Isolation of *Salmonella Spp*. in different meat samples of Kathmandu valley

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By

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ABSTRACT

The present study was conducted at Central Veterinary Laboratory, Tripureshwor, kathmandu from Ashoj to Mangsir, 2065 with the aims of determination of the isolation of *Salmonella spp.* in meat samples of kathmandu Valley. Altogether 250 meat samples including Goat (75), Chicken (125), and Buffallo (50) were collected from different retail shops of kathmandu Valley. All these samples were processed using standard procedures for the isolation and identification of *Salmonella spp.* from meat samples. All *Salmonella* isolates were tested to different antibiotics using standard protocol for the determination of antibiotic susceptibility profile.

The isolation of *Salmonella spp*. detected case was found to be 9.2%. The detection rate was found highest in the month of Ashoj (11.11%).The isolation of *Salmonella spp* detected case in retail shops of kathmandu (11.01%) was higher than that of Bhaktapur (8.77%) and Latitpur (6.66%). The isolation of *Salmonella spp* detected in chicken (11.2%) was higher than buffalo (8%) and goat (6.66%) meat samples. Among 23 isolates of *Salmonella,* 39.13% were *S. gallinarum,* 34.78% were *S. pullorum* and 26.08% were *Salmonella spp*. belonging to Serogroups D and E. Among these serogroups, *Salmonella* belonging to serogroup D was the most predominant. Multidrug- resistant (MDR) was observed in only 4.34% of the *Salmonella* isolates. However, most of the isolates in addition to those pathogenic to human (i.e , belonging to Serogroup D and E) were resistant to the antibiotics Tetracycline and Nalidixic acid. All isolates were sensitive to Cephotaxime, Ciprofloxacin, Ofloxacin, and Chloramphenicol.

Key words: Salmonella, Serogroups, Multidrug-resistant, Pathogenic, Antibiotics.

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LIST OF ABBREVIATIONS

A/A	: Acid / Acid
Ado	: Adonitol
Alk/A	: Alkali / Acid
Ara	: Arabinose
Buff	: Buffalo
CVL	: Central Veterinary Laboratory
Dul	: Dulcitol
d	: 16-84% Strains Positive
Hrs	: Hours
Ino	: Inositol
Lac	: lactose
MA	: Mac Conkey Agar
Man	: Mannitol
MDR	: Multi drug Resistant
Min	: Minutes
ml	: Milliliters
MR	: Methyl Red
NA	: Nutrient Agar
No	: Number
SIM	: Sulphide Indole Motility
Tre	: Trehalose
TSI	: Triple Sugar Iron
UK	: United Kingdom
US	: United States
Vp	: Voges Proskauer
WHO	: World Health Organization
(w)	: Relatively Delayed or Work
XLD	: Xylose Lysine Deoxycholate Agar
Xyl	: Xylose

CHAPTER- I

1. INTRODUCTION

The term Meat is widely used to define flesh and offal including their natural associates skin and gristle, derived from carcass of any animal and bird normally used for human consumption. A meat product is defined as any food, which consists of meat or of which meat is an ingredient (Robinson, 2001).

Meat is a good source of protein, vitamins essential fatty acids and minerals like Zinc, Iron, Calcium, Phosphorous, etc. (Frazier and Westhoff, 1978).

Meat is an ideal culture medium for the growth of various microorganisms as it is rich in nitrogenous foods of various degrees of complexity, plentifully supplied with minerals and accessory growth factors, high in moisture (76.78% to 77.94%), protein (17.49% to 18.42%), lipid (0.47% to 0.82%), phospholipids (147.34 mg% to 206.1 mg%), total cholesterol (28.66 mg% to 34.32 mg%) and abundantly supplied with minerals and accessory growth factors, usually has some fermentable carbohydrates (glycogen). Lastly, it has a favorable p^H (5.7 to 7.2) for most microorganisms and is classified as low-acid foods, when classified on the basis of acidity (Frazier and Westhoff, 1978).

According to William C. Frazier and Dennis C. Westhoff 1993, the healthy inner flesh of meats has been reported to contain few or no organisms, though they have been found in Lymph nodes, bone marrow, and even flesh. The carcass of healthy animal slaughtered for meat and held in a refrigerated room is likely to have only nominal surface contamination while the inner tissues are sterile. Fresh meat cut from the chilled carcass has its surface contaminated with micro-organisms characteristic of the environment and the implements (saws or knives) used to cut meat. Each new surface of meat, resulting from a new cut, adds more microorganisms to the exposed tissue. *Staphylococcus spp, Streptococcus spp, Clostridium spp, and Salmonella spp* have been isolated from the lymph node of red meat animals. Normal slaughtering practices would remove the lymph node from the edible parts. The important contamination,

however, comes from external sources during bleeding, handling and processing.

In Nepal, lack of appropriate slaughtering facilities and unsatisfactory slaughtering techniques are causing unnecessary losses in meat as well as its invaluable by products. Animals particularly buffaloes are slaughtered in Kathmandu, in slaughtering places which are frequently polluted with street dust, garbage, human excreta, animal blood, intestinal contents and dirty effluents and which are not protected against dogs, rodents and insects. Meat products under such conditions are generally deteriorated due to the bacterial infections and which causes food poisoning time to time. Due to lack of meat inspection act and in absence of meat inspection, meat from unhealthy or parasitic infected animals may be source for infection and spreading diseases to human as well as animals. Besides meat quality is adversely affected by careless handling conditions in the slaughtering places as well as in the meat markets or shops (Joshi D. D. 1991).

Food borne diseases caused by non-typhoid Salmonella represents an important public health problem worldwide. In most part of the world, *S. typhimurium* is the most common species. Some others are *S. enteritidis, S. haldar, S. Heidelberg, S. agona, S. Virchow, S. newport and S. anatum* (Ananthanarayan and Paniker, 2000). Salmonellosis in humans is generally contracted through the consumption of contaminated food of animal origin mainly meat, poultry eggs & milk, although many other foods including green vegetables contaminated from manure have been implicated in its transmission (WHO, 2004).

Multi-drug resistant (MDR) strains of Salmonella are now encountered frequently and the cases of MDR have increased considerably in recent years. It is reported that some strains of Salmonella have developed MDR (WHO, 2004). *Salmonella gallinarum,* the causative agent of fowl typhoid are resistant to antibiotics- penicillin, ampicillin, tetracycline and Cloxacillin (NARC, 2053/54).

Several reports have been published on microbiology of meat from different parts of the world with different organism pattern. However we do not have such type of enough information regarding meat microbiology of Nepal. So, it is hoped that, this research work would be informative and helpful for both planners, policy-makers and also those who are interested to know about microbiology quality of meat.

CHAPTER-II

2. OBJECTIVES

2.1 GENERAL OBJECTIVE

- To isolate *Salmonella species* from different meat samples of Kathmandu Valley

2.2 SPECIFIC OBJECTIVES

- To isolate and identify *Salmonella species* from different meat samples of different retail shops of Kathmandu Valley.
- To isolate different sero groups of Salmonella.
- Antibiotic Sensitivity testing of the isolates of Salmonella by disc diffusion method.

CHAPTER III

3. LITERATURE REVIEW

3.1 SALMONELLA

Salmonella is one of the important pathogenic members of family "*Enterobacteriaceae*" responsible for causing various infections and food poisoning in human and animals. *Salmonella species* are gram negative rods with size of 2-4x6µm. they are non-acid fast, non capsulated and non spore forming. Most serotypes are motile with peritrichous flagella. These non-motile bacteria are susceptible to cause infection in poultry birds with high mortality rate. *Salmonella species* are aerogenic, non lactose fermenter, urease negative, indole negative, methyl red positive, Voges-Proskauer negative and citrate variable (Mackie and Mc Cartney 1989).

Several species of Salmonella are pathogenic, some producing mild gastroenteritis, other producing a severe and often fatal food poisoning, which is called Salmonellosis. Salmonella grow between 15-45^{o}C, and at pH range of 4 to 8. with the exception of limited number of human host adapted serotypes (also referred to as the typhoidal *Salmonellae*), the members of the genus Salmonella are regarded as zoonotic or potentially zoonotic (Acha and Szyfres, 2001).

3.2 HISTORY

The Salmonella group previously was also called the TPE group, the so called typhus-paratyphus enteritis group. It comprises the typhus bacillus, *Salmonella typhi*, previously called Eberth- Gaffkey bacillus or Eberthela typhi after the name of two scientists Eberth (1880) observed into the mesenteric lymph node and spleen of typhoid patient, and Gaffkey (1884) isolated the organism. In 1896, it was demonstrated that the serum from an animal immunized with the typhoid bacillus agglutinated (clumped) the typhoid bacterial cells, and it was shown that the serum of patients afflicted with typhoid likewise agglutinated the typhoid bacillus. Sero diagnosis of typhoid was thus made possible by 1896 (Kenneth,2006).

3.3 NOMENCLATURE

The classification of Salmonella has been controversial for many years. According to the latest nomenclature, which reflects recent advances in taxonomy, the genus Salmonella consists of only two species. *S. enterica* and *S. bongori* (Le minor and Popoff, 1987). *Salmonella enterica* is divided into six subspecies (OIE, 2004). These subspecies are:

Previous nomenclature	current nomenclature
Subspecies I	Subspecies *enterica*
Subspecies II	Subspecies *salamae*
Subspecies IIIa	Subspecies *arizonae*
Subspecies IIIb	Subspecies *diarizonae*
Subspecies IV	Subspecies *houtenae*
Subspecies V	Subspecies *indica*

For the serovars of *S. bongori*, the symbol V was retained to avoid confusion with the serovars name of *S. enterica* subspecies *enterica*. Strains of *Salmonella* are classified into serovars on the basis of extensive diversity of lipopolysaccharides (LPS) antigen (O) flagellar protein antigen (H) in accordance with the Kauffmann-White scheme, currently approximately 2500 serovars are recognized. The most common serovars that cause infection in humans and food animals belong to subspecies *enterica* (OIE, 2004).

3.4 KAUFFMANN-WHITE CLASSIFICATION

This scheme, first developed in 1934, classifies the Salmonella into different O groups or O sero groups, each of which contains a number of serotypes possessing a common O antigen not found in other O groups. The O groups first defined were designated by capital letters A to Z and those discovered later by number (51-67) of the characteristic O antigen. It is now considered more

correct to designated each O group by its characteristics more correct to designate each O group by its characteristics O factor, ie to abandon letters A-Z used to designate early O groups. Hence, O groups become; O3 (A), O4 (B), O7 (C1), O8 (C2-C3), O9, 12 (D1), O9, 46 (D2), O3, 10(E1) etc. (Ewing 1986, Popoff Le minor 1992). Groups O2 to O3, 10(A-E1) contain nearly all the *Salmonellae* that are important pathogens in man and animals. Within each group the different serotypes are distinguished by their particular H antigen or combination of H antigens. Serotypes that shares the same antigenic formula may be distinguished from one another by biochemical tests.

Table 1: Antigenic Formulae of some representative serotype of *Salmonella* (Kauffmann-White classification (Mackie and Mc Cartney, 1989).

	O-antigen			**H-antigens**	
Serogroup	**group**	**Serotype name**	**O antigens and Vi**	**phase 1**	**phase 2**
2	A	Paratyphi A	1, 2, 12	a	(1, 5)
4	B	Paratyphi B	1, 4, (5), 12	b	1, 2
		Stanley	1, 4, (5), 12, 27	d	1, 2
		Schwarzengrund	1,4,12, 27	d	1, 7
		Saintpaul	1, 4, (5), 12	e, h	1, 2
		Derby	1, 4, (5), 12	f, g	(1, 2)
		Agona	1,4,12	f, g, s	-
		Typhimurium	1, 4, (5) ,12	i	1,2
		Bredeney	1,4, 12, 27	l,v	1,7
		Brandenburg	1, 4,12	l, v	e,n,z 15
		Heidelberg	1,4,(5), 12	r	1,2
7	C1	Choleraesusis	6,7	c	1,5
		Paratyphi C	6,7 (Vi)	c	1,5
		Typhisuis	6,7	c,g,m,(p),	1,5
		Montevideo	6,7,14	s	(1,2,7)

		Thompson	6,7,14	k	1,5
		Virchow	6,7	r	1,2
		Infantis	6,7,14	r	1,5
		Mbandaka	6,7,14	z10	e,n,z15
8	C2-C3	Muenchen	6,8	d	1,2
		Newport	6,8,20	e,h	1,2
		Hadar	6,8	z10	e,n,x
		Miami	1,9,12	a	1,5
		Sendai	1,9,12	a	1,5
9	D1	Typhi	9,12(Vi)	d	-
		Enteritidis	1,9,12	g,m	(1,7)
		Dublin	1,9,12(Vi)	g,p	-
		Panama	1,9,12	l, v	1,5
		Gallinarum	1,9,12	-	-
3, 10	E1	Anatum	3,10,(15),(15,34)	e,h	1,6
		Weltevreden	3,10,(15)	r	z6
1,3,19	E4	Sentfenberg	1,3,19	g,(s),t	-
11	F	Rubislaw	11	r	e,n,x
13	G	Kedougou	1,13,23	i	l,w

Note: Somatice factors associated with phage conversion are underlined.

Antigens in brackets [X] are not always present.

3.5 ANTIGENIC STRUCTURE

In the Kauffmann-White classification the genus *Salmonella* is subdivided into more than 2300 serotypes containing different combinations of antigen (Mackie and Mc Cartney, 1996)

Somatic (O) or Cell Wall Antigen

These somatic antigen represent the side chains of repeating sugar projecting outward from the lipopolysaccharide layer on the surface of bacterial cell wall. Over 60different antigen have been recognized and they are designated by Arabic numerals. O antigens are heat stable being unaffected by heating for 2.5 hours at 100^{o}C and alcohol stable (Mackie and Mc Cartney, 1996).

H-antigen/ Flagellar antigen

H-antigen is flagellar antigen. Many Salmonella are diphasic that is they can occur in two antigenic forms referred as Phase I and Phase II. Phase I antigens are either numbered or given a letter if known to occur in both phases. The definitive identification of a diphasic Salmonella always requires the identification of the H-antigens of both phases.

Surface (K or Vi) antigen

Vi antigen is surface antigen and helpful in phase typing of *S. typhi, S. paratyphi C and S. dublins* as these serotype possess this antigen (Bergey's manual 1990).

"O" and "Vi" agglutinins produce fine granular agglutination while H agglutinin bring about a large flocculent agglutination.

3.6 CULTURAL AND BIOCHEMICAL CHARACTERISTICS OF SALMONELLA

3.6.1 Cultural Characteristics

Salmonella is member of *Enterobacteriaceae* group, therefore it follows all the characters common to *Enterobacteria* ie rod shape, aerobic, facultative anaerobic, catalase +ve, oxidase –ve, production of gas by carbohydrate break down etc. in nutrient and blood agar, colonies of most strains are moderately large (e.g 2-3 mm in diameter), grey-white, moist, circular discs with a smooth convex surface and entire edge. Their size and degree of opacity varies with the serotype (Collee et al, 1996).

The organism are aerobes, the optimum temperature for growth is 37^{o}C. They grow readily on ordinary nutrient media. Grow as non-lactose fermentative colonies on Mac Conkey Agar. In selective media like Bismuth Sulfite Agar colonies of *Salmonella* are metallic black in color with 1-2 mm diameter size while in XLD, colonies are black centered with 1-2mm diameter in size (Banwart JG 1987).

3.6.2 Biochemical Characteristics

In general, Salmonella is catalase +ve, oxidase –ve, produce gas from glucose at 37^{o}C. produce acid from carbohydrate breakdown ie sugars like Dulcitol, Arabinose, Maltose, Trehalose, Xylose, etc. with variation in reaction according to different types, use citrate as carbon sources, MR +ve, Vp –ve. Protein reactions shown by Salmonella are –ve gelatin hydrolysis, -ve indole reaction, -ve urea hydrolyzation, production of H_2S in TSI etc. (Joshi V, 2003).

Few biochemical characteristics are different between *S. pullorum and S. gallinarum* (Christensen et al, 1992). Both of them ferment arabinose, dextrose, galactose, mannitol, mannose, rhamnose and xylose to produce acid with or without gas. *S. gallinarum* decarboxylates ornithine, whereas *S. gallinarum* does not (Shiva Prasad, 1997). *S. pullorum and S. gallinarum* produce a red slant with a yellow butt and shows delayed blackening from H_2S production. *S. gallinarum* does not form gas in triple sugar iron agar, but *S. pullorum* may show weak gas production (Doughlas et al, 1998).

3.7 SALMONELLOSIS

Salmonellosis is a diseases caused by the ingestion of *Salmonella spp* by human or animals.

Following ingestion, symptoms may or may not develop depending upon the dose, level, serotype and Virulence of the strain and host resistance (Bryan, 1968). *Salmonella species* is the potent organism that causes several losses in poultry by infecting with several disease .*S. pullorum* diseases and fowl typhoid is the two specific manifestations of Salmonellosis caused by non-motile bacteria, *Salmonella pullorum* and *Salmonella gallinarum*.

A broad group of diseases called paratyphoid infections are also caused by Salmonella organism that are motile. The micro organisms are highly prevalent in human excretions, poultry meat, pork and beef origin meat. Probably these are the major source of Salmonellosis problem in human and poultry.

3.7.1 Over view of salmonellosis in human

Salmonellosis in humans is generally contracted through the consumption of contaminated food of animal origin (mainly meat, poultry, egg & milk), although many other foods, including green vegetables contaminated from manure, have been implicated in its transmission. The causative organisms pass through the food chain from primary production to households or food-service establishments and institutions. In addition to acquiring infection from contaminated food, human cases have also occurred where individuals have had contact with infected animals, including domestic animals such as cats & dogs. Domestic animals probably acquire the infection in the same ways as humans, i.e through consumption of contaminated raw meat, poultry or poultry-derived products. The evolution of specific Salmonella serotypes in intensive animal husbandry and subsequently in humans has been observed over the past three decades. *S. enteritidis* caused the most recent epidemic, which peaked in humans in 1992 in many European countries. Its current slight decline sets the scene for re-emergence of *S. typhimurim* as the most important serotype in human Salmonellosis. Another possible scenario is that these two particular strains with epidemic potential will dominate in many countries in the Foreseeable Future (WHO, 2004).

3.7.2 Overview of Salmonellosis in Poultry & animal

3.7.2.1 Pullorum Diseases

It is an acute or chronic infection bacterial disease affecting primarily chickens and turkeys but most domestic and wild fowl can be infected as well. The disease is caused by the bacteria *Salmonella pullorum,* non-motile bacteria.

Most outbreaks of acute pullorum diseases in chicken result from infection while they are in the hatchery. Pullorum disease is highly fatal to young chicks

or adults but may die soon after hatching without exhibiting any observable signs.

Mortality rate in such outbreaks may approach 90% if untreated. Survivors are usually stunted and unthrifty. Infection in young birds may be indicated by droopiness, ruffled feathers, a child appearance with birds huddling near a source of heat, labored, breathing difficulties and presence of a white diarrhoea with a "pested-down" appearance around the vent white diarrhoea symptoms instigated the term "Bacillary White Diarrhoea" (Pandey, 2003).

3.7.2.2 Fowl Typhoid

The disease is caused by the non-motile bacterium *Salmonella gallinarum*. Hence, *Salmonella gallinarum* is attributed to septicemia of acute or chronic in chicken (Pandey et al 1991). The poultry bird infected from the fowl typhoid shows the similar symptoms to the pullorum disease. Post-mortem finding of the liver, kidneys and spleen may be enlarged and discolored.

Fowl typhoid has increased in most part of the world. In areas where the disease is common, it has become one of the most important diseases in poultry (Silva et al, 1981). Fowl typhoid is endemic in Nepal. The mortality rate due to the disease may vary from moderate to very high. Although, *Salmonella gallinarum* is pathogenic to both chicks and adults under natural condition, virulence of the isolates may vary depending upon strain variation environment situation, individual susceptibility and cultural condition of the isolates.

3.8 SALMONELLOSIS IN GLOBAL PERSPECTIVES

From the data obtained from US department of agricultural, food safety and inspection service (FSIS) *S. enterica* serotype *enteritidis* in broiler chicken carcass rinses collected from 2000 to 2005 showed that the annual number of isolates increased > 4 fold and the proportion of chicken slaughter establishment with *S. enteritidis* positive rinses increased nearly 3 fold. During the six year study period, 280 (0.5%) *Salmonella entritidis* were recovered from 51, 327 broiler rinses. The number of establishment testing positive increased

from 17 (9%) of 197 in 2000 to 47 (25% of 187 in 2005. the most predominant phage type in broiler chickens and in the chicken slaughter establishment with *S. enteritidis* positive rinses were PT8 and PT13 (Sean et al, 2006).

A case study on Salmonella outbreaks in a middle town of USA reported that 125 cases of food borne Salmonellosis resulted from cross contaminated food items served in a picnic. 125 people out of 175 people who attended the picnic developed diarrhoea after having smorgasbord prepared by a bar restaurant. The stool culture report showed the presence of *S. infantis, S. agona, S. schwarzengruna*. The incidence took place in Minnisota USA in September 1973 (Levy et al, 1975).

Mohamed Osama N. et al, (2001) conducted an experimental and surveillance study for the diagnosis of Salmonellosis in meat of slaughtered animal. The study evaluated meat juice as a sample from infected mice with *S. typhimurium* for detection of anti Salmonella antibody by ELISA techniques. Studied showed that meat juice as efficient as blood in diagnosis of Salmonellosis. The method can be applied efficiently for surveillance study for screening programs.

A study was carried out regarding the Salmonella incidence in minced meat produced in a European Union approved slaughtering and cutting plant. Throughout 21 mounts, 297 pool samples (1,485 individual samples) of mixed minced meat (beef and pork) were examined according to council directive 94/65/EC and to ISO 6579. Salmonella were detected in 47 (15.8%) of the pool samples. After separation of the positive pools, 93 individual samples were determined to be Salmonella positive, representing 6.3% of the total 1,485 samples. Serotype resulted in most isolates (69.9%) being identified as *Salmonella typhimurium* (Stock and Andreas, 2001).

Victoria Atanassova (2002) recorded the isolation of *Salmonella spp*. in foreign chicken meat from different European countries. During the study period of Feb 2000 to March 2001, a total of 2006 samples of frozen chicken meat were examined for isolation of *Salmonella spp* using classical cultural detection as well as RFLP_PCR techniques. 15.7% ie 453 samples were found to contaminate with *Salmonella spp* of all isolates, 199 were characteristics as

S. enteritidis (43.9%) 112 isolates as *Salmonella hador* (24.7%) 78 isolates as *S. typhimurium* (17.2%) and 64 isolates belonged to other *Salmonella spp.*

Berchier et al (1987) reported that the presence of Salmonella at slaughter time in a poultry processing plant, industries water, scaled tank water, pre-chilled water, defeathered carcass, finished carcass, poultry meal and feces of living birds were investigated. *Salmonella spp.* were not isolated from industrial water but they were isolated from all other material of 9 serotypes detected *S. typhimurium* was predominant (Prasai, 2000).

Szazades et al 1996 investigated the evaluation of *Salmonella spp.* in a slaughter house laboratory during 17 years. During the last 17 years (1978 to 1994), 3261 Salmonella strains were isolated by an enrichment method from 84,349 samples originated from the slaughter house and from the territory of the country. 3.87% of the samples proved to be positive. The isolated *Salmonella spp*. belonged into 52 serotypes. Positive Salmonella samples were classified into the following 8 sample groups (1): *Salmonella spp*. were found in 0.91% in the samples of the complementary bacteriological examination. In spite of the lower incidence, the food safety importance of this sample group is constant. (2): phase investigation of swab samples was positive in 9.83% of the cases. Since, 1989 the sampling has been carried out according to the HACCP (Hazard Analysis and Critical Control Point) system. Higher number of positive samples could be traced back to the deliberate exploration of the possible places of Salmonella incidence, (3): important role has been attached to the examination of frying savage in case of clearing up the hygienic situation in a production unit, similar to the phase investigation. (4): results of the mass of products preserved by maturation, drying and party by starter cultures (sausages, salami, and other “dry products”) and (5): finished products indicate the changes in the incidence of *Salmonella spp.* during processing of products. Inspite of the 11.92% incidences of *Salmonella spp.* in the mass samples, that of finished products was 2.19%. (6): of the 19,63. heat treated meat products only two samples (0.01%) contained *Salmonella spp.* which indicates a high level product safety because an appropriate heat treatment means practically an insuperable obstacle for the Salmonella. (7): 15.93% *Salmonella spp.*

contamination of poultry slaughter house samples indicates an important food hygienic problem. (8): 4.45% of the regional samples (territory of the country) were *Salmonella spp* positive of them. “Flamed sausage” examined in the previous years, was an important group, its *Salmonella spp.* contamination was 8.78% indicating the important food safety problems of this product. In the samples, most frequently the incidence of *Salmonella infantis* (17.30%), *Salmonella derby* (13.46%), *Salmonella typhimurium* (9.69%), as well as *Salmonella anatum* (6.16%), *Salmonella Heidelberg* (5.61%), *Salmonella agona* (4.88%), *Salmonella cholerasesuis* (4.63%), *Salmonella enteritidis* (3.74%), *Salmonella virchow* (3.71%), *Salmonella hadar* (3.34%), and *Salmonella bredeney* (3.13%) were observed.

Bok et al, (1986) studied on the incidence of food borne pathogens on retail broilers. They found that out of total 718 isolates 158 isolates were identified as potential human pathogens. Salmonella was the major pathogenic isolate (86%) of total pathogens, 136 isolates.

Cross contamination of food is one of the 10 main factors that contribute to food borne illness. People handling both cooked and raw foods can transfer microorganisms from the raw to cooked product. With no further treatment, this is a potential health hazard. The housewife who cuts up raw vegetables for salad may transfer *Salmonella spp.* from the raw chicken to the raw vegetables. The meat department in a retail store may use the same knife and block to cut fish, cold meat, chicken, beef and other types of food. It is evident that a potential health hazard can result (Banwart, 1987).

Tellez et al, (2001) reported about *Salmonella enteritidis* colonization in the intestinal tract of chicken causing food borne illness in humans and reduction of *S. enteritidis* colonization in the intestinal tract of chicken can reduce potential carcass contamination during slaughter.

Sansoni L. et al (2001) isolated *Salmonella spp.* from human and environment sources in province of Verona in a 2-year study i.e. between 1996-1997. The data collected revealed the widespread presence of *Salmonella spp.* in water sources. They also found the putative role of water sources in human infection

in the province of Verona confirmed by the fact that main serotype in circulation both in river and in man are same. Findings also showed high isolation in coinciding in both water and human cases within summer autumn period.

Morgan-Jones (1980) reported the occurrence of Salmonella during the rearing of broiler birds. They found that Salmonella were not isolated from empty cleaned and fumigated houses and only on one occasion from feed. Salmonella were isolated from the environment of the chicks and spasmodically from litter water troughs and dust. They concluded that water in water troughs rather the food, appeared to be the major oral route of infection or re-infection of birds during rearing (Karki, 1995).

Salmonellosis infections were 55.1% of the reported food borne disease cases reported from 1993 to 1996 in Korea (Bajk and Roh, 1998).

Of the reported food borne outbreaks in Europe caused by an identical agent, more than one third were confirmed to be caused by *S. enteritidis*. Food associated with *S. enteritidis* outbreaks include egg and egg products (68.2%), cake and ice creams (8%) and poultry and poultry products (3%). Other vehicles include meat and meat products (4%), mixed foods (4%), fish and shell fish (2%), and milk and milk products (3%). In *S. typhimurium* outbreaks, eggs products (39%) meat and meat products (10%) were reported as the vehicles of infection. A large no of other Salmonella serotypes were also involved in an outbreak in Europe, but specific serotypes were not reported (WHO, 2001).

A total of 825 samples of retail raw meats (chicken, turkey, pork and beef) were examined for the presence of *E. coli* and *Salmonella* serovars. The samples were randomly obtained from 59 stores of four supermarkets in the Washington D.C. from June 1999 to July 2000. Of the 212 chicken samples, 82 (38.7%) yielded *E. coli*, while 19% of the beef samples, 16.3% of the pork samples and 11.9% of the turkey samples were positive for E. coli. Only 25 (3%) of the retail meat samples tested were positive for Salmonella (Zhao et al, 2001).

3.9 SALMONELLOSIS IN NATIONAL PERSPECTIVES

In Nepal, Salmonellosis has caused a burning problem for poultry raiser as well as for consumers. More than 2,200 serovar of Salmonella has been identified so far all over the world and few of them cause disease in poultry e.g. *S. pullorum, S. gallinarum, S. enteritidis, S. typhimurium, S. typhi, S. arizone, S. anatis, & S. Virchow*, and some cause food poisoning in human eg. *S.enteritidis, S. typhimurium, S. Virchow* . Salmonellosis, a zoonotic and egg born disease has great importance as most of the chickens are distributed from recent established hatcheries or brought from neighbouring countries which play significant role in disease transmission in the country. (Prasai 2000).

Joshi (1991) studied the current practices of livestock slaughtering and meat marketing in Kathmandu, Lalitpur and Bhaktapur. He reported that total buffalo slaughtering places of kathmandu, Lalitpur and Bhaktapur are 81, 22 and 20 respectively and total poultry slaughtering places of kathmandu, Lalitpur and Bhaktapur are 107, 30 and 5 respectively. He also reported that total number of buffalo meat shops in Kathmandu, Lalitpur and Bhaktapur are 181, 39 and 48 respectively and total poultry meat shops in Kathmandu, Lalitpur and Bhaktapur are 115, 30 and 13 respectively. In this study, he also reported that the average number of buffaloes slaughtered per day in Kathmandu, Lalitpur and Bhaktapur are 324, 88 and 80 respectively and average number of poultry slaughtered per day in Kathmandu, Lalitpur and Bhaktapur are 1070, 300 and 150 respectively. In this study, total 111 butchers were interviewed about their knowledge of meat borne diseases. On an average, 35.1% had the knowledge of meat borne and 64.9% had no knowledge about it.

Different samples of the poultry were studied and bacterial analysis was done. *Salmonella spp.* was found to be the most predominant organism followed by *E. coli, Staphylococcus aureus* and *Streptococcus spp* (annual report of NARC in the fiscal year 2042/2043).

According to the annual report of NARC in the fiscal year 2044/2045, *E. coli* was found to be the most predominant organisms followed by *enterobacter*,

Pasteurella spp and *Citrobacter spp* from the different samples of the goats. Besides *Pasteurella* and *enterobacter spp* were reported to be the predominated from the cow and buffalo samples. *Klebsiella, Streptococcus and Enterobacter spp* were found from the samples of 200 lab animals. In the same year 652 poultry samples were analyzed for the bacterial analysis. From the study it was reported that Salmonella (31%) was found to be the most predominant organisms followed by *E. coli* (30%), *Staphylococcus* (5%), and *enterobacter* (0.6%), serological test for *Salmonella spp* confirmed that 0.68% were positive for the *pullorum spp*.

Two hundred samples were collected from the apparently healthy and sick birds. Out of hundreds healthy birds, only two were sero positive for Salmonella infection. Both sero positive birds were slaughtered for the collection of samples for *Salmonella spp* culture. Two isolates of *S. pullorum* were found in the culture of livers collected from both sero positive cases. On the other hand, out of one hundred sick birds ten were positive for *Salmonella gallinarum*. (Annual report of Nepal Agricultural Research Council, NARC, fiscal Year 2053/2054).

The problem of Salmonellosis is important not only for chicken raises but also for public health as far as human cases of enteric fever are concerned. Enteric fever is an endemic disease in Kathmandu Valley. The disease flares upto an epidemic proportional from time to time (Malla, FB and Shakya , GM, 1984).

A survey was carried out between June and November 1990 to find out the isolation of Salmonella in dairy product sold in street of Kathmandu Valley. A total of 200 samples of various dairy products were collected Salmonella was isolated from 3% samples. Variety wise 12.5% in ice-cream and 2.1% in sweet item (Joshi DD et al, 1990).

A survey work carried out to assess the occurrence of micro organism in cheese collected from retail shop in Kathmandu city market. Among other bacteria *Salmonella typhi* was found to be present in 11% of total samples (Tuladhar, E and Sharma, A.P.,1997).

In annual bulletin of CFRL 1997/98 Salmonella was reported from the raw

frozen pork samples in Prasuma Factory during the processing of momo (momo is an item of mined meat). *Salmonella spp* was also detected in raw pieces, on surface meat and in mixing dough. In the same report Salmonella was also reported from 3 samples of raw momo out of 29 samples.

A general survey of hygienic quality of ethnic Newari meat varieties was conducted out. Samples were collected from different restaurant of Kathmandu valley. Coliforms were present in 55% samples. In a variety homed 'sekuwa' (an under cooked meat variety) *Salmonella spp* was detected in 3% samples, in 'kachila(raw meat variety) this percentage was 5% while in momo it was 7% (Shrestha,H.et .al 1999)

Manandhar (2000) recorded the sero-isolation of *Salmonella spp*, using ELISA(enzyme linked immunosorbent assay) in human's and chicken in related areas of Philippines, total 128 human and 128 chicken serum samples were collected. Sero isolation ratio was found significantly higher (p<_0.05) for *S. enteritidis* in> 40 year age group and for *S. abortusequi* in early age groups. There was no difference in sero isolation or exposed and unexposed groups for *S. enteritidis*.

According to annual report of NARC (2000/2001) Pradhan A conducted a study about calf diarrhea with particular reference to *E-coli* infection 30fecal samples were collected form calves affected with diarrhea in Kandaghari Gothatar .*E-coli* in 66.60% cases and *Streptococcus spp*, in 10% cases .*bacillus spp*. In 10% cases and Salmonella in 6.6% cases were isolated.

In a study conducted at CVL and ADCC(central veterinary laboratory and animal disease control section) out of 268 post-mortem chicken samples cultured 259 samples were found to be infected *E-coli* was made predominant isolates (73.13%), followed by *Salmonella spp*, *Streptococcus spp* (7.08%) and *S, aureus* (2.61%). Among *Salmonella spp, S. gallinarum* and *S. pullorlun* were identified, 95.34% of *Salmonella spp* were sensitive to chloramphenicol while Ampicillin and cloxacillin were found to be least effective drugs (Pandey, 2003).

3.10 ANTIBIOTIC SUSCEPTIBILITY TESTING

Antimicrobial susceptibility testing is an in vitro method for estimating the activity of drugs which will assist clinician in selecting an antimicrobial drug effective in inhibiting the growth of an infecting microorganisms in vivo. The primary goal of antimicrobial susceptibility testing is to determine whether the bacterial etiology of concern is capable of expressing resistance to the antimicrobial agents that are posterities choices as therapeutic agents for managing the infection.

World Health organization (WHO) recommended modified Kirby-Bauer disk diffusion technique is used by most laboratories to lest routinely for antimicrobial susceptibility. Using this lest, antimicrobial resistance is detected by allowing the antibiotics to diffuse from a point source, commonly in the form of an impregnated filter paper discs, into an agar medium that has been seeded with the test organism, visible growth of bacteria occurs on the surface of the agar where the concentration of antibiotic has fallen below its inhibitory level for the test strain. Following incubation the diameter of the zone of inhibition around each disc measured in millimeters. (Collee et al,1996).

3.11 BACTERIAL RESISTANCE TO ANTIBIOTICS IN GLOBAL SCENARIO:

The study on the antibiotic resistance of 3600 strains of *Salmonella spp* of animal origin . 98 sero types were identified and the most common 3 sero types were *S. saintpaul, Salmonella typhimurium* and *S.dublin.* The resistance patterns to various antibiotics Ampicillin, Streptomycin, kanamycin, Neomycin, Framycetin, Gentamycin Chloramphenicol, Tetracycline and Colistin were studied. Out of 3624strains, 1025 were found resistant to one or more antibiotics 509 were resistant to one anitibiotic,218 to two, 117 to three, 92 four, 59 to five, 23 to six, 6 to seven and 1 to eight. Resistant to Tetracycline was the most common (16% strains were resistant). The incidence of multiple resistances appeared to be increasing. (Gledel et al 1977).

The study was conducted to estimate the anti microbial resistance of Salmonella isolated from raw chicken between November 2003 to April 2004. A total of

120 chicken carcasses were collected from 36 different sale points and examined for the presence of Salmonella .Salmonella was isolated from 75 (62.5%) of the examined samples. Out of 90 isolates obtained, 21 seotypes were identified, the most prevalent being *S. kentuchy* (13%) *S. muenster* (133%), *S. bvan*caster (8.8%), *S. enteritidis* (6.6%) and *S. hadar* (6.6%) . All Salmonella were tested against 16 selected antimicrobial agents. Out of 71 resistant isolates, 33 (46.5%) showed multiple resistance to five or more antibiotics. Reistance to Ampicilin, Trimethoprim, Trimethoprim –sulphamethoxaZole, Tetracycline and sulphonamides were most frequent. (Rianatou et al, 2006).

Of 200 ground meat samples purchased in the Washington ,D.C area, 41(20%) contained Salmonella , with a total of 13 serotypes .84% of the isolated were resistant to at least one antibiotic, and 53% were resistant to at least three antibiotics.16% of the isolated were resistant to ceftriaxone, the drug of choice for treating Salmonellosis in children. Bacteriophage typing identified four isolate of *Salmonella enterica* serotype t*yphimurium* definitive type 104(DT104),one of DT104b, and two of DT208.Five isolates of *S.enterica* serotype *agona* had resistance to 9 antibiotics, and the two isolate of serotype *typhimurium* DT208 were resistant to 12 antibiotics .(White et al,2001)

In a study conducted between 1994-1996, the relative frequency of Salmonella strains from hospitalized patients in Southern Israel was found to be changed. The most prevalent isolate was found to be *S. typhimurium* (DT104) and *S. agona* followed by *S. enteritidis*. In addition to the R-type ACT (i.e Resistance to Ampicillin ,Chloramphenicol, and Tetracycline) the *S. typhimurium* (DT104) possesses a chromosomally encoded resistance to Quinolone drug, Nalidixic acid .In 1996,27% of the *S.typhimurium,* DT104 were of R-type ACTN. *S. enteritidis* exhibited to over 95% susceptibility to at least 8 of the most commonly used antibiotic but non of the isolates were resistant to Quinolone and Fluroquinolone .(Metzer. et al,1998)

Arora etal (1987) Studied on detection of Upper respiratory tract bacterial carriers in poultry. On bacteriological examination they isolated *E.coli, Klebsiella pneumoniae, Citrobacter intermedius, Citrobacter freundii, Enterobacter cloacae ,Proteus mirabilis, Pseudomonas aeruginosa and*

Acenetobacter spp. They found that the isolates were sensitive to gentamycin, kanamycin ,chloramphenical, furadantin and ampicillin on in vitro sensitivity test. However, *Pseudomonas aeruginosa* was resistant to the antibiotics except for gentamycin and chemotherapeutic agents used .

3.11.1 Mechanism of Antimicrobial Resistance

It is important to note that resistance genes and mechanism existed long before antibiotics were used. For example, antibiotic resistance bacteria have been isolated from deep within glaciers in Canada's high Aretic regions, estimated at 2000 years old. The original source of resistant genes was probably soil microorganisms, some of which can produce antibiotics in active form and thus environmental bacteria are exposed.

There are many different mechanism by which microorganism might exhibit resistance to drugs (Brooks et al, 2004).

1. Microorganism produce enzymes that destroy the active drugs. Examples: Staphylococci resistant to penicillin G produce a beta- lactamase that destroys the active drug .Other beta- lactamares are produced by gram negative rods.

2. Microorganism develop an altered structural target for the drug examples: Erythromycin-_resistant organism have an altered receptor on the 50's subunit of the ribosome, resulting from methylation of a 23's ribosomal RNA. Resistance to some penicillins and Cephalosporins may be a function of the loss or alteration of penicillin binding proteins (PBPs) .

3. Microorganisms developed and altered metabolic pathway that by passes the reaction inhibited by the drug. Example: Some Sulphonamide – resistant bacteria do not require extra cellular para – amino banzoic acid (PABA) but, like mammalian, cells can utilize performed folic acid.

4. Micro Organism change their permeability to the drug. example: Tetracycline accumulate in susceptible bacteria but not in resistant bacteria. Streptococci have a natural permeability barrier to aminoglycosides.

5. Micro-Organisms developed and altered enzyme that can still perform it's metabolic function but is much less affected by the drug. Example: in Trimethoprim – resistant bacteria, the dihydrofolic acid reductase is inhibited for less efficiently than in Trimethoprim – susceptible bacteria.

Multiple drug resistant :

Multi drug resistant *Salmonella gallinarum* isolated from the broilers were studied and was considered as a major problem in the poultry birds in Buenos Aires (Mairorini et al, 1993). White et al. (1997) reported antimicrobial resistance of *Salmonella gallinarun* isolates in France. During the multi drug profile analysis and antibiotics susceptibility testing of endemic strains of multi resistant Salmonella, it was confirmed that plasmid was resistance of the isolates in Great Britain (Evans and Davies 1996)

The multi Drug resistant Salmonella was a potential case of infection in poultry birds in India (Chaudhary 1996) The isolates of multi drug resistant Salmonella strains were more prevalent in Tanzania to commercial chicken, than local chicken (Gyles,1989) Conjugative plasmid mediated multiple drug resistant Salmonella infections is an emerging problem in Bangladesh (Rahman and Albert 1998)

R. Factors:

One of the earliest examples was in Japan in 1959. Previously sensitive *E. Coli* become resistant to multiple antibiotics through acquisition of a conjugative plasmid (R-factor) from resistant Salmonella and Shigella isolates. A number of R-factors have now been characterized including RP4, encoding resistant to ampicillin, kanamycin, tetracycline and neomycin, found in *P. aeruginosa* and other gram negative bacteria, R1, encoding resistance to ampicillin, kanamycin, sulphonamides, chloramphenicol and streptomycin, found in gram negative bacteria and PSH6, encoding resistance to gentamicin , trimethoprim and kanamycin, found in *S. aureus* .

Mobile Gene Cassettes and Integrons:

Many gram negative resistance genes are located in gene cassettes one or more of these cassettes can be integrated into a specific position on the chromosome termed as integron. Thus, integrons are genetic elements that recognize and capture multiple mobile gene cassettes (Smith, 2004). Although integrons by themselves are not motile, due to their presence in plasmids and transposons, they can be transferred horizontally.

Chromosomal Multiple Antibiotic Resistance (Mar) Locus:

The multiple-antibiotic resistance (mar) locus was first described in Escherichia coil by Stuart levy and collogues at TUFts University and has since been recognized in other enteric bacteria. The locus consists of two divergently transcribed units, marc, and mar RAB. (Smith, 2004)

3.12 THE RATIONAL USE OF ANTIBIOTICS IN POULTRY PRODUCTION AND THE PROBLEM OF DRUG RESISTANCE

The increasing use of antibiotics in few decades in the poultry production for the prevention and treatment of infectious diseases and for growth promotion has provided an intense selective pressure in favor of genus coding for drug resistance and consequently resistance is now common in many bacterial species (Dutta, 1984).

A large amount of drugs are being used globally annually to secure sufficient quantities of food to feed fast growing population. The use of antibiotics helped for the fast growth of food animal industry worldwide. Thus, the use of anitbiotics is considered essential for the efficient production of foods of animal origin (WHO, 1985).

It is generally accepted that certain antibiotics such as penicillin, tetracycline, virginiamycin, bambermycin, avoparcin, bacitracin, etc. are capable of stimulating the growth rate or keep efficiency or both. The indiscriminate use, however, may lead to the emergence of resistance and residues (Huber, 1971)

and alteration of the intestinal micro-flora of exposed individuals (WHO, 1963).

In the United States, many antibiotics are incorporated in food at low levels to promote growth and protect against diseases. (Tanner, 1993)

In a study Speele (1984) stated that chicken increased up to 12.2% in weight gain and 7.1% feed efficiency upto 4 weeks of age.

In the United States, more than 40% of all antibiotics produced are used for animals (Stalheim, 1987). Correspondingly, more than 80% of the meat and eggs consumed by the public have been produced with the aid of modicated feeds (Marteniuk, et al. 1988)

Mixing of 20 ppm antibiotics with feed does not give rise to drug residues in food. However, fooding 100-200 ppm antibiotics may result in reside in food and development of antibiotic resistant organism (WHO, 1969).

CHAPTER- IV

4. MATERIALS AND METHODS

The present study was conducted at Central Veterinary Laboratory (CVL), Tripureshwor, Kathmandu. The study was carried out from Ashoj to Mangshir, 2065 (September 17 to December 15, 2008). During this period of three months, altogether 250 meat samples including chicken (125), goat (75) and buffallo (50) were collected from different localities of Kathmandu valley. These samples were collected and processed according to the standard laboratory methods (OIE, 2004).

4.1 MATERIALS

All the materials required for present work are listed in the appendix I.

4.2 METHODOLOGY

4.2.1 Sample Size and Site

The sample size (n) of the study was 250, which included chicken (125), goat (75) and buffalo (50) randomly. From Kathmandu city, a total of 118 meat samples were processed including 63 chickens, 30 goat and 25 buffalo meat. Similarly from Lalitpur local meat shops, 75 meat samples were processed including 37 chicken, 25 goat and 13 buffalo meat and from Bhaktapur local meat shops, 57 samples were processed including 25 chicken, 20 goat and 12 buffalo meat.

4.2.2 Sample Collection

The different meat samples were collected in sterile plastic bags and transported to the lab. in an ice-cold box within 2 hours of collection. About 25 grams of each samples were collected from different meat shops.

4.2.3 Sample Processing

4.2.3.1 Mincing

Before proceeding twenty five grams of each meat samples (chicken, goat and buffalo) were minced separately. It was done using a sterile sharp scissor and sterile blade. Bacteriological culture was performed first followed by the routine microscopic observation.

4.2.3.2 Pre-Enrichment

5 gm of the minced meat sample was placed in MC-Cartney bottle containing 25 ml of buffered peptone water for pre-enrichment and those bottles containing samples were incubated at 37ºc for 18 hours.

4.2.3.3 Enrichment

One milliter of each sample from pre-enriched vial was added to the test tube containing 10 ml of selenite F Broth and the tubes were incubated at 42ºC for 18 hours.

4.2.4 Primary Culture on Selective Media

One loopful of sample from Selenite F. Broth was taken and streaked on XLD agar media and the cultured plates were incubated at 42ºc for 24 hours.

4.2.5 Colony Characteristics

After an overnight incubation, XLD plates were examined for the presence of *Salmonella spp* like colonies, suspected colonies were marked and sub-cultured in Mac Conkey agar plates.

4.2.6 Culture on Mac Conkey Agar and Nutrient Agar Plates

From XLD media plates, black colonies or red colonies with black center or colonies with pink color were selected without touching the nearby colonies and streaked on MaC conkey agar plates to obtain the isolated colony. The isolated colonies were sub-cultured in nutrient agar plates for performing various bio-chemical tests and antibiotic sensitivity tests.

4.2.7 Study of Morphology

Gram's staining of the pure isolate from NA was done. The protocol of Gram's staining is included in the Appendix III.

4.2.8 Agglutination Test with Poly 'O' Sera

Pure colonies from the NA plates were transferred to a glass slide containing 1-2 drops of poly 'O' sera and the colony was emulsified properly and observed for agglutination. If agglutination occurred, the bacteria were confirmed to be *Salmonella spp*.

4.2.9 Biochemical Test

The biochemical test such as catalase test, oxidase test, indole test, methyl red test, Voges proskauer test, citrate utilization test, triple sugar iron (TSI) test, urease test, motility test (hanging drop method), sulphide production test and gas production test were carried out by standard method.(Cheesbrough, 2000).

4.2.10 Sugar Utilization Tests

After performing various biochemical tests, those isolates which were identified as *Salmonella spp.* were subjected to sugar utilization tests. For this, the different species of Salmonella were identified by inoculating the pure culture from NA into 1% sugar solutions prepared in peptone water and incubated at 37ºC for 24 hours. The utilization of sugar was indicated by the change in colour of sugar solution into pink after the addition of Andrade's indicator.

Table 2: Sugar Utilization Tests of some of species of Salmonella

Tests performed	1	2	3	4	5	6	7	8
Motility	+	-	+	+	+	+	+	+
Carbohydrates: Acid from Adonitol	-	-	-	-	-	-	-	-
Arabinose	-	+	+	-	+	+	+	+
Dulcitol	(d)	-	+	(d)	+	+	-	+
Meso-Inositol	-	-	-	-	(d)	-	-	-
Lactose	-	-	-	-	-	-	+	-
Maltose	+	(d)	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+
Rhamnose	-	+	(+)	+	+	+	+	+
Salicin	-	-	-	-	-	-	-	+
Sucrose	-	-	-	-	-	-	-	-
Trehalose	+	+	+	-	+	+	+	+
Xylose	(d)	+	+	+	+	+	+	+

Note:

1. *S. typhi*
2. *S. pullorum*
3. *S. gallinarum*
4. *S. cholerasuis*
5. *S. Kauffmannii*
6. *S. salamae*
7. *S. arizonae*
8. *S. houtenae*

The composition and preparation of biochemical media and reagents used in the biochemical test are mentioned in the appendix II. The procedure for performing bio chemical tests are mentioned in Appendix IV.

4.2.11 Antibiotic Susceptibility Testing

The antimicrobial susceptibility testing of the isolates towards various antimicrobial discs was done by modified kirby-Bauer disk diffusion method as recommended by national committee for clinical laboratory standards (NCCLS) using diagnostic sensitivity testing (DST) media.

- DST media was prepared and sterilized as instructed by the manufacturer.

- The pH of the medium 7.2- 7.4 and the depth of the medium at 4 mm (about 25 ml per plate) were maintained in petri dish.
- Using a sterile inoculating wire loop, a single isolated colony of which the sensitivity pattern is to be determined was touched and inoculated into nutrient broth tube and was incubated at 37ºc for 24 hrs.

After incubation, the turbidity of the suspension was matched with the turbidity standard of the MC-farland tube number 0.5

Using a sterile swab, a plate of DST was inoculated with the bacterial suspension using carpet culture technique. The plate was left for about 5 minutes to let the agar surface dry.

Using sterile forceps, appropriate antimicrobial discs (6 mm diameter) was placed, evenly distributed on the inoculated plates, not more than 6 discs were placed on a 90 mm diameter petri dish.

After overnight incubation, the plates were examined to ensure confluent growth and the diameter of each zone of inhibition in mm was measured and compared with standardized zone interpretative chart provided by the company.

The preparation and composition of diagnostic sensitivity testing (DST) media are shown in appendix I and II. The detailed about antibiotic discs used and its interpretive chart are mentioned in the appendix I and V.

4.2.12 Serotyping

The isolates of Salmonella that were motile and which produced black colony on XLD media were sereotype at WARUN institute for the identification of the 'O' antigen group.

4.2.13 Quality Control

To obtain reliable microbiological result, it is necessary to maintain quality control. Quality of each test was maintained by using standard procedures through out the study period from sample collection to sample transportation, handling, media preparation. The quality of each agar plates prepared was

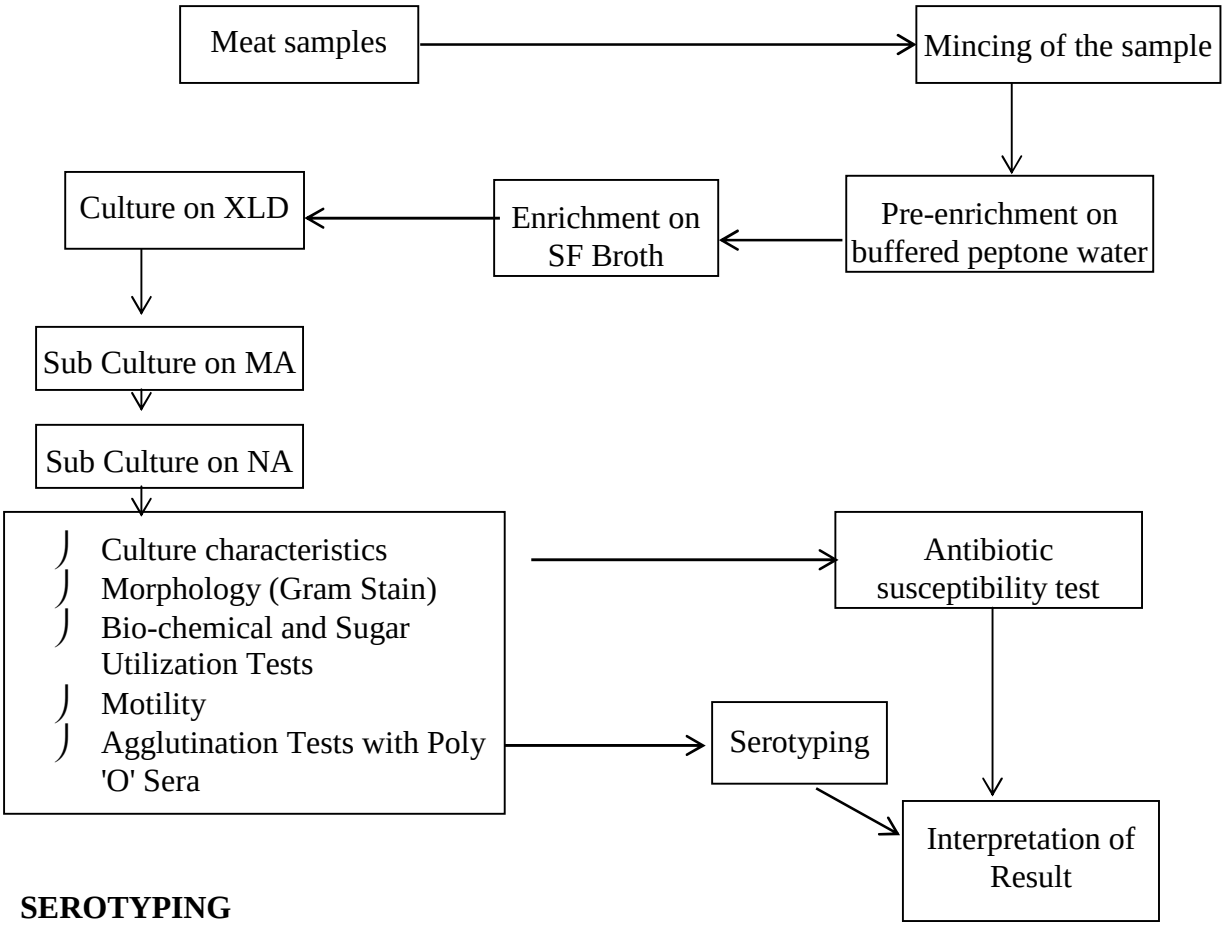
tested by incubating one plate of each lot on the incubator. Control strains of *S. pullorum* and *S. gallinarum* available at CVL were used as positive control for the standardization of the kirby-Bauer test. Quality of sensitivity tests was maintained by maintaining the thickness of DST at 4 mm and the pH at 7.2-7.4. Similarly, antibiotics discs containing the correct amount as indicated were used. Strict aseptic conditions were maintained while carrying out all the procedures.

4.2.14 Data Analysis

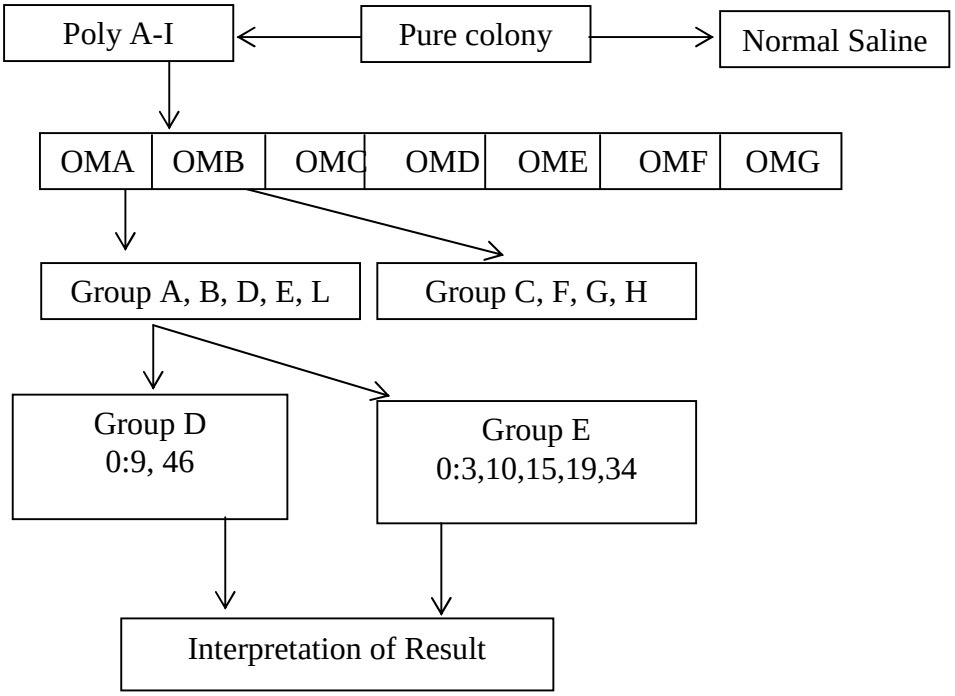
Chi-square test was used to determine significant of isolation of Salmonella from meat sample and presence of different types of *Salmonella species* in the meat sample.(Appendix VI) .

Data Analysis Software: Statistical Programme for Social Sciences (SPSS); Microsoft Excel

STUDY DESIGN



SEROTYPING



CHAPTER – V

5. RESULTS

5.1 COLONY CHARACTERISTICS OF ISOLATES ON XLD

Salmonella spp isolated from 23 meat samples showed the following characteristics colony on XLD media.

Table 3: Colony characteristics of *Salmonella spp* on XLD media.

No. of isolates	Size (mm)	Color	Margin	Elevation	Consistency
17	1-2	Red	Entire	Raised	Soft
6	2-3	Black	Entire	Raised	Soft

5.2 COLONY CHARACTERISTICS OF ISOLATES ON NA

The black and red colony of *Salmonella spp* formed on XLD media when sub cultured on NA showed the following colony characteristics:

Table 4: Colony characteristics of *Salmonella spp*. on NA media

No. of isolates	Size (mm)	Color	Margin	Elevation	Consistency
17	1-2	Creamy white	Entire	Raised	Soft
6	2-3	Creamy white	Entire	Raised	Soft

5.3 MORPHOLOGY

All isolates from NA agar plate were gram negative short rods

5.4 MOTILITY (HANGING DROP METHOD)

Seventeen isolates from NA agar plate having colony size 1-2 mm in diameter were non-motile and rests were motile.

5.5 AGGLUTINATION TESTS WITH POLY 'O' SERA

All the isolates showed agglutination when tested with poly 'O' sera.

5.6 BIOCHEMICAL TESTS

5.6.1 Catalase and Oxidase Tests

All isolates were catalase positive and oxidase negative.

5.6.2 Other Biochemical Tests

Table 5: Biochemical tests performed for the identification of isolates

Organism code	Indole	MR	VP	Citrate	Urease	TSI	H_2S	Motility
A	-	+	-	+	-	Alk/A, no gas	+++	Motile
B	-	+	-	-	-	Alk/ A, no gas	+	Non-motile

Note: organism code A includes 6 isolates that produced black colony on XLD media and B includes 17 isolates that produced red colony on XLD + (Trace H_2S production), +++ (whole media turned black due to H_2S production.

5.6.3 Sugar Fermentation Tests

Table 6: Sugar fermentation tests performed for the identification of *Salmonella* isolates

Organism code	Sugars								Identified Organism
	Ara	Dul	Lac	Man	Tre	Xyl	Ado	Ino	
A (6)	+	+	-	+	+	+	-	-	Other *Salmonella spp*.
B (9)	+	+	-	+	+	+	-	-	*S. gallinarum*
B (8)	+	-	-	+	+	+	-	-	*S. pullorum*

Note: 6 isolates included in A differs from 9 isolates included in B (9) and 8 isolates included in B (8) due to formation of black colony on XLD. 9 isolates included in B (9) differs from 8 isolate included in B (8) due to dulcitol positive.

5.7 MONTHLY ISOLATION OF *SALMONELLA SPECIES*

The present study was of three month duration from September 17 to December 15(Ashoj to Mangshir). During this period, altogether 250 meat samples were collected and processed for the isolation of *Salmonella species*. The isolation rate was highest in the month of Ashoj i.e 11.11%(8/72) (Table 7/ Fig 2).

Table 7: Monthly isolation of *Salmonella spp* in meat samples of Kathmandu Valley

Month	*Salmonella spp* positive cases	Percentage	Total sample
Ashoj	8	11.11%	72
Kartik	9	9.00%	100
Mangshir	6	7.69%	78
Total	**23**	**9.20%**	**250**

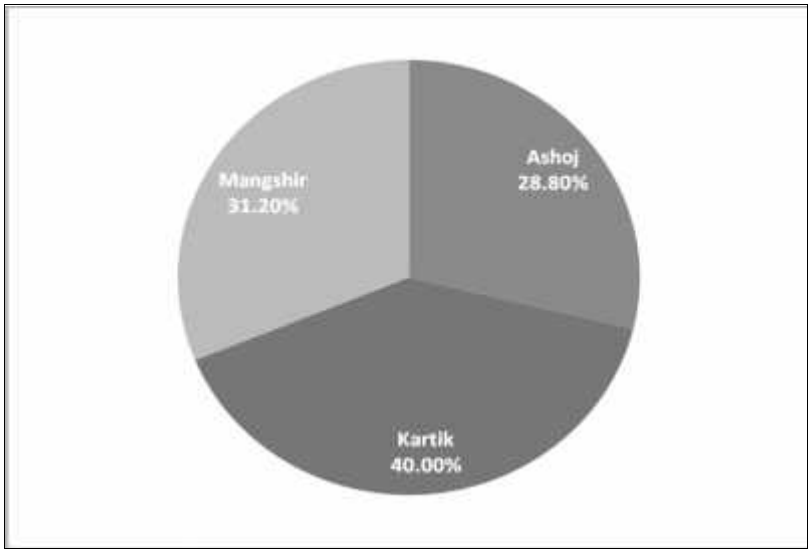


Figure 10: Total meat samples collected in different months of Kathmandu Valley

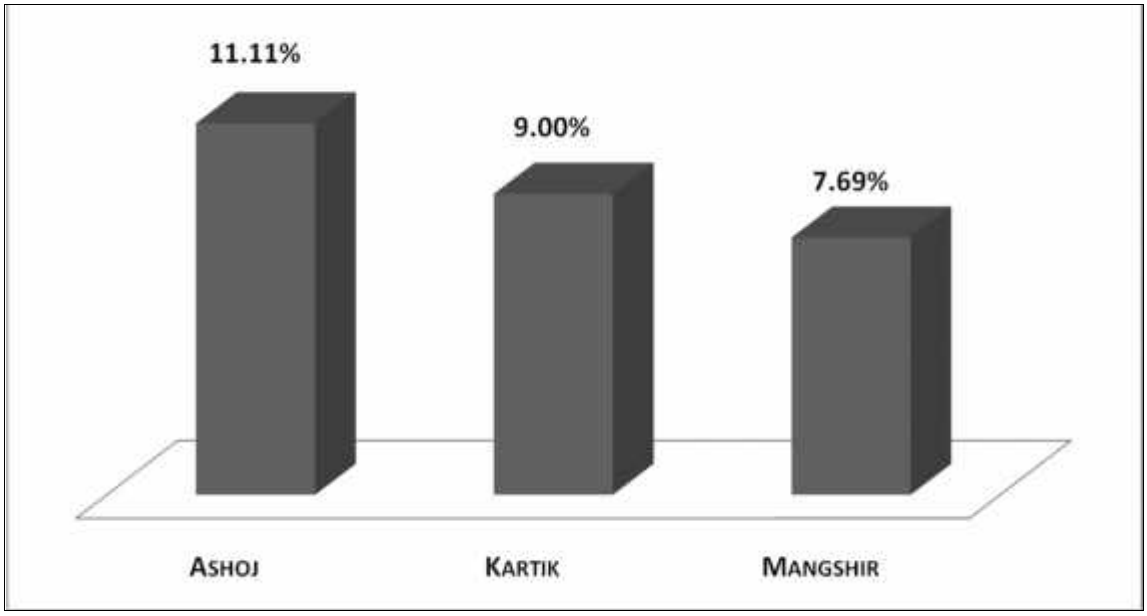


Figure 11: Monthly isolation of *Salmonella spp* in meat different samples of Kathmandu Valley

5.8 ANIMAL-WISE ISOLATION OF *SALMONELLA SPECIES*

In the study, different animal meat samples were included. Altogether 125 chicken, 75 goat and 50 buffalo meat samples were analyzed for Salmonella *spp* contamination. The result revealed that isolation of *Salmonella spp* contamination was seen highest in chicken (11.20%) followed by buffalo (8.00%) and goat (6.67%) (Table 8/fig 4)

Table 8: Animal wise isolation of *Salmonella spp* in meat sample of Kathmandu Valley

Animals	***Salmonella spp* positive cases**	**Percentage**	**Total sample**
Goat	5	6.67%	75
Buffalo	4	8.00%	50
Chicken	14	11.20%	125
Total	**23**	**9.20%**	**250**

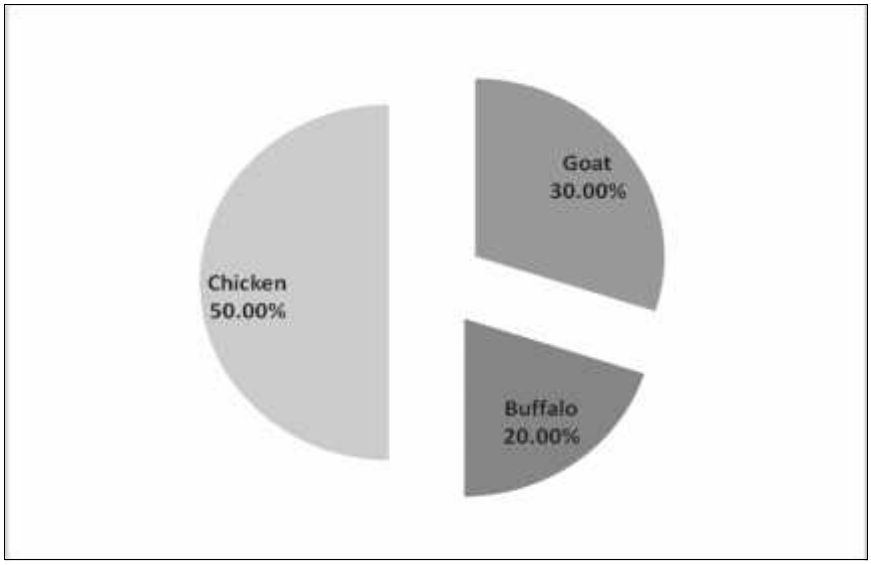


Figure 12: Total meat samples collected from different animals in Kathmandu Valley

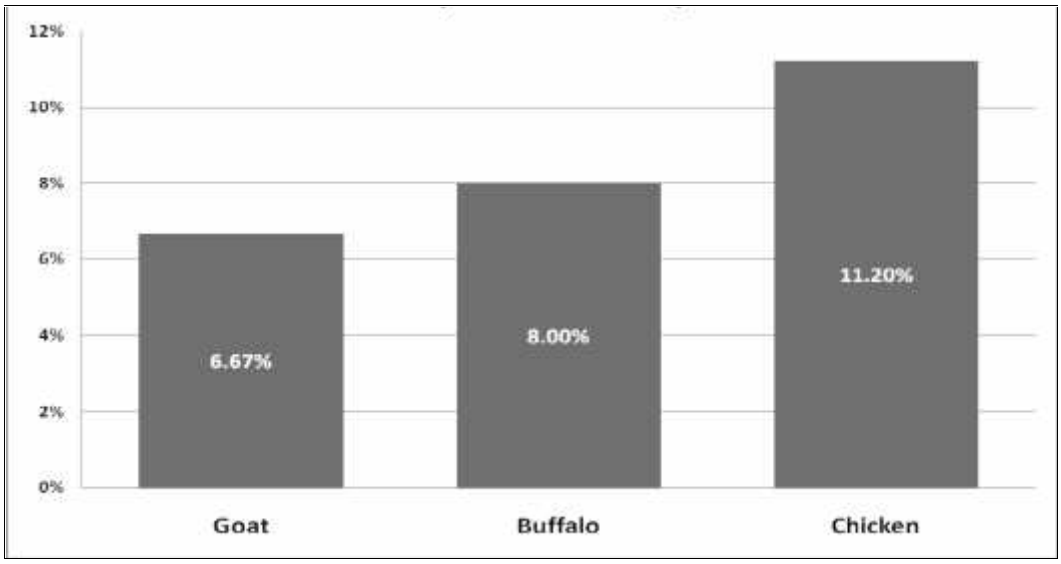


Figure 13: Animal wise isolation of *Salmonella spp* in meat samples of Kathmandu Valley

5.9: LOCATION WISE DISTRIBUTION OF *SALMONELLA SPP* IN MEAT SAMPLES

In the study, meat samples were collected from 23 different places of Kathmandu including more than 23 retail meat shops. Sampling was done randomly. Similarly, 9 different places of Latitpur and 5 different places of Bhaktapur were selected randomly for collecting chicken, goat and buffalo

meat samples. The isolation rate of Salmonella was highest in Kathmandu 11.02% than Bhaktapur 8.77% and Latitpur 6.67%.(Table 9/ Fig 6)

Table 9: Distribution of Salmonella in meat samples of different localities of Kathmandu Valley

Location	***Salmonella spp* positive cases**	**Percentage**	**Total sample**
Kathmandu	13	11.02%	118
Lalitpur	5	6.67%	75
Bhaktapur	5	8.77%	57
	23	**9.20%**	**250**

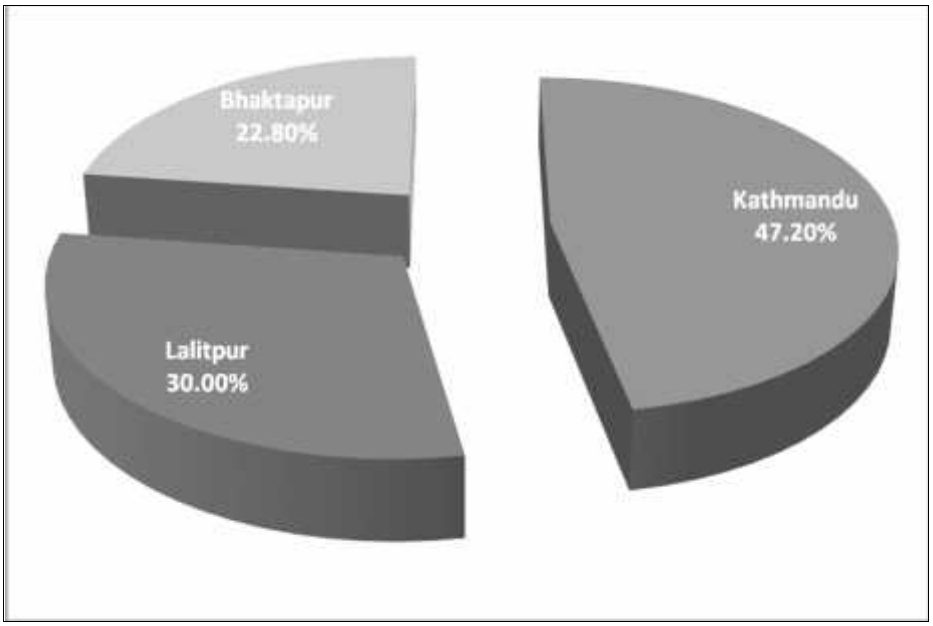


Figure 14: Total meat sample collected from different cities of Kathmandu Valley

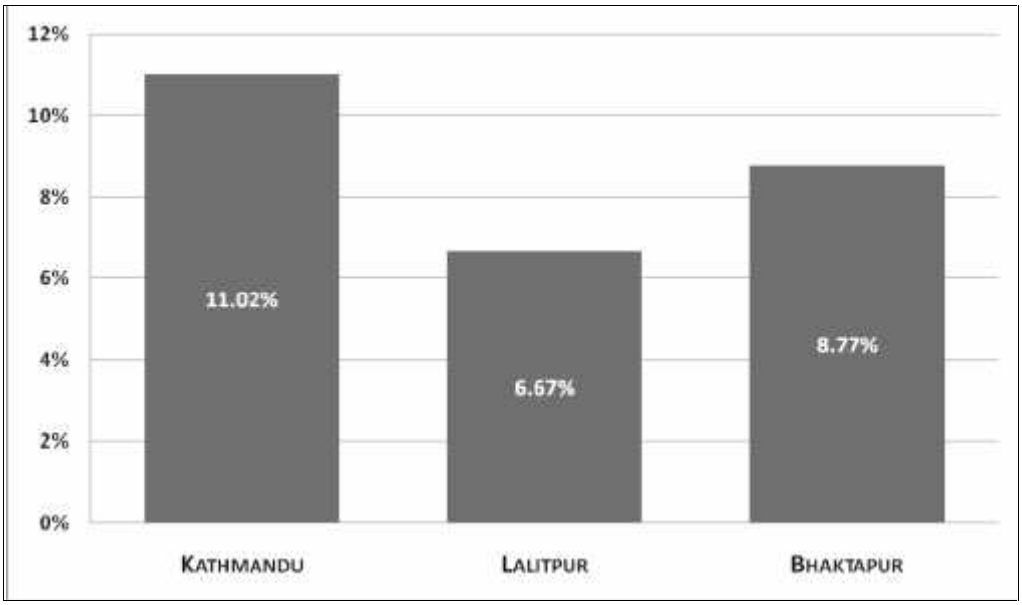


Figure 15: Location wise distribution of *Salmonella spp* in meat samples collected in Kathmandu Valley

5.10 SPECIES WISE DISTRIBUTION OF SALMONELLA

Overall the isolation rate of *Salmonella spp* was found to be 9.20% (23/250). Two different species of Salmonella were isolated and identified viz; 39.13% *Salmonella gallinarum*, 34.78% *Salmonella pullorum* and 26.09% isolate was other *Salmonella* species.(Table 10/ Fig 7and 8)

Table10: Isolation of different species of Salmonella in meat samples of Kathmandu Valley

***Salmonella spp* positive cases**		***Salmonella gallinarum***		***Salmonella pullorum***		**Other *Salmonella spp* (D,E)**	
Total +ve cases	**%**	**Total cases**	**%**	**Total cases**	**%**	**Total cases**	**%**
23	9.20	9	39.13	8	34.78	6	26.09

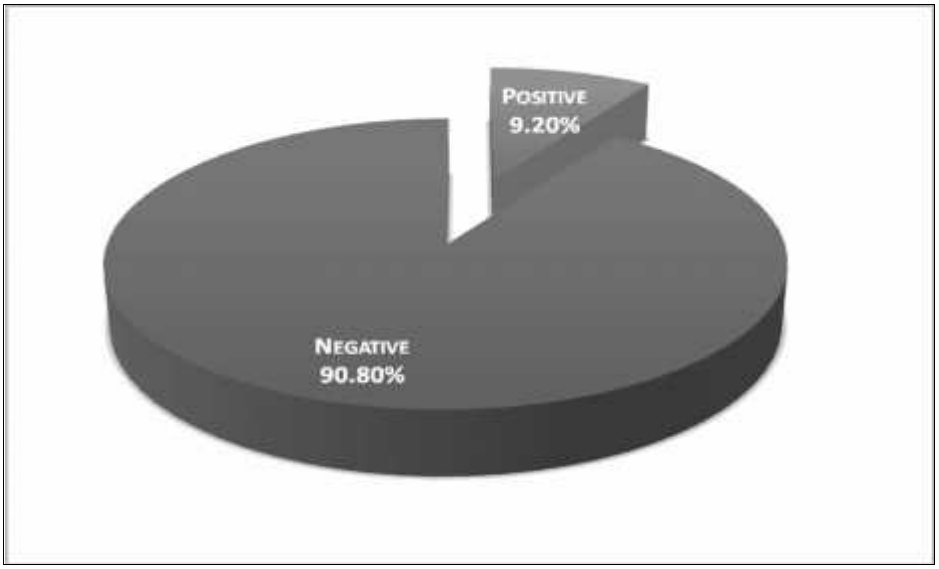


Figure 16: Total positive cases of Salmonella in meat sample of Kathmandu Valley

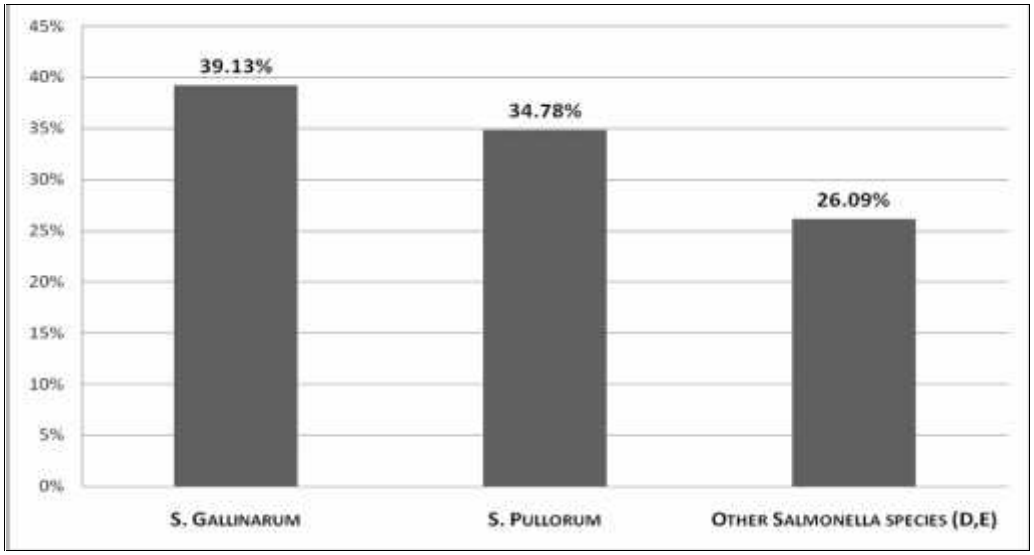


Figure 17: Isolation of different *Salmonella spp* in meat samples of Kathmandu Valley

5.11 DISTRIBUTION OF DIFFERENT SPECIES OF SALMONELLA IN DIFFERENT ANIMALS.

Among different species of Salmonella, non-zoonotic species *S.gallinarum* was found to be highest in the buffalo and goat meat sample than chicken meat samples . *S.pullorum* was found highest in chicken meat sample than goat and Buffalo meat samples. The isolation of other *Salmonella spp* was also found

highest in chicken meat samples . This study showed that the goat meat sample was not contaminated with other Salmonella (serogroup D,E).(Table 11/ Fig 9)

Table 11: Species & animal wise isolation of Salmonella in meat samples of Ktm Valley

Meat samples	*S. pullorum*		*S. gallinarum*		Other *Salmonella spp*		Sample size
	+ve cases	%	+ve cases	%	+ve cases	%	
Goat	2	2.67	3	4.00	0	0.00	75
Buffalo	1	2.00	2	4.00	1	2.00	50
Chicken	5	4.00	4	3.20	5	4.00	125
	8		**9**		**6**		**250**

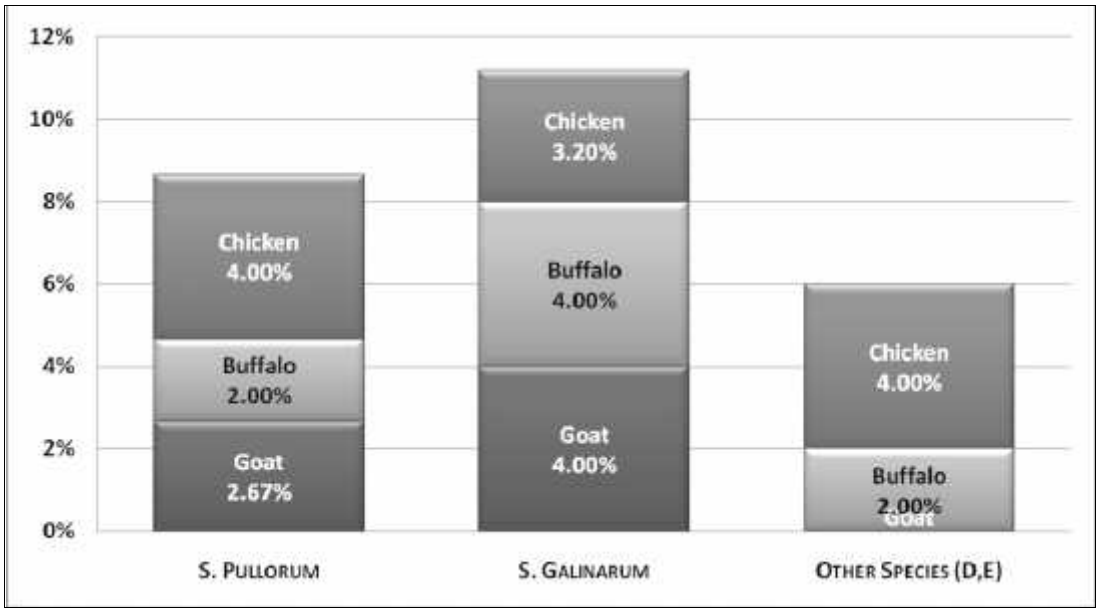


Figure 18: Species & animal wise isolation of different *Salmonella spp* in meat samples of Kathmandu Valley

5.12 ANTIBIOTIC SUSCEPTIBILITY PATTERN ON SALMONELLA ISOLATED FROM MEAT

Among the common antibiotics used against the Salmonella isolated from meat, most of them were found 100% effective to antibiotics like cephotaxime, ciprofloxacin, enrolfloxacin, oxfloxacin, chloramphenicol. Resistance to

cotrimoxazole, amoxicillin and gentamycin was seen in only one isolate. Most of the isolates were found to be resistant to nalidixic acid (19/23) and tetracycline (12/23) (Table 12).

Table 12: Antibiotic susceptibility pattern of Salmonella

S.N.	Antibiotics used	Drug potency (mcg/discs)	Total no. of isolates	Resistant	Intermediate susceptible	Susceptible
1.	Cephotaxime	30	23	0	0	23
2.	Cotrimoxazole	25	23	1	0	22
3.	Chloramphenical	30	23	0	0	23
4.	Ciprofloxacin	5	23	0	0	23
5.	Ofloxacin	5	23	0	0	23
6.	Enrolfloxacin	10	23	0	0	23
7.	Tetracycline	30	23	12	6	5
8.	Amoxycillin	10	23	1	3	19
9.	Nalidixic acid	30	23	19	1	3
10.	Gentamycin	10	23	1	0	22

5.13 ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *SALMONELLA GALLINARUM*

S. gallinarum was found to be more resistant towards nalidixic acid. Among the 10 antimicrobials tested, most of the antibiotics were 100% effective and only one isolate was resistant to cotrimoxazole, amoxicillin and gentamycin and out of 9, 5 isolates were resistant to tetracycline (Table 13)

Table 13: Antibiotic susceptibility pattern of *Salmonella gallinarum*

S.N.	Antibiotics used	Drug potency (mcg/discs)	Total no. of isolates	Resistant	Intermediate susceptible	Susceptible
1.	Cephotaxime	30	9	0	0	9
2.	Cotrimoxazole	25	9	1	0	8
3.	Chloramphenical	30	9	0	0	9
4.	Ciprofloxacin	5	9	0	0	9
5.	Ofloxacin	5	9	0	0	9
6.	Enrolfloxacin	10	9	0	0	9
7.	Tetracycline	30	9	5	2	2
8.	Amoxycillin	10	9	1	0	8
9.	Nalidixic acid	30	9	7	0	2
10.	Gentamycin	10	9	1	0	8

5.14 ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *SALMONELLA PULLORUM*

Out of 23 *Salmonella spp* isolated from meat samples only 8 species of *S. pullorum* was identified. Out of 8, 7 isolates were resistant to nalidixic acid. Most of the antibiotics were 100% effective and 4 isolates were resistant to tetracycline. (Table 14)

Table 14: Antibiotic susceptibility pattern of *Salmonella pullorum*

S.N.	Antibiotics used	Drug potency (mcg/discs)	Total no. of isolates	Resistant	Intermediate susceptible	Susceptible
1.	Cephotaxime	30	8	0	0	8
2.	Cotrimoxazole	25	8	0	0	8
3.	Chloramphenicol	30	8	0	0	8
4.	Ciprofloxacin	5	8	0	0	8
5.	Ofloxacin	5	8	0	0	8
6.	Enrolfloxacin	10	8	0	0	8
7.	Tetracycline	30	8	4	3	1
8.	Amoxycillin	10	8	0	0	8
9.	Nalidixic acid	30	8	7	1	0
10.	Gentamycin	10	8	0	0	8

5.15 ANTIBIOTIC SUSCEPTIBILITY PATTERN OF OTHER *SALMONELLA SPECIES*

Other *Salmonella spp*. were found to be 100% susceptible to cephotaxime, ciprofloxacin, enrolfloxacin, ofloxacin, chloramphenicol, cotriomoxazole and gentamycin. Among the 10 antimicrobials tested, the least drug of choice was found to be nalidixic acid followed by tetracycline. (Table 15)

Table 15: Antibiotic susceptibility pattern of other *Salmonella spp.*

S.N.	Antibiotics used	Drug potency (mcg/discs)	Total no. of isolates	Resistant	Intermediate susceptible	Susceptible
1.	Cephotaxime	30	6	0	0	6
2.	Cotrimoxazole	25	6	0	0	6
3.	Chloramphenicol	30	6	0	0	6
4.	Ciprofloxacin	5	6	0	0	6
5.	Ofloxacin	5	6	0	0	6
6.	Enrolfloxacin	10	6	0	0	6
7.	Tetracycline	30	6	3	1	2
8.	Amoxycillin	10	6	0	3	3
9.	Nalidixic acid	30	6	5	0	1
10.	Gentamycin	10	6	0	0	6

5.16 SEROTYPING OF *SALMONELLA SPP.*

Six *Salmonella spp*. that produced black colony on XLD and that could not be identified by various sugar fermentation tests were taken for sero-typing at institute AFRIMS (Armed Force Research Institute of Medical Sciences). Two different sero groups were identified being serogroup D in highest number (4/6) (Table 16).

Table 16: Sero-typing of *Salmonella spp.*

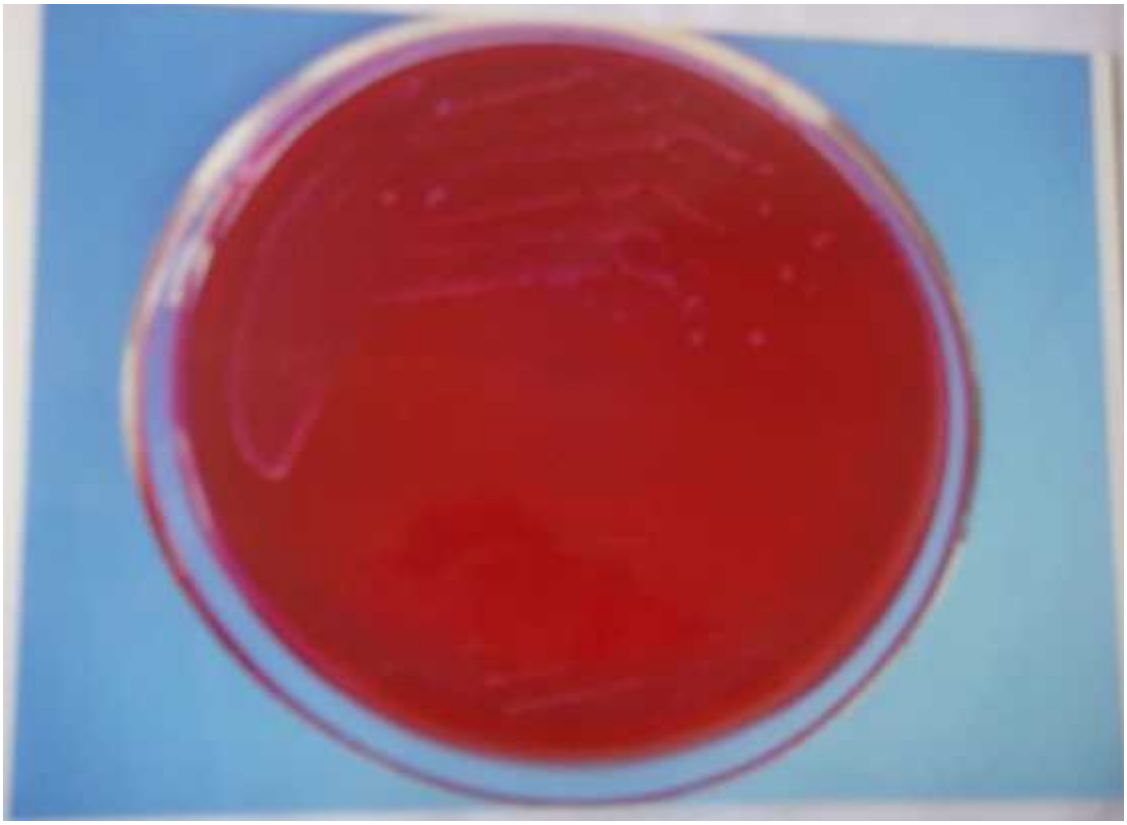
O-antigen group	Total no. of isolates	%
D	4	66.66
E	2	33.33

5.17 ANALYSIS OF MDR ISOLATES

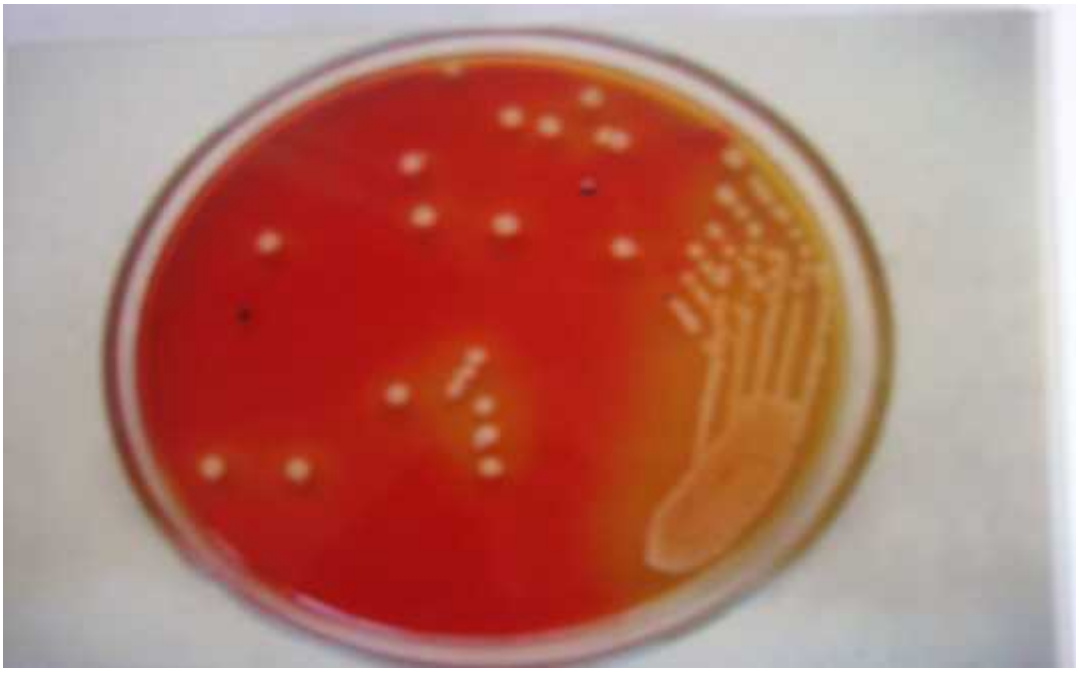
None of the *Salmonella spp*. was sensitive to the 10 antimicrobials tested. 13 of 23 species were resistant to one drug and 9 of 23 were resistant to two drugs. Only 4.34% (1/23) were found to be MDR strain (resistant to 2 or more drugs) (table 17).

Table 17: Resistance pattern and distribution of MDR strains of *Salmonella spp.*

S.N.	Organism	Total no. of isolates	Resistant to			
			1 drug	2 drugs	>2 drugs (MDR)	%
1.	*S. gallinarum*	9	4	4	1	11.11
2.	*S. pullorum*	8	5	3	0	0
3.	Other *Salmonella spp.*	6	4	2	0	0

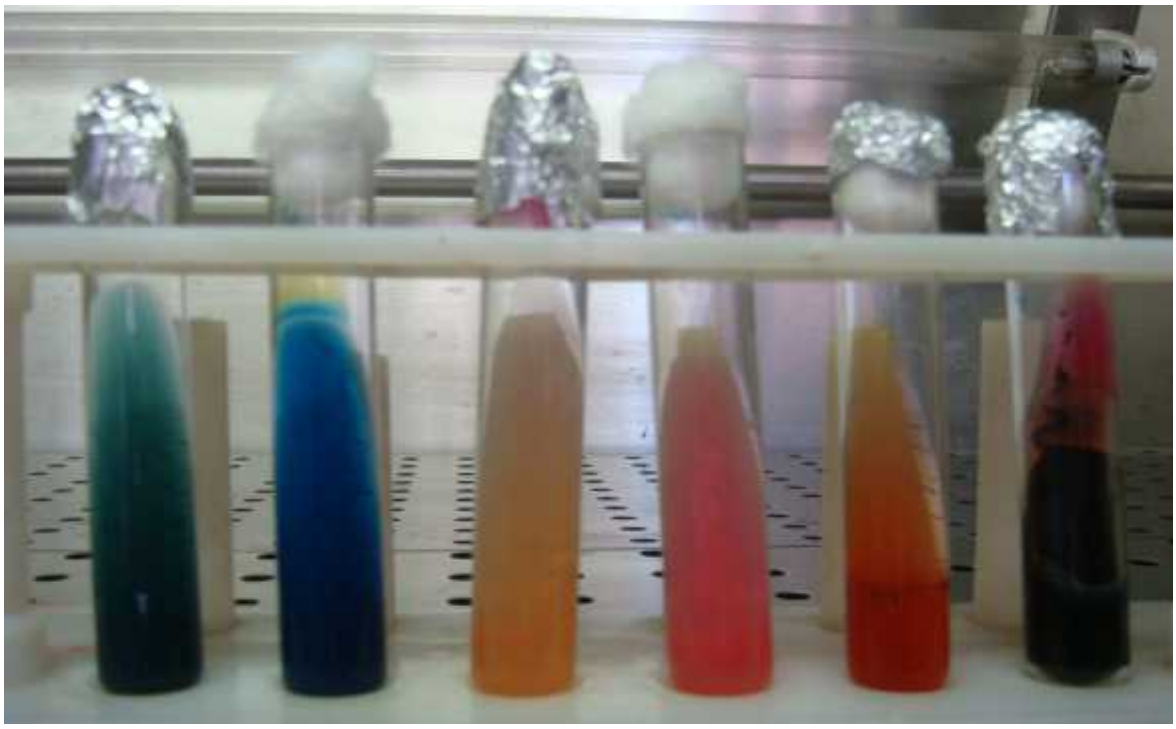


Photograph 1: Pure culture of *Salmonella* gallinarum on XLD agar plate



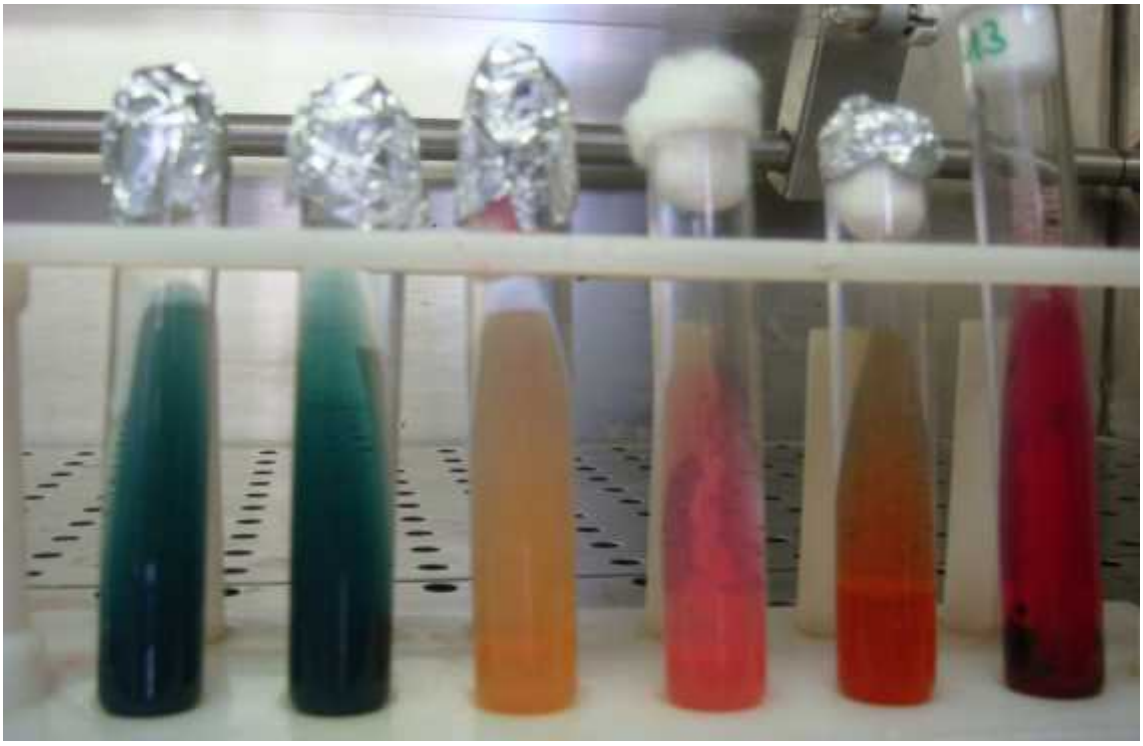
Photograph 2: Culture of *Salmonella* Serogroup D or E) on XLD agar plate

Photograph 3: Agglutination of *Salmonella* spp. with poly 'O' sera.



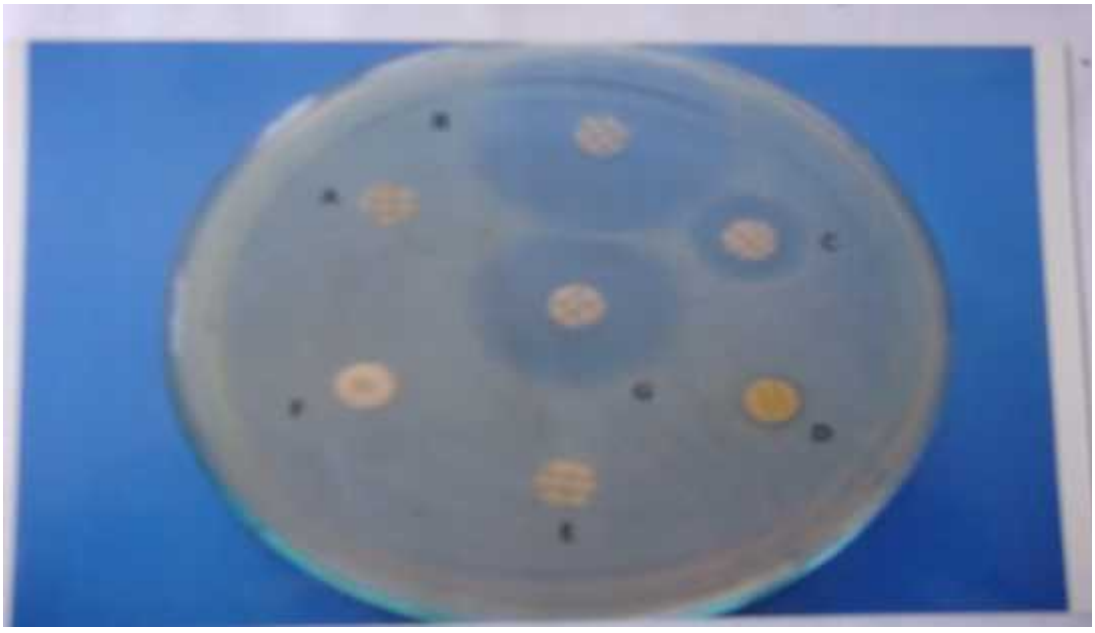
Photograph 4: Biochemical test of *Salmonella* spp. (Serogroup D or E)

A. Citrate Control B. Citrate Positive C. Urease Control
D. Urease Negative E. Control TSI F. Alk/A, H_2S Positive



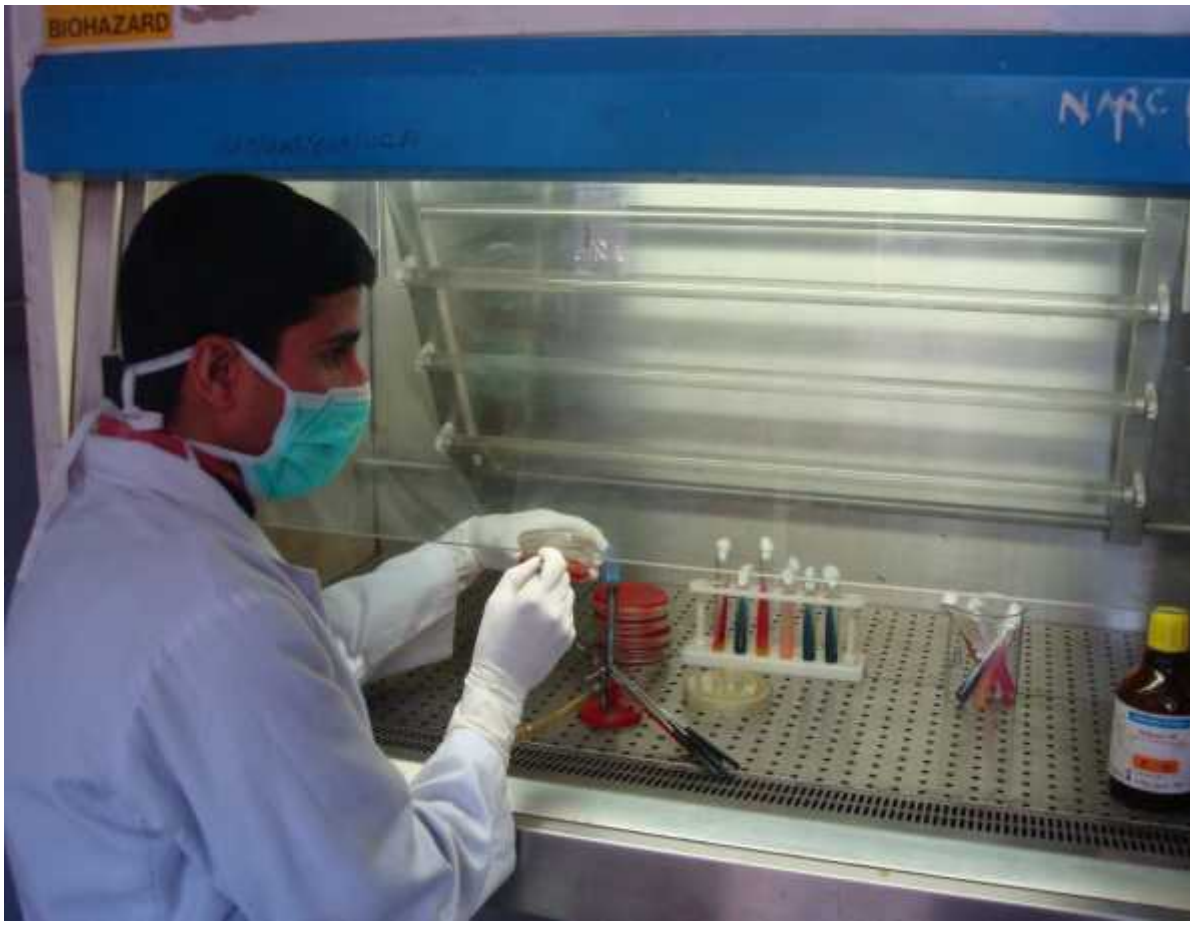
Photograph 5: Biochemical tests of *S. gallinarum*

A. Citrate Control B. Citrate Negative C. Urease Control
D. Urease Negative E. Control TSI F. Alk/A, H_2S Positive



Photograph 6: Antibiotic susceptibility test of *S. gallinarum* (MDR strain)

A. Nalidixic acid B. Ciprofloxacin C. Gentamycin
D. Tetraacycline E. Amoxycillin F. Cotrimoxazole
G. Cephotaxime
(A, C, D, E and F resistant, B and G Sensitive)



Photograph 7: Investigator processing the sample.

CHAPTER-VI

6.1 DISCUSSION

The number of organism present in the meat and meat products at any given time depends on its handling, storage time and temperature. Salmonella is responsible for the major cause of food borne illness worldwide and poultry meat is the main vehicle. This study reports the isolation of *Salmonella spp.* in different animal meat of Kathmandu valley).

In this study, among 250 meat samples microbiologically analyzed for the contamination of Salmonella, 23 samples (9.2%) were found to be Salmonella positive. The chi-square analysis result also revealed that, there is significant isolation of Salmonella in the different meat samples (χ^2=166.464 at d.f 1 and P value < 0.5).The isolation rate was found lower than that found by Maharjan et al (2000), which showed 11.4% isolation of *Salmonella spp* in raw meat samples. However, Shrestha et al. (2000), Shrestha (2005) and Shrestha K (2008) found 13%, 13.5% and 13% isolation of Salmonella in poultry meat respectively which were almost similar but this present study shows that there has been significant decrease in the isolation rate of Salmonella than previous year. This may be due to improvement in animal slaughtering places this year.

In contrast to this study conducted at CVL and ADCS by Pandey (2003), 25.07% Salmonella positive cases were detected from the post-mortem chicken samples. The contradiction may be due to the differences in the meat sample collected i.e. in this study samples were not the post mortem but were from the retail meat shops of Kathmandu valley. The isolation rate of *Salmonella spp* (9.2%) was found higher in this study as it is compared with the reports of foreign countries. The isolation rate was 1%, 5.6%, 5.5% and 0% in Ireland, Turkey, Denmark and Spain respectively (Shrestha K. 2008).

The isolation rate of *Salmonella* spp isolated cases in meat of these developed countries seems to be lower which may be due to the implementation of Animal Slaughterhouse and meat inspection act as well as HACCP Principle.

Shrestha (2005) had conducted similar types of study on poultry meat and found high isolation rate (24.48%) in the month of Bhadra. But this study has conducted from Ashoj, and found higher isolation rate in the month of Ashoj (11.11%). The reason behind this may be due to the warm and humid storage temperature which is suitable for the growth of micro-organisms. Similar type of result was also found by Shrestha K (2008) i.e. 11.63% isolation rate in the month of Ashoj, Banatvala et al. (1999); Delarocque et al. (1998), Joshi (2003), Maharjan et al. (2006) also found similar type of result.

In the present study, the isolation of *Salmonella spp.* isolated cases were found higher in Kathmandu (11.01%) than Bhaktapur (8.77%) and Lalitpur (6.66%) as shown in table 9. This result is similar to the result obtained by Karki (1995); and Shrestha K (2008). In a study carried out by Joshi (2001), he reported that the environmental sanitary conditions of the meat shops were not satisfactory and standard. Out of 70 slaughtering places, the environment is good while in 70% and 15.7% were average and poor respectively. Out of 70 butchers, only 14.3% had the knowledge of environmental pollution due to the poor sanitary conditions in slaughtering places. Lack of water and poor sanitary condition in slaughtering places may be the reason for high isolation rate of Salmonella contamination in poultry meat of Kathmandu and Bhaktapur area which is similar to the conducted present study.

In the study conducted by Joshi D.D., Joshi V. and Mishra P.N. (2005) the isolation rate of Salmonella was found to be 11.3% including 3.2% *S. pullorum*, 0.81% *S. gallinarum*, 1.62% *S. typhi*, 0.81% *S. choleraesuis* and 4.8% Salmonella of subgenus I or II type. The sample collected were buff, chicken and chevon meat samples. The isolation rate of Salmonella found on buff, chicken and cheovan were 13.5%, 14.5% and 3.2% respectively. The highest isolation rate was seen in chicken meat sample which is similar to the present study. The reason behind getting highest frequency of Salmonella in chicken meat samples than buff and goat may be due to the poor sanitary conditions in most of the chicken meat shops and slaughtering places.

All the isolates were analyzed and categorized based on their cultural, morphological as well as biochemical characteristics. From 23 meat samples,

Salmonella were isolated and identified. *S. gallinarum* was isolated from 9 meat samples where as *S. pullorum* was isolated from 8 meat samples and sero-group (D and E) was isolated in 6 meat samples The chi-square analysis result also revealed that, there is significant isolation of different species of Salmonella in the different meat samples (χ^2=577.360 at d.f 3,P value < 0.5). In a similar study, there are reports of isolation of *S. gallinarum* and *S. pullorum* from poultry meat samples of Kathmandu valley as found by Shrestha (2005), Manandhar et al. (2004) and Shrestha K (2008). When the percentage of isolation is compared with the present study, it can be said that the process of slaughtering diseased has decreased this year. The reason behind getting almost similar frequency of *S. pullorum* and *S. gallinarum* in poultry meat sample might be due to pullorum disease which is usually confined to the chicks at first 2-3 weeks of age and occasionally occurs in adults (Shivprasad, 1997) and most of the samples processed in this study might be of age group less than 2-3 weeks. The reason behind getting almost similar result of isolation *S. gallinarum* and pullorum in goat meat sample with chicken meat sample may be due to contamination from the same chopping board and knief used in the same meat shop. Although *S. gallinarum* and *S. pullorum* were isolated in meat sample, these are considered to be non-pathogenic for humans. However, they may cause significant loss to the poultry farmers.

The 6 isolates were found neither *S. gallinarum* nor *S. pullorum* based on their cultural tests mainly citrate utilization tests and H2S production in SIM media. (Both *S. gallinarum* and *S. pullorum* are) citrate negative but other 6 showed the utilization of citrate). These 6 isolates were further categorized by serotyping. Serotyping result revealed that they belonged to two groups D and E as shown in table 16. It was found that *Salmonella spp*. belonging to serogroup D had highest frequency.

S. enteritidis, a member of sero group D is the common isolates of poultry meat as reported by Shrestha (2005); Manandhar et al. (2004) and Joshi (2003). Isolation of *S. enteritidis* in foreign countries is also maximum in poultry meat being 51.7% in Albania, 29.3% in Korea and 30% in Brazil (Beli et al. 2000), Chung et al. 2003: Fuzihara et al. 2000). The Salmonella isolates belonging to

sero-group D in the present study is *S. enteritidis* which is being more prevalent in chicken meat of Nepal as well as of the foreign countries. However, for more confirmed identification, complete serotyping should be done.

So far literature concern, Salmonella belonging to serogroup E was only reported by Shrestha K (2008) to be present in poultry meat of Nepal. The present study also found Salmonella serogroup E positive cases and to my knowledge it is the second report in Nepal and it has opened the pathway for the further research. However, there are reports of isolation of Salmonella belonging to sero groups E in the poultry meat of foreign countries.

Although with low isolation rate Maharjan et al (2006) and Manandhar et al. (2004) had isolated *S. typhi* also from meat samples of Kathmandu valley. In the present study *S. typhi*, *S. paratyphi* A and B and S. cholerasuis were not isolated from any of the meat samples. It is good as a point for consumer's health.

In this study, antibiotic susceptibility test was performed for all 23 isolates of Salmonella. All isolates were found to be sensitive to the antibiotics like cephotaxime were found sensitive to the antibiotics like cephotaxime (third generation cephalosporins), fluoroquinolones such as ciprofloxacin, ofloxacin and enrol floxacin and broad spectrum antibiotics like chloramphenicol. Out of 23 isolates only 1 (*S. gallinarum*) showed resistance to cotrimoxazole, gentamycin and amoxycillin. In the study, nalidixic acid was found to be least effective drug followed by tetracycline. This finding is also similar to the result of NARC (053/054) also which showed that tetracycline resistant is more among *S. gallinarum* and *S. pullorum*.

Aarestrup et al. (2006) in his study isolated *Salmonella enterica* serotype schwarzengrund from food and food animals in Denmark and found to be resistance to nalidixic acid. The study conducted by abouzeed et al (2000); Capita et al. (2003); Metzer et al (1998), Winokur et al. (2000) also showed that tetracycline and nalidixic acid were the least effective drug to treat *Salmonella spp.* These findings also supports the findings of the present study.

According to Kurutepe et al. (2005) multi-drug resistant organisms (MDR) are defined as the organisms which are resistance to 2 or more of the antimicrobial agents. Based on this definition of MDR only 1 isolate (*S. gallinarum*) was found to be MDR. Resistance to tetracycline and nalidixic acid was most common in the case of Salmonella belonging to sero groups D and E. However, the antibiotic susceptibility pattern shows that amoxicillin (10 mcg) is also going to be resistant. Many studies had showed that isolation of MDR *Salmonella spp* in poultry meat of foreign countries is significantly higher (Aboozeed et al. 2000: Capita et al. 2003, Jorgensen et al. 2002; White et al. 2003) till date. But it is in increasing tendency which alarms the concerned authorities.

6.2 CONCLUSION

In this study, 250 meat samples including 125 chicken, 75 goat and 50 buffalo meat samples were processed and Salmonella was isolated from 23 (9.2%) meat samples. The chi-square analysis result also revealed that, there is significant isolation of different species of Salmonella in the different meat samples (χ^2=577.360 at d.f 3).

Among the isolates, 39.13% *S. gallinarum*, 34.78% *S. pullorum* and 26.08% were other *Salmonella spp.* belonging to sero group D and E. Various studies show that MDR Salmonella is increasing globally in recent years. In the present study 4.34% MDR *Salmonella spp*. was observed. The present study was carried out with the aim of isolation of MDR Salmonella in meat samples of Kathmandu valley. For the second time in Nepal, isolation of Salmonella belonging to sero group E in addition to those belonging to serogroup D in meat sample is another interesting finding of this study.

Based on the evidence of this study, it can be concluded that from the health and hygiene point of view, the meat sold in retail shops of Kathmandu valley should be improved in the meat quality.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATIONS

7.1 SUMMARY

Altogether, 250 meat samples (chicken 125, goat 75 and buffalo 50) collected from different localities of retail shops of Kathmandu valley were microbiologically analyzed for the presence of Salmonella. Out of 250 samples, 23 (9.2%) samples were found to be Salmonella positive. Statistically, the isolation rate of Salmonella in different meat samples is also significant (χ^2=166.464 at d.f 1), this also supports the result.

The study was of three month duration from Ashoj to Mangshir. The isolation rate was higher in the month of Ashoj. When the isolation rate is compared with the different animal meat samples, chicken meat samples were found to be more contaminated with *Salmonella spp* than that of goat and buffalo.

Out of 118, (11.01%) samples were found to be positive for *Salmonella spp*. in meat samples of Kathmandu which is higher than Bhaktapur (8.77%) and Lalitpur (6.66%) meat samples.

Among the 23 isolates of Salmonella, 9 (39.13%) isolates were identified as *S. gallinarum*, and 8 (34.78%) *S. pullorum*. Sero typing was done for remaining 6 isolates and it was found to be within two different 'o' antigen groups- 4 belonging to sero group D (66.66%), and 2 to serogroup E (33.39%). The *Salmonella spp.* belonging to sero group D was most predominant.

These 23 isolates were tested against 10 antimicrobials and it was found that among these antibiotics, nalidixic acid, tetracycline and amoxycillin was least effective to *Salmonella spp*. All these isolates were highly sensitive (100%) to cephotaxime, fluoroquinolones, chloramphenicol. MDR pattern was observed in 1 (4.34%) out of 23 isolates.

7.2 RECOMMENDATIONS

- Findings of this study showed that there is as light variation in the month wise isolation of Salmonella isolated positive cases, so the study should be carried out including all seasons.

- The study was conducted on only central veterinary laboratory and the samples collected were from only Kathmandu valley, so the study should also be carried out in the whole country Nepal. For the Nepalese awareness.

- Due to lack of laboratory facilities, complete serotyping of all isolates could not be determined so it is recommended for complete serotyping.

- As the multi-drug resistant Salmonella is found to be an emerging problem in meat of Kathmandu valley. It would be better if the concerned authorities check for the standard dose of antimicrobial to be used in animal feed.

- Implementation of animal slaughtering house and meat inspection act is necessary.

- Consumer's awareness programme should be conducted by distributing pamphlets or through radio or through other means.

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APPENDIX-I

LIST OF EQUIPMENTS AND MATERIALS USED DURING THE STUDY

A. EQUIPMENTS

Autoclave	Hot air oven
Refrigerator	Incubator
Laminar flow Safety Hood	Microscope
Weighing machine	Bunsen Burner

B. MICROBIOLOGICAL MEDIA

Selenite F Broth	Buffered Peptone water
Nutrient agar	Xylose Lysine Deoxycholate agar
Mac Conkey agar	MR-Vp medium
Sulphur Indole Motility agar	Nutrient Broth
Diagnostic Sensitivity test agar	Triple Sugar Iron
Urease medium	Simmons Citrate agar

C. CHEMICAL AND REAGENTS

Arabinose	Xylose
Dulcitol	Adonitol
Mannitol	Meso-inositol
Trehalose	Lactose
3%Hydrogen peroxide	Barrit’s reagent
Crystal Violet	Kovac’s reagent
Gram’s iodine	Lysol
Absolute (95%) alcohol	Andrade’s Indicator
Safranin	Polyvalent ‘O’-Sera of *Salmonella*
Normal Saline	

D. ANTIBIOTICS DISC

All the antibiotics discs used for the susceptibility tests were from Hi- Media laboratories Pvt, Limited Bambay,India. The antibiotics used were as follows:

Amoxicillin(10mcg)	Gentamicin(10mcg)
Cefotaxime(30mcg)	Nalidixic acid(30mcg)
Enrolfloxacin(10mcg)	Chloramphenicol(30mcg)
Ciprofloxicin(5mcg)	Tetracycline(30mcg)
Ofloxacin(5mcg)	Cotrimoxazole(25mcg)

E.SERO-TEST

All the serum used for sero-typing were from S and R reagents Laboratory, Bangkok, Thailand. The serum used were as follows

Polyvalent A-I serum	(lot.2668, Exp.02.10)
Sero Group OMA	(lot.2469, Exp.09.08)
Sero Group D	(lot.2238, Exp.09.08)
Sero Group E	(lot.2376, Exp.09.08)

E. MISCELLANEOUS

Scissors, Forceps, Sterile Blade, Conical flask, Cotton, Distilled water, Droppers, Glass slide, Cover slips, Immersion oil, Inoculating loop, Inoculating wire, Lysol, Measuring cylinder, Petri plates, Pipettes, Spatula, Test tubes, Cotton swab.

APPENDIX-II

A. COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA

The media used were from two companies:

a. Hi-Media Laboratories Pvt. Limited, Bombay, India

b. Oxoid Unipath Ltd. Basingstoke, Hampshire, England

(All compositions are given in grams per liter and at 25^{o}c Temperature)

1. Buffered Peptone Water (Hi-Media Laboratories)

Ingredients	gm/liter
Protease peptone	10.0
Sodium chloride	5.0
Disodium phosphate	3.50
Monopotassium phosphate	1.50
Final pH	7.2± 0.2

Direction: 20 grams of the medium was suspended in 1000ml of distilled water and then boiled to dissolve completely.Then the medium was sterilized by autoclaving at 121^{o}c(15 lbs pressure) for 15 minutes.

2. Selenite F Broth (Hi-Media Laboratories)

Ingredients	gm\ liter
Casein enzymic hydrolysate	5.0
Lactose	4.0
Sodium phosphate	10.0
Sodium hydrogen Selenite	4.o
Final pH	7.2± 0.2

Direction: 23 grams of the medium was suspended in 1000 ml of distilled water and then heated with frequent agitation until the media boils. The medium was immediately transferred to the sterile vials and placed in waterbath at 50^{o}c for 30 minutes.

3. Xylose Lysine Deoxycholate Agar (XLD) (Oxoid, England)

Ingredients	gm\liter
Yeast extract	3.0
L-Lysine	5.0
Lactose	7.50
Sucrose	7.50
Xylose	3.50
Sodium chloride	5.0
Sodium Deoxycholate	2.50
Sodium thiosulphate	6.80
Ferric ammonium citrate	0.80
Phenol red	0.08
Agar	15.0
Final pH	7.4± 0.2

Direction: 56.68 gm of the medium was suspended in 1000 ml of distilled water and then heated with frequent agitation until the medium boils. The medium was immediately transferred to a waterbath 50^{o}c for 30 minutes and poured into the sterile Petriplates.

4. Mac Conkey Agar(Oxoid, England)

Ingredients	gm / liter
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0
pH	7.4± 0.2

Direction: 52 grams of the medium was suspended in 1000 ml of distilled water

and then boiled to dissolve completely.Then the medium was sterilized by autoclaving at 121^{o}c (15 lbs pressure) for 15 minutes.

5. Nutrient Agar(Oxoid, England)

Ingredients	**gm/ liter**
Peptone	10.0
Sodium chloride	5.0
Beef extract	10.0
Yeast extract	1.5
Agar	12.0
Final pH (25^{o}c)	7.4± 0.2

Direction; 37 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^{o}c (15 lbs pressure) for 15 minutes.

6. Diagnostic Sensitivity Test Agar(DST) (Oxoid, England)

Ingredients	**gm/ liter**
Proteose peptone	10.0
Veal infusion solids	10.0
Dextrose	2.0
Sodium chloride	3.0
Disodium phosphate	2.0
Sodium acetate	1.0
Adenine sulphate	0.01
Guanine hydrochloride	0.01
Uracil	0.01
Xanthine	0.0002
Agar	15.0
Final pH(25^{o}c)	7.4 ± 0.2

Direction: 43.0 grams of the medium was suspended in 1000 ml of distilled water and boiled completely. It was then sterilized by autoclaving at 121^{o}c for 15 minutes.Then the medium was poured in the sterile petriplates.

B. COMPOSITION AND PREPARATION OF DIFFERENT BIOCHEMICAL TESTS MEDIA

1. MR-Vp medium (Hi-Media Laboratories)

Ingredients	gm/ liter
Peptone	5.0
Dextrose	5.0
Dipotassium phosphate	5.0
Final pH(25^{o}c)	6.9± 0.2

Direction: 15 grams powder was dissolved in 1000 ml of distilled water and mixed well.3 ml of medium was distributed in each test tube and autoclaved at 121^{o}c for 15 minutes.

2. Sulphide Indole Motility (SIM) medium (Oxoid, England)

Ingredients	gm/ liter
Tryptone	20.0
Peptone	6.1
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Agar	3.5
Final pH (at25oc)	7.3± 0.2

Direction: 30 grams of the medium was suspended in 1000 ml of distilled water and dissolved completely. Then it was distributed in tubes to a depth of about 3 inches and sterilized by autoclaving at 121^{o}c for 15 minutes.

3. Simmon's Citrate Agar (Oxoid,England)

Ingredients	gm/ liter
Magnesium sulphate	0.2
Ammonium dihydrogen Phosphate	0.2
Sodium ammonium Phosphate	1.0
Sodium citrate,tribasic	2.0
Sodium chloride	5.0
Agar	15.0
Bromothymol Blue	0.08
Final pH (at 25^{o}c)	6.8± 0.2

Direction : 23 grams of the medium was dissolved in 1000 ml of distilled water.3 ml medium was distributed in test tubes and sterilized by autoclaving at 121^{o}c for 15 minutes. After autoclaving, tubes containing medium were tilted to form slant.

4. Triple Sugar Iron Agar(TSI) (Oxoid,England)

Ingredients	gm/liter
Lab-lemco powder	3.0
Yeast extract	3.0
Peptone	20.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
Ferric citrate	0.3
Sodium chloride	5.0
Sodium thiosulphate	0.3
Phenol red	0.025
Agar	12.0
Final pH(at 25^{o}c)	7.4± 0.2

Direction: 65 grams of the medium was dissolved in 1000 ml of distolled water and sterilized by autoclaving at 15 lbs(121^{o}c) for 15 minutes.The medium was allowed to set in sloped form with a butt about 1 inch of length.

5. Urea Broth Base

Ingredients	**gm/ liter**
Monopotassium phosphate	9.1
Dipotassium phosphate	9.5
Yeat extract	0.1
Phenol red	0.01
Sterile 40% urea solution	5 ml

Directon : As directed by manufacturing company,1.87grams of the medium was suspended in 95 ml of distilled water and sterilized by autoclaving at 121^{o}c for 15 minutes.After cooling to about 55oc , 5 ml of sterile urea solution was added aseptically, mixed well and distributed 5 ml amount in sterile test tubes.

C. COMPOSITION AND PREPARATION OF DIFFERENT STAINING AND TESTS REAGENTS.

1. For gram's Stain

(a) Crystal Violet Solution

Crystal Violet	20.0 g
Ammonium Oxalate	9.0
Ethanol	95 ml
Distilled water (D/W) to make 1 liter.	

Direction : In a clean piece of paper, 20 gm of crystal violet was weighed and transferred to a clean brown bottle.Then, 95 ml of ethanol was added and mixed until the dye was completelydissolved.To the mixture, 9 gm of ammonium oxalate dissolved in 200 ml of D/W was added. Final volume was made 1 liter by adding D/W.

(b) Lugol's Iodine

Potassium Iodine	20.0 g
Iodine	10.0 g
Distilled water	1000 ml

Direction : To 250 ml of D/W, 20 gm of potassium iodide was dissolved.Then 10 gm of iodine was mixed to it until it was dissolved completely.Final volume was made 1 litre by adding D/W.

(c) Acetone- alcohol Decoloriser

Acetone	500 ml
Ethanol	475 ml
Distilled water	25 ml

Direction : To 25 ml of D/W, 475 ml of absolute alcohol was added, mixed well and transferred into a clean bottle.Then immediately 500 ml acetone was added to the bottle and mixed well.

(d) Saffrain (counter stain)

Safrain	10.0 g
Distilled water	1000ml

Direction : In a clean piece of paper,10 gm of safrain was weighed and transferred to a clean bottle.Then 1 litre distilled water was added to the bottle and mixed well until safrain dissolved completely.

2. Biochemical Test Reagents

(a) Catalase Reagent (For catalase test)

Hydrogen peroxide	3 ml
Distilled water	97 ml

Direction : To 97 ml of D/W ,3 ml of hydrogen peroxide was added and mixed well.

(b) Oxidase Reagent (Impregnated in what's man No.1 filter paper)

(For oxidase test)

Tetramethyl *p*-phenylene diamine dihydrochloride	1gm
Distilled water	100 ml

Direction ; This reagent solution was made by dissolving 1 gm of TPD in 100 ml D/W. To that solution strips of what's man No.1 filter paper were soaked and drained for about 30 seconds.Then these strips were freeze dried and stored in dark bottle tightly sealed with a screw cap.

(c) Kovac's Indole Reagent (Indole test)

Isoamyl alcohol	30 ml
p-dimethyl aminobenzaldehyde	2.0 g
conc.Hydrochloric acid	10 ml.

Direction : In 30 ml of isoamylalcohol, 2 gm of p-dimethyl aminobenzaldehyde was dissolved and transferred to a clean bottle.Then to it 10 ml of conc.Hcl was added to the bottle and mixed well.

(d) Methyl red Solution (Methyl red test)

Methyl red	0.05 g
Ethyl alcohol(absolute)	28 ml
Distilled water	22 ml

Direction : In 28 ml of ethanol, 0.05 gm of methyl red was dissolved and transferred to a clean brown bottle.Then to it 22 ml D/W was added to the bottle and mixed well.

(e) Barritt's Reagent (for Voges- Proskauer Test)

Solution A

α-napthol	5.0 g
Ethyl alcohol	100 ml

Direction : To 25 ml of ethanol, 5 gm of α-napthol was dissolved and transferred to a clean brown bottle.Then the final volume was made 100 ml by adding distilled water.

Solution B

Potassium hydroxide	40.0 g
Distilled water	1000 ml

Direction : To 25 ml of D/W, 40gm of KOH was dissolved and transferred to a clean brown bottle.Then the final volume was made 100 ml by adding distilled water.

(f) Andrade's Indicator

Acid Fuschin	5.0 g
Distilled water	1000 ml
1 N NaoH	150-180 ml

Direction : Acid Fuschin was dissolved in didtilled water and then 150 ml of alkali solution was added .It was mixed properly and allowed to stand at room temperature for 24 hours in rotor .If the dye has not been decolorized then 10 ml alkali was added further, mixed well and left for next 24 hours. The ultimate desired color is straw yellow.

APPENDIX-III

GRAM-STAIN PROCRDURE

First devised by Hans Christian Gram during the late 19th century, the Gram stain can be used effectively to divide all bacterial species into two large groups :those that take up the basic dye ,crystal violet (Gram positive) and those that allow the crystal dye to wash out easily with the decolorizer alcohol or acetone (Gram – negative). The following steps are involved in Gram-stain;

1. A thin film of the material to be examined was prepared and dried on a glass slide.
2. The material on the slide was heat fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain with out drying for 1 minute.
4. The slide was rinsed with tap water, shaking off excess.
5. The slide was flooded with iodine solution and allowed to remain on the surface with out drying for twice as long as the crystal violet was in contact with the slide surface.
6. The slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear requires more aggressive decolorizing.
8. The slide was flooded with counter stain (safranin) for 1minute and washed off with tap water.
9. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100 X.

APPENDIX-IV

METHODOLOGY OF BIOCHEMICAL TESTS USED FOR IDENTIFICATION OF BACTERIA

A. Catalase Test

This test is performed to demonstrate the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide. During aerobic respiration, in the presence of oxygen, micro organisms produce hydrogen peroxide, which is lethal to the cell itself. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being *Streptococcus Spp.*

Procedure: A small amount of culture from nutrient agar plate was taken in a clean glass slide and about 2-3 drops of 3% H_2O_2 was put on the surface of the slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (eg. Blood agar) or if an iron wire loop is used.

B. Oxidase Test

This test is performed for the detection of cytochrome Oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl *p-phenylene* diamine dihydrochloride, the cytochrome Oxidase oxidizes it in to a deep purple colored end product indophenol which is detected in the test. The test is used for screening species of *Neisseria, Alcaligenes, Aeromonas, Vibrio, Campylobacter* and *pseudomonas* which give positive reactions and for excluding the enterobacteriaceae, all species of which give negative reaction.

Procedure: A piece of filter paper was soaked with few drops of Oxidase reagent (whatman's No. 1 filter paper impregnated with 1% *Tetramethyl p-phenylene diamine dihydrochloride.)* then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue purple color with 10 seconds.

C. Indole Production Test

This test detects the ability of the organism to produce an enzyme 'tryptophanase' which oxidizes tryptophan to form indolic metabolites: indole, skatola (methyl indole) and indole acetic acid. The enzyme tryptophanase catalyses the deamination reaction attacking the tryptophan molecule in its side chain and leaving the aromatic ring intact in the form of indole.

Procedure: A smooth bacterial colony was stabbed on SIM (Sulphide Indole Motility) medium by a sterile stab-wire and the inoculated media was incubated at 37^{o} c for 24 hours. After 24 hours incubation, 2-3 drops of kovac's reagent was added. Appearance of red color on the top of media indicates indole positive. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in indole.

D. Methyl Red Test

This test is performed to test the ability of an organism to produce and maintain stable acid end product from the fermentation of glucose to give a red color with the indicator methyl red and to over come the buffering capacity of the system. Medium used in the study was Clark and Lubs medium (MR/Vp broth, pH 6.9). Methyl red is an indicator which is already acid and will denote changes in degree of acidity by color reactions of over a pH range of 4.4 to 6.0.

Procedure: A pure colony of the test organism was inoculated in to 2 ml of MRVp medium and was incubated at 30^{o} c for 24 hours. After incubation, about 5 drops of methyl red reagent was added and mixed well. The positive test was indicated by the development of bright red color, indicating acidity and negative with yellow color.

E. Voges-Proskauer (VP) Test

The principle of this test is to determine the ability of some organisms to produce a acetyl methyl carbinol, a neutral end product (acetoin) or its reduction product 2,3-butanidiol during fermentation of carbohydrates. An organism of the

entrobacteriaceae group is usually either methyl red positive and Voges – proskauer – negative or methyl red negative and Voges – proskauer positive.

Procedure: A pure colony of the test organism was inoculated in to 2 ml of MRVP medium and was incubated at 37^{o}c for 24 hours. After incubation, about 5 drops of barritt's reagent was added and shaken well for maximum aeration and kept for 15 minutes, positive test is indicated by the development of pink red color.

F. Citrate Utilization Test

This test is performed t detect whether an organism utilizes citrate as a sole source of carbon for metabolism with resulting alkalinity. The medium used for citrate fermentation (Simmons Citrate Medium) also contains inorganic ammonium salts. Organisms capable of utilizing citrate as its sole carbon source also utilizes the ammonium salts present in the medium as its sole Nitrogen source, the ammonium salts are broken down to ammonia with resulting alkalinity.

Procedure: A loopful of test organism was streaked on the slant area of Simmon's Citrate agar medium and incubated at 37^{o}c for 24 hours. A positive test was indicated by the growth of organism and change of media by green to blue, due to alkaline reaction. The pH indicator bromothymol blue has pH a range of 6.0 to 7.6, that is above pH 7.6, a blue color develops due to alkalinity of the medium.

G. Motility Test

This test is done to determine if an organism was motile or non-motile. Bacteria are motile by means of flagella. Flagella occur primarily among the bacilli, how ever a few cocci forms are motile. Motile bacteria may contain single flagella. The motility media used for motility test are semisolid, making motility interpretations macroscopic.

Procedure: Motility of organism was tested by hanging drop and cultural method. In cultural method, the test organism was stabbed in the SIM medium and incubated at 37^{o}c for 48 hours. Motile organisms migrate from the stabline and deffuse in to the medium causing turbidity. Where as non-motile bacteria show the growth along the stabline, and the surrounding media remains colorless and clear color.

In hanging drop method, 1-2 drops of bacterial suspension was added to the centre of concavity slid. Grease was applied to four corners of coverslips and placed over the slid. The slid was then observed under 10X to observe motility.]

H. Triple Sugar Iron (TSI) Agar Test

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (Glucose, Sucrose and Lactose in concentrations of 0.1%, 1%, 1.0% respectively), with or with out the production of gas (Indicated by cracks in the medium as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium). A pH indicator (phenol red) included in the medium can detect acid production from fermentation of these carbohydrates and it gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

Procedure: The test organism was streaked and stabbed on the surface of TSI and incubated at 37^{o}c for 24 hours. Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slat and butt indicates sucrose or lactose fermentation. The results are interpreted as follows:

a. Yellow (Acid)/Yellow (Acid), Gas, H_2S ⟶ Lactose/Sucrose Fermenter, H_2S producer.

b. Red (Alkanine)/Yellow (Acid), No Gas, No H_2S ⟶ Only Glucose, Not Lactose/Sucrose Fermenter, Not Aerogenic, No H_2S production.

c. Red (Alkanine)/No Change ⟶ Glucose, Lactose and Sucrose Non – Fermenter.

d. Yellow (Acid)/No Change ⟶ Glucose-Oxidiser.

e. No Change / No Change ⟶ Non Fermenter.

I. Urea Hydrolysis Test

This test demonstrates the urease activity present in certain bacteria which decomposes urea releasing ammonia and carbon dioxide. Ammonia thus produced changes the color of indicator (Phenol Red) incorporated in the medium.

Procedure: The test organism was inoculated in a medium containing urea and the indicator phenol red. The inoculated medium was incubated at 37^{o}c over night. Positive organism shows pink red color due to the break down of the urea to ammonia. With the release of ammonia the medium becomes alkaline as shown by a change in color of the indicator to pink.

APPENDIX -V

ZONE SIZE INTERPREATIVE CHART

Antimicrobial Agents used	Symbol	Disc Content	Resistant (mm or less)	Intermediate (mm)	Susceptible (mm or more)
Amoxycillin	Ac	10mcg	13	14-17	18
Cephotaxime	Ce	30mcg	14	15-22	23
Ciprofloxacin	Cf	5mcg	15	16-20	21
Chloramphenicol	C	30mcg	12	13-17	18
Cotrimoxazole	Co	25mcg	10	11-15	16
Enrolfloxacin	Ex	10mcg	12	13-16	17
Gentamicin	G	10mcg	12	13-14	15
Nalidixic Acid	Na	30mcg	13	14-18	19
Ofloxacin	Of	5mcg	12	13-15	16
Tetracycline	T	30mcg	15	15-18	19

(Source: Product information guide, Hi-Media Laboratories Pvt. Limited, Bombay, India)

APPENDIX - VI

DATA ANALYSIS (CHI-SQUARE TEST)

1. Association of Isolation of Salmonella in Meat Samples.

Test statistics is χ^2

H_o: There is no significant isolation of Salmonella in the meat samples.

H_1: There is significant isolation of Salmonella in the meat samples.

	Observed N	**Expected N**	**Residual (O-E)**	**(O-E)2**	**(O-E)2 / E**
Negative	227	125	102	10404	83.232
Positive	23	125	-102	10404	83.232
Total	**250**			**20808**	**166.464**

From $\chi^2 = \frac{(O\,Z\,E)^2}{E}$ we find $\chi^2 = 166.464$

Thus, χ^2 cal (166.464) > χ^2 tab at ~ = 0.05 and d.f. = 1 i.e. 3.84

Hence, χ^2 cal > χ^2 tab, H_o is rejected i.e. H_1 is accepted. i.e there is significant isolation of Salmonella in the meat samples.

2. Association of isolation of different species of Salmonella in the meat sample.

Test Statistics is χ^2

H_o: There is no significant isolation of different species of Salmonella in the meat sample.

H_1: There is significant isolationof different species of Salmonella in the meat sample.

	Observed N	**Expected N**	**Residual (O-E)**	**$(O-E)^2$**	**$(O-E)^2$ / E**
Not present	227	62.5	164.5	27060.25	432.964
S Gallinarum	9	62.5	-53.5	2862.25	45.796
S Pullorum	8	62.5	-54.5	2970.25	47.524
Other Salmonella spp	6	62.5	-56.5	3192.25	51.076
Total	**250**			**36085**	**577.36**

From $\chi^2 = \frac{(O\,Z\,E)^2}{E}$, we find $\chi^2 = 577.360$

Thus, χ^2 cal (577.360) > χ^2 tab at α = 0.05 and d.f. = 3 i.e. 7.815

Hence, χ^2 cal > χ^2 tab, H_o is rejected i.e. H_1 is accepted i.e. there is significant isolation of different species of Salmonella in the meat sample.

Data Analysis Software: Statistical Programme for Social Sciences (SPSS); Microsoft Excel