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Susceptibility profile of Zimbabwean livestock fecal *Escherichia coli* isolates to veterinary antibiotics: Implications for standardization of antimicrobial resistance surveillance in livestock production

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ABSTRACT

Susceptibility patterns of Escherichia coli to Veterinary Critically Important Antimicrobials (VCIAs) are poorly understood in most developing countries. We determined those patterns on n = 180 livestock fecal isolates from Chikomba district by disk diffusion method. Multiple Antibiotic Resistance (MAR) indices for the isolates were determined for risk analysis. Chi-square was used to test how antibiotic susceptibility level associated with animal species and farming scale. Resistance to Tetracycline and Ampicillin was high across animal species (above 70%). Moderate levels of resistance (30% to 54%) to Erythromycin, Trimethoprim and Chloramphenicol were detected across livestock species. Resistance levels were low (below 30 %) for Ciprofloxacin and Gentamicin. Resistance to Gentamicin, Tetracycline, Ciprofloxacin, Ampicillin, Chloramphenicol and Ceftazidime was associated with animal species ($P < 0.05$). Antibiotic susceptibility patterns were independent of farming scale ($P > 0.05$). Frequencies of isolates within each risk zone depended on animal species ($P < 0.05$), contrary to farming systems ($P > 0.05$). Multi-Drug Resistance was 73%, where most isolates were resistant to 5 antibiotics (23%) and none exhibited resistance to all antibiotics. Only 55% of isolates from cattle and over 80% from pigs and layers were within the high risk zone. Twenty nine of the isolates were extended spectrum beta lactamase (ESBL) positive. Higher ESBL frequencies (44%) were observed within Large Scale Commercial Farms (LSCF) followed by for Small Scale Commercial Farms (SSCF) (25%) and Resettlement farms (A1) (14%). Similarly, ESBL prevalence varied by livestock species ($P > 0.05$), as follows: pig (39%), layers (32%) and beef (12%). Our study suggests high incidences of multi-drug resistance in livestock which need AMR surveillance strategies.

Introduction

Escherichia coli are gram-negative bacteria that inhabit the guts of many animals. There is much documented work on the incidences of resistance of such bacteria to antimicrobial agents (Founou *et al.*, 2018; Raiz *et al.*, 2011). This tendency is enhanced by their potential to produce Extended Spectrum Beta Lactamase (ESBL) enzymes which can hydrolyze beta lactam derived antibiotics such as Penicillins, Cephalosporins and Aztreonam (Massot *et al.*, 2017; Trot, 2013). As a result of this, *Escherichia coli* often exhibit multi-drug resistance characteristics, rendering antibiotics ineffective in treating disease conditions (Massot *et al.*, 2017; Rossolini *et al.*, 2007; O'Neill, 2015). Notwithstanding this, animal health is faced with challenges of limited drug alternatives in the treatment of conditions as a result of exposure to these bacteria. Given the high incidences of *Escherichia coli* diseases such as Colibacilosis, the sustainability of the livestock production sector will under serious threat.

Increased antimicrobial use, particularly in livestock production has been reported to be the major cause of antimicrobial resistance (AMR), worldwide (Landers *et al.*, 2012; O'Neill, 2015; Tiseo *et al.*, 2020). Such antimicrobial agents are normally

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administered to animals for therapeutic or prophylactic reasons and to some extend for promoting animal growth (Founou *et al*., 2018; Van Den Bogaard *et al*., 2001). Non-therapeutic use of antimicrobials in animal production, although it has been banned in the European Union (Maron *et al*., 2013; European Medicines Agency, 2021), reports on this practice are still evident in many countries (Anderson *et al*., 2020; Tiseo *et al*., 2020) In many African countries, including Zimbabwe, there are high incidences of misuse and/or overuse of antimicrobial drugs since they are freely accessed over-the-counter without prescriptions (Anderson *et al*., 2020; Ferreira, 2017; Ndhlovu and Masika, 2016; O'Neill, 2015). A number of studies (Roschanski *et al*., 2016; Gonggrijp, 2016; Irrgang *et al*., 2016) support the availability of a positive correlation between misuse of antimicrobials and the development of antimicrobial resistance in many microoganisms. As a result of this, new antibiotic-resistant bacterial species may be selected for, either from the normal commensal and pathogenic bacteria. These may be easily disseminated to humans through either the food chain or waste disposal and spread the danger to public health safety (Animal Health Australia, 2015; FAO, 2004; O'Neill, 2015).

This danger of antimicrobial resistance is aggravated if it occurs on VCIA due to their limited therapeutic alternatives (Ndhlovu and Masika, 2016). Conducting regular clinical diagnosis of antimicrobial resistance involving such antimicrobials is therefore an essential endeavor. Despite AMR being a global threat, there is a severe lack of AMR surveillance within the livestock production sector, particularly involving VCIAs, in Zimbabwe (Government of Zimbabwe, 2017) and in Africa at large (Founou, 2018), due to undermining of its dangers and lack of priority with regards to funding of for regular clinical diagnosis of this danger.

More so, there are limited studies that have used an integrated approach to capture the issue of antimicrobial resistance by farming scale, livestock species and antimicrobials classes especially the veterinary critically important antimicrobials. This is despite the importance of this phenomenon in standardization of antimicrobial resistance surveillance through effective understanding of the possible sources and its developmental patterns (O'Neill, 2015). Therefore, our aim was to assess the susceptibility patterns of livestock fecal *Escherichia coli* isolates to VCIA by sensitivity testing, between faming systems, livestock species, and antimicrobial classes.

Materials and Methods

Site description

The study was undertaken at Marondera University of Agricultural Sciences and Technology (-18°11'6.97" S 31°33'6.95" E). Livestock fecal samples were collected in Chikomba District (latitude: -18.8885^0 or 18^0 53'18.5" south and longitude: 31.0975^0 or 31^0 5'51" east) of Mashonaland East province, which is located in the North Eastern side of Zimbabwe.

Ethics

Formal permission to conduct the study in Chikomba District was sought from the District Veterinary Officer. The methodology of this study was approved by the Human Research Ethics committee of Marondera University of Agricultural Sciences and Technology (20-01-2019; 08/19/Humans). Participation was based on participant's desire and volition to participate in the study after reading, understanding and signing the contents of a consent form.

Sample collection

The online Raosoft sample size calculator which was set at 95% level of confidence and 5% margin of error was initially used for determination of a representative sample size. A total of n= 180 fresh livestock fecal samples (fresh as defined as still soft and warm) were collected from 10 wards purposively selected from a total of 26 wards under livestock production for the period between April 2019 and May 2019. Ward selection was based on the availability of all farming systems. Household selection criterion was based on whether a farmer uses antimicrobials in livestock (using procedures from Anderson et al., 2020), coupled with willingness to participate. This involved collection of n=60 samples for each animal species (pig, poultry and cattle), from all farming systems (A1, SSCF and LSCF). Samples were collected in sterile polythene bags. They were transported in cool boxes containing ice packs and stored at 4°C prior to laboratory analysis.

Isolation of *E. coli* and Antimicrobial Sensitivity testing procedure

Isolation procedures were undertaken according to CLSI. Serial dilutions were done by transferring 1ml of sample into 9ml of peptone water. The dilutions were done up to 10^{-7}. Samples were selected for culturing within 24 hours on MacConkey agar selective media. To produce pure cultures, isolated colonies were picked and sub-cultured by streaking on selective media i.e XLD and MacConkey agar. Morphological characteristics such a rose pink color,

circular, convex, smooth and viscous colonies were used for identifying *E. coli* colonies. The colonies were the same, indicating pure culture formation. Seventy, non-duplicated isolates which were shown to exhibit *E. coli* properties through their morphological characteristics were randomly selected from the samples and stored at -20^{0}c prior to identification through their biochemical profiles.

Isolates were tested using the disk diffusion technique, based on Clinical Laboratory Standards Institute (CLSI) guidelines (Clinical Laboratory Standards Institute, 2011) on Mueller Hinton Agar (New England Biolabs, Mildrand, South Africa). A panel of 9 commercial antibiotic mast disks (New England Biolabs, Mildrand, South Africa), involving Cefotaxime (30 µg), Erythromycin (15 µg), Gentamicin (10 µg), Tetracycline (30 µg), Trimethoprim (5 µg), Ciprofloxacin (5 µg), Ampicillin (10 µg), Chloramphenicol (30µg) and Ceftazidime (30 µg), were purposively selected from VCIA list (World Organization for Animal Health, 2015). These were tested against n = 56 layers, n = 36 beef cattle and n = 48 pig *Escherichia coli* isolates, which were confirmed through their biochemical profiles (gram staining, lactose fermentation tests, motility tests, citrate tests, indole tests, catalase tests) according to Bergy *et al.* (2009), in 150 mm agar plates. *Escherichia coli* ATCC 25922 was used as the quality control strain. Diameter of clear zones around each antibiotic disk were measured and recorded. Inhibition zones were interpreted according to the standard levels of susceptibility (susceptible, intermediate and resistant).

ESBL production screening tests

An initial ESBL screen test was undertaken by disk diffusion method. A combination of Ceftazidime (CAZ) (30 µg) and Cefotaxime (CTX) (30 µg) was employed in the screening procedure to improve the sensitivity of detection. Screening results were interpreted according to Clinical Laboratory Standards Institute (2011) guidelines, which recommend conducting ESBL testing if the inhibition zones are as follows: ≤ 22 and ≤ 27 for Ceftazidime and Cefotaxime respectively. The phenotypic confirmatory disc diffusion test was used for ESBL confirmation on isolates (n = 92) that exhibited resistance to a combination of the antibiotic disks used for screening. Cefepime-clavulanic acid (FEL) (30 + 10 µg) and Cefepime (FEP) (30 µg) alone (New England Biolabs, Mildrand, South Africa) were used. *Escherichia coli* ATCC 25922 (New England Biolabs, Mildrand, South Africa) was the quality control strain. An increase in zone diameter of ≥5 mm for Cefepime-clavulanic acid from Cefepime alone was considered phenotypic confirmation of ESBL production.

Result interpretation and analysis

The results were interpreted using the CLSI cut off values with three levels of susceptibility: resistant, intermediate and susceptible. Multiple antibiotic resistance (MAR) index was calculated according to Krumperman (1983) using the formula **a/b**, where **a** represented the number of antibiotics to which a particular isolate was resistant to and **b** was the total number of antibiotics tested. Analysis of that was extended by animal species and farming system in order to scrutinize their associative health risk levels. Chi-square analysis was done to assess the level of association between categorical variables using the SAS statistical software, version 6. Statistical significance was determined at $p < 0.05$.

Results

***Escherichia coli* isolation patterns**

Out of the 180 samples that were collected for laboratory analysis, only 78% of the isolates were confirmed to be *Escherichia coli* through their biochemical profiles. Isolation rates by animal species were 40% for layers, 34% for pigs and 26% for beef cattle.

Overall susceptibility patterns across all farming systems and livestock species

The overall proportions of isolates showing a non-susceptibility pattern to a panel of 9 antibiotics are shown in Figure 1. Generally, *E. coli* isolates exhibited high resistance to Tetracycline (95%) and Ampicillin (80%), moderate resistance to Ceftazidime (45%), Cefotaxime (41%), Erythromycin (32%), Trimethoprim (34%) and Chloramphenicol (32%) and minimum resistance to Ciprofloxacin (16%) and Gentamicin (18%).

Susceptibility patterns of *Escherichia coli* isolated from different animal species to VCIAs

The susceptibility profile of *Escherichia coli* by animal species is shown in Table 1. High resistances to Tetracycline (≥ 94%) and Ampicillin (≥ 75%) were detected across all animal species. Moderate levels were shown on Cefotaxime, Erythromycin, Trimethoprim, Chloramphenicol and Ceftazidime. Resistance to Ciprofloxacin and Gentamicin was low. Gentamicin, Tetracycline, Ciprofloxacin Ampicillin, Chloramphenicol and Ceftazidime resistance was associated with animal species ($p < 0.05$).

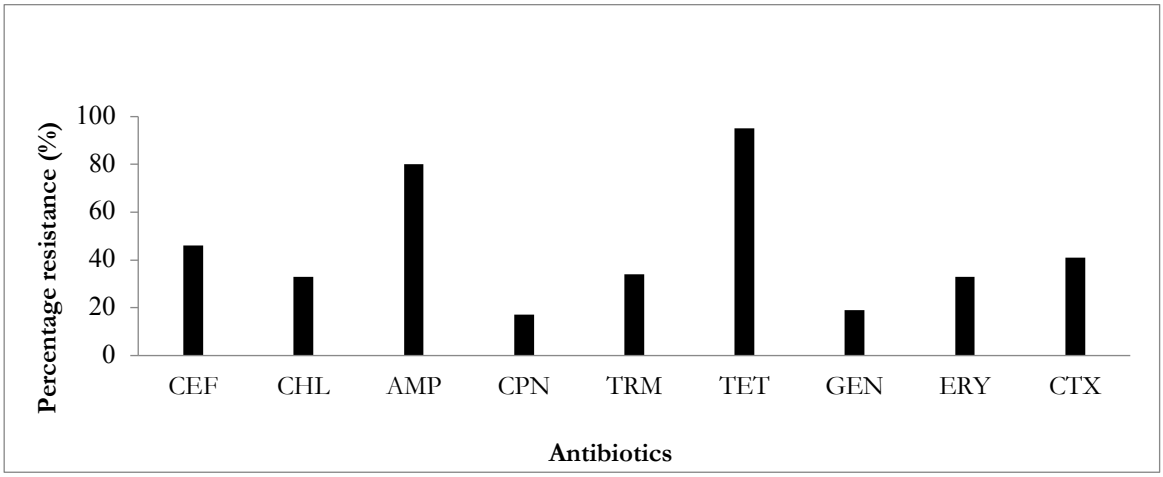

Figure 1. Percentage of isolates (n=140) showing non-susceptibility of *E. coli* to 9 selected antibiotics. CEF, Ceftazidime; CHL, Chloramphenicol; AMP, Ampicillin; CPN, Ciprofloxacin; TRM, Trimethoprim; TET, Tetracycline; GEN, Gentamicin; ERY, Erythromycin; CTX, Cefotaxime.

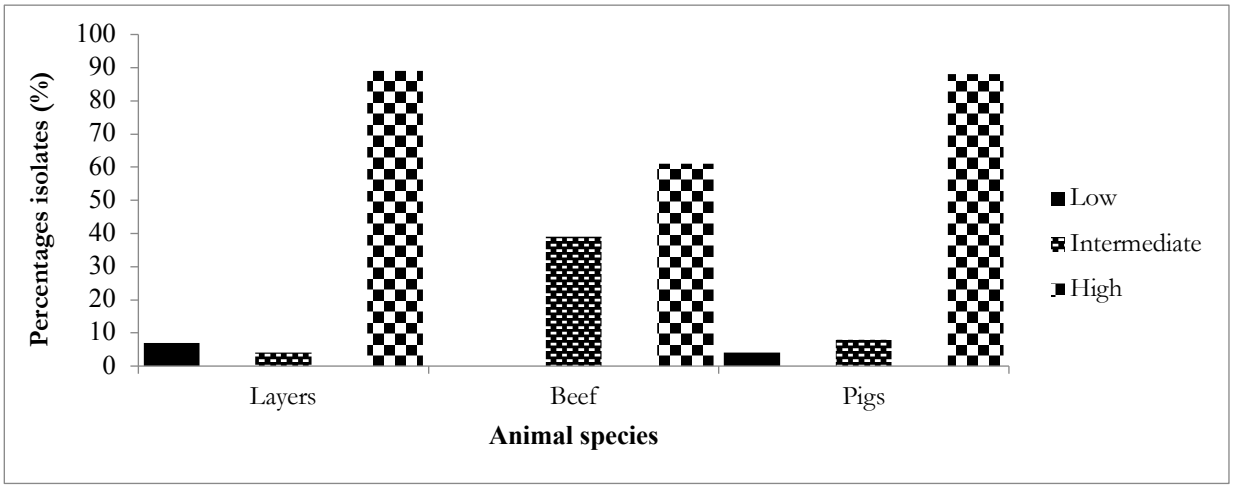

Figure 2. Proportions of n=140 isolates in risk zones (Low, Intermediate and High risk zone) by animal species.

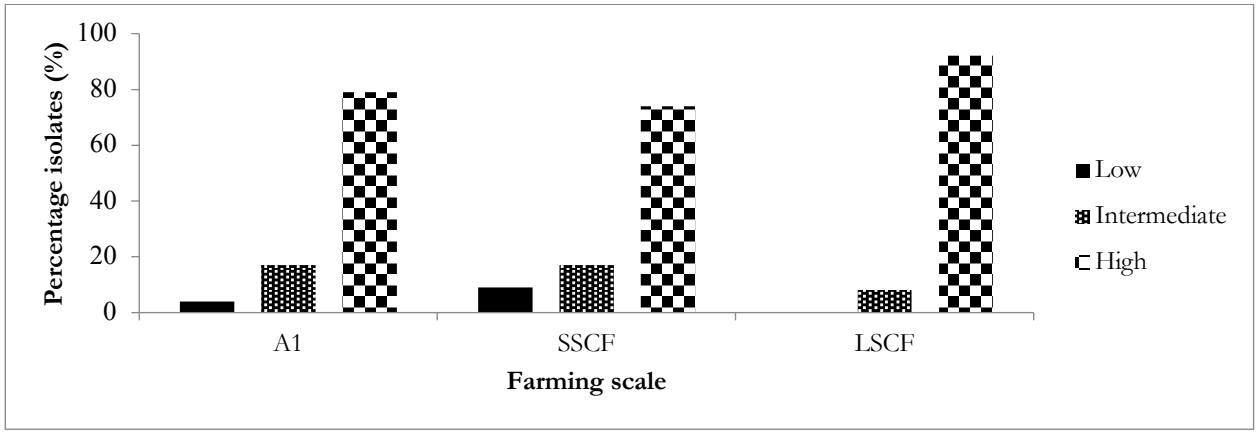

Figure 3. Proportions n=140 isolates in risk zones (Low, Intermediate and High risk zone) by farming scale.

Susceptibility patterns of *E. coli* isolated from different farming scales to VCIAs

The susceptibility patterns of *E. coli* by farming scale are shown in Table 2. No significant associations were detected between susceptibility pattern and farming scale ($p > 0.05$). Resistance to Tetracyclines, Ampicillin, Ceftazidime, Cefotaxime and Gentamicin was low for A1 farms moderately high for SSCF and high for LSCF. LSCF maintained high resistances on Trimethoprim and Ciprofloxacin.

MAR and risk analysis

MAR indices of the isolates are presented in Table 3. MAR index ranged from 0.1 to 0.8 for all isolates. Multi-Drug Resistance (MDR) (resistance of an isolate to at least 3 antibiotics) was 73%. Most of the isolates (23%) were resistant to 5 antibiotics. None of the isolates were resistant to all antibiotics. Only 5% of the isolates were resistant to one antibiotic.

Multiple antibiotic resistance risk analysis by animal species

MAR indices were categorized into Low risk zone (MAR < 0.2), Intermediate risk zone (MAR 0.2-0.25) and the High risk zone (MAR ≥ 0.3; Figure 2). Frequencies of animals in each risk zones and type of animal were associated (χ^2, 13.0; p = 0.001). Over 80% of pig and layer isolates were in the high risk zone. No beef isolates were in the low risk zone.

Multiple antibiotic resistance and risk analysis by farming scale

The proportions of isolates within each of the risk zones for each farming scale are shown in Figure 3. There was no detected association between farming scale and number of isolates within the risk zones (χ^2, 14.1; p = 0.482). LSCF had more (90%) isolates in the high risk zone, followed by A1 (75%) and SSCF (72%). No isolates from LSCF were in the low risk zone and less than 20% from A1and SSCF were in this zone.

ESBL prevalence by farming scale and animal species

A total of n = 40 samples (29%) were ESBL positive. ESBL prevalence and farming scale were associated (χ^2, 13.8; p = 0.008). LSCF had the highest level of ESBLs (44%), followed by SSCF (25%) and A1 farms (14%). There was no significant association between ESBL prevalence and animal species (χ^2, 7.8; p = 0.098). The highest prevalence of ESBLs was observed on pig samples (39%) followed by layer samples (32%) and beef samples (12%).

Table 1. Isolates showing non-susceptibility of *E. coli* to VCIAS by animal species.

Antimicrobial agent	Poultry (n=56) (%)			Cattle (n=36) (%)			Pig (n=48) (%)			Statistical test (Chi-square)	
	R	I	S	R	I	S	R	I	S	χ2	P-value
CTX (30µg)											
ERY (15µg)	46	36	18	39	33	28	38	46	16	11.2	0.08
GEN (10µg)	39	18	43	33	17	50	25	29	46	11.8	0.06
TET (30µg)	25	14	61	28	0	72	25	24	51	15.1	0.01
TRM (5µg	96	0	4	94	0	6	96	4	0	12.9	0.04
CPN (5µg)	36	35	39	28	39	33	38	24	38	12.4	0.13
AMP (10µg)	29	18	53	0	16	84	17	13	70	16.5	0.01
CHL (30µg)	86	4	10	78	17	5	75	8	17	13.1	0.04
CEF(30µg)	36	14	50	22	56	22	38	4	100	13.1	0.04
	46	18	36	33	32	35	54	4	42	14.2	0.02

CEF, Ceftazidime; CHL, Chloramphenicol; AMP, Ampicillin; CPN, Ciprofloxacin; TRM, Trimethoprim; TET, Tetracycline; GEN, Gentamicin; ERY, Erythromycin; CTX, Cefotaxime. R, Resistance; I, Intermediate; S, Susceptible. P values less than 0.05 are significant

Table 2. Isolates showing non-susceptibility of *E. coli* to VCIAs by farming scale.

Antimicrobial class	A1 (n=42) (%)			SSCF (n=48) (%)			LSCF (n=50) (%)			Statistical test (Chi-square)	
	R	I	S	R	I	S	R	I	S	χ2	P- value
CTX(30µg)											
ERY(15µg)	30	48	22	35	39	26	58	29	13	4.9	0.55
GEN (10µg)	39	30	31	22	4	74	38	29	33	11.9	0.06
TET (30µg)	18	17	65	18	17	65	21	25	54	1.0	0.98
TRM (5µg)	87	9	4	100	0	0	100	0	0	6.4	0.37
CPN (5µg)	30	22	48	22	35	43	50	29	21	8.2	0.40
AMP(10µg)	4	13	83	26	17	57	21	17	62	4.9	0.54
CHL (30µg)	74	13	13	78	9	13	88	4	6	1.8	0.93
CEF (30µg)	48	9	43	26	22	52	25	8	67	5.7	0.45
	30	35	35	35	30	35	71	0	29	16.2	0.01

CEF, Ceftazidime; CHL, Chloramphenicol; AMP, Ampicillin; CPN, Ciprofloxacin; TRM, Trimethoprim; TET, Tetracycline; GEN, Gentamicin; ERY, Erythromycin; CTX, Cefotaxime; R, Resistance; I, Intermediate; S, Susceptible. P values less than 0.05 are significant.

Table 3. Multiple antibiotic resistance frequencies.

MAR index	Number of isolates	Percentage of isolates
0	0	0
0.1	6	5
0.2	20	14
0.3	30	21
0.4	28	20
0.5	32	23
0.6	16	11
0.7	4	3
0.8	4	3

MAR index, Multiple Antibiotic Resistance index.

Discussion

High *E. coli* resistance to Tetracycline (95%), moderately high to Chloramphenicol (32%) and low to Ciprofloxacin (16%) observed in this study concurs with earlier reports (Saidi *et al.*, 2013; Adekosan *et al.*, 2015). Considering that antimicrobial resistance is a function of antimicrobial usage (Ferreira, 2017; O'Neill, 2015; Sabir *et al.*, 2013), our finding suggests a wide use of Tetracyclines within farming systems. Tetracycline use in human medicine is limited (Sabir *et al.*, 2013) despite its crucial role in animal health. This being a cheap and easily accessible antibiotic in many countries especially in Africa, high resistance patterns are discouraging as it indicates a promising challenge of treating common livestock infections.

Moderate levels of resistance to Chloramphenicol are typical of its limited use at sub- therapeutic levels (Raida *et al.*, 2004). Although Ciproflaxacin resistance was not common in this study, a few recorded cases indicate an emerging tendency of *Escherichia coli* to develop resistance against such antimicrobial agents. High resistance to Ampicillin and moderate resistance to Erythromycin, Trimethoprim and Cefotaxime have also been reported (Amer *et al.*, 2018). Since Penicillins and Cephalosporins are beta lactam antibiotics (Rossolini *et al.*, 2007) and wide use of Penicillins in Zimbabwe (Anderson *et al.*, 2020; Ndhlovu and Masika, 2016), might have induced resistance to Cephalosporins by default.

At an animal species level, resistance to tetracycline was high for layers and pigs and low for beef, which is in line with the reports of (Baywater *et al.*, 2004). That suggests a need to reduce the use of Tetracyclines as first line of treatment in livestock. Such an intervention strategy may therefore be prioritized in poultry production. Since Ampicillin and Trimethoprim resistance was generally higher for layer isolates, prescribers may find it necessary to reduce their use as first line of treatment in such animals (Loannis, 2017). Encouragingly, low resistance levels to Gentamicin across all animal species observed in this study places hope on the success of treating *E. coli* infections. Within farming scales, resistance to Tetracycline, Ampicillin, Gentamicin and Cefotaxime, was low for A1 isolates and equally high for SSCF and LSCF. Higher levels of animal production normally correspond to increased antimicrobial use and resistance (Markland *et al.*, 2019; Quinn *et al.*, 2011). Such a pattern therefore implies heavy use antimicrobials in LSCF and need for intervention.

Much of our isolates were resistant to 5 antibiotics, which agrees with a local study by (Saidi et al., 2013). This indicates a serious multi-drug resistance incidence within farming systems. Most of the isolates from pigs and layers were found within the high risk zone compared to those for beef cattle. Most of the isolates exhibiting MAR characteristics reflect a source where antibiotics were severely used (Krumperman, 1983). The general tendencies of using antibiotics at sub-therapeutic level in pigs and poultry than beef (O'Neill, 2015; Van Den Bogaard *et al.*, 2001) might explain the observed MAR index patterns. Such data therefore suggests that pigs and poultry are more risky sources of MAR bacteria, like LSCF. Moderately high levels of multi-drug resistance in A1 farms could be a result of poor antimicrobial usage practices (Government of Zimbabwe, 2017; Anderson *et al.*, 2020).

In clinical terms, the diagnosis of extended spectrum beta lactamase-producing bacteria increases the essence of antimicrobial resistance diagnosis (Raiz *et al.*, 2011). We dictated 29% ESBL positive isolates, confirming what was reported by a local study (Mbanga *et al.*, 2014). This presents an emerging clinical challenge with regards to the use of beta lactam antibiotics and some non-beta lactam antibiotics such as Flouoroquinolones, Aminoglycosides as reported by Jabeen *et al.* (2005).

Conclusions

There is a serious *E. coli* resistance to VCIAs in livestock. This reflected as high resistance to Tetracycline and Ampicillin, moderate resistance to Cephalosporins, Erythromycin, Chloramphenicol and minimum resistance to Ciprofloxacin and Gentamicin. This resistance was higher in LSCF, moderately higher in SSCF. Isolates from poultry were resistant to most of the antibiotics. Most of the isolates exhibited resistance to 5 antibiotics. Pig and poultry samples were more risky sources of antimicrobial resistance. This was also evident for isolates from all farming systems. AMR surveillance

is therefore warranted to ensure sustainable use of the available antimicrobials in Zimbabwe.

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