentage of *Salmonella* positive was found during the cutting period, 25.0% (25/100). *Salmonella* positive results before cutting and after disinfecting were 3.0% (3/100) and 16.0% (16/100), respectively. As can be seen from Table 15, samples from hands, knives and shackles were less frequently positive than samples from cutting boards. No contamination was found on plastic curtains at during any round of sampling occasion.

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Table 15: Number and percentage of salmonellae positive samples in environmental samples

Swab technique	Before cutting No. of Positive (%) (95%CI)	During cutting No. of Positive (%) (95%CI)	After disinfecting No. of Positive (%) (95%CI)	Total
Cutting board	0 (0) (0-16.8)	11 (55.0) (31.5-76.9)	12 (60.0) (36.1-80.9)	23(38.3) (26.1-51.8)
Plastic curtain	0 (0) (0-16.8)	0 (0) (0-16.8)	0 (0) (0-16.8)	0 (0) (0-5.9)
Knife	1(5.0) (0.1-24.8)	6 (30.0) (11.8-54.2)	1 (5.0) (0.1-24.8)	8 (13.3) (5.9-24.5)
Shackle	0 (0) (0-16.8)	0 (0) (0-16.8)	1 (5.0) (0.1-24.8)	1 (1.6) (0-8.9)
Hands of staff	2 (10.0) (1.2-31.6)	8 (40.0) (19.1-63.9)	2 (10.0) (1.2-31.6)	12(20.0) (10.7-32.3)
Total	**3(3.0)** **(0.6-8.5)**	**25(25.0)** **(16.8-34.6)**	**16(16.0)** **(9.4-24.6)**	**44(14.7)** **(10.8-19.1)**

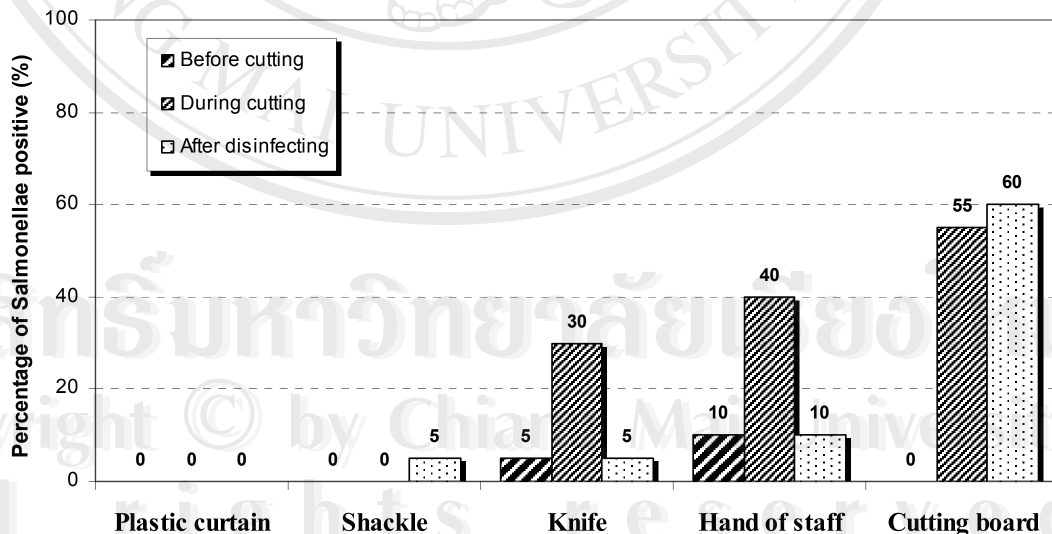

Figure 12: Percentage of salmonellae positive environmental samples at three time intervals in the cutting unit of the slaughterhouse

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4.3 *Salmonella* serotypes in meat products and environmental samples

A total of 331 positive samples were identified among 846 samples from cut, transported, retail pork and environmental samples. The three most frequent serogroups were salmonellae belonging to serogroup C (45.0%), B (34.1%) and D (13.9%). In this study, serogroup A was not found (Table 16).

Table 16: Number and percentage of salmonellae serogroup in pork and environment

Type of Sample	SEROGROUP					Total
	B	**C**	**D**	**E**	**F-67**	**(%)**
Cutting pork	34 (35.4)	45 (46.8)	4 (4.1)	8 (8.3)	5 (5.2)	96 (29.0)
Transported pork	43 (35.2)	48 (39.3)	3 (2.4)	22 (18.0)	6 (4.9)	122 (36.9)
Retail pork	20 (28.9)	34 (49.2)	2 (2.8)	13 (18.8)	-	69 (20.8)
Environment	16 (36.3)	22 (50.0)	2 (2.8)	3 (6.8)	1 (2.2)	44 (13.3)
Shackles	-	1 (100)	-	-	-	1 (2.2)
Knives	4 (50)	3 (37.5)	1 (12.5)	-	-	8 (18.2)
Hands of staff	6 (50.0)	4 (33.3)	-	2 (16.6)	-	12 (27.3)
Cutting boards	6 (20.0)	14 (60.8)	1 (4.3)	1 (4.3)	1 (4.3)	23 (52.3)
Total (%)	**113 (34.1)**	**149 (45.0)**	**11 (3.3)**	**46 (13.9)**	**12 (3.6)**	**331**

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The five most predominant serotypes were *S.* Rissen (45.3 %), *S.* Typhimurium (16.3%), *S.* Krefeld (10.6), *S.* Stanley (6.3%) and *S.* Lagos (6.0%). Also *S.* Panama, *S.* Weltevrenden, *S.* Agama, *S.* Gloucester, *S.* Tumodi, *S.* Anatum were identified (Table 17). *S.* Rissen was the most predominant in every sample type (cut, transported, retail and environmental samples) (Figure 13). The largest variation in serotypes was found in "transported pork" (Table 18). On nine (45%) occasions (farms), there was one serotype of *Salmonella* found in samples from the slaughterhouse and also most of the serotypes also were isolated from pork, e.g. farm ID 2. On other occasions (50%), there was more than one serovar in the environmental samples and also found in pork product samples e.g. farm ID 1. The summary detail of *Salmonella* serotypes isolated from the environment and pork samples are shown in Table 18.

Table 17: The 11 most frequent serovars of *Salmonella* isolated from pork and environmental samples

Serovar	Number of isolates (%)
Rissen	150 (45.3)
Typhimurium	54 (16.3)
Krefeld	35 (10.6)
Stanley	21 (6.3)
Lagos	20 (6.0)
Panama	11 (3.3)
Weltevrenden	8 (2.4)
Agama	7 (2.1)
Gloucester	4 (1.2)
Tumodi	4 (1.2)
Anatum	3 (0.9)
Other	14 (4.2)
Total	**331**

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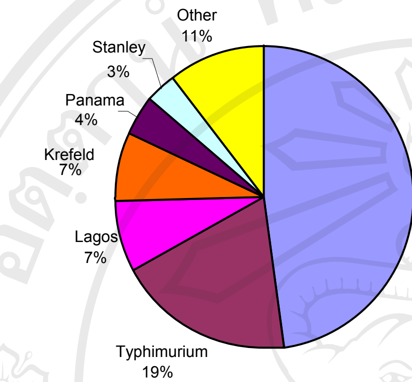
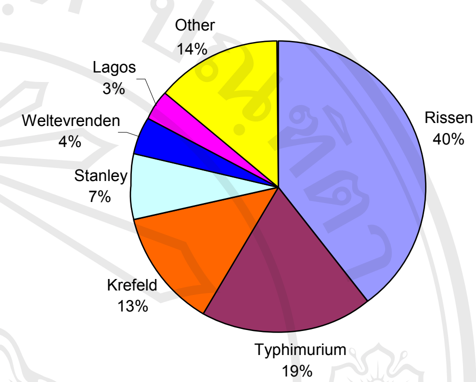
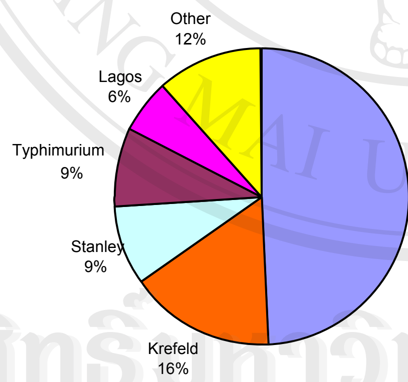
Cut pork (n = 173)



Transported pork (n = 173)



Retail pork (n = 200)



Environment (n = 300)

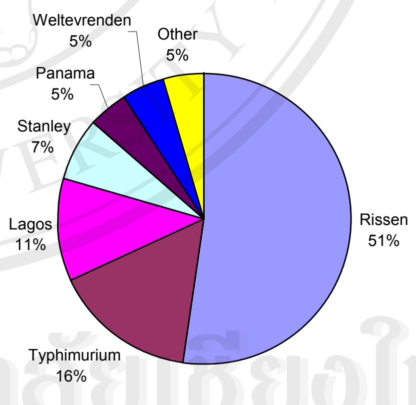


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Figure 13: Distribution of the most common *Salmonella* serovars among the different samples

Table 18: Detail of *Salmonella* serotypes isolated from the environment and pork samples

Farm ID	Environment*(Type of sample)			Pork**(Number of sample positives)		
	Before cutting	**During cutting**	**After disinfecting**	**Cut**	**Transported**	**Retail**
1		Panama (B,K)	Rissen (B)	Panama (3)	Rissen (3)	Panama (2)
				Rissen (1)	Panama (2)	Rissen (1)
2		Rissen (B)	Rissen (B)	Rissen (5)	Rissen (5)	Rissen (3)
				Anatum (1)	Krefeld (2)	Anatum (1)
				Krefeld (1)		
3			*F-67**** (B)	*F-67**** (5)	*F-67**** (6)	Lagos (1)
				Rissen (2)	Rissen (3)	
4		Typhimurium (S)	Typhimurium (B)	Rissen (2)	Rissen (2)	Tumodi (1)
		Weltevrenden (B)		Typhimurium (1)	Typhimurium (2)	Krefeld (1)
				Panama (1)	Tumodi (2)	
				Tumodi (1)	Panama (1)	
					Lagos (1)	
5		Rissen (K)		Rissen (1)	Rissen (1)	Rissen (3)
		Lagos (S)		Lagos (1)	Typhimurium (1)	
6		Rissen (B)	Rissen (B)	Rissen (4)	Rissen (5)	Rissen (2)

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Table 18 *(Countd.)*

Farm ID	Environment*(Type of sample)			Pork**(Number of sample positives)		
	Before cutting	**During cutting**	**After disinfecting**	**Cut**	**Transported**	**Retail**
7	Rissen (S)			Agama (1)	Rissen (3)	Typhimurium (2) Rissen (1)
8		Rissen (B) Rissen (S)		Krefeld (5) Rissen (2) Lagos (1)	Krefeld (5) Rissen (3)	Krefeld (8) Rissen (2)
9	Lagos (S)	Rissen (B,K)		Rissen (2) Lagos (2)	Krefeld (5) Rissen (1) Lagos (1) Typhimurium (1)	Lagos (3) Krefeld (2)
10		Rissen (K)	Rissen (B)		Rissen (4) Krefeld (1)	Rissen (2)
11		Typhimurium (B,S)		Typhimurium (10)	Typhimurium (10)	Rissen (3) Typhimurium (1)
12		Lagos (B)		Rissen (1)	Rissen (4) Weltevrenden (4)	Rissen (2) Weltevrenden (1)
13	Lagos (K)	Weltevrenden (S)	Krefeld (S) Rissen (B)	Typhimurium (2) Krefeld (1) Rissen (1)	Typhimurium (3) Krefeld (3) Rissen (2)	

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Table 18 *(Countd.)*

Farm ID	Environment*(Type of sample)			Pork**(Number of sample positives)		
	Before cutting	**During cutting**	**After disinfecting**	**Cut**	**Transported**	**Retail**
14		Lagos (B)	Typhimurium (K)	Typhimurium (2) Lagos (2) Gloucester (1) Agama (1)	Typhimurium (2) Lagos (1) Gloucester (2) Agama (1)	Agama (1)
15		Typhimurium (S)	Rissen (B)	Typhimurium (2) Lagos (1)	Typhimurium (3) Lagos (1) Agama (1) Gloucester (1)	Agama (2) Stanley (2)
16					Stanley (1)	
17		Rissen (B,S)	Rissen (B)	Rissen (8)	Rissen (8)	Rissen (9) Typhimurium (1)
18			Rissen (B,S)	Rissen (4)	Stanley (3) Rissen (2)	Rissen (5)
19		Stanley (B,S,K)	Rissen (H) Typhimurium (B)	Rissen (6) Stanley (1)	Stanley (5) Rissen (1)	Stanley (1) Rissen (1) Typhimurium (1)

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Table 18 *(Countd.)*

Farm ID	Environment*(Type of sample)			Pork**(Number of sample positives)		
	Before cutting	**During cutting**	**After disinfecting**	**Cut**	**Transported**	**Retail**
20			Rissen (B)	Rissen (6) Stanley (1) Typhimurium (1)	Stanley (2) Typhimurium (1) Anatum (1) Weltevrenden (1)	Stanley (2) Typhimurium (1)

* Environmental samples (B = Cutting board; S = Hands of staff; H = Shackle; K = Knife)
**Pork Samples (Number of sample positives)
***Salmonella spp.* in serogroup F-67.

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5. DISCUSSION AND CONCLUSIONS

5.1 Discussion

Pig carcasses, when entering the cutting process after overnight chilling at 4°C were contaminated with *Salmonella* in 13.3% of cases (Chantong, 2005). Contamination rates in the subsequent stages of 1) cutting in the slaughterhouse, 2) of transportation to sale outlets and 3) in retail products from there on had considerably increased. Respective rates at the 3 stages were 55.5%, 70.5% and 34.5%. Finisher pigs entered the slaughterhouse from their farms with already extreme high *Salmonella* rates, transport and lairage stress further increased the rates and contamination was continued during the slaughter process. Final washing and chilling of carcasses failed to reduce *Salmonella* contamination to acceptable low levels before carcasses were cut into meat portions products which entered markets. Temperatures in the chilling room, in the cutting room and in the transport truck were different (-5°C to 18°C in chilling rooms, 20°C to 25°C in the cutting area and 6°C to 8°C in the transport vehicle). These temperatures were higher than recommended by the company, being -10°C, 18°C and 4°C, respectively. Because salmonellae can grow between 5°C and 47°C, the temperatures of meat and meat products are preferably kept under 5°C during storage and transport, to minimize growth of *Salmonella* (Broch *et al*., 1996; Lo Fo Wong *et al*., 2002). Thus the high presence of *Salmonella* determined in this study for meat products eventually offered at markets might have been on the one hand the result of temperatures which were not kept low enough, as from the cutting room.

Multiplication of bacteria also depends on the time for growth in the different processing stages. Thus, in this study times of “cut pork” were significantly shorter than times of “transport pork”. As contamination of meat products at transport was 70.5%; an increase of 15% over contamination at cutting, transport without doubt does impact the increment of salmonellae. Packaging of wholesale quantities of meats in plastic or foam trays and subsequent transportation are further candidate

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factors that can positively increase chances of further contamination of pork products by *Salmonella* at this stage of the marketing chain. This is contrary to findings reported by Berend *et al.* (1998a) in the Netherlands, who did not detect differences between the percentages of *Salmonella*-positive retail-ready-pork in butcher shops and supermarkets compared to those at the end of the cutting lines. In this study, samples collected in supermarkets came from separate meat parts such as bones, bellies, ribs, collars, loins, ground pork, shoulder meats, hams, and fillets; the variations in contamination in these parts mirrored those in the carcasses they were cut from. The procedure of cutting one carcass into a variety of different meats probably increases and spreads contamination on these parts.

Cross-contamination at cutting and packaging, apart from the subsequent carry-over effect of unhygienic conditions at transport, was the essential factor of contamination of meat parts. The high contamination rates of "cut products" clearly points to unhygienic handling during these processing stages. The sample collection process, both for meats during cutting and after transportation supports this identification of the cutting and the transport stages of processing as particularly unsatisfactory in regard to hygiene. As in each case several parts such as belly, loin, shoulder, fillet and neck from one carcass were pooled into one sample, the chance of identifying contamination was higher than when a single meat sample would have been investigated. This result is supported by the study of Berend *et al*. (1998a) who found that when surface area samples of meats were investigated, the chances of detecting contamination was higher than when carcass swabs were investigated.

Cutting done by more than one person increased the probability of cross-contamination. The amount of cross-contamination during cutting though is primarily influenced by the contamination levels of slaughter carcasses than by contamination from the environment: the higher the carcasses are contaminated, the more cross-contamination during cutting occurs (Berend *et al.,* 1997).

In this study, the prevalence of 34.5% of *Salmonella* in retail products was higher than the prevalence of 24% reported by Boonmar *et al.* (1997) in their study of 100 samples from retail shops or supermarkets in Thailand. Bangtrakulnonth *et al.*

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(1999) from investigations of processed pork products from retail markets and supermarkets also found largely variable *Salmonella* prevalences, ranging from 54.6% in meat balls, 13.2% in pork sausages, 17.7% in pork balls to 6.3% in other meat products. Moreover, these authors found no significant difference between contamination rates in meats from open/wet (retail) markets and from supermarkets. One reason for the higher *Salmonella* prevalences established in this study might be the type of product investigated. Pork meat associated with bones (de-boned, ribs) had higher contamination levels than meat from other parts of a pig, e.g. collar or ground pork.

Results of studies for *Salmonella* contamination differ widely between countries. In a study on the occurrence and epidemiology of *Salmonella* in European pig slaughterhouses, in one out of the 5 investigated countries no *Salmonella* contamination at all was detected, while the overall prevalence of *Salmonella* was 5.3% in 3485 post-slaughter samples (Hald *et al.,* 2003); this is lower than the prevalence of *Salmonella* contamination in Mexico, Belgium and in this study. In the study from Mexico, the contamination level in all samples was overall 91.8 % (Escartin *et al*., 1995). In the western part of Belgium, Botteldoom *et al.* (2003) determined contamination of *Salmonella* in pigs from 5 slaughterhouses at 37%, with high variations between slaughterhouses and also between sampling days in the same abattoir. This latter finding corresponds with this study, where the prevalence varied day by day, ranging from 0-100%, depending on the hour of sampling (increase of contamination with hours), or with the supplying farm as described by Berends *et al*. (1998b).

The proportion of *Salmonella*- positive environmental samples during cutting time of 25% was high compared to the time before and after cutting (3.0% and 16.0% respectively). Swanenburg *et al.* (2001) also found the highest prevalence of *Salmonella* during cutting in all their 3 investigated slaughterhouses.

In this study the most frequent contaminated samples were swabs from the cutting board (55%), followed by hands and knives (40% and 30%, respectively). No contamination was detected on the plastic curtain and on the shackles, these two

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points only were in short contact with the pig carcasses. There positive samples were from the chilling room at a low temperature (0-4°C)

Bottledoorn *et al.* (2003) and Hald *et al.* (2003) also demonstrated contamination of slaughterhouse environments before the start of any slaughter activity. 25% and 7.9% contamination rates clearly indicate incomplete or inefficient cleaning. With slaughtering, contamination levels increase during the day. Also in this study, *Salmonella* were present in slaughterhouses both before cutting and after cleaning was finalized. Some contamination is said to be unavoidable when slaughter work is conducted routinely (Berends *et al*., 1997).

In this study, most of the *Salmonella* serotypes found in the slaughterhouse environmental samples were also isolated from the pork products (cut, transported and retail pork products). Respective *Salmonella*-positive samples in the "cut pork" were positively associated with the number of *Salmonella* in "transported pork". Contamination was carried over from cutting over transportation to retail with particular unhygienic factors increasing contamination during transport, and cooling at retail decreasing contamination again. Lo Fo Wong *et al*. (2002) also point out that *Salmonella* contamination at the manufacturing and retail level of pork production essentially depends on the incoming raw materials.

The most frequent serogroup in pork and environment samples was group C. The two most frequently isolated serotypes from the animal-related samples were *S.* Rissen and *S.* Typhimurium; these were also the most prevalent serotypes in the environment of the slaughterhouse. Between sampling days, some variation in the prevalence of the most prevalent serotypes existed, pointing to differences on the farms of origin of the slaughtered pigs. The five most prevalent serotypes isolated from pork and from environmental samples of the slaughterhouse were *S.* Rissen (45.3%), *S.* Typhimurium (16.3%), *S.* Krefeld (10.6%), *S.* Stanley (6.3%) and *S.* Lagos (6.0%). *S.* Derby, associated with pigs in Thailand (Bangtrakulnonth *et al*., 2004a), was not detected in this study. The proportions of serotypes reported by Bangtrakulnonth *et al*. (2004a) for Thailand for a selection of investigated foods did differed between food products, with *S.* Anatum and *S.* Rissen occurring as the most

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frequent serotypes in non-pork raw foods. However, *S.* Rissen also was identified the most common serovar in subset studies of this total "pig chain investigation program". Dorn-In (2005) demonstrated the common occurrence of *S.* Rissen in finisher pigs at farms and Chantong (2005) at the slaughter of these pigs at the slaughterhouse.

In order to reduce contamination levels, common hygiene recommendations are to continuously clean equipment while slaughtering or processing meat ("cleaning in place") and to give personal hygiene highest attention. Such elementary hygienic measures are not implemented at the study slaughterhouse; contaminated carcasses rather are constantly being brought into cutting lines and interim cleaning and disinfection of surfaces and utensils is only done during work breaks and at the end of the working day. If implemented, these measures have shown to prevent about 10% of all cross-contamination that takes place during a working day (Berends *et al*., 1998a). Measures against cross-contamination at individual slaughter stages are of highest importance (Berends *et al*., 1997; Warriner *et al*., 2002; Hald *et al*., 2003; Botteldon *et al*., 2003). A comprehensive quality safety and assurance scheme like HACCP (Hazard Analysis Critical Control Point), or GMP (Good Manufacturing Practice) which also includes strong staff educational programs would increase the level of awareness regarding food hygiene in slaughterhouses and at retail level: even more effectively: Avoidance and/or reduction of Salmonella are at the core of such programs (Borch *et al*., 1996; Legnani *et al*., 2004; Van der Gaag *et al*., 2004a).

5.2 Conclusions

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Recent trends in global food production, processing, distribution and food preparation are creating an increasing demand for food safety research in order to ensure a safer global food supply for national, and, where applicable, for international markets. Many campaigns in this direction are initiated in Thailand. One of these projects is known as "Clean Food - Good Taste". In order to protect consumers as well as to promote good tourism in Thailand, the project has aimed at assuring good sanitation of all restaurants and street vendors in Thailand since 1989. The year 2004

in Thailand also was declared "Food Safety Year". Several food safety management programs including GMP, HACCP, and ISO9000 have been implemented with most large, small, and medium food industry enterprises (SMEs) in the country.

Results of this study on the *Salmonella* problem in pork products point out that major problem still remain. Considering that the study was done in an integrated pig production chain of an important food production company in Thailand, the problem in lesser "controlled" establishment may be even greater. Company "control" though apparently was incomplete throughout the production chain. The biggest problem was detected at farm level; unacceptable high *Salmonella* contamination rates in finisher pigs on their farms of origin from thereon were perpetuated throughout the slaughtering and meat cutting lines, ending up at sale level. Hygiene deficits during cutting, cooling and transportation of meats added to the problem. The important thing for food safety is to trace food products from production to consumption and backwards. The present study demonstrated that such traceability schemes are possible and valuable in Thailand. In order to develop a food chain system in the country, it is important to realize that the retail level is the last "check-point" at which contaminated end-products can be identified. All steps in a production chain though have to be integrated to derive a true "farm to fork" food safety system. *Salmonella* and other food-borne microorganisms stand out centrally in such monitoring systems. The importance of *Salmonella* present throughout the entire pork production chain, the high levels of *Salmonella* at different stages of this chain and the involvement of human-pathogen *Salmonella* serotypes clearly demonstrate the urgency to more focus on microbiological food contaminants.

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APPENDICES

Appendix A: Equipment and Materials

Apparatus and glass wares

1. Apparatus for dry sterilization (oven) or wet sterilization (autoclave) (memmert, Model BE 600)
2. Incubator, capable of operating at 37 ^{o}C ± 1 ^{o}C (memmert, Model BE 800)
3. Water bath or Incubator, capable of operating at 41.5 ^{o}C ± 1 ^{o}C (Termarks, Model B 8054)
4. Water bath, capable of operating at 44.5 ^{o}C to 47 ^{o}C (Termarks, Model B 8054)
5. Water bath or Incubator, capable of operating at 37 ^{o}C ± 1 ^{o}C (Termarks, Model B 8054)
6. Sterile loop, of diameter approximately 3 mm or 10 µl diameter, or sterile pipettes
7. pH-meter (EUTECH INSTUMENT, Model :Waterproof pH Testers)
8. Graduated pipettes or automatic pipettes
9. Petri dish
10. Scale with weight of 2,000 g capacity, sensitivity of 0.1g
11. Test or culture tube rack
12. Vortex mixer (VORTEX, Model G-560E)
13. Sterile scissor, scalpel and forceps
14. Bunsen burner
15. Plastic bags
16. Marker
17. Stomacher (TUL instrument, Model 22)
18. Stomacher bags
19. pH paper
20. Thermometer

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21. Glass slides

Culture media and reagents

1. Non-selective pre-enrichment medium: Buffered peptone water (Merck, Germany)
2. First selective enrichment medium: Rappaport-Vassiliadis medium with soya (RVS broth) (Merck, Germany)
3. Second selective enrichment medium: Muller-Kauffmann tetrathionate novobiocin broth (MKTTn broth) (Merck, Germany)
4. Solid selective plating-out medium
5. 4.1 Xylose lysine deoxycholate agar (XLD agar) (Merck, Germany)
6. 4.2 Brilliant green phenol red Lactose Sucrose agar (BPLS agar) (Merck, Germany)
7. Nutrient agar (Merck, Germany)
8. Triple sugar/iron agar
9. Urea agar
10. L-Lysine decarboxylation
11. Reagent for detection of β-galactosidase (paper discs)
12. Reagent for Voges-Proskauer(VP) reaction
13. Reagent for indole reaction
14. Semi-solid nutrient agar
15. Sterile distilled water
16. Physiological saline solution, 0.85%
17. *Salmonella* O Polyvalent A-E
18. *Salmonella* O Polyvalent F-67
19. *Salmonella* O Group A
20. *Salmonella* O Group B
21. *Salmonella* O Group C
22. *Salmonella* O Group D
23. *Salmonella* O Group E

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24. *Salmonella* Vi
25. *Salmonella* O4
26. *Salmonella* O5
27. *Salmonella* O6_1,6_2,7
28. *Salmonella* O7
29. *Salmonella* O8
30. *Salmonella* O9
31. *Salmonella* O10
32. *Salmonella* O15
33. *Salmonella* O19
34. *Salmonella* O20
35. *Salmonella* O27
36. *Salmonella* O34
37. *Salmonella* O46
38. Flagellar antiserum for *Salmonella*

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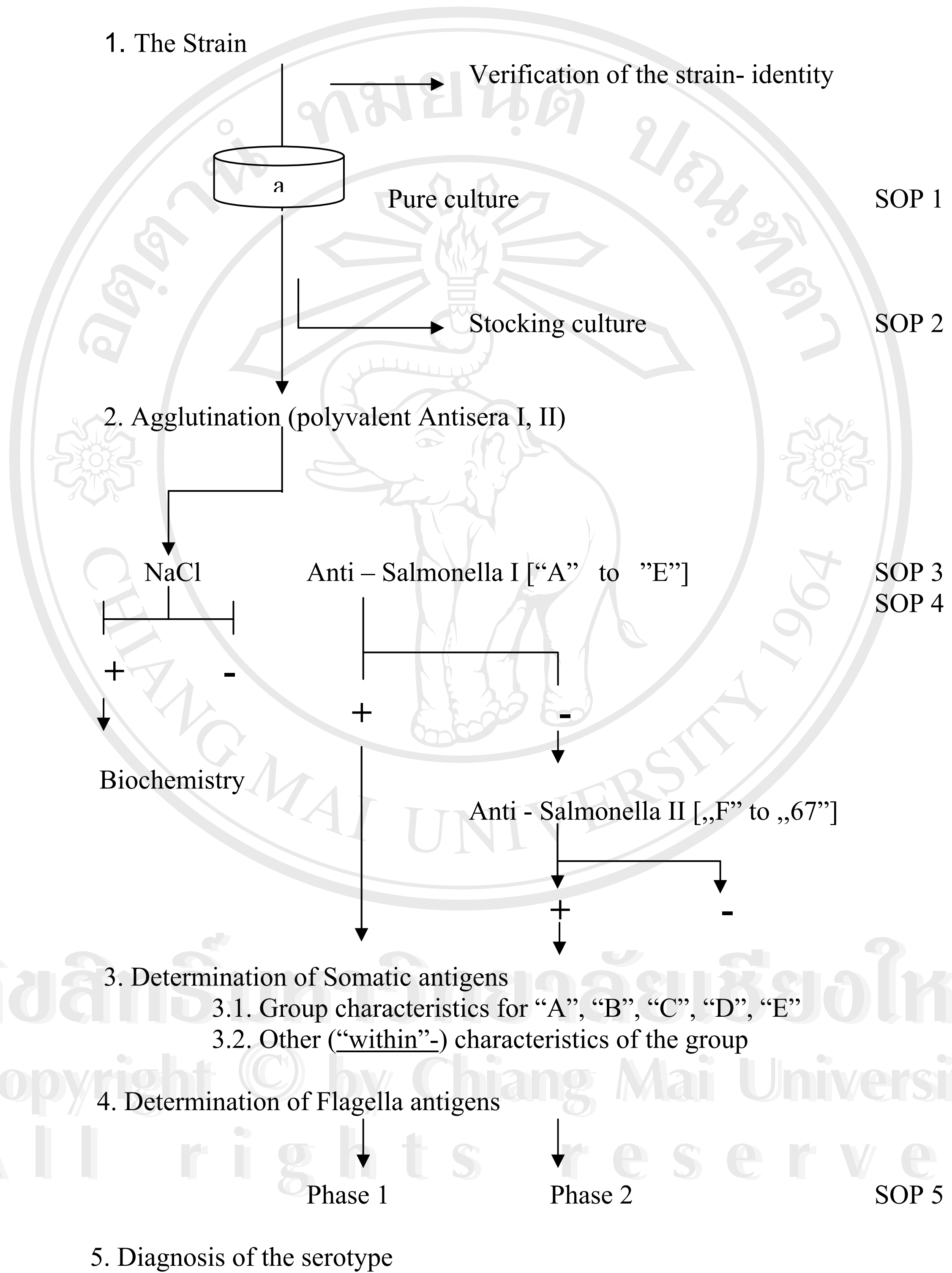
Appendix B: Standard Operation Prescription of Salmonellae Serotyping



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based on both, somatic and flagellar antigens.
If no diagnosis possible, give the tested antigens as "-"

Serotyping of somatic antigens

A particular serovar would be determined by an individual combination of several antigens (on the surface- O- or on the flagella –H-)

1. Determination of main groups (A, B, C, D, E)

For testing, use the instruction of the manufacturer of the antigen sera. (here: manufacturer SIFIN, Germany)

1. Starting with the observation "Polyvalent serum I (A-E): positive"
2. Sequence of testing (based on the percentage of the occurrence in Thailand)
3. Test the main groups until you get a positive reaction.
4. Stop testing if one group reacts positive; don't test for the other ones.

	+	-	Finished
2.1 Multi-*Salmonella* group B			
2.2 Multi-*Salmonella* group C			
2.3 Multi-*Salmonella* group E			
2.4 Multi-*Salmonella* group D			
2.5 Multi-*Salmonella* group A			

2. Second step: Characterization of the sub-groups using antisera "within" the main groups

- Gather all strains belonging to one particular main group, e.g. "B"
- Use the sequence of somatic antigen sera for the determination of Salmonella within the main groups as demonstrated below
- This following sequence is given in alphabetic order, not depending on the importance (i.e.: occurrence) of the main group
- In practical performance use the sequence B, C, E, D, A

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2.1. Within Group "A"

Probability: very low

Necessary antisera: Antiserum against O2

Procedure and sequence of testing: Group O2 covers all members of main group A

2.2. Within Group "B"

Probability: high

Necessary antisera: Antiserum against O4, O5, O27

Procedure and sequence of testing:

O4	+	+	+
O5	+	-	-
O27	n.n.	+	-
Diagnosis:	O 4,5,12	O 4,12,27	O 4,12

(n.n.): not necessary

Possible combinations:

O 4,5,12	O 4,12,27	O 4,12
O 1,4,5,12	O 1,4,1,27	O 1,4,12

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2.3. Within Group C

Probability: high

Necessary antisera: Antiserum against O7, O8, O20, $O6_1,6_2,7$ (= O6)

Procedure and sequence of testing:

O7	+	-	-	-
O8	n.n.	+	+	+
$O6_1,6_2,7$	n.n.	+	-	-
O20	n.n.	n.n.	+	-
Diagnosis:	O 6,7	O 6,8	O 8,20	O 8

(n.n.): not necessary

Possible combinations:

O 6,7	O 6,8	O 8,20	O 8
O 6,7,Vi			
O 6,7,14			

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2.4. Within Group D

Probability: low, but not zero

Necessary antisera: Antiserum against O9, O46, Vi, O27

Procedure and sequence of testing:

O9 (+) and Vi (-) determines D

In order of the in group antisera

O9	+	+	-	+	+
Vi	-	+	+	-	-
O46	-	n.n.	n.n.	+	-/+
O27	-	n.n.	n.n.	n.n.	+
Diagnosis:	O9,12	S.Typhi	S.Typhi S.Paratyph.C (spec. Tests)	O 9,46	O 9,12,46,27

(n.n.): not necessary

Possible combinations:

O 9,12 | O 9,46 | O 9,12,46,27

O 9,12,Vi

O 1,9,12

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2.5. Within Group E

Probability: high

Necessary antisera: Antiserum against O10, O15, O34, O19

Procedure and sequence of testing:

O10	+	-	-	-	+	-
O15	n.n.	+	+	-	n.n.	+
O34	n.n.	-	+	n.n.	n.n.	-
O19	-	-	n.n.	+	+	+
Diagn.	O 3,10	O 3,15	O 3,15,34	O 1,3,19	O 1,3,10,19	O1,3,15,19

(n.n.): not necessary

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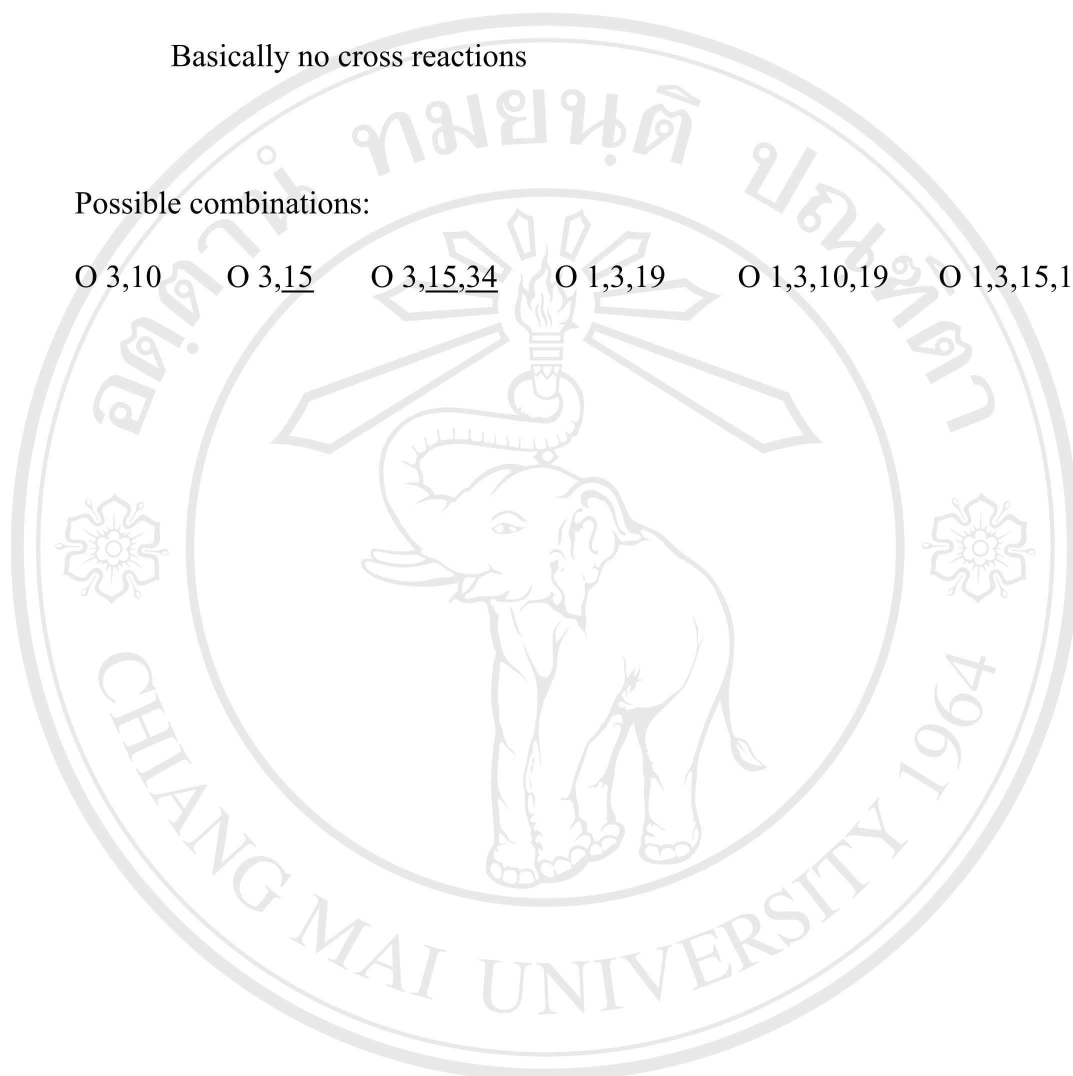
2.6. Groups O 11 (F) to O 67

Basically no cross reactions

Possible combinations:

O 3,10 O 3,15 O 3,15,34 O 1,3,19 O 1,3,10,19 O 1,3,15,19



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Determination of flagellar antigens

This procedure should be done after transfer of the isolate to the “motility”-agar while sticking to the strain you tested for somatic antigens.

1. Procedure

Having identified somatic main- and subgroups of the isolates,

- Gather all isolates belonging to one group, e.g. main group B
- Gather all isolates belonging to one subgroup, e.g. O 4,5,12, in case you start with Members of group B
- Look for identical sources of the isolates in order to get all information you can get in order to spare time and resources
- In every case ensure that the typing of flagellar antigens happens within the true group. Otherwise the whole procedure fails.
- Use simultaneously the Tables of LeMinor and Popoff (available at the Center), find out which combinations are possible in the case you are looking for. Use the list prepared in Berlin, which might serve as a help for orientation.
- Look for the possible H- antigen- combinations and start with the phase 1 antigens. Note: A strain must not express the antigens of phase 1.
- Prepare a list continuing the somatic antigen, note carefully every outcome of every test, also the negative outcome.
- Never test alone, testing and recording must be done carefully.

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2. Preparation and choice of strains for testing

- In the very beginning, use colonies from the slant, simultaneously transferring some material to a new stock
- Transfer material to the motility agar (recipe: SOP 5)

- If an expected combination (e.g. the 2nd phase), does not appear, the strain might be in the first phase only or vice versa. Proceed then with the challenge test
- Note, a strain must not basically be in the flagellar phase 1

3. Phase challenge procedure

A strain must be hindered to express the phase, we do already know. So the antigens are to be blocked by the particular H- antiserum to force it to develop the other phase.

E.g.:

- 1,4,12 has expressed the phase 1 antigen "I", but no reaction with H "1" and "2", which would mean S. Typhimurium.
- Take another motility agar, streak some I antigen on the surface and let the strain "swim". It will express the other flagella, which were not blocked.
- Not all antisera are capable of inducing the other flagellar phase. Each antiserum being capable is indicated for that purpose.

Determination of virulence antigens

See Vi antigens under O-antigens (group D)

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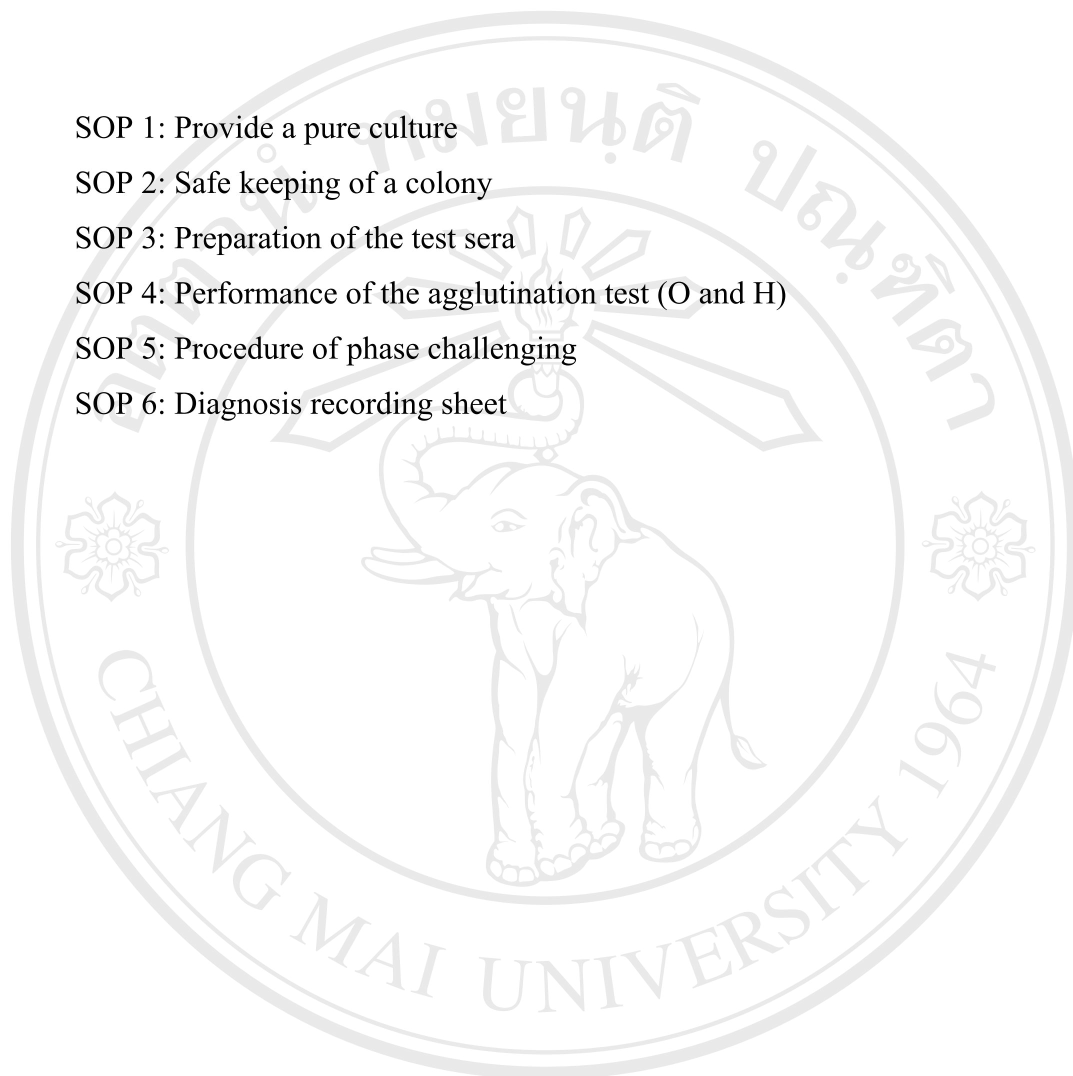
Appendix C: Standard Operation Prescriptions incl. Recipes



SOP 1: Provide a pure culture

SOP 2: Safe keeping of a colony

SOP 3: Preparation of the test sera

SOP 4: Performance of the agglutination test (O and H)

SOP 5: Procedure of phase challenging

SOP 6: Diagnosis recording sheet

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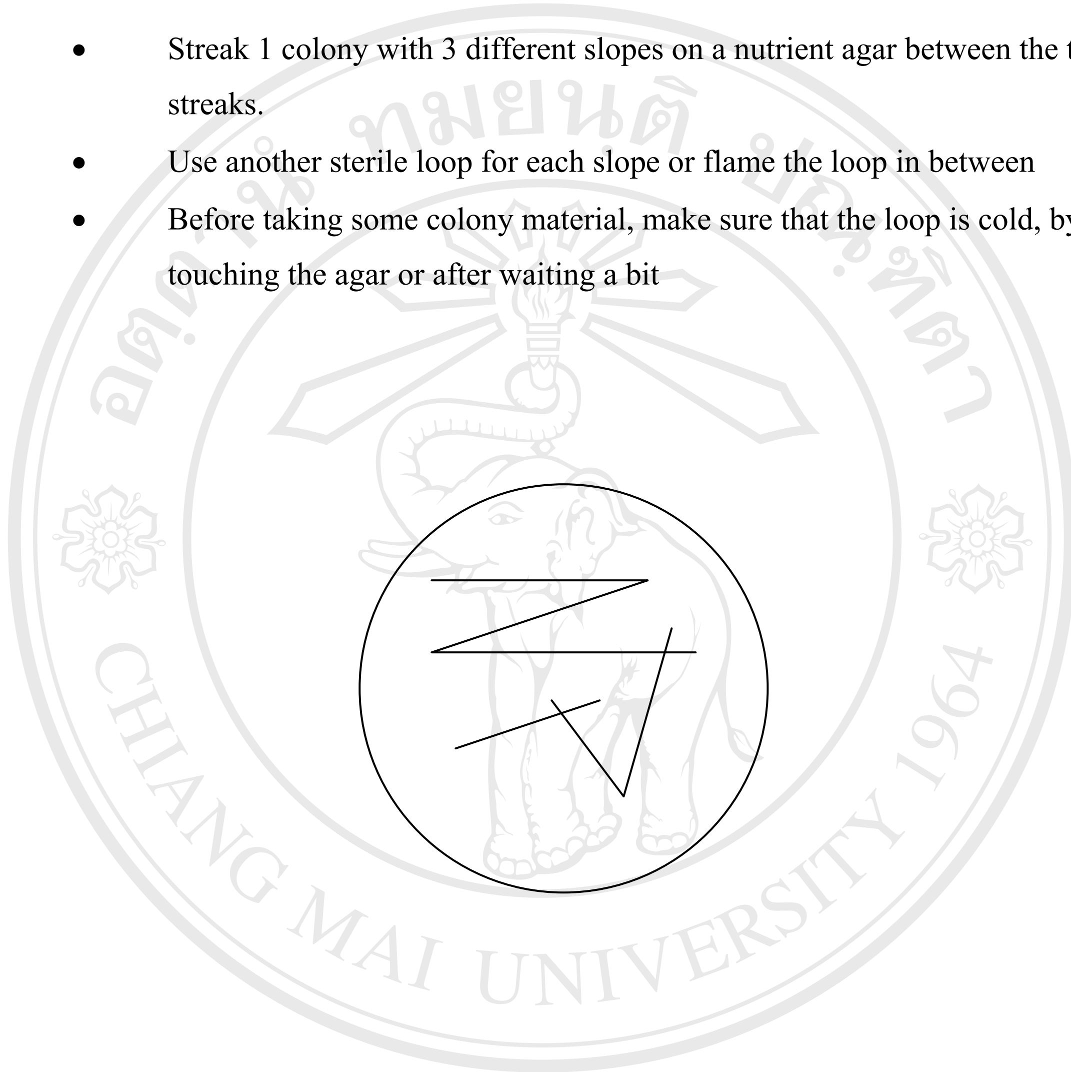
SOP 1: Provide a pure culture

- Streak 1 colony with 3 different slopes on a nutrient agar between the three streaks.
- Use another sterile loop for each slope or flame the loop in between
- Before taking some colony material, make sure that the loop is cold, by touching the agar or after waiting a bit



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SOP 2: Safe keeping of the colony

- Verify the pure character of your streak from yesterday (SOP 1)
- Choose 1 colony for testing with polyvalent I, then with polyvalent II
- Leave enough colony material from the very identical colony in case of a positive result
- Pick up material from this very same colony and streak it on the slant

Recipe slant for stocking the culture

- Nutrient agar as used normally
- Tubes with a lid
- Pour in liquefied agar (7 ml)
- Sterilise in the tube
- Let it cool in an oblique or nearly horizontal position
- If not possible: streak the strain on one sector of an agar plate (use at max. 4 sectors in order to avoid confusion, describe the sector carefully)

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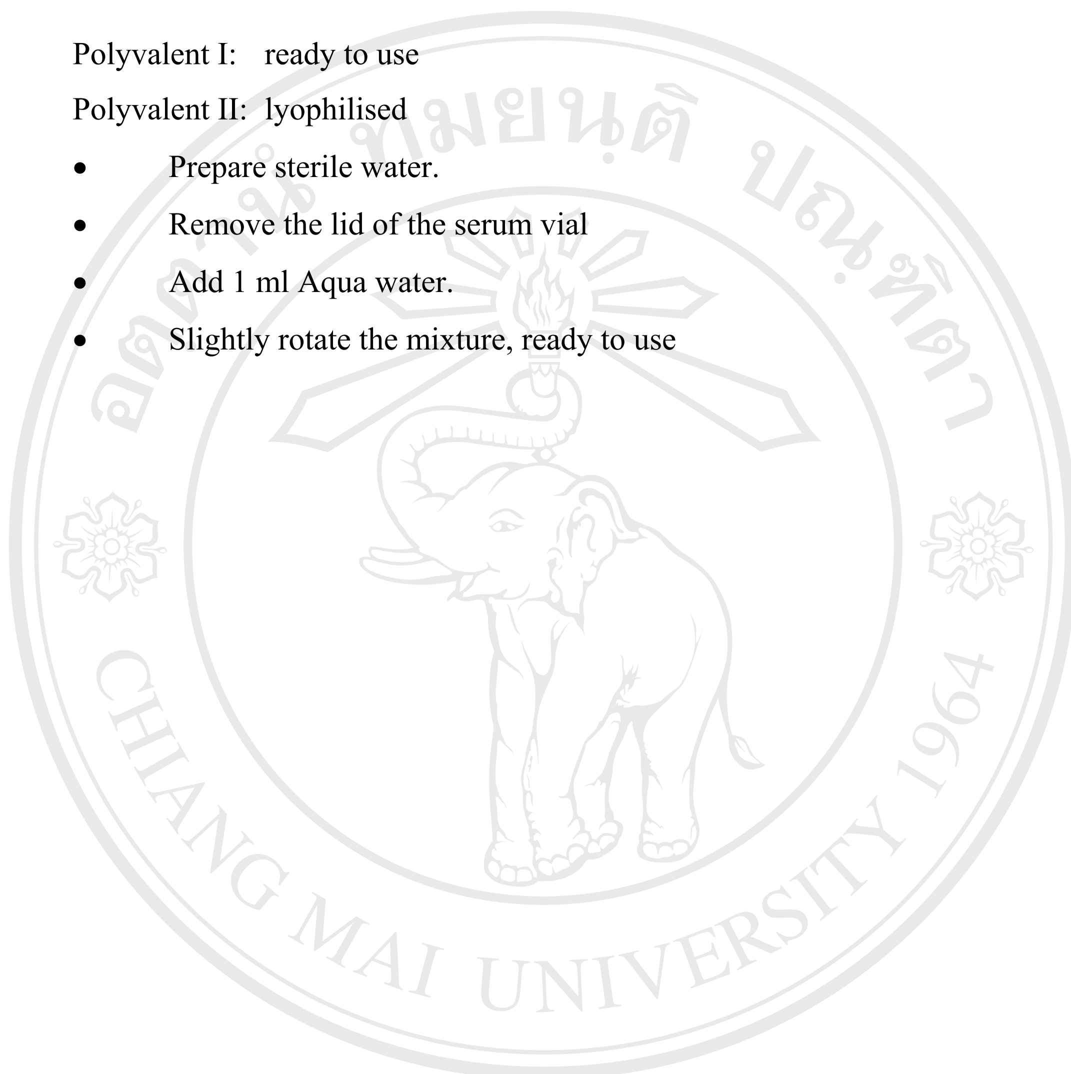
SOP 3: Preparation of test sera

Polyvalent I: ready to use

Polyvalent II: lyophilised

- Prepare sterile water.
- Remove the lid of the serum vial
- Add 1 ml Aqua water.
- Slightly rotate the mixture, ready to use



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SOP 4: Performance of agglutination

- Take a colony and make sure that some material is left for the slant
- Mix one drop of anti-Salmonella with some material of the suspicious colony by slowly drawing colony material into the drop with the aid of a loop

Results:

- Firstly, a slightly milky suspension develops
- Testing for O- antigens: There appears a distinct agglutination.
- Testing for H- antigens: More milky, there appears a "scar" on the surface

Interpretation of the results:

- Positive reaction: visible agglutination after 1-20 slight rotation. May happen instantly.
- Negative reaction: after 2 min. of rotation the liquid still remains milky

Troubleshooting:

- In case of negative results: repeat the test with the loop streaking across several colonies
- If nothing works: enrich several colonies again in a selective broth in every case: Make sure that your main group as a start for all is correct. So, in case of subgrouping, consider starting with the determined main group. If you are in the wrong main group, nothing works.
- if nothing works: Go into the selective enrichment broth again taking a streak across several colonies, streak on selective solid media, look for suspicious colonies and repeat the whole procedure

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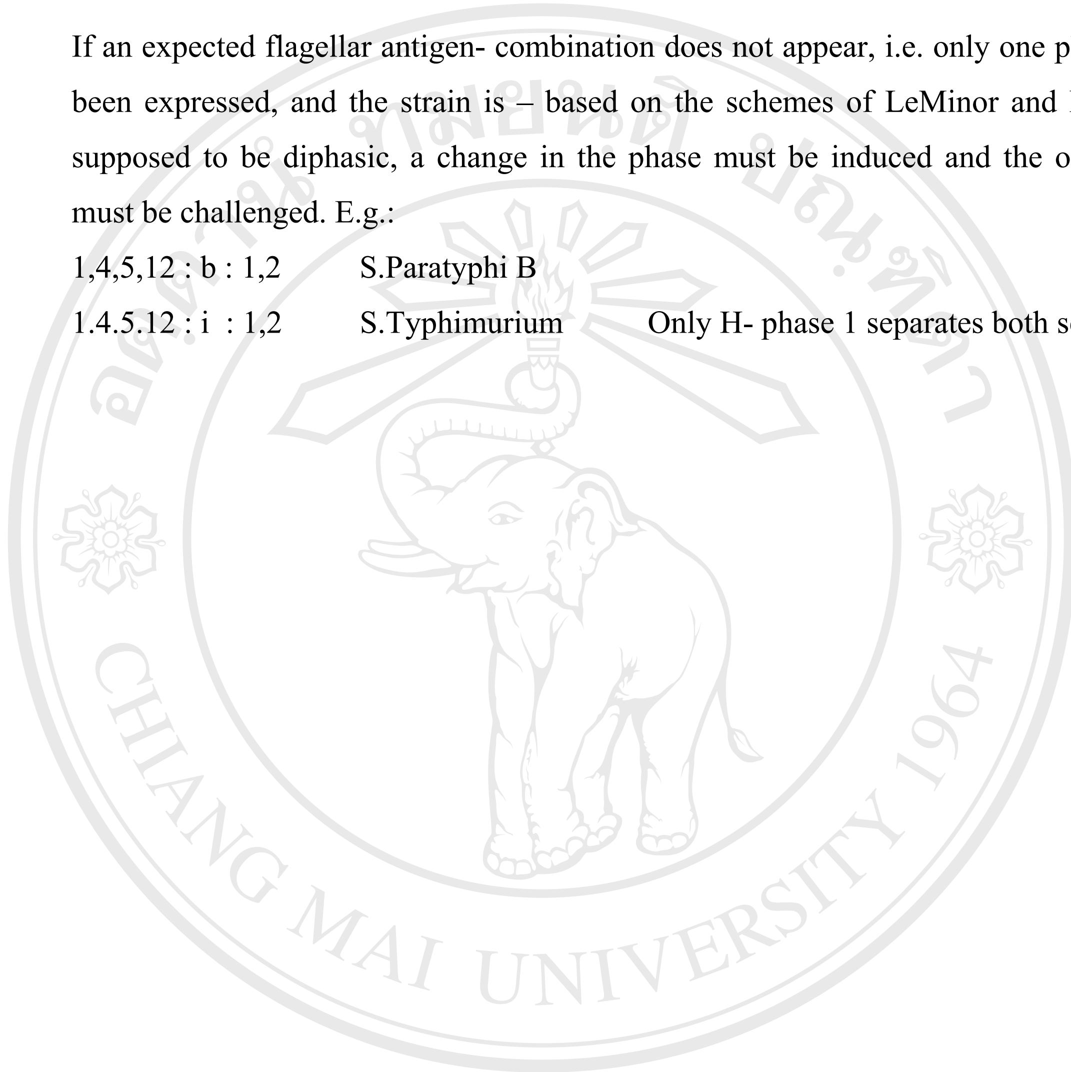
SOP 5: Procedure of phase challenging in case of expected diphasic serotypes

If an expected flagellar antigen- combination does not appear, i.e. only one phase has been expressed, and the strain is – based on the schemes of LeMinor and Popoff - supposed to be diphasic, a change in the phase must be induced and the other one must be challenged. E.g.:

1,4,5,12 : b : 1,2	S.Paratyphi B	
1.4.5.12 : i : 1,2	S.Typhimurium	Only H- phase 1 separates both serotypes



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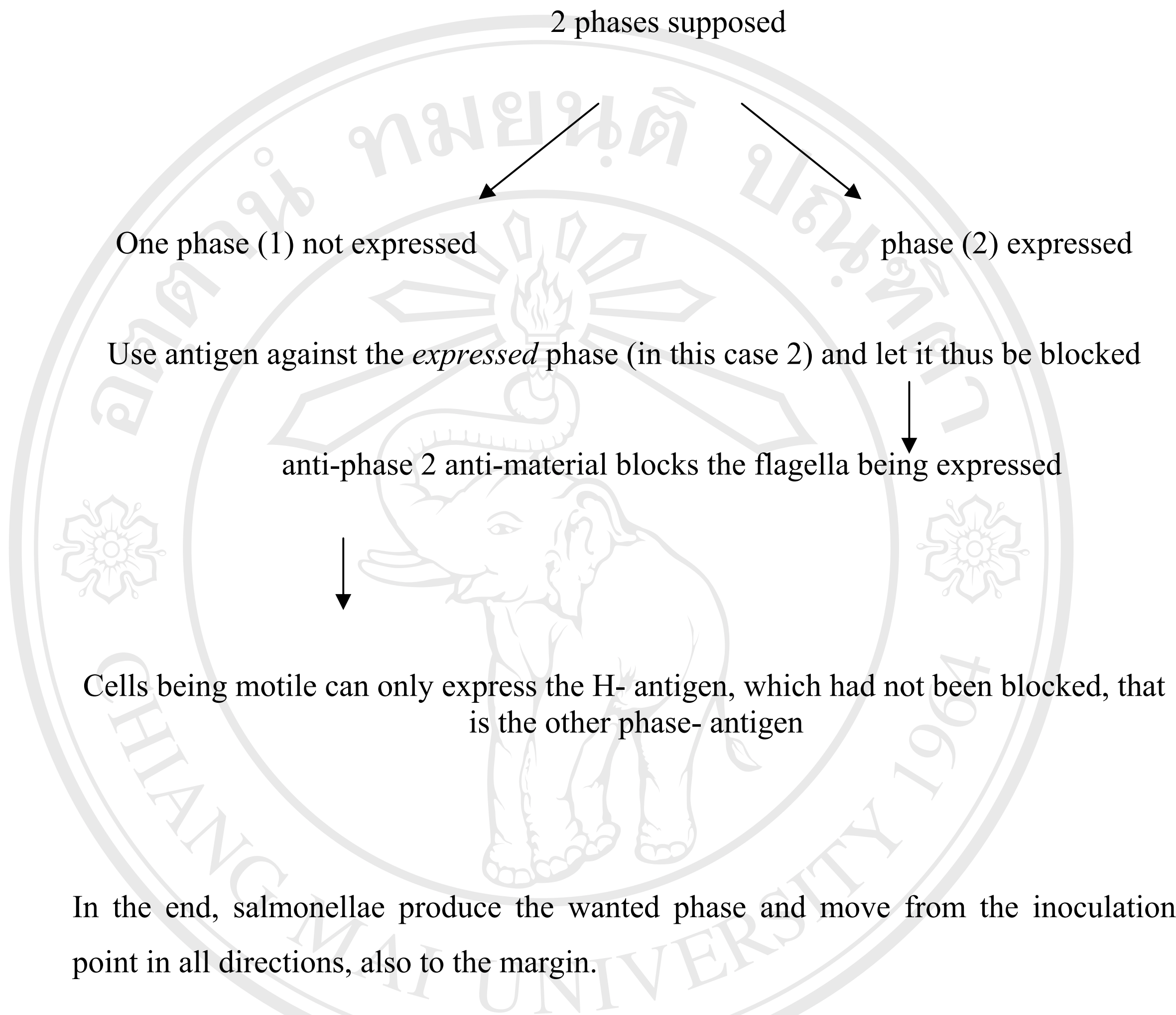
5.1. Phase challenge test



In the end, salmonellae produce the wanted phase and move from the inoculation point in all directions, also to the margin.

Cave: Serum must not contain antibodies against the phase that is expected to develop;

Serum must not contain O- antibodies against the very strain: In that case, R- variants may occur (Pietzsch)

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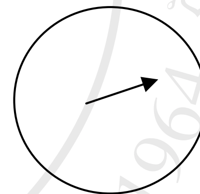
5.2. Procedure

- Superscribe the Petri dishes with the very strain number you have in use to avoid confusion because of the wrong strain number
- Use Petri dishes with motility agar (recipe see below)
- Give 0,1 ml of the particular antiserum on the surface of the motility agar
- Spread it with a glass spreader
- Place colony material right in the centre of the Petri dish
- Incubate face down at 35 – 37 min. for 16 – 20 h
- For testing, take material for the very margin of the Petri dish.
- The strain can be picked up at the margin of the Petri dish
- Flagella of the other phase should have developed, test for the other phase



5.3. Recipe for motility agar

- 16.5 g nutrient broth I (Sifin)
- 2o g nutrient agar I (Sifin)
- Water to 1000 ml, cooking
- Autoclaving at 121 °C for 12 min.
- Storage of the agar: Preferably in glass tubes in order to avoid exsiccation

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6. Sheet for data recording

Note: This sheet is an original and should not be copied. If you do, declare the copy as a copy in order to have the original up to date.

Project
Responsible
Begin of testing
Number of strains

Stage	Pig No	Strain-No.	Main O	Somatic sub	Flagella Phase 1	Flagella phase 2	Phase challenge	Diagnosis
1								
2								
3								

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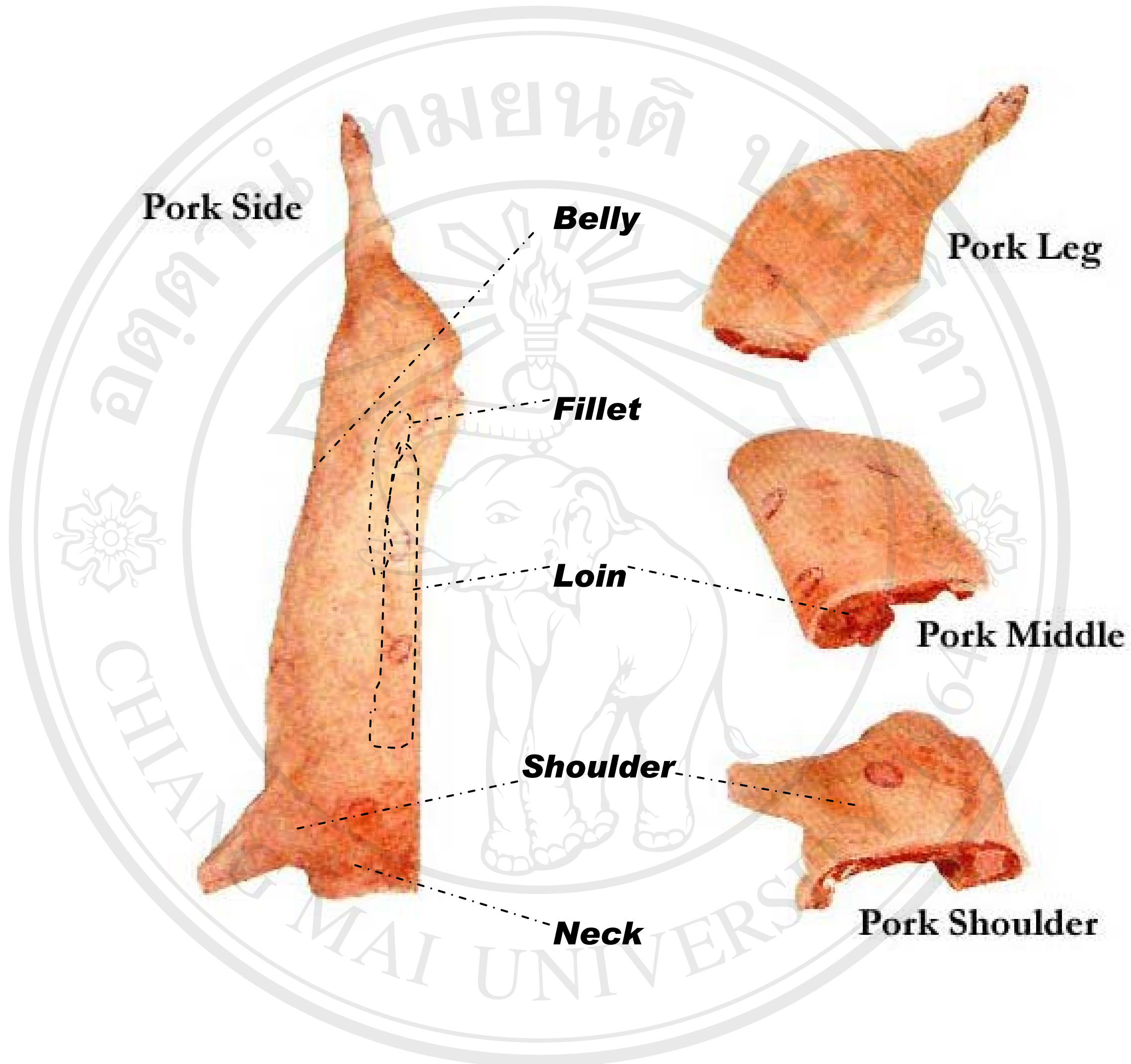
Appendix D: Position of sample collection



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Appendix E: Map of Chiang Mai province, Thailand

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Appendix F: Number of swine slaughtered by region, 1994-2003

Year	Northern	North - Eastern	Central Plain	Southern	Whole Kingdom
1994	887,458	812,211	1,602,492	782,294	4,084,455
1995	894,073	789,809	1,458,746	769,176	3,911,804
1996	963,055	702,785	1,355,536	702,303	3,723,679
1997	905,478	750,759	1,453,098	725,231	3,834,566
1998	985,584	737,267	1,410,792	697,705	3,741,348
1999	880,422	721,210	1,453,953	587,669	3,643,254
2000	940,590	761,301	1,395,071	568,281	3,665,243
2001	1,178,287	968,500	1,720,173	587,382	4,454,342
2002	1,030,392	786,055	1,626,968	529,030	3,972,445
2003	897,951	860,648	1,763,681	554,869	4,167,149

Source: Department of Livestock Development

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Appendix G: Import and export values (Bath) in pigs and pork products of Thailand, 2003-2005

Month	2003		2004		2005	
	Import	Export	Import	Export	Import	Export
January	15,868,205	86,835,772	6,629,068	87,693,157	6,028,877	76,568,564
February	10,240,313	76,692,624	6,414,343	79,981,970	4,797,585	111,215,063
March	16,676,803	97,380,729	4,264,398	89,322,739	7,875,878	130,436,829
April	5,614,277	109,529,286	6,132,637	73,018,163	6,209,409	119,333,013
May	11,961,193	81,951,179	7,482,295	80,198,198	12,872,115	145,439,043
June	7,560,168	88,600,814	14,655,567	83,927,228	23,566,031	149,044,969
July	14,917,379	68,105,653	10,474,822	89,331,856		
August	11,820,724	104,537,656	7,973,073	143,553,358		
September	15,196,480	129,453,044	9,183,616	125,805,600		
October	10,854,420	91,877,685	360,250	594,600		
November	10,074,023	103,567,771	8,459,125	124,822,930		
December	9,457,445	140,889,337	7,936,893	109,760,917		

Source: Department of Livestock Development

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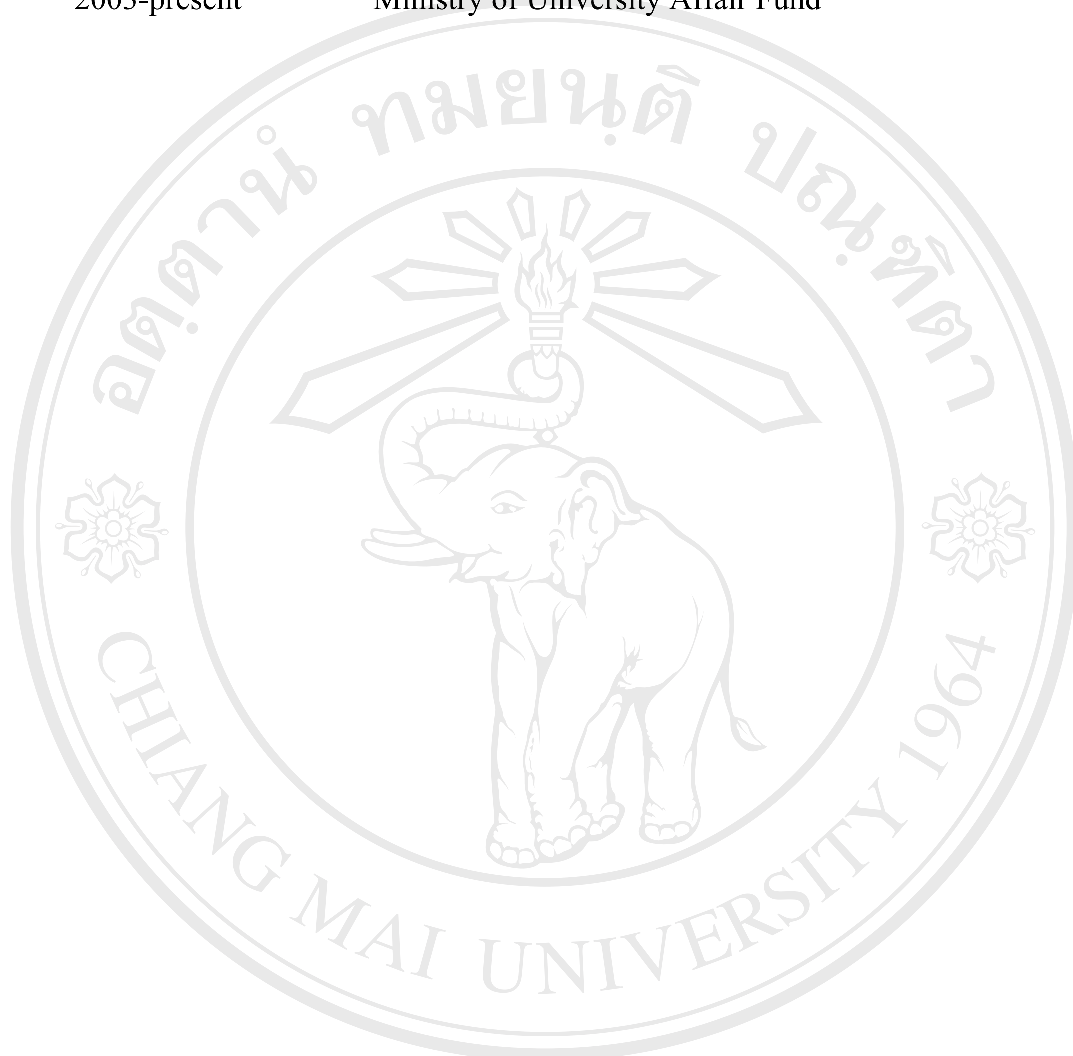
Awards/Scholarship

2003-present Ministry of University Affair Fund



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DECLARATION

I, the under signed, declare that the thesis is my original work and has not been presented for a degree in any university.

Name	ARSOOTH SANGUANKIAT
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Date of Submission	*September 19, 2005*

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