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Shalu Choudhary
College of Applied Education and Health Sciences, Meerut, Uttar Pradesh, India

Ayush Tomar
College of Applied Education and Health Sciences, Meerut, Uttar Pradesh, India

Kaish Ansari
College of Applied Education and Health Sciences, Meerut, Uttar Pradesh, India

Nisha Dhillon
College of Applied Education and Health Sciences, Meerut, Uttar Pradesh, India

Lalit Kumar
College of Applied Education and Health Sciences, Meerut, Uttar Pradesh, India

Megha Singh
College of Applied Education and Health Sciences, Meerut, Uttar Pradesh, India

Prakrati Tyagi
College of Applied Education and Health Sciences, Meerut, Uttar Pradesh, India

Sonal Chaudhary
College of Applied Education and Health Sciences, Meerut, Uttar Pradesh, India

Corresponding Author:
Shalu Choudhary
College of Applied Education and Health Sciences, Meerut, Uttar Pradesh, India

Antimicrobial activity of certain drugs against the different isolates found in bovine fecal samples

Shalu Choudhary, Ayush Tomar, Kaish Ansari, Nisha Dhillon, Lalit Kumar, Megha Singh, Prakrati Tyagi and Sonal Chaudhary

Abstract
There are different types of bovine animal excreta having a wide range of micro-organisms such as *Salmonella, E.coli, Bacillus, Clostridium, Bacteroids, Cytophage* etc. Among these microorganisms, *Salmonella* and *E.coli* are the most common bacteria available in bovine fecal samples. The aim of this study was to check the antimicrobial activity of certain drugs against the above-mentioned bacteria. For determining the antimicrobial activity, the bacteria were isolated on Eosin Methylene Blue agar media (EMB agar). Eosin methylene blue agar is used as a selective media for the growth of gram-negative bacteria. The bacteria isolated from the selective media were identified on the basis of their morphological studies and the characterization of bacteria is done by the help of morphological, and biochemical analysis. It was identified that the isolated bacteria were *Salmonella enterica* and *E. coli*. The antibiotic Ampicilin and Fosfomycin gave the maximum inhibitory concentration against these bacteria.

Keywords: Antibiotics, ampicillin, bovine feaces, ciprofloxacin, *E. coli,* fosfomycin *Salmonella*

1. Introduction
Animal excreta are undigested of consumed food materials. Total of 24 different minerals such as nitrogen, potassium and sulfur are found in animal excreta. It is also used in organic farming in many countries. Animal excreta provide increased resistance against pests and diseases. Animal excreta also have been used for cooking purpose in rural areas (Munshi *et al.*, 2018) [1]. It also stimulates plant growth and other beneficial activities such as phosphorus solubilization (Rajeswari *et al*., 2016). A wide variety of microbiomes found in fecal matter of bovine animals which are mainly responsible for food spoilage, water borne diseases (Hailu and Kebede, 2016) [2]. From ancient times, the microbes played a key role in the production of food especially in dairy and beverage industry. Microorganisms also used for nitrogen fixation in legumes, for fermented vegetables (Nain *et al.*, 2020) [3]. In industrialized countries, farmers are increasingly resistant towards the utilization of bovine excreta in the agricultural field. Different sources for the isolation of *Salmonella* and *E.coli* such as sewage water, spider, human beings and bovine etc. Bovine sample are easily available and easy to isolates the microbes. The gastroinstenial tract of animals contains enteric microbes that shown toxious affects. *Salmonella and E. coli* are common pathogens of the human and animal gut, but some species also capable produce infectious disease (Kibert *et al*., 2011). Pathogenic bacteria such as *E.coli* and *Salmonella* present in bovine gastrointestinal tract and cause outbreak of disease by consumption of contaminated food and water.
Microbial communities such as bacteria and fungi are very helpful in decomposition of organic matter, composting, degradation and recycling of agricultural and livestock wastes (*Devanoori*, 2020). Hervibores bovine animal excreta, passed from its gastrointestinal tract excrete undigested residues of consumed matter. Bovine animal excreta contain nutrients such as nitrogen, and phosphorus, organic matter, pathogens and volatile compounds (Teo and Teoh, 2011) [4]. Variety of pathogenic microbial species has been identified in different animal species, which are distributed across the world. Excreta of animal contains undigested residue of food material. In animal excreta the numerous species of gram negative bacteria such as *salmonella* and *E.coli*, etc. are present (Iovine *et al.*, 2015) [5]. It also contains facultative gram-negative bacteria (like *Klebsiella*) and some identified & non-identified fungi (*Aspergillus spp.*) and yeast (*Candid spp.)* (Seyedmousavi *et al.*,2018; Freitas *et al*., 2014) [6, 7]. Salmonella present in the environment, able to easily survive and proliferate, but not capable in causing diseases (López *et al.*, 2016) [8]. *E. coli* measures as 2.0 micrometer long and 0.25-1.0 micrometer in diameter and *Salmonella* is 2 to 5 micrometer long & 0.7 -1.5 micrometer in diameter. (Tenaillon *et al.*, 2010) [9].

Salmonella are commensal bacteria & *E.coli* is intracellular pathogens. *Salmonella* and *E. coli* both are coliform bacteria. Some strains of these bacteria cause infection such as cholera & food poisioning. *Escherichia coli* and *Salmonella* diverged from a common ancestor. *Salmonella enterica* is a micro-organism with high zoonotic and affecting different animal species (Atwill *et al*., 2012) [10]. After eating food or drinking water contaminated with the Salmonella typhi bacteria or E.coli, the bacteria move down into the digestive system, where they will quickly multiply. Left untreated, the bacteria can get into the blood stream and spread to other areas of the body. If organs and tissues became damage as the result of the new infection, it can cause serious complications such as internal bleeding (S. Rajan, 2017) [27]. Spreading of many classical human diseases such as Cholera, typhoid fever has been controlled through improvement in personal hygiene.
Many Salmonella isolates exhibited resistance to tetracycline, nalidixic acid and streptomycin. Most *E.coli* isolates were susceptible to all antimicrobial drugs. Many E.coli isolates exhibited resistance to tetracycline, streptomycin and gentamycin (Musgrave *et al.*, 2006) [26]. In this study, Salmonella enterica serovars from different origin were isolated and their antimicrobial susceptibility was determined. Different types of antibiotics used against isolated bacteria. The aim and objective of this research is the isolation, identification, morphological and biochemical characterization of micro-organisms from animal excreta whereas to check the antimicrobial activity of drugs against isolated micro-organisms.

2. Material and Method

2.1 Collection of samples

In this research, the sample of different types of animal excreta (horse, buffalo, cow and from goat) were collected in different sterilized container. All samples were instantly transported to the microbiology laboratories. Each sample (10.0g) was homogenized with 40ml of sterile distilled water to prepare a stock solution.

2.2 Isolation of bacteria

The eosine methylene blue media was prepared for the isolation of bacteria. It's a selective media for gram negative bacteria, this media contains dyes that is toxic for gram positive bacteria, so that it inhibits the growth of gram-positive bacteria. The animal's excreta were collected in a sterilized container from different animals. The bovine sample was serially diluted by taking 10 test tubes labelled from 0-9 containing 9 mL autoclaved distilled water. 1 gm of sample was dissolved in 1mL of autoclaved distilled water in separate test tube from which 1mL was transferred to test tube with dilution factor 10^{-3}, 10^{-5} and 10^{-7} were spread on EMB (Eosine methylene blue agar) plates for the growth of bacteria. After that the petri plates were incubated at 37^0 C for 24 hours in an incubator.

2.2.1 Sub-Culturing of isolates

Subculturing was accomplished by streak plate method on EMB agar plates to obtain pure or single culture of the specific colony and incubated at 37°C for 24 hours.

2.3 Characterization and identification of micro-organisms:

After isolating the pure colony of bacteria were identified by gram staining method. On the basis of this, the morphological characteristics were done.

2.3.1. Morphological Characterization of colony

Microorganism produces definite patterns on culture media plates as they grow and divide. Morphological characterization is done on basis of size, shape, colour, texture and the general structure of an individual colony of a particular microorganism on a culture media plate that support its growth. Colonial morphology which can also be referred to as cultural characteristics pertains to the microscopic appearance of a bacterium on different kinds of growth culture media. In this study, the morphology of isolated colonies was varied such as irregular, spindle, round, and entire in shape and these colonies were appeared brown, white, milky, and orange in colour.

2.3.2 Gram's Staining of bacteria

In this staining, the bacteria are classified into two groups on the basis of colour that bacteria are gram positive (purple colour) and gram negative (pink colour). We used four reagents in this staining such as crystal violet (primary stain), gram's iodine (mordent dye), decolorized, and safranin (counter stain). A smear was made on a clean slide and heat fixed, 1-2 drop of crystal violet for 60seconds were added and washed by distilled water drop wise. Then 1-2 drop of gram's iodine for 60seconds were added and washed by distilled water. Decolourizer or ethanol for 20-30 seconds were added and washed by distilled water. Then Safranin was added fir 60seconds and washed. Extra water was removed with the help of tissue paper and left the slide to dry. Examine the slide under microscope at 100X.

2.3.4. Biochemical Characterization of bacteria

For biochemical characterization three methods were performed for identification of bacteria.

2.3.3.1. Catalase test

In this test, 1-2 drops of hydrogen peroxide solution were poured on slide. Isolated colony of bacteria was taken with the help of a sterile inoculating loop and immersed in the hydrogen peroxide solution. The slide was left for bubbling.

2.3.3.2. Coagulase Test

In this test, one drops of normal saline solution was taken on a clean slide and a single isolated colony was mixed in saline solution and left for few minutes for cloting.

2.3.3.3. IMVIC Test

IMVIC reactions are a set of four useful reactions that are commonly employed in the identification of members of family Enterobacteriaceae. A series of tests used to study the physiological characteristics of bacteria from the family Enterobacteriaceae, especially Escherichia and Enterobacter. They are designed to differentiate Gram – negative intestinal bacilli of family Enterobacteriaceae which contains a large number of genera that are biochemically and genetically related to one another. IMVIC tests consist of four different tests each of the letters in "IMVIC" stands for one of these tests. I- Indole, M – Methyl red, V- Voges- Proskauer, C-Citrate.

2.3.3.3.1. Indole test

Some bacteria can produce indole from amino acid tryptophan using the enzyme tryptophanase. Production of indole is detected using Ehrlich's reagent or Kovac's reagent. Indole reacts with the aldehyde in the reagent to give a red colour.

An alcoholic layer concentrates the red colour as a ring at the top.

2.3.3.3.2. Methyl red (MR) test
This is to detect the ability of an organism to produce and maintain stable acid end products from glucose fermentation. Some bacteria produce large amounts of acids from glucose fermentation that they overcome the buffering action of the system. Methyl Red is a pH indicator, which remains red in colour at a pH of 4.4 or less.

2.3.3.3.3. Voges Proskauer (VP) Test
While MR test is useful in detecting mixed acid producers, VP test detects butylene glycol Acetyl-methyl carbinol (acetone) is an intermediate in the production of butylene glycol. In these test two reagents, 40% KOH and alpha-naphthol were added to test broth after incubation and exposed to atmospheric oxygen. If acetone is present, it is oxidized in the presence of air and KOH to diacetyl. Diacetyl then reacts with guanidine components of peptone, in the presence of alpha- naphthol to produce red colour. Role of alpha-naphthol is that of a catalyst and a colour intensifier.

2.3.3.3.4. Citrate utilization Test
This test detects the ability of an organism to utilize citrate as the sole source of carbon and energy. Bacteria are inoculated on a medium containing sodium citrate and a pH indicator bromothymol blue. The medium also contains inorganic ammonium salts, which is utilized as sole source of nitrogen. Utilization of citrate involves the enzyme citritase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and CO_2. Production of Na_2CO_3 as well as NH_3 from utilization of sodium citrate and ammonium salt respectively results in alkaline pH. This results in change of medium's colour from green to blue.

2.4 Antibiotic Sensitivity Test
The antibiotic sensitivity test was done using the different antibiotic drugs such as Ciprofloxacin, Tigecycline, clositin, ampicillin, fosfomycin, and doxycycline. The bacterial suspension of *salmonella* and *E.coli* were prepared and 0.1ml of sample was speeded separately on the EMB media. Then antibiotic disc was applied over it. Kept the plates on incubator at 37^0 C for 24 hours.

2.5 Natural Sensitivity Test
Many fruit extracts have been shown to be effective at killing or inhibiting the growth of bacteria. Some fruit extracts have been shown to inhibit the adhesion of bacteria. These include bel fruit, black currant, grapes, and cranberry. Natural antibiotics are the most common drugs used to kill or inhibit bacterial growth. Bel fruit has been widely recognized for it is antibacterial and anti-inflammatory properties. It can reduce the growth of many diseases causing bacteria. Bel fruit is effective in killing several harmful, including Salmonella and *E.coli*.

3. Result
Microbial antibiotic resistance has been confessing as an emerging worldwide problem in human beings in developed and developing countries. In this study, we identified that the presence of *Salmonella* with high amount in different types of animal excreta (Cow, goat, Buffalo, Horse) as compared to other bacterial microbiota such as *E.coli, Pseudomonas* and also some unidentified microbes.

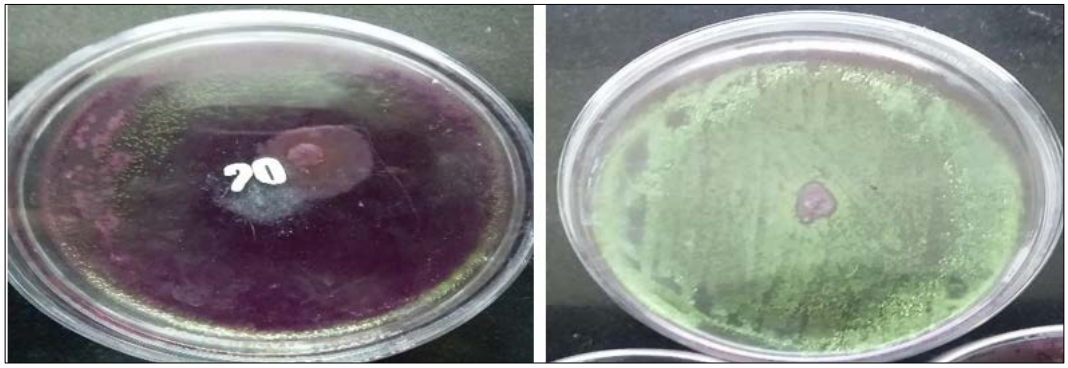
3.1 Collection of samples: collected the bovine sample of different animals such as cow bovine, goat bovine, buffalo bovine, and horse bovine sample (figure 1) in sterilized containers and transferred it into laboratory.

Fig 1: Samples of different animal bovine samples in sterilize container. (a) Cow bovine sample, (b) Goat bovine sample, (c) Horse bovine sample, and (d) Buffalo bovine sample.

3.2 Isolation of bacteria: Isolated bacteria grow on eosin methylene blue (EMB Agar). These media are differential media for gram negative bacteria. On this media, the *Salmonella* gives pink colour colony and *E.coli* gives metallic green sheen colony(figure2). The growth of *Salmonella* and *E.coli* is equal in all plates, but in the sample of horse produce high population of *Salmonella* on agar plates as compared to other plates. Isolated colony is given in table 1.

Table 1: Composition of Eosin methylene blue (EMB agar)

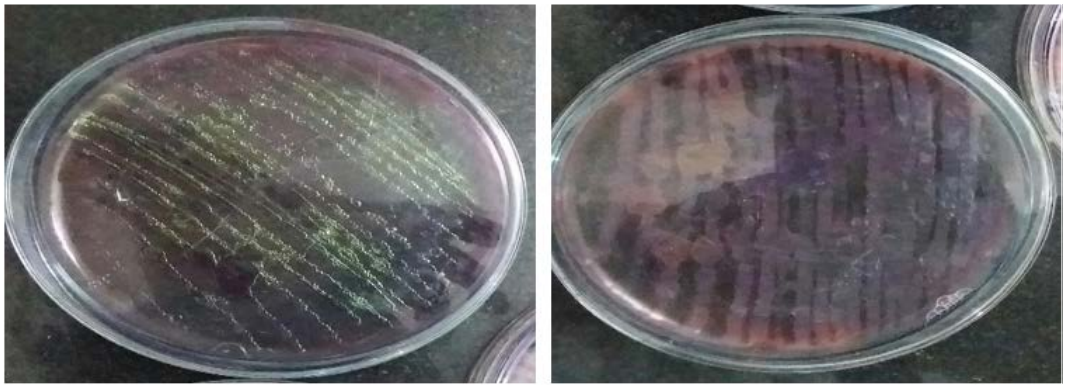
Peptone	10.0gm
Lactose	05.0gm
Dipotassium	02.0gm
Methylene blue	00.55gm
Agar	15.00gm
Distilled water	1000ml



A B

Fig 2: Subculturing of isolated bacteria: (A) *E.coli* and (B) *Salmonella*

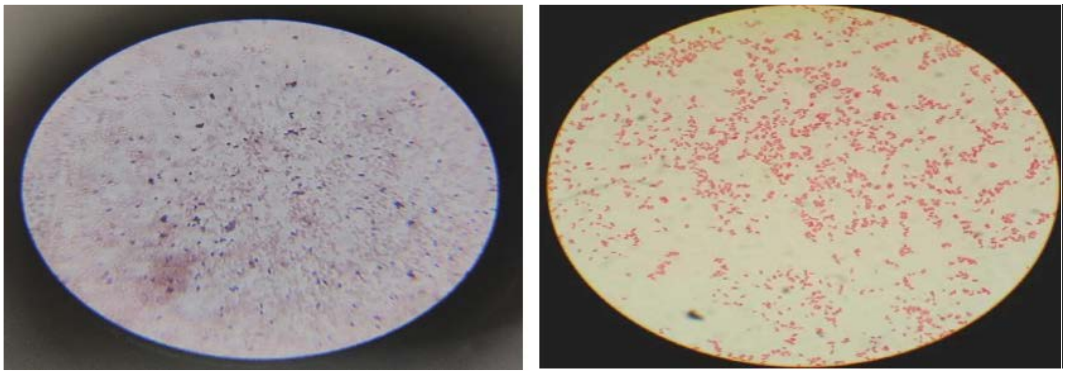
3.3 Subculturing the isolated colony: After isolation of the bacteria, single colony of *Salmonella* and *E. coli* individually streak on EMB agar plates (figure 2).



A B

Fig 3: Subculturing of isolated bacteria: (A) *E.coli* and (B) *Salmonella*

3.4 Morphological Characterization of bacteria:
Isolated bacteria given different structure and morphology in identification and microscopic examination. After microscopy, Salmonella and *E.coli* were gram negative in rod shape (figure 3). Colony characteristics of these bacteria is differed in margine, elevation and texture (table 3). On the basis of culture characteristics and gram staining, the large numbers of salmonella bacteria were founded in horse than cow, goat and buffalo (Table No 2). *E.coli* was present in all animal excreta. The *Salmonella* gives pink colour colony and *E.coli* gives metallic green sheen on EMB media.



A B

Fig 4: Gram Staining of Isolated Bacteria (A) *E.coli* and (B)*Salmonella*

Table 2: Bacterial growth on different Animal Excreta

Bacteria	Cow	Ox	Buffalo	Horse
E.coli	+	+	+	+
Salmonella	+	+	+	++

+ Determines the moderate growth
++ Determines the high growth

Table 3: Morphological Characteristics of *E.coli* and *Salmonella.*

S. No.	Gram's Stain	Bacteria	Shape	Margine	Colony Colour	Elevation	Texture
1.	Pink colour	*E.coli*	Rod	Entire	Metallic green sheen	Slightly raised	Off white, dry
2.	Pink colour	Salmonella	Rod	Smooth	Pink	Convex	Slimy, moist

3.5 Biochemical Characterization of micro-organisms:
In biochemical characterization, *Salmonella* and *E.coli* given positive & negative reaction in different test (table 4). *Salmonella* and *E.coli* shown catalase positive, coagulase negative, indole positive, MR positive, citrate negative & in citrate E.coli shown negative and salmonella given positive reaction (figure 4).

Table 4: Examination of biochemical test.

S. No.	Biochemical test	*E.coli*	*Salmonella*
1.	Catalase test	Positive	Positive
2.	Coagulase test	Negative	Negative
3.	Indole test	Positive	Positive
4.	MR test	Positive	Positive
5.	VP test	Negative	Positive
6.	Citrate test	Negative	Negative

A B C D E

Fig 5: Biochemical test: A) Catalase test, B) Coagulase test, C) Citrate & Indole test, D) VP test and E) MR test.

3.6 Effect of Antibiotics: Different types of antibiotics were used against isolated bacteria *Salmonella* and *E.coli* the antibiotics like Ciprofloxacin, Clositin, were work against non-tyophoid *Salmonella.* while Ampicillin, Tigecycline, Fosfpmycin, and Doxycycline shown resistant against non-typhoid *Salmonella.* the antibiotics Tigecycline Ampicillin, and Doxycycline shown resistant against *E.coli.* the antibiotics Ciprofloxacin, Clositin, and Fosfomycin, shown susceptibility against *E.coli*. The Antibiotics Ciprofloxacin and Clositin shown senstivity against *E.coli* and *Salmonella* whereas Tigecycline, Ampicillin, and Doxycycline shown resistant against E.coli and Salmonella (table 5).

Table 5: Effect of Antibiotic on *E. coli* and *Salmonella.*

Bacteria	Ciprofloxacin	Tigecycline	Clositin	Ampicillin	Fosfomycin	Doxycycline
E.coli	+	-	+	-	+	-
Salmonella	+	-	+	-	-	-

- Determines the resistant against *E. coli* and *Salmonella*
+ Determines the susceptibility against *E. coli* and Salmonella

Fig 6: (i) Antibiotic Sensitivity of *E.coli* on antibiotic Ciprofloxacin and Fosfomycin

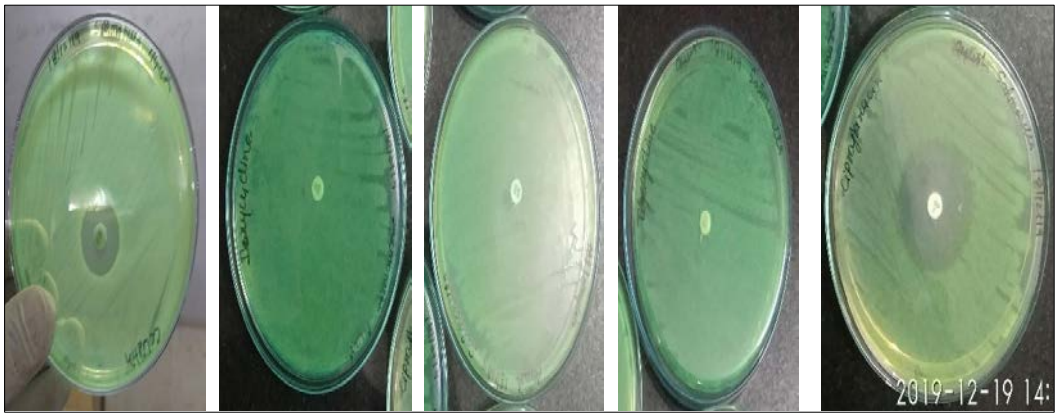


Fig 7: (ii) Antibiotics Sensitivity of Salmonella on antibiotics Clositin, Doxycycline, Fosfomycin, Tigecycline and Ciprofloxacin.

4. Discussion

According to the standard operational procedure, Antibiotics play a major role for the prevention of bacterial infections; it inhibits or kill the growth of microbes. Basically, we used antibiotics to prevent the infection. The used of antibiotics we clarify that whom antibiotics has resistance power against it & which antibiotics suggest for further infection. Natural antibiotics also play a key role to kill or inhibit the growth of bacteria. So, we used some antibiotics for checked the antibacterial activity of bacteria.

Salmonella and *E.coli* has increased worldwide. In this study, isolation rate of *E.Coli* was found maximum than *Salmonella*. This information is confirmed with reports by other researchers (Kibert *et al.*, 2011). In some clinical samples, *E.coli* showed high resistance power to tetracycline, amoxicillin. The resistance rate recorded as compare to other study (Khan *et al.*, 2002) [17] and low resistance power of antibiotics. Antimicrobial resistance rate was found higher as compared to susceptibility patterns reported from previous studies (Kibert *et al.*, 2011). *Salmonella* was sensitive to Tigecycline, and ampicillin. Similar reports conducted in different part of world (Bharathi *et al*., 2008) [21]. In some studies norfloxacin, ciprofloxacin, were found to be the most effective against *Salmonella* & *E.coli*. Furthermore, in other study, a high rate of antimicrobial resistance was recorded, and the report of studies done elsewhere.

5. Conclusion

In the present study, animals were the main reservoirs *Salmonella* or other microbes. Non-typhoid *Salmonella* has importance on zoonoses and health impact. The highest percentage of *Salmonella spp.* were isolated from different animal excreta. It was found that various species of gram positive bacteria were present in animal excreta such as *Bacillus*, *Enterococcus*, *Diplococcus* and some gram negative bacteria such as *Salmonella*, *E.coli* and *Pseudomonas* etc. Some identified & non-identified fungi and yeast were also present in animal excreta. Some bacterial antibiotics play a major role to inhibit the growth of microorganism. Further, this research would be helpful in the investigation of bovine gastrointestinal micro flora and their antimicrobial susceptibility. So that's why we can use these antibiotics in these infections which caused by *Salmonella* & *E.coli*.

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