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# Cross Sectional Study on Non-typhoidal *Salmonella* in Freshly Slaughtered Goat Samples From Rewa, Madhya Pradesh, India

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## ABSTRACT

**Background:** Non-typhoidal *Salmonella* (NTS), is a leading cause of food borne infections of zoonotic significance, for which different food animals are considered reservoir and source of infection. Goats are mostly slaughtered at small slaughterhouses with poor hygienic conditions making chevon a likely transmission source for salmonellosis.

**Methods:** This cross-sectional study was carried out to determine the prevalence of NTS in goats from Rewa city of Madhya Pradesh and surrounding areas. A total of 240 samples from freshly slaughtered goat carcasses, including meat (n=120) and intestinal content (n=120) were collected from different butcher shops and processed for isolation and identification of *Salmonellae* by culture, biochemical methods and molecular methods.

**Result:** A total of 10.43% prevalence of *Salmonella* (only one serotype *S. typhimurium)* was found including 14.17% positivity in meat and 6.67% in intestinal content of sampled goats. AMR pattern indicated that the 68% of isolated *Salmonella* were Multi Drug Resistant and showed high resistance towards different antibiotics including third generation cephalosporins. Invasive and virulence markers like *invA* and *spiA* were present in all the isolates but *spvR* and *spvC* were not detected. Significant correlation was detected between phenotypic and genotypic (*blaTEM* and *ampC* gene) drug resistance pattern. A significant Pearson square correlation (0.468) ($p<0.0001$) was found between the occurrence of *Salmonella* in intestinal content and meat but percent positivity of cross contaminated meat was also found very high (52.94%).

**Key words:** Cross contamination, MDR, Non-Typhoidal *Salmonella*, Slaughter house.

## INTRODUCTION

Non-typhoidal salmonellosis (NTS) is a major food borne zoonotic infection worldwide for which food animals are considered as the major reservoirs and source of infection to humans (Eng *et al.,* 2015). *Salmonella* have been isolated from different variety of foods such as poultry, beef, pork, eggs, milk, cheese, sea-foods and their food products; from some other cold-blooded animals and also from fruits, juices and vegetables (Flockhart *et al.* 2017; Malar *et al.* 2023). The transmission of non-typhoidal *Salmonella* infection to humans is sporadic and outbreak occurs through the ingestion of food or water contaminated with waste of infected animals, by direct contact with infected animals or by consumption of food from infected animals (Adem, 2022; Authority, 2018; Ehuwa *et al.,* 2021).

In India goats are one of the main meat-producing animals, whose meat (chevon) has huge domestic demand. Goat rearing under intensive and semi-intensive system for economic commercial production has become more common since past couple of years. However, the goat meat production is still mostly unorganized and slaughtering facilities are not up to the required standards. Unhygienic and improperly cooked meat products are common vehicles for epidemics of gastroenteritis infection. On one hand, infected animal may intrinsically harbor the infective bacteria in variable levels in muscle tissue and on other hand, meat mostly gets cross contamination from outside. Extrinsic contributors of carcass and meat contamination include poor slaughtering facilities and meat handling practices, which play a very important role in the spread of meat borne infections.

A variable range of prevalence from very high up to 60% to very low up to 0.1% of various *Salmonella* serotypes, among slaughtered goats in different countries including India (Hoelzer *et al.,* 2011) have been reported. Goat meat contaminated with faeces could be a source for human salmonellosis. The prevalence of *Salmonella* in faeces is

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recognized as an important risk factor for carcass contamination. Processing factors also play a significant role in carcass contamination (Sodagari *et al.,* 2020). Meat processing may transfer microbiota of gut including intestinal pathogens from the caecum, cloaca or faeces directly to the meat and organ surfaces of the carcass. Microbiota transfer may also be indirect, from personnel as well as slaughter equipment.

Antimicrobial resistance (AMR) is a silent pandemic going on and affecting global health with at least million infections and deaths (CDC, 2019). The World Health Organization (WHO) has designated antibiotic resistance as one of the three most important threats to public health in the 21st century (Amann *et al.,* 2019). The emergence of antibiotic-resistant *Salmonella* has become a serious problem due to ineffective treatment of salmonellosis by several antibiotics.

In India *NTS* are being reported from various animal and plant sources, which showing increased prevalence of genes for AMR and biofilm formation including blaTEM, blaSHV and blaCTX-M (Chakraborty *et al.,* 2020; Rabins *et al.,* 2018; Hassan *et al.,* 2020). By considering the infection potential, virulence and antibiotic resistance in food borne *Salmonella*, it is very important to determine the *Salmonella* burden in different animal sources spatially so that specific suitable preventive strategies may be formulated to counter its spread in the community. Accordingly, this study was designed with the objective of a cross sectional study to detect the prevalence of *Salmonella* in meat and intestinal content samples collected from slaughtered goat carcasses in the butcher shops of Rewa city of Madhya Pradesh, followed by characterization of different virulence markers and drug resistant pattern of isolated *Salmonella*e spp. Percent positivity of *Salmonellae* in meat and intestinal content of the same carcass may help in understanding the correlation between them as a source of infection as well as understanding the extent of cross contamination during slaughter practices.

## MATERIALS AND METHODS

### Place of work

The research was conducted at the College of Veterinary Science and Animal Husbandry in Rewa, Madhya Pradesh. The goal of the current study was to isolate and identify Salmonella from goat (meat and intestinal content) in and surrounding areas of Rewa city, Madhya Pradesh.

### Sample collection

A total of 240 goat samples including 120 neck meat sample and 120 intestinal content (of same animal) were collected randomly from goat carcasses freshly slaughtered at Small animal slaughterhouse and butcher shops of in and surrounding areas of Rewa city, Madhya Pradesh from June to December 2022. The samples were collected at the time of slaughter aseptically into sterile containers, labelled clearly, placed into cooler boxes and then transported immediately to the laboratory and preferably be processed within four to six hours of collection. Standard protocol of isolation and identification of *Salmonella* sp. described in Bacteriological Analytical Manual (BAM), U.S. Food and Drug Administration (USFDA) was followed with necessary modifications (Wallace *et al.,* 2023).

### Processing of samples and primary isolation

Intestinal content (1 gm) and meat sample 25 gm was triturated using sterilized pestle and mortar in 1ml distilled

**Table 1:** Primer (sequences and relevant information) used in the study.

| Genes | Primer sequence (5' to 3') | Size (bp) | Tm (°C) | Reference |
|---|---|---|---|---|
| *invA* | F: GTG AAA TTA TCG CCACGT TCG GGC AA | 284 | 62 | Rahn *et al.* (1992) |
| | R: TCA TCG CAC CGT CAAAGG AAC C | | | |
| *Spy* | F: TTG TTC ACT TTT TAC CCC TGA A | 401 | 57 | De Freitas *et al.* (2010) |
| | R: CCC TGA CAG CCG TTA GAT ATT | | | |
| *Sdf I* | F: TGT GTT TTA TCT GAT GCA AGA GG | 304 | 57 | De Freitas *et al.* (2010) |
| | R: TGA ACT ACG TTC GTT CTT CTG G | | | |
| *spiA* | F: CCAGGGGTCGTTAGTGTATTGCGTGAGATG | 550 | 55 | Ochman *et al.* (1996) |
| | R: CGCGTAACAAAGAACCCGTAGTGATGGATT | | | |
| spvC | F: AAGGTCGTTCAACAAGCC | 252 | 54 | Hai *et al.* (2020) |
| | R: CATTTCACCACCATCACG | | | |
| spvR | F: ATGGATTTCATTAATAAAAAATTA | 894 | 55 | Huang *et al.* (2005) |
| | R: TCAGAAGGTGGACTGTTTCAGTTT | | | |
| ampC | F: AAC ATG GGG TAT CAG GGA GAT G | 380 | 58 | Pérez-Pérez and Hanson (2002) |
| | R: CAA AGC GCG TAA CCG GAT TGG | | | |
| *tetA* | F: GCTACATCCTGCTTGCCTTC | 210 | 58 | Carlson *et al.* (1999) |
| | R: CATAGATCGCCGTGAAGAGG | | | |
| *bla*TEM | F: GGTCCTCCGATCGTTGTCAG | 310 | 68 | Carlson *et al.* (1999) |
| | R: TTCATCCATAGTTGCCTGACT | | | |

water separately and the homogenate was inoculated in the ratio of 1:10 of pre-enrichment media [Buffered peptone water (BPW)] and incubated for 16 to 20 hours at 37°C. Later for enrichment the samples were inoculated to the selective enrichment broth that was Rapaport Vasiliadis medium and incubated at 42°C for 18-24 hrs and later a loop full of inoculum was streaked on Maconkeys agar, Xylose Lysine Deoxycholate (XLD) Agar, Hektoen enteric agar **(**HEA) and Brilliant Green Agar (BGA) plates and incubated at 37°C for 24 hr. Culture plates were examined for the presence of typical colonies based on morphological characteristics.

**Phenotypic confirmation of *Salmonella***

For phenotypic confirmation, biochemical tests included were Catalase test, Oxidase test, Iodole production, Methyl red, Voges Proskauer, Citrate utilization, Urease production and Triple sugar iron test. Phenotypically confirmed *Salmonella* isolates were preserved by sub-culturing the pure colony onto nutrient agar and subsequently stored in 10% glycerol supplemented Trypticase Soya Broth (TSB) and stored at -80°C for further analysis.

**Molecular confirmation of *Salmonella***

DNA was extracted using the boiling and snap chill method. Sediment of overnight culture of *Salmonella* on nutrient broth was re-suspended into 200 µl of distilled water and kept in a boiling water bath at 100°C for 10 minutes. Then it was immediately placed straight onto ice (-20°C) for 10 min and centrifuged at 13000 rpm for 5 minutes. The sediment was precipitated by ethanol, followed by transfer of 120 µl of supernatant into a clean microcentrifuge tube and stored at 4°C until further analysis.

Purity and concentration of DNA was checked by Nanodrop 2000C. DNA concentrations were determined and corrected to 50 ng/ul for further molecular research. DNA samples (with an optical density ratio of 1.8 to 2 at 260/280 nm), were used for PCR. All the *Salmonella* isolates were first screened for the genus confirmation by genus specific primer for *invA* gene that is also a virulence marker for invasiveness of bacteria. The PCR was carried out for the *invA* gene primers product size 284 bp as described by Rahn *et al.* 1992 (Table 1).

The PCR was carried out in the thermal cycler (Veriti ®) with pre-heated lid (Lid temp. 105°C). Reaction mixture and cycling conditions were followed as mentioned in the Table 2. *Salmonella enterica* subsp. *enterica* serovar cholerae suis ATCC 10708 was used as a positive control reference strain and for negative control nuclease-free water was added in place of DNA template.

**Serotyping of Isolate**

Serotype detection was carried out by multiplex PCR for the most commonly occurring *Salmonella i.e.*, Enteritidis and *Salmonella* Typhimurium. The oligonucleotide primer *Salmonella* Typhimurium *Spy* (401bp) *Salmonella* Entritidis *Sdf* (304 bp) were used (De Freitas *et al.,* 2010) (Table 1). Reaction mixture was similar to table no 2 and cycling condition included as initial activation at 94°C for 5 min, followed by 35 cycles of denaturation 94°C for 30 sec, annealing at 57°C (1 min), Extension at 72°C (1 min) and final extension of 30 sec at 72°C, with holding at 4°C for 10 min.

**Detection of virulence gene**

All the *Salmonella* isolates were screened for the presence of virulence *spiA,* spvR and spvC gene which are the marker for virulence, biofilm formation invasiveness and immune suppression. Optimum PCR reaction mixture and thermal cycling conditions for *Salmonella spiA* virulence genes were referred from Ochman *et al.* (1996) and for the spvR and spvC genes the reaction mixture of 12.5 volume was optimized as table no 2 while the cycling condition included, initial denaturation at 94°C for 5 min, followed by 30 cycles of the denaturation at 94°C for 30 sec, annealing (48°C for 30s for *spvR* and 52°C for 30s for *spvC*) Extension at 72°C for 1min and final extension was at 72°C (30 sec) and holding at 4°C for 10 min. The amplified products were run in agarose gel electrophoresis through 1% agarose gel (Hai *et al.,* 2020, Huang *et al.,* 2005) (Table 1).

**Antibiotic susceptibility test**

*Salmonella* isolates were tested for antibiotic susceptibility test following the method of agar disc diffusion (Hudzicki, 2009). A total of 14 discs consisting of 7 groups were tested for the senstivity test and results were recorded as per the

**Table 2:** PCR reaction mixture and cycling conditions for *Inv A* gene.

| Reaction mixture | | Cycling conditions | | |
|---|---|---|---|---|
| Composition | Amount | Process | Temperature and time | Cycles |
| Master mix | 6.25µl | Initial activation | 94°C, 5 minutes | 1 |
| Each of the forward and reverse primers (20 µM), | 1.0µl | Denaturation | 94°C, 30 seconds | 35 cycles |
| Nuclease-free water | 3.25 µl | Annealing | 60°C, 30 seconds | |
| DNA template | 1 µl | Extension | 72°C, 30 seconds | |
| Total | 12.5µl | Final extension | 72°C, 1 min | 1 |
| | | Storage at | 4°C | Till further use |

criteria of Clinical Laboratory Standards Institute 2021 (CLSI 2021). The isolates were further tested for presence of *bla*TEM gene for 800 bp PCR product using specific primers as described by Dallenne *et al.* (2010). The AmpC and tetA genes were tested using methodology described by Pérez-Pérez and Hanson, 2002 and Carlson *et al.* 1999 (Table 1)

**Statistical analysis**

All data were summarized by using descriptive statistics and tables in the Microsoft excel sheet and used for calculation of prevalence and percent positivity. The significance of prevalence determination in meat and intestinal content sample was determined by Pearson's chi square test. A comparison of prevalence of antibiotic-resistant *Salmonella* in meat sample and intestinal content sample were analysed using R software version 3.2.2. Statistically associations between variables were considered significant only when the p value is less than 0.05.

Per cent positivity of cross contaminated meat that is the fraction of difference in number of *Salmonella* positive meat samples and intestinal content samples, to number of positive meat sample was calculated as following formula:

**Per cent positivity of cross contaminated meat**

$$\frac{[(\text{No of positive meat samples} - \text{No of positive intestinal content sample})]}{\text{No of positive meat samples}} \times 100$$

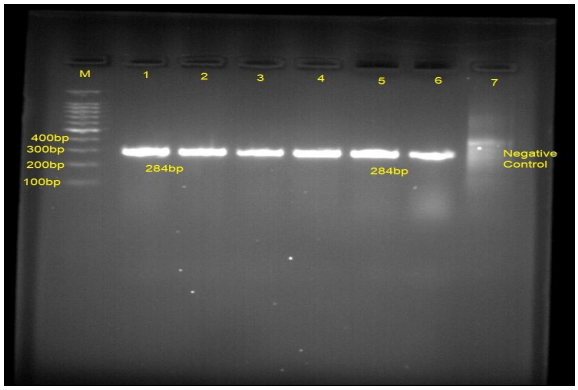
## RESULTS AND DISCUSSION

Among a total of 240 samples 10.42% (25/240) prevalence for *Salmonella* was found including 6.67% (8/120) from intestinal samples and 14.17% (17/120) from neck meat showing highly significant (Pearson's chi square test, P>.001) (Table 3). The phenotypic confirmation was done by confirming the specific colony characteristics on selective media including XLD, BGA and HEA agar plates. The presumptive colonies confirmed by biochemical characterization by IMViC which showed-ve, -ve, +ve, +ve reactions and on TSI agar slants were typical of *Salmonella* ie alkaline slant and acidic butt and produced $H_2S$ and isolates were negative for urease production test. All *Salmonella* isolates exhibited swimming and swarming motility. The molecular confirmation by PCR for *invA* gene given 284 bp specific band on agarose gel electrophoresis (Fig 1). Multiplex PCR for serotype identification indicated that all the 25 isolates were of *Salmonella enterica* serovars Typhimurirm (25/240) 10.4% (Fig 2). Out of all the virulence markers studied, all the isolates were found to carry *spiA* gene, with the 500 bp band as shown in Fig 3, but no isolate was positive for other virulence genes *spvR* and *spvC*.

Antimicrobial resistance was assessed using phenotypic (disc diffusion method) and genotypic (PCR for drug resistance gene) approaches. Highest senstivity of the *Salmonella* was found towards Co-Trimoxazole and Streptomycin that is 86% followed by 68% for Cefotaxime and Imepenum, Trimethoprim (59%), Azithromycin (55%), Tetracycline (33%), Ceftazidime (27%). All the isolates were resistant to Ampicillin, followed by Ciprofloxacin (77%), Tetracyclin (68%), Cefoperazone, Cefixime, Gentamicin (59%) (Fig 4). Phenotypic drug resistance was matching with the genotypically as 80 % isolates were positive for the

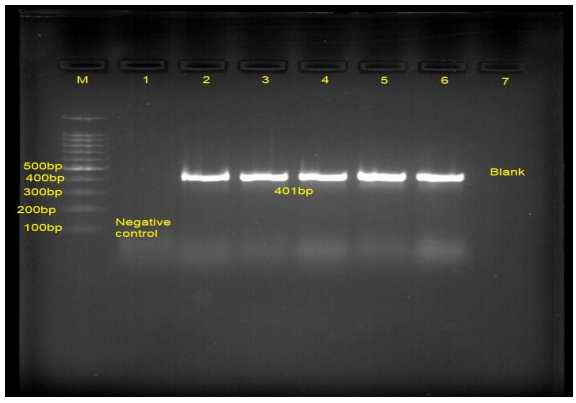
**Table 3:** Prevalence of *Salmonella* in test samples.

| Sample source | No of samples (n) | Positive samples (%) | Total prevalence (%) | *Salmonella* isolate | $\chi^2$ value, P=0.0001 |
|---|---|---|---|---|---|
| Goat intestinal content | 120 | 8 | 6.67 | *S. Typhimurium* | 64.398[a] |
| Chevon | 120 | 17 | 14.17 | *S. Typhimurium* | 93.081[a] |
| Total | 240 | 25 | 10.42 | | |



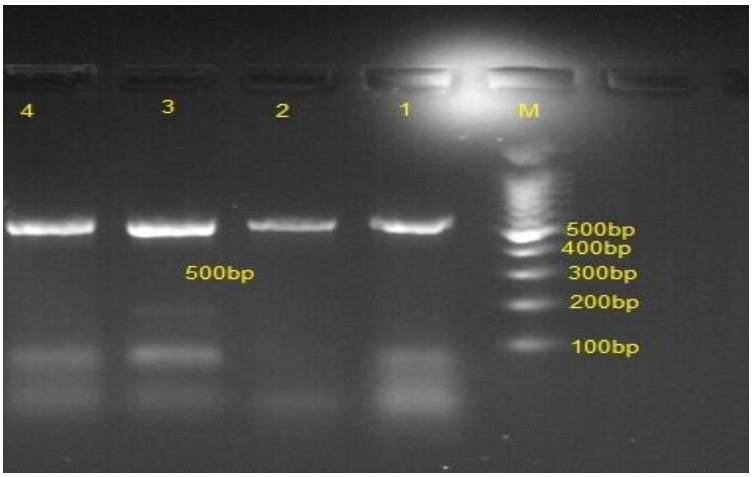

LANE M: Molecular weight marker(100bp), Lane 1: Positive control of *Salmonella*, L 2through L 6: Isolates of *Salmonella*, L 7: Negative control.

**Fig 1:** Agarose Gel Electrophoresis showing amplification products of genus specific (InvA gene) PCR.




Lane M: Molecular weight marker 100 bp, Lane 1: Negative control, Lane 2: Positive control of *S. Typhimurium*, Lane 3-6: *Salmonella* Isolates samples of *S. Typhimurium* from different source, L7: Blank.

**Fig 2:** Agarose Gel electrophoresis showing amplification products (S. Typhimurium- spy gene, 401bp) of serotype specific multiplex PCR.




Lane M: Molecular weight marker (100bp), Lane1 positive control L2-L3: isolated *Salmonella* samples

**Fig 3:** Agarose Gel electrophoresis showing amplification products of virulence *spiA* gene (500bp) specific PCR in isolated *Salmonella.*

*blaTEM* gene, while for *AmpC* and *tetA* genes the positivity was 20% and 0%.

Emergence of MDR *Salmonella* is one of the major concern and in this study the AMR pattern of isolated *Salmonella* indicated that the 68% of isolated *Salmonella* were multi drug resistant and showed resistance to one or more antibiotics of more than three group of antibiotics.

**Correlation between the presence of *Salmonella* in meat and intestinal content**

At the time of slaughter meat and intestinal content of the same goat were collected, to understand the correlation between the occurrence of *Salmonella* in intestinal content and meat at a time. The results showed a significant correlation (0.468) at the 0.01 level (2-tailed) by pearson square method. It indicates the possibility of meat contamination due to internal infection (Table 4).

This study was conducted to detect the presence of *Salmonella* infection in goat intestinal content and its meat which revealed overall 10.42 % occurrences of *Salmonella* (6.67% (8/120) from intestinal content, 14.17% (17/120) in meat samples. Mahindroo *et al.,* 2019, reported the 7.7% prevalence of NTS from goat feces which is correspond to this study while in disagreement the goat meat prevalence was 1.57% only in comparatively (14.17%). In this study, prevalence of *Salmonella* was higher in comparison to the prevalence reported by other studies, 0.7% Molla *et al.* (2006), 2.5% Zubair and Ibrahim, (2012), 3.3% Dabassa and Bacha, (2012), 7.38% Kuma *et al.* (2017), 9% Naik *et al.* (2015) and lower than 17.6% Chandra *et al.* (2006) and 20.5% Paul *et al.* (2021). The variation in the difference in prevalence may be due to variation in hygienic conditions of the slaughter houses, sanitization of knife and chopping

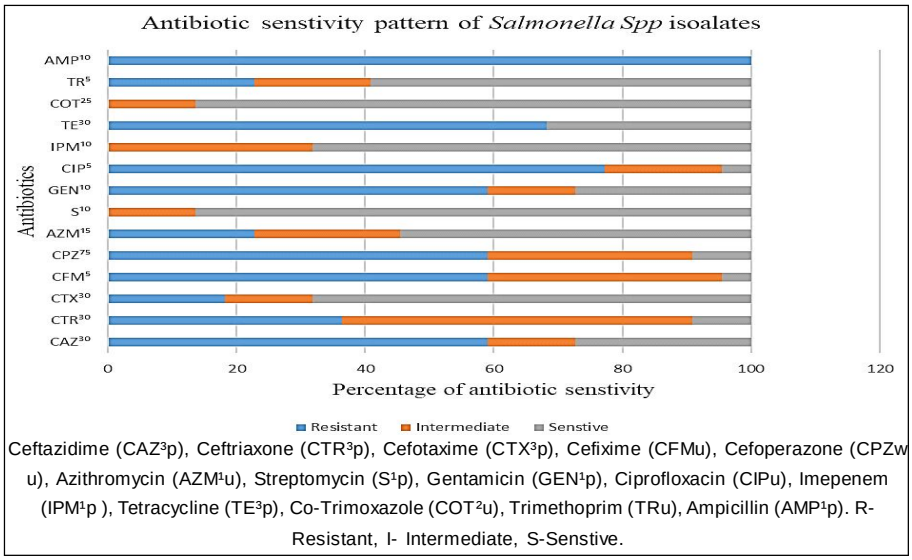



**Fig 4:** Antibiotic resistance pattern of isoalted *Salmonella spp* (*S. Typhimurium*).

**Table 4:** Correlations between the presence of *Salmonella* in meat and intestinal content.

| | | Meat | Intestinal content |
|---|---|---|---|
| Meat | Pearson Correlation | 1 | .468** |
| | Sig. (2-tailed) | | 0 |
| | Sum of Squares and Cross-products | 14.65 | 4.894 |
| | Covariance | 0.12 | 0.04 |
| | N | 123 | 123 |
| Intestinal content | Pearson Correlation | .468** | 1 |
| | Sig. (2-tailed) | 0 | |
| | Sum of Squares and Cross-products | 4.894 | 7.48 |
| | Covariance | 0.04 | 0.061 |
| | N | 123 | 123 |

**. Correlation is significant at the 0.01 level (2-tailed).

board used which may be the source of contamination and uses of antibiotics growth promoter in the goat feed for prevention of disease could be the reason. The only serotype detected was *S*. Typhimurium, which is one of the most commonly occurring serotype.

Isolated *Salmonellae* were screened for the presence of virulence genes such as *invA*, *spiA, spvR* and *spvC* which involve both biofilm formation and virulence. Only *invA* and *spiA* gene were positive in isolated *Salmonellae,* indicating their pathogenic potential. The *invA* gene is most commonly used for the confirmation of *Salmonellae* by PCR but it is also a potential virulence factor indicating invasiveness of the bacteria as it encodes essential proteins in bacterial cell membranes. The location of the gene is the *Salmonella* Pathogenicity Island (SPI) in the DNA region is related to the pathogenicity of *Salmonella enterica* and is present in almost all serotypes (Lou *et al.,* 2019). spvC gene was not present in any of the isolates and this gene is generally associated with the suppression of innate immunity in infected hosts and the systemic spread of *Salmonella* (Wu *et al.,* 2016).

The isolated *Salmonella* showed high resistant to some antibiotics like cephalosporin groups which are critically important for clinical infections therapy in animal as well as humans. Presence of MDR *Salmonella* in tested samples is of high concern particularly at emergency situations like food borne *Salmonella* infection outbreaks (Kolhe *et al.,* 2020).

At the time of slaughter meat and intestinal content sample were collected from same goat, to understand the correlation between the occurrence of *Salmonella* in intestinal content and meat. Significant correlation between *Salmonella* positivity in meat and intestinal content, indicate the possibility of meat contamination due to internal infection. However, the positivity of *Salmonella* was comparatively higher in meat than the intestinal content. It may be assumed that in absence of gastrointestinal infection, meat might be cross contaminated during various slaughtering processes. As in during sample collection it was observed that in most of slaughtering premises there were lack of hygiene and practices were not as per Good Production Practices (GPP). The recorded observations while collecting the samples

indicated that in more than 70.8% places, the knife hygiene (disinfection of knife) for subsequent slaughter was not being followed. It may be the factor contributing in the cross contamination of meat. However other factors like various slaughtering practices might contribute in further contamination of meat. This might hugely contribute to the cross contamination and increased risk for human infection, even more than risk posed by consumption of pathogen harboring animal. Proper sanitation and use of clean water during the slaughtering and processing of meat can protect the meat from contamination (Mkangara 2023).

Occupational exposure in persons engaged with handling the infected animals and their products should be identified and awareness among occupational groups need to be emphasized. There is a need of spreading awareness about food safety at every stratum of society, and its negligence might be devastative in terms of AMR emergence and occurrence of outbreaks.

## CONCLUSION

This study showed 10.43% prevalence of *Salmonella* in the goat samples. Despite both the samples were collected from the same animal at the time of slaughter, the prevalence in meat samples was higher (14.17%) in comparison to intestinal content (6.67%). This might be attributed to the cross contamination during slaughtering. The percentage positivity of cross contaminated meat was 52.94% which was very high, making it a matter of great concern as this cross contamination might increase the risk of occupational exposure as well as food borne transmissions. Detected *Salmonellae* have genes for invasiveness, virulence and drug resistance, indicating the severity of infection which may be difficult to control. The high percentage of MDR in isolates is a valuable information for clinical therapy particularly during outbreak emergencies.

### FURTHER RESEARCH

Further epidemiological risk attribution of the goat food products towards zoonotic transmission of nontyphoidal *Salmonella* needed to be done so that proper prevention and control measure can be developed and implemented.

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### Data availability statement

All the data related to study is available and will be provided on demand.

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### Conflict of interest

The authors have declared no conflict of interests exist.

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