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

ABDOAL WAHAB M. M. MASUD KAMMON

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

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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ABDOALWAHAB M. M. MASUD KAMMON

2003

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-Vasiliadis 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 *invA* 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
MENGUNGKAN KAEDAH PENGKULTURAN DAN TEKNIK
TINDAKBALAS BERANTAI POLIMERASE

OLEH

ABDOALWAHAB M. M. MASUD KAMMON

2003

Penyelia: Prof.Madya. Dr. Saleha Abdul Aziz


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 Rapaport-Vasiliadiis 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 merupakan "gold standard" dengan menggunakan statistik Kappa.

Sebanyak 27 (24.77%) isolat \textit{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 persetujuan (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 \textit{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 \textit{invA} (primer 139 dan 141), dipastikan sebagai \textit{Salmonella} yang spesifik atas gel agarosa yang diwarnai dengan etidium bromida. Dengan PCR, 24% (13 daripada 54) dan 13% (7 daripada 54) \textit{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 \textit{Salmonella} apabila ujian PCR dijalankan. Primer yang tertentu digunakan sebagai pengenalpastian \textit{Salmonella enteritidis}. Tiada isolat \textit{Salmonella} yang dikenalpasti sebagai \textit{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. DIA8ALM 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 digunakan dalam kajian ini belum pernah diauji ke atas swab kloaka dan sampel najis daripada ternakan ayam. Kemungkinan dengan kaedah estrak DNA yang diperbaiki mungkin dapat mengatasi masalah perencat dan DNA yang rendah. PCR digunakan bersama dengan kaedah kultur untuk pengenalpastian *Salmonella* terutama apabila serovar perlu ditentukan.
ACKNOWLEDGMENTS

I thank Allah for the support he has provided me during this research effort. I would like to express my gratitude and thanks to my supervisor, Dr. Saleha Abdul Aziz and my co-supervisor Dr. Abdul Rahman Omar, for their valuable concern, guidance, advice, and support throughout this research. I would also like to express my appreciation to Dr. Latfah Hassan and Dr. Nadzri Salem for their suggestions and recommendations on the statistical analyses used in this study.

I would also like to express my gratitude to Puan Nor Zaleha from the Faculty of Food Science and Biotechnology for providing the primer-set ST11-ST15 and Salmonella enteritidis isolates as positive control.

I would also like to thank Dr. Mohd Khusahry, and Puan Nor Asia from MARDI for giving me the permission to collect my samples.

My sincere thanks are also extended to the entire faculty, post graduate students and technicians at Veterinary Public Health and Biologics Laboratories.

Finally, my appreciation and my utmost gratitude are expressed to my wife, for her support and encouragement and to my parents for their prayers.
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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DECLARATION

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.

Abdoalwahab M. M. Masud
Kammon
Date: 28/5/2003
TABLE OF CONTENTS

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

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

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGA</td>
<td>Brilliant Green Agar</td>
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<tr>
<td>BGN</td>
<td>Brilliant Green with Novobiocin</td>
</tr>
<tr>
<td>BPLS</td>
<td>Brilliant Green Phenol-red Lactose Sucrose</td>
</tr>
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<td>BPW</td>
<td>Buffered Peptone Water</td>
</tr>
<tr>
<td>BS</td>
<td>Bismuth Sulfite Agar</td>
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<tr>
<td>CDDR</td>
<td>Canadian Communicable Diseases Report</td>
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<td>CFU</td>
<td>Colony Forming Units</td>
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<td>DIASALM</td>
<td>Diagnostic <em>Salmonella</em> Medium</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
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<td>ESWR</td>
<td>Euro Surveillance Weekly Report</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>HE</td>
<td>Hekton Enteric Agar</td>
</tr>
<tr>
<td>ISO</td>
<td>International Standards Organization</td>
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<tr>
<td>LIA</td>
<td>Lysine Iron Agar</td>
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<tr>
<td>MMWR</td>
<td>Morbidity and Mortality Weekly Report</td>
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<td>MSRV</td>
<td>Modified Semi-solid Rappaport Vassiliadis</td>
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<td>NBGL</td>
<td>Novobiocin Brilliant Green Glycerol Lactose</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PFGE</td>
<td>Pulsed Field Gel Electrophoresis</td>
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<tr>
<td>RV</td>
<td>Rappaport Vassiliadis</td>
</tr>
<tr>
<td>SC</td>
<td>Selenite Cystine</td>
</tr>
<tr>
<td>SEM</td>
<td><em>Salmonella</em> Enrichment Medium</td>
</tr>
<tr>
<td>SIM</td>
<td>Sulfide Indol Motility</td>
</tr>
<tr>
<td>SPI</td>
<td><em>Salmonella</em> Pathogenicity Island</td>
</tr>
<tr>
<td>SS</td>
<td><em>Salmonella</em> Shigella Agar</td>
</tr>
<tr>
<td>TSI</td>
<td>Triple Sugar Iron Agar</td>
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<tr>
<td>USAHA</td>
<td>United States Animal Health Association</td>
</tr>
<tr>
<td>VRI</td>
<td>Veterinary Research Institute</td>
</tr>
<tr>
<td>XLD</td>
<td>Xylose Lysine Desoxycholate Agar</td>
</tr>
<tr>
<td>XLT-4</td>
<td>Xylose Lysine Tergitol 4 Agar</td>
</tr>
</tbody>
</table>
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 aerobic non-sporeforming rods, and belong to the family Enterobacteriaceae. According to the latest nomenclature, the genus Salmonella consists of only two species: Salmonella 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 °C is recommended. After incubation, an aliquot of the pre-enrichment broth is transferred into 10 ml of selective enrichment