UNIVERSITI PUTRA MALAYSIA

DETECTION OF *SALMONELLA* IN POULTRY USING CONVENTIONAL CULTURE METHODS AND POLYMERASE CHAIN REACTION TECHNIQUE

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BY

ABDOALWAHAB M. M. MASUD KAMMON

A project paper submitted in Fulfillment of the requirements for the degree of Master of Veterinary Medicine in the Faculty of Veterinary Medicine

Universiti Putra Malaysia

2003

DEDICATION

I would like to dedicate this work to my parents, my wife, my sons Mohamed and Muhanad, and my brothers and sisters.

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine, Universiti Putra Malaysia in Partial fulfilment of the requirements for the degree of Master of Veterinary Medicine

DETECTION OF *SALMONELLA* IN POULTRY USING CONVENTIONAL CULTURE METHODS AND POLYMERASE CHAIN REACTION TECHNIQUE

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Supervisor: Assoc. Prof. Dr. Saleha Abdul Aziz

A study was carried out to evaluate three culture media and PCR for the detection of *Salmonella spp*. to improve *Salmonella* monitoring program. A total of 109 samples were collected from two farms. Sixty four samples were collected from farm A. These included 16 cloacal swabs collected from broilers before slaughtering, 18 intestinal swabs and 20 caecal swabs collected from broilers after evisceration, and 10 cloacal swabs collected from village chickens. Forty five samples were collected from farm B, which included 15 cloacal swabs from each of village chickens, turkeys, and guinea fowls.

Samples were pre-enriched in BPW and investigated by plating them on XLT4 agar after enrichment in selenite cystine broth, BPLS agar after enrichment in Rappaport-Vasilliadis broth, and DIASALM directly after pre-enrichment in BPW. Suspected positive colonies were confirmed biochemically and serologically. DIASALM and BPLS agar were comparatively evaluated against XLT4 agar as the "gold standard" using Kappa statistic to determine the level of agreement between them.

A total of 27 (24.77%) *Salmonella* were detected from the 109 samples. Isolation rates for XLT4, DIASALM, and BPLS were 20.20% (22 out of 109), 17.43% (19 out of 109), and 13.8% (15 out of 109), respectively. The sensitivity and agreement (Kappa statistic) with the "gold standard" for each evaluated detection method were: 70.4% and 0.69 (substantial) for DIASALM and 55.56% and 0.58 (moderate) for BPLS.

For the detection of *Salmonella spp*. by PCR, bacterial chromosomal DNA was extracted by boiling. Amplicons (429 bp) and (284 bp) derived from primers to the genomic random fragment (primers ST11 and ST15) and *inv*A genes (primers 139 and 141) respectively, were confirmed as *Salmonella* specific on ethidium bromide-stained agarose gels. Using PCR assay *Salmonella* was detected 24% (13 out of 54) and 13% (7 out of 54) in broilers in farm A using primers ST11-ST15 and 139-141, respectively. Poultry species in farm B were negative for *Salmonella* by

PCR. A specific primer was used for the detection of *Salmonella enteritidis*. None of *Salmonella* detected was *Salmonella enteritidis*.

This study concluded that XLT4 agar is the most sensitive medium and is very specific for the isolation of *Salmonella* from chicken feces. DIASALM is a good medium for the isolation of *Salmonella*. The inability of PCR to successfully detect *Salmonella* specific products from all the samples that were positive for isolation is not clear. However, this would be partly explained by the presence of inhibitor factors in the DNA preparations. In addition, the primer set ST11-ST15 used in this study has not before been tested on cloacal swabs and fecal samples from poultry. Perhaps, with improved DNA extraction method may overcome the inhibitory problem and also low yield of DNA. PCR should be used together with cultivation for the detection of *Salmonella* especially when the serovar is to be determined.

ABSTRAK

Abstrak daripada kertas projek yang dibentangkan kepada Fakulti Perubatan Veterinar, Universiti Putra Malaysia adalah sebahagian daripada keperluan memenuhi Ijazah Sarjana dalam Perubatan Veterinar.

PENGENALPASTIAN *SALMONELLA* DALAM TERNAKAN AYAM MENGGUNAKAN KAEDAH PENGKULTURAN DAN TEKNIK TINDAKBALAS BERANTAI POLIMERASE

OLEH

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Kajian ini dijalankan untuk menilai tiga media pengkulturan dan pengenalpastian *Salmonella spp*. Menggunakan tindakbalas beranti polimerase (PCR) bagi memperbaiki pemantauan program *Salmonella*. Jumlah sampel sebanyak 109 diambil daripada due ladang. Enom puluh empat sampel diambil daripada ladang A. Ia meliputi 16 sampel swab kloaka diambil daripada ayam pedaging sebelum disembelih, 18 sampel daripada usus dan 20 swab sekum juga diambil daripada ayam pedaging selepas eviserasi dan 10 sampel swab kloaka diambil daripada ayam kampung. Empat puluh lima sampel diambil daripada ladang B di mana ia termasuk 15 sampel swab kloaka daripada setiap ayam kampung, ayam belanda dan ayam peru.

Sampel dimasukkan ke dalam air pepton bufer (BPW) sebagai media pra-pengkayaan dan kemudian di platkan ke atas agar XLT4 selepas

dikayakan dalam cecair Selenite Cystine, ke atas agar BPLS daripada pengkayaan cecair Rapapport-Vasilliadis dan ke atas DIASALM agar secara terus daripada BPW. Koloni positif dikenalpasti melalui ujian biokima dan serologi. Keupayaan agar DIASALM dan BPLS diuji terhadap XLT4 agar yang merupakaan "gold standard" dengan menggunakan statistik Kappa.

Sebanyak 27 (24.77%) isolat *Salmonella* dikenalpasti daripada 109 sampel. Kadar pemencilan untuk XLT4, DIASALM dan BPLS agar mesing-mesing adalah 20.20% (22 daripada 109), 17.43% (19 daripada 109) dan 13.8% (15 daripada 109). Kepekaan dan persetujnan (statistik Kappa) dengan "gold standard" adalah : 70.4% dan 0.69 (agak tinggi) bagi DIASALM dan 55.56% dan 0.58 (sederhana) bagi BPLS.

Untuk pengenalpastian Salmonella spp. oleh PCR, kromosom DNA bakteria diestrak secara pendidihan tak langsung. Amplikon (429 bp) dan (284 bp) diperolehi daripada primer kepada serpihan genom secara rawak (primer ST11 dan ST15) dan dalam gen *inv*A (primer 139 dan 141), dipastikan sebagai *Salmonella* yang spesifik atas gel agarosa yang diwarnai dengan etidium bromida. Dengan PCR, 24% (13 daripada 54) dan 13% (7 daripada 54) *Salmonella* dikenalpasti pada ayam pedaging di ladang A dengan menggunakan primer ST11-ST15 dan 139-141. Spesis ternakan ayam dalam ladang B adalah negatif bagi *Salmonella* apabila ujian PCR dijalankan. Primer yang tertentu digunakan sebagai pengenalpastian *Salmonella enteritidis*. Tiada isolat *Salmonella* yang dikenalpasti sebagai *Salmonella enteritidis*.

Dapat disimpulkan daripada kajian ini bahawa agar XLT4 adalah media yang paling peka dan merupakan ia media yang sesuai untuk pemencilan *Salmonella* daripada najis ayam. DIASALM juga didapati media yang baik untuk pemencilan *Salmonella*. Ketidakbolehan ujian PCR untuk mengenalpasti *Salmonella* secara spesifik daripada semua sampel yang positif pada pemencilan mesih tidak jelas. Bagaimanapun ia mungkin disebabkan kehadiran faktor perencat dalam penyediaan DNA. Set primer ST11-ST15 yang digunakanan dalam kajian ini belum pernah diauji ke atas swab kloaka dan sampel najis daripada ternakan ayam. Kemungkinan dengan kaedah estrak DNA yang diperbaiki mungkin depat mengatasi masalah perencat dan DNA yang rendah. PCR digunakan bersama dengan kaedah kultur untuk pengenalpastian *Salmonella* terutama apabila serovar perlu ditentukan.

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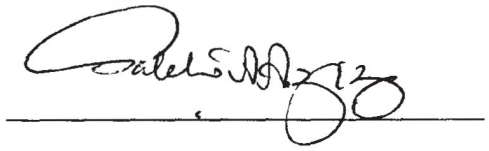
It is hereby certified that I have read this project paper entitled "Detection of *Salmonella* in Poultry using conventional culture methods and PCR technique" by Abdoalwahab M M. Masud Kammon and in my opinion it is satisfactory in term of scope, quality and presentations as fulfillment of the requirement for the degree of Master of Veterinary Medicine, VPD 5908 Project



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I hereby declare that the project paper is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any degree at UPM or other institutions.

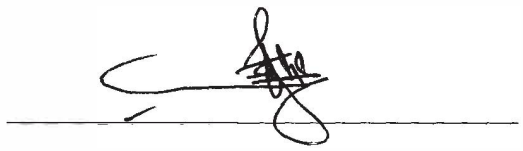

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TABLE OF CONTENTS

		Page
Dedication		2
Abstract		3
Abstrak		6
Acknowledgments		9
Approval		10
Declaration		11
Table of Contents		12
List of Tables		14
List of Figures		15
List of Abbreviations		16
CHAPTER		
I	INTRODUCTION	18
II	LITRATURE REVIEW	22
	Salmonella	22
	Isolation of *Salmonella*	23
	Pre-enrichment	24
	Enrichment media	25
	Selective plating media	27
	Polymerase Chain Reaction (PCR)	29
	Advantages and disadvantages of PCR	30
	DNA extraction procedures	31
	Detection of *Salmonella* by polymerase chain reaction (PCR)	31
	Control of *Salmonella*	37
III	MATERIALS AND METHODS	39
	Collection of samples	39
	Isolation and identification	40
	Statistical analyses	42
	Extraction of bacterial chromosomal DNA	44
	Detection of *Salmonella* using PCR	44
	Primers for PCR	44
	PCR assay	45
	Detection of PCR products	46
IV	RESULTS	47
	Detection of *Salmonella* by cultivation	47
	Detection of *Salmonella* by PCR	57
	Optimization of PCR assay	57
	Sensitivity of PCR assay	57
V	DISCUSSION	63
VI	CONCLUSION	66

REFERENCES 68
APPENDIX I 75
APPENDIX II 78
APPENDIX III 79
VITA 80

LIST OF TABLES

TABLE		PAGE
1.	Primers used to detect of *Salmonella* species by PCR.	34
2.	*Salmonella* bacteriological testing in Malaysia.	38
3.	Number and type of samples taken from each farm.	39
4.	Evaluation of a method using a 2×2 table.	42
5.	Primers used for the detection of *Salmonella* at genus level and serovar level (*Salmonella enteritidis*).	45
6.	Isolation of *Salmonella* from broilers in farm A.	47
7.	Isolation of *Salmonella* from different poultry species in farm B.	48
8.	Isolation of *Salmonella spp.* by different media.	48
9.	Comparison in performances of DIASALM and BPLS standardized against XLT4.	50
10.	Comparison in test performance of DIASALM and BPLS against XLT4 at (95%) confidence interval.	50

LIST OF FIGURES

FIGURE		PAGE
1.	Organization of genes in SPI1 at centisome 63.	33
2.	Diagramatic representation showing the genetic organization of the sefABC operon and location and orientation of the oligonucleotide primers (#808 and #809).	36
3.	Growth of *Salmonella* on XLT4 agar.	51
4.	Growth of *Salmonella* on BPLS agar.	52
5.	Growth of *Salmonella* on DIASALM.	53
6.	Biochemical reactions of *Salmonella* on LIA, Urea, TSI, and SIM.	54
7.	Comparison in performance parameters of DIASALM and BPLS against XLT4.	55
8.	Kappa statistic of test agreement between DIASALM, BPLS, and XLT4.	56
9.	Optimization of PCR products using DNA from *Salmonella entertidis* and three different primers.	59
10.	*Salmonella*- specific DNA products amplified by PCR using primer ST11-ST15.	60
11.	Electrophoresis of PCR products amplified by 139-141 primer.	61
12.	The result of using internal positive control.	62

LIST OF ABBREVIATIONS

BGA	Brilliant Green Agar
BGN	Brilliant Green with Novobiocin
BPLS	Brilliant Green Phenol-red Lactose Sucrose
BPW	Buffered Peptone Water
BS	Bismuth Sulfite Agar
CCDR	Canadian Communicable Diseases Report
CFU	Colony Forming Units
DIASALM	Diagnostic *Salmonella* Medium
DNA	Deoxyribonucleic Acid
ESWR	Euro Surveillance Weekly Report
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
HE	Hekton Enteric Agar
ISO	International Standards Organization
LIA	Lysine Iron Agar
MMWR	Morbidity and Mortality Weekly Report
MSRV	Modified Semi-solid Rappaport Vassiliadis
NBGL	Novobiocin Brilliant Green Glycerol Lactose

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RV	Rappaport Vassiliadis
SC	Selenite Cystine
SEM	*Salmonella* Enrichment Medium
SIM	Sulfide Indol Motility
SPI	*Salmonella* Pathogenicity Island
SS	*Salmonella* Shigella Agar
TSI	Triple Sugar Iron Agar
USAHA	United States Animal Health Association
VRI	Veterinary Research Institute
XLD	Xylose Lysine Desoxycholate Agar
XLT-4	Xylose Lysine Tergitol 4 Agar

CHAPTER I

INTRODUCTION

Salmonella is responsible for food borne outbreaks of human gastrointestinal disease, as well as heavy economic losses in poultry industry

There are three types of poultry diseases caused by *Salmonella*, namely the pullorum disease, fowl typhoid and fowl paratyphoid Among *Salmonella* serotypes in poultry, *Salmonella pullorum* and *Salmonella gallinarum* lead to high mortality due to septicemia They are rarely isolated from humans and have little public health significance This day in USA and many countries Salmonella pullorum has rarely been isolated and there has been no isolation of *Salmonella gallinarum* since 1988 in any type of poultry (USAHA, 2002) Fowl paratyphoid is caused by *Salmonella enteritidis*, and many other serotypes leading to omphalitis, peritonitis, and pericarditis in young chickens (Miek Desmidt *et al* , 1997)

Salmonella is often present in the intestinal tracts of birds, is readily acquired from feed and environmental sources, and contaminates body parts of fowl on the farm (Chambers *et al* , 1998, Caldwell 1995)

Poultry products are being identified as important sources of *Salmonella* that cause illness in humans During the last decade, there has been a world-wide increase in cases of *Salmonella enteritidis* infection in

poultry, causing an increasing number of human gastroenteritis cases (Miek Desmidt *et al.*, 1997).

In 2000, a total of 32,021 cases of *Salmonella* isolates were reported in United States. Of the 2,449 known *Salmonella* serotypes, the two most commonly reported in 2000 were *Salmonella typhimurium* and *Salmonella enteritidis* (MMWR). Sixty eight confirmed cases of *Salmonella enteritidis* PT 14b have been reported in UK since September 2002 (ESWR). In Canada, during the first quarter of 2000 (1 January to 30 April), 134 cases of travel-related gastroenteritis were identified. Seventy one (53%) were cases of *Salmonella enteritidis* infection. These cases were related to travel overseas, especially to warmer climates. However, only one case of the 2700 travel-related gastroenteritis in Canadians was reported to be visit Malaysia during that period (CCDR).

In Malaysia, there are several reports on the prevalence of *Salmonella*. A significant increase of cases of *Salmonella enteritidis*, both in humans and poultry has been reported (Longanathan and Maznah 1994). Mokhtar *et al*. (1996), in his report on the serotypes found in animals and livestock products and feeds in Malaysia for the period from 1991 to 1995, found that 2170 serotypes of *Salmonella* was frequently isolated from poultry (26.84%) and *Salmonella enteritidis* accounted for 36% of the total isolates from poultry. This increase of cases of *Salmonella enteritidis* in animals was accompanied with increase of cases of *Salmonella enteritidis* in humans during the period from 1989 to 1994 (Rohani *et al*., 1995). These results

indicate that animal products are very important sources of salmonellosis in humans

The primary motivation for controlling *Salmonella* infections in poultry was to reduce disease losses in poultry flocks Public health concerns, political pressures and consumer demands have made prevention of food-borne *Salmonella* transmission of disease to humans an urgent priority for poultry producers (Dhillon *et al.*, 1999). Since the prevention of *Salmonella* infection is very important for poultry health and for the food industry, and this prevention can be achieved only by good monitoring and screening programs Therefore, poultry industries have to routinely monitor *Salmonella* to assess contamination risks in their production as well as processing chain and it is desirable that the monitoring method can be applied to large numbers of samples at low cost. Although there are many forms for monitoring of salmonellosis, such as bacteriology and serology, it is not a simple decision on which form of monitoring is the most appropriate for poultry flocks (Davies *et al* , 1997)

Salmonella control program in Malaysia and ASEAN region still are not highly structured as in Europe or USA. Except for Singapore, which requires *Salmonella* free certificate prior to entry into the country, the other ASEAN countries do not impose any specific conditions for poultry importation (Loganathan and Maznah 1994). In addition, poultry industry in Malaysia need to be aware of emerging diseases to ensure that the industry can meet the challenges of either the Agreement on Agriculture of the World

Trade Organization (WTO) or the ASEAN Free Trade Agreement (AFTA), which to be fully implemented in year 2003 (Hussein 2000). In Malaysia, with the exception of the mandatory testing for export farms and voluntary testing of breeder farms, there are no special *Salmonella* control programs. Therefore, current testing program in Malaysia is required to be improved and more rapid and sensitive methods for the identification of *Salmonella* in various types of samples are needed. It is important to evaluate any new test before it is adopted in the local poultry industries for screening and monitoring of pathogen.

The objectives of the study were:

1. To determine the level of agreement between three culture methods for the isolation and identification of *Salmonella* from poultry.
2. To investigate the capability of polymerase chain reaction (PCR) assay using two different primers specific for the detection of *Salmonella* in clinical samples of poultry.

CHAPTER II

LITERATURE REVIEW

Salmonella

Salmonella was discovered in 1885 by Dr. D. E Salmon. *Salmonella* are gram-negative an aerobic non-sporeforming rods, and belong to the family *Enterobacteriacae*. According to the latest nomenclature, the genus *Salmonella* consists of only two species: *Salmonell .enterica* and *Salmonella bongori. Salmonella enterica* species are subdivided into six subspecies: *enterica, salamae, arizonae, diarizonae, houtenae* and *indica* (Grimont *et al*., 2000). Strains of *Salmonella* are classified into serotypes on the basis of O and H antigens in accordance with the Kauffman/White scheme. Currently 2,449 serovars are recognized (MMWR).

Acha and Szyfres (1987) classified *Salmonella* serovars into three groups based on their adaptation to either human or animals. Group one, includes *Salmonella typhi* and *Salmonella paratyphi* which causes typhoid fever and paratyphoid fever in humans. Group two, includes serovars which infrequently cause disease in humans but are more specific to cause disease in animals, such as *Salmonella choleraesuis*, *Salmonella Dublin*, *Salmonella sendai*, *Salmonella pullorum*, and *Salmonella gallinarum*. Group three, includes the other serovars in which *Salmonella typhimurium* and

Salmonella enteritidis are the most important, causing a typhoid-like disease in mice and gastroenteritis in humans. However, all members of the third group are pathogenic to both humans and animals.

Isolation of *Salmonella*

Food-poisoning *Salmonella* colonize the chicken gastrointestinal tract while other *Salmonella* serotypes, particularly those which produce systemic diseases such as *S. pullorum* and *S. gallinarum* poorly colonize the alimentary tract (Barrow *et al*., 1988). Brownell *et al*. (1969) reported that the caecum is the primary predilection site of *Salmonella* colonization. The cloaca is often commonly colonized (Chambers *et al*., 1998). The poultry have been known to carry many of *Salmonella* serotypes. It is more difficult to detect an asymptomatic carrier because such carriers only periodically shed the organism in the feces (Hirsh 1990).

The continued concern for monitoring poultry elicited development of several types of *Salmonella* sampling procedures. These include different sampling methods which have been compared and used to estimate the prevalence of *Salmonella* among flocks, such as sampling of caecal contents and caecal tonsils (Brownell *et al*., 1969), poultry tissues and cloacal swabs (Stephen *et al*., 1975; Olga *et al*., 1979), litter and drag swabs (Kingston 1981), either wet or dry drag swabs (Byrd *et al*., 1997), protective foot covers (Caldwell *et al*., 1998), crop swabs (Chambers *et al*., 1998), and feces and pairs of socks (Gradel *et al*., 2002).

The recovery of *Salmonella* depends on many factors such as the type, amount and sources of samples, the use of pre-enrichment and the type used, the enrichment media and incubation time, and the selective plating media (Waltman 2000)

Pre-enrichment

The optimal recovery of *Salmonella* can be achieved by using pre-enrichment in a non-selective broth, and followed by selective enrichment in broth and streaking on a selective agar media This procedure is recommended by several international organizations (FAO 1979, and FDA 1992) and authors (Cox *et al* , 1981, Goossens *et al* , 1984, June *et al* , 1996)

It has been recognized that the isolation of *Salmonella* is probably best achieved by a combination of pre-enrichment followed by selective enrichment (D'Aoust *et al* , 1981), and the buffered peptone water (BPW) as a pre-enrichment medium, is the medium of choice (Henk van der Zee and Huis in't Veld 2000)

The numbers of *Salmonella* in feces or cloacal swab from asymptomatic birds are usually very low, and it is necessary to use pre-enrichment media to assist the isolation The incubation of pre-enrichment for 18-24 hours at 35-37 ^{o}C is recommended After incubation, an aliquot of the pre-enrichment broth is transferred into 10 ml of selective enrichment