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Abstract

This study determined the antibiotic resistance patterns of *Escherichia coli* and *Staphylococcus aureus* from the raw meat and faeces of three game species from three different farms across South Africa. The Kirby-Bauer disk diffusion method was used according to the Clinical and Laboratory Standards Institute 2018 guidelines. *E. coli* was tested against ampicillin, ceftazidime, chloramphenicol, streptomycin, sulphafurazole and tetracycline. *S. aureus* was tested against tetracycline, erthromycin, vancomycin, penicillin, oxacillin and cefoxitin. There were no significant differences in the *E. coli* antibiotic resistance profiles between the meat and faecal samples (except towards ceftazidime where 5% of the meat isolates were resistant and 0% of the faecal isolates). The *S. aureus* meat isolates showed high (75%) resistance towards penicillin and on average, 13% were resistant to oxacillin/ cefoxitin, indicating methicillin resistance. The results from this study indicate that there is incidence of antibiotic resistant bacteria from the faeces and meat of wildlife species across South Africa, suggesting that cross contamination of the meat occurred during slaughter by antibiotic resistant bacteria from the abattoir personnel or equipment and or from carcass faecal matter. In addition, the results highlight the importance of food safety and hygiene procedures during slaughter to prevent cross-contamination of antibiotic resistant bacteria, as well as pathogens, onto raw meat.

Keywords

Antimicrobial resistance; game; bacteria; pathogen

Introduction

Various studies have demonstrated that wild animals and their surrounding environments are significant reservoirs of antibiotic resistance genes and antibiotic resistant bacteria (Cantas et al. 2013; Costa et al. 2008; Karesh et al. 2012). This is a growing public health issue, due to

increased wildlife contact with humans, as well as livestock and domestic animals due to co-habitation on the same farm (van den Honert et al. 2018). In addition, there is a rising trend in the consumption of game meat over recent years (Cantas et al. 2013; Dias et al. 2015). Majority of these wild animals are slaughtered in field abattoirs (van Shalkwyk & Hoffman, 2010). This can potentially heighten the risk that bacteria from the gut and skin of animals can cross-contaminate meat during the slaughter process, typically due to inadequate hygiene conditions and handling (Bakhtiary et al. 2016; van Shalkwyk and Hoffman, 2010). Schlegová et al. (2004) has stated that bacteria which contaminate meat can be the source of foodborne diseases and also possibly a cause of drug resistant human pathogenic bacteria (Schlegová et al. 2004).

Although studies are limited, especially on bacteria from wildlife, several studies have been conducted on the antibiotic resistance patterns within a meat processing facility and conclude that cross-contamination of antibiotic resistant bacteria onto the meat does occur and originates from various sources such as the animal faeces, staff hands or facility equipment and machinery (Amir et al. 2017; Aslam et al. 2003; Schlegová et al. 2004).

This study evaluated the antibiotic resistance patterns of *Escherichia coli* and *Staphylococcus aureus* isolated from the meat and faeces of three commonly hunted/harvested game species from different regions in South Africa.

Methods

Ethics number

All animals were hunted and sampled according to the standard operating procedure approved by the Stellenbosch University Animal Care and Use Committee (ethics number: SU-ACUM14-001SOP).

Study area and sample collection

All three farms are private game farms where the wildlife are free to roam with the other wildlife species on the farm. The wildlife graze and drink on the farm's natural resources and are given supplementary feed when necessary, especially in times of drought when the grass has become depleted.

Meat (the *infraspinatus* muscle) and faecal (from the ileum of the small intestine) samples were collected from the same animal in order to directly compare the antibiotic resistance profiles of the meat and faeces of each animal. All samples from the same farm were collected on the same day. After sample collection, all samples were stored frozen at -20℃ until analysis commenced.

Impala (*Aepyceros melampus*) meat (n=5) and faecal (n=5) samples were collected from five different impala from a farm in Modimolle, Limpopo, South Africa. Bontebok (*Damaliscus pygargus*) meat (n=5) and faecal (n=5) samples were collected from five different bontebok from a farm in Wellington, Western Cape, South Africa. Springbok (*Antidorcas marsupialis*) meat (n=5) and faecal (n=5) samples were collected from five different springbok from a farm in Witsand, Western Cape, South Africa.

Isolation and species confirmation of bacteria

Samples were defrosted at room temperature until thawed before analysis commenced. The bacteria were isolated from the samples using a series of plating on selective agar media. The inoculated agar plates were incubated overnight at 35℃. For *E. coli*, Violet Red Bile Dextrose agar (Merck Bioloab, South Africa) and then Eosin Methylene Blue agar was used to isolate the bacteria from the faecal and meat samples. Baird Parker agar (Oxoid, Hampshire, England) was used to isolate *S. aureus* from the meat samples. *E. coli* characteristic growth on Violet Red Bile Dextrose agar is purple/red colonies surrounded by a halo and on Eosin Methylene Blue agar, characteristic growth is colonies with a dark purple center with a green metallic sheen. *S. aureus* characteristic growth on Baird Parker agar is black colonies with a clear halo.

Gram's stain and various biochemical tests, the citrate test, catalase test and Staphylase test (Oxoid, Hampshire, England) were used to confirm colony identity.

After pure cultures were obtained, colonies from the selective agar plates were streaked onto Nutrient agar (Merck Biolab, Modderfontein, South Africa) plates and incubated overnight at 35℃, which were then used for the antibiotic susceptibility test (AST).

Antibiotic susceptibility testing

All samples were tested for antibiotic susceptibility in triplicate as each animal was sub-sampled three times in the isolation step. Fresh cultures grown overnight on Nutrient agar (Merck Biolab, Modderfontein, South Africa) were used for the antibiotic susceptibility analysis. The disk diffusion method was used according to the Clinical & Laboratory Standards Institute (CLSI) 2018 guidelines (M100S) using Mueller-Hinton agar (Merck Biolab, Modderfontein, South Africa). For *E. coli*, the antibiotic discs (Oxoid, South Africa) ampicillin 10µg, chloramphenicol 30 µg, ceftazidime 30 µg, streptomycin 10 µg, sulphafurazole 300 µg and tetracycline 30 µg were tested. For *S. aureus*, the antibiotic discs (Oxoid, Hampshire, England) cefoxitin 30 µg, erythromycin 15 µg, oxacillin 1 µg, penicillin 10U, tetracycline 30 µg and vancomycin 30 µg were tested. The quality control strains *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 (Thermo Fisher Scientific, Lake Charles, Louisiana) were used as quality controls and an uninoculated agar plate was used as a negative control. The results of the disc diffusion test classified the isolates as resistant, intermediately resistant or susceptible to the selection of antibiotics, according to the zone diameter specifications as listed by the CLSI 2018 guidelines.

Antibiotic resistant gene detection

A crude extraction method using lysis buffer and boiling was used to extract DNA from fresh overnight broth cultures of the isolated *E. coli* meat and faecal samples. The ZymoBiomics

DNA kit (Inqaba Biotec, Muckleneuk, South Africa) was used according to the manufacturer's instructions to extract DNA from fresh overnight broth cultures of the isolated *S. aureus* meat samples.

Polymerase chain reaction (PCR) was used to detect various antibiotic resistant genes which are commonly associated with phenotypic antibiotic resistance. The antibiotic resistant genes detected in the *E. coli* isolates were: *tet*A at 502bp and *tet*B at 930bp (tetracycline resistance), *sul*1 at 433bp and *sul*2 at 721bp (sulphonamide resistance), *bla*CMY at 1000bp (ampicillin resistance) and *aad*A at 525bp (streptomycin resistance). The antibiotic resistant genes detected in the *S. aureus* isolates were: *tet*K at 1515bp, *tet*L at 229bp and *tet*M at 406bp (tetracycline resistance), *van*A at 732bp and *van*B at 647bp (vancomycin resistance) and *bla*Z at 498bp (penicillin resistance). All reactions were performed in duplicate. The primers and reaction conditions used in this study are described by van den Honert *et al.* (2020), except the *bla*Z gene, which is the most common gene encoding production of beta-lactamases to hydrolyse penicillin. The *bla*Z gene was detected using the following primers and reaction conditions: forward primer sequence: 5'-AAGAGATTTGCCTATGCTTC-3', reverse primer sequence: 5'-GCTTGACCACTTTTATCAGC-3'; 5min initial denaturation at 94 ℃ followed by 35 cycles of 94 ℃ for 30s, 55 ℃ for 30s and 72 ℃ for 10min and a final extension step of 72 ℃ for 10min (Russi et al., 2015).

Gel electrophoresis was performed using 1.2% agarose gel (Lonza SeaKem, Rockland, ME, USA) stained with EZ-Vision® in-gel solution DNA dye (Amresco, Solon, OH, USA). Gel visualisation was performed using the Bio-Rad Gel Doc XR+ System (Bio-Rad, Hercules, CA, USA) in combination with Image Lab Software V5.2.1.

Statistical analysis

Statistical analysis was performed on the *E. coli* isolate results to determine if there were any significant differences between the meat and faecal sample antibiotic resistant levels. The

statistical analysis was performed using Statistica 13.2 software (StatSoft, USA). The data was analysed using one-way analysis of variance (ANOVA). The main effect was meat versus faecal sample. Significant results were identified by least significant means (LSM) by using a 95% confidence interval ($p \leq 0.05$).

Results and Discussion

Antibiotic resistant bacteria and some of the associated antibiotic resistance genes were found in both the faecal and meat samples of the wildlife species from all three of the farms (Figs. 1-2). Table 1 shows the phenotype-genotype correlations of the *E. coli* and *S. aureus* isolates. Antibiotic resistant genes were detected in all the isolates which were classified as phenotypically resistant to the various antibiotics.

Escherichia coli was isolated from 22 (49%) and 45 (100%) of the meat and faecal samples, respectively. A summary of the antibiotic susceptibility test results for the *E. coli* isolates is shown in Figure 1. There were no significant differences in the *E. coli* antibiotic resistance patterns between the meat and faecal samples, except for ceftazidime. Thus it can be speculated that contamination of the game meat occurred from *E. coli* both from the carcass faeces and from the surrounding environment, equipment and/ or the slaughter personnel (Gouws et al. 2017)

The presence of *E. coli* (49%) on the meat samples indicates that faecal contamination from the carcass occurred on the meat of these animals during the slaughter process (Aslam et al. 2003). The presence of *S. aureus* on the meat samples (36%) indicates that unhygienic practices and thus cross-contamination occurred onto the meat from the hide and/ or from the meat handlers. Ultimately, the presence of *S. aureus* on raw meat is indicative of poor hygiene conditions in the food chain, mainly due to contamination by food handlers and equipment (Naas et al. 2019). The detection of *E. coli* and *S. aureus* from the meat samples was expected and the frequency of isolation is similar to those found by Schlegová et al. (2004), who detected

E. coli and *S. aureus* in meat samples of beef in a slaughterhouse in 67% and 24% of samples, respectively.

The *E. coli* isolates were most frequently resistant to streptomycin, followed by ampicillin, sulphafurazole, tetracycline and then ceftazidime. No *E. coli* isolates were resistant to chloramphenicol.

The *E. coli* isolates were resistant to streptomycin (average 9% meat; 5% faecal) where a high percentage were intermediately resistant (average 59% meat; 68% faecal) (Fig. 1). Other studies have reported that streptomycin resistance is common in food animals due to its extensive use in both agricultural and clinical settings (Boerlin et al. 2005; Bryan et al. 2004; Kozak et al., 2009; Wilkerson et al. 2004). In addition, streptomycin is present in the soil, produced by organisms such as *Streptomyces griseus*, and could confer a natural low-level resistance to the grazing wildlife (Overbeek et al. 2002, Wegst-Uhrich et al. 2014).

Moreover, the faecal and meat *E. coli* isolates were notably resistant to ampicillin (average 18% meat; 20% faecal). More specifically, the high average of ampicillin resistance was mainly attributed to the faecal (100% resistant) and meat (73% resistant) samples from the bontebok species from the Wellington farm (data not shown). The higher ampicillin resistance seen in the bontebok *E. coli* isolates can be attributed to the fact that this farm which hosts the bontebok was previously a dairy and sheep farm about thirty years ago. The penicillin antibiotic class, which includes ampicillin, is the most widely used antibiotic class in sheep farming (Wegst-Uhrich et al. 2014). The application of antibiotics during the dairy and sheep farming period could have stimulated the development of antibiotic resistant bacteria within the soil which could be transferred to the grazing wildlife (Wegst-Uhrich et al. 2014).

Furthermore, the *E. coli* isolates had low levels of resistance towards sulphafurazole (average 9% meat; 2% faecal) and tetracycline (average 9% meat; 4% faecal). This is consistent with Li

et al.'s (2007) study which reported similar resistant levels of *E. coli* isolates from game meat, with sulphafurazole resistance at 7.9% and tetracycline resistance at 13%.

No *E. coli* meat or faecal isolates were resistant to chloramphenicol. Low resistance was expected as chloramphenicol is not permitted for food animal use in South Africa and many other countries (Rawat and Nair, 2010).

The only significant difference in antibiotic resistance levels between the *E. coli* isolates from the meat and faecal samples was towards ceftazidime, where 5% of the meat isolates and 0% of the faecal isolates were classified as resistant. Resistance to ceftazidime indicates suspicion for extended-spectrum β–lactamase (ESBL) production (Overdevest et al. 2011). However, additional phenotypic confirmatory tests would still need to be performed to confirm ESBL-production (Dahms et al. 2015; Henton et al. 2011; Overdevest et al. 2011). Other studies have also speculated that environmental ESBL *E. coli* is a result of human influence, as the majority of ESBLs are reported from human clinical isolates due to the direct use of novel sub-classes of β-lactam antibiotics (Guenther et al. 2011; Skurnik et al. 2006). Thus it can be speculated that contamination of the meat occurred predominantly from human influence, most likely during the skinning and evisceration steps, as also found by Schlegová et al. (2004), via resistant genotype and phenotype analysis.

S. aureus was isolated from 16 of meat samples (36%). A summary of the antibiotic susceptibility test results for the *S. aureus* isolates is shown in Figure 2. The *S. aureus* isolates were most frequently resistant to penicillin, followed by oxacillin/ cefoxitin, tetracycline and then erythromycin. No isolates were classified as resistant to vancomycin.

There were 12 *S. aureus* isolates from the game meat which were resistant to penicillin (75%) (Fig. 2). This was anticipated, as resistance to penicillin is now widespread in humans and animals since the 1960s, in both community, hospital and meat staphylococcal isolates (Appelbaum, 2007; Chambers and DeLeo, 2009; Lowy, 2003; Schlegová et al. 2004).

Methicillin resistant *S. aureus* (MRSA) was only detected in two of the bontebok meat isolates (13% average) (indicated by oxacillin and confirmed by cefoxitin). Although genetic confirmation to confirm methicillin resistance should be performed by detection of the *mec*A gene. Other studies have concluded that contamination of meat with MRSA can result from cross contamination of the carcass from the animal itself or from the people involved in the meat handling during slaughter and processing (Gilmore et al. 2008).

The *S. aureus* meat isolates were classified as tetracycline resistant in 2 (12%) of the samples. Furthermore, 1 (6%) of the *S. aureus* isolates from the game meat were classified as resistant to erythromycin, although 5 (31%) were classified as intermediately resistant. Other studies have found varying frequencies of resistance to erythromycin (4.3-30%) and tetracycline (~50%) of *S. aureus* from meat (retail non-game meat), where tetracycline resistance is generally more common than erythromycin resistance (Kelman et al. 2011).

None of the *S. aureus* meat isolates were classified as resistant to vancomycin. Other studies have also reported negligible to very low levels (0%-3%) of vancomycin resistant *S. aureus* from raw commercial meat samples (Das and Mazumder, 2016; Jackson et al. 2013; Pesavento et al. 2005).

At least one of the selected antibiotic resistant genes were detected in all samples which showed to have a corresponding phenotypic antibiotic resistance pattern. There were seven samples where the antibiotic resistant gene was detected but the phenotypic method classified the isolates as susceptible. This occurred for ampicillin (4) from the *E. coli* isolates and for tetracycline (2) and vancomycin (1) from the *S. aureus* isolates. Some possible explanations for these resistance genes being detected in these samples could be that they are inactive genes, meaning that they are present but are not active because there is no antibiotic resistance selective pressure to phenotypically express the gene. Alternatively, PCR can be considered a more sensitive method to the disc diffusion phenotypic method, as resistance is dependent on

the size of the zone of inhibition, which is determined by the CLSI committee on an annual basis, whereas resistance in PCR is determined simply by the detection of a resistance gene (Gilmore et al. 2008).

Conclusion

Antibiotic resistant bacteria were detected in the faecal content and on the raw meat of the wildlife species, with ampicillin and streptomycin resistance being the most prevalent in *E. coli* from both sample types. The *S. aureus* isolates from the game meat showed high resistance to penicillin but fairly low resistance to the other five antibiotics.

Although it seems unlikely that antibiotic resistant bacteria would be found in wildlife, movement of antibiotic resistance genes and resistant bacteria can reach these more isolated environments from pollution of human and farm animal environments as well as via supplementary feed and water sources. Contamination via humans during the slaughter and processing steps can also be a source of antibiotic resistant bacteria onto the raw meat.

The *E. coli* isolated from the meat and faeces of the same animal showed to have similar antibiotic resistance patterns except towards ceftazidime, where there was a significant difference in resistance frequencies between the meat and faecal samples. These results indicate that cross contamination of the meat occurred from bacteria both from the carcass and from human origin.

In order to prevent cross-contamination of harmful and/ or antibiotic resistant bacteria from the hides or faeces onto raw meat, various precautionary steps can be put in place. For example, exposed muscle must avoid contact with workers hands and the animal's skin as best as possible. Workers hands are important sources of contamination during processing and thus hand washing is essential in preventing contamination of the carcass. Furthermore, it is important to clean all equipment and meat processing machinery to reduce the effect of cross-contamination.

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356

357 **Figure Legends**

358 Figure 1. Antibiotic susceptibility profiles of *E. coli* isolates from meat (n=22) and faecal (n=45)
359 samples from springbok, bontebok and impala to ampicillin (AMP) $p>0.05$, ceftazidime (CAZ)
360 $p\leq0.05$, chloramphenicol (C) $p>0.05$, streptomycin (ST) $p>0.05$, sulphafurazole (SF) $p>0.05$ and
361 tetracycline (TE) $p>0.05$.

362

363 Figure 2. Antibiotic susceptibility profile of *S. aureus* isolates (n=16) from meat samples from
364 springbok and bontebok to tetracycline (TE), erthromycin (E), vancomycin (VA), penicillin (P)
365 and oxacillin (OX) / cefoxitin (FOX).

366

367 **Tables**

Table 1. Phenotype-genotype antibiotic resistance patterns of *E. coli* and *S. aureus*

Location	**Animal**	**Phenotypic resistance[1]**	**Genotypic resistance**					
Escherichia coli			***bla*CMY**	***sul*1**	***sul*2**	***aad*A**	***tetA***	***tetB***
Witsand	Springbok	AMP(S), SF(R), ST(R), TE(R)	-	+	+	+	-	+
Witsand	Springbok	AMP(I), SF(I), ST(I), TE(S)	-	-	-	+	-	-
Witsand	Springbok	AMP(I), SF(I), ST(I), TE(S)	-	-	-	+	-	-
Witsand	Springbok[2]	AMP(S), SF(S), ST(R), TE(S)	+	-	-	+	-	-
Witsand	Springbok[2]	AMP(S), SF(S), ST(I), TE(S)	+	-	-	+	-	-
Modimolle	Impala	AMP(I), SF(I), ST(R), TE(S)	-	-	-	+	-	-
Modimolle	Impala	AMP(S), SF(S), ST(I), TE (S)	+	-	-	+	-	-
Modimolle	Impala[2]	AMP(R), SF(S), ST(S), TE(S)	+	-	-	-	-	-
Modimolle	Impala[2]	AMP(S), SF(S), ST(S), TE(S)	-	-	-	-	-	-
Modimolle	Impala[2]	AMP(S), SF(S), ST(I), TE(S)	-	-	-	+	-	-
Wellington	Bontebok	AMP(R), SF(I), ST(I), TE(S)	+	-	-	+	-	-
Wellington	Bontebok	AMP(R), SF(S), ST(I), TE(S)	+	-	-	+	-	-
Wellington	Bontebok	AMP(R), SF(S), ST(I), TE(S)	+	-	-	+	-	-
Wellington	Bontebok	AMP(S), SF(S), ST(I), TE(S)	+	-	-	+	-	-
Wellington	Bontebok[2]	AMP(R), SF(S), ST(S), TE(S)	+	-	-	-	-	-
Staphylococcus aureus			***tet*L**	***tet*K**	***tet*M**	***van*A**	***van*B**	***bla*Z**
Witsand	Springbok[2]	TE(S), VA(S), P (R)	+	-	-	+	-	+
Witsand	Springbok[2]	TE(S), VA(S), P (R)	+	-	-	-	-	+
Witsand	Springbok[2]	TE(S), VA(S), P (R)	-	-	-	-	-	+
Witsand	Springbok[2]	TE(S), VA(S), P (R)	-	-	-	-	-	+
Wellington	Bontebok[2]	TE(S), VA(S), P (R)	-	-	-	-	-	+

[1]*E. coli*: AMP, ampicillin (*bla*CMY gene); SF, sulphonamide (*sul*1 and *sul*2 genes); ST, streptomycin (*aad*A gene); TE, tetracycline (*tet*A and *tet*B genes). *S. aureus*: TE: tetracycline (*tet*L, *tet*K and *tet*M genes); VA: vancomycin (*van*A and *van*B genes); P: penicillin (*bla*Z gene)
S, susceptible; I, intermediate; R, resistant
[2]Meat samples (all others are faecal samples)

Figures

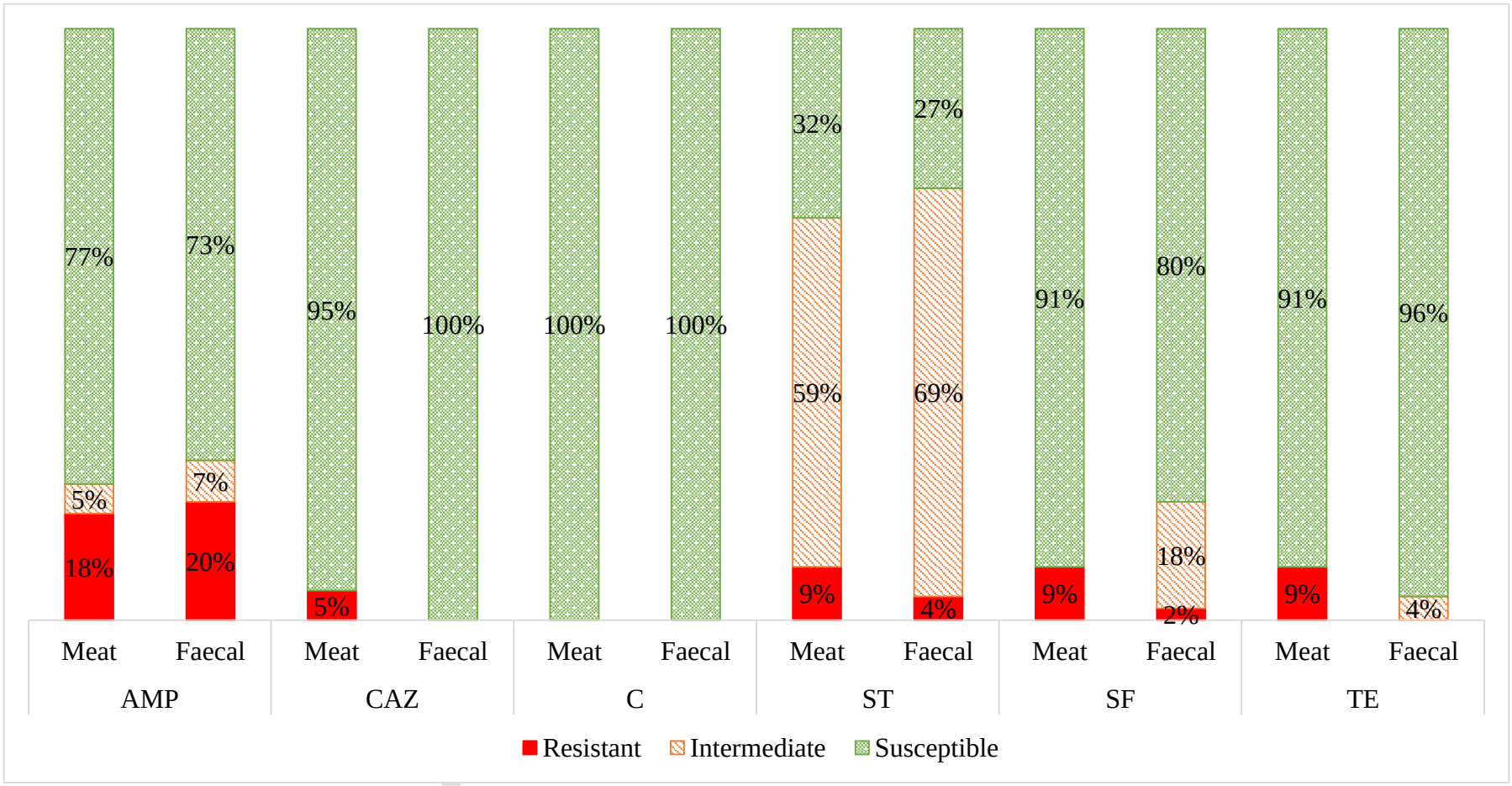
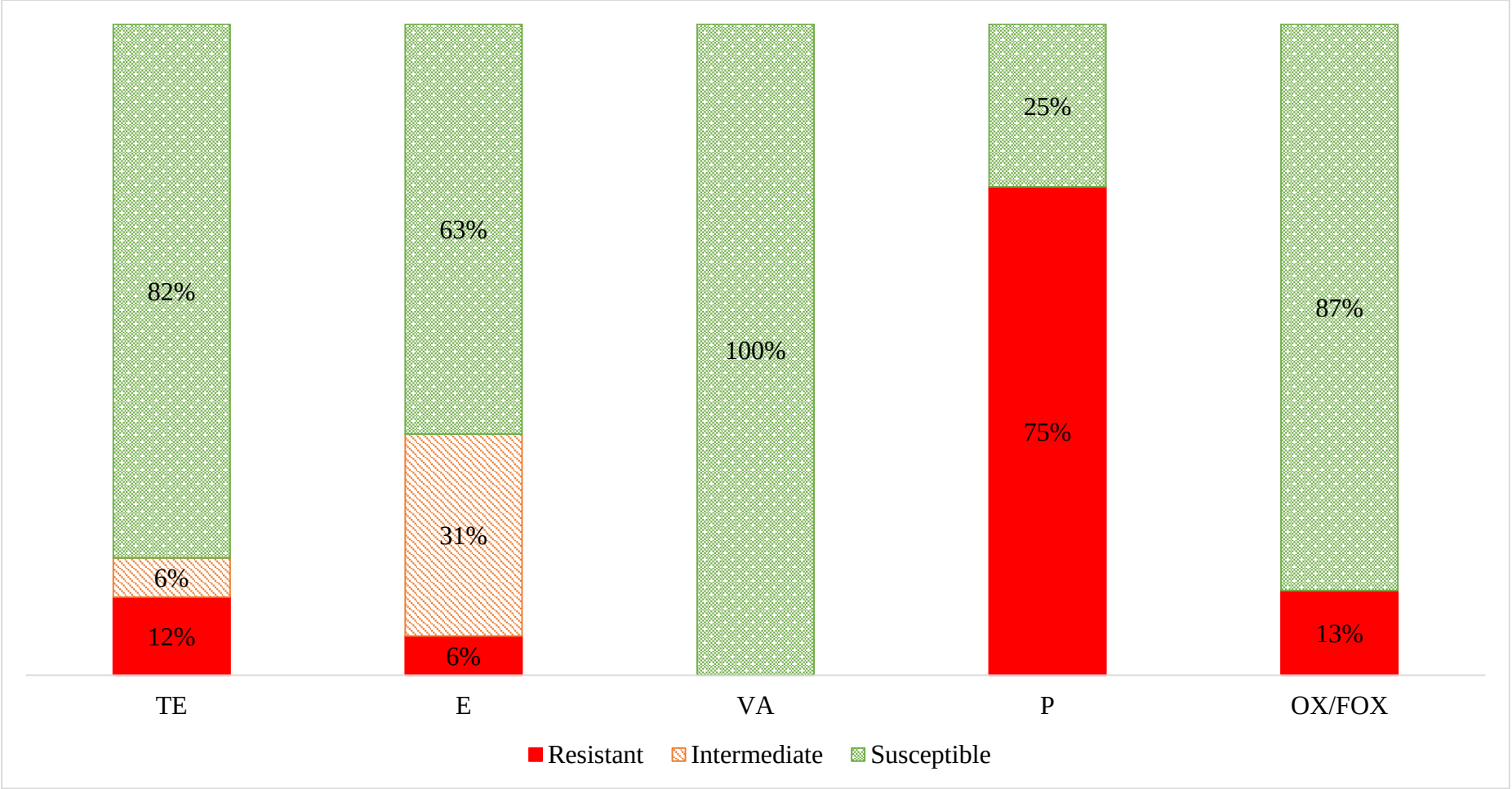


Figure 1



371

372 Figure 2

373