PREVALENCE AND ANTIMICROBIAL RESISTANCE OF *SALMONELLA*
IN IMPORTED CHICKEN CARCASSES IN BHUTAN

NARAPATI DAHAL

MASTER OF VETERINARY PUBLIC HEALTH

CHIANG MAI UNIVERSITY AND FREIE UNIVERSITÄT BERLIN
SEPTEMBER 2007
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NARAPATI DAHAL

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FREIE UNIVERSITÄT BERLIN IN PARTIAL FULFILLMENT
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THE REQUIREMENTS FOR THE DEGREE OF MASTER OF VETERINARY 
PUBLIC HEALTH

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21 September 2007

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Narapati Dahal
ABSTRACT

Salmonellosis is one of the most common and widely distributed foodborne diseases, and presence of antimicrobial resistant Salmonella in poultry and poultry products is a global public health problem. Bhutan imports chicken meat from India and its microbiological quality is not known. Therefore, a cross-sectional study was conducted from November 2006 to April 2007 with an aim to find the prevalence and antimicrobial resistance of Salmonella in imported chicken carcasses. During the same period, hygienic status of the chicken meat was determined by Aerobic Plate Count. The methods followed were ISO 6579: 2002 for Salmonella isolation, manufacturer’s instruction (Sifin, Germany) for serotype identification, ISO 4833: 2003 for Aerobic Plate Count, and the Disk Diffusion method (NCCLS 2000) for antimicrobial sensitivity testing.

Of the 400 samples analyzed (p-50% 95%CI error 5%), prevalence of Salmonella was 13% [CI: 9.94, 16.79] with Salmonella Enteritidis as the most frequently isolated serotype (84.62%), followed by Salmonella Typhimurium (15.38%). The isolation of Salmonella during winter and late spring was significantly
different ($p<0.001$). Broiler carcasses were 10.62 times (OR 10.62) more likely to yield *Salmonella* in the hot season as compared to the winter season. The level of hygienic status of carcasses ($n=32$) was found acceptable as compared to the international standards (median $4.408 \log_{10} \text{cfu g}^{-1}$, IQR 0.510).

Among seven antimicrobial tested ($n=52$), resistance was highest for nalidixic acid (96.15%) followed by amoxicillin (11.54%) and cephalexin (5.77%). Ciprofloxacin and sulpho-trimethoprim showed resistance of 1.92% each. While gentamicin was sensitive to all the isolates tested, chloramphenicol had a sensitivity of 98.08%. The isolates were resistant to a maximum of three antimicrobials. All eight *Salmonella* Typhimurium isolates were resistant to nalidixic acid with one isolate showing simultaneous resistance to cephalexin. *Salmonella* Enteritidis was resistant to five of the seven antimicrobials tested with simultaneous multidrug resistance in up to three antimicrobials. Overall, five resistance patterns were observed among 52 *Salmonella* isolates. In conclusion, comprehensive policies to ensure safe chicken meat import are recommended.
ชื่อเรื่องวิทยานิพนธ์ ควมชุกและการต่อต้านจุลชีพของเชื้อซัลโมเนลลาจากซากไก่นำไปในภูฏาน

ผู้เขียน นาย นราประดิ ดาฮาล

ปริญญา คัดวทยาศาสตร์สุขาภิบาลชีวภาพ

คณะกรรมการที่ปรึกษาวิทยานิพนธ์ คร. อุปโภคเบิกโลก ประธานกรรมการ (FU-Berlin)
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บทคัดย่อ

โรคที่เกิดจากเชื้อซัลโมเนลลาเป็นโรคที่พบได้ง่ายและระบาตอบ้องกว้างขวางชนิดหนึ่งของโรคที่เกิดกับอาหาร การติดต่อผู้ป่วยมาจากเชื้อซัลโมเนลลาในสัตว์ปีกและผลิตภัณฑ์สัตว์ปีกถือว่าเป็นปัญหาที่สำคัญของโลก ภูฏานนำเข้าเนื้อไก่จากอินเดียซึ่งไม่ทราบถึงคุณภาพด้านจุลินทรีย์ ด้วยเหตุนี้จึงทำการศึกษาตั้งแต่เดือนพฤศจิกายน พ.ศ. 2549 ถึงเดือนเมษายน พ.ศ. 2550โดยมีเป้าหมายเพื่อหาความชุก และการติดต่อผู้ป่วยมาจากเชื้อซัลโมเนลลาในสัตว์ปีกนำไปในซากไก่ช่วงเวลาดังกล่าวได้ตรวจสอบสภาพสุขอนามัยของเนื้อไก่ด้วยวิธี Aerobic Plate Count การแยกเชื้อซัลโมเนลลาในสัตว์ปีกไปในตามการปฏิบัติของ ISO 6579: 2002 การจำแนกเชื้อได้ใช้วิธี disk diffusion เพื่อทดสอบการต่อต้านยาของเชื้อได้ตามการรีวิวของ NCCLS 2000

จากการวิเคราะห์ 400 ตัวอย่าง (p-50% 95% CI error 5%), พบความชุกของเชื้อซัลโมเนลลา 13% [CI: 9.94, 16.79] Salmonella Enteritidis เป็นเชื้อที่แยกได้มากที่สุด (84.62%) ตามด้วยเชื้อ Salmonella Typhimurium (15.38%) เชื้อซัลโมเนลลาในช่วงหน้าหนาวมีความน่าจะจะมีผู้ป่วยมีมากกว่าช่วงหน้าร้อนที่อยู่ในบึง (p< 0.001) โดยพบเชื้อซัลโมเนลลาในเนื้อไก่เป็น 10.62 เท่า (OR 10.62) ในช่วงที่ร้อนมากกว่าในฤดูร้อน สภาพสุขอนามัยของซากไก่ (n=32) พบว่ายอมรับได้เมื่อเปรียบเทียบกับมาตรฐานสากล (median 4.408 log 10 cfu g⁻¹, IQR 0.510)
จากการตรวจสอบการดื้อยา 7 ชนิด (n=52) พบว่าเชื้อที่สูงคือ nalidixic acid (96.15%) ตามด้วย amoxicillin (11.54%) cephalexin (5.77%) ส่วน ciprofloxacin และ sulpha-trimethoprim พบว่าเชื้อต่ออย่างแย่จัดว่า 1.92%. โดยไม่มีเชื้อตัวไหนที่แยกได้เชื้อดื้อยา gentamicin ส่วนเชื้อมีความไวต่อchloramphenicol มาที่สุด (98.08%) เชื้อที่แยกได้คือมากที่สุดต่อยาปฏิชีวนะ 3 ชนิด Salmonella Typhimurium ที่แยกได้ 8 isolates คือต่อ nalidixic acid และมี 1 isolate ที่แสดงการดื้อต่อ cephalaxin ส่วน Salmonella Enteritidis คือต่อยาปฏิชีวนะ 5 ใน 7 ตัว ที่ใช้ทดสอบและคือต่อการใช้ยาปฏิชีวนะรวมถึง 3 ชนิด โดยรวมพบว่ามีรูปแบบการดื้อยาอยู่ 5 แบบ จาก Salmonella ที่แยกได้ 2 isolates ข้อเสนอแนะให้มีนโยบายที่รัดกุมเพื่อประกันความปลอดภัยของเนื้อไก่
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<td>BAFRA</td>
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<td>BPLS</td>
<td>Brilliant-green phenol-red lactose sucrose</td>
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<td>CAC</td>
<td>Codex Alimentarius Commission</td>
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<tr>
<td>masl</td>
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<tr>
<td>SQF</td>
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<td>ml</td>
<td>Milliliters</td>
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1. INTRODUCTION AND OBJECTIVES

1.1 Bhutan overview

Situated on the southern slopes of the eastern Himalayas, Bhutan is a small country located between altitudes 26° 40’ and 28° 20’ N and between longitudes 88° 45’ and 92° 7’ E, in the eastern Hindu Kush Himalayan region (Walter et al., 2001) with an area of 40 076 Km² (Ministry of Agriculture, 2002). The kingdom is entirely landlocked, bordered by the Tibetan Autonomous Region of China in the north and by the Indian states of Sikkim, Arunachal Pradesh, West Bengal and Assam in the west, east and south (Phuntshok, 1999). Its physical features are characterized by high, rugged mountains and an intricate network of deep valleys, ravines and depressions earmarking watercourses, drainage basins, waterfalls, human settlements, glacial lakes and moraines (Ministry of Agriculture, 2002). The land rises from an elevation of about 100 meters above sea level (masl) in the south to more than 7550 masl in the north. The country can be classified into agro-ecological zones based on the agro-climatic condition determined by altitude, rainfall and topography within three broad geographical zones: the Southern Foothills, the Inner Himalayas and the High Himalayas. Livestock and associated farming systems differ from one agro-ecological zone to the other based on altitude, temperature and rainfall distribution. Their combined effects greatly influence agricultural activities.

Bhutan is one of the least densely populated countries with 69.1% of the population still residing in the rural areas. The human population estimate is 672 425 (Ministry of Health, 2006), of which around 85% are engaged in subsistence, mixed livestock and arable agriculture, although only 7.8% of the total land area is under permanent cultivation. It is estimated that about 79% of the country’s population is dependent upon agriculture (Ministry of Home Affairs, 2002) and over 90% of the households own livestock (RNR Statistics, 2004). Development in Bhutan is guided by principles which emphasize the need to ensure the preservation of natural resources and cultural heritage which allows development from subsistence to a
modern economy to proceed in a sustainable manner. The Renewable Natural Resources sector of the Royal Government of Bhutan represented by Ministry of Agriculture, cover agriculture, livestock and forestry and it remains the single most important sector accounting for 32.7% of the gross domestic product in 2003. (RNR Statistics, 2004).

![Map of Bhutan showing district boundaries, major highways and border entry points](image)

Fig. 1: Map of Bhutan showing district boundaries, major highways and border entry points

1.2 Scientific justifications

1.2.1 The poultry industry

Although poultry rearing has been an integral part of the Bhutanese farming system, commercial poultry was introduced only in the early sixties with the introduction of White Leghorn and Rhode Island Red breeds through the agency of
the government poultry farms (Rai, 1987). There was a consistent effort by the government for poultry development throughout the late sixties and seventies for improving the quality of birds, egg production and encouragement of private entrepreneurship through the distribution of improved birds from the government breeding units (Tenzing, 1978). However, the development of the poultry industry in Bhutan has been very slow and the major reasons involved are a lack of compounded feed within the country (Rai, 1987), a lack of appropriate farm technology (Satyanarayana, 1981), poor market access and pricing policies (Win, 1990), poor knowledge and management practices (Tamang, 1990).

Today, with the set up of the feed mills and commercial poultry farms at the few urban centers, the total poultry population is approximated at 1.5-2 million heads of which over 70% population is in backyard farms (RNR Statistics, 2004). About 55.1% of the households rear poultry in backyard farming system mainly for the purpose of eggs. There are no organized poultry slaughter houses in the country. The commercial farms operate manual poultry slaughter and sell their products either through the farm outlets or sell it through the meat sale counters in the urban centers. An increasing number of farmers are taking up back-yard poultry farming and are encouraged to do so through the national policy of food self-sufficiency.

Production from these commercial farms does not meet the market demand for chicken meat in Bhutan. Backyard production is mainly focused on egg production for household consumption and it also provides a supplementary income to the farming communities (RNR statistics, 2004). Development of private entrepreneurship in poultry production is still very slow, partly due to the fact that Bhutan is a Buddhist state and people avoid killing. The system of distributing the improved breeds of poultry to the backyard farmers from the government breeding units is continuing. As the economic returns are being realized by the farmers, it is expected that the backyard production will shift its course from the present system of household egg consumption to catering to the needs of urban meat demand. Local poultry production in the year 2005 was approximately 18.6 metric tons which is only 1.4% of the total poultry meat consumption in the country. Therefore, as a result of
the low production of poultry and poultry products at present, the essential demand for chicken meat in the Bhutanese market is met through the imports. Bhutan imports day-old chicks, eggs and chicken meat mainly from India. The annual import of poultry meat is approximately 1334.6 metric tons, valued at 2.4 million US dollars in 2005 (BAFRA, 2005).

1.2.2 Food safety

The preamble of the Codex Alimentarius Commission states that adequate, safe, sound and wholesome food is a vital element for the achievement of acceptable standards of living and that the right to a standard of living adequate for the health and wellbeing of the individual and his family is proclaimed in the Universal Declaration of Human Rights of the United Nations. Bhutan is a member of The Codex Alimentarius, The World Animal Health Organization (OIE), and The World Health Organization (WHO). Bhutan is aware of the importance of The World Trade Organization (WTO) and its central role in shaping the commercial and trading relationship among nations in the international market place, and has expressed interest in the multilateral trading system by having begun the accession process to join The World Trade Organization since 1999 (Tobgay, 2006). Economically, Bhutan is in a way compelled to integrate into the global trading system. Further, to accommodate the growing needs and aspirations of the people, and with due emphasis from the Codex Alimentarius, Bhutan is in the process of developing the national standards for foods and food products. A few major constraints for development in the food safety area are the lack of basic infrastructure and the appropriate technology, and an acute shortage of trained personnel in the food safety and quality control. The food production system in Bhutan is still a livelihood enterprise, and commercial food producing companies are very few.

Routine meat inspection and quality control is a responsibility of The Bhutan Agriculture and Food Regulatory Authority (BAFRA), an independent organization under the Ministry of Agriculture. Being recently established, the organization has inadequately trained personnel in the broader areas of public health and the regulatory
system, as well as in the key policy-making positions. A majority of the meat inspectors among the organization are diploma graduates with short-term training in meat inspection. The laboratories are not fully equipped, both in terms of facilities as well as in manpower. Therefore, with the lack of resources such as skilled manpower and aiding laboratory facilities to efficiently carry out the regulatory works, the quality of meat and meat products imported into the country is unknown, and has to rely on the information provided at the source.

In a few instances, there were incidences of *Salmonella* mass food poisoning such as in school picnics, attributed to chicken meat consumption. Although *Salmonella* in poultry meat has been recognized as a major source, no systematic studies have been done with respect to the *Salmonella* in imported chicken meat in Bhutan. In late 2006, a team of officials from the Department of Livestock (DOL) in Bhutan visited Arambagh Hatcheries; poultry slaughter firm in West Bengal in India, and have recommended the import of well-packed frozen products. Although strict import conditions have been laid down, such as conforming to the recognized standard operating procedures and processing standards like HACCP and Safe Quality Food (SQF) (*Press release, 2006*), it essentially takes more than 8-10 hours to get the products to the consumers. The country does not have bulk meat storage facilities as of now. The retail shops own a deep freezer for storage and the shops are of the open type. Therefore, it is probable that there is a risk of Salmonellosis in humans through chicken meat consumption in the country.

1.2.3 Antimicrobial resistance

In recent years problems related to *Salmonella* have increased significantly, both in terms of the incidence and severity of cases of human Salmonellosis. Since the beginning of the 1990s, strains of *Salmonella* which are resistant to a range of antimicrobials including the first choice agents for treatment of humans have emerged and are threatening to become a serious public health problem. Drug resistant *Salmonella* emerge in response to antimicrobial usage in humans and in food animals and selective pressure from the use of antimicrobials is a major driving force behind
the emergence of resistance. Multi-drug resistance to critically important antimicrobials is compounding the problem (WHO, 2005). There are reports of high prevalence of resistance in Salmonella isolates from countries such as Taiwan (Lauderdale et al., 2006), India (Mandal et al., 2004, 2006), The Netherlands (Duijkeren et al., 2003), resistant isolates from France (Weill et al., 2006), Canada (Poppe et al., 2006), and Ethiopia (Molla et al., 2003). Similarly, there are various reports of multi-drug resistant Salmonella organisms isolated from chickens in India. One of the studies indicated a rise in the antibiotic resistance in Salmonella Typhi (Gautam et al., 2002). In recent years, antibiotic resistance in Salmonella has assumed alarming proportions (Murugkar et al., 2005) and the isolates were resistant to at least one of the 15 antibiotics tested. Typhoid fever alone is estimated to have caused 21.6 million illnesses and 216 500 deaths globally in 2000 (Bahn et al., 2005) and the incidence is high in South Central and Southeast Asia. It has been reported that livestock and their products can contribute to as much as 96% of the total Salmonella infection in humans. The source of live poultry as well as poultry meat import to Bhutan is India. The presence of these organisms in the imported meat for consumption is a safety concern of the general Bhutanese population. The presence of resistant organisms in the imported products is a therapeutic concern for the physicians which might pose prolonged treatment in cases of outbreaks, delayed recovery or treatment failure. Information on the antimicrobial resistance pattern of the Salmonella isolates from chicken meat could be useful for successful treatment, as well as planning strategic use of drugs to minimize resistance in the future.

1.2.4 Antimicrobial selection

Antimicrobial resistance is a global public health problem. Although all countries are affected, the extent of the problem in the developing nations is unknown (Ang et al., 2004). The most widely used antibiotics for treatment of Salmonellosis in humans is a group of fluoroquinolones and third-generation cephalosporins. The earlier drugs chloramphenicol, ampicillin, amoxicillin and trimethoprim-sulfamethoxazole are occasionally used as alternatives (WHO, 2005).
The most commonly used antimicrobial agents for either chemoprophylaxis or therapy for control of bacterial diseases in poultry in India includes sulfadiazine, sulphamethoxy pyridazine, neomycin, furazolidone, ciprofloxacin, enrofloxacin, nitrofurantoin, colistin, ampicillin and cloxacillin (Prakash et al., 2005). In many of the studies carried out in India, non-typhoidal Salmonella species, the typhoidal species and the avian isolates were found resistant to amoxicillin, nalidixic acid, cotrimoxazole, chloramphenicol, ciprofloxacin, sulphadiazine, sulphamethoxy pyridazine, neomycin, furazolidone, doxycycline, ampicillin, tetracycline, chlorotetracycline, kanamycin, gentamicin, amikacin, ceftizoxime and ceftriaxone (Gautam et al., 2002, Tankhiwale et al., 2003a, Taneja et al., 2005, Prakash et al., 2005, Achla et al., 2005, Ray et al., 2006, Das et al., 2006).

Typhoid has been listed as a priority disease in Bhutan and there were 2948 reported cases in 2005 (Ministry of Health, 2006). Currently, the most common antimicrobials used for treatment of Salmonellosis in humans in Bhutan include ciprofloxacin, cephalexin, chloramphenicol, nalidixic acid, amoxicillin, gentamicin and cotrimoxazole, and all of these antimicrobials are listed in the Essential Drugs Program (EDP) of the Department of Health (DOH). Therefore, the Salmonella isolates are tested with antimicrobial sensitivity testing to see if they are resistant to these antimicrobials used in Bhutan.

1.3 Significance of the study

The prevalence of Salmonella in the imported chicken meat in Bhutan has never been studied, and is therefore unknown. Quantification of the disease will invariably help to measure the prevalence of Salmonella and document the isolates occurring in the imported chicken carcasses. The study will help provide information on the bacterial load of the imported chicken meat, and will form the basis for future quality control of the meat in Bhutan.

A proper documentation of the meat quality will form a basis for control and prevention of foodborne disease in the wake of rising consumer safety and the
globalization of food products. This will also provide the basis to develop the national standards for the import of chicken meat and scrutinize the import regulations of the country. The identification of antimicrobial resistant serotypes of *Salmonella* will help in instituting an appropriate therapy and provide information on the auxiliary emergence of drug resistance.

Recommendations based on the research findings will be submitted to the Bhutan Agriculture and Food Regulatory Authority on the status of imported chicken meat, in terms of *Salmonella* identification and bacterial load, for improvement in the import meat quality. The information will prove essential for developing sound food safety standards. Besides, the research will also kickstart the procedure for isolation and identification of *Salmonella* in meat and meat products following an internationally recognized protocol in the country. A similar proposal will be submitted to the Department of Health on the findings of antimicrobial resistance. Since the study is the first of its kind in the country, it is expected to provide basic information on the status of *Salmonella* in chicken meat imported in Bhutan. The information is also expected to contribute to the Department of Health.

In a holistic view, the research is expected to initiate similar studies in both imported as well as locally produced meat and meat products. To some extent, it will also develop public awareness on food safety and the demand for safe food in the country.

### 1.4 Objectives

i. To find out the prevalence of *Salmonella* spp. in imported chicken carcasses

ii. Antimicrobial sensitivity testing of the isolates to determine resistance pattern by Disc Diffusion method.

iii. Identification of *Salmonella* serotypes isolated

iv. Enumeration of the bacterial load in the chicken carcass by Total Plate Count.
2. LITERATURE REVIEW

2.1 Evolution of *Salmonella*

It is speculated that the genera of *Escherichia coli* and *Salmonella* diverged from a common ancestor about the time of the emergence of mammals, and emerge as mammalian and avian pathogens through the acquisition of pathogenicity islands and of a virulence plasmid, through variation in lipopolysaccharide antigens, through development of mechanism for flagellar antigen phase shifting, and in other ways (Wray *et al.*, 2001). Some writers estimate *Salmonella* diverged from the genus *Escherichia* 120–160 million years ago (Lawrence, 1999, Cotter *et al.*, 2000). Baumler *et al.*, found that the close DNA relatedness among *Salmonella* serotypes is evidence for their clonal origin, and based on the degree of sequence divergence, it can be estimated that a common ancestor of the genus existed about 25 to 40 million years ago (Baumler *et al.*, 1998). In 1892 Loeffler described the causative agent of murine typhoid, (then known as *Bacillus Typhi*) that caused an epidemic typhoid fever-like disease in mice (Santos *et al.*, 2001). Recently, *Salmonella* Typhi was identified in ancient skeletal material, thereby incriminating typhoid fever for the plague that devastated Athens in 430-426 B.C. It is hypothesized that accumulation of single mutations, insertions or deletions with the genome of modern-time *Salmonella* Typhi appears to have generated many pseudogenes, suggesting its recent evolutionary origin (Papagrigorakis *et al.*, 2007).

2.2 Genus *Salmonella*

*Salmonella* organisms are facultative anaerobic gram-negative rods within the family of Enterobacteriaceae (Yan *et al.*, 2003). Classically, the members of this genus are motile by peritrichous flagella except *Salmonella* Pullorum and *Salmonella* Gallinarum, which lack flagella, however, the long standing fact that *Salmonella* Pullorum is non motile has been disproved and it has been shown that the motility can be induced under special medium conditions (Holt *et al.*, 1997). *Salmonella* grow
optimally at 35°C to 37°C, catabolize a variety of carbohydrates into acid and
gas, use citrate as the sole carbon source, produce H$_2$S and decarboxylate lysine and
ornithine to cadaverine and putrescine respectively (Barbara et al., 2000). Historically
Salmonella catabolized glucose and lysine, but failed to metabolize lactose, sucrose
and urea, however due to the widespread exchange of genetic elements between
compatible bacterial strains in the environment, atypical Salmonella biotypes that
cannot decarboxylate lysine (Muramatsu et al., 1992, Morita et al., 2006) or that
readily use lactose (Falcao et al., 1975, Kohbata et al., 1983, Glosnicka et al., 1987)
sucrose (Johnson et al., 1976, Reid et al., 1993) and urea, have been isolated. They
are chemo-organotrophic organisms, having both a respiratory and a fermentative
type of metabolism (John et al., 1994). Many serotypes in the group are closely related
to each other by somatic and flagellar antigens and most strains show diphasic
variation of the flagellar antigens.

The genus Salmonella comprises two species: (1) Salmonella enterica, which
is divided into six subspecies: Salmonella enterica subspecies enterica (I), Salmonella
enterica subspecies salamae (II), Salmonella enterica subspecies arizonae (IIIa),
Salmonella enterica subspecies diarizonae (IIIb), Salmonella enterica subspecies
houtenae (IV) and Salmonella enterica subspecies indica (VI); and (2) Salmonella
bongori (formerly called Salmonella enterica subspecies bongori V). Species and
subspecies can be distinguished on the basis of differential characters, and through
antigenic formulae, into 2501 serovars (Solari et al., 2003). However, a recent report
from the Centre for Infectious Disease Research and Policy classifies members of the
Salmonella species into more than 2541 serotypes (serovars) according to their
somatic (O) and flagellar (H) antigens (CIDRAP, 2006). The antigenic formulae of
Salmonella serotypes are defined and maintained by the World Health Organization
(WHO) Collaborating Centre for Reference and Research on Salmonella at the
Pasteur Institute, Paris, France (WHO collaborating centre), and new serotypes are
listed in the annual updates of the Kauffmann-White scheme (Brenner et al., 2000).
2.3 Current nomenclature

Several schemes based on biochemical characteristics, DNA homology, and enzyme electrophoretic patterns have been used for the taxonomic classification of *Salmonella* (Barbara et al., 2000). *Salmonella* nomenclature is complex, and scientists use different systems to refer to and communicate about this genus. The current usage often combines several nomenclatural systems that inconsistently divide the genus into species, subspecies, subgenera, groups, subgroups, and serotypes (serovars) which causes confusion (Brenner et al., 2000). The genus *Salmonella* has two systems of nomenclature in circulation. One system proposed by Le Minor and Popoff in the 1980s, which has received a wide acceptance although it does not conform to the rules of bacteriological code, and the other which conforms to the bacteriological code, but used by the minority. The present problem is that two systems of nomenclature are in the use for the members of the genus *Salmonella* (Tindall et al., 2005).

The nomenclature of the genus *Salmonella* has evolved from the initial one serotype-one species concept proposed by Kauffmann on the basis of the serologic identification of O (somatic) and H (flagellar) antigens. A capsular polysaccharide, the Vi antigen is present on *Salmonella* Typhi and few other serovars of *Salmonella*, including *Salmonella* Dublin (Heyndrickx et al., 2005). The defining development in *Salmonella* taxonomy occurred in 1973 when Corsa et al. demonstrated by DNA-DNA hybridization that all serotypes and subgenera I, II and IV of *Salmonella* and all serotypes of “Arizona” were related at the species level, thus belonging to a single species. The single exception subsequently described is *Salmonella bongori* previously known as subspecies V which by DNA-DNA hybridization is a distinct species. In 1986 the subcommittee of Enterobacteriaceae of the International Committee on Systematic Bacteriology at the XIV International Congress of Microbiology unanimously recommended the change of type species of *Salmonella* to *Salmonella enterica*, a name coined by Kauffmann and Edwards in 1952, however the Judicial committee denied with the fact that the *Salmonella* Typhi might be overlooked as it is one of the most important human pathogens (Brenner et al., 2000).
The current nomenclature used by CDC is based on the recommendations from the WHO collaborating centre and it adequately addresses the concern and requirements of clinical and public health microbiologists (Deb and Kapoor, 2005). The nomenclature is summarized in the table 1. According to the CDC system, the genus *Salmonella* contains two species, each of which contains multiple serotypes (Brenner et al., 2000) as shown in table 2.

Table 1: *Salmonella* species, subspecies, serotypes and their usual habitats, Kauffmann-White scheme

<table>
<thead>
<tr>
<th><em>Salmonella</em> species and subspecies</th>
<th>No. of serovars within subspecies</th>
<th>Usual habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. enterica subsp. Enterica (I)</td>
<td>1454</td>
<td>Warm blooded animals</td>
</tr>
<tr>
<td>S. enterica subsp. Salamae (II)</td>
<td>489</td>
<td>Cold blooded animals and the environment</td>
</tr>
<tr>
<td>S. enterica subsp. Arizonae (IIIa)</td>
<td>94</td>
<td>Cold blooded animals and the environment</td>
</tr>
<tr>
<td>S. enterica subsp. Diarizonae (IIIb)</td>
<td>324</td>
<td>Cold blooded animals and the environment</td>
</tr>
<tr>
<td>S. enterica subsp. Hautenae (IV)</td>
<td>70</td>
<td>Cold blooded animals and the environment</td>
</tr>
<tr>
<td>S. enterica subsp. Indica (VI)</td>
<td>12</td>
<td>Cold blooded animals and the environment</td>
</tr>
<tr>
<td>S. bongori (V)</td>
<td>20</td>
<td>Cold blooded animals and the environment</td>
</tr>
</tbody>
</table>
Table 2: *Salmonella* nomenclature in use at CDC, 2000.

<table>
<thead>
<tr>
<th>Taxonomic position</th>
<th>Nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genus (italics)</td>
<td><em>Salmonella</em></td>
</tr>
<tr>
<td>Species (italics)</td>
<td>• <em>enterica</em>, which includes subspecies I, II, IIIa, IIIb, IV and VI</td>
</tr>
<tr>
<td></td>
<td>• <em>bongori</em> (formerly subspecies V)</td>
</tr>
<tr>
<td>Serotype (capitalized, not italicized)</td>
<td>• The first time a serotype is mentioned in the text; the name should be preceded by the word “serotype” or “ser”.</td>
</tr>
<tr>
<td></td>
<td>• Serotypes are named in subspecies I and designated by antigenic formulae in subspecies II to IV, and VI and S. <em>bongori</em></td>
</tr>
<tr>
<td></td>
<td>• Members of subspecies II, IV and VI and S. <em>bongori</em> retain their names if named before 1966</td>
</tr>
</tbody>
</table>

### 2.4 Morphology

Salmonellae are Gram-negative, straight rods not exceeding 1.5 micrometers in width. They are facultative anaerobes usually motile by peritrichous flagella ([European Commission, 2000a](#)). Most salmonellae form common fimbriae and most of them possess type-1 fimbriae associated with mannose-sensitive adhesive properties. These fimbriae are composed of fimbrillin subunits containing a high proportion of hydrophobic amino-acids ([Old et al., 1998](#)). *Salmonella* are routinely classified by serotype on the basis of expression of three surface antigens, the somatic O antigen, the flagella H1 and H2 antigens and the capsular Vi antigen, according to the Kauffmann-White scheme ([Scott et al., 2002](#)). The absence of flagella may consequently affect complete identification of the serotype; *Salmonella enterica* serovar Typhimurium exhibits morphological differences dependent on the peptone constituents of the culture medium however, in media containing soy-based peptone as the primary nutrient, *Salmonella* displays a normal flagellated morphology ([Victoria et al., 2006](#)).
2.5 Serotyping

*Salmonella* express flagellar, polysaccharide and capsular antigens which determine strain pathogenicity and therefore variation of these antigens has formed the basis for *Salmonella* serotyping. The Kauffmann-White scheme, first published in 1929, divides *Salmonella* into more than 2500 serotypes according to their antigenic formulae (*Mortimer et al.*, 2004). Today, 57 O antigens and 117 H antigens have been identified and more than 2500 serotypes have been described. Some of the H antigens share common antigen factors. These antigens are clustered in five complexes, the E, G, L, Z4 and 1 complex. *Salmonella* H antigens are expressed in different phases. Most serotypes are diphasic, i.e. they express two flagella antigens, and a minor part are monophasic, i.e. express one flagella antigen. *Salmonella* Gallinarum is the only serotype in the Kauffmann-White scheme that does not express any flagella antigen and is therefore non-motile (*Sonne-Hansen et al.*, 2005). *Salmonella* serotyping methods recognize 63 distinct phase 1 flagellar antigenic factors and 37 phase 2 flagellar antigenic factors, although the latter are not always present. Some antigenic factors, denoted by square brackets in formulae, may be present or absent without affecting serotype designation. Serotyping methods are stable, reproducible and have high typability, yet there are several drawbacks, particularly the dependence on availability of antisera, considering the ethics, cost and quality control measures necessary to maintain such a supply (*Mortimer et al.*, 2004).

*Salmonella* isolates can be differentiated from one another in a wide variety of ways, and the number of *Salmonella* continues to increase. Epidemiologically, it is important to be able to distinguish *Salmonella* isolates, because definitive typing of *Salmonella* isolates may assist in tracing the source of an outbreak and monitoring trends in antimicrobial resistance associated with a particular type (*Yan et al.*, 2003). In addition to the conventional antigen-based serotyping, there are advanced techniques for serotyping currently being used to enhance the tracing of the individual isolates. The following techniques are currently available for serotyping.
(1) Conventional method
(2) Phage typing
(3) Molecular typing
   a. Restriction digestion based techniques
   b. Amplification based techniques
   c. Nucleotide sequencing based techniques

2.5.1 Conventional serotyping

Conventional serotyping of *Salmonella* is the most commonly used method to differentiate strains, which are epidemiologically the smallest bacterial unit from which isolates share the same phenotypic and genotypic traits (Yan *et al.*, 2003). In most clinical studies, initial serotyping is done using polyvalent O antisera to allow *Salmonella* isolates to be grouped into different O groups designated in capitalized letters. Many *Salmonella* show diphasic production of flagellar antigens and each strain can spontaneously and reversibly vary between these two phases with different sets of H antigens. In phase 1 or the specific phase, the different antigens are designated by small letters, and in phase 2 or the group phase, the antigens first discovered are numbered. In a single cell, usually only one antigen is expressed at a time (Yan *et al.*, 2003). Conventional serotyping using the autoagglutination method has some limitations, such as limitations in detection of Vi antigens (Wain *et al.*, 2005), strains which are not typeable (Rasschaert *et al.*, 2005); it only allows detection of a single antigen-antibody reaction at a time, requires well-experienced technologists to perform, consumes relatively high amount of reagents, and takes a longer time (Cai *et al.*, 2005).

2.6 *Salmonella*: disease and pathogenesis

*Salmonella* are well-known pathogens, highly adaptive and potentially pathogenic for humans and/or animals. *Salmonella* infections are capable of producing serious infections that are often foodborne and present as gastroenteritis. However, a small percentage of these infections may become invasive and result in
bacteremia and serious extra intestinal disease (Fluit, 2005). The main reservoirs for non-typhoidal *Salmonella* are animals such as poultry, livestock, pets and reptiles. *Salmonella Typhi* and *Salmonella Paratyphi* colonize only in humans, so they can be acquired only from close contact with a person who has typhoid fever, from a chronic carrier, or from water or food contaminated by human feces (CIDRAP, 2006).

While certain serovars of *Salmonella enterica* cause disease in humans and a variety of animals, other serovars are highly restricted to a specific host. *Salmonella* infections range from gastrointestinal infections that are accompanied by inflammation of intestinal epithelia, diarrhea and vomiting, to typhoid fever, a life threatening infection (Hensel, 2004). The outcome of *Salmonella* infections is determined by the host and the status of the bacterium. Whereas, age, genetic and environmental factors mainly determine the status of the host, the status of the bacterium is determined by so-called virulence factors (Alphons et al., 2005).

Serotypes adapted to man, such as *Salmonella Typhi* and *Salmonella Paratyphi*, usually cause severe diseases in humans as a septicaemic typhoidic syndrome (enteric fever). These serotypes are not usually pathogenic to animals. Serotypes that are highly adapted to animal hosts, such as *Salmonella Gallinarum* (poultry) or *Salmonella Abortus-ovis* (sheep), usually produce very mild symptoms in man. However, *Salmonella Choleraesuis* which has the pig as a primary host also causes severe systemic illness. In the same way, *Salmonella Dublin*, which has a preference for bovines, is primarily responsible for the systemic form of Salmonellosis. In young calves this disease causes high mortality, and in adult cattle it results in fever, reduced milk yield, diarrhea, abortion, and occasionally death. Ubiquitous serotypes, such as *Salmonella Enteritidis* or *Salmonella Typhimurium*, which affect both man and animals, generally cause gastrointestinal infections usually less severe than enteric fever. However, they also have the capacity to produce typhoid-like infections in mice and in humans, or asymptomatic intestinal colonization in chickens (Velge et al., 2005).
Salmonella avoid host defense in the stomach and reach the intestines, (CIDRAP, 2006) and the bacteria interact with the non-phagocytic cells such as the epithelial cells of the intestinal mucosa (Hensel, 2004). They adhere to the intestinal epithelial cells by adhesive structures (fimbriae) that promote binding and invade epithelial cells to provoke gastroenteritis. The organisms have virulence factors such as virulence-plasmids, toxins, fimbriae and flagella that help in establishing an infection (Alphons et al., 2005).

The mechanism of pathogenesis has been described in the following steps

a) **Bacterial mediated endocytosis:** A highly coordinated series of interactions between proteins released by salmonellae and proteins of the host cell causes host cellular surface membrane ruffling and engulfment of bacteria in cellular vacuoles.

b) **Neutrophil recruitment and migration:** Salmonellae associated with gastroenteritis induce a secretory response in intestinal epithelium and initiate recruitment and transmigration of neutrophils into the intestinal lumen.

c) **Epithelial cell cytokine secretion:** In tissue culture models of Salmonella Enteritidis, translocation of SPI-1 proteins into intestinal epithelial cells leads to synthesis and polarized secretion of inflammatory mediators and neutrophil chemoattractants.

d) **Fluid and electrolyte secretion:** Several translocated SPI-1 proteins contribute to intestinal inflammation and fluid secretion. Intestinal inflammation probably contributes to fluid secretion and diarrhea by disrupting the epithelial barrier and increasing water flux by an exudative mechanism. Innate immune system activation also contributes to intestinal inflammation.

e) **Systemic infection:** Salmonella Typhi invades macrophages and the migration of infected macrophages to reticuloendothelial organs via the lymphatic system and blood produces systemic illness with less diarrhea.
2.6.1 Salmonellosis in humans

With respect to human disease, *Salmonella* serotypes can be divided into three groups that cause distinctive clinical syndromes, typhoid fever, bacteremia and enteritis (Santos et al., 2001). The non-typhoid *Salmonella* serotypes can cause protean manifestations in humans, including acute gastroenteritis, bacteremia, and extraintestinal localized infections involving many organs (Chiu et al., 2004). Within *Salmonella enterica* subspecies I (*Salmonella enterica* subspecies *entericae*), the most common O-antigen serogroups are A, B, C1, C2, D and E. Strains within these serogroups cause approximately 99% of *Salmonella* infections in humans and warm-blooded animals. Serotypes in other subspecies are usually isolated from cold-blooded animals and the environment but rarely from humans (Velge et al., 2005).

Following ingestion of contaminated food or water, the pathogenesis of both typhoid and *Salmonella* enteritis begins with the intestinal phase, while only typhoid progresses to a systemic phase (Brown et al., 2005). Transmission of this disease within the human population is generally a result of poor sanitation of water and food supplies in developing nations. The broad host-range *Salmonella* serovars are prevalent within warm-blooded animal populations that make up the human food supply, and bacterial transmission generally results from consumption of raw or undercooked food products (Jones, 2005).

The vast majority of *Salmonella* infections are transmitted from animals to humans through food and occasionally from person to person through the fecal-oral route. In general, *Salmonella* cause one or more of four broad clinical syndromes such as gastro-enteritis, enteric fever, septicemia with associated focal lesions, and asymptomatic long-term carriage.

2.6.2 Salmonellosis in animals

*Salmonella* serotypes have a broad host range (Santos et al., 2003), prevalent in the warm-blooded animal population (Jones, 2005), including rodents (Porwollik et
snakes (Solari et al., 2003), and free living terrestrial and aquatic turtles (Vila et al., 2006), and the pathogenicity of Salmonella serovars is known to be specific for animal species (Ishibashi et al., 1996). Some serotypes are highly adapted to animal hosts, such as Salmonella Gallinarum in poultry and Salmonella Abortussuis in sheep. Many nontyphoidal Salmonella strains such as Salmonella Typhimurium and Salmonella Enteritidis infect a wide range of animal host including poultry, cattle and pigs (Ohl and Miller, 2001). These serotypes generally cause self limiting gastrointestinal infections usually less severe than enteric fever in humans. However, they also have the capacity to produce typhoid-like infections in mice and in humans or asymptomatic intestinal colonization in chickens (Velge et al., 2005).

2.7 Salmonella: A public health perspective

Salmonellosis is an important global public health problem causing substantial morbidity, and thus also has a significant economic impact. Although most infections cause mild to moderate self-limited disease, serious infections leading to deaths do occur (Jong and Ekdahl, 2006). In spite of the improvement in hygiene, food processing, education of food handlers and information to the consumers, foodborne diseases still dominate as the most important public health problem in most countries (Dominguez et al., 2002). Many foods, particularly those of animal origin, have been identified as vehicles for transmission of these pathogens to human beings and spreading them to the processing and kitchen environment (Uyttendaele et al., 1998). In developed countries food is recognized as the most frequently implicated vehicle of transmission and causes heavy financial burden on health care systems (Jordan et al., 2006). In the United States alone, an estimated 1.4 million non-typhoidal Salmonella infections, resulting in 168 000 visits to physicians, 15 000 hospitalizations and 580 deaths occur annually and the total cost associated with Salmonella is estimated at US$ 3 billion annually (WHO, 2005). Apart from the foodborne infections, the other major epidemiological development in Salmonellosis is the emergence of multiple-antibiotic resistant Salmonella, particularly in the developing countries (Okeke et al., 2005).
2.7.1 Global overview

In many countries incidence of human *Salmonella* infection has increased drastically over the years. The two most commonly isolated serotypes of concern and mostly implicated in disease outbreaks are *Salmonella enterica* serotype Typhimurium and *Salmonella enterica* serotype Enteritidis (Sadeyen *et al.*, 2004, Chiu *et al.*, 2004, Buck *et al.*, 2004). Besides the importance of this micro-organism for public health, another aspect is the cost generated by human Salmonellosis. During 1999, the cost linked to foodborne Salmonellosis ranged between 560 million and 2.8 billion € in Europe, where *Salmonella* was estimated to be responsible for nearly 166 000 cases (Korsak *et al.*, 2006). It is reported that the rate of Salmonellosis in the United States is between 15 to 20 cases per 100 000 people and approximately 10% of the Salmonellosis cases are caused by consumption of poultry meat (Oscar, 2004). The *Salmonella* species is one of the eight micro-organisms in the European Union Zoonoses Monitoring Directive, which shows it is a disease considered to have a high impact on human health in the Union (Jong and Ekdahl, 2006). The Enter-net surveillance program reported *Salmonella enterica* serotypes Enteritidis and Typhimurium, the most predominant organisms identified by the participating countries making up over 80% of all isolates during the period of 1998-2003. It also reported that for all *Salmonella* the general trend is declining with a reduction of 35.3% in 2003 over 1998 (Eurosurveillance, 2004).

2.7.2 Status in developing countries

Ensuring consumer health concerns by greater involvement of the health sector, development of Codex standards, guidelines and incorporation of the work of the Commission into the national legislation to promote food safety and fair trading practices are reflected in the priorities of the Codex Alimentarius Commission in the developing countries (Moy and Schlundt, 2005). The Food and Agriculture Organization of the United Nations and the World Health Organization jointly state that “illness due to contaminated food was perhaps the most widespread health problem in the contemporary world,” and “an important cause of reduced economic
productivity” (Kaferstein, 2003). With the increasing population in the developing world, there is an increasing demand for meat and meat products which will force the present resource driven system of livestock production to a demand driven system (Zessin, 2006) which will increase the disease transmission risks. There is a multifactorial risk of foodborne hazards in the developing countries due to poor sanitation and inadequate access to potable water (Henson, 2003b).

Poultry products have always topped the incidence of Salmonellosis in many developing countries including India, Egypt, Brazil and Zimbabwe (Henson, 2003b) and is the most seriously perceived food risks in chicken meat, even in the developed countries (Yeung, 2001).

The reported prevalence of Salmonella in chicken carcasses in South Asian countries varies from country to country. Studies in northern Thailand revealed 57% prevalence in chicken meat at the market during 2002-2003 (Padungtod et al., 2006), 14.5% prevalence in Kathmandu, Nepal (Maharjan et al., 2006), and 42.63% prevalence in Ho Chi Minh city, Vietnam (Bao, 2005). Sero-prevalence of poultry Salmonella in Bangladesh has been reported to be 23.46% (Sikder et al., 2005). Not much literature has been available on the prevalence of Salmonella in chicken carcasses from India, few researches report negligible (Vaidya et al., 2005) to as low as 5% (Rahman et al., 2004a), to a prevalence of 69% (Bajaj et al., 2003). However, the overall annual incidence of foodborne Salmonellosis in India is nearly 6 per 1000 inhabitants (Henson, 2003b).

2.7.3 Salmonella in food animals in India

In economically weaker developing Asian nations including India, in view of limited adherence to very high standards of hygiene, the probability of bacterial contamination of food commodities at various stages of processing and handling is very high (Goel et al., 2002). Indian food processing industries are not as developed as in the Western hemisphere, lack of proper cold chains, inadequate power supply, and low consumer perception of the risks of foodborne illness are great deterrents in
achieving food safety (Marthi, 1999). *Salmonella*, a major public health problem in India is noted to be in increasing trend (Bhattacharya et al., 2007, Shahane et al., 2007). In studies conducted in northern India, the prevalence of *Salmonella* in live and slaughtered goats was 17.6% and 46% by indirect ELISA (Chandra et al., 2005, 2007), and 3% in sheep meat in central India (Yadav et al., 2006). There are reports of *Salmonella* outbreaks in Japanese quails (Mathew et al., 1990), poultry (Praksah et al., 2005) and other species of animals including does and ewes (Verma et al., 1998).

2.7.4 *Salmonella* in Bhutan

Although there are no publications available, there are records of *Salmonella* isolation from different sources including poultry and food items in Bhutan (unpublished lab records). Outbreaks in poultry and pheasant farms have been recorded earlier. There are frequent outbreaks of Salmonellosis reported in humans, either through the water sources or from the food items (Kuensel, May 2007). There is no surveillance of *Salmonella* presently carried out in the country. Implementation and monitoring of *Salmonella* surveillance in food and food products will be an immense responsibility of the concerned agencies in the near future in Bhutan.

2.7.5 *Salmonella* in poultry products

Poultry meat and its derivatives are among the food products that cause the most concern to public health authorities, owing to the associated risks of bacterial food poisoning (Luiz et al., 2004). The modernization of chicken farms and globalization of the bird breeding trade also have played a role in infection (Velge et al., 2005). *Salmonella enterica* serovar Enteritidis is transmitted to the human food supply through eggs of hens that appear healthy (Porwollik et al., 2005). Contamination with *Salmonella* in poultry products can occur at multiple steps along the food chain, which includes production, processing, distribution, retail marketing, handling and preparation (Cui et al., 2005a). *Salmonella* accounts for 19% in the fresh and frozen poultry products in South Africa (Nierop et al., 2005), 3.1% and 2.8% in chicken and turkey meat respectively in Ireland (Jordan et al., 2006), 6.5% in Albania
(Beli et al., 2001), 60% in Portugal (Antunes et al., 2003) and 49% in Spain (Capita et al., 2003).

2.7.6 Health and economic impact

While it is recognized that the prevalence of foodborne illnesses in developing countries is considerable, in most countries there is limited data through which the incidence of particular diseases and trends can be assessed over time (Henson 2003b). The growing movement of people, live animals, and food products across borders; rapid urbanization in developing countries; increasing numbers of immune-compromised people; changes in food handling and consumption; and the emergence of new or antibiotic-resistant pathogens all contribute to increasing food safety risks (Unnevehr, 2003). Food safety requirements in export markets have a profound impact on the way that supply chains for agricultural and food products in developing countries operate. Food safety regulations and standards are increasingly influencing the ability of developing countries to access markets for agricultural and food products, particularly in industrialized countries (Henson, 2003a).

Food safety is of particular concern in developing countries not only because of the high prevalence of the foodborne illness and other hazards associated with food, but also because of the considerable economic and social cost that, in turn, reflects prevailing levels of economic development. The majority of trade-limiting factors in developing countries relate to economics, poor infrastructure and lagging skills; food safety is still mainly the responsibility of the consumers. Improving food safety along western standards, however, may carry considerable costs and price food out of reach of the poor (Veen, 2005).

The Center for Disease Control and Prevention has estimated that *Salmonella* infections were responsible for 1.4 million annual illnesses, resulting in nearly 600 deaths in 2003 in United States (CDC, 2003). The proportion of illnesses attributed to *Salmonella*-contaminated meat and poultry is unknown. More severe cases of Salmonellosis tend to occur in the very old, the very young, and the immuno-
compromised. The estimated annual costs in dollars in 1998 and 2003 of medical care and lost productivity due to food-borne *Salmonella* infections in the United States were $2.3 and $2.9 billion (Frenzen *et al.*, 1999, ERS-USDA, 2003).

All together 150,165 cases of human *Salmonellosis* have been reported in 14 member states of the European Union in the year 2000 (European Commission, 2000b), and 192,703 cases in 2004 (Forshell *et al.*, 2006). In the year 2000, 16,983 laboratory confirmed cases of *Salmonellosis* were reported in the United Kingdom, most commonly associated with the consumption of chicken and undercooked egg dishes (UK zoonoses report, 2000). In the year 2001, China recorded that 17.9% of the total food poisoning was caused by *Salmonella* spp. (FAO/WHO, 2004). The trend in *Salmonellosis* in Australia has been increasing over the time, both in the number of cases recorded as well as in rate per population (Sumner *et al.*, 2004).

Apart from the impact on the health of the individuals, economic losses on international and national trade due to *Salmonella* in the poultry products and product recalls have direct economic and public perception effects on the processing industries. The recalls also have a negative effect on the demand of the product and effect a move towards non-meat products. In the United States, although the incidence has decreased, *Salmonella* accounts for 5% of USDA recalls (Kramer *et al.*, 2005).

**2.8 Antimicrobial resistance**

Drug resistance in foodborne bacterial enteric pathogens is an almost inevitable consequence of the use of antimicrobial drugs in food-producing animals, and specifically in the developing countries by use of medicines in humans (Threlfall *et al.*, 2000, Bogaard and Stobberingh, 2000). A major concern is that the high levels of antibiotic resistance are a result of the use of antibiotics in food animals. A recent estimate in the United States suggests that 24.6 million pounds of antibiotics are given to animals each year as growth promoters at sub-therapeutic amounts in their feed compared to 3 million pounds consumed by humans (White *et al.*, 2001). In recent years the emergence and global dissemination of multi-drug resistant typhoidal strains
has posed major public health problems in the developing countries, and over the past decade it has assumed epidemic proportions in South Asia (Okeke et al., 2005). Antimicrobial resistance among non typhoid Salmonella serotypes has been a serious problem worldwide. The identification of antimicrobial-resistant Salmonella in food has raised concerns on treatment of foodborne Salmonellosis especially the development of ceftriaxone and ciprofloxacin-resistant Salmonella, as these are important in treating Salmonella infections in children and adults, respectively. The extent of global food trade and the intercontinental transmission of resistant Salmonella via foods underscores the potential impact that local geographical agricultural antimicrobial use may have on consumer health worldwide (Butaye et al., 2006). Conventional antimicrobial agents, such as ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole had been the drug of choice in the treatment of Salmonellosis before the 1980s. However, multi-drug resistance, with rates of resistance to these antimicrobial agents of more than 50% has been reported in many areas of the world. Extended-spectrum cephalosporins and fluoroquinolones are increasingly reported after 1991 (Chiu et al., 2004). The possible emergence and spread of Salmonella strains resistant to antibiotics commonly used as treatment are concerns, because these infections can be invasive and difficult to treat by the drugs of choice for invasive Salmonella disease (Paterson, 2006). In developing countries, household subsistence farming is common, which means that a large proportion of the population has close contact with food animals; therefore, if resistant organisms are common in animals, the chance that they will be transmitted to human beings is more likely (Okeke et al., 2005). Some research studies indicate that the costs associated with antimicrobial resistance are higher by several times (Howard and Scott, 2005).

The emergence of Salmonella strains that are resistant to commonly used antimicrobials should be particularly noted by clinicians, microbiologists and those responsible for control of communicable diseases, as well as food producers including the food industry. Control of drug resistant Salmonella is most efficiently achieved through the reduction of antimicrobial use. Prudent usage in food animals should be combined with good husbandry, good abattoir practice and good hygiene at all stages in the food production chain, from processing plants to kitchens and food service
establishments. Although some countries have succeeded in reducing the frequency of \textit{Salmonella} in poultry dramatically, it is unlikely that the eradication of \textit{Salmonella} in domestic animals is possible in the foreseeable future. The increased occurrence of drug-resistant pathogens in food of animal origin emphasizes the general need for cooking such foods thoroughly prior to consumption. Education of food handlers in the principle of safe food handling is an essential step towards reducing the incidence of foodborne diseases resulting from cross-contamination during processing and preparation of foods (WHO, 2005).

2.8.1 Global trends in resistance pattern

Antimicrobial resistance is one of the biggest challenges facing global public health. Although antimicrobial drugs have saved many lives and eased the suffering of many millions, poverty, ignorance, poor sanitation, hunger and malnutrition, inadequate access to drugs, poor and inadequate health care systems, civil conflicts and bad governance (Byarugaba, 2004), misdiagnosis, counterfeit drugs and lack of education in developing countries have tremendously limited the benefits of these drugs in controlling infectious diseases (Walia, 2006). Most non-typhoidal \textit{Salmonella} infections manifest as potentially self-limiting diarrhea. Antimicrobial resistance is clinically relevant because 3-10\% of these infections can progress to life-threatening bacteraemia, particularly in young and immuno-compromised patients. In Indonesia, \textit{Salmonella} Paratyphi isolates recovered between 1995 and 2001 were universally susceptible to commonly used antimicrobials, \textit{Salmonella} Enteritidis isolates were resistant to most of the antimicrobials tested, with the exceptions of fluoroquinolones. A similar study in Zimbabwe reported much lower rates of resistance among \textit{Salmonella} Enteritidis, and more than 50\% of non-typhoidal \textit{Salmonella} isolates from children in Kenya were multi-drug resistant (Okeke \textit{et al.}, 2005). One of the studies in Spain reported high percentages of resistance of \textit{Salmonella} isolates to sulfadiazine, neomycin, tetracycline and streptomycin, which might be the result of use of antibiotics as a prophylaxis, growth promoter or treatment (Carraminana \textit{et al.}, 2004). A similar study in Alberta, Canada indicated high resistance of \textit{Salmonella} isolates from food and food animals to ampicillin, streptomycin, sulfamethoxazole and
tetracycline (Johnson et al., 2005). In Ethiopia, resistance pattern of Salmonella isolates from chickens indicated large proportions of strains resistant to a variety of drugs (Molla et al., 2003). Over the past decade in Nepal, increasing antibiotic resistance in Salmonella enterica has lead to a shift in the antibiotics used against this organism from chloramphenicol and ampicillin to trimethoprim-sulfamethoxazole, fluoroquinolones and ceftriaxone, where only a 16-40% positive response to treatment has been achieved (Pokharel et al., 2006). In a study in the United States Salmonella isolated from pre-harvest turkey production sources were resistant to multiple antibiotics (Nayak et al., 2004). In another study, of 380 Salmonella isolates from animal origin in the US, 82% of the isolates were resistant to at least one antimicrobial, and 70% to three or more antimicrobials. Resistance was most often observed to tetracycline, followed by streptomycin, sulfamethoxazole, ampicillin, chloramphenicol, kanamycin, amoxicillin/clavulanic acid, and ceftiofur (Zhao et al., 2007). From 1999 to 2003, 34 411 Salmonella were isolated from animals in the USA, of which 10.9% were found to be resistant to ceftiofur, a third generation cephalosporin used in animals, whilst only 0.3% were resistant to ceftriaxone, a third generation cephalosporin used in human medicine. There was an increase in ceftiofur resistance (Frye and Fedorka-Cray, 2007). Increased antibiotic resistance among Salmonella is not only in the percentage isolates resistant to a particular antibiotic, but also the development of resistance against newer antibiotics (Fluit, 2005).

In a study in Nepal, 35 multi-drug-resistant strains out of 132 strains of Salmonella Typhi were observed showing simultaneous resistance to ampicillin, chloramphenicol, and co-trimoxazole. Although there were no isolates resistant to ciprofloxacin, 69.23% of 52 isolates tested for minimum inhibitory concentration of ciprofloxacin showed reduced susceptibility and 76% of 112 strains tested for nalidixic acid were resistant (Khanal B et al., 2007). There are reports of Salmonella resistant strains isolated from The Netherlands (Duijkeren et al., 2003, 2006), France (Weill et al., 2006), Portugal (Antunes et al., 2003) and many other countries.

Between the year 1999 and 2004, the number of publications reporting Salmonella resistant to β-lactams antibiotics has increased drastically. In 2004,
Salmonella resistant to extended spectrum cephalosporins were identified in 43 countries (Arlet et al., 2006). In a retrospective study in Korea, the resistance rate against chloramphenicol showed mild increase, but the ampicillin, trimethoprim/sulfamethoxazole, kanamycin or nalidixic acid remained at a similar level over 9 years (Yoo et al., 2004). Because the majority of human cases of non-typhoidal Salmonellosis are acquired through the consumption of contaminated food and water, data on the proportions of serotypes and their resistance patterns in different countries are important for global public health management, as food consumption practices vary in different countries and increasing global travel and food trade increase the likelihood of acquiring infections from non-domestic sources (Lauderdale et al., 2006).

2.8.2 Resistance pattern in India

In developing countries like India, easy availability of a wide range of drugs coupled with inadequate health services result in increased proportions of drugs used as self-medication compared to prescribed drugs resulting in impending health problems and antimicrobials resistance. Approximately, 78% of Salmonella Typhi isolates collected from infected patients between 1990 and 1991 demonstrated resistance to chloramphenicol, ampicillin and trimethoprim/sulfamethoxazole. Approximately 81% of the Salmonella enterica serotype Typhi isolates from northern India were resistant to chloramphenicol (Sharma et al., 2005b). A study in Calcutta in India revealed all Salmonella enterica serogroups were uniformly resistant to commonly used drugs with an exception to norfloxacin and ciprofloxacin (Saha et al., 2001). In a few of the studies, a changing pattern of the multi-drug resistant Salmonella isolates was noted (Madhulika et al., 2004, Das and Bhattacharya, 2006). In a recent study in England, Scotland and Wales, it was found that 70% of typhoid cases in returning travelers originated from India or Pakistan, with the highest level of antimicrobial resistance from the Indian subcontinent (Cooke et al., 2007). A study in south India revealed that Salmonella strains from egg and egg-storing trays were resistant to ampicillin, neomycin, polymyxin-B and tetracycline, with 8.9% resistance level to ciprofloxacin (Suresh et al., 2006). Although resistance patterns in Salmonella
have been increasingly observed, re-emergence of chloramphenicol sensitivity has been noted in few of the studies (Sood et al., 1999, Tankhiwale et al., 2003a, Achla et al., 2005, Mohanty et al., 2006).

2.8.3 Status in Bhutan

Although the Department of Health does antimicrobial sensitivity testing of the Salmonella isolates, the procedure is rather treatment based and the published data are not available.

2.9 Prevention and control of Salmonella: overview

Prevention and control measures should be designed based on the two distinct categories, Salmonella infections that have a direct negative impact on the poultry population, and Salmonella infections of importance to public health (Breytenbach, 2004). Prevention of Salmonellosis by the implementation of hygiene measures is difficult and use of antibiotics may give rise to the emergence of resistance problems (Mastroeni and Menager, 2003). Reducing Salmonella prevalence requires a multi-hurdle approach at all stages of breeding, hatching, grow-out, transportation and processing. No silver bullets can be added at a single point in production or processing that will completely eliminate Salmonella on chickens. Therefore, some researchers describe “competitive exclusion” as the most effective and harmless method to control Salmonella in poultry (Ragione and Woodward, 2003, Schneitz, 2005). Attenuated DNA recombinant live Salmonella vaccines (Kang et al., 2002), combined with comprehensive control strategy in animals, feed and animal food stuffs with restrictions on the infected flocks until they have been cleaned up from infections and mandatory testing before slaughter like that being implemented in Sweden (Boqvist and Vagsholm, 2005), will help reduce Salmonellosis. The prevention of paratyphoid Salmonella infection which has greater public health consequences requires a comprehensive control strategy including regular monitoring, strict biosecurity, sourcing feed containing no animal protein, and vaccination (Breytenbach, 2004).
2.9.1 Prevention and control of *Salmonella* in end products

Ensuring safe food production requires knowledge on the nature and origin of the animals, animal feed, the health status animals at the farm, the use of veterinary medicinal products, the results of any analysis of the samples taken at the farm and slaughter data regarding ante-mortem and post-mortem findings and the risks associated with post-harvest production stages (Snijders and Knapen, 2002), or no part of the food chain can be regarded alone but has to be seen as part of the whole. A holistic approach to fresh meat storage and retailing must therefore start with the living animal and cannot end with the sale of the meat. It must also include the consumers (Nowak *et al.*, 2006). Additional measures to control secondary contamination could be prevention of contamination by cleaning and disinfection, hygiene of personnel and proper processing (Sinell, 1995). Growth of micro-organisms in meat and poultry products can be controlled by maintaining a cold chain at 10°C, especially for *Salmonella* during transport and storage (Coleman *et al.*, 2003).

Inactivation of potential microbiological pathogens in the end-product may appear attractive, as it reduces the spoilage micro-organisms too (Farkas, 1998), however, other options such as antimicrobial packaging (Quintavalla and Vicini, 2002), vacuum packaging, intelligent packaging and smart active labeling with freshness and spoilage indicators (Nowak *et al.*, 2006) might prove better choices for consumer health protection. Provided the initial production stages have low bacteria and/or are pathogen free, the approach to preventing spoilage of fresh meat is to keep the products at low temperatures, where in most countries the storage temperature is prescribed by law. Enabling rapid identification of microbial contamination to allow rapid response (Doyle and Erickson, 2006), knowledge and attitude of the consumers (Woteki *et al.*, 2001), personal hygiene of food handlers (Nowak *et al.*, 2006), consumer perception of food safety (Redmond and Griffith, 2004), and continuous further education are equally important to achieve food safety practices (Fischer *et al.*, 2006).
3. MATERIAL AND METHODS

3.1 Study area and period

The research was focused on Thimphu, the capital city of Bhutan. Over 80% of the imported meat is sold through the retail market in the city and transported further to the other nearby towns such as Paro and Punakha. The study population included only the broiler carcasses, as the import of other poultry meat such as goose and ducks is not popular. The study was carried out between November 2006 and April 2007. The isolation of *Salmonella* species, antimicrobial resistance and Total Plate Count was done at the National Centre for Animal Health, Thimphu, Bhutan. The identification by sero-typing of the *Salmonella* isolates was done at the Veterinary Public Health Centre for Asia Pacific, Chiang Mai University, Thailand during April to June 2007.

3.2 Sample size determination

The total annual import of chicken carcasses into Bhutan was approximately 1334.6 metric tons in 2005, which was approximated at 3 33 650 carcasses. With the confidence interval of 95% and maximum allowable error of 5%, the total sample size was determined using win Episcope Version 2.0 (EPIDECON). The expected prevalence of Salmonellosis is 50% used for sample size determination. Using the following formula from win Episcope

\[ SD = \sqrt{P(1-P)} \]

\[ = \sqrt{0.50 * (1-0.50)} \]

\[ = 0.5 \]

Where P is the expected prevalence of 50% and the sample size n is calculated using the above standard deviation (SD).
n = \left[t \times \text{SD}/L\right]^2
\left[1.96 \times 0.5/0.05\right]^2
= 384.16 \approx 385

Where t is Student’s t-value of 1.96 with the desired level of 95% confidence, and L is the allowable absolute error of 5%. Keeping 5% of the above sample size as risk in preventing sample loss during analysis, the total sample size was estimated at 400 samples.

The Total Plate Count for enumeration of the bacterial load was done for two samples from each sample lot. Samples were collected every week and each sample lot of 25 chicken carcasses constituted two samples for Total Plate Count using the pour plate method as described in ISO 4833: 2003.

3.3 Sampling strategy

Random samples of chicken were collected directly from the containers every week when the consignment arrived at the destination in Thimphu. Whole chicken carcasses were purchased after the consignments were inspected by the meat inspectors and transported to the laboratory in cool boxes. Samples were processed for Total Plate Count and Salmonella isolation within 2 hours of collection. A chicken breast (25 g) was used for isolation and identification of Salmonella species, and breast skin samples (10 g) for enumeration of the total bacterial load in the carcass. Approximately 25 samples were analyzed every week for Salmonella isolation and 2 samples for Total Plate Count for a period of 16 weeks. The sample information details recorded were the average temperature at the collection point and weight of the lot every week, plus further details such as manufactured date, batch number, date received and the date of sample analysis.
3.4 Study design

A cross-sectional study to find out the prevalence of *Salmonella* in imported chicken by analyzing the chicken carcass samples. Isolation and identification of *Salmonella* contamination in the carcass sample was done, and the hygienic status of the meat was determined by Total Plate Count of each sample lot during the sampling period. Isolates of *Salmonella* were tested for antimicrobial resistance to find out the resistance pattern in the organisms. The antimicrobials for resistance testing were selected based on the present therapeutic use of those antimicrobials in human and commercial poultry production. The *Salmonella* isolates were tested against ciprofloxacin, cephalexin, chloramphenicol, nalidixic acid, amoxicillin, gentamycin and sulfa-trimethoprim.

3.5 Analysis method

3.5.1 *Salmonella* isolation procedure

The study was conducted utilizing the conventional methods for the detection of *Salmonella* following the standard guidelines from ISO 6579:2002 (Microbiology of food and animal feeding stuffs horizontal method for the detection of *Salmonella* spp.)

3.5.1.1 Non-selective pre-enrichment

A weight of 25 grams of chicken breast cut into fine pieces was transferred into a stomacher bag with 225 ml of buffered peptone water. The sample mixture was shaken approximately for 2 minutes and the samples incubated at 37 °C ± 1 °C for 18 ± 2 hours.

3.5.1.2 Selective enrichment

The pre-enrichment broth after incubation was mixed and 0.1 ml of the broth was transferred into a tube containing 10 ml of Rappaport-Vassiliadis medium with
soya (RVS broth). Another 1 ml of the pre-enrichment broth was transferred into a tube containing 10 ml of Muller-Kauffmann tetraphionate novobiocin broth (MKTTn broth). The inoculated RVS broth was incubated at 41.5 °C±1 °C for 24 ± 3 hours and the inoculated MKTTn broth at 37 °C±1 °C for 24 ± 3 hours.

3.5.1.3 Plating out and identification

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

Typical colonies of *Salmonella* grow on XLD agar with a black centre and a lightly transparent zone of reddish color due to the color change of the indicator. *Salmonella* H2S negative variants, e.g. *Salmonella* Paratyphi A grown on XLD agar are pink with a darker pink centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening. Typical colonies of *Salmonella* grown on BPLS agar have a reddish color and translucent colony.

3.5.1.4 Confirmation

Five typical colonies per plate grown on the XLD agar and BPLS agar were transferred and inoculated on triple sugar iron agar (TSI), incubated at 37°C±1°C for 24 ± 3 hours. In fewer than five typical or suspected colonies per petri dish observed, all suspected colonies were streaked on the surface of pre-dried nutrient agar plates, in a manner which allowed well-isolated colonies to develop. The inoculated plates were incubated at 37°C±1°C for 24 ± 3 hours. Thus the pure culture obtained was used for biochemical and serological confirmation.
3.5.1.4.1 Biochemical confirmation

By means of an inoculating loop, the following biochemical confirmation media were inoculated with the pure culture obtained in nutrient agar plates.

3.5.1.4.2 Triple sugar iron agar (TSI agar)

The agar slant surface was streaked and the butt stabbed and incubated at 37°C ± 1 for 24 ± 3 hours. The results were recorded as follows.

Table 3: Observation of TSI agar for presence of *Salmonella*

<table>
<thead>
<tr>
<th>Area of reaction</th>
<th>Result</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butt</td>
<td>Yellow</td>
<td>Glucose positive (glucose used)</td>
</tr>
<tr>
<td></td>
<td>Red or unchanged</td>
<td>Glucose negative (glucose not used)</td>
</tr>
<tr>
<td></td>
<td>Black</td>
<td>Formation of hydrogen sulfide</td>
</tr>
<tr>
<td></td>
<td>Bubbles or cracks</td>
<td>Gas formation from glucose</td>
</tr>
<tr>
<td>Slant surface</td>
<td>Yellow</td>
<td>Lactose and/or sucrose positive (lactose and/or sucrose used)</td>
</tr>
<tr>
<td></td>
<td>Red or unchanged</td>
<td>Lactose and sucrose negative (neither lactose nor sucrose used)</td>
</tr>
</tbody>
</table>

Typical *Salmonella* cultures show alkaline (red) slants and acid (yellow) butts with gas formation (bubbles) and (in about 90% of the cases) formation of hydrogen sulfide (blackening of the agar). When lactose-positive *Salmonella* is isolated the TSI agar slant is yellow. Thus, preliminary confirmation of *Salmonella* cultures is not based on the results of the TSI agar test only.
3.5.1.4.3 Urea agar

The agar slant surface was streaked and incubated at 37°C ± 1°C for 24 ± 3 hours and examination was done at intervals. If the reaction is positive, splitting of urea liberates ammonia, which changes the color of phenol red to rose pink, and later to deep cerise (moderate red). The reaction is often apparent after 2 to 4 hours.

3.5.1.4.4 L-lysine decarboxylation medium

Inoculate just below the surface of the liquid medium and incubate at 37°C ± 1°C for 24 ± 3 hours. Turbidity and a purple color after incubation indicate a positive reaction. A yellow color indicates a negative reaction.

3.5.1.4.5 Detection of β-galactosidase

Suspend a loop-full of the suspected colony in a tube containing 0.25 ml of the saline solution. Add 1 drop of toluene and shake the tube. Put the tube in a water bath set at 37°C and leave for several minutes (approximately 5 minutes). Add 0.25 ml of the reagent for detection of β-galactosidase and mix. Replace the tube in the water bath set at 37°C and leave for 24 ± 3 hours, examining the tube at intervals. A yellow color indicates a positive reaction. The reaction is often apparent after 20 minutes.

3.5.1.4.6 Medium for Voges-Proskauer reaction

Suspend a loop-full of the suspected colony in a sterile tube containing 3 ml of the VP medium and incubate at 37°C ± 1°C for 24 ± 3 hours. After incubation add 2 drops of creatine solution, 2 drops of the ethanolic solution of 1-naphthol and then 2 drops of potassium hydroxide solution. Shake after addition of each reagent. The formation of pink to bright red color within 15 minute indicates a positive reaction.
3.5.1.4.7 Medium for indole reaction

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

Interpretation of biochemical reaction

Table 4: Interpretation of biochemical test

<table>
<thead>
<tr>
<th>Test</th>
<th>S. Typhi</th>
<th>S. Paratyphi A</th>
<th>S. Paratyphi B</th>
<th>S. Paratyphi C</th>
<th>Other strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSI Acid from glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TSI gas from glucose</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TSI acid from lactose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TSI acid from sucrose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TSI H2S produced</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urea hydrolysis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lysine decarboxylation</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-galactosidase reaction</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Voges-Proskauer reaction</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Production of indole</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
PRE-ENRICHMENT

Incubation 18 h ± 2 h at 37°C ± 1°C

+ 0.1 ml of culture + 10 ml of RVS broth

Incubation 24 h ± 3 h at 41.5°C ± 1°C

+ 1 ml culture + 10 ml MKTTn broth

Incubation 24 h ± 3 h at 37°C ± 1°C

PLATING - OUT

Loop-full of suspect colonies

XLD medium and BPLS agar Incubation

24 h ± 3 h at 37°C ± 1°C

From each plate test a characteristic colony. If negative, test the other four marked colonies

CONFIRMATION

Nutrient agar Incubation

24 h ± 3 h at 37°C ± 1°C

Biochemical confirmation

Serological confirmation

Results expression

Fig. 2: Flow diagram showing procedure for isolation of Salmonella spp. (ISO 6579: 2002)
3.5.2 Enumeration of microorganisms by Pour Plate technique

The study was conducted utilizing the method for horizontal enumeration of microorganisms using a colony count technique in a solid medium after aerobiosis incubation at 30°C as described in the ISO 4833: 2003. It is intended to indicate the level of microorganisms in the meat product. A total of two samples every week from each batch of samples for a period of 16 weeks were analyzed, which amounted to a total of 32 samples for Total Plate Count.

3.5.2.1 Preparation of sample

Preparation of the sample diluent, peptone salt solution (maximum recovery diluent), was carried out according to the procedure outlined in the ISO 6887-1. 10 g chicken breast skin sample was weighed carefully and mixed with 90 ml of diluent in a blender for 2 minutes. This mixture was considered to be a $10^{-1}$ dilution. 1 ml of the mixture was transferred to the tube containing 10 ml of diluent and labeled $10^{-2}$ dilution. Further dilutions were made by transferring 1 ml of the succeeding dilutions to the tubes containing 10 ml diluent to achieve ten-fold dilutions.

3.5.2.2 Inoculation and incubation

1. Take two sterile petri dishes and transfer to each dish by means of a sterile pipette 1 ml of the initial suspension ($10^{-1}$ dilution).
2. Take two other sterile petri dishes and transfer to each dish, by means of a sterile pipette, 1 ml of the $10^{-2}$ dilution.
3. Repeat the procedure with the further dilutions, using a new sterile pipette for each decimal dilution until the last test intended dilution.
4. Pour about 12 ml to 15 ml of the plate count agar at 44°C to 47°C into each petri dish. The time elapsing between the end of the preparation of the initial suspension and the time when the medium is poured shall not exceed 15 minutes.
5. Carefully mix the inoculum with the medium by rotating the petri dishes and allow the mixture to solidify by leaving the petri dishes standing on a cool horizontal surface.

6. After complete solidification, overlay with 4 ml of the medium tempered at 44°C to 47°C on to the surface of the inoculated medium and allow to solidify.

7. After solidification, invert the prepared dishes and place them in an incubator at 30°C ± 1°C for 72 ± 3 hours. Do not stack the dishes more than six high. Stacks of dishes should be separated from one another and from the walls and top of the incubator.

3.5.2.3 Counting of colonies and recording of the results

After an incubation of 72 ± 3 hours at 30°C ± 1°C, the colonies were counted visually under a subdued light source. Examination of the plates was done as soon as they were removed from the incubator. The plates containing between 30 to 300 colonies were counted and recorded. The pinpoint colonies were included in the count and the spread colonies were counted as a single colony. Distinction was made between the mistaking particles of the undissolved or the precipitating matter and the pinpoint colonies by observing under the magnification of a colony counter. The results were recorded in Microsoft excel spreadsheet to determine the bacterial load in the original 10 g of sample by using calculation.
10 g sample + 90 ml maximum recovery diluent

Prepare a $10^{-1}$ dilution of sample

Homogenize by stomaching

Prepare further dilutions in peptone salt diluent

Inoculate 1 ml of each dilution into an empty Petri dish and add approximately 12 ml to 15 ml of plate count agar at 44°C to 47°C

Mix by gentle rotation and allow to settle

Overlay with 4 ml medium and allow to settle

Incubate at $30^\circ C \pm 1^\circ C$ for $72 \pm 3$ hours

Count the colonies and enumerate bacterial load per gram

Fig. 3: Flow chart showing enumeration of microorganisms using pour plate technique
3.5.3 Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was done by the agar disk diffusion method as described by NCCLS 2000, now known as the Clinical and Laboratory Standards Institute (CLSI). The pure Salmonella isolates confirmed by the biochemical testing procedure as described in ISO 6579: 2002 were tested for antimicrobial susceptibility.

3.5.3.1 Preparation of Mueller-Hinton agar

The Mueller-Hinton agar was prepared as per the instructions provided by the manufacturer. After autoclaving the media at 121°C for 15 minutes, it was cooled to 50°C and approximately 30 ml to 50 ml was poured into the 15 x 150 mm Petri dishes. The depth of the agar in the petri dishes was maintained approximately at 4 mm. The freshly prepared plates were used on the same day. The pH of the medium was regularly tested for its consistency.

3.5.3.2 Turbidity standards (McFarland)

McFarland 0.5 turbidity standards were prepared as per the standard guidelines described by the Clinical and Laboratory Standards Institute (CLSI). A volume of 0.5 ml of a 1.175% (wt/vol) barium chloride dihydrate (BaCl2•2H2O) solution was added to 99.5 ml of 0.18 mol/L (1% vol/vol) sulfuric acid with constant stirring to maintain the suspension. The turbidity standard was then aliquoted into test tubes 4 ml each, identical to those used to prepare the inoculum suspension. The McFarland standard tubes were sealed with parafilm to prevent evaporation. McFarland standards then were stored in the dark at room temperature (22° to 25°C). Before each use, the standards were shaken well, mixing the fine white precipitate of barium sulfate in the tube. The accuracy of the density of a prepared McFarland standard was checked by using a spectrophotometer with a 1-cm light path; for freshly prepared 0.5 McFarland standards, the absorbance at a wavelength of 625 nm was 0.88. The McFarland standards were replaced every week with a fresh preparation.
3.5.3.3 Inoculum preparation

Growth method

1. At least 3-5 well isolated colonies of the same morphological type were selected from the agar plate culture. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 4 ml tryptic soy broth.
2. The broth culture was either directly adjusted to the McFarland standards or by incubation at 35°C until it achieved or exceeded the turbidity of the 0.5 McFarland standards (usually 2-6 hours).
3. The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity optically comparable to the point of the 0.5 McFarland standards.

3.5.3.4 Inoculation of test plates

1. Optimally within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab then was rotated several times pressed firmly on the inside wall of the tube above the fluid level. This removed excess inoculum from the swab.
2. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step the rim of the agar was swabbed. The procedure was done under laminar flow to avoid contamination.
3. The lid was left ajar for 3-5 minutes but no more than 15 minutes, to allow for any access surface moisture to be absorbed before applying the drug impregnated disks.
3.5.3.5 Application of disks to inoculated agar plates

1. The predetermined battery of antimicrobial disks was dispensed onto the surface of the inoculated agar plate. Each disk was pressed down individually to ensure complete contact with the agar surface. The disk placed in the agar surface was not closer than 24 mm from center to center. A total of 7 disks were placed on one 150 mm plate.

2. The plates were inverted and placed in an incubator set to 35°C within 15 minutes after the disks were applied. The plates should not be incubated in an increased CO₂ atmosphere; because the interpretive standards were developed by using ambient air incubation-CO₂ will significantly alter the size of the inhibitory zones of some agents.

3.5.3.6 Reading plates and interpreting results

1. After 16-18 hours of incubation, each plate was examined. The resulting zone of inhibition was uniformly circular with a confluent lawn of growth. The diameters of the zones of complete inhibition (judged by the unaided eye) were measured, including the diameter of the disk. Zones are measured to the nearest whole millimeter, using sliding calipers, which are held on the back of the inverted petri plate. The petri plate was held a few inches above a black, nonreflecting background and illuminated with reflected light. The results were recorded at 16-18 hours post incubation. Transmitted light from the colony counter was used to examine the zones for light growth wherever indicated, within apparent zones of inhibition.

2. The zone margin was taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, was ignored.

3. The sizes of zones of inhibition were interpreted by referring to zone diameter interpretive standards from NCCLS 2000, and equivalent minimal inhibitory concentration (MIC) breakpoints for enterobacteriaceae, and organisms are
reported as susceptible, intermediate or resistant to the agents that have been tested.

3.6 Data management and analysis

Data management, entry and analysis were done using the program Excel, version 2003 (Microsoft® Office Excel 2003, Professional Edition). Descriptive statistics was used to describe the result of prevalence analysis. Prevalence was estimated as the number of samples detected positive to Salmonella isolation from the total sample analyzed. For Total Plate Count all numeric analyses were made using logarithm bacterial count values in Excel spreadsheets. Counts expressed as colony forming units (cfu/g) was transformed into log_{10} prior to statistical analysis using the Statistical Package for Social Sciences (SPSS, version 11.0). The prevalence and confidence intervals for antimicrobial resistance were estimated using win Episcope (win Episcope® version 2.0) and Epicalc 2000 (version 1.02) software. The resistance pattern of Salmonella isolates was analyzed using Excels spreadsheet.
4. RESULTS

4.1 Source and characteristics of samples

The import of broiler chicken to Bhutan is from Arambagh Hatcherries, Illam Bazaar, West Bengal in India. The firm is certified by the Ministry of Food Processing Industries, and Export Inspection Council, Ministry of Commerce, Government of India. The chicken carcasses are frozen (-18°C) and are transported in sealed cool boxes from the slaughter house to the destination in Thimphu. Individual carcasses are packed in tamper-proof plastic bags and labeled with the date of manufacture, batch number and expiry date. The supply of chicken meat as of April 2007 was limited to Thimphu city, from where it was further distributed to other nearby towns for safety and quality reasons. However, consignments delivered en-route within Bhutan was regulated and was under full knowledge of regulatory authorities. The import of the chicken meat is based on the specific safety criteria laid down by the Department of Livestock, Bhutan (appendix B).

There are three importers supplying chicken meat to Bhutan, and all of them supply to Thimphu city. The sample collection was done randomly from all three importers as the consignment reached the destination. Weekly, 25 samples were analyzed and individual carcasses presented a sample for analysis.

Fig. 4: Frozen individual packages of broiler carcasses from Arambagh hatcherries, India.
4.2 Prevalence of *Salmonella* in the broiler carcasses.

A total of 400 broiler carcasses were processed for isolation of *Salmonella* organisms during November, 2006 to April 2007. Of the 400 samples tested, *Salmonella* was detected from 52 (13%, 95%CI: 9.94, 16.79) carcasses using the method described by ISO 6579:2002. (Microbiology of food and animal feeding stuffs horizontal method for the detection of *Salmonella* spp.). There were two serotypes observed, *Salmonella* Enteritidis and *Salmonella* Typhimurium. *Salmonella* Enteritidis was the most frequently isolated serotype 44 (84.62%) followed by *Salmonella* Typhimurium 8 (15.38%).

![Distribution of Salmonella serovars in broiler carcasses](image)

Fig. 5: Distribution of *Salmonella* serovars in broiler carcasses

4.2.1 Batch-wise variation in *Salmonella* isolation

Among all the different batches of broiler carcasses analyzed, the 16th batch revealed maximum isolation of *Salmonella* species (fig. 6), corresponding to late spring. *Salmonella* was not detected in five batches (8, 9, 10, 14 and 15) which were analyzed during peak winter season (December 2006 - February 2007). Comparison was also made between the weekly weight of the chicken carcasses and the number of positive *Salmonella* cases. There was no relationship to indicate variation in number of *Salmonella* isolates with respect to weight of the chicken carcasses.
4.2.2 Seasonal variation in *Salmonella* isolation

There were a total of 350 samples analyzed during winter (November to February) and 50 samples in early spring (March to April). There was a significant seasonal difference ($p < .001$) in the isolation of *Salmonella* during winter and late spring (Table 5). The imported broiler carcasses were 10.62 times (Odds ratio 10.62) more likely to yield *Salmonella* in the hot season as compared to the winter season.

Table 5: Table showing the seasonal variation in *Salmonella* isolation

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Nos. Positive</th>
<th>Positive (%) [95% CI]</th>
<th>p-value</th>
<th>OR [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter</td>
<td>350</td>
<td>28</td>
<td>8.0 [5.47, 11.48]</td>
<td>0.001</td>
<td>10.62 [5.40, 20.87]</td>
</tr>
<tr>
<td>Spring</td>
<td>50</td>
<td>24</td>
<td>48.00 [33.88, 62.42]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3 Results of the level of microbiological contamination of poultry carcasses

Two samples randomly selected from a batch of 25 carcasses every week were subjected to Total Plate Count following guidelines ISO 4833: 2003 (horizontal enumeration of microorganisms using a colony count technique in a solid medium after aerobiosis incubation at 30°C). A total of 32 samples were analyzed from 16 different batches. APC at 30°C were transformed to $\log_{10}$ number of colony forming units per gram ($\log_{10}$ cfu g$^{-1}$). The median value estimated is $4.408 \log_{10}$ cfu g$^{-1}$. The result is summarized as shown in table 6.

![Histogram showing the distribution of Total Plate Count data.](image)

**Table 6: Selected statistical values for Total Plate Count.**

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>4.408</td>
</tr>
<tr>
<td>25% range</td>
<td>4.128</td>
</tr>
<tr>
<td>75% range</td>
<td>4.638</td>
</tr>
<tr>
<td>Minimum</td>
<td>2.8</td>
</tr>
<tr>
<td>Maximum</td>
<td>5.2</td>
</tr>
<tr>
<td>Inter quartile range</td>
<td>0.510</td>
</tr>
</tbody>
</table>
4.3.1 Variation in microbial load in relation to temperature during processing

Average temperature was recorded for every batch of samples during processing. The temperature of the samples ranged from a minimum of -5.5°C to a maximum of 7°C during processing. The data were grouped in relation to the processing temperature, below 4°C and above 4°C. A graph (fig. 8) was plotted against the temperature of the samples during processing and the Total Plate Count (log cfu/g). Comparison was made using non-parametric test (Mann-Whitney test) to compare the statistical difference in Total Plate Count at a temperature below 4°C and a temperature above 4°C. There is no significant difference (p>0.05) between the growth of aerobic organisms between -5.5°C to 4°C and 4°C to 7°C.

Table 7: Variation in median log APC data at temperature below and above 4°C.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>n</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤4.0°C</td>
<td>28</td>
<td>4.417</td>
<td>2.8</td>
<td>5.2</td>
</tr>
<tr>
<td>≥4.01°C</td>
<td>4</td>
<td>4.408</td>
<td>4.4</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Fig. 8: The variation in log cfu/g in relation to temperature during processing.
4.4 Results of antimicrobial resistance testing

A total of 52 isolates (n=52) were tested against seven commonly used antimicrobials viz. cephalexin 30 µg (CL30), nalidixic acid 30 µg (NA30), chloramphenicol 30 µg (C30), ciprofloxacin 5 µg (CIP5), gentamycin 10 µg (CN10), amoxycillin 10 µg (AML10) and sulfa-trimethoprim 25 µg (SXT25) following NCCLS 2000 guidelines. The results of the antimicrobial sensitivity test are shown in the table 8.

4.4.1 Levels of antimicrobial resistance

Among all the antimicrobials tested, nalidixic acid was the most resisted drug followed by amoxicillin and cephalaxin. Gentamicin and chloramphenicol showed maximum sensitivity and were the only antimicrobials not resistant to any of the isolates tested.

Table 8: Levels of antimicrobial resistance of *Salmonella* isolates (n=52).

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No*</td>
<td>Percent [95%CI]</td>
<td>No*</td>
</tr>
<tr>
<td>CL30</td>
<td>3</td>
<td>5.77 [1.50, 16.92]</td>
<td>6</td>
</tr>
<tr>
<td>NA30</td>
<td>50</td>
<td>96.15 [85.67, 99.33]</td>
<td>0</td>
</tr>
<tr>
<td>C30</td>
<td>0</td>
<td>0 [0.0, 5.13]</td>
<td>1</td>
</tr>
<tr>
<td>CIP5</td>
<td>1</td>
<td>1.92 [0.10, 11.58]</td>
<td>10</td>
</tr>
<tr>
<td>CN10</td>
<td>0</td>
<td>0 [0.0, 5.13]</td>
<td>0</td>
</tr>
<tr>
<td>AML10</td>
<td>6</td>
<td>11.54 [4.78, 24.13]</td>
<td>1</td>
</tr>
<tr>
<td>SXT25</td>
<td>1</td>
<td>1.92 [0.10, 11.58]</td>
<td>1</td>
</tr>
</tbody>
</table>

No* is the number of isolates resistant, intermediate and sensitive.
4.4.2 Antimicrobial resistance by serotypes

Level of antimicrobial resistance by serotypes was calculated and is shown in the table below (table 9).

Table 9: Level of antimicrobial resistance by serotypes (n=52).

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>S. Enteritidis (n=44)</th>
<th>S. Typhimurium (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephalexin</td>
<td>2 (4.54)</td>
<td>1 (12.5)</td>
</tr>
<tr>
<td>Nalidixic Acid</td>
<td>42 (95.45)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1 (2.27)</td>
<td>0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amoxycillin</td>
<td>6 (13.63)</td>
<td>0</td>
</tr>
<tr>
<td>Sulfathiazole</td>
<td>1 (2.27)</td>
<td>0</td>
</tr>
</tbody>
</table>

Figures in the parenthesis indicate percentage of resistance

4.4.3 Analysis for multi-drug resistance

![Graph showing percentage of isolates resistant to one or more drugs](image)

Fig. 9: Graph showing percentage of isolates resistant to one or more drugs
Table 10: Percentage of isolates not resistant and resistant to one or more types of antimicrobial

<table>
<thead>
<tr>
<th></th>
<th>Number of isolates</th>
<th>% isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>None resistant</td>
<td>2</td>
<td>3.85</td>
</tr>
<tr>
<td>Resistant to ≥ 1 Antimicrobial</td>
<td>50</td>
<td>96.15</td>
</tr>
</tbody>
</table>

4.4.4 Resistance pattern

Table 11: Resistant pattern exhibited by *Salmonella* serotypes

<table>
<thead>
<tr>
<th>Resistant type</th>
<th>Resistance pattern (isolates with same pattern)</th>
<th>Serotype(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 type of Antimicrobial</td>
<td>NA(41)</td>
<td>Enteritidis (34)*, Typhimurium (7)</td>
</tr>
<tr>
<td>2 types of Antimicrobial</td>
<td>NA+ CL (2)</td>
<td>Enteritidis (1), Typhimurium (1)</td>
</tr>
<tr>
<td>3 types of Antimicrobial</td>
<td>NA+AML+CL (1)</td>
<td>Enteritidis (1)</td>
</tr>
<tr>
<td></td>
<td>NA+CIP+SXT (1)</td>
<td>Enteritidis (1)</td>
</tr>
</tbody>
</table>

NA (Nalidixic acid), CL (Cephalexin), AML (Amoxycillin), SXT (Sulphathrimethoprim), CIP (Ciprofloxacin). *Number of isolates of this serotype expressing specified resistance profile
5. DISCUSSIONS AND CONCLUSIONS

5.1 Prevalence of *Salmonella*

While it is widely acknowledged that *Salmonella* is the major cause of foodborne illness in poultry and its products, epidemiological data are needed to inform public health authorities about the nature and magnitude of the problem and to monitor trends over time. Raw poultry products are contaminated with harmful, pathogenic and spoilage bacteria by infected stocks, cross contamination, improper handling and storage or improper cooking of poultry, which can lead to human foodborne illness and loss of product shelf life (Myint, 2004). Food safety regulations on meat and poultry are important issues and there should be a comprehensive plan to address hazards by improving the food safety at the animal production and intermediate stages before the slaughter plant, food safety during transportation, storage and retail sale, and to educate consumers to follow safe food handling practices such as proper storage, preparation, and cooking of meat and poultry products. However, in this study, there is a lack of knowledge at the animal production stages, of strategies followed by the exporting firm to reduce *Salmonella* in the live poultry, of slaughter technology and the level of food safety practiced by the industry.

This was the first time that a study on prevalence and antimicrobial resistance of *Salmonella* in imported chicken carcasses was done in Bhutan. Although there is scarce literature available on prevalence and antimicrobial resistance of *Salmonella* in chicken meat on the Indian side, no published prevalence and antimicrobial resistance studies existed in Bhutan prior to this study. Hence, the results of this study are discussed, compared and contrasted with similar studies in other countries.

This study found an overall *Salmonella* prevalence of 13% for imported broiler carcasses at the consumer level. Although a prevalence of 14.7% *Salmonella* in live poultry birds (Murungkar *et al.*, 2005) was earlier reported from India, some
report indicate negligible prevalence in poultry carcasses (Vaidya et al., 2004). However, the level of contamination in this study is consistent with the recent literature reporting 14.5% from Nepal (Maharjan et al., 2006), 19.2% from South Africa (Nierop et al., 2005), and 12% from Turkey (Ozbey and Ertas, 2006). The low prevalence of *Salmonella* in imported chicken carcasses as compared to many other countries such as Malaysia (35.5%), Spain (55%), Portugal (60%) and Thailand (57%) may be due to the fact that the actual prevalence of *Salmonella* is low or the processing industry decontaminate the final products or the producers use antimicrobials in the production chain.

Among serotypes observed in this study, *Salmonella* Enteritidis was most common followed by *Salmonella* Typhimurium. These two serotypes are commonly reported from poultry in the Indian subcontinent (Rahman et al., 2004b, Sharma et al., 2005a).

5.1.1 Batch-wise variation in *Salmonella* isolation

The batch-wise prevalence of *Salmonella* was plotted in a graph to observe the variation in isolation (fig 6). There were no isolations from 8th, 9th, 10th, 14th and 15th batches, which correspond to the peak winter season (December-February). However, there was maximum isolation observed (24/50) in the 16th batch of samples, which were analyzed in the 15th and 16th week, corresponding to the spring (hot) season (April). These differences in isolation might be due to the fact that *Salmonella* is more prevalent in the hotter season (Fossler, 2005, Liljebjelke et al., 2005). However, no correlation was seen with regard to batch weight of the chicken carcasses with number of *Salmonella* positive cases.

5.1.2 Seasonal variation in *Salmonella* isolation

There was a major variation in seasonal prevalence of *Salmonella* in winter and early summer (late spring) months. A total of 350 samples were analyzed during winter and 50 samples during spring. There was a significant seasonal difference
(p<.001) in the isolation of *Salmonella*. The imported broiler carcasses were 10.62 times more likely to yield *Salmonella* in the hot season as compared to the winter season. Similarly in a study in Nepal, the prevalence of *Salmonella* was found highest during the months of April and May (Maharjan *et al.*, 2006).

The import consignment is sealed in plastic cool boxes and transported in company-owned freezer vans till the entry point in Bhutan. The consignment is then transferred and further transported in conventional vans. The temperatures rise as high as 38°C during late spring and early summer in India and parts of Bhutan, and maintenance of a cold chain during transport is practically difficult. This might be due to the fact that prevalence of *Salmonella* is higher during hot dry and monsoon seasons. (Mohanty *et al.*, 2006).

### 5.2 Microbiological contamination of poultry carcasses

Bhutan does not have established standards for microbial contamination of fresh frozen chicken meat. However, with the exception of the presence of *Salmonella*, the level of microbial contamination of the material studied met the compulsory standards prescribed in many other developing and developed countries (Vietnam 10⁶ cfu/g, Ireland 10⁴-10⁵ cfu/g, United Kingdom 10⁴-10⁵ cfu/g, Thailand 10⁵ cfu/g, Australia 10⁶ cfu/g). The presence of *Salmonella* in the chicken meat is a particular health risk for the consumer and is related to the presence of epidemiologically important serotypes (Bennasar *et al.*, 2000) found in this study. Since the isolation and serotyping of *Salmonella* is not performed on routine sanitary examination of the imported consignment, each case of the isolation of *Salmonella* could pose a substantial threat to the general consumers.

*Salmonella* present in the chicken meat might originate from farms or contaminate the raw material during slaughter, processing, handling and transport. Since the cold chain maintenance during transport and consequently the temperature of the product determines its microbiological status, there is a need to maintain the intrinsic parameters of the product the least favorable for microbial growth.
5.2.1 Variation in microbial load in relation to processing temperature

The transport of the chicken approximately takes 8-10 hours from the slaughterhouse till the destination in Bhutan. The temperature during the transport varies and is difficult to maintain at the recommended temperature of the producer (-18°C or below) during transport.

In an individual study, it was reported no significant difference in Total Plate Count on minced chicken meat stored at 3°C for 182 days and eggs stored at 5°C for over a day respectively (Linton et al., 2004, Reu et al., 2005). A similar comparison was made between the microbial counts observed below 4°C and between 4-7°C (table 7). The present study found that although there are variations in the microbial count below 4°C and above 4°C, there is no significant difference (p>0.05) between the growth of aerobic organisms between -5.5°C to 4°C and 4°C to 7°C.

5.3 Antimicrobial resistance

Approaches to prevent and control Salmonellosis in the food animal industry by various means such as improved biosecurity, vaccination, use of competitive exclusion products, and the introduction of novel immuno-potentiatiors with limited success has necessitated the use of antimicrobial chemotherapy in the treatment and control of Salmonellosis (Zhao et al., 2007). The use of antimicrobials in food animals has resulted in the development of antimicrobial resistance (White et al., 2001), through mutation and acquisition of resistance encoding genes (Fluit, 2005). The situation in India may be exaggerated by easy accessibility of antimicrobials at a cheaper price and their extensive use in poultry production (Prakash et al., 2005). Another major setback might be the quality and potency of locally produced antimicrobial drugs; for example, there are over 80 different brands of the fluoroquinolone ciprofloxacin in India (Hart and Kariuki, 1998). Thus there is widespread availability and uncontrolled use of antibiotics.
5.3.1 Level of antimicrobial resistance

In this study, approx. 96% of the isolates were found resistant to nalidixic acid (table 8) followed by amoxicillin (11.54%) and cephalexin (5.77%). Sulphamethprimethoprim and ciprofloxacin resistance was 1.92% each. Chloramphenicol and gentamicin was sensitive to all of the isolates tested. While the resistance to nalidixic acid is consistent with the prevalence of 92-96% reported from India (Laxmi et al., 2006), gentamicin was completely sensitive to all of the isolates tested. Chloramphenicol sensitivity was approximately 98%. The Salmonella isolates in India from 1996-99 and 2001 were reported 100% chloramphenicol sensitive, with sensitivity as high as 79% in 2000 (Mandal et al., 2004a). This re-emergence of chloramphenicol sensitivity could be attributed to the limited use of the antimicrobial during the last decade in India (Khan and Shukla, 2004). The high prevalence of nalidixic acid resistance among poultry isolates (89%) was also reported from France in 2000 (Cailhol et al., 2005). Resistances to trimethoprim-sulfamethoxazole among poultry isolates are reported from Senegal (Bada-Alamedji et al., 2006), Mexico (Zaidi et al., 2006) and USA (Zhao et al., 2006). However, trimethoprim-sulfamethoxazole resistance was comparatively lower in this study. Among fluoroquinolone, resistance to ciprofloxacin was found comparatively lower in the present study as compared to 35% resistance in USA (Cui et al., 2005b), 10.2 to 16.8% in Germany (Malorny et al., 2003) and 9.6% in Austria (Mayrhofer et al., 2004). Resistance to cephalexin was also lower than reported earlier in India (Murugkar et al., 2005).

5.3.2 Level of antimicrobial resistance by serotypes

Antibiotic resistance has been reported to be more common in Salmonella Typhimurium than the other serovars in India (Rahman, 2002). In the present study, 100% of Salmonella Typhimurium were found resistant to nalidixic acid and 12.5% to cephalexin (table 9). Salmonella Typhimurium is sensitive to all other antimicrobials tested. Increasing incidence of nalidixic acid resistant Salmonella Typhimurium is also reported from France (Corre et al., 1999) and the USA (Stevenson et al., 2006).
Several studies have indicated increased resistance to nalidixic acid and decreased susceptibility to fluoroquinolone in *Salmonella* species from food animals and infections in humans (Corre *et al.*, 1999, Mølbak *et al.*, 2002, Aarestrup *et al.*, 2003, Choi *et al.*, 2005). The present observation of 95.45% resistant *Salmonella* Enteritidis to nalidixic acid could be the result of mass use of quinolones in broiler farms in India. National Antimicrobial Resistance Monitoring System (NARMS) reported 84.9% resistance to nalidixic acid in 2004 (NARMS, 2004). Resistance to amoxicillin (13.63%), cephalexin (4.54%), ciprofloxacin and sulfa-trimethoprim (2.27% each) observed in this study are lower than earlier reported from North-eastern India (Murugkar *et al.*, 2005).

5.3.3 Multi-drug resistance

Of the 52 *Salmonella* isolates subjected to antimicrobial testing, 2(3.85%) were sensitive and 50 (96.15%) were resistant to one or more drugs tested. 78.85% of the isolates were resistant to one type of antimicrobial, 13.46% to two types and 3.85% to three types (fig. 9). While *Salmonella* Typhimurium from chicken breast showed resistance to 10 antimicrobials in the US (NARMS, 2004), the isolates of *Salmonella* Typhimurium in this study were found resistant to nalidixic acid and cephalexin. *Salmonella* Enteritidis was resistant to cephalexin, nalidixic acid, ciprofloxacin, amoxycillin and sulfa-trimethoprim with varying degrees of resistance (table 9). Multi-drug resistant *Salmonella* Typhimurium was reported in the past few decades and is frequently reported from the Indian subcontinent (Rahman *et al.*, 2004b). Two isolates of *Salmonella* Enteritidis are found resistant to three drugs, one to cephalexin, nalidixic acid and amoxicillin and another with nalidixic acid, sulfa-trimethoprim and ciprofloxacin. A higher proportion of antibiotic resistance in *Salmonella* Enteritidis has been reported from southern Brazil (Oliveira *et al.*, 2005).

5.3.4 Resistance pattern

Overall, five resistance patterns were observed among 52 *Salmonella* isolates (table 11). *Salmonella* Enteritidis was resistant to five of the seven antimicrobials
tested with simultaneous multi-drug resistance to up to three antimicrobials. A similar study in Turkey showed higher multi-drug resistance in *Salmonella* Enteritidis isolates from chicken as compared to human and egg isolates (Icgen et al, 2002). In contrast, one isolate of *Salmonella* Typhimurium showed resistance to two antimicrobials while the rest were resistant to nalidixic acid alone. *Salmonella* Typhimurium was also found to be comparatively resistant to as many as 5 drugs tested in United States (Berrang et al., 2006).

5.4 Recommendations

The current study reveals presence of *Salmonella* in the fresh frozen imported broiler carcasses from Arambagh Hatcheries in India. Despite the fact that the microbiological quality is acceptable, isolation of antimicrobial resistant *Salmonella* serotypes including multi-drug resistance poses a concern to public health authorities and the general Bhutanese consumers. In view of this research finding, there is a need to develop comprehensive policies to ensure safe food. The following recommendations should prove useful to ensure the microbiological quality of import chicken meat.

5.4.1 Setting import standards

The processing industry bears the responsibility of meeting food quality and safety regulatory requirements. Besides the demand for quality certification of the end products, the standards should incorporate pre-harvest and post production processes. This should include the animal disease situation, the use of antimicrobials, growth promoters and other chemicals during the pre-harvest process, hygienic standards, slaughter and facilities for handling, processing, production, storage, transport of meat with details of ante and post-mortem inspection, and qualifications of officers responsible for the inspection process. The production process should be supported by documentary evidence over a period of time. These standards and statements should be supported by a legal framework to instill a sense of responsibility to all those involved in the food business.
5.4.2 Sanitary examination of the product

An important step to ensure that exporting country certificates are authentic and accurate and that products meet the import standards is by sanitary examination on arrival of the consignment. This might call for review of our national standards and their applications to our own products. The current practice of physical inspection of the consignment should be supplemented with random samples of chicken meat for microbiological analysis and adopt routine inspection activities. The information generated should be disseminated to the concerned agencies for monitoring and surveillance.

5.4.3 Collaboration and resource utilization

Strengthening food control services in Bhutan requires better infrastructure with adequate amenities, skilled manpower and a capable system in place. Lack of these resources and the inability to invest as the Government has other priorities, calls for unanimity in sharing skills and facilities between the different organizations. Therefore, the veterinary public health division within the Department of Livestock should play a crucial role in collaboration with the Department of Public Health, Bhutan Agriculture and Regulatory Authority, the Department of Trade and Custom authorities and the private entrepreneurs.

It is evident from this research that the presence of *Salmonella* in imported chicken poses a risk to the consumers. An effective national *Salmonella* surveillance system should be set up for monitoring and control of *Salmonella* in food. Improved laboratory capacity could be achieved through collaboration with WHO Global *Salmonella* Surv program. Collaboration with national reference centres such as Central Research Institute in India and Centre for Antimicrobial Resistance Monitoring in foodborne pathogens, WHO Global Salmonella Surveillance Asian Regional Centre in Thailand will certainly assist in serotyping of the *Salmonella* isolates.
5.4.4 Cold chain

Maintenance of cold chains plays an important role in the integrity and assurance of the quality of the products, especially when the consignment comes from a long distance transport. A majority of the consumers are either unaware, or believe that sellers respect all the food safety requirements. Therefore, it becomes the duty of the food control authorities to verify and assure that the cold chain is maintained at the temperature indicated on the label and required by the producer. Since it becomes a huge investment for the private business entrepreneurs, the government should also consider establishing a cold storage in Phuentsholing for en-route storage of the products. Nevertheless, the food control authorities should monitor the maintenance of the cold chain and educate the business entrepreneurs involved in food business in a timely manner.

5.4.5 Public education

Although there is a general practice of cooking the chicken products before consumption in Bhutan, specific information on food quality is generally less known to the consumer. Awareness campaigns and notifications as and when necessary should be organized and incorporated in the routine process by the Public Health Department, Bhutan Agriculture and Food Regulatory Authority, and should also emphasize on educating the food business entrepreneurs and people involved in the food business.
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APPENDICES

Appendix A: Regulations of the Bhutan Agriculture and Regulatory Authority (BAFRA) on minimum standard requirements for the operation of meat shop/sale counter.

- The meat shops must be located in areas which are free from objectionable odors, smoke, dust or other contaminants.
- In order to avoid the harboring of bacteria and other sources of contamination, all internal finishes must be made of corrosion-resistant material, and be smooth, impervious, and easy to clean and disinfect. Joints, doors and windows must be fitted so as to prevent the entry of insects and rodents.
- The floor must be made of concrete, non-absorbent, washable, non-slip, without crevices, and must be easy to clean and disinfect. Floors must slope sufficiently for liquids to drain to trapped outlets and to proper sewerage. At all times, they must be kept clean and dry.
- The walls must be water-proof, non-absorbent, washable and painted white in color from floor to ceiling. They must be kept clean all the time. Angles between walls, between walls and floors and between walls and ceilings must be sealed and covered to facilitate cleaning.
- Ceilings must be made of concrete or ply-board and painted white in color. They must be impervious to dirt and dust and must be kept clean all the time.
- The meat shops dealing in fish and chicken must have a minimum dimension of 96 sq. ft with a minimum of 8 ft. breadth in the front (sale counter). Those dealing in fish, chicken and pork must have a minimum of 150 sq. ft with a minimum of 10 ft. breadth of front part. The minimum height from floor to the ceiling must be 8 ft.
• The meat shops dealing only in beef must have minimum of 8 ft. x 12 ft. for every 500 kg of beef dealt in a day. The minimum height from floor to the ceiling must be 8 ft.

• Meat shops must have a continuous water supply. In places where water supply is not continuous, adequate overhead water storage tanks of enough capacity must be provided. The water supply must be safe, clean and under adequate pressure. Water used for making ice must be potable and ice must be manufactured, handled, stored and used so as to protect it from contamination.

• Meat shops must have hand-washing facilities, including water, soap and suitable hygienic means of drying hands.

• Meat shops must have proper drainage facilities to drain out the meat effluent to closed sewerage drains.

• Display counters must be covered with continuous thin aluminum sheeting or tiles that can be cleaned easily. The display counter must be made at a level of 1.25 meter from the floor level. It must slope sufficiently from outside towards inside for liquids to drain into a small continuous drain running from one end of the display counter to the other. This small drain must be connected to a proper sewerage drain.

• Dimensions of display counters must be of 2 ft. x 2 ft. for each item. If all types of meat are kept on the same continuous display counter, a partition wall of sufficient height must be provided in order to avoid contact between the two different items. The beef display counter must also have partition walls between each item and different organs must be displayed separately.

• Displaying of meat on plastic mats or on any cloths that are kept on display counters is strictly prohibited.

• Maximum number or weight of varieties of meat allowed for display are fixed as follows:
  - Chickens: 3 nos.
  - Fish: 3 nos.
  - Pork/Beef: 3 pieces of 2-3 kg
• The rest of the stock must be stored in a deep freezer. If the display counter is a refrigerated glass cabin, the pieces or quantity of meat displayed can be increased according to the capacity of the glass cabin.
• Watering of meat kept on display counters is strictly prohibited.
• A standard deep freezer(s) of enough storage capacity as per the quantity of meat sold must be available for the storage of the meat that is being held for sale. Different types of meat must be stored separately in the freezer.
• Deep freezers must not be loaded beyond their designed capacity. They must be functioning all the time and the required temperature must be maintained. (deep freezers must be maintained at or below -18°C). Deep freezers must be washed and cleaned frequently and regularly.
• Holding of meat in bamboo baskets or stacking on the floor or keeping immersed in water with or without ice is strictly prohibited.
• Beef and pork must be hung or placed in suitable corrosion-resistant trays, in such a manner as to permit adequate circulation of air around the meat. Keeping of meat stacked on the floor is strictly prohibited.
• Meat must be hung in a manner that precludes drips from one piece falling onto another. Meat must not come in contact with walls and must be hung at least 1 meter above the floor level.
• Stomach and intestines must be transported only when thoroughly cleaned or scalded and heads only when skinned or scalded or de-haired.
• Stomach and intestines must be displayed separately or kept hung. Keeping of improperly cleaned stomach and intestines on display counter or keeping them on the floor of meat shop is strictly prohibited.
• The hook used for hanging meat should be preferably made of stainless steel or if made of iron, it should be painted.
• In beef and pork stalls there must be a sufficient number of fans to maintain enough circulation of cold air. A cold room facility must be set up especially those dealing in huge quantities of meat.
• A clean cutting table must be provided for chopping meat. The minimum height of the cutting table should be 1 meter. Cutting of meat on the floor is strictly prohibited.

• All equipment like knives, etc must be kept cleaned all the time and stored in a proper and clean place.

• All types of meat sold must be wrapped in clean suitable packing materials sufficient enough to protect the meat from contamination. Packing materials must be stored and used in a clean and sanitary manner.

• Meat must not be placed in any means of transport that has not been cleaned before loading and, if necessary, disinfected and then the disinfectant washed off with clean water. Where human labor is used for transportation, the health requirements for food handlers should apply.

• Vehicles intended for the transport of meat must be equipped so that meat does not come in contact with the floor.

• Meat shop owners must issue cash receipt to the customers for every transaction conducted.

• A certificate issued by the Assistant Regulatory Inspector certifying the suitability of meat as “Fit for Human Consumption” must be displayed at a place where the customer can easily read it.

• Only one salesperson in small and up to three salespersons in large meat shops are allowed to handle and sell meat from the counter. All salespersons must be registered with the local office of the Assistant Regulatory Inspector, QCRS. For registration, the owners must submit their name(s) and photocopy of citizen ID card.

• No other person other than those registered with the Assistant Regulatory Inspector, QCRS is allowed to handle and sell meat.

• Salespersons must wear a white apron with full arm sleeves along with half-arm plastic/rubber gloves that can be easily washed. He/she must make sure that the apron and gloves are kept clean all the time.

• Salespersons must display the ID card issued by QCRS identifying their permission to handle and sell meat while in meat shops.
• The owner must notify the local Assistant Regulatory Inspector, QCRS, one month in advance for a change of salesperson(s) for issuing ID cards granting him/her permission to handle and sell meat.

• Good personnel hygiene must be observed by salesperson(s). Smoking, chewing of tobacco and doma are not allowed while in meat shop. He/she must wash hands every time after entering the meat shop.

• Person(s) handling or selling meat must have a valid medical certificate with an X-ray result stating he/she is free from any communicable diseases issued by the relevant Government Medical Authority. The certificate must be renewed or a new one obtained at six monthly intervals.

• Any salesperson affected with open wounds, sores, diarrhea, colds, respiratory ailments or any other illness must not be allowed to handle or sell meat until he/she is fully recovered.

• It is the responsibility of meat shop owners to keep his/her premises and surroundings clean.
Appendix B: Regulations of the Department of Livestock for the import of poultry meat into Bhutan

- All poultry meat must be processed with proper packaging.
- Processed poultry meat must be certified from the government of India and recognized by the Ministry of Food Processing Industries (MFPI) and the Export Inspection Council India (EIC). It must conform to the recognized standard operating procedures and processing standards like HACCP and Safe Quality Food (SQF).
- Only whole-dressed chicken (without the head, feet, and internal organs) and portioned chicken will be allowed.
- It should be packed in tamper-proof and transparent food grade plastic packets. The packet should be marked with expiry date, approximate weight, and seal of the HACCP and SQF.
- All consignments should be transported in refrigerated vehicles to Bhutan and the quantity of each consignment should be specified.
- It must be stored at the required temperature during transportation and also at the retail sales counter.
- The price of whole dressed chicken should be sold at a price which is comparable to the price that existed before the ban was imposed.
- Each consignment should be accompanied with appropriate certification from government veterinarians in the area.
- The consignments are to be sealed before departure from the hatchery and should only be opened in the border town of Bhutan in the presence of regulatory official (BAFRA) to prevent any handling en-route.
- The supplying firms should strictly adhere to the current regulatory norms implemented by BAFRA.
DECLARATION

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

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