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Analysis of Multidrug Resistance Profile of *Escherichia coli* from Clinical Samples from Companion Animals and Bird Retrospect to Five-year (2015-2019) Literature Data

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Abstract: Based on WHO 2017 data of death rate among the children of aging under five years, a diarrheal disease in post-neonates and sepsis in neonate posse second and third major concern, respectively. Though Group B *Streptococci* infection is a primary etiological agent, *Escherichia coli* infection is the major cause of mortality. Multidrug resistance in *E. coli* was studied from companion animals, addressing zoonotic disease transfer possibilities to their handler or in the community via direct or indirect contact or through contaminated food or water. Out of 100 samples cultured, 78 bacterial pathogens were isolated, from which 29 (38%) isolates were *E. coli*, identified using IMViC and confirmed by Vitek2. Antibiotic susceptibility against 42 antibiotics belonging to 12 different antimicrobial categories was performed by the Kirby-Bauer method of disk diffusion assay. By using WHONET software, an antibiogram was deduced and found that 23 (80%) isolates were multidrug-resistant (MDR) and 4 (13%) were possible extensively drug-resistant (possible XDR). Comparison of resistance data to the literature data of the period 2015-2019 supplemented with details of susceptibility either in the form of disc diffusion test or minimum inhibitory concentration (MIC) was carried out to understand the current scenario of drug resistance in *E. coli* of non-human origin.

Keywords: multidrug resistance; MDR; *Escherichia coli*; animal pathogen; zoonotic transfer; antimicrobial susceptibility.

1. Introduction

Escherichia coli is an opportunistic pathogen reportedly accounting for approximately 8.8% of the total mortality rate in India [1] due to Multiple Drug Resistance (MDR) or Extensively Drug Resistance (XDR) urinary tract infections as well as *E. coli* associated diarrhea [2]. The presence of Extended Spectrum Beta-Lactamase positive or pathogenic MDR *E. coli* in samples from companion animals [3], milk of cows, pigs [4], poultry species [5,6], and even in the captive wildlife found in zoos and wildlife enclosures [2] was studied previously. Pathogens can transfer between human and their companion animals, birds, or livestock [7] even at their healthy state [8] is a known fact. One recent multi-country study was conducted to amputate antibiotics' usage and their corresponding resistance to *E. coli*. According to this study, the use of aminoglycosides, carbapenem, fluoroquinolones, and glycopeptide antibiotics are lower in India than in other countries. However, the percentage of resistance against them is higher. Even the study of a significant relationship between MDR or XDR *E. coli* infection and mortality rate in India found that as compared to non-MDR *E. coli*

infections, the odds of mortality were 2.63 times higher for MDR *E. coli*, 2.23 times higher for ESBL positive *E. coli,* and 2.43 times higher for XDR *E. coli* (at 95% Confidence Interval) [9]. According to the Veterinary Guideline (VET08), the Clinical and Laboratory Standard Institute, resistant or intermediate isolates may pose epidemiological threats to the particular region [10]. Hence, this study is designed to isolate *E. coli* and study their antibiotic sensitivity to categorize isolates to understand the current scenario in the locality as a part of one health approach.

2. Materials and Methods

A study design includes analyzing the pattern of antimicrobial resistance in *Escherichia coli* isolated from wounded or infectious companion animals and birds, including cattle and dogs in the South Gujarat region. No animals were killed or harmed during sampling. Samples were either procured from a microbiological laboratory or collected under a veterinarian's supervision from the clinic or in the stable by a trained laboratory technician. All the data obtained is solely for research purposes only. No treatment was deduced from the research to the animals.

Samples collected, including urine, feces, milk, tissue, a swab from infection site or wound, nasal cavity, or pus, were brought up in the icebox to the laboratory. Isolation of aerobic bacterial pathogens was performed within three hours as per standard procedures by streaking on Nutrient agar, MacConkey's agar, Blood agar, and Chocolate agar followed by incubation at 37ºC for 24 - 48 h.

Primary identification of all the isolates was carried out by standard microbial culture-based tests including morphological, colonial characteristics, IMViC test, and Sugar fermentation tests, followed by specialized tests – Haemolysis test Blood Agar (BA) and Eosin Methylene Blue (EMB) agar. Results of biochemical tests were subjected to an online taxonomic identification tool - "ABIS /REGNUM PROKARYOTAE" [11] to get probable isolate details, which were then confirmed by automated identification system VITEK 2. *Escherichia coli* NCIM 2645 and *Escherichia coli* NCIM 2065 strains were used as positive control while samples from infectious animals, yet giving no bacterial growth, serve as a negative control for samples.

The antimicrobial susceptibility of each isolate was carried out in Muller – Hinton (MH) Agar by Kirby-Bauer method of disk diffusion test [12]. Single disks of 42 different antibiotics (HiMedia) belonging to 12 different antimicrobial classes were tested following CLSI (Clinical Laboratory Standard Institute) Vet08 Guideline [10], and zone diameter in millimeter were noted down after incubation at 35 ºC ± 2 ºC for 16-18 hours. A new manual library was prepared in the WHONET 2019 software [13], to which antimicrobial susceptibility data were feed to obtain the sensitivity results. Characterization of each isolates based on the susceptibility pattern into multidrug resistance (MDR) or extensively drug resistance (XDR) was done by following the definition given by Magiorakas *et al*. [14] and WHONET 2019 software.

For the literature analysis, research papers from PubMed were screened for 2015 to 2019, showing antimicrobial resistance analysis from domestic or wild animals or birds along with food sources to judge the current antimicrobial resistance scenario in *E. coli*. Inclusion criteria were the research paper supplemented with the susceptibility data either in the form of disk diffusion or minimum inhibitory concentration (MIC) to deduce the category of resistance phenotype by following the definition of Magiorakas *et al*. [14].

3. Results and Discussion

3.1. Sampling, isolation, and identification of a pathogen.

In the first stage of sampling, 100 bacterial isolates were obtained, both gram-negative and gram-positive bacteria, out of which N=35 (46%) isolates were gram-negative pathogens. (Figure 1) They were further categorized as *Escherichia coli* (N=29, 38%) using microbial culture-based techniques as follows: positive catalase test, negative oxidase test, positive indole production, positive methyl red test, negative Voges-Proskauer test, negative citrate test (IMViC), positive hemolysis on blood agar and metallic sheath on EMB (Eosin Methylene Blue) agar with motile, gram-negative bacilli or cocci bacilli. These results, along with sugar fermentation tests, were subjected to an online taxonomic identification tool – ABIS/Regnum Prokaryotae [11] for probable isolate identification and confirmation of isolates were done by VITEK® 2 (BioMériux, USA). Other gram-negative isolates were *Pseudomonas aeruginosa* (N=2, 3%), *Acinetobacter sp*. (N=1, 1%), *Enterobacter sp*. (N=1, 1%) and *Klebsiella aerogenes* (N=2, 3l%).

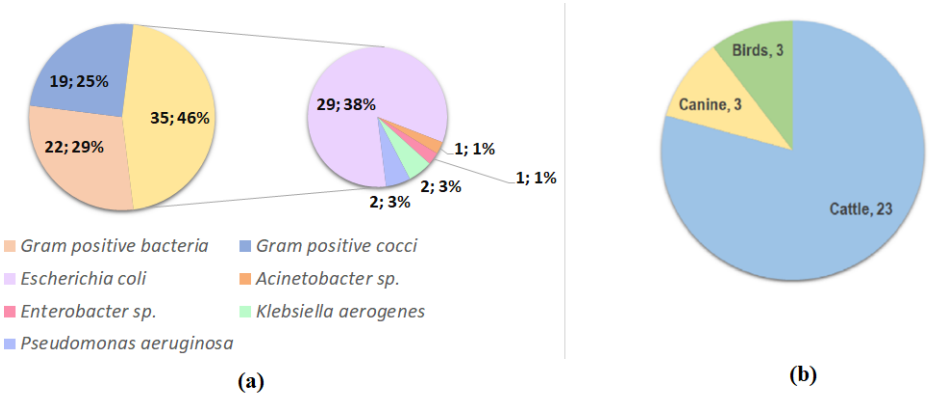


Figure 1. Isolate details: (**a**) Total percentage of isolates (samples, N=100); (**b**) Number of *Escherichia coli* isolates (N=29) according to the source of isolation.

3.2. Antibiotic susceptibility analysis in WHONET 2019.

By using an updated definition of WHONET 2019 Software, from the zone of inhibition (in mm) (Figure 2), sensitivity results were obtained, showing that 63.6% isolates were possible ESBL (Extended Spectrum Beta-lactamase) producers (Figure 3). Maximum resistance was found against streptogramin class of antibiotic combination – Quinupristin/Dalfopristin (60%), which is known to be used to treat infection of various enteric bacteria including *E. coli* and *Shigella flexneri* along with *Staphylococci* and Vancomycin-Resistant *Enterococcus faecium* [15]. Nearly 50% of isolates were resistant to Cefaperazone (55.2%), clarithromycin (53.8%), ampicillin (48.3%), followed by fluoroquinolone (Ciprofloxacin, Levofloxacin), tetracyclines (Doxycycline), and trimethoprim-Sulfamethoxazole (34.5% each). These antibiotics with veterinary usage approval showed little lower resistance than other studies from India [16, 17, 18]. However, the presence of carbapenem-resistant from *E. coli* isolates from bovine mastitis alongside MRSA (Methicillin-Resistant *Staphylococcus aureus*) is supporting our findings of Carbapenem-resistant *E. coli* isolates (13.8%), which is still a higher case than previously reported by Bandyopadhyay *et al*., 2015 [19]. Colistin was resistant in 9.1% isolates, with a minimum 3.4% resistance in aminoglycosides and chloramphenicol. Based on these sensitivity data, the percentages of MDR and possible XDR *E. coli* isolates were 80% (N=23) and 13%

(N=4), respectively (Figure 4), which is relatively higher for animal data than the findings in earlier studies [20]. A study on pattern analysis in *E. coli* from domestic and wild fecal samples related to human septage and sewage water showed resistance against tetracycline, cephalothin, and sulfamethoxazole, and streptomycin [21] was common, supporting our research data.

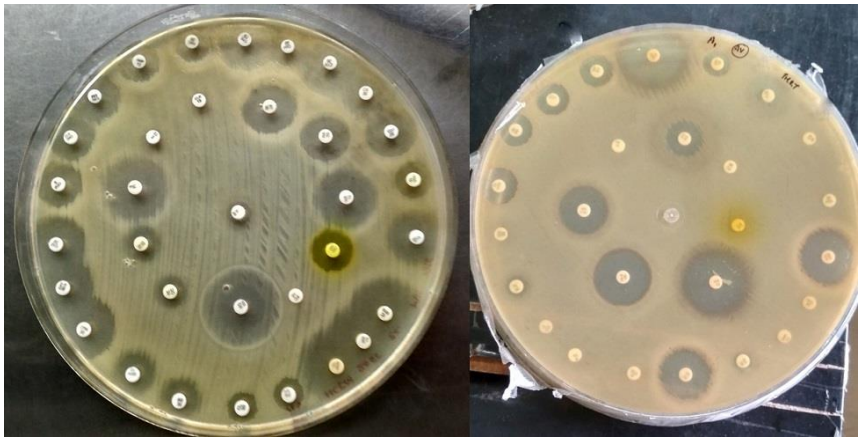
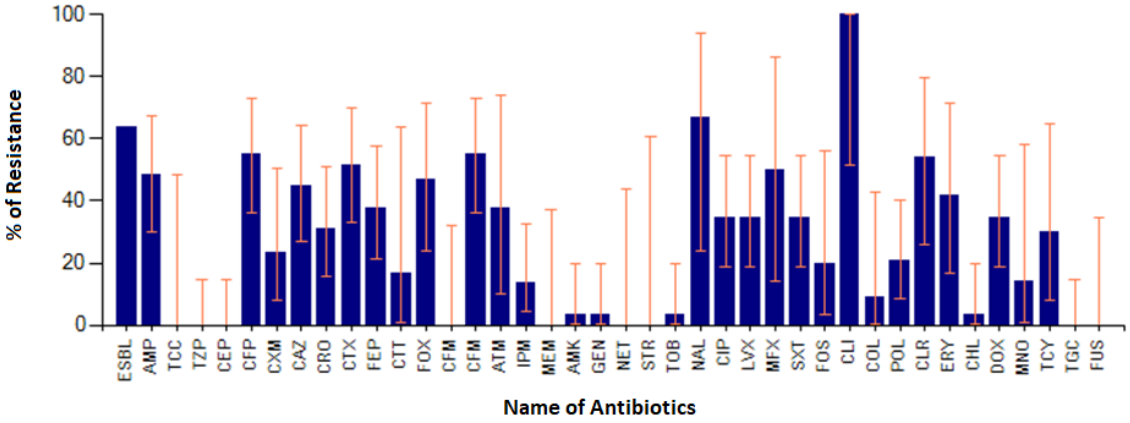


Figure 2. Antibiotic susceptibility results by disk diffusion method in *Escherichia coli* isolates.



KEY: AMP: Ampicillin, TCC: Ticarcillin-Clavulanic acid, TZP: Piperacillin-Tazobactam, CEP: Cephalothin, CFP: cefpirome, CXM: Cefuroxime, CAZ: Ceftazidime, CRO: Ceftriaxone, CTX: Cefotaxime, FEP: Cefepime, CTT: Cefotetan, FOX: Cefoxitin, CFM: Cefixime, ATM: Aztreonam, IPM: Imipenem, MEM: Meropenem, AMK: Amikacin, GEN: Gentamicin, NET: Netillin/Netilmicin, STR: Streptomycin, TOB: Tobramycin, NAL: Nalidixic acid, CIP: Ciprofloxacin, LVX: Levofloxacin, MFX: Moxifloxacin, SXT: Trimethoprim/Sulfamethoxazole, FOS: Fosfomycin, CLI: Clindamycin, COL: Colistin, POL: Polymyxin B, CLR: Clarithromycin, ERY: Erythromycin, CHL: Chloramphenicol, DOX: Doxycycline, MNO: Minocycline, TCY: Tetracycline, TGC: Tigecycline, FUS: Fusidic acid

Figure 3. Percentage antibiotic resistance in *Escherichia coli.*

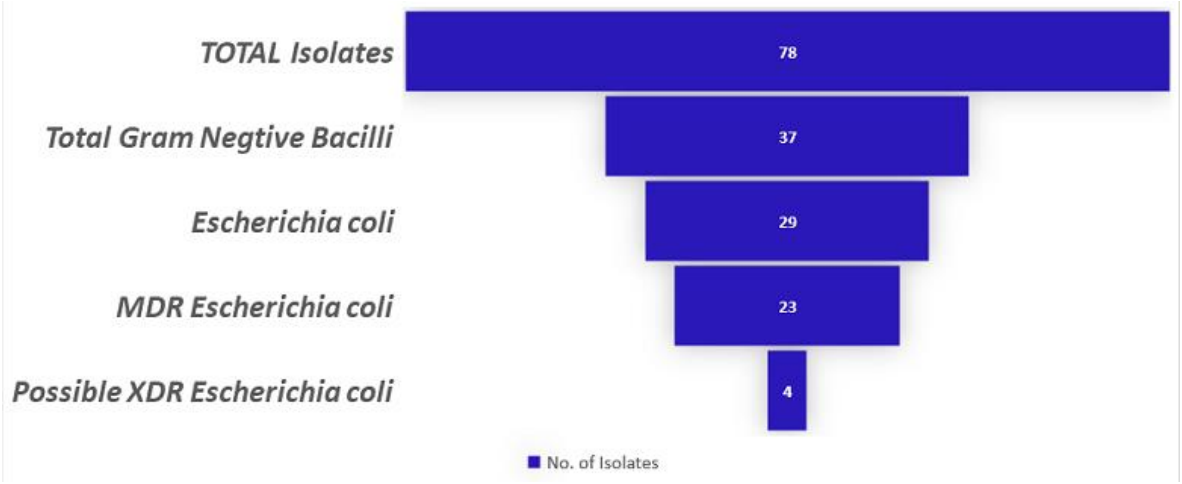


Figure 4. Categorization of *Escherichia coli* isolates into MDR and possible XDR using WHONET software by following the definition given by Magiorakas *et al.* [14].

3.3. Retrospective literature analysis (2015-2019).

Literature available on NCBI PubMed platform was screened for antimicrobial resistance in domestic or wild animals and birds or food sources. Twenty-two research papers (Table 1) were selected satisfying the following inclusion criteria: research papers published between the years 2015 and 2019 with antimicrobial susceptibility records for *Escherichia coli* either in the form of the zone of inhibition (ZOI) or minimum inhibitory concentration (MIC).

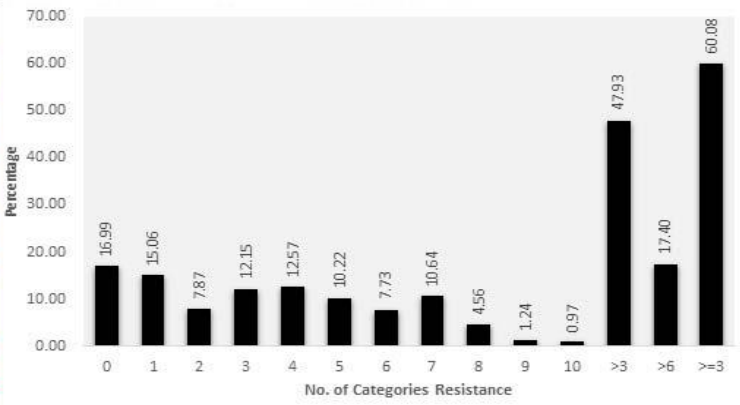
Table 1. Literature studied involving antimicrobial resistance analysis in domestic and wild animals and birds.

No	Year	Title	Reference
1	2015	Isolation of *Escherichia coli* and *Salmonella sp.* from free-ranging wild animals	[22]
2	2015	Application of swine manure on agricultural fields contributes to extended-spectrum ß-lactamase producing *Escherichia coli* spread in Taiwan, China	[23]
3	2015	Prevalence of Extended-Spectrum Cephalosporin - Resistant *Escherichia coli* in a farrowing farm: ST1121 clone harbouring IncHI2 plasmid contributes to the dissemination of blaCMY-2	[24]
4	2015	Widespread distribution of CTX-M and plasmid-mediated AmpC β-lactamases in *Escherichia coli* from Brazilian chicken meat	[25]
5	2016	Emergence of antimicrobial-resistant *Escherichia coli* of animal origin spreading in humans	[26]
6	2016	Diversity of *Escherichia coli* strains involved in vertebral osteomyelitis and arthritis in broilers in Brazil	[27]
7	2016	Extended-spectrum β-lactamase producing *Escherichia coli* isolated from wild birds in Saskatoon, Canada	[28]
8	2017	Virulence and transcriptome profile of multidrug-resistant *Escherichia coli* from chicken	[29]
9	2017	Probable secondary transmission of antimicrobial-resistant *Escherichia coli* between people living with and without pets	[30]
10	2017	Occurrence of *Escherichia coli* O157:H7 in cattle feces and contamination of carcass and various contact surfaces in abattoir and butcher shops of Hawassa, Ethiopia	[31]
11	2017	Characteristics of *Escherichia coli* isolated from broiler chickens with colibacillosis in commercial farms from a common hatchery	[32]
12	2017	Biofilm formation potential of heat-resistant *Escherichia coli* dairy isolates and the complete genome of multidrug-resistant, heat-resistant strain FAM21845	[33]
13	2017	Identification of atypical enteropathogenic *Escherichia coli* O98 from golden snub-nosed monkeys with Diarrhoea in China	[34]
14	2017	Characterization and zoonotic impact of Shiga toxin-producing *Escherichia coli* in some wild bird species	[35]
15	2017	Plasmid-mediated novel blaNDM-17 gene encoding a carbapenemase with enhanced activity in a sequence type 48 *Escherichia coli* Strain	[36]
16	2017	*Colis*tin resistance gene mcr-1 and its variant in *Escherichia coli* isolates from chickens in China	[37]
17	2017	High prevalence of CTX-M-15-Type ESBL-Producing E. *coli* from migratory avian species in Pakistan	[38]
18	2018	Molecular analysis of Shiga toxin-producing *Escherichia coli* o157:H7 and non-o157 strains isolated from calves	[39]
19	2018	Antibiotic-resistant *Escherichia coli* and class-1 integrons in humans, domestic animals, and wild primates in rural Uganda	[40]
20	2019	Comparative genomic analysis of 127 *E. coli* strains isolated from domestic animals diarrhea in China	[41]
21	2019	High incidence of multidrug-resistant *Escherichia coli* co-harbouring mcr-1 and blaCTX-M-15 recovered from pigs	[42]
22	2019	Virulence gene profiles, antimicrobial resistance, and phylogenetic groups of fecal *Escherichia coli* strains isolated from broiler chickens in Algeria	[43]

These twenty-two research paper includes 724 samples of animal pathogen whose drug susceptibility data were taken into account and antibiotics were categorized under following classes same as used in this same research: aminopenicillin, aminopenicillin + inhibitors, cephalosporins, aminoglycosides, phenicol, tetracyclines, folate inhibitors, monobactam, quinolones, fluoroquinolones, polymyxins, and macrolides. Manual screening for categorizing literature data by following the same definition reported that more than 60% of isolates were MDR. Additionally, 17.40% of isolates were resistant to more than six antimicrobial categories with 123 pan-susceptible and 172 isolates resistant to less than two antimicrobial categories (Figure 5).

Categories Resistant	No. of Isolates
0	123
1	109
2	57
3	88
4	91
5	74
6	56
7	77
8	33
9	9
10	7
>3	347
>6	126
>=3	435

(a)



(b)

Figure 5. Drug resistance isolates from literature data: (a) Numbers of isolate resistant to the respective numbers of antibiotic category. Resistance against a maximum of ten categories of antimicrobials was noted out of a total of 724 isolates; 435 isolates were found resistant to more than or equal to 3 categories (MDR), while 126 isolates were resistant to more than six antimicrobial categories. (b) Graphical representation of resistance isolates in percentage.

By considering MDR isolates, there were 164 different resistant patterns found for 429 isolates. Over the five-year period, the percentage resistance in beta-lactam class of antibiotics were found increased tremendously in birds (12.50 % - 92.65%), cattle (4.75% - 48.15%), chicken (37.50% - 85.19%), sheep - goat (2.50% - 21.82%) and even swine (35.71% - 40.28%) (Figure 6). Same were the cases with tetracyclines (9.52% - 96.97%) and sulphonamides/folate pathway inhibitors (9.52% - 87.88%) in different species. However, a sharp decrease in tetracycline resistance in swine (96.97% - 66.67%) was reported in 2019. In chicken, aminoglycosides resistance was recorded highest in 2017 (81.74%), which later on found decreased to 55.88% (2018). The opposite was the case with the quinolone resistance, which decreased at first from 80.00% (2016) to 77.39% (2017), which again increased tremendously to 97.06% (2018). Polymyxin resistance was 55.65 % and 69.70% in chicken (2017) and swine (2018), respectively.

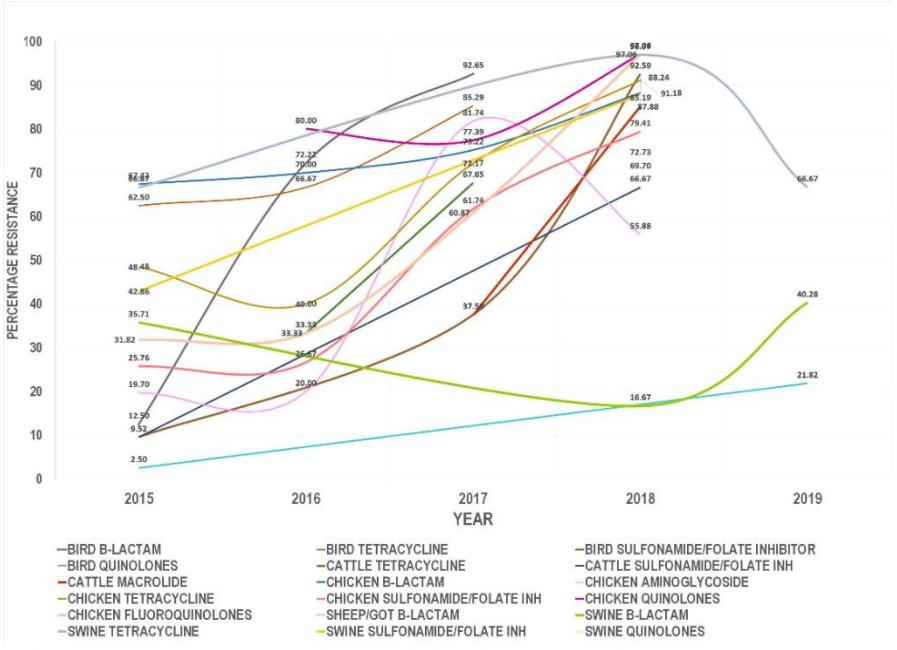


Figure 6. Comparative analysis of antibiotic resistance from different sources – birds, cattle, chicken, and swine based on the five-year (2015-2019) Literature data.

4. Conclusions

Globally, there is a wide usage of antimicrobial agents as growth promoters besides maintaining the livestock's health. Center for Disease Dynamics Economics and Policy (CDDEP) estimated that 131,109 tons of antimicrobial consumption in 2013 globally in livestock would be increased by 52% by 2030 mainly due to over-usage in the middle-income countries like India to meet the consumer demands [44]. Overuse of these antimicrobial agents contributes to the spread of drug-resistant pathogens from animals to humans. Although *E. coli* being an innocuous resident of the intestinal tract of warm-blooded animals, including humans, it is well known for causing diarrheal and extra-intestinal diseases. On 9th December 2020, WHO published data showing the top 10 causes of death worldwide responsible for 55% of total death worldwide. According to WHO neonatal conditions stood first and third, respectively, for lower and lower-middle-income countries in this list. Additionally, it is also a significant part of communicable top disease conditions [45]. Though Group B *Streptococci* infection is a primary etiological agent, *E. coli* infection is the major cause of mortality [46]. Several deaths among the children under five years, Diarrheal disease in post-neonates, and sepsis in neonate posse second and third major concern, respectively [47]. As compared to other countries, in India, antibiotic resistance is higher in comparison to their usage. [9]. Additionally, carbapenem and third-generation cephalosporin-resistant *E. coli* is included in the WHO priority pathogen list 1 (Critical) [48]. Hence, it needs to be addressed. Increasing drug resistance in animals and birds also increases the risk of zoonosis. Previous studies on effects of irrational use or overexploitation of antibiotics at subinhibitory concentrations had provided the basics of induction of biofilm formation in MDR clinical isolates [49] that can be rarely resolved in patients until removal of the colonized surface from the body resulting in increased morbidity and mortality [50]. Thus, understanding the frequency of the antibiotic resistance occurring together can help in deciding antibiotic policies both in human as well as animals as a prerequisite step to develop one health approach in addition to the generating base for future genotypic characterization of isolates to identify the causatives responsible for the spread of resistance genes across human population or animal as host and human interface.

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Conflicts of Interest

The authors declare no conflict of interest.

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