

**DEVELOPMENT OF A SPECIFIC AND SENSITIVE
METHOD FOR DETECTION OF *SALMONELLA* IN
CHICKENS USING POLYMERASE CHAIN REACTION
TECHNIQUE**

by

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APPROVAL

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Declaration

The contents of this thesis are the work of the author. The thesis has not been previously submitted for the award of a degree to any other University.

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ABSTRACT

Conventional microbiological techniques used in the detection and identification of *Salmonella* infections in chicken samples are relatively lengthy. In Zambia today, the situation has even been made worse by the indiscriminate use of antibiotics. Detection of *Salmonella* from such treated birds has recently been very poor and hence the need to develop other quick and reliable systems in the identification of *Salmonella* infections in poultry in Zambia.

DNA detection with polymerase chain reaction (PCR) as a means of identifying *Salmonella* infection in chickens has been developed. Amplification of *Salmonella* was evaluated using the known nucleotide sequences within the *InvA* and *phoE* genes of *Salmonella typhimurium*. Targeted genes of *Salmonella* were successfully amplified from a single colony of the bacteria by applying the PCR technique. With the two pairs of primers (*InvA*, *phoE*), a 284-base pair (bp) and 365-bp fragments, respectively, from *Salmonella* gene could be amplified specifically. Specificity and selectivity of the primers (*InvA* and *phoE*) was tested on both *Salmonella gallinarum* and *Salmonella typhimurium* as well as on other 10 *Salmonella* DNA. It was interesting

to note that both *Salmonella gallinarum* and *Salmonella typhimurium* and other 10 *Salmonella* strains which were tested for specificity in PCR produced specific amplified bands whose molecular weight was same as that of *S. typhimurium* band obtained with either the *InvA* or *phoE* gene primers. On the other hand, other bacteria other than *Salmonella* bacteria did not generate any specific bands when applied in PCR using the *InvA* and *phoE* gene primers. The amplification of both the *InvA* and *phoE* genes of *Salmonella* by the single colony method confirmed that *Salmonella* did contain genes which were potential targets for PCR.

Once the specificity and applicability of the *InvA* and *phoE* gene primers had been confirmed, the *InvA* gene primers were then successfully used to detect both *S. gallinarum* and *S. typhimurium* DNA from experimentally infected chicks. Using the *InvA* primers, it was possible to detect *S. gallinarum* and *S. typhimurium* in 15 out of 20 (75 per cent) and in 16 out of 20 (80 per cent) organ samples respectively, barely 21 hours after experimental infection. PCR was therefore shown to be more sensitive in the detection of *Salmonella* than the conventional isolation methods as it was possible to detect in total 50 out of 150 (33.3 per cent) organ samples compared to 24 out of 150 (16

per cent) by bacterial isolation methods. Detection by PCR still appeared more sensitive even in late stages of infection as *S. gallinarum* DNA could be detected from samples which were negative for bacterial isolation on three, seven and 14 days after infection. The size of the amplified band was a 284-bp fragment.

Having succeeded in amplifying *S. gallinarum* from experimental infection, the PCR was then used to detect *Salmonella* in naturally infected chickens using the *phoE* gene primers. Results obtained show that the PCR was more sensitive in detecting *Salmonella* DNA from clinical samples as it was possible to detect *Salmonella* in 10 out of 17 (58.8 per cent) spleen samples compared to 7 out of 17 (41.1 per cent) by isolation in enriched media. The size of the amplified band was a 365-bp fragment.

PCR technique was also used for detection of *Salmonella* in chicken pen samples. When compared with conventional isolation methods, PCR was shown to be more sensitive as it was possible to detect *Salmonella* in 13 out of 48 (27.1 per cent) faeces compared with 9 out of 48 (18 per cent) by conventional bacterial isolation methods.

In order to enable detection of *Salmonella* organisms directly from chicken eggs, an attempt was made to inoculate the egg samples directly into the PCR mixture. By direct PCR amplification method, DNA could be amplified from 20 out of 45 (44.5 per cent) samples from chicken yolks. Detection by bacteria isolation methods on the other hand, was only possible in 6 out of 45 (13.3 per cent). The PCR was once again shown to be more sensitive when compared with conventional bacterial isolation methods. The size of the amplified band was a 365-bp DNA fragment. Direct PCR amplification of *Salmonella* gene from the yolk of chicken eggs and embryos is a new finding. Detection of *Salmonella* directly from chicken yolk samples is a great scientific contribution as the need for DNA isolation and purification was completely eliminated. The chicken yolk samples were found free of PCR inhibitors, a fact which makes detection of *Salmonella* by PCR from these samples more efficient and quicker. The total time required for the detection of *Salmonella* organisms from eggs using PCR technique was only 6 hours compared to 3 days by conventional bacterial isolation procedures.

The complete sequence of the *InvA* and *phoE* genes of *Salmonella* were firstly elucidated and compared with those of known *InvA* and *phoE* genes of *S. typhimurium*. Except for one base pair at position 259, all the other bases showed greater homology to the *InvA* genes of *S. typhimurium* indicating that the amplified band was a *Salmonella* gene. Similarly, the sequence of the *phoE* gene showed great homology to that of *S. typhimurium*. In the case of *phoE* gene, all base pairs were identical except for five at position No. 211, 212, 213, 214, and 235.

From the results obtained in this study, it is clear that:

1. A sensitive and faster method for the detection of *Salmonella* from poultry has been developed. The culturing of bacteria which normally takes 3 days was eliminated and the period required for identification of *Salmonella* infection by PCR was only a day.
2. There was no need for primary bacterial isolation from clinical samples in order to run the PCR test as DNA for PCR was obtained directly from infected chicken organs. Extraction of *Salmonella* DNA directly from infected chicken organs is also a new finding.

3. Direct PCR amplification of *Salmonella* from the yolk of chicken eggs and embryos is a new finding requiring further exploitation.

CHAPTER ONE

INTRODUCTION

In the case of poultry, *Salmonella gallinarum* (*S. gallinarum*) appears to be the most important among all infections due to *Salmonella*. *S. gallinarum* is a bacterial serovar belonging to the species *Salmonella choleraesuis* of the genus *Salmonella*, family Enterobacteriaceae (Bergey's Manual of Determinative Bacteriology, 1994). *Salmonella gallinarum* is causative agent of fowl typhoid (FT) which is a septicaemic disease of mainly domestic birds. Fowl typhoid caused by *S. gallinarum* are of the greatest economic importance for poultry industry not only in Zambia but also in all poultry-producing countries in the world.

Infection due to *S. gallinarum* takes place in the intestinal tract, with localisation in the intestinal wall, liver, and spleen. This is often followed by bacteremia and high mortality in young chicks or followed by chronic disease in some adult chickens. The mortality may be moderate or very high, depending largely on the virulence of the inciting organisms (Pomeroy and Nagaraja. 1994). The mortality in young

chicks has been reported to be 100 percent in serious outbreaks, while in adult chickens mortality is highly variable, ranging from no losses to about 40 percent. As regards morbidity, this is often influenced by a number of factors including age, nutrition, management and frequency of exposure. The greatest losses are often observed during the second week after hatching, with a sharp decline during the third and fourth week of age.

Salmonella organisms are often shed in faeces of infected birds to cause infection to the incontact chicks. The organisms are also shed in eggs to cause transovarian transmission to newly hatched birds. The incidence of transovarian transmission is reported to be more than 30 percent.

In Zambia, the first report of avian salmonellosis date back to 1931 when four outbreaks of fowl typhoid were recorded in native bred fowls (Anon 1927-1975). In the following years through 1939, the disease was reported to be 'becoming a serious menace' and it was believed that the disease was introduced in the country through imported chicks (Anon 1927). The disease was reported to have continued with a fluctuating incidence records from the year 1940 through 1973 (Gasper

and Hrabeta. 1977). During 1976-79, there were reports of serious outbreaks of the disease observed in chickens, turkeys and guineafowls (Sharma *et al.* 1991). Based on this study, the Department of Veterinary and Tsetse Control Services introduced systematic measures for the control of fowl typhoid during 1977-79. The measures instituted included testing of breeding flocks in all the hatcheries by whole blood agglutination test, strict quarantine and depopulation of infected flocks. These measures taken by Government somehow controlled the disease until after 1980 when isolated cases were reported. During the period 1976-1985, Sharma *et al.* (1991) isolated 806 *Salmonella* cultures consisting 14 different serotypes from poultry in Zambia. The highest prevalence of *Salmonella* was observed in chicks (98.13 per cent) followed by turkey (0.62 percent). *S. gallinarum* was isolated from 781 specimen. This revelation suggested that *Salmonella* was still a problem in poultry in Zambia. Recent studies by Tuchili *et al.* (1996) also revealed the persistence of *Salmonella* in poultry in Zambia as they were able to isolate and characterise 23 strains of *Salmonella* from poultry. The identified serovars were *S. gallinarum* (11 strains), *S. agona* (7 strains), *S. haifa* (1 strain), *S. alamo* (1 strain), *S. virginia* (1 strain), *S.*

dublin (1 strain), and *S. infantis* (1 strain). The commonest serovar was again shown to be *S. gallinarum* which accounted for 47.8 percent.

Although the poultry industry is currently developing very rapidly with small scale farmers leading the way, this development is being hindered by poultry salmonellosis. The disease has not been effectively controlled since it was first reported in 1931 and this has been partly because of numerous problems which includes the use of less reliable diagnostic methods. Diagnostic methods of *Salmonella* infection currently in use in Zambia are very similar to those used in other countries. Diagnosis of *Salmonella* infection in birds has been made by isolating the organisms by conventional culture methods, by demonstrating the antigen of the organism in the tissue or by demonstrating the antibodies specific to the organism in the serum of birds. For many years, the test of choice in the diagnosis of *Salmonella* has been the slide agglutination test which was originally developed by Runnels *et al.* (1927) for use with serum and adapted by Schaffer *et al.* (1931) for whole blood by using stained antigen. The test is easy to perform and has assisted many countries in eliminating *Salmonella* infections in poultry. However, the test can yield erratic results, which

can be dependent on antigen quality. The test has also been found difficult to quantify and is not applicable for use with eggs (Barrow *et al.* 1992). Another serious problem with serological tests is that immunological response to infection with *Salmonella* appears to be greater in adult chickens and very low in young birds (Williams and Whittemore 1975). This obviously makes it extremely difficult to detect the infection in young birds, particularly if a method of low sensitivity is applied. The breed of chickens may also affect the response, for example the White Leghorn has been shown to produce lower agglutinating antibody titres to *Salmonella* bacterin than the Rhode Island Red (Garren and Hill 1959). It has also been shown that under natural conditions of infection, only a low level of agglutinins is produced in the serum as cited by Williams and Whittemore (1979). In majority cases, serologic evidence of infection are confirmed by bacteriological examination. Other problems associated with serological tests are attributed to some atypical reactions, which may be from infections other than *Salmonella*. Such reactions often cause problems of interpretation. A variety of bacteria possessing antigens in common with or closely related to those of *Salmonella* may infect birds

and produce an agglutinin response. Gerrard *et al.* (1948) found out that infections with coliforms, micrococci, and streptococci-particularly those belonging to Lancefield group D, were responsible for large percentage of non *pullorum/gallinarum* reactions in chickens. In recent years particularly in developed countries, there has been an increasing application of other tests like the Enzyme Linked Immunosorbent Assay (ELISA), latex agglutination and immunodiffusion. Many of these tests are very rapid but have also been found to suffer from a lack of specificity which has almost limited their acceptance (D'Aust *et al.* 1988 a and b, Curiale *et al.* 1990, St. Clair and Klenk 1990).

On the other hand, the laboratory procedures for *Salmonella* identification by conventional culture methods have been found to be labourious and time consuming, normally taking at least 2-3 days to obtain a definitive result (Edward and Ewing 1986, Kelly *et al.* 1985). Most of the *Salmonella* detection systems require pre-enrichment, followed by selective enrichment. Recent attempts to shorten the enrichment phase in the identification of *Salmonella* have been met with varying degrees of success as cited by Rahn *et al.* (1992).

Therefore, there is a great need to develop rapid and sensitive methods for detection of *Salmonella* infection in birds and eggs.

Advances in nucleic acid technology during the past few years have yielded practical nucleic acid-based tests for identifying infectious agents. The PCR, which is an elegantly simple molecular technique, has been developed for detection of specific regions of chromosomal DNA for diagnosis of human sickle cell anaemia (Saiki *et al.* 1985). Multiple copies of targeted DNA sequence can efficiently be obtained by use of this technique to make way for easier analysis of genetic information retained in cells or micro-organisms. Because of its swiftness and relative simplicity PCR technique has been widely used for sequencing and analysing the genes of viruses and certain groups of bacteria (Bej *et al.* 1990 a and b, Saris *et al.* 1990, Wu *et al.* 1992, Oyofe *et al.* 1992, Jackwood *et al.* 1992). The PCR has been set to revolutionize pathogen diagnosis. The method depends upon knowledge of particular gene sequence in the pathogen to be detected.

For the genus *Salmonella*, Galan and Curtis (1989) have characterised a group of *S. typhimurium* genes (*InvA, B, C, D*) that allow *S. typhimurium* to enter cultured epithelial cells. Other characterisation

of *S. typhimurium* genes was also done by Spierings *et al.* (1992), who showed that *S. typhimurium* did contain the *phoE* gene. Using these genes (*InvA*, *phoE*) it was possible to detect *S. typhimurium* DNA in experimental studies (Rahn *et al.* 1992, Spierings *et al.* 1992). However, most of these successful PCR amplifications were carried out using bacterial cells isolated from clinical samples. These methods have been found not to be very efficient as they also require the primary isolation of the bacteria which is a time consuming procedure. In order to reduce on time, attempts have been made to amplify *Salmonella typhimurium* DNA directly from chicken litter and carcasses but no meaningful success has been recorded. PCR has therefore, not been successfully used to detect *Salmonella* from chicken clinical samples.

As to other *Salmonella* serovars, however, it is not known whether the PCR technique aiming at the *InvA* and *phoE* genes is applicable to their detection or not. The present study aims at the elucidation of this question initially.

Another question to be elucidated is the applicability of PCR technique on the clinical specimens for diagnosis of *Salmonella*. The PCR technique developed for genus *Salmonella* so far enables

amplification of the *InvA* and *phoE* genes only when the starting materials are the DNA molecules extracted and purified from colonies of bacteria cultivated and isolated from infected tissues by conventional culture methods. However, the procedures to obtain such starting materials are laborious and time consuming.

PCR technique will become applicable for diagnosis of clinical cases only when 'direct' amplification of genes becomes possible by using infected crude chicken tissues or eggs as starting material, instead of cultivation, isolation and purification of DNA from infected tissues.

A Further question to be solved is the specificity and sensitivity of the PCR technique when compared with conventional bacterial culture methods. In order to get an answer to the above questions, the research was initiated with four major objectives.

1. to examine the specificity and applicability of the PCR technique using *S. typhimurium* *InvA* and *phoE* genes for detection and amplification of *Salmonella* genes, for the specific detection
2. to develop a PCR technique which enables the detection and amplification of *Salmonella* genes, directly from infected tissues and eggs,

3. to compare the PCR technique with a conventional bacterial isolation technique for the specificity and sensitivity in diagnosing *Salmonella* infections in chicks,

4. and to elucidate complete sequences of the *InvA* and *phoE* genes of the amplified bands and to compare them with those of *S. typhimurium* genes.

CHAPTER TWO

LITERATURE REVIEW

2. 0. Definition of *Salmonella* genus

The definition of *Salmonella* has been controversial for very many years. However, the *Salmonella* sub-committee (1934) defined *Salmonella* on the basis of their biochemical behaviour as well as their antigenic differences. As a result of this, the first 36 *Salmonella* species were published by the *Salmonella* sub-committee in 1934. More *Salmonella* species were presented at the International Congress in New York (*Salmonella* Sub-committee 1939, 1940). These presentations were, respectively, published in the Third and Fourth Proceedings of the International Congress of Microbiology (1940 and 1949). At the end of 1947, 140 *Salmonella* species were recognised and validly published in the proceedings of these congresses. The use of 'species' names for *Salmonella* serovars was generally accepted. However, Le Minor and Rhohde (1974) argued that 'scientifically none of the methods of nomenclature of *Salmonella* was satisfactory' and that 'the International

Enterobacteriaceae sub-committee had not given clear guidance of the naming of the different types'. Le Minor *et al.* (1974) suggested therefore that the use of 'species' for *Salmonella* remain in use as long as these serovar names were not taxonomically equated with species. These observations were supported by the earlier work of Crosa *et al.* (1973) and later by that of Stoleru *et al.* (1976) who demonstrated through the DNA relatedness tests that representative strains of biotypically typical *Salmonella* serovars (subgenus I), biochemically atypical *Salmonella* serovars (subgenus II and IV) and *Salmonella arizona* (subgenus III) belonged to a single genetic species. Five subgroups were distinguishable within this single genetic *Salmonella* species. The distinguished subgroups corresponded to *Salmonella* subgenera I, II, III (*S. arizona* with monophasic flagella antigens), III (*S. arizona* with diphasic flagella antigens), and IV.

Since genetic studies of *Salmonella* had demonstrated that representative strains of biotypically typical *Salmonella* strains belonged to a single genetic species (Stoleru *et al.* 1976), Le Minor (1982) proposed then that the logical definition of *Salmonella* should be as a single diverse species and that these genetic closely related strains be

treated as serovars rather than species, for example *Salmonella choleraesuis* serovar *gallinarum*, and hence their names should not be italicised.

But recently, *Salmonella* genus has been shown to comprise only two species on the basis of their biochemical characteristics (Bergey's Manual of Determinative Bacteriology 1994). All of the *Salmonella* (including Arizona) serovars therefore, belong to two species. *Salmonella bongori* contains less than 10 serovars that are extremely rare. The more than 2500 remaining serovars are all part of *Salmonella choleraesuis*, which could be divided phenotypically and genetically, into 6 subspecies. All serovars in subspecies *choleraesuis* are named, whereas serovars in other subspecies are not named. On the basis of biochemical behaviour, *Salmonella choleraesuis* is divided into several subspecies (subsp. *arizonae*, subsp. *choleraesuis*, subspecies *diarizonae*, subspecies *houtanae*, subspecies *indica*). On the other hand, *Salmonella choleraesuis* subspecies *choleraesuis* is also divided into five serovars (serovar *choleraesuis*, serovar *gallinarum*, serovar *paratyphi* A., serovar *pullorum*, serovar *typhi*). It has therefore been recommended that diagnostic laboratories should report named *Salmonella* serovars by

antigenic formula and subspecies. Some examples are : *Salmonella typhi* or *Salmonella* serovar *typhi*, *Salmonella typhimurium* or *Salmonella* serovar *typhimurium*. Since all the serovars that have been used in this study belong to *Salmonella choleraesuis*, it was decided that this system of nomenclature be adopted and as such, all *Salmonella* serovars used in this study will be referred to as : *S. typhimurium*, *S. gallinarum*, *S. agona*, *S. haifa*, *S. alamo*, *S. virginia*, *S. dublin*, *S. infantis*, *S. enteritidis* and *S. brown*.

2. 1. Salmonellosis in poultry

Avian salmonellosis is the term used to describe a large group of acute or chronic diseases of fowl caused by any one or more members of the bacterial genus *Salmonella*, which is a member of the large family *Enterobacteriaceae* (Snoeyenbos and Williams 1991). The genus *Salmonella* named for the late eminent American veterinarian, Daniel E. Salmon, is composed of over 2500 serotypes, which includes the group previously classified as *Arizona hinshawii*. Domestic poultry are known to constitute the largest single reservoir of *Salmonella* organisms existing in nature. Infection with the host adapted serotypes *S.*

gallinarum and *S. pullorum* often causes mortality in chickens over two weeks of age although mortality due to food poisoning *Salmonella* appears to be rare at this age (Cooper *et al.* 1989). In the case of very young birds however, Williams and Whittemore. (1975) reported that, if infection occurs during the first three days of life, mortality due to salmonellosis may reach 80 per cent. When the infection of poultry is purely due to *S. pullorum*, the disease is often referred to as pullorum disease (PD) and when *S. gallinarum* becomes the sole causative agent, the disease is then known as fowl typhoid (FT). Previously, infections due to *S. pullorum* was known as 'bacillary white diarrhoea', a name used to designate the disease until the term 'pullorum disease' was proposed in 1929. *S. pullorum* and *S. gallinarum* are considered biotypes of the same serotype but the disease caused are all called pullorum and fowl typhoid. The etiologic agent of PD was discovered by Rettger in 1899 and was described as a 'fatal septicaemia of young chicks' (Rettger, 1900). In another report, Rettger (1909) designated the disease as 'white diarrhoea' and shortly thereafter expanded the term to 'bacillary white diarrhoea' to distinguish it from other diseases of chicks.

Transmission of *S. pullorum/gallinarum* has generally been accepted as being through eggs. It is therefore an egg-borne infection. The cycle involves an infected hen laying infected eggs, from which infected chicks would hatch. Infected birds tend to remain so throughout life. As many as a third of the eggs laid by infected hens contain *S. pullorum/gallinarum*, mainly as a result of contamination of the ovum. Transmission of infection during hatching from infected to uninfected chicks can result in extensive dissemination of the bacteria. Cannibalism of infected birds and egg eating have also been cited as other ways of transmitting the infection (Snoeyenbos 1991). Other epidemiological investigations have also demonstrated the possibility of transmission of infection in incubators and hatcheries.

Although the chicken appears to be the natural host of *S. pullorum/gallinarum*, the turkey has also proved to be an important host. The high degree of adaptation of *S. pullorum/gallinarum* for the chicken, and to a lesser degree for the turkey, appears to have severely restricted pathogenicity for other hosts (Snoeyenbos 1991). In these hosts, infection is usually life long. Infections in other species have usually been minor and of life long-term significance. A substantial

percentage of chickens and turkeys that survive retain the infection with or without the presence of lesions. As regards morbidity and mortality, these are highly variable in chickens and turkeys and are often influenced by a number of factors including age, nutrition, management, strain type and frequency of exposure. Mortality may therefore range from no losses to 100 per cent in case of serious outbreaks. The greatest losses are often observed during the second week after hatching, with a sharp decline during the third and fourth week of age. General symptoms of infected birds usually include anorexia, huddling, pasty vents, dehydration, droopy wings, somnolence and sudden death. If birds are hatched from infected eggs, moribund and dead birds may be observed in the incubator or within a short time after hatching. In some cases, evidence of the disease is only observed 5-10 days post hatching. Mortality often reaches the peak during second or third week of life. Evans *et al.* (1955) reported blindness associated with salmonellosis in chicks and isolated *S. pullorum* from the anterior chamber of the eye and from the tibiotarsal joint. Similar results have recently been reported by Mayahi *et al.* (1995), who observed caseous exudate in the anterior and posterior chambers of affected eyes. Microscopically, cornea showed

vacuolation and heavy infiltration of neutrophils near the sclera-corneal junction. Localisation of *Salmonella* infection in the joints and adjacent synovial sheaths of chicks has also been reported (Ferguson *et al.* 1961). In the case of adult birds, PD does not manifest characteristics of an acute infection. Infection may therefore be present in a flock for a long time without producing distinct signs. As a result of this, such infected birds may not be detected by their physical appearance. In such flocks, the common signs include reduction in egg production, fertility and low hatchability.

From an economic point of view, avian salmonellosis has been ranked as one of the most important egg-borne bacterial disease of poultry and this is largely because of an ever increasing expansion of poultry industry throughout the world. Marsh (1976) estimated the economic loss to the U.S. poultry industry from paratyphoid (PT) infection at approximately 77 million U.S. dollars annually. Other economic losses due to the disease were reported in Australia where a severe outbreak due to *S. typhimurium* led to losses of many hundred thousands of dollars in an integrated poultry organisation (Jackson *et al.*

1971). The disease is therefore a problem of major economic concern and losses due to mortality and control programmes could be enormous.

2. 2. Paratyphoid infections

Paratyphoid is a disease caused by any one of a large number of different *Salmonella* serotypes but excluding *S. pullorum/gallinarum*. These *Salmonella* serotypes can readily infect many animal hosts and man. Asymptomatic carriage is commonly the result of infection. Gastroenteritis ('scours') or, depending upon animal species or serotype (or both), a specific disease syndrome may result. Williams (1980) described five clinical patterns. The first, called primary salmonellosis, is due to a particular pathogen in a given species resulting in a recognised clinical picture. The serotype may be host-adapted or nearly so. Secondary salmonellosis is associated with another disease, physiological state, or other stress situation. The third form is that of the chronic carrier (usually convalescent) who, although clinically well, excretes bacteria for weeks or occasionally months. The fourth is that of temporary carrier, often observed in slaughter animals. There are short periods of excretion that are reinforced by new lots of *Salmonella*-

contaminated feed or occasionally, a contaminated environment. The fifth form is designated a latent infection demonstrated by isolation of *Salmonella* from mesenteric lymph nodes or ovaries in case of poultry at the time of slaughter. This latter form may be part of the carrier-state syndrome resulting from repeated ingestion of contaminated feed (Williams 1980). Among the domestic animals and wild mammals, paratyphoid pathogens have been found to be common. Cattle, sheep, goats, pigs, dogs, cats, horses, mink, foxes and reptiles are among the many animal species that may be chronically infected, while healthy carriers shed the organisms in large numbers in faeces (Nagaraja *et al.* 1991). In these animals, paratyphoid usually occurs as an acute disease only in the very young or in old debilitated animals under extreme stress conditions. Rats and mice are frequently intestinal carriers of paratyphoid organisms, particularly *S. typhimurium* and *S. enteritidis*. Sato *et al.* (1970) found wild rats to be responsible for the spread of *Salmonella* infection to poultry flocks. In other studies Goyal and Singh (1970) found out that rodents, free-flying birds, and lizards were the main sources of *Salmonella* on a poultry farm.

2. 3. Public health significance of poultry salmonellosis

Contamination of foods and feeds by *Salmonella*, and hence salmonellosis in man, has always played, and still continue to play, a predominant role in the field of foodborne diseases, throughout the world (Bryan 1981). Epidemiological studies have shown that symptomless *Salmonella* excretors in food animals are a far more important source of food contamination than overtly diseased animals (Bryan 1981). Infection of animals, frequently resulting in clinical healthy carriers of pathogenic micro-organisms, is primarily attributable to contaminated feeds and secondarily to the existence of contaminated cycles, in which water, soil, dust and air, as well as insects, rodents, birds and man play an important role (Kampelmacher 1987). These routes of infection have been reported to exist in poultry houses, despite all efforts which may have been taken to eliminate or prevent introduction of pathogens (Kampelmacher 1987). It is a known fact that *Salmonella* spreads rapidly in poultry houses probably due to spillage of drinking water and a favourable ambient temperature, which encourages its multiplication in moistened litter and feed (Kampelmacher 1987). Contamination of poultry houses and instruments with particular strains

of *Salmonella* particularly *S. enteritidis* presents a high risk to the workers handling such contaminated materials. Contaminated eggs with *Salmonella* strains particularly *S. enteritidis* presents the greatest risks to the human populations all over the world. In 1990, the number of *Salmonella* infections reported annually to the Centre for Disease Control (CDC) in the United States of America had been increasing gradually and progressively for the past 30 years and was then reported to number 40,000 cases per year (Bradshaw *et al.* 1990).

The implication of eggs as a major source of *S. enteritidis* infection in humans has become an important international public health issue (Centre for Disease Control, 1988, 1990, St. Louis *et al.* 1988, Coyle *et al.*, 1988, Cowden *et al.* 1989). *S. enteritidis* has been isolated from the contents of clean, intact eggs produced by both naturally and experimentally infected laying hens (Humphrey *et al.* 1989a, 1989b, Timoney *et al.* 1989, Gast and Beard 1990). The costs for an estimated two million cases of *Salmonella* infections resulting from the consumption of contaminated meat and poultry in the United States of America was estimated at \$1.0 billion annually (Roberts 1988). The cost was based on medical expenses and lost productivity. Similar costs

were reported for West Germany and for England and Wales (Sockett. 1991). In recent years, the importance of poultry as a vehicle of human salmonellosis has generally paralleled the market increase in global consumption of poultry meat. At the same time the rate of *Salmonella* contamination appears to be on the increase.

2. 4. Description of non flagellate *Salmonella*

Majority of *Salmonella* strains are differentiated by biochemical tests such as ornithine decarboxylase. While *S. gallinarum* decarboxylate ornithine, *S. pullorum* does not. Both *S. gallinarum* and *S. pullorum* are described by the same antigenic formula (1, 9, 12: - : -) and as such can not be distinguished serologically. Strains of *S. gallinarum* and *S. pullorum* are unusual among *salmonellae* in being non-motile and non-flagellate. Their permanent non-flagellate status is thought to result from deletion and multiple mutations in the structural and other flagella genes (Iino and Lederberg 1964). That there are no reports of spontaneously motile or flagellate strains of these serotypes reaffirms with Iino's hypothesis. *S. gallinarum* and *S. pullorum* strains are also unusual with regard to the production of fimbriae. Thus, whereas the

majority of naturally occurring strains of most serotypes of *Salmonella* form type-1 fimbriae which bestow on the bacteria diverse adhesive properties (Duguid and Old. 1966, Old *et al.* 1968, Duguid *et al.* 1980), strains of *gallinarum* and *pullorum* form type-2 fimbriae which, whilst resembling type-1 fimbriae morphologically, lack their adhesiveness (Duguid *et al.* 1966).

2. 5. Serological behaviour of *Salmonella*

The work of Weil and Felix as reported by Kauffmann. (1965), independent of that of Smith and Reagh, on the antigenic structure of *Proteus* strains, first drew attention to the differences between somatic and flagella antigens. Kauffmann. (1965) reported the work of Weil and Felix who had demonstrated that, *Proteus* cultures occurred in two forms, the swarming form called the 'H' form (Mit Hauch) and the non-swarming form, called the 'O' form (Ohne Hauch). The 'H' form possessed both 'H' and 'O' antigens, the former being the flagellae antigens, and the latter the somatic or cellular antigens. The non-flagellated form contained no 'H' antigens. The flagellae antigen is heat-labile (thermolabile), but the 'O' antigen of both the 'O' and 'OH' forms is

considered to be heat resistant (thermostable), as it resists boiling for two and half hours. To completely destroy the antigenic effect of 'H' antigen, cultures containing it must be boiled for two and half hours, heating for a shorter period than this will only destroy the agglutinable and agglutinin-binding properties, but not the antigenic power of the antigen.

Both antigens, which have been designated as 'O' and 'H' antigens in the *Salmonella* group, stimulate entirely different antibodies in animals. The 'H' antigen gives a 'floculla', loose agglutination, which occurs rapidly and is easily shaken up. This is in contrast to the 'O' agglutination, which is 'granular', firm, slower to form, and is difficult to disturb. Metaphorically, the 'H' agglutination is gray and cloud-like, the 'O' agglutination is like white hail stones, although it often may consist of ragged fragments. Kauffmann. (1965) reported that, by direct microscopic examination of hanging drop preparations, Arkwright had demonstrated that qualitative differences did exist in 'H' and 'O' agglutination. In 'H' agglutination there was a complete immobility of the bacteria caused by an adherence of their flagella, and resulting in the formation of a loose conglomerate. 'O' agglutination, termed polar

agglutination, was caused by the bacteria adhering at their poles. This was first recognised by Mandelbaum, who also introduced microscopic agglutination for diagnostic purposes. In polar agglutination characteristic figures such as stars, chains and festoons are formed, these retain their motility, swimming around in the field of vision, the flagella being unaffected. The point of attack of the 'H' agglutinins is the flagella, that of the 'O' agglutinins the poles of the bacterial body. The flagella of Enterobacteriaceae are organs of active locomotion and not artificial products of a motility of the bacterial cell itself. While the 'O' antigens are carbohydrate compounds, the 'H' antigens consist of proteins.

In addition to the 'O' and 'H' antigens, Kauffmann. (1965) further demonstrated the existence of the 'K' antigens which were present on the bacteria and were either envelope or capsular and occurred in many groups of Enterobacteriaceae. The term 'K' antigen denotes a group of different capsular and envelope antigens, which are designated A, B, L, Vi, and which have different biochemical properties. There is no sharp line of demarcation between capsular and envelope antigens, the transition being gradual, and in consequence both capsular and envelope

antigens are grouped together under the term 'K' antigens. The designation 'K' is derived from the German word 'Kapsel', the capsules being the first described antigens in this group of antigens.

2. 6. 0. Antigenic variation within the *Salmonella* genus

Kauffmann. (1965) once stated that 'the principle of species diagnosis cannot be properly understood without reference to the phenomenon of antigenic variation'. The mosaic-like architecture of the *Salmonella* species is governed by the occurrence of variations causing certain antigenic alterations. In other words, in the serology of the genus *Salmonella* we are often dealing not with stable unalterable antigenic structures, but with organisms liable to explosive variation. In practical work, knowledge of this variation is indispensable, if uniform results, with proper interpretation, are to be obtained. Kauffmann. (1965) demonstrated the existence of the following kinds of antigenic variations within the *Salmonella* genus.

2. 6. 1. H-O variation

This is due to loss of 'H' antigens and often a transition of 'OH' forms to 'O' forms. Such a change is rare and once it occurs it is irreversible. Certain serovars like *S. gallinarum/pullorum* only exist in the 'O' form.

2. 6. 2. S-T-R variation

This is due to loss of 'O' antigens, transition of 'S' forms to 'T' or 'R' forms. With regard to 'T' forms, these do not contain the normal 'O' antigen and are regarded as transient, serological forms between the 'S' and 'R' forms. The 'T' forms are morphologically smooth forms and cannot be differentiated macroscopically from the 'S' forms. The 'S' form (smooth form) contains the 'O' antigen characteristic of the strain concerned.

2. 6. 3. 'R'-form (rough form)

This in contrast is lacking the normal 'O' antigen. Colonial morphology may frequently draw attention to 'R' forms, since the colonies are dull and rough and easily distinguished from the light

smooth 'S' colonies. Occasionally, serologically rough forms may look smooth, and morphologically rough forms may still contain smooth antigens.

2. 7. Cultural and biochemical behaviour of *Salmonella*

In the 11th Edition of Medical Microbiology, Cruickshank (1965), described *Salmonella* organisms as aerobe and facultative anaerobe bacteria, temperature range, 15°C-41°C, optimum, 37°C, which grow well on ordinary media. Colonies that form on agar are moderately large. However, Kauffmann (1965) reported of the existence of dwarf colonies first described by Jacobsen as '*Bacteria typhi mutabile*'. Colonies of *Salmonella* are often thick, greyish-white on blood agar, moist, circular disks, dome-shaped and smooth, the opacity and size vary with different strains. Stock laboratory cultures are known to show a mixture of smooth colonies with rough colonies which in the extreme are irregular, dull, effuse and dry.

Colonies on MacConkey's medium are often pale or colourless, since *Salmonella* does not ferment lactose. On Desoxycholate-citrate medium, colonies are also pale or colourless. To show the importance

of biochemical tests in identifying *Salmonella*, Edward and Ewing (1986) stated that, 'for investigators in most laboratories of bacteriology, a study of biochemical reactions of cultures remain the only method available for recognition and differentiation of the genera and species of Enterobacteriaceae'. Whereas it is convenient, even desirable in many instances, to perform serologic examinations at an early stage, identification should not be made on the basis of serologic tests alone. Micro-organisms such as *Pseudomonas*, *Actinobacter*, *Aeromonas* and *Vibrio* may be mistaken for members of one or another of the genera of Enterobacteriaceae. Tests for the reduction of nitrate, use of oxidation-fermentation (OF) medium, the oxidase test, decarboxylase tests, and flagella stains all are useful in the differentiation of the above mentioned bacteria from Enterobacteriaceae and from each other. Members of the genera *Aeromonas*, *Plesiomonas* and *Vibrio* utilise glucose fermentatively, but are oxidase positive and members of these genera yield different patterns of reactions in the decarboxylase tests.

Biochemical differentiation of members of the genus *Salmonella* from related bacteria has been facilitated by the introduction of several tests and methods (Bergey's Manual of Determinative Bacteriology

1994). Some of the tests were earlier described by Alfredson *et al.* (1972) who used tartaric acid isomers and citric acid in the biotyping of *S. typhimurium*. Many investigators in different laboratories have since evaluated most of the biochemical tests used to differentiate members of the *Salmonella* genus from other genera like *Citrobacter freundii*. Medium like potassium cyanide (KCN), in a definite, prescribed concentration has been shown to be of considerable value in the differentiation of members of the genus *Salmonella* (including the *arizona* group) from *C. freundii* (Bergey's Manual of Determinative Bacteriology 1994). This medium is of particular value in differentiation of cultures of *C. freundii* that ferment lactose slowly or fail to utilise lactose, i.e. strains formerly classified as Bethesda-Ballerup bacteria.

2. 8. Viability of *Salmonella*

The thermal death point of the majority of *Salmonella* is about 56°C. The majority of individual bacilli die within a few hours when subjected to drying. In water the bacilli gradually die but may survive for some time, thus in sewerage-polluted sea and fresh water, viable

bacilli have been found after four weeks. Cruickshank. (1965) noted that in soil, survival may occasionally occur for six weeks or longer, while in culture, the organisms survive for longer periods. Compared with other Gram-negative rods, *Salmonella* are relatively resistant to various environmental factors. Majority of *Salmonella* serovars grow at temperature ranging from 8°C to 45°C, at water activities above 0.94, and in a pH range of 4 to 8 (WHO Expert Committee 1988). *Salmonella* organisms are also able to multiply in an environment with a low level of or no oxygen. It appears that the organisms do not survive high temperatures and tend to die at temperatures above 70°C. Some strains of *Salmonella* e.g. *S. senftenberg* have higher resistance to heat at lower water activities.

Salmonella organisms have been shown to be resistant to drying even for years, especially in dried faeces, dust, and dry feed (Yarnych and Butko. 1984). Certain manipulations like salting and smoking have been known to have a limited effect on the survival of *Salmonella*, several months survival has been observed in brine containing more than 20 per cent of salt, especially in commodities with a high protein or fat content such as certain salted sausages. Because of their relatively

high resistance, *Salmonella* organisms have been isolated in various types of foods e.g. eggs, sausages, biscuits, chocolates and mayonnaise. However, majority of *Salmonella* organisms are easily killed by disinfectants that contain chlorine, lactic acid, iodides and many other common disinfectants.

2. 9. 0. Identification of *Salmonella* in clinical specimens

2. 9. 1 Serological identification of *Salmonella*

For many years, the test of choice in avian salmonellosis diagnosis has been the slide agglutination test which was originally developed by Runnels *et al.* (1927) for use with serum and adapted by Schaffer *et al.* (1931) for whole blood by using stained antigen. As the need to control PD became apparent, many investigators worked very hard to develop, evaluate, refine and modify many of these diagnostic systems. The practical application of a macroscopic tube agglutination test (TA) for detection of carriers of *Salmonella* organisms was reported by Johnes (1913). Extensive evaluation of these tests [Whole blood (WB), (TA)] was carried out before they were widely used. After evaluation of these tests, Gwatkin *et al.* (1941) concluded that the tests

were satisfactory for testing chickens. However, Winter *et al.* (1952) found out that the whole-blood (WB) test was not satisfactory for testing turkeys. Williams and Whittemore (1971) developed a microagglutination (MA) test system using tetrazolium-stained antigen, which was found to be as dependable as the tube agglutination (TA). Although most of these serological tests have been used and found useful, their reliability depends on antigen quality. These tests have also been found difficult to quantify and may not be applicable for use with eggs (Barrow *et al.* 1992). Another serious problem with serological tests is that immunological response to infection with *Salmonella* appears to be greater in adult chickens and very low in young birds (Williams and Whittemore 1975). This makes it extremely difficult to detect the infection in young birds, particularly if a method of low sensitivity is applied. The breed of chickens may also affect the response, for example the White Leghorn has been shown to produce lower agglutinating antibody titres to *Salmonella* bacterin than the Rhode Island Red (Garren and Hill 1959). Tests like the ELISA, latex agglutination and immunodiffusion have been used in the diagnosis of salmonellosis in poultry. Although the tests are very rapid, they have

been found to suffer from a lack of specificity, a factor which has limited their acceptance (D'Aust *et al.* 1988 a and b, Curiale *et al.* 1990, St. Clair and Klenk 1990). In the case of *Salmonella* cultures isolated from birds, Barker and Bleach (1980) suggested that slide agglutination with polyvalent 'O' and polyvalent 'H' (phases 1 and 2) serum should be performed on all colonies suggestive of *Salmonella*. If both are negative, then almost certainly the organism is not a *Salmonella*. If the 'O' is negative and the 'H' positive, the organism may be due to *S. typhi* which is human adapted, when the 'O' antigen may be masked by the Vi antigen. *S. typhi*, however, differs biochemically from the other *Salmonella* but, in case of doubt, antisera Vi must be checked. If a positive result is obtained, the organisms must be grown on agar containing $1/800$ phenol which will inhibit the Vi antigen, and slide agglutination should be repeated with polyvalent 'O' serum. If both are positive;

1. Slide agglutination using composite 'H' phase 2 serum must be performed. If no agglutination occurs, then the organism is in the specific phase 1.

2. If agglutination occurs, the organism is in the non-specific phase 2. Conversion to phase 1 should be carried out by Craigie tube or other methods.
3. If positive, agglutination with the specific 'O'-group sera must be performed. In practice, 'O'-groups B (factor 4), C1 (factor 7), C2 (factor 8) and D (factor 9) sera are used for the commonest species.
4. Once the group has been determined, slide agglutination with specific 'H' phase 1 sera for the commonest species within that group, having first confirmed that the organism is motile, must be performed. If possible with one of the specific 'H' phase 1 sera, confirmation should be done by the tube agglutination. If, however, there are several similar serotypes, then the organisms should be sent to a Reference Laboratory where the organisms could be converted to phase 2 and the antigens identified using specific phase 2 (factor) sera.
5. If unable to identify the organism after it has been converted to phase 1 (using the sera available), then it can be assumed that the organism is not one of the common species, and should be sent to a Reference Laboratory for full identification.

2. 9. 2. Cultural examination

According to the British Veterinary Association (1976), in the live animals, confirmation of clinical salmonellosis is obtained by cultural examination of the faeces or rectal swabs. After post-mortem examination, heart, liver, spleen, cecal tonsils and intestines should be cultured using enrichment media, e.g. selenite broth, tetrathionate broth, and selective media like desoxycholate citrate agar (DCA), and brilliant green agar. When abortion occurs, the foetus and placenta should also be cultured. *S. abortus-ovis* grows very poorly, producing very small colonies, and it is advisable to prolong incubation for up to 72 hours, the use of selenite broth is contraindicated because it appears to have an inhibitory effect on *S. abortus-ovis* and also *S. cholerae-suis*.

The heart blood and liver of chickens are usually cultured but if more than five chicks are available only the livers are cultured. When there is a history and/or lesions suggestive of fowl typhoid, the liver, spleen, heart blood and ovarian follicles and intestinal contents are plated onto DCA and into selenite broth. If after 24 hours of incubation, colonies suggestive of *Salmonella* appear, then serological tests should

be performed. These cultural tests will take minimum of at least 3 days to get a definitive diagnosis.

2. 10. Current methods used in the diagnosis of salmonellosis in poultry

Although the whole blood agglutination test has been widely used to detect *Salmonella* antibodies in chicken, this has been found not to be applicable for the detection of the same when used with eggs (Barrow *et al.* 1992). Sometimes, detection of salmonellosis in poultry is based on clinical manifestations of the disease which are usually non pathognomonic (Stuart and Pullen 1946), and the diagnosis is thus based on clinical suspicion and supported by positive culture and serologic findings of paired serum samples. The isolation of *Salmonella* from various body fluids using very tedious methods provides almost a conclusive diagnosis, but it is a time consuming process and it is not always possible to carry it out (Stuart and Pullen. 1946, Zuerlein and Smith. 1985).

For antigen detection, in the last few years, there has been an increasing application of other tests such as latex agglutination (Metzler

and Nachamkin. 1988), and ELISA (Barrow *et al.* 1992, Cooper *et al.* 1989). The tube agglutination Widal test has traditionally been used in the diagnosis of typhoid fever, but several reports have seriously questioned its reliability, especially in endemic areas (Levine *et al.* 1978, Yolken 1982). Thus, many more other tests have been tried and have been reported to have higher sensitivity and specificity than the Widal test, but they still required more extensive evaluation (Edelman and Levine 1986). Some of these other tests include the counterimmuno electrophoresis (Sundaraj *et al.* 1983), radio immunoassay (Tsang *et al.* 1981), the indirect fluorescent-antibody test (Doshi and Taylor 1984), ELISA (Nardiello *et al.* 1984, Beasley *et al.* 1981) and the immunomagnetic monoclonal antibody-based assays (Luk and Linderberg 1991). Many of these tests have been shown to be rapid but have also been found to suffer from a lack of specificity, a factor which has limited their acceptance as reported by Rahn *et al.* (1992). Detection of *Salmonella* from poultry has also been made difficult by the indiscriminate use of antibiotics by some farmers, especially in developing countries (Falade *et al.* 1989). Immune response to infection

with *Salmonella* in young birds has also been reported to be low (Williams and Whittemore 1975).

2. 11. Problems associated with current diagnostic methods for *Salmonella*

The *Salmonella* group of bacteria still remains an important enteric pathogens causing fowl typhoid, pullorum disease in poultry, gastro-enteritis ('food poisoning') and enteric fever in man (Cohen and Tauxe 1986), the laboratory procedures for *Salmonella* identification by conventional culture methods are still laborious and time-consuming, normally taking at least 2-3 days to obtain a definitive result (Edward and Ewing 1986, Kelly *et al.* 1985). Because of this, for the identification of *S. gallinarum*, serological diagnosis had been relied on. The slide agglutination test originally developed by Runnels *et al.* (1927) has been used extensively but this test can, however, sometimes yield erratic results. The agglutination test has also been found not to be applicable for the detection of bacteria when used with eggs (Barrow *et al.* 1992).

Because of the difficulties encountered in antibody testing for salmonellosis, especially in relation to non-specific cross-reactions, other tests which include the DNA hybridisation (Flowers *et al.* 1987) have been sought. However, many of these rapid tests have been found to suffer from a lack of specificity (D'Aoust and Sewell. 1988 a and b, Metzler and Nachamkin 1988, Curiale *et al.* 1990, D'Aoust *et al.* 1990, St. Clair and Klenk 1990) which has limited their acceptance. These tests though very rapid, all require pre-enrichment.

Because of numerous problems encountered in the cultural and antibody testing for salmonellosis, scientists have continued to develop other rapid and sensitive methods for detection of *Salmonella* which has mainly relied on serological tests for decades.

2. 12. Use of polymerase chain reaction (PCR) technique in diagnosis of various kinds of infections

Because of recent advances in molecular technology, and in particular, nucleic acid technology, many practical DNA based assays have been developed. Among the most promising is the polymerase chain reaction (PCR). The PCR has already been shown to be capable

of detecting DNA fragments specific to the bacterial or viral pathogens in question. The PCR technique is steadily being accepted as a very powerful diagnostic tool in the clinical diagnosis of various infections (Bej *et al.* 1990 a and b, Saris *et al.* 1990, Wernars *et al.* 1991, Oyofu *et al.* 1992). The PCR has rapidly become a valuable technique for molecular biologists. Marx (1988) reviewed a number of applications of the PCR in a variety of settings. Higuchi *et al.* (1988) detected mitochondrial DNA from single hairs, a finding which makes DNA a useful tool in forensic medicine. Further more, the PCR has been used to detect viral infections in humans particularly for those for which serology or culture are difficult. The technique has also been used to detect HIV and HTLV-1 directly from peripheral blood mononuclear cells of seropositive persons (Ou *et al.* 1988, Kwok *et al.* 1988, Loche and Mach. 1988). PCR has also been used in the study of human genetics and evolutionary biology as reported by White *et al.* (1989). Saiki *et al.* (1985) used PCR application to enhance the sensitivity of prenatal diagnosis of sickle cell anaemia. Using PCR, prenatal diagnosis was reported to take less than one day compared with several weeks using Southern blotting and hybridisation approaches. Other

workers have also used PCR to detect micro-organisms in a wide range of samples (Saiki *et al.* 1988, Burg *et al.* 1989, Olive, 1989, Rosa and Schwan, 1989, Whipple *et al.* 1992, Bej *et al.* 1990 a and b, Saris *et al.* 1990, Oyofe *et al.* 1992). In other experiments, PCR was used effectively to detect bacteria such as *Shigella* (Flankel *et al.* 1990), *Bordetella* (Glare *et al.* 1990), and *Listeria* (Bessen *et al.* 1990). In order to increase the sensitivity of the PCR, other tests like the booster polymerase chain reaction have also been developed (Ruano *et al.* 1989, 1990, Saulier and Andremont 1992).

2. 13. Principles of PCR

PCR has been defined as being an *in-vitro* method for producing large amounts of specific DNA fragment of defined length and sequence from small amounts of a complex template (Saiki *et al.* 1985). The PCR takes advantage of an enzyme that uses a defined segment in a strand of DNA as a template for assembling a complimentary strand. In early PCR applications, the enzyme used was the *Klenov* fragment of *Escherichia coli* DNA polymerase 1, but this heat-labile enzyme had to be added in each subsequent cycle because the enzyme was inactivated

during each denaturation step. The *Klenov* fragment has since been replaced by a more thermostable DNA polymerase of *Thermus aquaticus* (*Taq*), a development that has permitted automation of the procedure because all reaction components can be combined at the beginning of the test reaction. Another advantage of the *Taq* is that it has improved the specificity, yield, sensitivity, and length of target DNA that can be amplified (Saiki *et al.* 1988).

The PCR is a test based on the enzymatic amplification of a DNA fragment that is flanked by two oligonucleotide primers that hybridise to opposite strands of the target sequence (White *et al.* 1989). The primers are oriented with their 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complimentary sequences and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The three step cycle takes three steps:-

1. Denaturation of the target double stranded DNA.
2. Annealing of primers.
3. Primer extension.

A cycle usually takes 2-5 minutes and is repeated 20-40 times (Mullis and Faloona. 1987). The PCR reaction mixture contains buffers, nucleotides, primers, *Taq* DNA polymerase, and target DNA from the sample to be analysed.

The first step of denaturation separates the complimentary strands of DNA held together in the duplex by hydrogen bonds. Heating the reaction between 95-100°C for a minute is sufficient. In the second step, the primers are annealed or attached to the dissociated DNA strands. Each primer is complimentary to one of the original DNA strands, to either the left (5') or right (3') side of the sequence of interest. The primers are present in such vast molar excess that they are more likely to anneal to the dissociated strands than the strands are to reanneal to each other. Once annealing has occurred and in the presence of the *Taq* DNA polymerase, the number of strands essentially doubles at the end of each cycle. Approximately after 30 cycles, a single copy of DNA can be increased up to one million copies. Visualisation of the amplified DNA can be done by using gel electrophoresis and ethidium bromide staining.

2. 14. Use of PCR in diagnosis of *Salmonella* infection in poultry

In recent years, the (PCR) has received a lot of attention in the detection of *Salmonella*. PCR has so far been shown to offer a new strategy in the detection of experimental infections due to *S. enteritidis* and *S. typhimurium* (Cohen *et al.* 1994, Rahn *et al.* 1992). Rahn *et al.* (1992) used the PCR method to detect genus *Salmonella* irrespective of differences in the species, strains or serovar. In experimental study, Rahn *et al.* (1992) used the PCR method to detect all but four of 630 *Salmonella* strains of 112 serovars with 99.4 per cent sensitivity by using the *InvA* gene sequence of *S. typhimurium* as a primer. In another study, Cohen *et al.* (1994) used oligonucleotide primers specific for all members of the genus *Salmonella* to detect *S. enteritidis* from poultry faeces inoculated with known numbers of colony forming units of *S. enteritidis*. In an effort to improve the detection levels of *Salmonella*, Widjoatmodjo *et al.* (1991) developed the Magnetic Immuno PCR Assay (MIPA) for the detection of *Salmonella*. The assay utilises magnetic particles coated with monoclonal antibodies against *Salmonella* to extract the bacteria from the sample. In another report, Widjoatmodjo *et al.* (1992) used the Magnetic Polymerase Chain

Reaction Assay for direct detection of *salmonellae* in faecal samples. A panel of monoclonal antibodies specific for *Salmonella* serogroups had to be used to extract *Salmonella* from clinical samples.

2. 15. Problems associated with the current PCR technique for detection of *Salmonella* infection in poultry

Despite all these new DNA techniques, rapid detection of *Salmonella* from clinical specimens still remains a big problem because most of the PCR amplifications to date are performed on either purified DNA using the phenol-chloroform method or using single bacterial colonies isolated by conventional methods from experimental infections. Although Rahn *et al.* (1992) detected all but four of 630 *Salmonella* strains of 112 serovars with 99.4 percent sensitivity, this procedure required the primary isolation of the bacteria from suspected samples, isolation and purification of the bacteria DNA before PCR amplification. The major problem with this particular procedure is that it is time consuming and there is also the likelihood of failure to isolate the bacteria needed for the PCR amplification, especially if a method of low sensitivity is used to isolate the bacteria. On the other hand,

although Widjoatmojo's procedures (Widjoatmojo *et al.* 1991, 1992) does not require primary isolation of the bacteria for PCR amplification, there is also a setback in this procedure that the monoclonal antibodies specific for *Salmonella* serogroups had to be prepared. This procedure of monoclonal antibody preparation appears to be more expensive and may not be cost effective.

CHAPTER THREE

MATERIALS AND METHODS

3. 1. MATERIALS

3 . 1. 1. Bacterial strains

Strain L-55 of *S. typhimurium* was obtained from The National Institute of Animal Health, Tsukuba, Ibaraki, Japan and strain GTZ-3 of *S. gallinarum* isolated from sick chicks in Zambia were used to experimentally infect the chicks during the study. Other laboratory strains used for selectivity tests were: *E. coli*, *Citrobacter*, *Klebsiella*, *Shigella*, *Proteus*, *Edwardsiella*, *Morganella*, *Yersinia*, *Serratia*, and *Enterobacter* which were also isolated from clinical samples and identified by routine laboratory tests (Bergey's Manual of Determinative Bacteriology 1994).

3. 1. 2. Antisera

Polyvalent 'O' and polyvalent 'H' antisera kits against genus *Salmonella* were purchased from Denka Seiken Co., Tokyo, Japan.

Polyvalent 'O' kit contained antiserum against serotype 'O' antigen common to genus *Salmonella* and specific antiserum against serotype O2, O4, O7, O8, O9, O12, O9, O46, O3, O10, O1,3,19, O1, O11, O13, O6, O14, O16, O18, O21, O35 of genus *Salmonella* and Vi factor, respectively. Polyvalent 'H' kit contained polyvalent antiserum against serotype 'H' antigen common to the genus *Salmonella* and specific antiserum against serotype a, b, c, d, e, h, G, i, k, L, r, y, e, n, Z, Z₄ Z₁₀, and Z₂₉, respectively. These antisera were used for serotyping all *Salmonella* isolates from test samples. In addition to the sera included in the above 2 kits, the antisera against the phases H:1, 2, H:1, 5, H:1, 6 and H:1,7 of genus *Salmonella* were also obtained from Denka Seiken Co., Tokyo, Japan and used for checking phase inversions among cultures of genus *Salmonella*.

3. 1. 3. Antigen for *S. pullorum*

Antigen for rapid agglutination test was purchased from Chiba Prefecture Serum Institute, Chiba, Japan. The antigen was used for

serological detection of the antibodies against *S. pullorum* and *S. gallinarum* in the serum of chickens.

3. 1. 4. Culture media

The media for culturing *Salmonella* and other bacteria were prepared as follows:

(i) **Luria-Bertani (LB) broth.** 10 g Tryptose Agar Base powder (Nissui Pharmaceutical Co., Tokyo), 5 g Yeast Extract (Nissui Pharmaceutical Co., Tokyo) and 10 g sodium chloride (Wako Pure Chemical Industries Osaka, Japan) were dissolved in 1 litre of distilled water, pH was adjusted to 7.0. It was then dispensed in 10 ml test tubes and autoclaved at 121°C for 15 minutes. LB broth was used for cultivation of both *S. gallinarum* and *S. typhimurium*.

(ii) **Selenite brilliant green (SBG) broth.** 19.7g SBG powder (Nissui Pharmaceutical Co., Tokyo) and 4g sodium selenite (Wako Pure Chemical Industries Ltd, Osaka, Japan) was dissolved in 1 litre of distilled water. Ten ml of the broth was dispensed into screw-capped

test tubes and sterilised in boiling water bath for 10 minutes. The broth was used as an enrichment media for the isolation of genus *Salmonella*.

(iii) Heart infusion agar. Four per cent (w/v) suspension of heart infusion agar (Nissui Pharmaceutical Co., Tokyo) was prepared in distilled water. It was autoclaved at 121°C for 15 minutes and 15-20 ml was poured into Petri dishes to make solid agar plates. This agar was used for determining the colony forming unit (CFU).

(iv) Brilliant Green (BG) agar. Fifty-six g of the powdered BG agar was dissolved in 1 litre of distilled water, was then sterilised by autoclaving at 121°C for 15 minutes and 15-20 ml was poured into Petri dishes. BG agar was used as a highly selective media for the isolation and identification of genus *Salmonella* from test samples.

(v) MacConkey agar. Five per cent (w/v) of MacConkey agar (Nissui Pharmaceutical Co., Tokyo) was prepared in distilled water. It was autoclaved at 121°C for 15 minutes and 20 ml was poured into Petri

dishes. MacConkey agar was used as a differential medium for the detection and isolation of coliforms.

(vi) Sulphide indole motility (SIM) medium. Three and half percent (w/v) of SIM medium (Nissui Pharmaceutical Co., Tokyo, Japan) was prepared in distilled water. It was autoclaved at 121°C for 15 minutes and 5 ml was dispensed into test tubes. SIM medium was used for motility examination of genus *Salmonella*.

(vii) Tryptone water. Ten grams of tryptone (Oxoid Ltd., Basingstoke, Hants, England) and 5g sodium chloride (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in 1 litre of distilled water and mixed well. This was sterilised by autoclaving at 121°C for 15 minutes. Tryptone water was used to test the production of indole by genus *Salmonella*.

(viii) Urea agar. Two hundred and forty mg of Urea Agar Base (Oxoid Ltd, Hants, England) was dissolved in 95 ml of distilled water. The solution was briefly boiled to dissolve completely. Sterilisation was

done by autoclaving at 115°C for 20 minutes. It was cooled to 50°C and 5 ml of filtered 40 per cent urea solution was aseptically introduced. The solution was mixed well and distributed into 10 ml amounts in sterile test tubes and allowed to settle in the slope position. Urea agar was used to test the production of urease by genus *Salmonella*.

(ix) Triple sugar iron (TSI) agar. Sixty-five grams of TSI Agar (Nissui Pharmaceutical Co., Tokyo) was dissolved in 1 litre of distilled water. Sterilisation was done by autoclaving at 121°C for 15 minutes. The solution was allowed to settle in slopes. TSI agar was used for the differentiation of family Enterobacteriaceae according to their ability to ferment lactose, sucrose, dextrose and to produce hydrogen sulphide.

(x) Simmons citrate agar. Twenty-three grams of Simmons citrate agar (Oxoid Basingstoke, Hants, England) was suspended in 1 litre of distilled water and boiled for a short time to dissolve. Sterilisation was done by autoclaving at 121°C for 15 minutes. The agar was used for the differentiation of the family Enterobacteriaceae based on whether or not citrate is utilised as the sole source of carbon.

(xi) Dorset's egg slopes. Ready made Dorset Egg Medium (Nissui Pharmaceutical Co., Tokyo) was used as Dorset egg slopes. The medium contained 800 ml whole egg suspension and 200 ml of 0.9 percent sodium chloride solution. It was dispensed in 3 ml amounts in test tubes, autoclaved and then slanted to provide slopes. This medium was used for storage of standard stock cultures.

3. 1. 5. Materials for PCR technique

(I) Tris(hydroxymethyl)aminomethane Trisethylenediaminetetraacetic acid salt (EDTA) (Tris-EDTA) buffer solution

This buffer solution was prepared according to Sambrook *et al.* (1989). Briefly, 10 mmol Tris (Wako Pure Chemical Industries, Osaka, Japan) and 1mmol EDTA (Wako Pure Chemical Industries, Osaka, Japan) were dissolved in 1 litre of distilled water, its pH adjusted to 8.0 and sterilised by autoclaving at 121 °C for 15 minutes.

(ii) Tris-sucrose solution. Six g of Tris and 250 g sucrose (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in 1 litre of distilled

water, pH adjusted to 8.0 and sterilised. Resultant solution contained 50 mmol Tris and 25 percent (w/v) sucrose in 1 litre.

(iii) Tris-lysozyme solution. 30.2 grams Tris and 20.0 g lysozyme (SIGMA, St. Louis, USA) was dissolved into 1 litre of distilled water. The solution was divided into small aliquots and kept at -25°C .

(iv) Lysis solution. This solution contained 0.1 per cent (v/v) Triton X-100 (Kodac, Rochester, N. Y, USA), 6.25 mmol per litre EDTA and 50 mmol per litre Tris. The pH was adjusted to 8.0. Lysis solution was used for lysing of bacterial cell wall.

(v) Sodium dodecyl sulphate (SDS) solution. Ten percent of SDS (Wako Pure Chemical Industries, Osaka, Japan) stock solution was prepared by dissolving 1 g of SDS in 100 ml of distilled water. The pH was adjusted to 8.0. This stock solution was used for lysis of bacterial cell membranes at a final concentration of 0.5 per cent (w/v).

(vi) Equilibrated phenol solution. This solution was prepared according to Sambrook *et al.* (1989). Briefly, the phenol stored at -20°C was allowed to warm to room temperature and then melted at 68°C in a water bath. To the melted phenol, an equal volume of 0.5 mol per litre Tris solution (pH 8.0) at room temperature was added and stirred on a magnetic stirrer for 15 minutes. When the two phases had separated after turning off the stirrer, the phenol in the lower phase was collected by aspiration. An equal volume of 0.1 mol per litre Tris solution (pH 8.0) was then added to the phenol. The mixture was again stirred on a magnetic stirrer for 15 minutes. The stirrer was turned off and the phenol in the lower phase was again collected by aspiration. Extraction was repeated until the pH of the phenol phase became higher than 7.8. One tenth volume of 0.1 mol per litre Tris solution (pH 8.0) containing 0.2 percent β -mercaptoethanol (Wako Pure Chemical Industries, Osaka, Japan) was added to the finally equilibrated phenol phase. The equilibrated phenol solution in this form was stored under 10 mmol per litre Tris solution (pH 8.0). The final product of equilibrated phenol solution was stored at 4°C in a sealed container

until used. This solution was used for extracting cellular DNA from bacterial organisms and tissues of infected chickens.

(vii) Phenol-chloroform-isoamylalcohol(PCI)solution. Chloroform (Wako Pure Chemical Industries, Osaka, Japan) and isoamyl alcohol (Wako Pure Chemical Industries, Osaka, Japan) were mixed in the ratio of 24 to 1. This solution was added to an equal volume of equilibrated phenol solution. The solution was stored in this form under 100 mmol per litre Tris solution (pH 8.0) in a bottle and stored at 4°C. The PCI solution was used to remove the proteins contaminating the whole DNA samples prepared from bacterial cultures and chicken tissues.

(viii) Water-saturated ether. Twenty ml of distilled water was added to 80 ml of polyoxyethylene (10) octylphenyl ether (Wako Pure Chemical Industries, Osaka, Japan) and mixed well. The ether phase in the supernatant was collected. It was used as water saturated ether.

(ix) **Ethanol.** Special grade (99.5 v/v per cent) ethanol (Wako Pure Chemical Industries, Osaka, Japan) was used for the purification of DNA.

(x) **Isopropanol.** This chemical was purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan and was used for precipitating the DNA and not leave it in solution.

(xi) **Mineral oil.** Mineral oil (Sigma, St. Louis, MO, USA) was used to cover the PCR mixture added with the sample in order to prevent evaporation.

(xii) **PCR mixture.** The PCR mixture used in the present study were obtained from TAKARA, Osaka, Japan. One hundred μl of this mixture contained 50 mmol Tris (pH 9.0), 50 mmol KCl, 1.25 mmol MgCl_2 , 100 μmol each of dATP, dGTP, dCTP, dTTP, 0.5 μl (5U/ μl) recombinant *Taq* DNA polymerase and 10 μl of the 10x PCR buffer supplied by the same company. Five μl (0.075 μmol) of the upstream and downstream primers were then added, respectively.

(xiii) Primers. The two pairs (upstream and downstream primers for *InvA* gene of *S. typhimurium* and the other 2 primers for *phoE* gene of *S. typhimurium*) used in this study were purchased in the form of commercial products from Nippon Gene Co., Tokyo, Japan. The upstream primer (No. 633112) for *InvA* gene had a base sequence of 5'-GTG AAA TTA TCG CGT TCG GGC AA-3', while down stream primer (No. 634122) for *InvA* gene had a base sequence of 5'-TCA TCG CAC CGT CAA AGG AAC C-3'. These primers were used in Experiment 3.3.3.1 and 3.3.3.1. On the other hand , the upstream primer (*phoE* 0714-1) for *phoE* gene had a base sequence of 5'- AGC GCCGCGGTACGG GCG ATA AA-3', while downstream primer (*phoE* 0714-2) for *phoE* gene had a base sequence of 5'-ATC ATCGTCATTAATGCCTAA CGT-3'. These primers were used in Experiment 3.5.7.

(xiv) High speed micro centrifuge. Model TM-150, (Tomy Seiko Co., Tokyo, Japan) was used.

(xv) **Thermocycler.** ASTEC System PC-700 (Astec Co., Shimen, Fukuoka, Japan) was used for gene amplification.

3. 1. 6. Materials for electrophoresis for molecular weight determination of the amplified genes

(i) **Tris-phosphoric acid-EDTA (TPE) buffer solution.** Prepared by adding 108 g Tris, 15.5 ml undiluted phosphoric acid and 40 ml of 0.5 mol per litre EDTA solution in 1 litre of distilled water. The pH was adjusted to 8.0.

(ii) **Bromo-phenol blue (BPB) buffer solution.** Prepared by adding 0.25 g of bromo-phenol blue powder (Wako Pure Chemical Industries, Osaka, Japan) and 5 ml glycerol (Wako Pure Chemical Industries, Osaka, Japan) in 95 ml of distilled water. This buffer solution was used for loading the amplified DNA samples.

(iii) **Agarose gel.** Both 1 percent and 2 percent gels were used in this study. One or 2 g of agarose (Wako Pure Chemical Industries, Osaka,

Japan) was added to 100 ml of distilled water. It was autoclaved for 5 minutes. Five tenth per cent (w/v) ethidium bromide (Wako Pure Chemical Industries, Osaka, Japan) was then added and the gel was cast onto the slab.

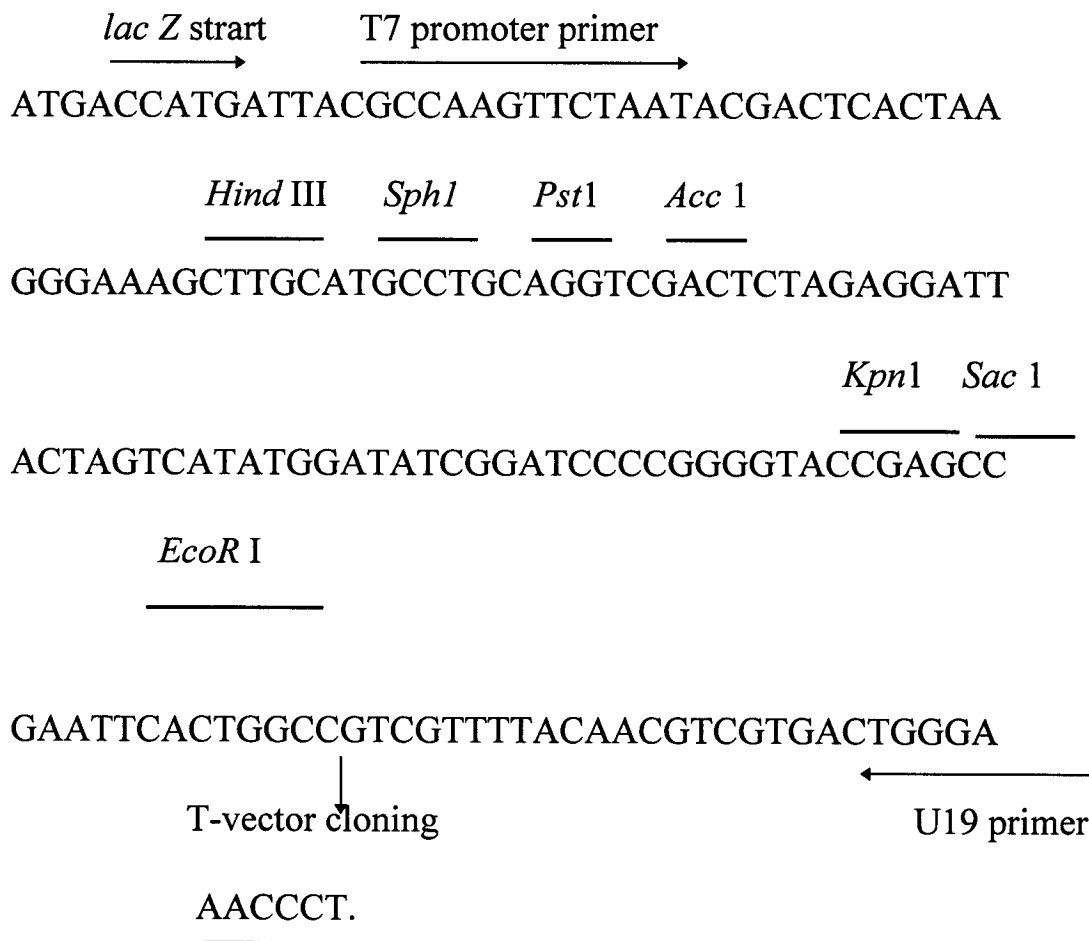
(iv) Electrophoresis tank. Mupid-2 (Cosmo Bio Co., Tokyo, Japan) was used to run the gel.

(v) Molecular weight marker. Molecular marker No.4, Lot No. 910049 whose molecular weight ranged from 194 base pairs (bp) to 1353 bp (Nippon Gene Co., Tokyo, Japan) was used to determine the molecular weight of the amplified genes of *S. gallinarum*.

(vi) Photographic equipment. ACMEL M-085 AUTO camera and its film No. 337, Hand-UV lamp (UVM-57) and the Transilluminator (CSF-20BF) (Nippon Polaroid, Tokyo, Japan) were used.

3. 1. 7. Materials for sequencing the amplified genes

(i) *E. coli* plasmid vector for gene insertion. The *pMOS* Blue T-vector (Amersham Life Science, Buckinghamshire, England) was used for insertion of PCR amplified *InvA* and *phoE* genes. This particular vector was chosen because it allows for direct cloning of PCR amplified genes. The system exploits the template-independent activity of thermostable polymerases which preferentially add a single adenosine nucleotide to the 3' end of double stranded DNA. Below is the genetic map and the possible insertion positions of the vector.



(ii) Competent *E. coli* for transformation with recombinant plasmid

Competent *E. coli* (Hb 101) used for cell transformation was supplied by Amersham LIFE SCIENCE.

(iii) Solution 1. This solution was prepared by dissolving 50 mmol glucose, 25 mmol Tris and 10 mmol EDTA in 1 litre of distilled water.

The pH was adjusted to 8.0. The solution was used to wash the bacterial cells before they were lysed and to remove all traces of the culture medium.

(iv) Solution 2. This solution was prepared by mixing and dissolving 0.2 mol NaOH and 10 g SDS in 1 litre of distilled water. It was then heated at 68°C to assist dissolving. pH was adjusted to 8.0 by adding drops of undiluted hydrochloric acid. The solution was used for lysis of the cell wall of the bacteria plasmid vector.

(v) Solution 3. This solution was prepared by mixing 60 ml of 5 mol per litre potassium acetate (Wako Pure Chemical Industries Ltd., Osaka, Japan) solution, 11.5 ml of glacial acetic acid (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 28.5 ml of the Tris-EDTA buffer solution containing 20 µg of deoxyribonuclease (DNAase) free pancreatic ribonuclease (RNAse) (20 µg/ml) (SIGMA, St. Louis, USA) in its 1 ml. The solution was used for neutralisation of the lysate.

(vi) Resuspension buffer solution (Buffer solution P1). This buffer solution was prepared by dissolving 6.055 g Tris, and 3.722 g EDTA in 800 ml of distilled water. The final volume was adjusted to 1 litre by adding distilled water. The pH was adjusted to 8.0 with hydrochloric acid. The buffer was used for complete removal of the SDS which could have inhibited the binding of the DNA to the resin of the plasmid DNA purification kit (QIAGEN INC., Chatsworth, California) if it remained in the lysate.

(vii) Lysis buffer solution (Buffer solution P2). This solution was prepared by dissolving 8 g NaOH pellets in 950 ml of distilled water and then 50 ml of 20 per cent SDS was added and the volume adjusted to 1 litre of distilled water. The solution was used for alkaline lysis of the plasmid DNA.

(viii) Neutralisation buffer solution (Buffer solution P3). It was prepared by dissolving 294.45 g potassium acetate (Wako Pure Chemical Industries, Osaka, Japan) in 500 ml of distilled water and its pH adjusted to 5.5 with glacial acetic acid. The volume was then

adjusted to 1 litre by adding distilled water. The solution was used for precipitating of bacterial cellular debris .

(ix) Equilibration buffer solution (Buffer solution QBT). It was prepared by dissolving 43.83 g NaCl and 10.4 g 3-[N-morpholino] propanesulfonic acid (MOPS) (Wako Pure Chemical Industries, Osaka, Japan) in 800 ml of distilled water. It's pH was then adjusted to 7.0. One hundred and fifty ml of pure ethanol and 15 ml of 10 percent Triton X-100 solution was added to the initial solution and the final volume adjusted to 1 litre. of distilled water. The buffer solution was used for complete removal of bacterial cellular debris contaminating the DNA preparations.

(x) Washing buffer solution (Buffer solution QC). The buffer solution was prepared by dissolving 58.44 g NaCl and 10.46 g MOPS in 800 ml of distilled water. The pH was adjusted to 7.0 and 150 ml pure ethanol was then added and the final volume adjusted to 1 litre with distilled water. The wash solution was used for removal of residual RNA from the resin.

(xi) Elution buffer solution (Buffer solution QF). This was prepared by dissolving 73.05 g NaCl and 6.055 g Tris in 800 ml of distilled water. After addition of 150 ml pure ethanol the pH was adjusted to 8.5 with hydrochloric acid. The final volume was then adjusted to 1 litre with distilled water. The buffer solution was used for elution of DNA from the QIAGEN-resin.

(xii) Tris-boric-EDTA (TBE) buffer solution. Ten time-concentrated solution was prepared by dissolving 108 g Tris, 55 g boric acid and 9.3 EDTA in distilled water. The final volume was adjusted to 1 litre by adding distilled water and was sterilised by autoclaving. It was diluted to give 1x solution by adding 100 ml of this solution to 900 ml of distilled water. The buffer solution was used for running the sequencing gel of the amplified *S. gallinarum* genes.

(xiii) Glycerol tolerant gel buffer solution. Twenty time-concentrated solution was prepared by dissolving 216 g Tris, 72 g Taurine and 4 g EDTA. This was added to 1 litre of distilled water and was then

autoclaved. It was diluted to give 1x solution by adding 100 ml of this solution to 900 ml of distilled water prior to be used. The solution was used for preparing sequencing gel.

(xiv) Sodium chloride-Tris-EDTA (STE) buffer solution. It was prepared by dissolving 100 mmol NaCl, 10 mmol Tris and 1 mmol EDTA (pH 8.0) in 1 litre of distilled water. The buffer was used for gel electrophoresis of the plasmid DNA.

(xv) SOC medium. This medium was prepared by suspending 20 g tryptone, 5 g yeast extract, 0.5 g NaCl and 6.8 g glucose (Wako Pure Chemical Industries, Osaka, Japan) in 950 ml of distilled water. It was used for enhancing the growth of the plasmid vector.

(xvi) Denaturing buffer. This buffer was prepared by dissolving 8 g NaOH and 74 g EDTA into 100 ml of distilled water (pH 8.0). It was used for denaturing the amplified *S. gallinarum* DNA before it was sequenced.

(xvii) 5-bromo-4-chloro-3-indolyn- β -galactoside (X-gal) solution.

This solution was prepared by dissolving 50 mg X-gal powder (Wako Pure Chemical Industries, Osaka, Japan) in 1 ml of dimethylformamide (Wako Pure Chemical Industries, Osaka, Japan) just before use. It was used as a chromogenic substrate during cell transformation

(xviii) Isopropyl β -D-thiogalactopyranoside (IPTG) solution. This was prepared by dissolving 2 g of IPTG (Wako Pure Chemical Industries, Osaka, Japan) in 8 ml of distilled water. It was used for inducing β -galactosidase during the DNA insertion into the plasmid vector.

(xix) X-gal/IPTG solution. This mixed solution was prepared by mixing 35 μ l of X-gal solution and 20 μ l of IPTG solution. It was used as an indicator for transformed cells.

(xx) L-antibiotics agar. The L-antibiotics agar plates were prepared by suspending 10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g nutrient agar (Wako Pure Chemical Industries, Osaka, Japan) in 1 litre

of distilled water. It was sterilised by autoclaving. Ampicillin was then added to a final concentration of 50 μg per ml and 15 μg per ml tetracycline. The plates were used for cultivation of the transformed cells.

(xxi) Enzymes. Both *EcoR* I and *Hind* III (BIO Labs, New England, USA) were used for restriction of *S. gallinarum* and *S. typhimurium* amplified genes once inserted into the *pMOS* Blue T-vector.

(xxii) Plasmid DNA purification kit. QIAquick-spin PCR purification kit (QIAGEN INC., Chatsworth, California) was used. This kit was used to purify the amplified *InvA* and *phoE* genes by removing cellular debris, DNA polymerase and SDS.

(xxiii) Ligation reaction mixture for gene cloning. The ligation mixture was part of the sequencing kit supplied by Amersham Life Science, Buckinghamshire, England. This mixture consisted of 1 μl 10x ligation buffer, 0.5 μl of 100 mmol per litre DTT, 0.5 μl of 10 mmol per litre ATP, 1 μl of 50 ng per μl of the *E. coli* plasmid vector [3.1.12 (I)] ,

0.5 μ l T4 DNA ligase and an appropriate amount of Tris-EDTA buffer for making up to a total 10 μ l.

(xxiv) DNA sequencing kit. A complete kit (SEQUENASETMVERSION 2.0 USB, Cleveland, Ohio) was used. This kit had been developed for dideoxy method (a manual method) for DNA base sequencing and consisted of a primer No. 70763(-40) 5'GTTTTCCCAGTCACGAC-3', labelling mix (dGTP)(5x concentrate), ddG termination mix (ACGT), sequencing extending mix (for dGTP), stop solution, enzyme dilution buffer, 10x restriction buffer solution and DNA polymerase (Sequenase Version 2.0 T7).

(xxv) Photographic equipment for radioactive gene products. A vacuum drier (ATTO), a film developer (Kodac M 35-X-OMAT) and a processor (K11-2750) (Kodac, Tokyo, Japan) were used.

3. 2. 0. METHODS

3. 2. 1. *In vitro* culture of *S. gallinarum*

3. 2. 1. 1. Subculture of standard strains of genus *Salmonella*

Strains L-55 and GTZ-3 of *S. gallinarum* were individually subcultured on MacConkey agar media. To maintain these two standard strains in pure state, a colony was occasionally picked from the culture on MacConkey agar and transferred to TSI agar for identification. Occasional serological identification was performed as well. The standard strains of genus *Salmonella* were stored in the form of Dorset's egg slope culture *in situ* at 4°C until used.

3. 2. .2. Chicken inoculation

3. 2. 2. 1. Preparation of bacteria suspension for chick inoculation

Isolated colonies of each serovar of *S. gallinarum* or *S. typhimurium* were picked from MacConkey agar and cultured either on BG agar or on MacConkey agar . After 18 hour culturing at 37°C, a portion or whole colony of bacteria was inoculated into 10 ml peptone water. The inoculated peptone water was further incubated at 37°C overnight, and was used for chicken inoculation.

3. 2. 3. *In vitro* culture of genus *Salmonella* and other bacteria originating from test-and field-specimens

(i) Processing of and culturing from chicken tissue samples. Pieces of tissues were collected aseptically from the hearts, kidneys, livers, spleens, and intestines from experimentally infected chicks and from chickens brought for diagnosis from the field. Samples were divided into two parts, one for bacterial isolation and the other for gene amplification. Chicken organ samples for gene amplification were stored in sterile plastic plates in plastic bags at -80°C until needed. Ten percent suspensions of the tissues were prepared for bacterial isolation. Two ml of these suspensions were then inoculated into 10 ml of SBG broth and were incubated at 41°C overnight for pre-enrichment culture. These were later subcultured on BG agar and incubated at 37°C overnight. Colonies that appeared red on BG agar were isolated, subcultured and stored for identification. Identification of bacterial isolates was performed as is described in Chapter 3.3.0.

(ii) Processing of and culturing from egg samples. Yolk was examined for the presence of *S. gallinarum* and other *Salmonella* serovars. A total of 870 eggs were divided into 87 groups consisting of 10 eggs each. Surfaces of the eggs were disinfected with 5 percent tincture of iodine solution first and then with 70 per cent ethyl alcohol. The yolks from each group were collected aseptically into a 100 ml sterile beaker to make a pool. Each pool was regarded as one yolk sample in the experiment. One ml of yolk samples were inoculated into 10 ml SBG broth and incubated at 41°C overnight for bacterial pre-enrichment culture. The bacteria grown in pre-enrichment culture were then cultured on BG agar at 37°C over night. Identification of bacterial isolates was performed as is described in Chapter 3.3.0.

(iii) Processing of and culturing from environmental samples. Two g of each sample originating from chicken faeces, floor litter, fluff of chicken, and the liver of a rat in a chicken farm was ground and 20 percent suspensions were made in peptone water. Twenty µl of the suspension was then subjected to pre-enrichment culture in SBG broth. The bacteria grown in the SBG broth were then subcultured on SBG

agar. Identification of bacterial isolates was done as is described in Chapter 3.3.0.

3. 3. 0. Identification of the bacterial isolates for the genus, species and serovar of genus *Salmonella*

(i) Identification by staining. One isolated colony from MacConkey agar was picked and stained by using Gram's stain for morphological identification of the bacteria

(ii) Serological identification. Suspected colonies for *Salmonella* on MacConkey agar was picked and inoculated onto TSI slopes and incubated overnight. Slide agglutination tests with polyvalent 'O' and polyvalent 'H' antisera were performed on all bacterial organisms suggestive of genus *Salmonella*. The bacterial organisms that caused agglutination to either polyvalent 'O' or polyvalent 'H' antiserum were subjected to further agglutination tests for serotyping by use of monovalent antisera against respective serotypes of genus *Salmonella* that were described in section 3.1.2.

(iii) Biochemical identification. Biochemical tests were utilised to classify the isolates of genus *Salmonella* into species, subspecies and serovars among the subspecies, according to the method described in Bergey's Manual of Determinative Bacteriology, 9th edition, 1994. Briefly, fermentation of several sugars such as lactose, sucrose, salicin, mannitol, dulcitol, maltose and dextrose, production of hydrogen sulphite in TSI agar, production of catalase and indole and other biochemical characteristics were utilised as parameters for the classification.

3. 3. 4. Experimental inoculation of chicks

Forty two, one-day old chicks obtained from *Salmonella* free parents, were divided into three groups, (A, B and C). Group A consisted of 15 chicks, Group B 16 chicks and Group C 11 chicks. Inoculation was made by oral administration of bacterial suspension at 2-4 days old. Group A and B were inoculated with 1×10^2 or 1×10^4 colony forming units (CFU) of *S. gallinarum*, at 2 days old while Group C received 1×10^7 CFU of *S. typhimurium* at four days old. Five

uninoculated chicks were included in each group, as controls. They were kept until the 14th day when the experiment ended.

3.4.0. Methods related to PCR technique

3.4.1. PCR technique

The PCR technique used in the present study was basically the same as that described by Rahn *et al* (1992). The technique was used with slight modifications by use of a thermocycler ASTEC System PC-700.

In the present technique, two kinds of selected gene targets (*InvA* and *phoE* genes of *S. typhimurium*) were copied in a three-step reaction consisting of (1) denaturing the target gene at elevated temperature (94°C), (2) cooling the reaction (at 53°C) to promote annealing of oligonucleotide primers (No. 633112 upstream primer and No. 634122 downstream primer for *InvA* gene and No. 0714-1 upstream primer and No. 0714-2 downstream primer for *phoE* gene) that are complementary to either strand of the target genes and (3) extending the bound primers by DNA polymerase (recombinant *Taq* DNA polymerase) action (at 72°C), resulting in a replication of the target sequence. The reaction was

repeated in successive 35 cycles of heating and cooling, referred to as thermocycles; each cycle doubled the sequences of target genes:

Precisely, the present technique consisted of the following steps

Step (i). Approximately 50 μ l 'PCR mixture' was dispensed into each reaction tube according to the number of samples to be amplified, plus positive and negative controls.

Step (ii). Five μ l of the samples to be amplified were added by the use of a digital micropipettor.

Step (iii). Sterile distilled water was added to make up to a total volume of 100 μ l.

Step (iv). Two drops of mineral oil were overlaid to prevent evaporation.

Step (v). Each tube was capped securely by use of a specific roller tool provided by the thermocycler company.

Step (vi). The tubes were placed in the thermocycler and thermocycling was performed according to the following sub-steps:

- Sub-step (a).** Initial heating of the samples to be amplified at 72°C for 7 minutes in order to allow for polymerization.
- Sub-step (b).** Heating at 94°C for 1 minute in order to denature the target gene of the samples.
- Sub-step (c).** Heating at 53°C for 2 minutes in order to anneal the primers.
- Sub-step (d).** Heating at 72°C for 3 minutes in order to extend the bound primers by Taq DNA polymerase action. The target gene sequences were replicated in this sub-step.
- Sub-step (e).** Sub-step **b** to **d** were repeated 35 times in total.
- Sub-step (f).** Heating at 72°C for 7 minutes before the samples were removed out of the thermocycler.
- Step (vii).** The reaction tubes which contained the final products were stored at 4°C until the final products were analysed by electrophoresis.

Positive and negative controls were included in the present technique. Positive controls consisted of the suspension of *S. typhimurium* organisms containing approximately 2×10^6 CFU, while negative control consisted of PCR mixture without any sample.

In order to avoid false positive results due to contamination of the DNA carried over from previous experiments or from environmental sources, the present PCR were carried out cautiously in a safety cabinet using single-use aliquots and positive displacement pipette.

3. 4. 2. Selectivity and Specificity of primers by 'Single colony method' in PCR technique

The experiment was designed to test the ability of both primers to detect and discriminate the *InvA* and *phoE* genes in a collection of both *Salmonella* serovars and other groups of bacteria other than *Salmonella* serovars. The tested bacteria other than *Salmonella* serovars included, *E. coli*, *Citrobacter*, *klebsiella*, *Shigella*, *Proteus*, *Edwardsiella*, *Morganella*, *Yersinia*, *Serratia* and *Enterobacter*. *Salmonella* serovars included, *S. agona*, *S. haifa*, *S. alamo*, *S. virginia*, *S. dublin*, *S. infantis*, *S. typhimurium*, *S. enteritidis*, *S. brown*, *S. gallinarum* and *S. typhimurium*. The 'single colony method' reported by Saris *et al.* (1990) was used with minor modifications. A loopful of the *Salmonella* organisms (approximately 2×10^6 CFU) were picked from one isolated colony (single colony) grown on MacConkey agar. The

organisms were directly suspended in 100 µl of PCR mixture (described in section 3.1.5 xii) and PCR amplification performed.

3. 4. 3. Extraction and purification of whole DNA from bacterial organisms, chicken tissues and environmental samples

(i) Extraction and purification from bacterial organisms

The organisms of *S. gallinarum* and *S. typhimurium* were cultivated in 20 ml of LB broth for 24 hours at 37°C until the maximum growth of the organisms, approximately 5×10^8 CFU/ml in total, were achieved. The whole broth cultures were subjected to mechanical shaking in a 37°C water bath for 24 hours, followed by chilling on ice for 10 minutes and centrifugation at 15,000 rpm for 5 minutes. The deposited organisms were washed once by use of 1 ml of 4°C Tris-EDTA buffer solution and centrifugation at 15,000 rpm for 5 minutes. The washed organisms were the subjected to lysis by the following serial steps; (1) suspension of the washed organisms in 165µl of the Tris-sucrose solution, (2) addition of 35 µl of the Tris-lysozyme solution, (3) incubation at 37°C for 30 minutes, (4) chilling on ice for 5 minutes, (5) addition of 265 µl of lysis solution, (6) chilling on ice for

20 minutes, (7) addition of 28 μ l of SDS solution, (8) thorough mixing and (9) incubation at 65°C for 10 minutes.

The lysate of bacterial cells were then subjected to extraction and purification of the DNA by the following serial steps; (1) chilling on ice of the lysate for 10 minutes, (2) addition of equal volume of equilibrated phenol solution, (3) mixing and centrifugation at 15,000 rpm for 5 minutes, (4) collection of the water phase which contained DNA, (5) repetition of steps 2 to 4, (6) addition of 560 μ l of PCI solution for purification of DNA product by removing contaminating proteins, (7) mixing and centrifugation at 15,000 rpm for 5 minutes, (8) collection of the aqueous phase, (9) addition of 560 μ l of water-saturated ether, (10) mixing and centrifugation, (11) collection of the water phase which contained further purified DNA extract, (12) transfer of 200 μ l of the water phase into a new microcentrifuge tube, (13) addition of 1 ml of 95 percent ethanol chilled to 4°C, (14) precipitation of DNA, (15) complete removal of ethanol by aspiration and evaporation and (18) resuspension of the final DNA product in 70 μ l of Tris-EDTA buffer solution. The resuspended DNA product was kept frozen at -20°C until needed.

(ii) Extraction and purification from frozen chicken tissue samples

Approximately 2 grams each of chicken tissue samples which were kept frozen at -81°C were placed in the mortars that had also been kept at -81°C and quickly ground to fine powders by use of pestles while they were frozen. The powders were suspended in 2 ml of sterile distilled water and their pH adjusted to 7.8 by adding 3 drops of 10 percent NaOH, since their pH were often low. One and a half ml's of the suspensions were transferred into microcentrifuge tubes, chilled and subjected to centrifugation at 15,000 rpm for 10 minutes. The deposits were resuspended in 1 ml Tris-EDTA buffer solution and subjected to the same centrifugation. DNA extraction and purification were done as is described in Chapter 3.4.3. (i).

(iii) Extraction and purification from environmental samples

Approximately 2 g's each of the environmental samples in dry state were ground to powder, suspended in 10 ml of lysis solution and allowed to settle overnight at room temperature. Three ml's each of the supernatants were collected and centrifuged at 15,000 rpm for 5

minutes. The supernatants were collected and stored at 4°C. DNA extraction and purification were done as is described in Chapter 3.4.3.

(i).

(iv) Direct amplification of *Salmonella* DNA from crude chicken homogenates

Chicken homogenates originating from experiment No. 3. 4. 3. (ii) were directly inoculated into the PCR mixture. Five microlitres of the chicken homogenate was suspended in PCR mixture. The PCR technique was performed as is described in Chapter 3. 4. 1.

3.4.4. Electrophoresis for determination of molecular weight of PCR amplified genes

Either 1 or 2 percent agarose gel was used. Ten µl of the final PCR products which were obtained in Chapter 3.3.3.1 Step 7 were transferred to new microcentrifuge tubes and 5 µl each of BPB buffer solution was added to them and mixed well. The electrophoresis tank was then filled with TPE buffer solution after which the gel was cast. The comb wells were carefully washed with TPE buffer solution to

remove air bubbles prior to the loading of the samples. When air bubbles had been completely removed, the samples were cautiously dropped into the comb wells using a positive displacement pipette. The electrophoresis tank was then connected to the electricity main supply. The electrophoresis was run at a constant voltage of 50V over a period of 1½ hours. After this the agarose gel which had already been stained with ethidium bromide was examined under the UV light using a hand UV lamp. The standard molecular marker was used to determine the molecular weight of the formed bands.

3. 4. 5. Methods for cloning *InvA* and *phoE* genes into *E. coli* plasmid

3. 4. 5. 1. Purification of the PCR amplified genes prior to cloning into *E. coli*

Prior to cloning into *E. coli* plasmid, the PCR amplified products were purified according to the following steps;

(i) Seventy five µl of the buffer supplied together with the QIAquick Spin Kit was added to 15 µl of the amplified products.

- (ii) QIAquick-spin column was then placed in a 2 ml microcentrifuge tube and the sample was then placed on top of the QIAquick-spin column.
- (iii) The microcentrifuge tube containing the column and PCR amplified product was then centrifuged at 15,000 rpm for 60 seconds.
- (iv) Flow-through fraction was then removed from the tube and discarded.
- (v) Seven hundred and fifty μ l of the buffer solution supplied together with the QIAquick-Spin Kit was placed on QIAquick-spin column and the whole centrifuge tube was centrifuged again in order to wash the PCR product remaining on the column.
- (vi) Flow-through fraction was removed from the tube and discarded.
- (vii) The same centrifugation was repeated once to remove residual ethanol completely which may have interfered with subsequent reactions.
- (viii) QIAquick-spin column was then placed in a clean 1.5 ml microcentrifuge tube.

(ix) Elution of DNA was done by adding 50 μl of 10 mmol per litre Tris (pH 8.5) or Tris-EDTA buffer solution to the tube and by centrifuging the tube at 15,000 rpm for 60 seconds.

(x) Resultant flow-through containing purified DNA was mixed with 20 μl of Tris-EDTA buffer solution and stored at -20°C until needed.

3. 4. 5. 2. Cloning of *InvA* and *phoE* genes into *E. coli* plasmid

Since direct cloning of the amplified DNA gene into the *E. coli* vector was opted for, it was necessary to inactivate further DNA polymerase activity. This was achieved by extracting the amplified PCR product with PCI solution. The 2 μl of the amplified PCR product was mixed with 5 μl of PCI solution. The mixture was homogenised for 1 minute and then centrifuged at 12,000 rpm for 1 minute. The aqueous phase contained the purified gene product of which DNA polymerase activity had been inactivated. Two μl of this product was transferred to a new microcentrifuge tube, to which 3.5 μl of ligation reaction mixture [3.1.7 (xxii)] was added, and were mixed well. The nuclease free water supplied together with the *pMOS* Blue T-vector Kit was then added to make up to 10 μl in total. The whole mixture was gently stirred with a

positive displacement pipette and incubated at 16°C for two hours to obtain the ligated or recombinant plasmid. Both the *InvA* and *phoE* genes were intended to be cloned into the *EcoR* I site of *E. coli* vector [3.1.7 (i)].

3. 4. 5. 3. Transformation of *E. coli* with recombinant plasmid

A frozen aliquot of the competent *E. coli* for uptake of recombinant plasmid DNA was thawed on ice after which the cells were evenly mixed by simple tapping with the finger. Twenty µl of the bacterial suspension was then pipetted into a pre-chilled microcentrifuge tube. To this, 1 µl of the recombinant plasmid (3.4.5.2) was added and mixed gently, after which the tube was left on ice for 30 minutes. The mixture was then heated at 42°C for exactly 40 seconds for enhancing the uptake of plasmid DNA by 'heat-shock'. It was critical that the *E. coli* received a sharp and distinct shock. The tube was returned to ice for 2 minutes and was added with 80 µl of SOC medium. The tube was then incubated at 37°C in a shaking water bath for 1 hour to allow the *E. coli* recover.

3. 4. 5. 4. Selection and cultivation of transformed *E. coli*

Transformed *E. coli* was selected based on its acquired antibiotic resistance. The culture of *E. coli* obtained at 3.4.5.3 were centrifuged at 15,000 rpm for 1 minute. The supernatant was discarded and the cells resuspended in 100 μ l of SOC medium. On the other hand, 30 μ l of X-gal/IPTG solution was spread on L-antibiotics agar plates using a glass rod and then left to soak and dry for 30 minutes. Onto this dried agar, 50 μ l of the *E. coli* culture was added and spread. The inoculated agar were then incubated at 37°C for overnight. White colonies produced on the agar were picked and cultured in LB broth containing 50 μ g ampicillin and 15 μ g tetracycline. The pMOS Blue T-vector gives white colour on L-antibiotics agar. The transformed *E. coli*, which were selected were further subcultured on L-antibiotics agar at 37°C for 6 hours. They were further cultivated in 50 ml of LB broth containing ampicillin and tetracycline for larger yield.

3. 4. 5. 5. Isolation and purification of recombinant plasmid DNA from transformed *E. coli*

The cultures of transformed *E. coli* obtained at chapter 3.4.5.4 were chilled at 4°C for 10 minutes and centrifuged at 12,000 rpm for 5 minutes. The deposited bacterial cells were suspended in 500 µl of lysis solution [described in Chapter 3.1.5. (iv) for lysis of bacterial cell walls. The lysate was centrifuged and the supernatant discarded. To the pellet, 100 µl of solution 1 was added and they were mixed well. To this suspension was added 200 µl of freshly prepared solution 2 for further lysis of bacterial cell walls and removal of contaminating lipids and proteins. The tubes containing the above solution were then tightly closed and the contents mixed by rapidly inverting the tubes five times. The tubes were then held on ice for 10 minutes, after which 150 µl ice cold solution 3 was added and vortexed gently for 10 seconds for neutralisation of the lysate. The tubes were held on ice for 5 minutes, after which the neutralised lysate was centrifuged at 12,000 rpm for 2 minutes at 4°C. The supernatant was transferred to a new microcentrifuge tube. Purification of plasmid DNA was performed by adding 400 µl PCI solution to the above supernatant, mixing and

centrifuging the mixture at 12,000 rpm for 2 minutes at 4°C. The supernatant containing plasmid DNA was harvested and transferred to a new microcentrifuge tube. For further purification of the plasmid DNA 600 µl of cold 100 per cent ethanol was added to the above tube, followed by incubation at room temperature for 2 minutes, centrifugation at 12,000 rpm for 5 minutes at 4°C. The supernatant was gently discarded by using disposable pipette tips. Tubes containing the deposited plasmid DNA were then left open and kept in an inverted position on a paper towel in order to allow all the fluid to drain away. Fluids adhering to the walls of the tubes were removed by using pipette tips. The isolated plasmid DNA was finally rinsed in 1 ml of cold 70 percent ethanol and allowed to dry in air. The purified plasmid DNA was resuspended in 50 µl of Tris-EDTA buffer solution containing 20 µg per ml of RNA which might have digested and removed the contaminating RNase in the final product. The final product was stored frozen at -20°C until needed.

3. 4. 5. 6. Enzymatic digestion of purified plasmid DNA

Five μl of purified plasmid DNA obtained at chapter 3.4.5.5 was mixed with 2.5 μl of 10x restriction buffer solution, 1 μl of *EcoRI*, 1 μl of *Hind* III and 5 μl of distilled water. The mixture was mixed by 15-time flicking with a finger. It was incubated at 37°C for overnight for enzymatic digestion.

3. 4. 5. 7. Electrophoresis for determination of molecular weight of the digested plasmid DNA

Either 1 or 2 percent agarose gel was used. Two μl of the digested plasmid and molecular weight marker were loaded alongside into the wells, respectively. The gel was run at a constant voltage of 100V for 1 hour. The gel was placed on a UV transilluminator and formed bands photographed using a Polaroid camera.

3. 5. 0. Methods for sequencing cloned *InvA* and *phoE* genes

3. 5. 1. Isolation and purification of plasmid DNA from transformed *E. coli*

Isolation and purification of plasmid DNA prior to sequencing of the cloned *InvA* and *phoE* genes were carried out as follows;

- (i)** Three ml of the transformed *E. coli* were centrifuged at 15,000 rpm for 5 minutes and the supernatant discarded.
- (ii)** The bacterial cell pellets from step (i) were then suspended in 0.3 ml buffer solution (P1).
- (iii)** Three hundred μ l of buffer solution (P2) was added to the above mixture, mixed and incubated at room temperature for 5 minutes.
- (iv)** After the incubation, 300 μ l of the chilled buffer solution (P3) was added, mixed and placed on ice for 10 minutes.
- (v)** The mixture was then centrifuged at 15,000 rpm for 15 minutes and the supernatant was promptly removed and retained for further purification.
- (vi)** On the other hand, QIAGEN-tip 20 was equilibrated with 1 ml Buffer Solution (QBT).

(vii) The supernatant obtained and stored at step (iv) was then applied on the equilibrated QIAGEN-tip 20.

(viii) The equilibrated QIAGEN-tip 20 was then washed with 1 ml Buffer QC.

(ix) Plasmid DNA was eluted from the QIAGEN-tip 20 with 0.8 ml Buffer QF.

(x) Eluted plasmid DNA was purified by precipitation with 700 μ l of isopropanol and centrifugation at 15,000 rpm for 30 minutes.

(xi) The precipitated plasmid DNA was finally washed by addition of 1 ml cold ethanol and centrifugation.

(xii) Supernatant (ethanol) was discarded and pelleted plasmid DNA dried by warm air steam.

(xiii) Dried plasmid DNA was resuspended in 50 μ l Tris-EDTA buffer solution and stored frozen at -20°C until needed.

3.5.2. Enzymatic digestion of purified plasmid DNA

Enzymatic digestion of the purified plasmid DNA for base sequencing was performed in the same manner as described in Chapter 3.4.5.6.

3. 5. 3. Purification of enzymatically digested plasmid DNA

Purification of enzymatically digested plasmid DNA was performed by denaturation, neutralisation and washing of the DNA according to the following serial steps;

- (i)** Ten μl of the digested plasmid DNA was denatured by addition of 4 μl of denaturing buffer and 26 μl of distilled water, followed by incubation at room temperature for 15 minutes.
- (ii)** The denatured plasmid DNA was then neutralised by addition of 16 μl of ammonium acetate solution.
- (iii)** The neutralised plasmid DNA was precipitated first by addition of 200 μl of cold ethanol, followed by incubation at -70°C for 5 minutes and sedimentation by centrifugation at 15,000 rpm for 10 minutes.
- (iv)** The once washed plasmid DNA was further washed by addition of 500 μl of cold 70 percent ethanol and sedimentation by centrifugation at 15,000 rpm for 5 minutes.
- (v)** The twice washed plasmid DNA was resuspended in 15 μl of Tris-EDTA buffer solution and stored at -20°C until needed.

3. 5. 4. Dideoxy method for sequencing *InvA* and *phoE* genes

Determination of base sequence of the digested plasmid DNA was performed by the dideoxy-chain termination procedure by use of a DNA sequencing kit. By the use of this method, template DNA is purified and is annealed to a synthetic oligonucleotide primer. The DNA synthesis is then carried out in two steps, the first is the labelling step and the second is the chain-termination step using dideoxynucleotides. In the first step, the primer is extended using limiting concentrations of the deoxynucleoside triphosphates, including radioactively labelled dATP. To start the procedure of sequencing, the following steps were taken:

(i) Annealing both the forward and reverse primers to the denatured DNA templates obtained at Chapter 3.5.3 and adding the sequenase enzyme. For each sample, two tubes were labelled, forward and reverse. To one tube was added 5 μ l denatured DNA, 1 μ l of primer T-7 and 2 μ l diluted sequence reaction buffer solution. Primer annealing was achieved by heating the tubes at 65°C for 2 minutes and left to cool slowly to less than 35°C over a period of 30 minutes.

(ii) Elongation of primers was carried out by mixing 0.5 μl of ATP^{35}S sequenase enzyme. For each sample, 8 tubes were prepared, four for each forward primer (ACGT) and four each reverse primer (ACGT). Five μl elongation mix was added to the forward DNA/primer and left at 8°C for 3 minutes. During this 3 minutes, 2 μl of each A, C, G, and T were pipetted into the respective tube and placed at 37°C . Immediately after the 3 minutes incubation of the elongation mix and DNA/primer, 3.5 μl of the elongation mix/DNA/primer were pipetted into each of the four separate tubes containing the dideoxy nucleotides and left at 37°C for 5 minutes.

(iii) To stop the elongation reaction 4 μl of stop mixture was added to each tube, capped and kept at -20°C until required for resolution on a sequencing gel. The same procedure was repeated for the reverse DNA/primer tube of each cloned DNA.

3. 5. 5. Sequencing gel electrophoresis

The TBE sequencing running gel was used. The gel was pre-warmed for 30 minutes before it was used. When ready, the gel solution was then cast between 44 x 38 cm glass plates. The gel was then left to

polymerise for 2 hours at room temperature. It was fixed into the electrophoresis apparatus and pre-run at 65 W for 1 hour before loading the samples. The sequenced samples were heated at 75°C for 2 minutes immediately before they were loaded into the wells. Three μl of the sample were loaded in each lane. The gel was run at a constant voltage of 1600 V for 3 hours.

3. 5. 6. Film exposure of the sequencing gel

When sequencing gel electrophoresis ended, the gel was carefully removed from the glass and cooled down. The gel was then transferred onto a filter paper and then dried (at 80°C) by using an ATTO Vacuum Drier. When the gel had been dried for 1 hour, it was film-exposed for overnight. The exposed film was then developed with Kodak M 35-X-OMAT Processor and was read with the help of a transilluminator.

CHAPTER FOUR

RESULTS

4. 1. 0. Specificity and applicability of the PCR technique using *S. typhimurium* *InvA* and *phoE* genes for detection of *S. gallinarum*

DNA from all bacteria other than *Salmonella* strains were tested in PCR by both the single colony method and amplification after DNA extraction from the bacterial organisms. With the two pairs of primers targeted at both the *InvA* and *phoE* genes, respectively, DNA from *E. coli*, *Citrobacter*, *Klebsiella*, *Shigella*, *Proteus*, *Edwardsiella*, *Morganella*, *Yersinia*, *Serratia* and *Enterobacter* was not amplified following the 35 cycles of PCR. In the case of *S. gallinarum* and *S. typhimurium* however, the amplification was completely specific in that the predicted molecular weight of the amplified gene was generated, regardless of the method of DNA preparation. Using the two primers, it was possible to generate genes with specific molecular weight of 284-bp and 365-bp, respectively. When a pair of primers targeted at the *S. typhimurium* *InvA* gene was used, both *S. gallinarum* and *S. typhimurium* DNA were specifically amplified. As was expected a 284-

bp DNA fragment was amplified when a single colony method was applied to PCR (Fig. 1, lane 2). The sizes and molecular weight of the amplified *S. gallinarum* DNA bands from chicken tissues (collected from chicks inoculated with 1×10^4 CFU *S. gallinarum*, (Fig. 1, lanes 6 to 13) were the same as that obtained when the single colony method was used (Fig. 1, lane 2). To evaluate the specificity of the primers for other *Salmonella* serovars, 9 other *Salmonella* serovars included in the study (*S. agona*, *S. haifa*, *S. alamo*, *S. virginia*, *S. dublin*, *S. infantis*, *S. typhimurium*, *S. enteritidis*, *S. brown*) were specifically amplified by the single colony method (Fig. 2, lanes 1-10) giving specific molecular weight as was expected. Even though sequencing of the amplified bands from these *Salmonella* serovars had not yet been done, the molecular weight was the same when compared with that of *S. typhimurium* *phoE* gene. The results therefore showed that the primers that were used in this study, were specific and selective for the genus *Salmonella* and not only for the specific identification of *S. gallinarum* and *S. typhimurium*.

4. 2. 0. Detection of *InvA* and *phoE* genes by PCR technique in chickens infected with *S. gallinarum* or other *Salmonella* serovars

4. 2. 1. Detection of *Salmonella* in experimentally infected chicks by PCR and isolation methods

The study was designed to evaluate the ability and also sensitivity of PCR to detect the *InvA* gene from chicken tissues infected with the genus *Salmonella*. These evaluations were carried out by using two pairs of primers homologous to target regions of the *InvA* gene in a PCR amplification procedure. Both *S. gallinarum* and *S. typhimurium* DNA were amplified from 15 out of 20 organ samples tested 21 hours after infection (Table 1). The results in Table 1 were obtained from group A chicks inoculated with 1×10^2 *S. gallinarum*. The isolation of *S. gallinarum* with pre-enriched media, on the other hand, was possible in only five cases out of 20. *S. gallinarum* DNA was not detected by PCR in two organs even when they were positive on isolation (Table 1 chick No. 1 and 10). An increase in the dosage of 1×10^4 with *S. gallinarum* (group B, total 16 chicks examined) resulted in similar results to those obtained in group A, namely, DNA was detected in 16 out of 20 samples by PCR 21 hours after infection whereas five

positives were obtained on bacterial isolation (Table 3). None of the organs from uninoculated control chicks resulted in specific DNA amplification.

In case of infection with *S. typhimurium* in a total of 11 chicks, *S. typhimurium* DNA could be detected from 17 samples out of 20 tested 21 hours after infection (Table 2).

When direct amplification from the chicken homogenates was done, there was no amplification made from any of the samples even when these were positive on bacterial isolation. No further attempt was made to amplify *Salmonella*, DNA from crude chicken samples.

4. 2. 2. Detection of *Salmonella* by PCR and isolation in naturally infected chickens

The *phoE* gene primers were evaluated for the detection of genus *Salmonella* in naturally infected chickens when applied in PCR. Liver, spleen, heart, kidney, and intestines were collected from sick or dead chickens and DNA was extracted from them. It was the extracted DNA that was used in PCR. Results are shown in Table 6. *Salmonella* was detected by PCR technique in 10 out of 17 (58.8 per cent) spleen

samples, in 22 out of 51 (43.1 per cent) liver samples, in 10 out of 21 (47.6 per cent) intestinal samples, and in 6 out of 11 (54.5 per cent) heart samples, giving an average of 51 per cent detection when compared to 31.3 per cent by bacterial isolation method. The size of the amplified fragment was, as expected, a 365-bp fragment (Fig. 5, lanes 1, 2, 3, 7, 8, 9, and Fig. 6, lanes 3, 4, 5). On the other hand, detection of *Salmonella* by bacterial isolation methods was possible in 7 out of 17 (41.1 per cent) spleen, in 17 out of 51 (33.3 per cent) liver, in 5 out of 21 (23.8 per cent) intestine, and in 3 out of 11 (23.8 per cent) heart samples. There was no isolation from two kidney samples (Table 6).

4. 2. 3. Detection of *Salmonella* by PCR and isolation in chicken pen samples

In order to establish the sources of infection in poultry farms under investigation, samples from chicken pens consisting of floor litter, fluff, and faecal samples were collected. Specific amplification of *Salmonella* DNA was possible in 13 out of 48 (27.1 per cent) faecal samples, in 3 out of 14 (21.4 per cent) chicken floor litter, and in 6 out of 17 (35.3 per cent) chicken fluff samples (Table 5 and Fig. 5, lanes 5,

6, 10, 11, and Fig. 7, lanes 2, 5, 8 and Fig. 8, lanes 2, 3, 4, and 5). Detection of *Salmonella* by PCR was also made from a rat liver (Table 5). On the other hand, detection of *Salmonella* by conventional bacterial isolation methods was only possible in 9 out of 48 (18.8 percent) fecal samples, and in 1 out of 17 (5.9 per cent) hatcher chick fluff (Table 5). There were no detection made from floor litter and rat liver by bacterial isolation methods.

4. 3. 0. Isolation and detection of *Salmonella phoE* gene by PCR technique from chicken eggs

Crude samples obtained from yolks of dead-in-shells, fresh table eggs and infertile eggs from chickens were directly inoculated into the PCR mixture. Results are presented in Figures 3, 4 and 9 and in Table 4. A 365-bp DNA fragment was amplified from the yolk of infertile chicken eggs and chicken embryos (Fig. 9, lanes 2, 4, 5, 7, 8, 9, and Fig. 3, lanes 1, 2, 3, 5, 6). There was no DNA band amplified from a control tube which contained only the PCR mixture (without DNA sample) (Fig 9, lane 1). By direct PCR amplification method DNA could be amplified from 20 out of 45 (44.4 per cent) samples from yolks of dead-

in-shells (Table 4). In infertile eggs, DNA detection was possible in 6 out of 21 (28.6 per cent) infertile eggs (Table 4). There was no detection made from fresh table eggs (Table 4 and Fig. 9 lanes 3 and 6). Results in Table 4 also show that *Salmonella* detection by direct PCR from the yolk of dead-in-shell eggs improved significantly when PCR was performed directly from enriched broth as it was possible to detect 23 out of 45 (51.1 per cent) dead-in-shells chicken embryos compared to 20 out of 45 (44.4 per cent), before enrichment. Direct amplification of *Salmonella* DNA from infertile egg yolks showed no improvement even after enrichment (Table 4). As was expected, the amplified band was a 365-bp fragment (Fig. 4 lanes 1, 3, 5, 7, 8, 9 and 10). Detection of *Salmonella* by isolation in pre-enrichment media was possible in 11 out of 45 (24.4 per cent) dead-in-shells chicken embryos and in 5 out of 21 (23.8 per cent) infertile eggs. Detection of *Salmonella* by direct bacterial isolation was possible in 6 out of 45 (13.3 per cent) dead-in-shells chicken embryos and in 2 out of 21 (9.5 percent) infertile eggs. There was no detection of *Salmonella* from fresh table eggs by either direct bacterial isolation or after enrichment.

4. 4. 0. Nucleotide sequence of *InvA* and *phoE* genes originating from chickens infected with *S. gallinarum*

4. 4. 1. Determination of *InvA* gene sequence and comparison with that of *S. typhimurium*

Once the *InvA* gene fragment had been amplified from *S. gallinarum* that was used to experimentally infect chickens, it was then purified and successfully cloned (Fig. 10) into the pMOS Blue T-vector. The cloning site of the vector was the *EcoRI* site. Transformation of *E. coli* with recombinant plasmid was necessary before selection and cultivation of the transformed *E. coli*. Recombinant plasmid DNA was then isolated from transformed *E. coli* and later purified and then digested by use of restriction enzymes. Using the DNA sequencing kit, the entire *InvA* gene (about 284-bp) was sequenced (Fig. 11). The results in Fig. 11 shows that except for 5 bases at position No. 211, 212, 213, 214, and 235, the comparison shows sequence homology to that of *S. typhimurium InvA* gene which is already known.

4. 4. 2. Determination of *phoE* gene sequence and comparison with that of *S. typhimurium*

PCR was used to amplify the *phoE* gene from organ tissues of chickens infected with *S. gallinarum*. The amplified gene was used for direct cloning into the *E. coli* vector (Fig. 10). The size of the amplified gene was a 365-bp fragment (Fig. 10). It was important to sequence this gene in order to ascertain the nature of the DNA and to compare it with that of *S. typhimurium phoE* gene which is already known. The procedures for cloning, transformation, purification, digestion and sequencing is exactly as is described in chapter 3.3.6. Using the dideoxy-chain termination procedure by use of a DNA sequencing kit, the entire *phoE* gene (365-bp) was sequenced. Except for one base pair, the amplified DNA band showed identity with *S. typhimurium phoE* gene (Fig. 12 at position 259).

Table 1. Isolation of *Salmonella gallinarum* and detection of *Salmonella gallinarum* DNA from organs of experimentally infected chicks (a)

Chick No.	Time after infection	Heart		Kidney		Liver		Spleen		Intestine	
		Isol.	PCR	Isol.	PCR	Isol.	PCR	Isol.	PCR	Isol.	PCR
1.	21 hr	+	-	+	+	-	+	+	+	-	+
2.		+	+	-	-	-	-	+	+	+	+
3.		-	+	+	+	-	+	-	-	-	+
4.		-	+	-	+	-	-	-	+	-	-
5.	3 d	-	+	-	+	-	-	-	+	+	+
6.		-	+	-	-	+	+	-	+	-	-
7.		-	+	-	-	+	+	-	-	-	-
8.		-	-	-	+	-	+	-	-	-	-
9.	7 d	-	+	+	+	+	+	+	+	+	+
10.		+	+	+	+	+	+	+	+	+	-
11.		+	+	-	+	+	+	+	+	-	+
12.		-	-	-	-	-	-	-	-	-	-
13.	14 d	-	+	-	+	-	+	+	+	-	+
14.		-	-	-	+	-	-	+	+	-	+
15.		-	-	-	+	-	+	-	-	-	-

(a) Two-day old chicks were inoculated orally with 1×10^2 CFU of *S. gallinarum* GTZ-3 strain.

Table 2. Isolation of *Salmonella typhimurium* and detection of *Salmonella typhimurium* DNA from organs of experimentally infected chicks (a)

Chick No.	Time after infection	Heart		Kidney		Liver		Spleen		Intestine	
		Isol.	PCR	Isol.	PCR	Isol.	PCR	Isol.	PCR	Isol.	PCR
16	21 hr	+	+	+	+	+	-	+	+	-	+
17		-	+	+	+	+	+	-	+	-	+
18		-	+	+	+	+	+	+	+	-	-
19		-	+	+	+	+	+	-	-	-	+
20	4 d	+	+	+	+	-	+	+	+	+	+
21		-	+	-	+	-	+	-	+	+	+
22		+	+	-	+	+	+	-	-	-	-
23		-	-	-	+	-	+	-	-	-	-
24	7 d	-	-	+	-	+	+	+	+	+	+
25		-	+	-	-	+	ND	-	-	-	+
26		-	-	+	-	+	-	-	-	-	+

(a) Four-day old chicks were inoculated orally with 1×10^7 CFU of *S. typhimurium* L55 strain. ND - Not done

Table 3. Isolation of *Salmonella gallinarum* and detection of *Salmonella gallinarum* DNA from organs of experimentally infected chicks (a)

Chick No.	Time after infection ¹	Heart		Kidney		Liver		Spleen		Intestine	
		Isol.	PCR	Isol.	PCR	Isol.	PCR	Isol.	PCR	Isol.	PCR
27	21 hr	-	+	-	+	-	+	+	+	-	+
28		-	+	-	+	-	+	-	+	+	+
29		-	+	-	+	-	+	+	+	+	+
30		-	+	-	-	-	-	-	-	+	+
31	3 d	-	+	-	+	+	+	+	+	+	+
32		+	+	-	+	+	+	+	+	-	-
33		-	+	+	+	-	+	+	+	-	-
34		-	-	-	-	-	-	+	+	+	+
35	7 d	+	+	+	+	+	+	+	+	+	+
36		+	+	+	+	+	+	+	+	+	+
37		-	+	-	-	+	+	-	+	-	-
38		-	-	-	-	-	-	-	-	-	-
39	14 d	+	+	+	+	+	+	+	+	+	-
40		-	+	+	+	+	+	+	+	-	+
41		+	+	-	-	-	-	-	-	-	-
42		-	-	-	-	-	-	-	-	-	-

(a) Two-day old chicks were inoculated orally 1×10^4 GTZ-3 strain.

Table 4. Isolation of *Salmonella* and detection of *Salmonella* DNA from egg yolks of chicken eggs

Specimen type	Bacteria isolation		Detection by PCR	
	direct	after enrichment	direct	after enrichment
Chicken embryo (dead-in-shell)	6/45 (13.3%)	11/45 (24.4%)	20/45 (44.4%)	23/45 (51.1%)
Infertile eggs (yolk)	2/21 (9.5%)	5/21 (23.8%)	6/21 (28.6%)	6/21 (28.6%)
Fresh table eggs (yolk)	0/21	0/21	0/21	0/21

Numerator - positive, denominator - number tested

Table 5. Isolation of *Salmonella* and detection of *Salmonella* DNA from hatcher chick fluff, floor litter, faeces and rat liver

Specimen type	Bacteria isolation	Detection by PCR
Faeces	9/48 (18.8%)	13/48 (27.1%)
Hatcher chick fluff	1/17 (5.9%)	6/17 (35.3%)
Floor litter	0/14	3/14 (21.4%)
Rat liver	0/1	1/1 (100%)

Numerator - positive, denominator - number tested

Table 6. Isolation of *Salmonella* and detection of *Salmonella* DNA from clinical specimens of chickens suspected for *Salmonella* infection

Sample type	Bacteria isolation	Detection by PCR
Spleen	7/17 (41.1%)	10/17 (58.8%)
Liver	17/51 (33.3%)	22/51 (43.1%)
Intestine	5/21 (23.8%)	10/21 (47.6%)
Heart	3/11 (23.8%)	6/11 (54.5%)
Kidney	0/2	0/2

Numerator - positive, denominator - number tested

Figures.

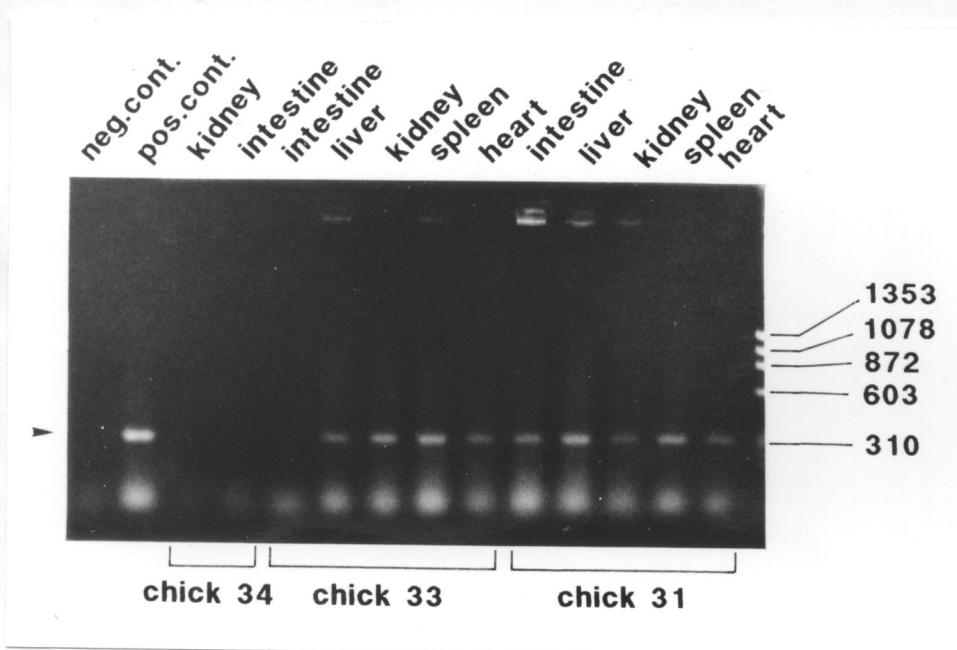


Fig. 1. PCR amplified products from *Salmonella gallinarum* colony and organs from chicks inoculated with 1×10^4 *S. gallinarum* (Group B). Amplified DNA bands from liver (lane 6), kidney (lane 7), spleen (lane 8) and heart (lane 9), and negative amplification in intestine (lane 5) of chick No. 33, three days after inoculation. Lanes 10 to 14 show DNA bands (chick No. 31) from the same organs as No. 33. Negative amplification in kidney (lane 3) and intestine (lane 4) of chick No. 34 is also shown. Lanes 1 and 2 show negative (DNA amplified from single colony) and positive (PCR mixture without extracted DNA) controls, respectively. Lane 15 is a molecular weight marker.

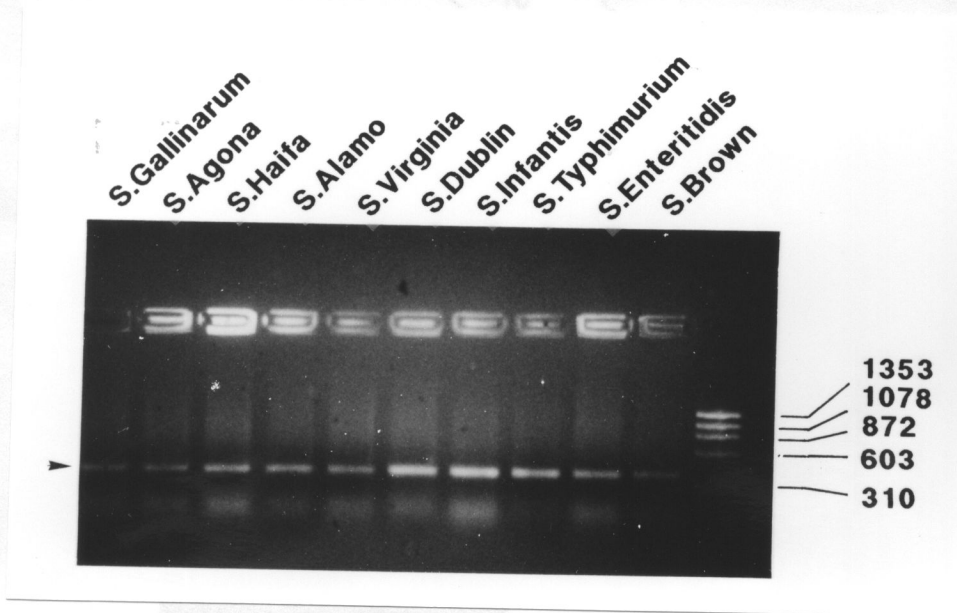


Fig. 2. Screening for the specificity of the *S. typhimurium* *phoE* primers by a single colony amplification method. Lanes 1 to 10 show specific DNA band amplifications of *S. gallinarum* (lane 1), *S. agona* (lane 2), *S. haifa* (lane 3), *S. alamo* (lane 4), *S. virginia* (lane 5), *S. dublin* (lane 6), *S. infantis* (lane 7), *S. typhimurium* (lane 8), *S. enteritidis* (lane 9) and *S. brown* (lane 10). Lane 11 is a DNA molecular marker.

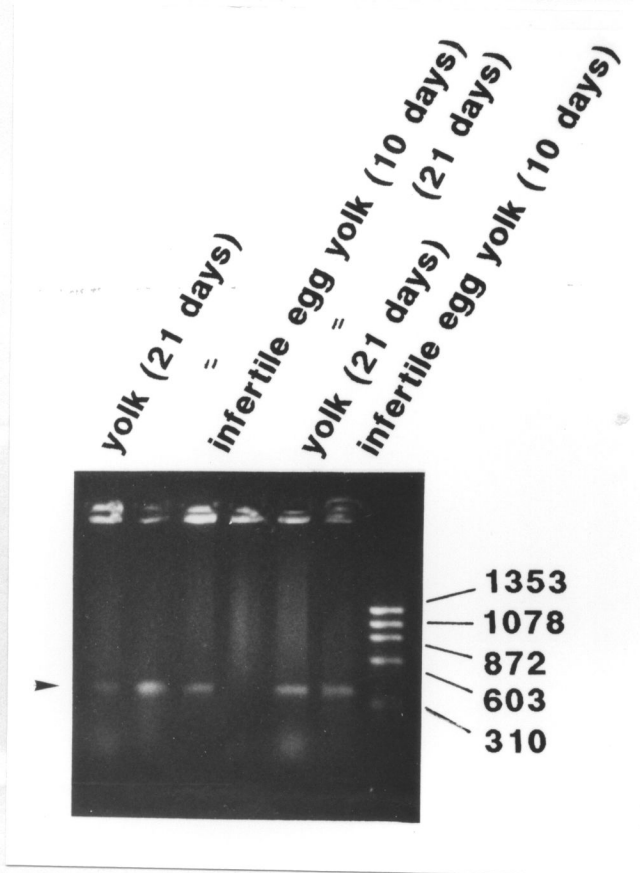


Fig. 3. Agarose gel electrophoresis showing results of direct PCR from yolk of chicken embryos and infertile eggs. Lanes 1, 2 and 3 show positive amplification of *Salmonella* DNA from yolks of chicken embryos while lane 4 shows a negative result from an infertile egg. Lanes 5 and 6 are also positive amplifications from yolks of chicken embryos and infertile egg. Lane 7 is a DNA marker.



Fig. 4. Direct amplification of a 365 bp *Salmonella* DNA fragment from yolks of dead-in-shell embryos after enrichment. Positive amplifications from yolks of chicken embryos (lanes 1, 3, 5 and 7) while lanes 2, 4 and 6 show negative amplification. Lanes 8, 9 and 10 are also positive amplifications from yolks of dead-in-shell embryos. Lane 11 shows a DNA molecular marker.

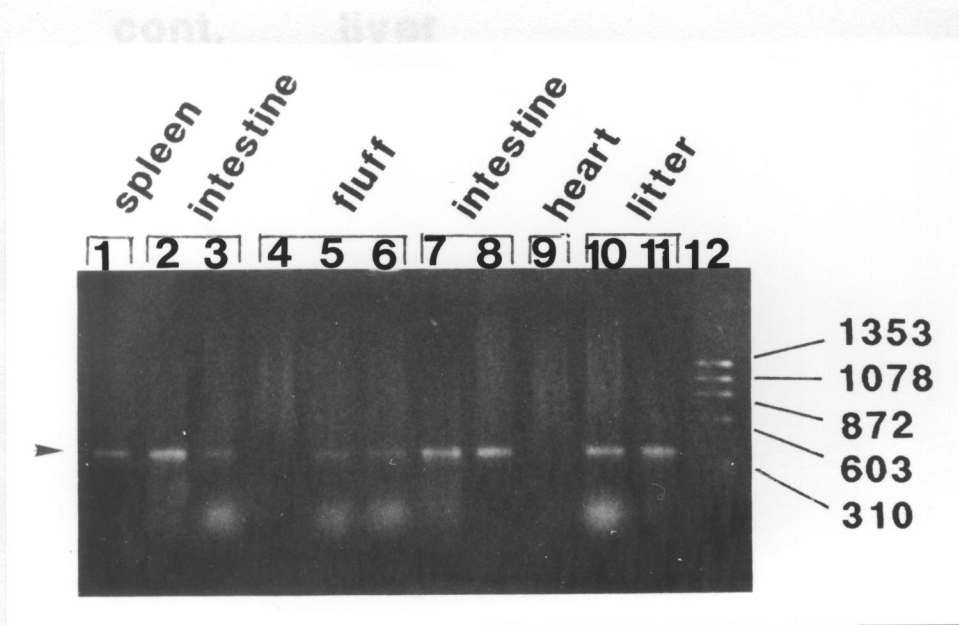


Fig. 5. Gel electrophoresis of PCR amplified *Salmonella* DNA from chickens naturally infected with *Salmonella* and amplified DNA from chicken pen samples. Amplified DNA bands from spleen (lane 1), intestine (lanes 2, 3, 7 and 8). Lane 9 is negative amplification from the heart. Lanes 5, 6, 10 and 11 are positive amplifications from chicken pen samples (fluff and chicken litter) while lane 4 is a negative amplification from chicken fluff. Lane 12 is a DNA molecular marker.

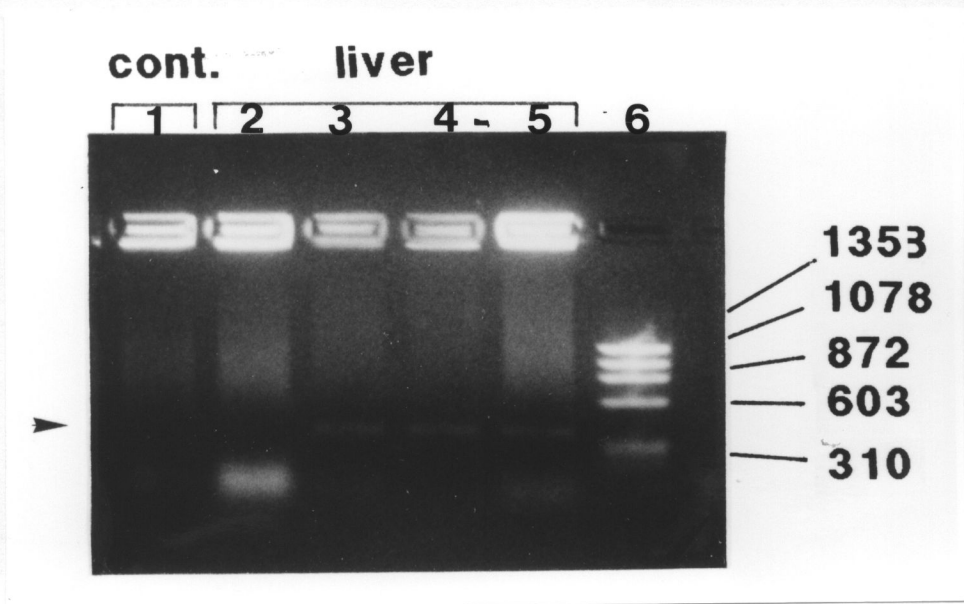


Fig. 7. Gel electrophoresis of amplified *Salmonella* DNA from

Fig. 6. PCR amplifications from livers of moribund chickens. Lane 1 shows a negative amplification from a control tube containing only the PCR mixture and lane 2 is a negative amplification from a chicken liver. Lanes 3, 4 and 5 are positive amplifications from chicken livers while lane 6 is a DNA molecular marker.

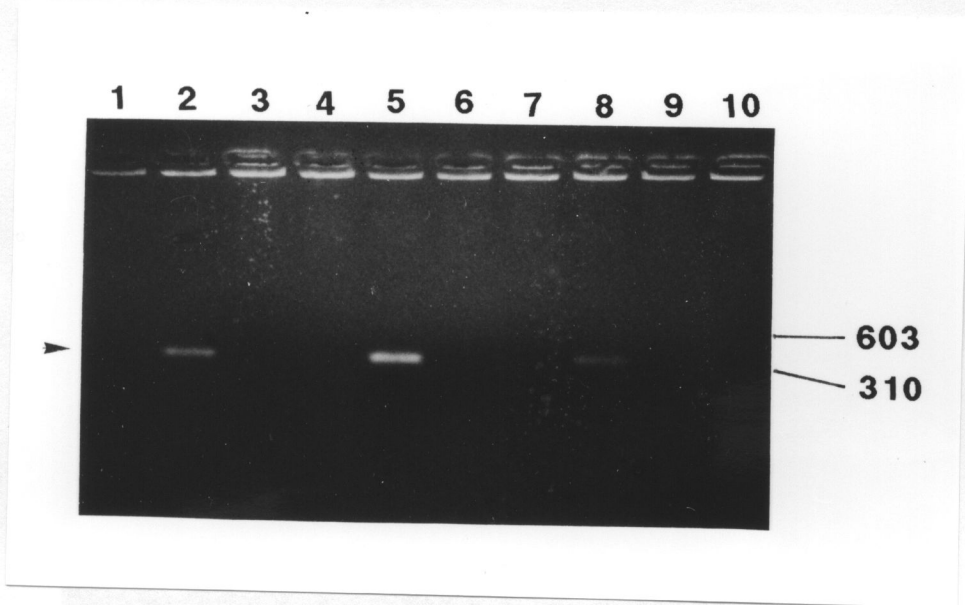


Fig. 7. Gel electrophoresis of amplified *Salmonella* DNA from pens and chicken pen samples. Amplified DNA bands from chicken floor litter (lane 2), chicken faeces (lane 5) and hatcher chick fluff (lane 8). Negative amplifications are from the floor litter (lanes 3 and 4), chicken faeces (lanes 6 and 7) and hatcher chick fluff (lanes 9 and 10). Lane 1 shows a negative control containing only the PCR mixture.

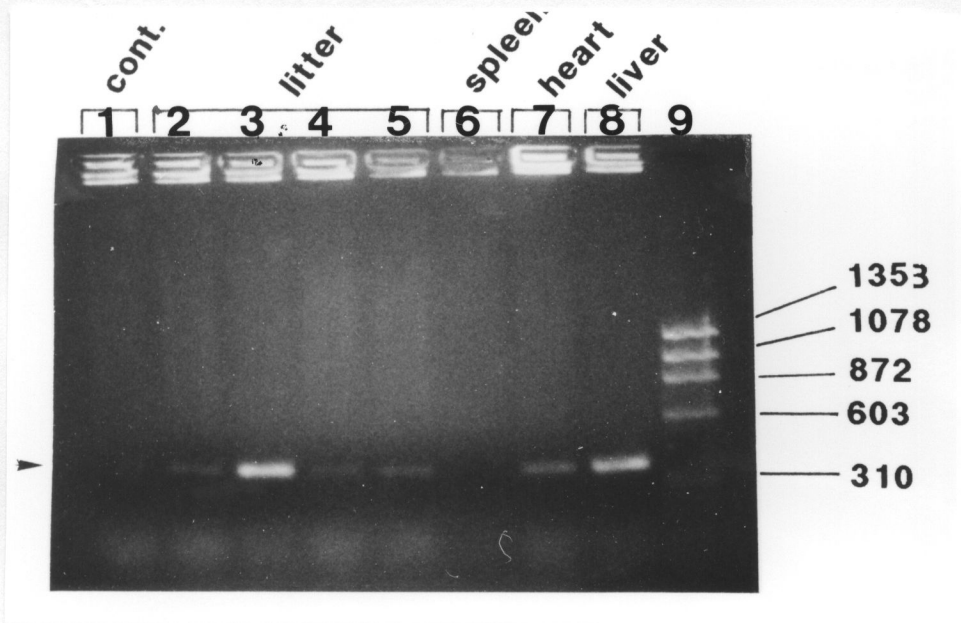


Fig. 8. *Salmonella* DNA amplifications from chicken organs and chicken pen samples. Lanes 2, 3, 4 and 5 show positive amplification from chicken litter while lanes 7 and 8 show *Salmonella* DNA amplification from heart and liver, respectively. Lane 6 is a negative amplification from the spleen while lane 1 is a negative control. Lane 9 is a DNA marker.

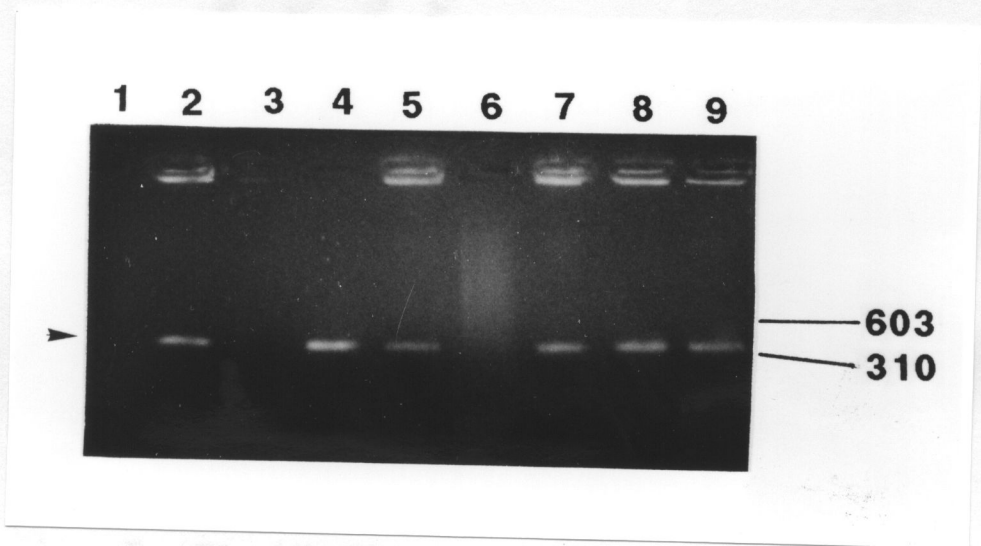


Fig. 9. Direct PCR amplification of *Salmonella* DNA from chicken egg yolks. Amplified *Salmonella* DNA bands from chicken yolk of dead-in-shell embryos (lanes 2, 4 and 5), infertile eggs (lanes 7, 8 and 9), and negative samples from fresh table eggs (lanes 3 and 6). Lane 1 shows a negative control which contained only the PCR mixture.

Upper- Sequence of *Salmonella typhimurium InvA* gene

Lower- Gene sequence of amplified *Salmonella gallinarum* DNA

1a TTCGTTATTGGCGATAGCCTGGCGGTGGGTTTGTGTTG

1b TTCGTTATTGGCGATAGCCTGGCGGTGGGTTTGTGTTG

38a TCTTCTCTATTGTCACCGTGGTCCAGTTTATCGTTATT

38b TCTTCTCTATTGTCACCGTGGTCCAGTTTATCGTTATT

76a ACCAAAGGTTTCAGAACGTGTCGCGGAAGTCGCGGCC

76b ACCAAAGGTTTCAGAACGTGTCGCGGAAGTCGCGGCC

112a CGATTTTCTCTGGATGGTATGCCCGGTAAACAGATGA

112b CGATTTTCTCTGGATGGTATGCCCGGTAAACAGATGA

149a GTATTGATGCCGATTTGAAGGCCGGTATTATTGATGC

149b GTATTGATGCCGATTTGAAGGCCGGTATTATTGATGC

186a GGATGCCGCGCGCGAACGGCGAAGCGTACTGGAAAG

186b GGATGCCGCGCGCGAACGGCGAAGGTACCTGGAAAG

222a GGAAAGCCAGCTTTAC

222b GGAAAGCCAGCTCTAC

Fig. 11. Nucleotide sequence of the PCR amplified *Salmonella gallinarum* DNA product. Sequencing was done with SEQUENASETM VERSION 2.0 using the dideoxy-chain termination procedure. Except for 5 bases at positions No. 211, 212, 213, 214 and position 235, all bases were identical to the already known sequence of *Salmonella typhimurium InvA* gene.

Upper Gene sequence of *Salmonella typhimurium* *phoE* gene
Lower Gene sequence of amplified *Salmonella* DNA from field samples.

1a GCCGAAGCCTGGGCTACGGGTGTTAAGTATGACGCT
1b GCCGAAGCCTGGGCTACGGGTGTTAAGTATGACGCT
37a AATGATATTTATATTGCGACCTTCTATTCAGAAACCC
37b AATGATATTTATATTGCGACCTTCTATTCAGAAACCC
74a GCAACATGAGCGCAGTTTCCGGCGGATTTGCCAATAA
74b GCAACATGAGCGCAGTTTCCGGCGGATTTGCCAATAA
112a AACCCAAAACCTTCGAAGCGGTTATCCAGTATCAGTTT
112b AACCCAAAACCTTCGAAGCGGTTATCCAGTATCAGTTT
149a GATTTTGGTCTGCGTCCGTCATTAGGCTATGTGCTGT
149b GATTTTGGTCTGCGTCCGTCATTAGGCTATGTGCTGT
186a CAAAAGGCAAAGATATTGAGGGCGTCGGCAGTGAGG
186b CAAAAGGCAAAGATATTGAGGGCGTCGGCAGTGAGG
222a ATTTGGTGAATTACATTGACGTTGGCGCAACCTATTA
222b ATTTGGTGAATTACATTGACGTTGGCGCAACCTATTA
259a CTCAACAAAAATATGTCGCGGTTTGTAGATTACAAA
259b TTCAACAAAAATATGTCGCGGTTTGTAGATTACAAA
295a ATCAATCAGCTTGATAGCGATAAC
295b ATCAATCAGCTTGATAGCGATAAC

Fig. 12. Comparison of nucleotide gene sequence between the already known sequence of *Salmonella typhimurium phoE* gene and the *Salmonella* amplified DNA from chicken samples. Except for one base pair at position 259, all the other bases showed identity.

CHAPTER FIVE

DISCUSSION

Specificity of conventional culture methods for identification of *Salmonella* organisms are already established. Most of these culture methods require laborious and time consuming processes, requiring pre-enrichment followed by selective enrichment. This study was therefore designed to evaluate the ability of the PCR method to detect both the *InvA* and *phoE* genes in chicken organs infected with *S. gallinarum* or other *Salmonella* serovars and to compare the sensitivity of this method with the conventional isolation methods. The distribution and characterisation of both the *InvA* and *phoE* genes of *S. typhimurium* has already been demonstrated (Galan *et al.* 1991, Spierings *et al.* 1992). When oligonucleotides were synthesised based on the nucleotide sequences encoding the fifth and eighth cell-surface-exposed region of *phoE* of *S. typhimurium* and used in PCR, the primers turned out to be specific for *Salmonella* (Spierings *et al.* 1992). In case of the *InvA* gene, Rahn *et al.* (1992) also demonstrated that the *InvA* gene contained sequences unique to *Salmonella* and showed that this gene was a suitable PCR target with potential diagnostic applications.

To test the specificity of the primers (*InvA* and *phoE*) DNA from other *Salmonella* isolates (*S. agona*, *S. haifa*, *S. alamo*, *S. virginia*, *S. dublin*, *S. infantis*, *S. typhimurium*, *S. enteritidis* and *S. brown*) was also amplified using the *InvA* and *phoE* gene primers of *S. typhimurium*. Selectivity of the two primers (*InvA* and *phoE*) was also tested on *S. gallinarum*, *E. coli*, *Citrobacter*, *Klebsiella*, *Shigella*, *Proteus*, *Edwardsiella*, *Morganella*, *Yersinia*, *Serratia* and *Enterobacter*. It is interesting to note that all the 10 *Salmonella* serovars which were tested for specificity in PCR produced specific amplified bands whose molecular weight was same as the *S. typhimurium* band obtained with either the *InvA* or *phoE* gene primers. On the other hand, bacteria other than *Salmonella* did not generate any specific bands when applied in PCR using the *InvA* and *phoE* gene primers. This result was in agreement with that of Rahn *et al.* (1992) who detected all but four of 630 *Salmonella* strains with a 99.4 per cent sensitivity when the *InvA* was used in PCR. Sensitivity of the PCR in detection of *Salmonella* was also evaluated on the DNA from chickens and chickenpen samples. The specificity and applicability of PCR technique using *S. typhimurium* *InvA* and *phoE* genes for detection and amplification of *Salmonella*

genes was verified by amplifying the DNA from *S. gallinarum* and *S. typhimurium* in PCR by the single colony method. DNA from *S. gallinarum* was specifically amplified. The PCR amplification was shown to be completely specific as the expected molecular weight of the amplicon was same as that of *S. typhimurium* *InvA* and *phoE* genes (284-bp and 365-bp, respectively) from which the primers were prepared. The amplification of the *InvA* and *phoE* genes by the single colony method confirmed that *S. gallinarum* did contain both genes (*InvA* and *phoE*). The presence of these genes in *S. gallinarum* suggested that these genes were potential targets for PCR. The PCR technique selectively amplified *S. gallinarum*. None of the bacteria other than *Salmonella* colonies could be amplified by the single colony method. Our results clearly indicate that both pairs of the primers (*InvA* and *phoE*) were selective and applicable for amplification of *S. gallinarum* from poultry samples.

Having demonstrated the presence of the *InvA* and *phoE* genes in *S. gallinarum*, a study was then initiated to develop an improved PCR technique for the detection of other *Salmonella* from the infected tissues and eggs. The emphasis in the first part of the study was to apply the

PCR with the *InvA* sequence gene to detect *S. gallinarum* and *S. typhimurium* DNA from experimentally infected chicks. With a pair of primers directed at the *InvA* gene it was possible to detect DNA of either *S. gallinarum* or *S. typhimurium* in 15 out of 20 barely 21 hours after experimental infection (Table 1). PCR was shown to be more sensitive than the conventional isolation procedures as it was possible to detect 50 out of 150 (33.3 per cent) organ samples compared to 24 out of 150 (16 per cent) by bacterial isolation methods (Table 1). Similar results were obtained when chicks were experimentally infected with *S. typhimurium*. In Table 2, results show that detection of *S. typhimurium* by PCR was more sensitive as it was possible to detect *Salmonella* DNA in 38 out of 110 (34.5 per cent) compared to 25 out of 110 (22.7 per cent) by bacterial isolation methods. Detection by PCR still appeared more sensitive even in late stages of infection as *S. gallinarum* DNA could be detected from samples which were negative for bacterial isolation on three, seven and 14 days after infection (Table 1, 2, 3). In the late stages of infection (14 days), it was possible to detect *Salmonella* in 10 out of 30 (33.3 per cent) tested samples compared to 1 out of 30 (3.3 per cent) by isolation in pre-enriched media. The size and

nucleotide sequence of the amplified DNA were in agreement with those reported by Rahn *et al.* (1992) and Galan and Curtis (1989), respectively, as it was possible to amplify a 284 bp fragment from the extracted DNA. The specificity of the test has been demonstrated as none of the bacteria other than *Salmonella* including total 49 species, 142 strains of Gram negative and positive bacteria yielded the specific amplification product (Rahn *et al.* 1992). The result in this study showed that DNA extraction improved when the pH of the organ homogenate was corrected to 7.8. There were a few exceptions in which some positive cases on bacterial isolation were negative in PCR amplification. This could possibly be attributed to the loss of DNA during extraction procedures. The PCR was also shown to be more sensitive than bacterial isolation even with pre-enrichment media. It was possible to detect *S. gallinarum* DNA both in the early and late stages of infection from samples in which a substantial number of bacteria could have been reduced by the defence mechanisms of the chick during the course of infection. The low level of bacterial isolation in the late stages of infection possibly indicates that isolation of *Salmonella* from chronically infected chickens, even when pre-

enrichment media is used may not be very reliable especially in antibiotic-treated chickens. PCR is therefore a very useful method to detect latent infection by microorganisms. Boyle and Blackwell (1991) reported that *herpes* virus DNA was successfully amplified in fish with latent infection.

The *Salmonella* DNA amplified in the present study was derived from *Salmonella* bacterial cells present in the experimentally infected organs. The differences between the DNA amplification and bacterial isolation detection limits could possibly be due to the fact that a number of bacteria are killed by the host defence during the course of infection but dead cells remained in tissues. DNA could also be amplified from the dead cells before it is rapidly degraded in host tissues by DNase. Since it has been reported that less than 0.1 pg of DNA was detectable in fish specimen when the PCR was used (Boyle and Blackwell 1991), this would lead to higher frequency of the detection of *Salmonella* DNA but poor isolation of bacteria from specimens in the present study.

In order to shorten the time spent on identification of *Salmonella* by bacterial isolation methods which normally takes about 3 days, amplification of *Salmonella* DNA directly from chick organ homogenate

was also tried but no specific DNA was detected. This result is supported by earlier results of Rahn *et al.* (1992) who failed to amplify *Salmonella* DNA directly from chicken litter and chicken carcass rinse samples. Detection of other infectious agents by PCR has only been successful after DNA extraction from samples (Beji *et al.* 1990 a and b, Boyle and Blackwell 1991, Cohen *et al.* 1994, Oyofu *et al.* 1992, Rahn *et al.* 1992, Saris *et al.* 1990, Wernars *et al.* 1991, Widjoatmodjo *et al.* 1991). The failure to directly amplify DNA from clinical samples could be partly due to the presence of certain inhibitors that have been found to affect the performance of the PCR. Haemoglobin (Mercier *et al.* 1990) and heparin (Beutler *et al.* 1990) have been cited as inhibitors of the PCR reaction. Because of these reasons, the PCR application detecting infectious agents directly from clinical specimens has not been widely used. Extraction of DNA from organs therefore seemed to be essential. The results shown in Tables 1, 2, and 3 confirm that the *InvA* gene of *Salmonella* could be used to detect *S. gallinarum* from clinical specimens only if the DNA extraction procedure is used. The PCR method has been found to be valuable in detecting *S. enteritidis* from faeces experimentally inoculated with a known number of the bacteria

(Cohen *et al.* 1994) only after DNA extraction from these samples. Probably what is significant about this study is the fact that *S. gallinarum* DNA was detected from organs of experimentally infected chicks 21 hours after the infection even when bacteria could not be isolated in bacterial culture.

Having succeeded in amplifying *S. gallinarum* from experimental infection, it was then decided that the same method be applied to detect other *Salmonella* serovars from chicken embryos, clinical and environmental samples by PCR technique and compare this with conventional culture methods. In order to do this, the *phoE* gene primers which have already been shown to be specific for the detection of *Salmonella* (Spierings *et al.* 1992) were evaluated in PCR to detect *Salmonella* from naturally infected chickens. The PCR technique was at the same time compared with the conventional bacterial isolation methods. Results obtained in Table 6 show that the PCR was more sensitive in detecting *Salmonella* DNA from clinical samples as it was possible to detect *Salmonella* in 10 out of 17 (58.8 per cent) spleen samples compared to 7 out of 17 (41.1 per cent) by isolation in enriched media. Similar results were obtained from heart samples where

Salmonella could be detected in 6 out of 11 (54.5 per cent) by PCR compared to 3 out of 11 (23.8 per cent) by isolation methods. Results from liver and heart samples also showed a similar trend as PCR was shown to be more sensitive in detecting *Salmonella* from clinical samples. The size of the amplified band was a 365-bp (Fig. 5, lanes 1, 2, 3, 7, 8, 9 and Fig. 6, lanes 3, 4, 5) which was in agreement with that obtained by Spierings *et al.* (1992) when they could detect the *phoE* gene in 132 out of 133 tested *Salmonella* strains.

Detection of *Salmonella* was also made in chicken pen samples by the use of PCR which was also compared with conventional bacteria isolation methods. Detection of *Salmonella* DNA from pen samples (environmental samples) was possible in 13 out of 48 (27.1 per cent) faeces, in 6 out of 17 (35.3 per cent) hatcher chick fluff, in 3 out of 14 (21.4 per cent) floor litter and in a rat liver by PCR. The detection of *Salmonella* by PCR was much better when this was compared with conventional isolation methods where detection was made in 9 out of 48 (18.8 per cent) faeces and in 1 out of 17 (5.9 per cent) hatcher chick fluff. There was no isolation from the floor litter and rat liver. The ability of PCR technique to detect *Salmonella* DNA from chicken faeces

is supported by the work of Cohen *et al.* (1994) who detected *S. enteritidis* DNA by PCR from faeces inoculated with a known number of bacteria. Direct amplification of *Salmonella* DNA from chicken litter could not be tried because of the presumably presence of inhibitors in the faeces as reported by Rahn *et al.* (1992) who could not amplify *Salmonella* DNA from chicken litter and chicken carcass rinse samples. DNA detection from these samples was therefore done only after DNA extraction. *Salmonella* DNA detection was also made from the liver of a rat. This detection probably indicates the possible existence of a reservoir of *Salmonella* infection in the rat population at this particular farm. Isolation of bacteria from environmental samples was poor as only one *Salmonella* isolate from the litter and chick fluff could be made by using this method. The failure to detect *Salmonella* by isolation method from PCR positive samples probably indicates the difficulties that may exist in tracing *Salmonella* infection when a method of low sensitivity is used.

The time consuming step in DNA technology is the isolation and purification of DNA from test samples. To test if isolation and purification of the DNA template from dead-in-shell, fresh table eggs

and infertile eggs was necessary for PCR, crude samples were directly inoculated into the PCR mixture. As results are clearly presented in Fig. 3, 4 and 9 and in Table 4, no purification of DNA was needed. Using crude samples in PCR, it was possible to detect *Salmonella* DNA in 20 out of 45 (44.5 per cent) tested samples from yolks of dead-in-shells. In infertile eggs, DNA was detected in 6 out of 21 (28.6 per cent) tested samples. There was no detection of *Salmonella* from fresh table eggs. Results presented in Table 4 also show that *Salmonella* detection by direct PCR from the yolk of dead-in-shell chicken eggs improved significantly when PCR was performed directly from enriched broth as it was possible to detect 23 out of 45 (51.1 per cent) tested samples compared to 20 out of 45 (44.4 percent), before enrichment. As was expected the amplified band was a 365-bp fragment (Fig. 9, lanes 2, 4, 5, 7, 8, 9 and Fig. 1, 3, 5, 6). When the PCR was compared with conventional bacterial isolation methods, it was shown to be more sensitive. The good performance of PCR has long been recognised to be poor when certain PCR inhibitors are present in the sample (Beutler *et al.* 1990, Mercier *et al.* 1990, Rahn *et al.* 1992). But in this study, there appeared to be no apparent inhibitors in the chicken egg yolk samples to

interfere with the PCR reactions as it was possible to amplify *Salmonella* DNA directly from chicken egg yolk without need for DNA isolation and purification. There was no detection made from fresh table eggs by both methods. The failure to detect *Salmonella* from these samples could possibly suggest that the bacteria multiplication might have been lowered below detectable levels by the low storage temperature of fresh table eggs practised at this farm. Direct PCR amplification of *Salmonella* DNA from the yolk of chicken eggs and embryos is the first report. The total time required for the detection of *Salmonella* from eggs using PCR is only 6 hours. This forms a significant contribution to our present knowledge.

To elucidate complete sequences of the *InvA* and *phoE* genes of *S. gallinarum* and to compare them with those of *S. typhimurium* genes, it was absolutely necessary to analyse the genetic relationship between the *S. gallinarum* PCR amplified DNA products and the already known *S. typhimurium* *InvA* and *phoE* genes. The *S. gallinarum* DNA amplified products were successfully cloned into the pMOS Blue T-vector (Fig. 10) and later sequenced. In the case of *InvA* gene, sequence was determined for a 284 bp and except for 5 bases at positions No. 211,

212, 213, 214, and 235 (Fig. 11), it showed homology to the already known *InvA* gene of *S. typhimurium*. The sequenced *phoE* gene of *Salmonella* from field samples also showed identity with *S. typhimurium* except for one base pair at position 259 (Fig. 12). This result is significant because it then therefore means that the *InvA* and *phoE* genes are well conserved among the *Salmonella* strains and that primers prepared from these genes could successfully be applied in the PCR for the detection of other *Salmonella* serovars in chickens. This assertion is further supported by the fact that the *phoE* gene primers that were used were able to selectively amplify all *Salmonella* serovars tested in this study.

5. 1. Conclusions

1 . From the results obtained in the experimental infection with *S. gallinarum* and *S. typhimurium*, it has been demonstrated that *S. gallinarum* can be detected from chicken samples by the PCR technique when *S. typhimurium* *InvA* and *phoE* gene primers are used. The study also showed that there was no need for primary bacterial isolation. The culturing procedure was therefore eliminated and hence shortening the

period required for identification of *Salmonella* infection. Confirmation of salmonellosis could therefore be done within a day of collection of samples compared to 3 days when culturing method is applied.

2. It was possible to demonstrate that the PCR was a more sensitive method in detecting *Salmonella* specific gene from organs of experimentally infected chicks as it was possible to detect *Salmonella* DNA not only in samples positive for bacterial isolation, but, also in negative samples.

3. Detection of *Salmonella* DNA even in late stages of infection proved that PCR can be a very useful tool in detecting latent infection by microorganisms.

4 . For the first time, PCR has been used to directly detect *Salmonella* DNA from yolk of chicken embryo without need for DNA extraction and primary bacterial isolation.

5. Detection of *Salmonella* DNA from clinical samples was also possible, but only after *Salmonella* DNA extraction from infected chicken organs. The PCR was once again shown to be more sensitive in the detection of *Salmonella*.

6 . Detection of *Salmonella* DNA from environmental samples was also possible, but only after DNA extraction from these samples. Moreover, time required to confirm the presence of *Salmonella* in environmental samples was only a day compared to 3 days by bacterial isolation methods.

7 . Genetic analysis of the amplified *Salmonella* DNA revealed that the amplified fragment was identical with *S. typhimurium* from which the *Salmonella* specific gene primers were prepared. The result is significant in that the *InvA* and *phoE* genes of *S. typhimurium* could be used to specifically amplify *Salmonella* isolates from clinical samples.

8 . The primers used were shown to be *Salmonella* genus specific. This assertion is supported by the ability of these primers to selectively amplify all *Salmonella* strains included in this study.

9 . The advantage of the PCR technique over the conventional bacterial isolation procedures is that it is extremely sensitive and this makes it possible to amplify even dead bacterial cells killed by host defence before they are rapidly degraded in host tissues by DNase during the course of infection.

10. The overall result has indicated the superiority of the PCR technique over the conventional bacterial isolation procedures in detecting *Salmonella* from clinical samples.

5 . 2. Recommendations for future work

Since not much work has been done in detecting *Salmonella* from poultry clinical samples using the PCR technique, it is recommended that;

1. Search for more primers that would be serovar specific rather than genus specific be done. This could be done by sequencing longer *Salmonella* DNA fragments which could possibly show serovar specific conserved regions. If such regions could be identified, then these could be used to prepare serovar specific primers.
2. Tracing the inhibitors of the PCR reactions from clinical samples. This could be done by determining the chemical composition of such clinical samples and assessing their possible roles in PCR inhibitions.

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