Veterinary Integrative Sciences 2024; 22(3): 815 - 822 DOI; 10.12982/VIS.2024.054

Vet Integr Sci

Veterinary Integrative Sciences

Faculty of Veterinary Medicine
Chiang Mai University

ISSN; 2629-9968 (online)

Website; www.vet.cmu.ac.th/cmvj

Research article

The sensitivity of extended-spectrum beta-lactamase-producing *Escherichia coli* isolated from animal feces to antibiotics and Vietnamese garlic (*Allium sativum* L.) aqueous extracts *in vitro*

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Abstract

This research was conducted to study the sensitivity of extended-spectrum beta-lactamase-producing *Escherichia coli* (ESBL-producing *E. coli*) isolated from pigs, dogs, chickens, and pheasants to antibiotics and extracts of Ly Son, Hai Duong, and Co Don garlics. The antibiotic susceptibility of bacteria was determined by the Kirby–Bauer test. Besides, the minimum inhibitory concentrations (MIC) and the minimum bactericidal concentration (MBC) of garlic extracts were determined by the dilution method. The results show that ESBL-producing *E. coli* isolated from chickens, dogs, pheasants, and pigs were resistant to 3/12, 5/12, 7/12, and 7/12 antibiotics, respectively. These strains were highly resistant to amoxicillin (100%), ampicillin (100%), and streptomycin (70-100%). Moreover, they were multi-resistant to 2-10 antibiotics. However, they were inhibited by Ly Son, Hai Duong, and Co Don garlic extracts with MIC 4.69-9.38 mg/mL, 2.35-18.75 mg/mL, and 4.69-18.75 mg/mL, respectively. MBC of Ly Son, Hai Duong, and Co Don garlic extracts ranged from 9.38 to >150 mg/mL. The ESBL-producing *E. coli* strains isolated from dogs were less sensitive to garlic extracts than those isolated from pigs, chickens, and pheasants. This study shows the potential of garlic to replace antibiotics in the prevention and treatment of animal diseases in the future.

Keywords: Antibacterial activity, ESBL-producing *E. coli*, Garlic

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Funding; This study was funded in part by the Vietnam Ministry of Education and Training, Code: B2022-TCT-17.

Article history; *received manuscript: 26 May 2023*
revised manuscript: 12 October 2023
accepted manuscript: 17 December 2023
published online: 10 January 2024

Academic editor: *Nguyen Trong Ngu*

INTRODUCTION

The disease caused by *E. coli* is a common disease in livestock species. Although the disease does not have a high mortality rate, it causes significant losses to both livestock and farmers. Antibiotics are considered a very effective treatment and control method for diseases caused by *E. coli* (Luppi, 2017). However, the overuse of antibiotics in treatment has led to the fact that many pathogenic strains of *E. coli* are capable of producing extended-spectrum beta-lactamase (ESBL) enzymes, causing antibiotic inactivation and making it difficult to treat the disease, leading to low treatment results. The percentage of dogs infected with ESBL-producing *E. coli* surveyed at Veterinary clinics in Portugal and Dak Lak province (Vietnam) was 13.02% (Carvalho et al., 2021) and 17.95 % (Le et al., 2021), respectively. According to the survey results of Le Minh et al. (2016), the rate of chickens infected with ESBL-producing *E. coli* in Vinh Long province of Vietnam was 61%.

To solve the problem of antibiotic resistance of bacteria, many plant species have been studied for their antibacterial activity to find natural antibacterial agents to replace antibiotics. Since ancient times, garlic has not only been used as a spice in cuisine in many countries around the world but is also known as a plant with antibacterial properties. Many studies showed that garlic extract has the ability to inhibit and kill many multidrug-resistant bacteria such as *E. coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Proteus* spp. (Durairaj et al., 2009; Ismail et al, 2020). In Vietnam, there are many kinds of garlic, such as Ly Son, Hai Duong, and Co Don. However, the studies on the antimicrobial activity of these kinds of garlic against bacteria, especially ESBL-producing *E. coli* strains isolated from animals, are limited. Therefore, this study was conducted to investigate the antibacterial activity of Ly Son garlic, Hai Duong garlic, and Co Don garlic against 40 strains of ESBL-producing *E. coli* isolated from animals in the Mekong Delta of Vietnam.

MATERIALS AND METHODS

Preparation of bacterial suspension

The study was carried out on 40 ESBL -producing *E. coli* strains isolated from the feces of dogs, pigs, chickens, and pheasants in the Mekong Delta of Vietnam. They were stored in the Laboratory of Veterinary Pharmacology, Faculty of Veterinary Medicine, College of Agriculture, Can Tho University. The turbidity of the bacterial suspension was determined by MacFarland Densitometer Den-1 (Biosan, England) with a turbidity of 0.5 (equivalent to a bacterial density of 10^8 CFU/mL).

Preparation of Aqueous Garlic Extracts

The dry matter content of garlic was determined by the drying method at 105 ºC of Asare (2004), cited by Teye et al. (2011). Fresh garlic (*Allium sativum* L.) bulbs (Ly Son, Hai Dương, and Co Don) were purchased from a supermarket in Can Tho city of Vietnam. Garlic bulbs are peeled and weighed fresh garlic equivalent to 15 g dry matter. Then, different kinds of garlic were homogenized aseptically using a sterile mortar and pestle. They were put into the volume flask and filled with sterile distilled water to 100 mL. After 15 minutes, the aqueous garlic extract was collected by sterile cellulose acetate membrane filters (Sartorius stedim biotech, Germany) with a pore size of 0.2 µm (Rahman et al., 2022).

Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed on Mueller-Hinton Agar (Merck, Germany) using the agar diffusion method. Total of 40 ESBL-producing *E. coli* isolated from pigs, dogs, chickens and pheasants were tested sensitivity to 12 antibiotics (Nam Khoa, Vietnam) including cefaclor (30 µg), colistin (10 µg), cefuroxime (30 µg), ampicillin (10 µg), amikacin (30 µg), enrofloxacin (5 µg), ofloxacin (5 µg), gentamycin (10 µg), amoxicillin (10µg), streptomycin (10 µg), kanamycin (30 µg), fosfomycin (200 µg). Inhibition zone diameters were interpreted according to CLSI 2021 guidelines.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration of garlic extract against 40 strains of ESBL-producing *E. coli* was determined by dilution in nutrient broth on a microtiter plate incorporating resazurin (Sarker et al., 2007). The resazurin indicator is blue in solution, and the wells with bacterial growth will change the color of the resazurin solution from blue to pink. The MIC was determined as the lowest concentration of garlic extract to inhibit microbial growth but did not kill them (no resazurin discoloration).

The resazurin solution was prepared with a concentration of 0.1% in sterile distilled water. This solution is mixed with a Vortex mixer to ensure that it is well-dissolved and homogenous.

Determination of minimum bactericidal concentration

The minimum bactericidal concentration was determined by the dilution method (Koeth, 2016). The presence or absence of bacteria on the agar plate was observed to determine the minimum bactericidal concentration of the garlic extract. The MBC was defined as the lowest concentration of garlic extract at which no colonies of *E. coli* were seen (Johnson et al., 2016).

RESULTS

The sensitivity of ESBL-producing *E. coli* to antibiotics

The results showed that ESBL-producing *E. coli* isolated from animals is highly resistant to amoxicillin 100%, ampicillin 100%, and streptomycin (70-100%). However, they were highly sensitive to amikacin 80-100% and fosfomycin 100% (Table 1). Moreover, ESBL-producing *E. coli* strains were multi-resistant to the antibiotics. ESBL-producing *E. coli* isolated from chickens, dogs, pheasants, and pigs were multi-resistant to 2-8, 4-9, 7-9, and 3-10 antibiotics, respectively (Table 2). These strains formed many multi-resistant phenotypes, with 4 phenotypes for chickens, 7 phenotypes for dogs, and 5 phenotypes for pheasants and pigs (Table 3).

Table 1 The antibiotic resistance ratio of ESBL-producing *E. coli*

Antibiotics	Ratio of *E. coli* isolated from animals (%)											
	Chickens (n=10)			Dogs (n=10)			Pheasants (n=10)			Pigs (n=10)		
	S	I	R	S	I	R	S	I	R	S	I	R
Ampicillin	0	0	100	0	0	100	0	0	100	0	0	100
Amoxicillin	0	0	100	0	0	100	0	0	100	0	0	100
Cefaclor	50	0	50	0	0	100	0	0	100	60	0	40
Cefuroxime	50	0	50	0	0	100	0	0	100	80	0	20
Streptomycin	0	20	80	0	30	70	0	0	100	0	20	80
Gentamicin	80	0	20	50	0	50	50	0	50	40	0	60
Kanamycin	80	0	20	50	0	50	70	20	10	20	20	60
Amikacin	80	0	20	100	0	0	90	0	10	100	0	0
Enrofloxacin	100	0	0	70	0	30	0	0	100	40	0	60
Ofloxacin	100	0	0	70	0	30	0	0	100	20	0	80
Colistin	0	100	0	0	90	10	0	60	40	0	60	40
Fosfomycin	100	0	0	100	0	0	100	0	0	100	0	0

S: Sensitive, I: Intermediate, R: Resistant

Table 2 The multidrug-resistance ratio of ESBL-producing *E. coli*

Number of antibiotics resistant	Ratio of *E. coli* isolated from animals (%)			
	Chickens(n=10)	Dogs (n=10)	Pheasants (n=10)	Pigs (n=10)
1	0	0	0	0
2	40	0	0	0
3	20	0	0	20
4	0	10	0	20
5	20	30	0	0
6	0	20	0	20
7	0	10	10	0
8	20	10	70	0
9	0	20	20	20
10	0	0	0	20

Table 3 The multidrug-resistant phenotype ratio of ESBL-producing *E. coli*

Multidrug-resistant phenotype	Ratio of *E. coli* isolated from animals (%)			
	Chickens (n=10)	Dog (n=10)	Pheasants (n=10)	Pigs (n=10)
Am+Ax	40	0	0	0
Am+Ax+Sm	20	0	0	20
Am+Ax+Cr+Cu	0	10	0	0
Am+Ax+Ef+Of	0	0	0	20
Am+Ax+Cr+Cu+Sm	20	30	0	0
Am+Ax+Cr+Co+Cu+Ge	0	10	0	0
Am+Ax+Cr+Cu+Ge+Kn	0	10	0	0
Am+Ax+Ge+Of+Kn+Sm	0	0	0	20
Am+Ax+Cr+Cu+Ge+Sm+Kn	0	10	0	0
Am+Ax+Cr+Cu+Ef+Of+Sm	0	0	10	0
Am+Ax+Ak+Cr+Cu+Ge+Sm+Kn	20	0	0	0
Am+Ax+Cr+Cu+Ef+Of+Sm+Kn	0	10	0	0
Am+Ax+Cr+Co+Cu+Ef+Of+Sm	0	0	40	0
Am+Ax+Cr+Cu+Ge+Ef+Of+Sm	0	0	30	0
Am+Ax+Ak+Cr+Cu+Ge+Ef+Of+Sm	0	0	10	0
Am+Ax+Cr+Cu+Ge+Ef+Of+Sm+Kn	0	20	10	0
Am+Ax+Cr+Co+Ge+Ef+Of+Sm+Kn	0	0	0	20
Am+Ax+Cr+Co+Cu+Ge+Ef+Of+Sm+Kn	0	0	0	20

Am: ampicillin, Ax: amoxicillin, Sm: streptomycin, Cr: cefaclor, Cu: cefuroxime, Ef: enrofloxacin, Of: ofloxacin, Co: colistin, Ge: gentamycin, Kn: kanamycin, Ak: amikaxin

The sensitivity of ESBL-producing *E. coli* to Vietnamese garlic extracts

The dry matter contents of Ly Son garlic, Hai Duong garlic, and Co Don garlic were 36.51%, 35.80%, and 38.81%, respectively. The results of determining the minimum inhibitory concentration in Table 4 showed that 40 strains of ESBL-producing *E. coli* were inhibited by garlic extracts at concentrations ranging from 2.34 to 18.75 mg/mL. Among the investigated garlic, Ly Son garlic extract (LSGE) had the best antibacterial activity against 40 ESBL-producing *E. coli* strains when the MIC values were 4.69 mg/mL and 9.38 mg/mL. Hai Duong garlic extract (HDGE) showed lower antibacterial activity than LSGE because the MIC value of HDGE ranged from 2.34-18.75 mg/mL; however, at the concentration of 2.34 mg/mL, the HDGE was only able to inhibit 7.50% (3/40) of the tested bacterial strains. Moreover, at the same concentration of 4.69 mg/mL, HDGE only inhibited 15% (6/40) ESBL-producing *E. coli* strains. Co Don garlic extract (CDGE) exhibited the lowest antibacterial activity when the MIC was 4.69-18.75 mg/mL. At the concentration of 4.69 mg/mL, CDGE inhibited only 17.50% (3/40), but at the concentration of 9.38 mg/mL and 18.75 mg/mL, this extract inhibited the bacteria surveyed with a ratio of 67.50% (27/40) and 15.00% (6/40), respectively.

The results in Table 4 show that strains of ESBL-producing *E. coli* could be killed by LSGE (18/40), CDGE (26/40), and HDGE (19/40) with MBC from 9.38 to 150 mg/mL. Generally, garlic extracts killed bacteria isolated from pigs with MBC<150 mg/mL, while these extracts did not kill bacteria isolated from dogs. Besides, MBC:MIC >4 ratio was high from 70.00%-82.50% (Table 5).

Table 4 MIC and MBC of garlic extracts on strains of ESBL-producing *E. coli*

Animals	ESBL-producing *E. coli*	MIC (mg/mL)			MBC (mg/mL)		
		LSGE	HDGE	CDGE	LSGE	HDGE	CDGE
Pig	P1	9.38	9.38	9.38	18.75	9.38	18.75
	P2	9.38	9.38	18.75	37.5	18.75	18.75
	P3	9.38	9.38	9.38	150.00	18.75	18.75
	P4	4.69	4.69	4.69	37.50	18.75	37.50
	P5	9.38	9.38	9.38	150.00	18.75	18.75
	P6	9.38	9.38	9.38	18.75	9.38	18.75
	P7	4.69	4.69	4.69	37.50	18.75	37.50
	P8	4.69	9.38	9.38	>150.00	75.00	37.50
	P9	9.38	9.38	18.75	37.5	18.75	18.75
	P10	4.69	9.38	9.38	>150.00	75.00	37.50
Chicken	C1	4.69	18.75	4.69	150.00	>150.00	37.50
	C2	4.69	2.34	9.38	>150.00	>150.00	150.00
	C3	9.38	9.38	9.38	>150.00	150.00	75.00
	C4	4.69	18.75	4.69	150.00	>150.00	37.50
	C5	4.69	2.34	9.38	>150.00	>150.00	150.00
	C6	4.69	2.34	9.38	>150.00	>150.00	150.00
	C7	4.69	18.75	4.69	150.00	>150.00	37.50
	C8	9.38	9.38	9.38	>150.00	150.00	75.00
	C9	4.69	4.69	4.69	>150.00	>150.00	150.00
	C10	4.69	4.69	4.69	>150.00	>150.00	150.00
Dog	D1	9.38	9.38	9.38	>150.00	>150.00	>150.00
	D2	9.38	9.38	9.38	>150.00	>150.00	>150.00
	D3	4.69	9.38	9.38	>150.00	>150.00	>150.00
	D4	4.69	4.69	9.38	>150.00	>150.00	>150.00
	D5	9.38	9.38	9.38	>150.00	>150.00	>150.00
	D6	9.38	9.38	9.38	>150.00	>150.00	>150.00
	D7	9.38	9.38	9.38	>150.00	>150.00	>150.00
	D8	9.38	9.38	9.38	>150.00	>150.00	>150.00
	D9	9.38	9.38	9.38	>150.00	>150.00	>150.00
	D10	9.38	9.38	18.75	>150.00	>150.00	>150.00
Pheasant	Ph1	9.38	9.38	18.75	150.00	>150.00	37.50
	Ph2	4.69	9.38	9.38	>150.00	37.50	37.50
	Ph3	9.38	9.38	18.75	18.75	150.00	>150.00
	Ph4	4.69	9.38	9.38	150.00	150.00	>150.00
	Ph5	4.69	9.38	9.38	75.00	>150.00	37.50
	Ph6	9.38	9.38	18.75	150.00	75.00	37.50
	Ph7	4.69	18.75	9.38	>150.00	75.00	>150.00
	Ph8	4.69	4.69	9.38	18.75	150.00	>150.00
	Ph9	9.38	9.38	9.38	37.50	37.50	75.00
	Ph10	9.38	9.38	9.38	>150.00	>150.00	>150.00

Table 5 The rate of MBC and MIC of garlic extracts

MBC:MIC	LSGE	HDGE	CDGE
≤4	17.50%	27.50%	30.00%
>4	82.50%	72.50%	70.00%

DISCUSSION

The survey results on antibiotic sensitivity of 40 strains of ESBL-producing *E. coli* in this study show the serious multi-resistant status of *E. coli* strains that cause diseases in animals. ESBL-producing *E. coli* isolated from chickens, dogs, pheasants, and pigs were resistant to 2-10 antibiotics with 18 multidrug-resistant phenotypes. The *E. coli* isolated from animals in this study showed high resistance to ampicillin, amoxicillin, and streptomycin . However, the results of this study show that ESBL-producing *E. coli* isolated from animals can be inhibited by garlic extracts . ESBL-producing *E. coli* strains isolated from dogs had low sensitivity to garlic extracts. Besides, to evaluate the bactericidal ability of garlic extracts, the MBC:MIC ratio needs to be determined. If the ratio MBC:MIC≤4, the effect was considered bactericidal, but if the ratio MBC:MIC>4, the effect was defined as bacteriostatic (Levison, 2004; Benjamin, 2012). Generally, garlic extracts had the bacteriostatic ability with the ratio MBC:MIC>4 from 70.00%-82.50% (Table 5).

Previous studies have shown that *E. coli* growth was inhibited by garlic extract (Chand, 2013; Ismail et al., 2020). Research by Durairaj et al. (2009) showed that multidrug-resistant *E. coli* could be inhibited by garlic extract at a concentration of 18 mg/mL. A study in Vietnam by Hai and Tho (2013) also showed that Hai Duong aqueous garlic extract has bactericidal ability against both pathogenic *E. coli* and ampicillin and kanamycin-resistant *E. coli* with the diameter of zone of inhibition is 20.8±0.91 mm, 22.7±0.92mm, and 22.7±0.92 mm, respectively. Tessema et al. (2006) reported that *E. coli* can be inhibited by 33.75 mg/mL of the crude preparation of garlic, but garlic did not reveal a bactericidal effect up to a concentration of 37.5 mg/mL. The results of Garba et al. (2013) showed that the minimum bactericidal concentration of garlic juice extract against *E. coli* was 200 mg/mL. An *in vivo* study by Thuy et al. (2023) showed that supplementation of garlic powder in the diet could reduce the density of *E. coli* in chicken feces at the 4^{th} and 10^{th} week age.

The chemical components with antibacterial activity in garlic are fat-soluble organosulfur compounds, including allicin, ajoenes, and allyl sulfide. These organosulfur compounds have been shown to be active against a wide range of bacteria, including multidrug-resistant (MDR) strains (Bhatwalkar et al., 2021). Allicin, an important component of garlic, has the ability to interfere with and inhibit RNA production and lipid synthesis of bacterial cells, resulting in protein synthesis being severely affected and stopped at all phases due to the absence of messenger RNA, ribosomal RNA, and carrier RNA. The deficiency of amino acids and proteins will inhibit the growth and development of the organism because amino acids and proteins are necessary for all parts of the cellular structure. In addition, when lipid synthesis is affected, resulting in the failure of the phospholipid biologic layers of the cell wall to form correctly, the synthesis of cell wall components of microbial strains is affected severely (Meriga et al., 2012). Reactive organosulfur compounds form disulfide bonds with free sulfhydryl enzyme groups and compromise bacterial membrane integrity (Bhatwalkar et al., 2021).

CONCLUSIONS

The fresh extracts of Vietnamese garlics (*Allium sativum* L.) including Ly Son, Co Don and Hai Duong garlics exhibited antibacterial activities. This study also evaluated the efficacy of the fresh extracts used against ESBL-producing strains of *E. coli* isolated from pigs, chickens, dogs, and pheasants. Based on these findings, garlics can act as a natural antibacterial compound used for animal health care in the future.

AUTHOR CONTRIBUTIONS

Bui Thi Le Minh and Nguyen Khanh Thuan: Conceptualization and design the experiment, investigation, supervision, editing and finalization.
Nguyen Ho Quang Minh, Huynh Ngoc Trang: Investigation, methodology, formal analysis, manuscript preparation.

ACKNOWLEDGEMENTS

This study was funded in part by the Vietnam Ministry of Education and Training, Code: B2023-TCT-17.

CONFLICT OF INTEREST

We have no conflict of interest.

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How to cite this article;

Bui Thi Le Minh, Nguyen Ho Quang Minh, Huynh Ngoc Trang, Nguyen Khanh Thuan. The sensitivity of extended spectrum beta-lactamase producing *Escherichia coli* isolated from animal feces to antibiotics and Vietnamese garlic (*Allium sativum* L.) aqueous extracts *in vitro*. Veterinary Integrative Sciences. 2024; 22(3): 815 - 822