Prevalence of Antibiotic-Resistant Bacteria in the Environment of Poultry Farms

Yuvaneswary Veloo (yuvanes@moh.gov.my)
National Institute for Health Malaysia
Syahidiah Syed Abu Thahir
National Institute of Health
Rafiza Shaharudin
National Institute of Health
Sakshaleni Rajendiran
National Institute of Health
Lim Kuang Hock
National Institute of Health
Vickneshwaran Muthu
Kementerian Kesihatan Malaysia
Norazah Ahmad
National Institute of Health

Research article

Keywords: antibiotics, resistance, environmental bacteria, poultry

Posted Date: July 20th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-42662/v1

Abstract

Background

The occurrence and spread of antibiotic-resistant bacteria (ARB) due to the high demand in poultry industries are of great public health concern. Indiscriminate and abusive use of various antibiotics on a large scale causes antibiotic resistance (AMR) in animal-associated bacteria that may be pathogenic to humans. These bacteria are widely disseminated in the environment via animal waste. This study was therefore designed to assess the prevalence of multiple antibiotic resistance (MAR) among the environmental bacteria in poultry farms and to determine the risk contamination category of poultry by calculating the multiple antibiotic resistance index (MARI).

Results

More than half (58.2%) of the 511 total bacteria had MAR, and a number of bacteria were resistant to cefazolin (86.8%), fusidic acid (84.6%), ampicillin (79.3%), clindamycin (65.5%) and erythromycin (63.7%). These antibiotics are listed under the WHO's criteria of critically and highly important antibiotics in human medicine. In this study, 39.53% of the MARI values, which indicate the contamination level in the environment, indicated a high risk, while 14.48% were ambiguous.

Conclusion

These results therefore have shown that MAR is present not only among humans and animals but also in environmental bacteria. The high prevalence of MAR and the MARI values, together with the resistance patterns of each bacterium, indicate various effects, including possible occupational risks among workers. This study provides an introduction to the AMR of bacteria in the environment. Further studies are needed to observe the horizontal transfer of the resistance gene and the overall mobile genetic elements in environmental bacteria.

Background

Antibiotics are used in large amounts in veterinary medicine for therapeutic purposes as well as to increase animal husbandry production by serving as growth promoters and prophylaxis agents against developing diseases [1, 2, 3]. The significance of the overuse and misuse of veterinary antibiotics is worrying, as it contributes to the increase in emergence and spread of antibiotic-resistant bacteria (ARB) causing infections in both humans and animals [4, 5].

Antibiotic usage patterns in animal husbandries vary across regions and countries. Noticeably, the quantity of antibiotics being sold in low- and middle-income countries is far greater than that in high-income countries [6, 7, 8]. Southeast Asia (SEA) is considered a hotspot area for antibiotic resistance

(AMR), as it is a bloc of fast-developing and interconnected economies [5, 9, 10, 11]. In recent decades, countries in SEA (Vietnam, Thailand, Indonesia) have been experiencing rapid growth in aquaculture and poultry production sectors, representing 48% of the global veterinary antibiotic market [11, 12, 13]. Prevention and therapy using antibiotics has become one of most favorable approaches for these countries to avoid any uncontrolled epidemic diseases that can jeopardize the economy [14].

The indiscriminate and abusive use of antibiotics can result in higher concentrations of antibiotic residues in the environment [15]. Resistance to antibiotics is a side effect associated with this pollution [15, 16]. Several authors have investigated the prevalence of AMR in numerous environmental samples. Carballo *et al*. observed between 33.3% and 66.7% resistance to five well-known antibiotics used in livestock farming, viz. tetracycline, chloramphenicol, nalidixic acid, sulfamethoxazole and ampicillin [17]. Similarly, Zhu *et al*. noted high levels of tetracycline in manure and soil samples collected from large commercial swine farms in China [18].

Undoubtedly, animal husbandries are an important component in understanding the interplay of ARB between humans, animals and the environment [19, 20, 21]. In summary, ARB diffuse between humans and animals through environmental sources. AMR can be transmitted from environmental bacteria to those that colonize humans and animals [22]. This paper aims to assess the prevalence of multiple antibiotic resistance (MAR) among environmental bacteria in poultry farms and to determine the risk contamination category of poultry by calculating the multiple antibiotic resistance index (MARI). The results provide preliminary findings on ARB distribution in the poultry environment, as this environment is one of the potential sources of ARB.

Results

Antibiotic resistance in environmental bacteria

A total of 511 bacterial isolates were sampled from soil and effluent samples. Figure 1 shows the AMR patterns in gram-negative and gram-positive bacterial isolates and their prevalence rates. Detailed information is provided in Table 1, which comprises a list of antibiotics tested for gram-negative and gram-positive bacteria, the total number of bacteria resistant to each antibiotic and the prevalence rate of this resistance, which was calculated based on the number of ARB divided by the total number of bacteria that were analyzed.

Table 1
Antibiotic resistance patterns in gram-negative and gram-positive bacterial isolates and the prevalence rate of the resistance

Antibiotics	Number of Resistance	Prevalence Rate (%)
Gram-Negative Bacteria		
Ampicillin, n = 213	169	79.3
Amoxicillin/clavulanic acid, n = 304	67	22.0
Ampicillin/sulbactam, n = 262	122	46.6
Piperacillin/tazobactam, n = 360	13	3.6
Cefazolin urine, n = 371	155	41.8
Cefazolin other, n = 136	118	86.8
Cefuroxime, n = 311	92	29.6
Cefuroxime axetil, n = 269	89	33.1
Cefoxitin, n = 311	113	36.3
Cefotaxime, n = 328	27	8.2
Ceftazidime, n = 370	30	8.1
Ceftriaxone, n = 325	25	7.7
Cefepime, n = 370	23	6.2
Aztreonam, n = 304	41	13.5
Meropenem, n = 329	6	1.8
Amikacin, n = 367	27	7.4
Gentamicin, n = 371	50	13.5
Ciprofloxacin, n = 371	60	16.2
Nitrofurantoin, n = 268	45	16.8
Trimethoprim/sulfamethoxazole, n = 367	146	39.8
Gram-Positive Bacteria		
Benzylpenicillin, n = 54	28	51.9
Ampicillin, n = 103	5	4.9
Oxacillin, n = 39	24	61.5

Antibiotics	Number of Resistance	Prevalence Rate (%)
Imipenem, n = 33	6	18.2
Gentamicin high level (synergy), n = 98	16	16.3
Streptomycin high level (synergy), n = 98	29	29.6
Gentamicin, n = 40	0	0
Ciprofloxacin, n = 136	21	15.4
Moxifloxacin, n = 56	7	12.5
Erythromycin, n = 135	86	63.7
Clindamycin, n = 58	38	65.5
Linezolid, n = 139	42	30.2
Teicoplanin, n = 136	13	9.6
Vancomycin, n = 139	24	17.3
Tetracycline, n = 139	91	65.5
Tigecycline, n = 137	0	0
Fosfomycin, n = 19	0	0
Fusidic acid, n = 39	33	84.6
Rifampicin, n = 39	9	23.1
Trimethoprim/sulfamethoxazole, n = 53	24	45.3

Based on the Mann-Whitney *U*-test, there was no significant difference between isolates from soils and effluents in resistance against antibiotics (U = 32144, *p*-value = 0.943). Therefore, the samples were further discussed as overall environmental samples. According to Fig. 1, gram-negative bacteria isolated from poultry farms were highly resistant to ampicillin (79.3%), cefazolin (86.8%), ampicillin/sulbactam (46.65%) and trimethoprim-sulfamethoxazole (39.8%). To our surprise, 1.8% of the isolates were resistant to meropenem, a drug used clinically to treat against extended-spectrum beta lactamase (ESBL) organisms.

For gram-positive bacteria, it was found that isolates were resistant to benzylpenicillin (51.9%), oxacillin (61.5%), erythromycin (63.7%), clindamycin (65.5%), tetracycline (65.5%) and fusidic acid (84.6%). Additionally, 17.3% of the isolates showed resistance against vancomycin, a drug of choice for treating

methicillin-resistant *Staphylococcus aureus* (MRSA). Meanwhile, 18.2% of the isolates were resistant to imipenem, which has broad coverage for gram-positive and gram-negative bacteria and anaerobes.

Prevalence of multidrug resistance in the poultry environment

A total of 372 isolates of gram-negative bacteria and 138 isolates of gram-positive bacteria were tested for antibiotic susceptibility. Figure 2 below shows the number of antibiotic-resistant gram-negative and gram-positive bacteria in each district. In total, 58.2% of the isolates were resistant to at least 3 or more antibiotics tested. District C had the highest percentage, 85.7%, followed by district E, at 69.7% (Fig. 2). There were 32 bacterial isolates with resistance to at least 10 antibiotics, 4 of which showed resistance to 16 antibiotics. Only 52 bacterial isolates (10.18%) were sensitive to all antibiotics tested.

A Kruskal-Wallis H test showed that there was a statistically significant difference in the resistance to different antibiotics among 9 districts ($\chi2(2) = 34.79$, $p < 0.001$). A significant difference was also observed among 33 farms ($\chi2(2) = 80.54$, $p < 0.001$). Details on the percentage of resistance in the farms and districts are available in Table 2.

Table 2
Details of farms, including the number of isolates with resistance against antibiotics

District	Farm	All Sensitive, n (%)	Resistant to 1 type of antibiotic, n (%)	Resistant to 2 types of antibiotics, n (%)	Resistant to 3 or more types of antibiotics, n (%)
A	1, n = 16	0(0.00)	1(6.25)	2(12.50	13(81.25)
	2, n = 11	0(0.00)	1(9.09)	1(9.09)	9(81.82)
	3, n = 12	3(25.00)	4(33.33)	1(8.33)	4(33.33)
	4, n = 12	3(25.00)	2(16.67)	2(16.67)	5(41.67)
B	5, n = 8	2(25.00)	0(0.00)	1(12.50)	5(62.50)
	6, n = 14	2(14.29)	2(14.29)	4(28.57)	6(42.86)
	7, n = 17	4(23.53)	2(11.76)	1(5.88)	10(58.82)
	8, n = 23	7(30.43)	4(17.39)	0(0.00)	12(52.17)
C	9, n = 17	1(5.88)	1(5.88)	2(11.76)	13(76.47)
	10, n = 22	0(0.00)	1(4.55)	1(4.55)	20(90.91)
D	11, n = 31	3(9.68)	8(25.81)	3(9.68)	17(54.84)
	12, n = 17	2(11.76)	6(35.29)	2(11.76)	7(41.18)
	13, n = 6	0(0.00)	3(50.00)	0(0.00)	3(50.00)
E	14, n = 13	1(7.69)	2(15.38)	2(15.38)	8(61.54)
	15, n = 11	3(27.27)	2(18.18)	0(0.00)	6(54.55)
	16, n = 11	1(9.09)	2(18.18)	3(27.27)	5(45.45)
	17, n = 14	3(21.43)	0(0.00)	2(14.29)	9(64.29)

District	Farm	All Sensitive, n (%)	Resistant to 1 type of antibiotic, n (%)	Resistant to 2 types of antibiotics, n (%)	Resistant to 3 or more types of antibiotics, n (%)
	18, n = 6	1(16.67)	2(33.33)	1(16.67)	2(33.33)
	19, n = 13	1(7.69)	4(30.77)	1(7.69)	7(53.85)
	20, n = 15	2(13.33)	1(6.67)	0(0.00)	12(80.00)
	21, n = 13	0(0.00)	0(0.00)	3(23.08)	10(76.92)
	22, n = 13	0(0.00)	0(0.00)	1(7.69)	12(92.31)
F	23, n = 28	1(3.57)	1(3.57)	3(10.71	23(82.14)
	24, n = 9	1(11.11)	2(22.22)	3(33.33)	3(33.33)
	25, n = 14	1(7.14)	4(28.57)	5(35.71)	4(28.57)
G	26, n = 21	5(23.81)	4(19.05)	5(23.81)	7(33.33)
	27, n = 15	0(0.00)	1(6.67)	4(26.67)	10(66.67)
	28, n = 20	0(0.00)	5(25.00)	5(25.00)	10(50.00)
H	29, n = 26	0(0.00)	8(30.77)	7(26.92)	11(42.31)
	30, n = 34	0(0.00)	8(23.53)	14(41.18)	12(35.29)
I	31, n = 9	1(11.11)	4(44.44)	2(22.22)	2(22.22)
	32, n = 9	3(33.33)	0(0.00)	2(22.22)	4(44.44)
	33, n = 11	2(18.18)	5(45.45)	1(9.09)	3(27.27)

Additionally, the Mann-Whitney *U*-test was conducted to analyze the effect of the types of chickens reared (broiler, free range) on the farms as well as the types of coop systems (closed system, open system)

being applied. This test showed that isolates from broiler chicken farms exhibited a significantly higher percentage of resistance than did isolates from free range chicken farms (U = 22515, $p < 0.0001$). Regarding the effect of coop system, the open system exhibited a significantly higher percentage of resistance than did the closed system (U = 25402, $p < 0.05$). The line graphs in Fig. 3 show the comparison of the number of isolates with different amounts of AMR between broiler and free range chicken farms, as well as in the number of isolates with different amounts of AMR between open and closed systems.

Multiple antibiotic resistance index determination

Many isolates acquired resistance to multiple antibiotics. The MARI was calculated, and Table 2 and Figure 4 present the MARI values of all the isolates from the poultry farms. In general, half of the isolates (54.01%) from the soil and effluent samples had a MARI value higher than 0.2. The MARI values ranged widely, from 0.00 to 0.89. There were 100 isolates with very high values of more than 0.40 (49.5%), 10 of which had extreme values of more than 0.79 (10.89%). There were 49 isolates whose values were between 0.20 and 0.25; this range is considered to indicate ambiguity, and these values need extra scrutiny [23].

Discussion

This study is a preliminary approach for exploring the percentage of resistance in bacteria isolated from poultry farming environments. There were high levels of resistance to certain antibiotics, those commonly used for human health. Some of these antibiotics are classified by the WHO as critically important antibiotics. The resistance rates were substantially high for some of those antibiotics, especially ampicillin (79.3%), erythromycin (63.7%), linezolid (30.2%), rifampicin (23.1), gentamicin (16.3%) and ciprofloxacin (15.4%). There was also a high level of resistance to antibiotics that are listed as highly important antibiotics in human medicine, including cefazolin (86.8%), fusidic acid (84.6%), clindamycin (65.5%), benzylpenicillin (51.9%) and trimethoprim-sulfamethoxazole (45.3%) [24].

According to Gumphol *et al.*, it was suspected that compared with high-income countries, low- and middle-income countries used higher amounts of antibiotics categorized as medically important [7]. This was supported by the findings of veterinary drug residues in food samples, which indicated that violation rates in developing countries were higher than those in developed countries [25].

In Malaysia, some studies have been conducted on ARB isolated from poultry [25–28]. Results similar to those in this study, which was centered in a poultry environment, were obtained. A high percentage of resistance to the antibiotics ampicillin, erythromycin and tetracycline, was found in both the poultry and the environment, and most of the bacteria isolated were resistant to multiple antibiotics. More than half of the isolates (58.2%) in this study showed MAR.

The MAR observed in this study may be attributed to the indiscriminate use of antibiotics in poultry production. The resistance percentage, which was significantly higher in broiler farms and closed systems, may have influenced the presence of multi-antibiotic-resistant bacteria and increased the likelihood of contamination on farms. Other studies have shown that poultry type can influence the contamination and the presence of multi-antibiotic-resistant bacteria [29, 30]. This was further proven by the analysis in this study, which showed that 39.53% of the MARI values indicated high risk MARI and 14.48% were ambiguous. This MARI value distribution is alarming, as it shows that isolates originated from high-risk source(s) of contamination with antibiotics, which may increase the possibility of transfer of mobile antibiotic genes between bacteria [31].

This study showed that poultry farms have been contaminated with ARB. Chicken type, coop system and location played important roles in determining the significance of this resistance. Environmental bacteria have been exposed to antibiotics for a very long time, and now, there are multi-antibiotic-resistant bacteria, whose presence can affect the effectiveness of antibiotics overall, not only in animal husbandry [32].

Study limitations and opportunities for future research

Some limitations were found in this study. First, we were not able to obtain complete information regarding the types of antibiotics used in the poultry farms. This is because we believe that most of the antibiotics were mixed in with the feed and other supplements. In addition, the antibiotics used are controlled by the supplier, who has full control over food and medication, and it was difficult to obtain this information from the supplier. Second, due to time and budget constraints, we were not able to quantify the presence of antibiotics in the environmental sample; therefore, we could not link the ARB with the presence of trace antibiotics. Third, the classification of the chickens and farms was broad. Despite these limitations, our study provided multidisciplinary data that were sufficient to analyze the prevalence of ARB in a poultry environment.

Conclusions

In conclusion, this study confirmed the presence of multi-antibiotic-resistant bacteria in poultry farms. The prevalence of MAR and the MAR index, together with the resistance patterns of each isolate, showed that AMR is present not only among humans and animals but also in environmental bacteria. Antibiotic usage in poultry farms, either directly or indirectly, could be one of the factors causing the increase in MAR, and there are always possibilities for various effects, including MAR in environmental bacteria as well as occupational-related risks.

Regulatory authorities play an important role in controlling the usage of antibiotics in farms. They are responsible for granting marketing authorization and quality control of the antibiotic agents. With proper and limited prescription of the antibiotics, including precise treatment regime, dose, treatment intervals and duration of treatment, the amount of antibiotics secreted could be controlled. We hope that the result

of this study contribute to strengthening control measures and policies surrounding antibiotic usage among animals and proper disposal of antibiotics.

Basically, this study is an introduction to the AMR of bacteria in the environment. Further studies need to be done to observe the horizontal transfer of the resistance gene and the overall mobile genetic elements.

Methods

Environmental sampling

A cross-sectional study was conducted from January 2018 to October 2019 in 9 districts of Selangor state. This study was conducted in poultry farms that were registered with the state Department of Veterinary Services. In total, there were 213 registered farms, and based on the calculation of sample size, 33 farms were randomly selected. The farms were categorized into 2 types of chicken breeding farms, namely, broiler farms and free-range farms. All the sampling were conducted after getting permission from state Department of Veterinary Services and the farm owners.

Soil and effluent samples were collected for this study by trained personnel. Soil samples were collected from 3 different locations within each farm, namely, at the coop and in areas where the chickens clustered together [33,34]. A metal spade was cleaned, disinfected with 75% alcohol and flamed prior to soil sample collection. Soil samples from all 3 locations were pooled together in sterile Ziplock plastic bags [35]. Effluent water, if available, was also sampled from the drainage or water-pooled area. Effluent samples were collected by using a scoop sterilized using the same method as for the spade and placed in a sterile Ziplock plastic bag. All the samples were transported at 4°C in an ice box back to the laboratory.

Isolation and enumeration of bacteria

All the samples were processed within 24 h. The soil sample was homogenized, and 10 g was weighed before it was dispensed into the first dilution bottle containing 90 mL of Difco^{TM} Peptone water. The effluent sample was used directly. This was recorded as a 10^{-1} dilution. The dilution bottles were vigorously shaken and then left to settle for a few minutes. Tenfold dilutions were made by transferring a 1 mL aliquot from the dilution bottle to a fresh 9 mL peptone water bottle, vortexing and continuing until a ten-fold dilution was reached [36]. One milliliter of each dilution was poured into petri dishes containing trypticase soy agar and spread using a disposable loop. All the agar plates were incubated at 37°C for 24 h. Based on morphology, approximately three colonies were selected from plates containing 30 to 300 isolates and then isolated and purified before testing for identification and susceptibility.

Identification and susceptibility testing of bacteria

Gram staining was conducted to classify the isolates into gram-negative and gram-positive bacteria, and for further identification and susceptibility testing, the VITEK®2 system was used. VITEX ®2 GN and VITEX ®2 GP cards (bioMérieux, Nurtingen, Germany) were used for bacterial identification, while AST-GN83 and AST-P592 cards (bioMérieux, Nurtingen, Germany) were used to determine the minimum inhibitory concentrations [37].

Statistical analysis

All statistical analyses were performed using Statistical Package for Social Sciences [(SPSS) IBM software version 20]. The resistance profile for each antibiotic was used to calculate the percentage of AMR in the poultry environment. The Kruskal-Wallis *H* test and Mann-Whitney *U*-test were conducted to compare the amount of AMR in the bacteria between the farms and districts. A *p*-value below 0.05 was considered statistically significant.

MARI of isolates

MARI was calculated using the formula by Blasco *et al*. [38]:

$MAR_{index} = a/b$

a: number of antibiotics to which each isolate was resistant

b: total number of antibiotics that were tested against an individual isolate.

MARI values less than 0.20 indicated a low risk of contamination, valued between 0.20 and 0.25 were categorized as ambiguous, and values above 0.25 indicated high risk [33,39].

Abbreviations

ARB: Antibiotic-resistant bacteria, MAR: Multiple antibiotic resistance, MARI: Multiple antibiotic resistance index, ESBL: Extended-spectrum beta lactamase.

Declarations

Ethics approval and consent to participate

Informed consent was obtained from all the participants.

Consent for publication

Not applicable.

Availability of data and materials

The dataset used and/or analyzed during the study is available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

A research grant under the Ministry of Health for 2018 provided funding to Yuvaneswary Veloo under grant number P42 00500 117 6018, Sustainable Environment and Climate Change. The funder had no role in design and conduct of the study, data collection, analysis and interpretation of the data. The funder had role in approval of the manuscript; and decision to submit the manuscript for publication.

Authors' contributions

YV, SAT, SR and LKH participated in the study design, sampling, laboratory work and statistical analysis. RS and NA supervised the study. All authors read and approved this manuscript.

Acknowledgments

We would like to thank the Director General of Health Malaysia for his permission to publish this article. We thank the zoonosis sector, Disease Control Division for initiating the idea for this project and the Department of Veterinary Services for their contributions to farm selection and data collection during sampling.

This project was funded by the Ministry of Health, Malaysia: NMRR -17-1198-36521, and the work was supported by the Institute for Medical Research, Malaysia. Facilities provided by the Environmental Health Research Centre, IMR, to run the laboratory testing were very much appreciated.

Author details

[1]Institute for Medical Research, National Institute for Health, Setia Alam, Malaysia. [2] Zoonosis Sector, Disease Control Division, Ministry of Health, Putrajaya, Malaysia.

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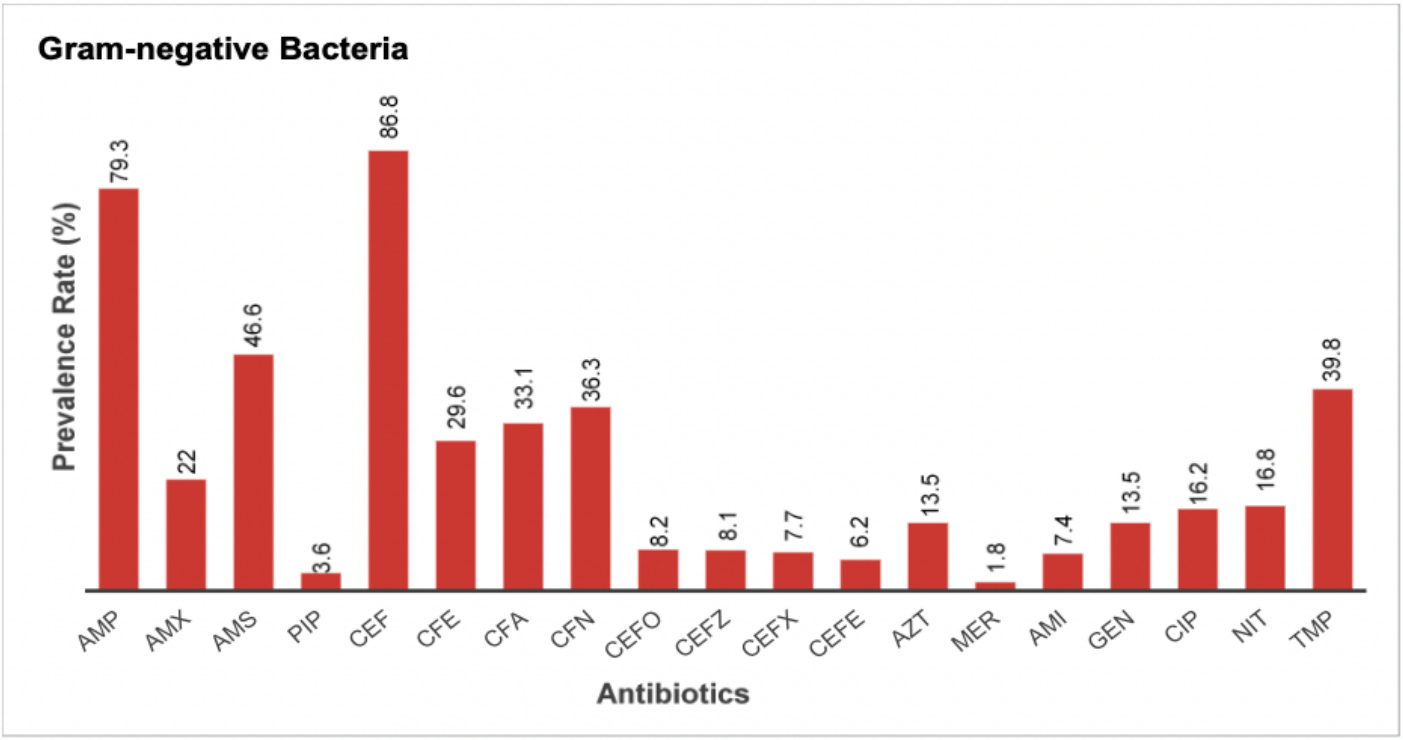
Figures



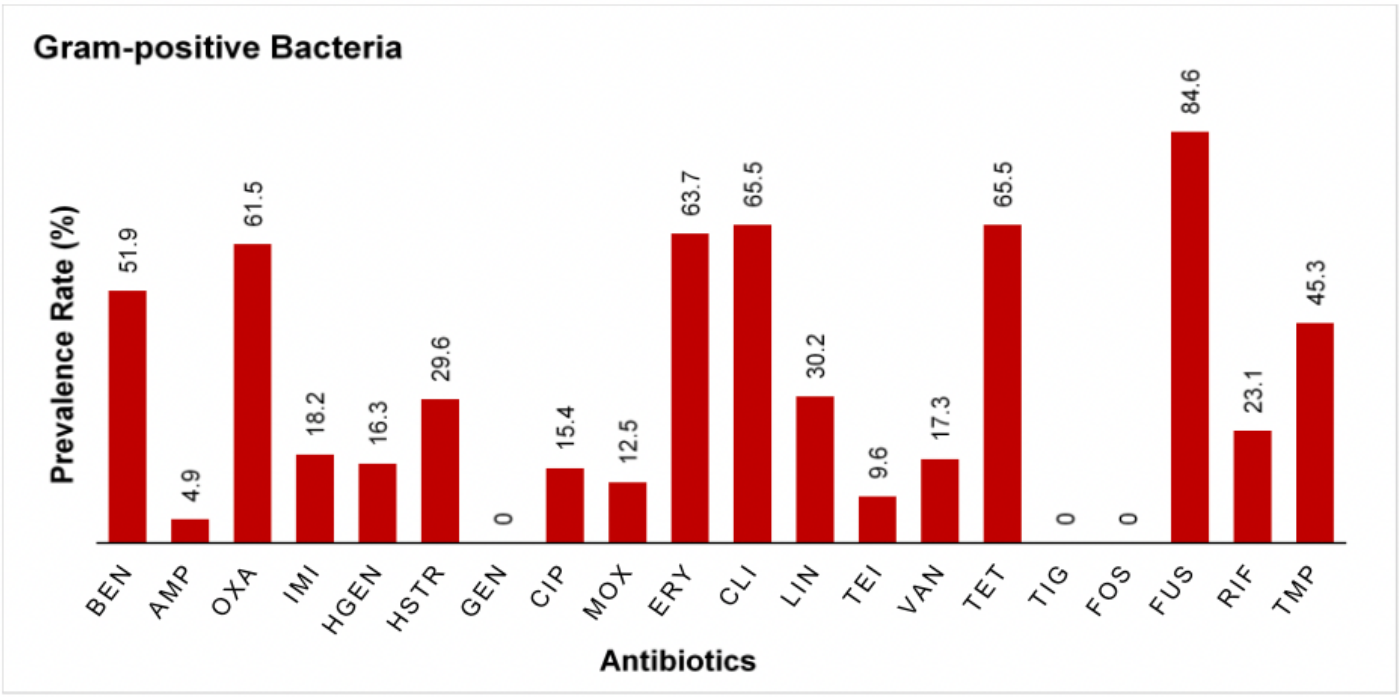


Figure 1

Antibiotic resistance patterns in gram-negative and gram-positive bacterial isolates and the prevalence rates of the antibiotic resistance. Antibiotics abbreviations: AMP, ampicillin; AMX, amoxicillin/clavulanic acid; AMS, ampicillin/sulbactam; PIP, piperacillin/tazobactam; CEF, cefazolin; CFE, cefuroxime; CPA, cefuroxime axetil; CFN, cefoxitin; CEFO, cefotaxime; CEFZ, ceftazidime; CEFX, ceftriaxone; CEFE, cefepime; AZT, aztreonam; MER, meropenem; AMI, amikacin; GEN, gentamicin; CIP, ciprofloxacin; NIT, nitrofurantoin;

TMP, trimethoprim//sulfamethoxazole; BEN, benzylpenicilin; OXA, oxacillin; IMI, imipenem; HGEN, high level gentamicin (synergy); HSTR, high level streptomycin (synergy); MOX, moxifloxacin; ERY, erythromycin; CLI, clindamycin; LIN, linezolid; TEI, teicoplanin; VAN, vancomycin; TET, tetracycline; TIG, tigecycline; FOS, fosfomycin; FUS, fusidic acid; RIF, rifampicin.

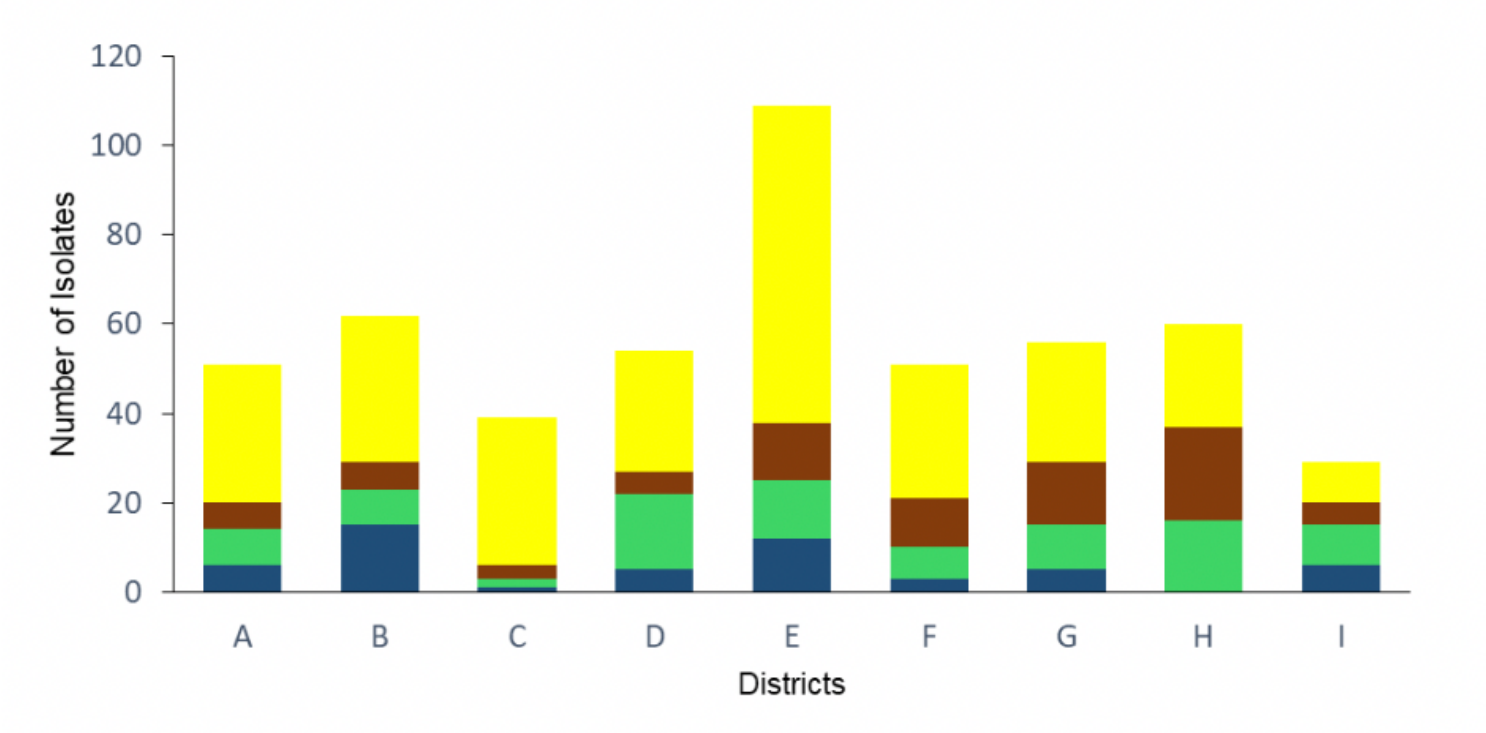


Figure 2

Number of antibiotic-resistance bacteria in each district. Blue = all sensitive, Green = sensitive to 1 antibiotic, Brown = sensitive to 2 antibiotics, Yellow = sensitive to 3 or more antibiotics.

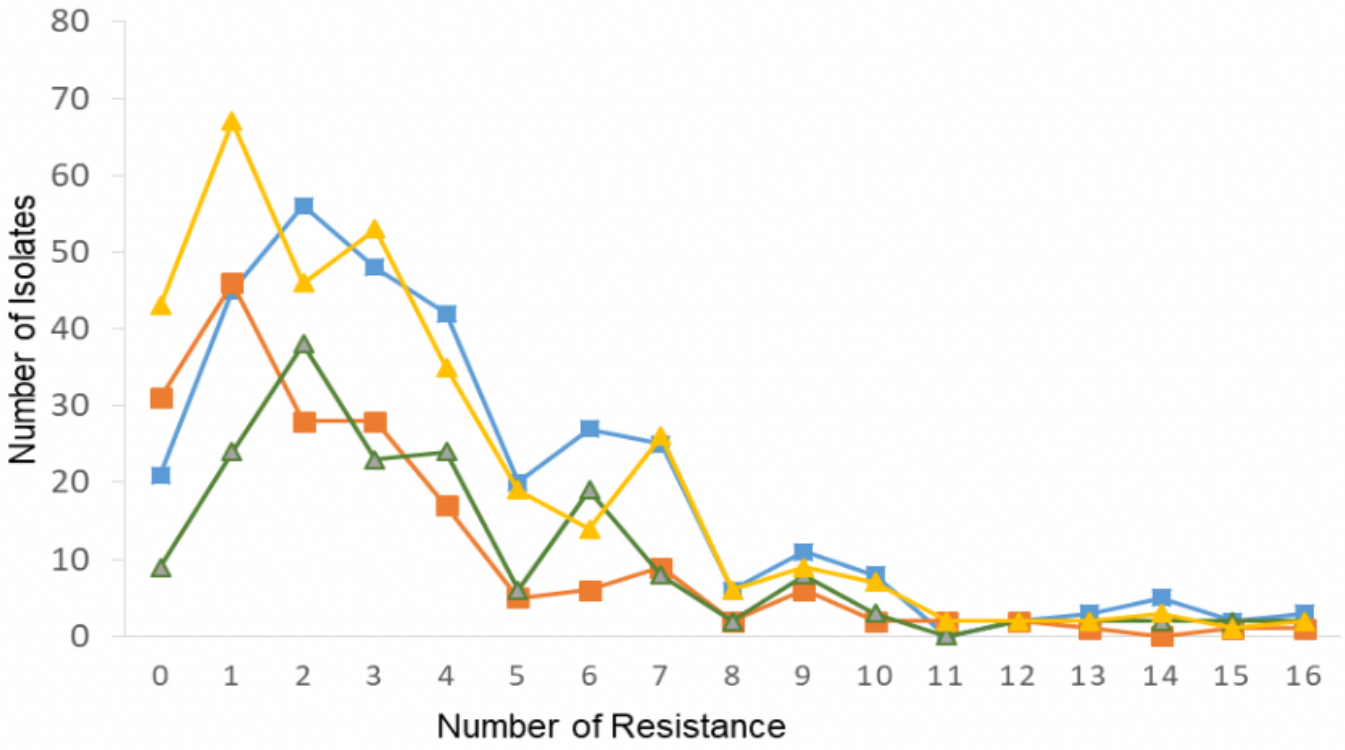


Figure 3

Number of bacterial isolates versus amount of antibiotic resistance based on type of chicken reared and system of the farms. Square with blue line = broiler chicken, Square with orange line = free range chicken, Triangle with green line = close system, Triangle with yellow line = open system.

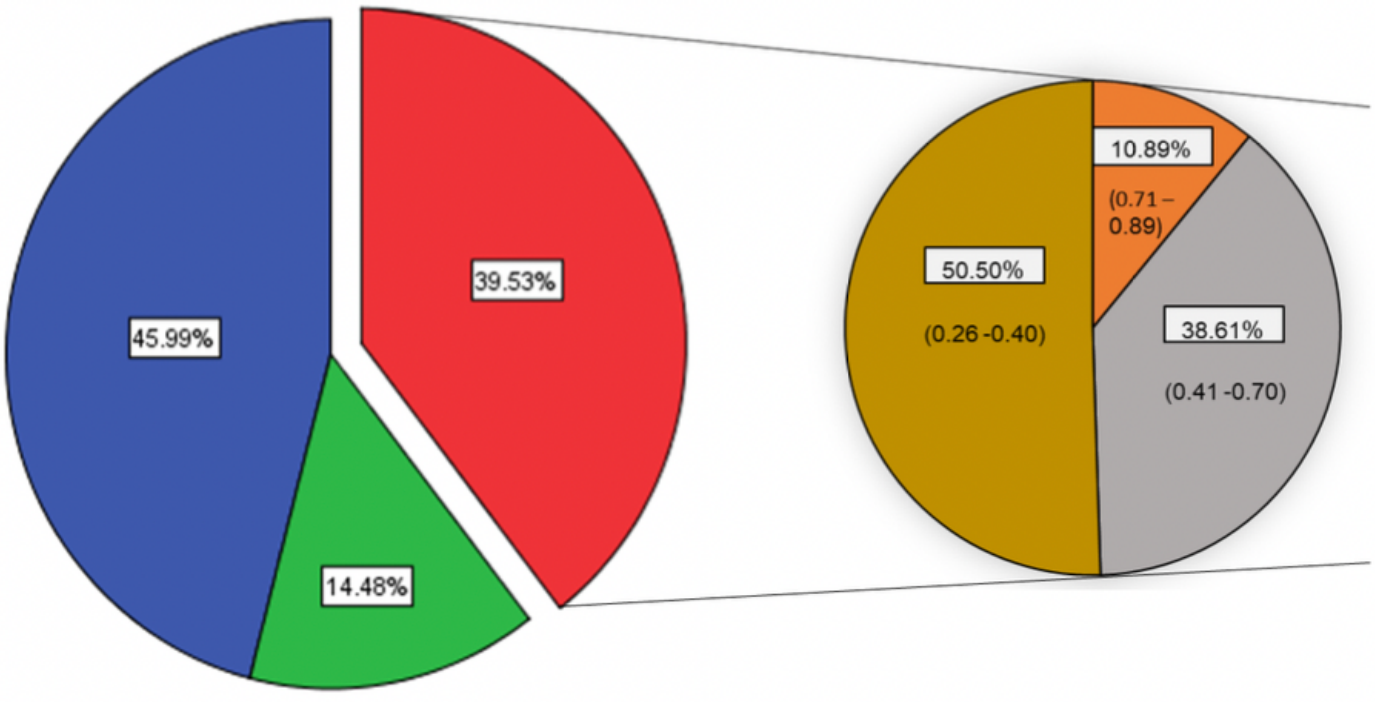


Figure 4

Percentage of MARI values indicating low risk, ambiguity and high risk. Blue = low risk (< 0.20), Green = ambiguity (0.20-0.25), Red = high risk (>0.25).

Supplementary Files

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