The foodborne diseases antimicrobial resistance development in food animals: A case of *Salmonella* isolates from diarrheic sheep in and around Gondar city, Ethiopia: A cross-sectional study

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Abstract

Background: In recent years, an increase in the occurrence of antimicrobial-resistant pathogens especially foodborne zoonotic bacteria has been observed in several countries. As a result, fatality rates are increasing due to those resistant bacteria in both human and animal populations, particularly in developing countries like Ethiopia where the risk of infection is high due to poor biosecurity measures, close animal-human interactions, and extensive use of antimicrobials for animal productions. One of those zoonotic diseases, which commonly contaminates food and having zoonotic characteristics is *Salmonella*.

Methodology: A cross-sectional study on samples collected from diarrheic sheep in and around Gondar city, to assess the risk of antibiotic resistance development of *Salmonella* in food animal production was conducted. A total of 80 positively isolated *Salmonellae* were taken from 165 diarrheic sheep and antibiotics resistance test was conducted by Kirby-Bauer Disc diffusion method. The disc diffusion test was performed using ten commonly used antibiotic discs in the study area.

Result: *Salmonellae* were 100% resistant for amoxicillin and ampicillin. In addition to this, the bacteria were sensitive for kanamycin, gentamicin, and ciprofloxacin with some level of intermediate resistance. *Salmonellae* were 75% sensitive with 25% resistance for chloramphenicol while trimethoprim, tetracycline, doxycycline and nalidixic acid showed 37.5%, 37.5%, 25% and 12.5% of resistance respectively. Generally, this study revealed that *Salmonellae* were developed a wide range of resistance to different antibiotics in the study area.

Conclusion: In general, majority of previously curative antibiotics are developed resistance against *Salmonella*. Therefore, the rational use of antibiotics, antimicrobial use governance in animal production, further studies, and integrative approaches among animal-human health professionals are recommended for the reduction and mitigation of health risks arising from antibiotic-resistant zoonotic pathogens like *Salmonellae*.

Keywords: Zoonotic disease; Food Animals; Salmonella; Antimicrobials; Antibiotic resistance; Sheep; Gondar
1. Introduction

Despite antimicrobials contribute a vital role for treatment, surgery and prophylactic activities, they are endangered by dramatically increasing antimicrobial resistance (AMR). Even though different factors aggravates AMR, overuse, and misuse of antimicrobials in different sectors plays a major role. Among the activities that increase AMR: using antibiotics in animal production, using drugs without professional oversight and poor diagnostic techniques are the leading factors [1, 2]. Since it affects both the animal and human health sectors, the risk of AMR in zoonotic diseases is severer than AMR in other diseases causative agents. Especially people in developing countries, living in the poor hygienic condition and consuming raw animal origin food are at the high risk of affecting by antimicrobial-resistant zoonotic diseases (1).

Like other bacteria, *Salmonella* is developing resistance against antibiotics based on either of the following resistance mechanisms: “production of enzymes that inactivate antimicrobial agents through degradation or structural modification, reduction of bacterial cell permeability to antibiotics, activation of antimicrobial efflux pumps, and modification of the cellular target for the drug” [3, 4].

The resistance of *Salmonella* against commonly used and previously curative antibiotics is dramatically increasing with wide geographic coverage [5, 6, 7, 8, and 9]. The death rate of patients, who were contracted by antibiotic-resistant *Salmonella* is high and the economic loss due to morbidity is increasing as well [10]. The Zoonotic nature of this bacteria makes the AMR against *Salmonella* worst, in which it can be transmitted easily from animal to human and the damage is exploited [11, 12]. The exposure of *Salmonella* to different antibiotics, while it is in the environment or inside animals, enable it to develop resistance. The resistant *Salmonella* then easily pass to human through the food chain and cause catastrophic effect in human health.

Even if the international tripartite organizations (WHO, FAO and OIE) and Codex Alimentarius are working against antibiotic resistance and set action plans to minimize antibiotic use along the food chain it is increasing at an alarming rate [13, 14]. Before this research, many other types of research were conducted to assess the resistance pattern of *Salmonella* both in human and animals, but due to “evolutionary nature of the bacteria and the dependency of patients to antibiotics”, the resistance level of *Salmonellae* to antibiotics are
increasing from time to time [15, 16, 17, 18, 6, 7, 8, 9]. Antibiotic resistance surveillance in food animal like sheep is strongly recommended for the mitigation of this global health crisis (AMR) and there was no surveillance has done yet in the study area.

Isolation of the common diseases causing agent (*Salmonella*) and conducting antibiotic sensitivity testing are the recommended activities to see the antibiotic resistance phenomenon of zoonotic diseases in animals. Such type of surveillance are the foundation for conducting further studies, formulating antimicrobial use policies and creating public awareness.

Therefore, the objective of this study was:

- To assess the level of zoonotic diseases antimicrobial resistance in food animals by taking *Salmonella* as an example.

### 2. Material and Methods

#### 2.1. Study area

The study was conducted in and around Gondar City, Northwest Ethiopia. The climate condition of Gondar is moderate with an average temperature of 19.3°C and an annual rainfall of 1151mm. It has an elevation of 2133m above sea level with a latitude and longitude of 12° 36’ N and 37° 28’ E respectively. Gondar city is located 740km away from Addis Ababa (the Capital city of Ethiopia). In the area, there is a large number of livestock populations reared in the different management systems and antibiotics were massively used for different purposes (prophylaxis, treatment, metaphylaxis, and growth promotion) in animal production.

#### 2.2. Study population

The antibiotic resistance test was performed on *Salmonella* isolates, collected from sheep in and around Gondar city, during the outbreak of diarrheal disease. All diarrheic sheep were sampled and *Salmonella* was isolated from the feces of those sheep. Since the owners were not agreed, some sheep were excluded from sampling.

#### 2.3. Study design

A cross-sectional study was conducted from September 2019 to March 2020. The study was performed for the purpose of isolating *Salmonella* and conducting antibiotic testing on the
isolates. The result of antibiotic resistance tests was used to understand the progress of zoonotic diseases AMR in food-producing animals.

2.4. Sample collection techniques and antibiotic resistance testing procedure

2.4.1. Sample collection techniques

Diarrheic fecal samples were collected from 165 lambs in sub-cities and districts of Gondar, such as Chank, Fenter, Welka, Kebele 16, and 18. After isolation and characterization processes, 80 samples were isolated positive for *Salmonella*. The samples were cultured in nutrient agar and antibiotic sensitivity tests were conducted with Mueller-Hinton agar (OXOID) in University of Gondar veterinary microbiology laboratory.

2.4.2. Isolation and identification process of *Salmonella*

Isolation of *Salmonella* was performed as recommended by the FDA [19, 20]. In brief, 1ml of the sample from the transport swab was inoculated in 9 ml of buffered peptone water and incubated at 37°C for 18 h for pre-enrichment. Further, for selective enrichment 0.1 ml of the pre-enriched inoculum were transferred to 10 ml of Rappaport-Vassiliadis broth and incubated at 42°C for 24 h. Enriched samples were inoculated on MacConkey Agar (MCA) a dual purpose (selective and differential) medium, by four flame techniques, and plates were incubated at 37°C for 24hour.

Gram’s staining was performed on pure colony [21] to determine the shape and arrangement of bacteria. The non-lactose fermenting colorless colony from the MacConkey Agar (*Salmonella*) were sub cultured onto *Salmonella* agar media which was used as a selective media for pathogenic *Salmonella* and incubated at 37°C for 24 hours for the appearance of the characteristic colorless colony with black center. Similarly, enrichment, a loop full of inoculums were streaked on xylose lysine desoxycholate (XLD) agar and incubated at 37°C for 24 hours. The presumptive *Salmonella* colony appearing slightly transparent red halo with a black center surrounded by a pink-red zone on XLD agar were screened, further for its biochemical characterization. Standard biochemical tests, Catalase test, Indole, Methyl red, Voges-Proskauer test, Citrate utilization, urease test, Triple sugar Iron test and Carbohydrate fermentation test were used as confirmation of identification [22, 23].
2.4.3. Preparation of 0.5 McFarland Standard

According to [19] 0.5ml of BaCl2 (1.175% BaCl2.2H2O) was added to 99.5 ml of H2SO4 (1%) and the standard was poured into plastic caped tubes of the same size and volume as those used in the bacterial suspension. The tubes were tightly sealed to prevent loss by evaporation and stored protected from light at room temperature and the standard was vigorously agitated in a vortex mixer before use.

2.4.4. Antimicrobial resistance testing of *Salmonella*

The isolated *Salmonella* was screened for in vitro antimicrobial resistance test using the agar disk diffusion method [24]. Ten different antibiotics (OXOID, England) discs with their concentrations given in parentheses was used in the antibiograms: such as kanamycin (k) (30µg), gentamicin (CN) (10µg), amoxicillin (Amx) (10µg), doxycycline (Do) (30µg), chloramphenicol (C) (30µg), tetracycline (TE) (30µg), ciprofloxacin (CIP) (5µg), trimethoprim (TMP) (5µg), nalidixic acid (NAL) (30µg) and ampicillin (AMP) (10µg).

An isolated colony from nutrient agar plates were transferred by sterilized inoculating loop into tubes containing 5 ml of the normal saline solution until it achieved 0.5 McFarland turbidity standards and then a sterile cotton swab was dipped into the adjusted suspension and pressed against the inside of the tube to remove excess water. The swab was then spread evenly over the entire surface of the plate of Mueller-Hinton agar (OXOID) to obtain uniform inoculums. The plates were allowed to dry for 5 minutes. Antibiotics impregnated discs were then applied to the surface of the inoculated plates with disc dispenser. Each disc was gently pressed down onto the agar by sterile forceps to ensure complete contact with the agar surface.

Even distribution of discs and minimum distance of 24 mm from the center to center and from the edge of the plate was ensured to prevent overlapping of the inhibition zones. Four antibiotic discs were placed in each Petri dish. Within 15 minutes of the application of the discs, the plates were inverted and incubated at 37°C. After 24 hours of incubation, the plates were examined, and the diameter of the zones of complete inhibition to the nearest whole millimeter was measured by a digital caliper. Based on the Clinical and Laboratory Standard Institute [25], the clear zones (inhibition zones) around the antibiotic discs (including the disc) were expressed as Susceptible (S), Intermediate (I), and Resistant (R).
1.5. Data analysis

The dataset for data analysis was prepared using Microsoft excel 2013 and descriptive statistics (graphs, numbers, tables, and percentages) were used to describe findings. Each antibiotic was tested for all 80 positively isolated *Salmonella*. The average inhibition zones of all 80 tests of each antibiotic were calculated and one single inhibition zone for each antibiotic was produced, for the purpose of easing analysis. One sample can be resistant to more than one antibiotic. The nature of resistance (multiple or single) and the number of resistant samples for each antibiotic are presented in table form.

3. Result

During the study, 165 total samples were examined. After a consecutive culture and biochemical test activities, 80 of them were tested positive for *Salmonella*. The resistance level of selected antibiotics was tested on each isolate. As the antibiotic sensitivity test result revealed, *Salmonellae* were completely (100%) resistant to Ampicillin and amoxicillin, whereas 87.5% of positive samples for *Salmonella* were susceptible to gentamicin and ciprofloxacin. In addition, 75% of *Salmonella* isolates were susceptible to kanamycin and chloramphenicol while nalidixic acid, trimethoprim, tetracycline, doxycycline were showed 50%, 37.5%, 37.5% and 25% level of resistance respectively (Figure 1).

*Figure 1: Over all antibiotic resistance level of Salmonella isolates against selected antibiotics in percent (%)*

According to the clinical and laboratory standard institute [25], antibiotic sensitivities are expressed in three modalities (sensitive, intermediate, and resistance). To categorize the sensitivity of *Salmonella* into these three modalities, CLSI uses the measurement result of the inhibition zone in millimeter. Table 1 shows the average inhibition zone of all 80 *Salmonella* positive samples by comparing them with the standard.

Table 1: Comparison of the inhibition zone of the standard with the average finding of this study.

| Antibiotics | Standards (in mm) | Finding of this study (in mm) |
|-------------|-------------------|------------------------------|
|             | Sensitive | Intermediate | Resistant | Sensitive | Intermediate | Resistant |
| Antibiotic         | MIC (µg) | Sensitivity (µg) | Sensitivity (µg) | Sensitivity (µg) | Sensitivity (µg) |
|--------------------|----------|------------------|------------------|------------------|------------------|
| Tetracycline       | ≥ 15     | 12-14            | ≤ 11             | 16               | 13               | 11               |
| Gentamicin         | ≥ 15     | 13-14            | ≤ 12             | 16               | 14               | N/A              |
| Kanamycin          | ≥ 18     | 14-17            | ≤ 13             | 18               | 15               | N/A              |
| Doxycycline        | ≥ 14     | 11-13            | ≤ 10             | 15               | 12               | 9                |
| Chloramphenicol    | ≥ 18     | 13-17            | ≤ 12             | 18               | 13               | 11               |
| Ciprofloxacin      | ≥ 30     | 21-30            | ≤ 20             | 30               | 26               | N/A              |
| Trimethoprim       | ≥ 16     | 11-15            | ≤ 10             | 16               | 13               | 10               |
| Nalidixic acid     | ≥ 19     | 14-18            | ≤ 13             | 19               | 16               | 13               |
| Amoxicillin        | ≥ 17     | 14-16            | ≤ 13             | N/A              | N/A              | 6                |
| Ampicillin         | ≥ 18     | 14-17            | ≤ 13             | N/A              | N/A              | 10               |

N/A Represents no test result was found during the study

All 80 *Salmonella* isolates showed multidrug-resistant. Only two antibiotics (ampicillin and amoxicillin) were resistant for all isolates, whereas all isolates were fully or intermediately susceptible for 3 antibiotics (gentamicin, kanamycin, and ciprofloxacin). The details of multidrug resistance are described in Table 2.

Table 2: The number of *Salmonella* isolates that was developed multi-drug resistance

| List of antibiotics                      | Number of *Salmonella* isolates (n=80) | Number of antibiotics (n=10) |
|-----------------------------------------|----------------------------------------|------------------------------|
| TE, DO, AMP, AMX, C, NAL and TMP       | 10                                     | 7                            |
| TMP, AMX, AMP, DO and TE               | 20                                     | 5                            |
| TMP, AMX, AMP and TE                   | 30                                     | 4                            |
| AMX and AMP                             | 80                                     | 2                            |

*AMX= Amoxicillin (10µg), DO=Doxycycline (30µg), C=Chloramphenicol (30µg), TE=Tetracycline (30µg), CIP=Ciprofloxacin (5µg), TMP=Trimethoprim (5µg), NAL=Nalidixic acid (30µg) and AMP=Ampicillin (10µg)*

4. Discussion

All (80) *Salmonella* isolates were found multidrug-resistant. In agreement with this study *Salmonella* were developed multidrug resistance in Modjo [18], Gondar [7], Jimma [9], and China [8]. This multidrug resistance might be due to the irrational use of antibiotics practiced in the area [26, 27] and or evolutionary nature of *Salmonella* species [16]. The multidrug resistance bacteria cause a dangerous health crisis than resistance against single antibiotics, especially the crisis is very bad when it is on zoonotic diseases. Since it easily
circulates between animals and humans, it is difficult to eradicate zoonotic resistance bacteria [5].

*Salmonella* isolates of this study were sensitive to gentamicin, kanamycin, and ciprofloxacin. Likewise, on *Salmonella* species isolated from diarrheic sheep in San Angelo, 100% sensitivity level of *Salmonella* isolates were registered to ciprofloxacin and gentamicin [28]. As reported by the same study, 99.37% of *Salmonella* isolates were sensitive to kanamycin [22]. Specifically, the sensitivity of *Salmonella* to gentamicin agrees with the study conducted in Harar [29] with 92.8% sensitivity level. In addition to this, the study done by [30] in Indonesia revealed that 92% of *Salmonella* isolates were sensitive to gentamicin. My finding also slightly agrees with the study done in Adis Abeba by [31] with the samples collected from diarrheic children, which was 50% sensitive, 50% intermediate and 0% resistance for Gentamycin and kanamycin. Standing from the result of this study, prudent use of these effective antibiotics is recommended to preserve a choice of treatment for the future.

In contrast to my finding, a high level of resistance to gentamicin (75.6%) was reported by [31]. In the same study area from samples collected from human diarrhea and by [33] resistance in Kenya with resistance level of 13.0% to kanamycin. Resistance to gentamicin which was reported by [31] and [33] might be due to extensive use of gentamicin in human medicine compared with veterinary medicine [34]. The difference can be also due to differences in geographical locations, antimicrobial use policies, and study time.

*Salmonella* isolates were fully developed resistance to amoxicillin and ampicillin. This finding is similar to the research result conducted in Harer [29] and West Showa [35], in which *Salmonella* isolates were 100% resistant for both ampicillin and amoxicillin. In the researches done in Nekemt [36] and Addis Ababa [31], *Salmonella* showed 90% and 80% resistance respectively to amoxicillin. Comparable to my findings, *Salmonella* was 90% resistant to ampicillin in Taiwan [37]. In addition to these reports, resistant *Salmonella* was most frequent to amoxicillin reported in Serbia [38] and to ampicillin in Nigeria [39].

Unlike the result of this study, ampicillin, and amoxicillin was effective to *Salmonella* in the same study area conduct in human hospitals [31]. The difference between these findings can be due to the difference in the study time, animal and human health professionals practice and
extensive use of antibiotics for prophylaxis, anaphylaxis, and metaphylaxis purposes in animal production.

Majority (75%) of the *Salmonella* isolates were susceptible to chloramphenicol with 12.5% intermediate and 12.5% resistant. In addition, half (50%) of the isolates were susceptible to nalidixic acid with 12.5% resistant and 37.5% intermediate. Similarly, *Salmonellae* collected from the diarrhea of feedlot cattle showed 19.1% resistance to chloramphenicol [40]. Comparable to this study, tetracycline revealed 42.41% sensitivity for *Salmonella* in the study conducted by [28] and in the opposite, *Salmonellae* were highly resistant (96.2%) to nalidixic acid in the research done by the same researcher [28]. In contrast to the present study, a high level of resistance to tetracycline (100%), ciprofloxacin (63.6%), and chloramphenicol (45%) were reported in Nigeria [40]. This difference might be due to different antimicrobial using practice between Ethiopia and Nigeria.

Similar to this study, high sensitivity pattern of *Salmonella* to ciprofloxacin and chloramphenicol were reported in Burkina Faso [39], and high level of resistance to tetracycline (95%) and a moderate level of resistance to gentamicin (37%) and nalidixic acid (32%) were reported [39]. This might be due to the widely used practice of these drugs in the country. On the other hand, the main difference among the outcome of the studies is due to the fact that there is strain variation in resistance to a given antibiotic. For instance, *Salmonella enteric serovar paratyphi*-A was Susceptible to gentamicin (100%), chloramphenicol (95%) and tetracycline (92.5%) and *Salmonella enteric serovar Typhi* was Susceptible to gentamicin (97.7%), chloramphenicol (97.5%) and tetracycline (93.2%) [40].

5. Conclusion and Recommendation

Antibiotic resistances for *Salmonellae* isolates are dramatically increasing in different corners of the world. The most aggravating practice for antimicrobial resistance development is the imprudent use of antibiotics in animal production. The high level of antibiotic resistance that was seen in this study can occur as a result of irrational antimicrobial use practices (prophylaxis, anaphylaxis, metaphylaxis and using drugs for growth promotion) in sheep production. Other zoonotic diseases causative agents are expected to develop the same level of resistance with *Salmonella*. The majority (7 out of 10) of the antibiotics tested for the sensitivity of *Salmonellae* have developed resistance. Even some antibiotics (ampicillin and
amoxicillin) were out of use from the treatment of Salmonellosis and others are on the way of losing its curative nature forever. More devastatingly, all resistant *Salmonellae* were developed multidrug resistance. This study showed that unless and other ways we take urgent and integrated measures, the whole antibiotics will be useless in the near future. In this rapid resistance development rate, the currently curable food animal origin zoonotic diseases will be out of control due to a lack of effective antibiotics.

Among the prospective actions that the researchers recommended are the followings. Firstly, multidisciplinary activities among different sectors, actors, and professionals have demanded the mitigation of antimicrobial resistance. Secondly, awareness creation about the severity of antimicrobial-resistant zoonotic diseases risk should be communicated to different stakeholders (animal producers, policymakers and antimicrobial use regulators). Thirdly, appropriate diagnostic equipment should be furnished for reliable and informative antimicrobial resistance surveillance activities. Lastly, further antimicrobial resistance investigations followed by new drug development and facilitating alternative antimicrobial uses, like vaccination and biosecurity measures in animal production are recommended.

6. **Declaration**

6.1. **Ethics approval and consent to participate**

The fecal the sample was collected from sheep that came to the veterinary clinic for the treatment of diarrheal cases in the study area. The researchers asked the owners agreed to take the feces of their sheep for research purposes. For the owners who was not volunteer, I didn’t take feces from their animals. In addition to the owner’s consent, the researchers got permission for access to collect data from the study area by both Wollo and Gondar Universities. Confidentiality of the sample and all other sources were maintained by the research ethics guidelines of the above two universities. So, in this study animals, humans, or tissue from animal or human was not involved directly, but only feces.

6.2. **Data availability statement**

The data used to support the findings of this study are included in the article in the frequency table.
6.3. Competing interest

The author declare that there is no competing interest.

6.4. Funding

Since the research is done based on personal initiatives, anyone didn’t fund my research.

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