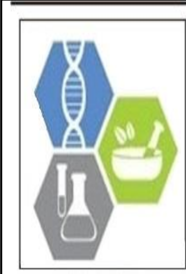

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Conventional and molecular detection of *Salmonella typhimurium* in sources of chicken origin and its antimicrobial resistance pattern in Mizoram (India)

Vanlalhruaii Hmar, Devajani Deka and Parimal Roychoudhury

Abstract
The study aimed to detect *Salmonella* by cultural and molecular methods, its serotyping and antimicrobial sensitivity pattern in different samples of chicken sources from Mizoram. A total of 400 samples comprised of raw meat (50), internal organs (50), egg (50) and cloacal swab (50), each from Aizawl and Kolasib district were subjected to isolation and identification of *Salmonella* by conventional bacteriological methods which involved pre enrichment, enrichment, selective plating on agar media and biochemical tests. The presumptive *Salmonella* isolates were subjected to*16S-rRNA* gene detection and subsequently to serotyping and antimicrobial sensitivity test. A total of 27 (6.75%) *Salmonella typhimurium* serovars were confirmed by PCR and serotyping. Highest numbers of *Salmonella* were recovered in XLD agar with PCR conformity. *Salmonella typhimurium* was detected highest in internal organs followed by cloacal swab, egg and meat. The serovars showed 100 per cent sensitivity towards Imipenem and highest resistance to Tetracycline (92.59%).

Keywords: *Salmonella typhimurium*, molecular detection, antimicrobial sensitivity, chicken sources, Mizoram

Introduction
Salmonella is identified as a leading cause of foodborne illness in humans and animals worldwide resulting in 16 million cases of typhoid fever, 1.3 billion cases of gastroenteritis and 3 million deaths annually [5]. Zoonotic transmission of nontyphoidal *Salmonella* serovars is predominantly associated with food of animal origin such as eggs, milk, poultry meat, beef and pork. The most frequently encountered *Salmonella* species from foods like poultry, pork and beef products are *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis [36].
Salmonella outbreaks associated with consumption of wide variety of contaminated food of poultry origin especially chicken meat, chicken egg and their products have been causing major public health threat [6, 14, 19, 38]. *Salmonella* in chicken flocks are usually transmitted through infected litter, fluff insects, faeces, feed, water, dust, equipment, fomites, diseased chicks and rodents [30]. In India, salmonellosis is endemic and causes heavy economic losses every year and its importance as potential zoonosis needs no emphasis [13, 32].
Salmonella is a gram negative, flagellated, rod-shaped and facultative anaerobic bacterium which is non-fastidious in nature and can multiply under various environmental conditions outside the living hosts. They are heat sensitive and can be killed at temperature of 70 °C or above. Currently, 2,610 different *Salmonella* serovars have been described according to the White-Kauffmann-Le Minor scheme [12]. Based on the difference in *16S-rRNA* sequence analysis, *Salmonella* serotypes are placed under 2 species namely; *S. enterica* and *S. bongori* and the classification is being currently used by World Health Organization (WHO) and Centre for Disease Control and Prevention (CDC) [31]. *Salmonella enterica* subspecies I is mainly isolated from warm-blooded animals and contribute for more than 99 per cent of clinical isolates [5]. Based on the host preferences, epidemiologic classification of *Salmonella* is done including the groups of host-restricted serotypes that infect only humans such as *S.* Typhi, host-adapted serotypes which are associated with one host species but can cause disease in other hosts serotypes such as *S.* Pullorum in birds and the remaining serotypes typically, *S.* Enteritidis, *S. typhimurium* and *S.* Heidelberg are the three most frequent serotypes recovered from humans each year [7].

Due to a variety of reasons, poultry meat and eggs are all time preferred food to other kinds of animal food products globally. However, most of these have been implicated as a major source of *Salmonella* infection in human [1]. Contamination of eggs can be influenced during egg production process, storage, handling and food preparation [15, 24]. Poultry meat products can be major vehicles of food borne salmonellosis because raw products may be initially contaminated with *Salmonella* as a spill over from the gastrointestinal tract due to improper slaughtering of birds and the birds are also contaminated from a variety of sources in farms and the contaminants are spread during processing. Scalding, defeathering, evisceration and giblet operations are major points of spread of the organism besides handling of poultry carcasses in markets and kitchens [29]. Every year, a large number of people are affected by the disease due to contaminated food consumption as reported by CDC [8] and WHO [37]. Antibiotic-resistant *Salmonella* has been emerging and become a serious public health issue worldwide [23]. The increasing incidence of antimicrobial resistance among *Salmonella* species causes therapeutic failure in animals and humans [3].

Mizoram is the southernmost landlocked hilly state in north east India and the poultry industry is dominated by village poultry production. In Mizoram, the estimated total egg production during 2016-17 was 397.908 lakh of which 265.694 lakh were of desi and 132.214 lakh were of improved variety. The total egg production is estimated to be increased by 4.27 per cent from 2015-16 with per capita availability of 32 eggs per year. Meat production from chicken (poultry) during 2016-17 was estimated at 2025.94 tonnes with a share of 13.70 per cent of the total meat production [28].

Keeping in view the above points, the present study was carried out to detect the *Salmonella* from the chicken sources by bacteriological and molecular methods, its serotype and antimicrobial sensitivity pattern from two districts of Mizoram namely Aizawl and Kolasib.

Materials and Methods

Collection of samples

A total 400 numbers of sample from chicken sources, 50 each from cloacal swab, internal organs, egg and raw meat were collected randomly from unorganised farms, backyard farms and retail markets at periodic intervals for a period of one year, June, 2018 – May, 2019 from Aizawl and Kolasib districts of Mizoram

Isolation and phenotypic characterization of *Salmonella*

The isolation and identification of *Salmonella* from internal organs and meat (ISO 6579:2002) and cloacal and egg swab (Bacteriological Analytical Manual) (BAM) of United States Food and Drug Administration (USFDA) employed four principal stages namely pre-enrichment, selective enrichment, selective plating and biochemical tests**.** Twenty five grams organs (intestine and liver), meat and egg contents were aseptically ground and added with 225 ml of buffered peptone water (BPW) and incubated at 37 ºC for 18 hours. One ml of pre-enriched broth was added to 10 ml selenite cysteine broth and tetra thionate broth and incubated at 37 ºC for 24 hours and to 10 ml Rappaport Vasiliadis broth incubating at 42 ºC for 24 hours. A loop-full of enriched culture from each broths was streaked onto selective agar plates of Xylose lysine deoxycholate agar (XLD), Brilliant green agar (BGA) and Hektoen enteric agar (HEA) and were checked for colonies with specific morphological characteristics following incubation at 37 ºC for 24 hours and the isolates were presumptively identified based on biochemical tests namely indole, methyl red, Vogues Proskauer, citrate (IMViC) and triple sugar iron (TSI).

Detection of species specific *16S-rRNA* gene of *Salmonella* by PCR

All the presumptively identified *Salmonella* isolates were processed for bacterial lysate preparation by using boiling and snap chilling method. A single colony of confirmed isolate was inoculated into 1ml of Luria Bertani (LB) broth and incubated at 37 ºC for 16-18 hours. After overnight incubation, cells were pelleted by centrifugation at 8000 rpm for 15 minutes at 4 ºC. Then the pellet was washed three times with sterile Normal Saline Solution (0.85%) and finally re-suspended in 500µl of nuclease free sterile distilled water. The cell suspension was heated in a boiling water bath for 10 minutes followed by immediate chilling. The cellular debris was sediment by centrifugation at 5000 rpm for 5 minutes. The supernatant was used as template for PCR assay.

The DNA lysates of presumptively identified *Salmonella* strains were subjected for detection of *16S-rRNA* species specific gene amplification by PCR using published primers (F-TAT CTG GCT ATC GCT GGC AGT G and R- TCC GCT AAT CTT TTG GCA ACC) (Whyte *et al.,* 2002). The PCR mixture consisted of PCR master mix (12.5µl), forward and reverse primer (1µl each), DNA template (4µl) and nuclease free water up to 25µl of total volume. The thermal cycling condition applied for 30 cycles of DNA amplification comprised of initial denaturation (95 ^{0}C for 5 minute), denaturation (94 ^{0}C for 45 second), annealing (59 ^{0}C for 1 minute), extension (72 ^{0}C for 45 second) and final extension (72 ^{0}C for 6 minute for one cycle). All the PCR products were analysed by agarose gel electrophoresis and the gel was visualised under UV transilluminator and documented by gel documentation system (Alpha imager).

Serotyping of *Salmonella*

All the *16S-r-RNA* positive *Salmonella* strains were sent to Central Research Institute, Kasauli, Himachal Pradesh, India for serotyping.

Detection of antibiotic sensitivity of *Salmonella*

All the *S. typhimurium* strains were subjected to *in vitro* antibiotic sensitivity testing by disc diffusion method [4] against a panel of 15 antibiotics namely; Chloramphenicol, Ampicillin, Trimethoprim, Ciprofloxacin, Tetracycline, Kanamycin, Ceftriaxone, Gentamicin, Streptomycin, Amoxyclav, Imipenem, Ticarcillin, Cefotaxime, Amikacin, and Cefoxitin as per Clinical and Laboratory Standard Institute (CLSI) guidelines [9]. The PCR positive isolates were grown in 5 ml of sterile LB broth under constant shaking at 37 °C for overnight. The overnight grown broth cultures were inoculated on Mueller Hinton agar plates by spread plating method and the antibiotic discs were placed aseptically after allowing absorption of inoculum for 10-15 minutes. Antibiotic discs were placed on inoculated agar surface at about two cm apart by using sterile forceps. The plates were incubated at 37 °C overnight and diameter of the zones of inhibition was measured.

Results

Out of 400 samples from different sources of chicken origin *viz.* raw meat (100), internal organs (100), egg (100) and cloacal swab (100) analysed from Aizawl and Kolasib districts, a total of 50 (12.50%) samples were found to be positive for *Salmonella* by the conventional bacteriological methods in which isolates showed characteristic colour changes in different broths (Rappaport, Tetrathionate and Selenite cysteine), characteristic colonies on different agars (Xylose lysine deoxycholate agar, Brilliant green agar and Hektoen enteric agar), Gram negative reaction and characteristic changes in different biochemical tests. The details of phenotypic characteristics of the *Salmonella* isolates are presented in Table1. Out of the three different agar media used for isolation of *Salmonella*, XLD was found to be most effectively isolating *Salmonella* (77.14%) followed by HEA (57.50%) and BGA (40.00%) as on subsequent PCR confirmation of *16S-rRNA* gene.

From the 50 numbers of phenotypically positive *Salmonella* strains irrespective of the media used, 27 numbers were confirmed as *Salmonella* by detecting the species specific *16S-rRNA* gene (480bp). (Table 2, Fig.3). All the PCR positive *Salmonella* strains were serotyped as *Salmonella typhimurium*.

Table 1: Cultural/ Biochemical characteristics of *Salmonella*

S. No	Cultural and Biochemical characteristics	Observations
1	Xylose lysine deoxycholate agar (XLD)	Black colonies
2	Brilliant Green Agar (BGA)	Pink colonies surrounded by Red medium
3	Hektoen enteric agar (HEA)	Black colonies surrounded by a narrow green or green blue margin
4	Gram Staining	Negative
5	Motility	Positive
6	Indole	Negative
7	Methy Red	Positive
8	VogesProskauer	Negative
9	Citrate	Positive
10	TSI	Positive -alkaline slant (red) and acidic butt (yellow) with or without H_2S production (blackening)

The overall detection rate of *S. typhimurium* was 6.75 per cent contributing to 8.00 and 5.50 per cent from Aizawl and Kolasib district, respectively (Table 2, Figure 1). Distribution of *S. typhimurium* isolated from different samples of chicken sources revealed highest in internal organs (11%) followed by cloacal swab (7%), egg (6%) and raw meat (3%). On Statistical analysis, Chi square test in pair wise comparison revealed no significant difference ($P>0.05$) in the occurrence of *Salmonella* between Aizawl and Kolasib district.

Table 2: Phenotypic and molecular detection of *Salmonella* in different samples of chicken sources from Aizawl and Kolasib districts.

S. No	District	Type of sample	Number of samples analysed	Number of samples positive for *Salmonella* spp. by phenotypic method	Number of samples positive for *Salmonella* by PCR (*16S-rRNA* gene)	% Prevalence of *Salmonella typhimurium*
1	Aizawl	Meat	50	4	2	4.00
2		Internal organs	50	10	6	12.00
3		Egg	50	6	4	8.00
4		Cloacal swab	50	7	4	8.00
Total			200	27	16	8.00
5	Kolasib	Meat	50	3	1	2.00
6		Internal organs	50	9	5	10.00
7		Egg	50	5	2	4.00
8		Cloacal swab	50	6	3	6.00
Total			200	23	11	5.50
Grand total			400	50	27	6.75

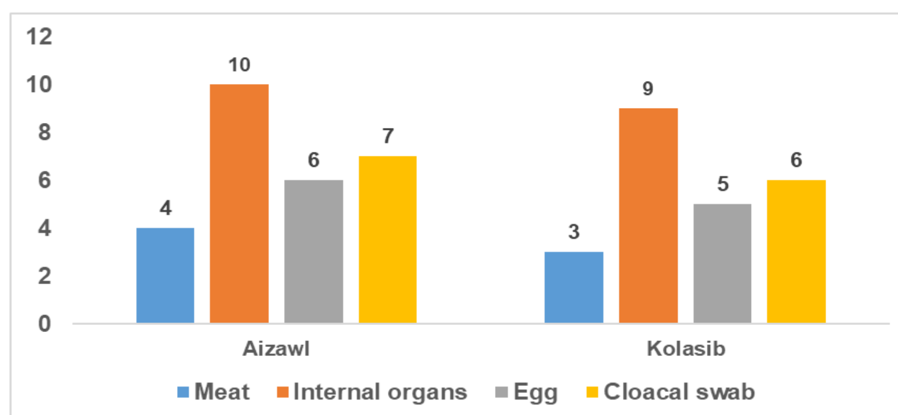

Fig 1: Phenotypic detection of *Salmonella* from different samples of chicken sources in Aizawl (n=200) and Kolasib Districts (n=200)

The occurrence rates of *Salmonella* from different samples were 4% and 2% in meat, 12% and 10% in internal organs, 8% and4% in egg and 8% and 6% in cloacal swab from Aizawl and Kolasib district, respectively (Table 2 and Fig. 1) with overall occurrence rates of 3% in meat, 11% in internal organs, 6% in egg and 7% in cloacal swab (Table 3 and Fig. 2).

Table 3: Overall prevalence of *Salmonella typhimurium* in different samples of chicken sources from Aizawl and Kolasib districts (N=400).

S. No	Type of sample	No of samples analysed	No of samples positive for *Salmonella typhimurium* (*16S- rRNA)*	% of prevalence for *Salmonella typhimurium*
1	Meat	100	3	3.00
2	Internal organs	100	11	11.00
3	Egg	100	6	6.00
4	Cloacal swab	100	7	7.00
Total		400	27	6.75

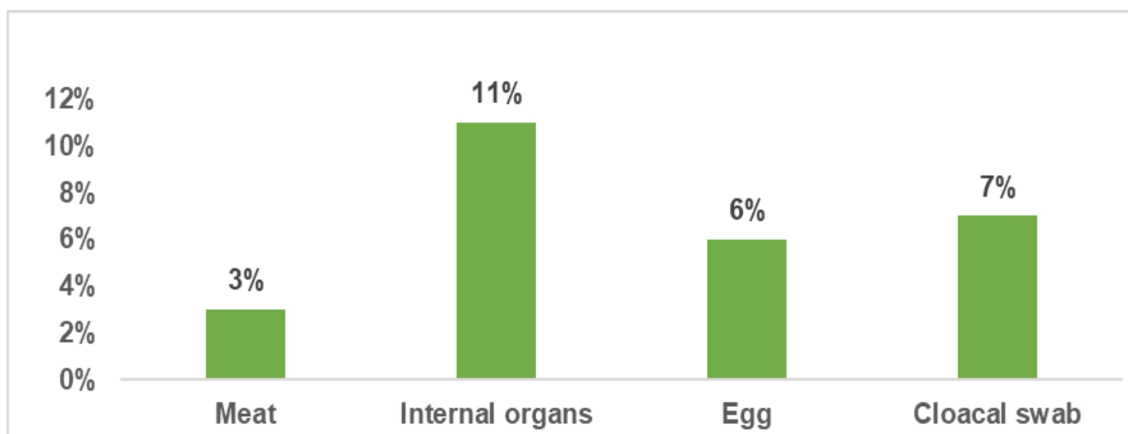

Fig 2: Overall prevalence of *Salmonella typhimurium* in different samples of chicken sources from Aizawl and Kolasib districts (N=400).

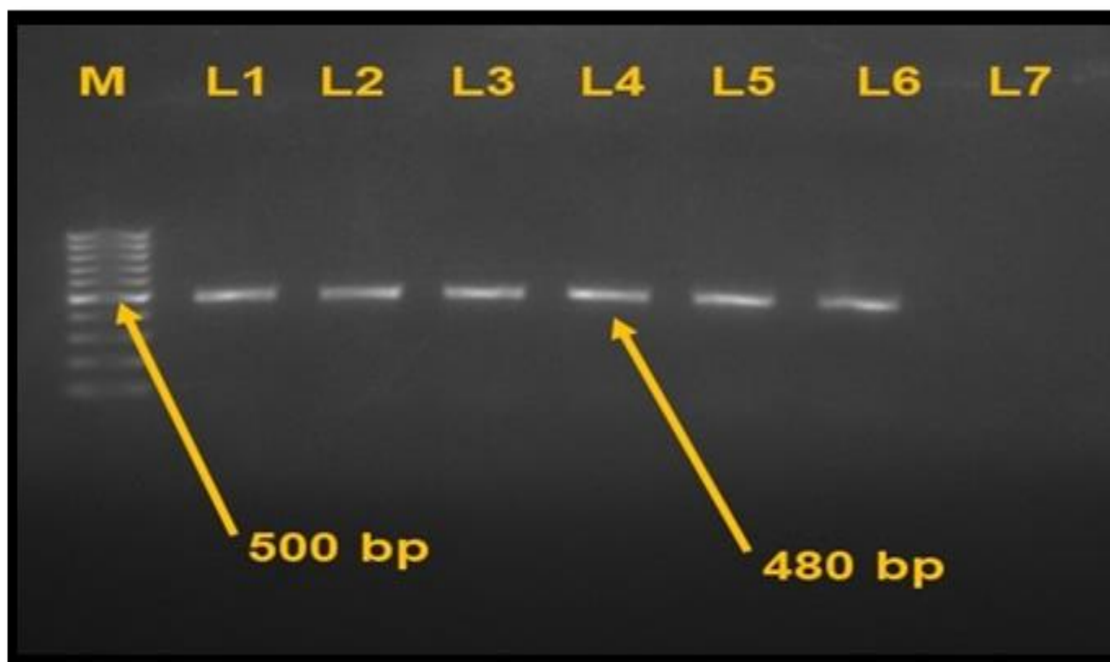

Fig 3: Agarose gel electrophoresis showing PCR amplicons of 16S-rRNA gene (480bp) obtained from *Salmonella* serovar; M: 100bp ladder; L1: Positive control; L2 to L6: Representative sample; L7: Negative control

Over all, the *S. typhimurium* serovars showed highest resistance to Tetracycline (92.59%) followed by Trimethoprim (44.44%), Ampicillin (40.74%), Streptomycin (25.92%), Gentamicin (22.22%), Kanamycin, Amoxyclav and Ticarcillin (18.51%), Ciprofloxacin, Cefotaxime, Amikacin and Cefoxitin (14.81%) and Chloramphenicol and Ceftriaxone (7.40%). Similarly, the *S. typhimurium* serovars isolated from Aizawl and Kolasib also showed highest resistance to Tetracycline (93.75% and 90.90%, respectively) (Table 4 and Fig. 4)**.**

Table 4: Antibiotic resistance pattern of *S. typhimurium* isolated from different samples of chicken sources in Aizawl and Kolasib districts

S. No	Antimicrobial agent	Overall Resistant (N=27)		Aizawl Resistant (n1=16)		Kolasib Resistant (n2=11)	
		No	(%)	No	(%)	No	(%)
1	Chloramphenicol (C)	2	7.40	1	6.25	1	9.09
2	Ampicillin (AMP)	11	40.74	7	43.75	4	36.36
3	Trimethopim (TR)	12	44.44	7	43.75	5	45.45
4	Ciprofloxacin (CIP)	4	14.81	1	6.25	3	27.27
5	Tetracycline (TE)	25	92.59	15	93.75	10	90.90
6	Kanamycin (K)	5	18.51	3	18.75	2	27.27
7	Ceftriaxone (CTR)	2	7.40	1	6.25	1	9.09
8	Gentamicin (GEN)	6	22.22	3	18.75	3	27.27
9	Streptomycin (S)	7	25.92	4	25.00	3	27.72
10	Amoxyclav (AMC)	4	18.51	2	12.50	2	18.18
11	Imipenem (IPM)	0	0.00	0	0.00	0	0.00
12	Ticarcillin (TI)	5	18.51	2	12.50	3	27.27
13	Cefotaxime (CTX)	4	14.81	2	12.50	2	18.18
14	Amikacin (AK)	4	14.81	2	12.50	2	18.18
15	Cefoxitin (CX)	4	14.81	2	12.50	2	18.18

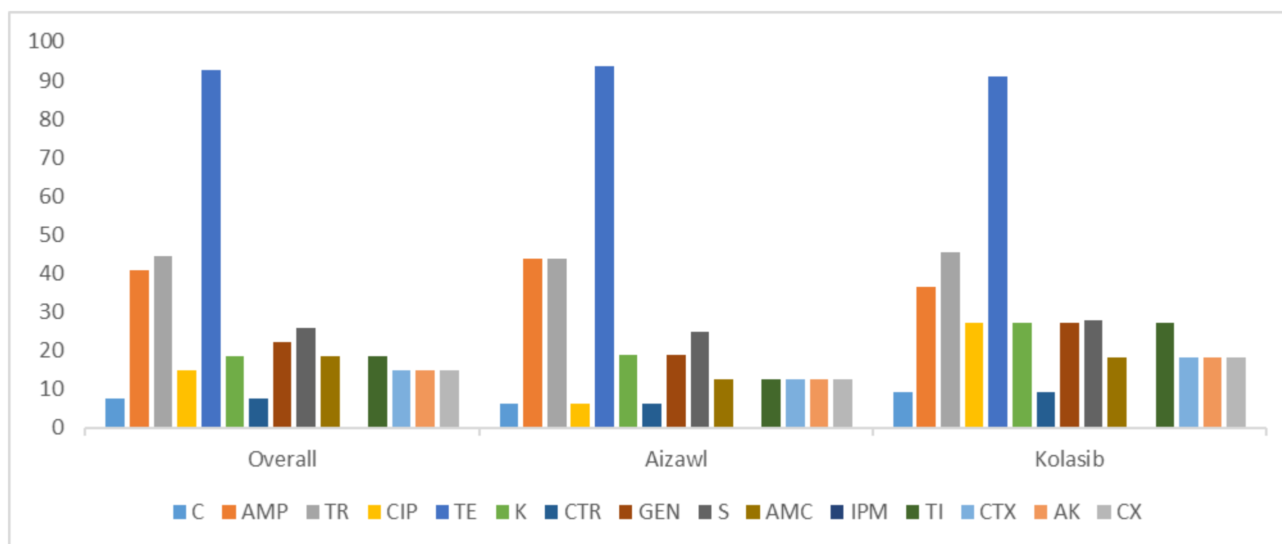

Fig 4: Antibiotic resistance pattern of *S. typhimurium* isolated from different samples of chicken sources

Discussion

Poultry is one of the most important reservoirs of *Salmonellae* that can be transmitted to humans through the dealing with chicken meat and or consumption of uncooked meat and eggs [28]. *Salmonella* outbreaks are commonly associated with consumption of contaminated food such as poultry meat and eggs which have been identified as the important vehicle for human salmonellosis [39]. *Salmonella* is one of the most important pathogens responsible for human food poisoning in developed and developing world where chicken and chicken products are considered as the prime source for this organism [21]. The detection of *Salmonella* is challenging as it has been reported to be the most common bacterial disease in the world [10]. There are several cultural methods and media in use for isolation and identification of *Salmonella*. These methods should promote the growth of even small numbers of injured cells and simultaneously reduce the growth of non-*Salmonella* organisms. In the present study, for isolation and identification of *Salmonella* from internal organs and meat of chicken, standard guidelines from ISO 6579:2002 were followed whereas the BAM, USFDA method was employed to isolate the organism from surface of egg and cloaca. The method included four principal stages such as pre-enrichment, selective enrichment, selective plating and biochemical test. The *Salmonella* organism was highest recovered from XLD followed by HEA and BGA when complied with PCR confirmation. Corrente *et al.,* [11] employed two culture media for isolation of *Salmonella* namely rambach agar (RA) and XLD with maximum recovery from XLD. Rall *et al.,* [33] isolated 29 numbers of *Salmonella* from 100 poultry carcasses using classic plating media combined and found positivity for *Salmonella* in BGA (13.80%), Salmonella-shigella agar (27.60%) and XLD (34.50%). Harsha *et al.,* [18] processed the surface swab samples of intact egg for isolation of *Salmonella* according (BAM, USFDA) and following pre-enrichment (BPW), enrichment (tetrathionate broth) and streaking onto the media such as XLD agar and HEA resulted in detection of *Salmonella* on egg shell ranging from 4 - 20.66%. Menghistu *et al.,* [25] followed the method of ISO 6579:2002 for isolation of *Salmonella* from chicken tissue samples and enriched broth culture from internal organs (intestine, liver, kidney, heart and spleen) was streaked into plates of MacConkey agar, Brilliant green phenol red lactose sucrose agar (BPLS agar) and HEA. However, the overall detection of the organism was as low (2.70%) in which liver and intestine accounted for highest. Kaushik *et al.,* [22] pre enriched chicken meat samples in BPW followed by enrichment in selenite cystine broth and selective plating on HEA and phenotypically detected 23.68 per cent samples positive for *Salmonella.*

All the 27 PCR positive isolates of *Salmonella* from Aizawl and Kolasib districts were *Salmonella enterica* serovar Typhimurium. Similar to the present finding, Anumolu and

Lakkini [2] detected 10.71% *S. typhimurium* from cloacal swab, egg swabs, poultry faeces and feed by PCR assay where as Irfan *et al.,* [20] isolated *S. enteria including S. typhimurium* from 507 samples of caecal content and faecal samples from chicken, emu and duck with the help of serotype specific PCR.

The emerging antimicrobial resistance in *S.* Typhi had been described in Africa and Asia in the late 1980s. The appearance of *S. typhimurium* DT104 raised public health concern threatening the lives of infected individuals [16]. The use of tetracycline in day old poultry and chicks through injection or drinking water to control infection of *Salmonella, Escherichia coli* and *Mycoplasma* showed high resistance to antimicrobial drugs [17]. The present study indicated 100 per cent sensitivity of the *S. typhimurium* serovars to Imipenem followed by Chloramphenicol and Ceftriaxone. On the other hand, the *S. typhimurium* serovars were found to be highest resistant to Tetracycline (92.59%) followed by Trimethoprim and Ampicillin. Irfan *et al*., (20) also recorded high sensitivity of *S. enterica* from caecal content and faeces to Chloramphenicol (96.87%) followed by Meropenem (84.37%) which was in the similar trend with the findings of present study and also detected high resistance to ampicillin (68.75%) and tetracycline (65.62%). Nagshetty *et al*., [27] examined 95 *Salmonella* isolates from environmental sources (sewage, water and food) and were sensitive to Imipenem. On the contrary to the present finding, Samanta *et al.* [34] detected resistance to Chloramphenicol, Ciprofloxacin, gentamicin, Levofloxacin, Norfloxacin and Oxytetracycline in 22 *Salmonella* isolates from cloacal swab, feed, drinking water and eggs. Similarly, Suresh *et al.,* [35] isolated 30, 90 and 7 of *Salmonella* from egg shell, egg content and egg storing tray and all the strains from egg and egg-storing trays were resistant to Ampicillin, Neomycin, Polymixin-B and Tetracycline, however, the strains were 100 per cent sensitive to Chloramphenicol and gentamycin. Kaushik *et al*., [22] reported 6.10 per cent *Salmonella typhimurium* from chicken meat in Patna, India and the strains were resistant to gentamicin, Ampicillin, Penicillin, Erythromycin, Vancomycin, Amikacin and Clindamycin with multidrug resistance.

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