Original Article

Possible cross-resistance and horizontal gene transfer associated with halquinol in *Escherichia coli* and *Salmonella enterica* isolated from pigs

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Abstract

The aims of this study were to determine the possible cross-resistance and horizontal gene transfer associated with halquinol in *Escherichia coli* and *Salmonella* spp. from pigs. Two of each *E. coli* and *Salmonella* isolates exhibiting low halquinol MIC (8 and 16 µg/ml, respectively) and susceptible to antibiotics tested were selected for *in vitro* stepwise-halquinol exposure experiment. Another *E. coli* (*n*=19) and *Salmonella* (*n*=6) isolates with high halquinol MICs (16-64 and 32-64 µg/ml, respectively) containing plasmids were used for transferability testing of halquinol resistance-encoding gene(s) using filter mating method. *In vitro* exposure to subinhibitory concentrations of halquinol promoted neither resistance to halquinol nor cross-resistance to antibiotics in *E. coli*. In contrast, spontaneous halquinol-resistant mutants of *Salmonella* were obtained with cross-resistance to trimethoprim. Horizontal transfer of halquinol resistance determinants was not detected in either *E. coli* or *Salmonella*. These findings demonstrate the possible species-specific responses to halquinol exposure and emphasize the importance of prudent antimicrobial use to limit resistance development and spread.

Keywords: antimicrobial resistance, *Escherichia coli*, halquinol, pig, *Salmonella*

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Introduction

In modern intensive animal production systems, antibiotics have been extensively formulated in animal feeds to promote growth, prevent diseases and treat bacterial infections. The widespread and imprudent use of antibiotics in food animal production is a key driver of antimicrobial resistance (AMR) in bacteria, including *Escherichia coli* and *Salmonella*. This undermines the effectiveness of antibiotic therapy in both human and veterinary medicine, posing a significant threat to animal and public health.

Most *E. coli* is non-pathogenic and referred to as a commensal of the gastrointestinal tract; however, some serotypes have contributed to various intestinal and extra-intestinal diseases in both humans and animals. Enteric colibacillosis disease caused by enterotoxigenic *E. coli* strains is one of the most important causes of postweaning and nursery diarrhea in pigs causing decreased growth rate, high mortality and morbidity rate leading to high economic losses (Luppi *et al.,* 2016). Both pathogenic and commensal *E. coli* isolates in pigs may disseminate to humans through food chains. At the same time, *Salmonella* spp. is among the most common causes of foodborne zoonoses, of which transmission to humans typically occur through the food chain, particularly via products from poultry, pigs, and cattle. In pigs, these pathogens can persist in the gastrointestinal tract without causing clinical signs, enabling their silent dissemination through the food chain.

In response to the reduction of antibiotic use in food animal production, biocides that are non-antibiotic antimicrobial agents (e.g., antiseptics and disinfectants) are gaining popularity as alternative agents for infection control. Certain biocides have been formulated in animal feeds for purposes like those of antibiotics. However, a particular concern has been raised regarding cross-resistance between biocides and antibiotics. In this case, prolonged use of a biocide at sub-therapeutic concentrations may trigger adaptive responses or mutations, either in chromosome or resistance genes on plasmids, leading to the selection of biocide-resistant bacterial strains that may also exhibit cross-resistance to antibiotics. To date, the occurrence of cross-resistance between biocides and antibiotics has been previously documented in several studies (Curiao *et al.,* 2016, Puangseree, *et al.,* 2021).

Halquinol is a non-antibiotic antimicrobial compound in the chloro-8-hydroxyquinoline group that inhibits the activity of RNA polymerase, thereby interfering RNA and protein synthesis of bacteria (Fraser and Creanor, 1975). Although not classified as biocide, halquinol is a non-antibiotic antimicrobial feed additive particularly commonly used in veterinary medicine, particularly in the pig and poultry industries. It is used both as a growth promoter and to control gastrointestinal disorders associated with *E. coli* and *Salmonella* in weaning and fattening pigs, primarily by improving gut health and reducing bacterial overgrowth. Currently, this antimicrobial agent is approved in many Asian and Latin American countries (e.g., Taiwan, Brazil, China, India, Vietnam, Thailand, and Indonesia etc.) (FAO and WHO, 2017).

In Thailand, halquinol has been approved exclusively for use in animals, specifically for the prevention and treatment of scours associated with *E. coli* and *Salmonella* in weaning and fattening pigs (FAO and WHO, 2017). This agent is classified as an intestinal anti-infective agent for veterinary use under the Anatomical Therapeutic Chemical Classification System for Veterinary Medicinal Products (ATCvet) and as a specially controlled drug for use in medicated premixes in animal feeds (JECFA, 2017, Rattanapanadda and Pakpong, 2022). Halquinol is commonly included in pig feed at concentrations ranging from 12 to 60 mg/kg for approximately 7 to 14 days, sometimes longer. This substance is usually diluted to sublethal concentrations downstream of its application, and following excretion, it enters the environment at sublethal concentrations. Subinhibitory exposure to halquinol may promote the selection of halquinol-resistant bacteria and contribute to cross-resistance with clinically important antibiotics. However, data on cross-resistance between halquinol and antibiotics in *E. coli* and *Salmonella* has not been reported to date. This study aimed to determine the possible cross-resistance between halquinol and antibiotics and horizontal transfer in *E. coli* and *Salmonella*.

Materials and Methods

Bacterial strains: The *E. coli* and *Salmonella* isolates used in this study originated from the bacterial stock of the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University. They were previously isolated from rectal swabs of pigs in 2018. The *E. coli* strains were isolated and confirmed biochemically (Quinn, 1994, Carter and Cole Jr., 2012) and a single colony of *E. coli* of each positive sample was collected. The *Salmonella* isolates were isolated by using ISO 6579:2002 (ISO, 2002). One to three *Salmonella* isolates were collected from each positive sample. All isolates were stored as 20% glycerol stocks at -80ºC.

Two *E. coli* (i.e., EC338 and EC339) and two *Salmonella* (i.e., SS38.1 and SS21.1) isolates exhibiting low halquinol Minimum Inhibitory Concentration (MIC) (EC338 and EC339, 8 µg/ml; SS38.1 and SS21.1, 16 µg/ml) were selected for stepwise-halquinol exposure experiment. EC338, EC339 and SS38.1 were susceptible to ampicillin, ciprofloxacin, chloramphenicol, gentamicin, streptomycin, sulfamethoxazole, tetracycline and trimethoprim. SS21.1 was susceptible to all the antibiotics except streptomycin and sulfamethoxazole.

Twenty-eight *E. coli* and 15 *Salmonella* isolates with high MIC halquinol (32-256 µg/ml and 32-64 µg/ml, respectively) were selected for detection of plasmid and then, those containing plasmids were included in conjugation experiments. All were resistant to multiple drugs (MDR, being resistant to 3 or more separate classes of antibiotics).

Antimicrobial susceptibility test: Susceptibilities to antibiotics and halquinol were examined by determination of MICs using a two-fold agar dilution technique according to the Clinical and Laboratory Standards Institute (CLSI) (NCCLS, 2002). The antimicrobial agents and clinical breakpoints were ampicillin (AMP, 32 µg/ml), ciprofloxacin (CIP, 4

µg/ml), chloramphenicol (CHP, 32 µg/ml), gentamicin (GEN, 16 µg/ml), streptomycin (STR, 32 µg/ml), sulfamethoxazole (SUL, 512 µg/ml), tetracycline (TET, 16 µg/ml), and trimethoprim (TRI, 16 µg/ml). All antibiotics were purchased from Sigma (St. Louis, MO, USA). *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 served as quality control strains.

Halquinol powder with 98.0076% purity and conforming to British Pharmacopoeia 80 Lot no. HLA-1387 was obtained from Vetcare Organics (Bangalore, India). It appears greenish. The optimum temperature for halquinol storage is 22-32°C.

Exposure experiments: The *E. coli* isolates EC338 and EC339 and the *Salmonella* isolates SS38.1 and SS21.1 were examined for the possible cross-resistance between halquinol and antibiotics (Fig. 1). *E. coli* K12 MG1655 was additionally included in this experiment.

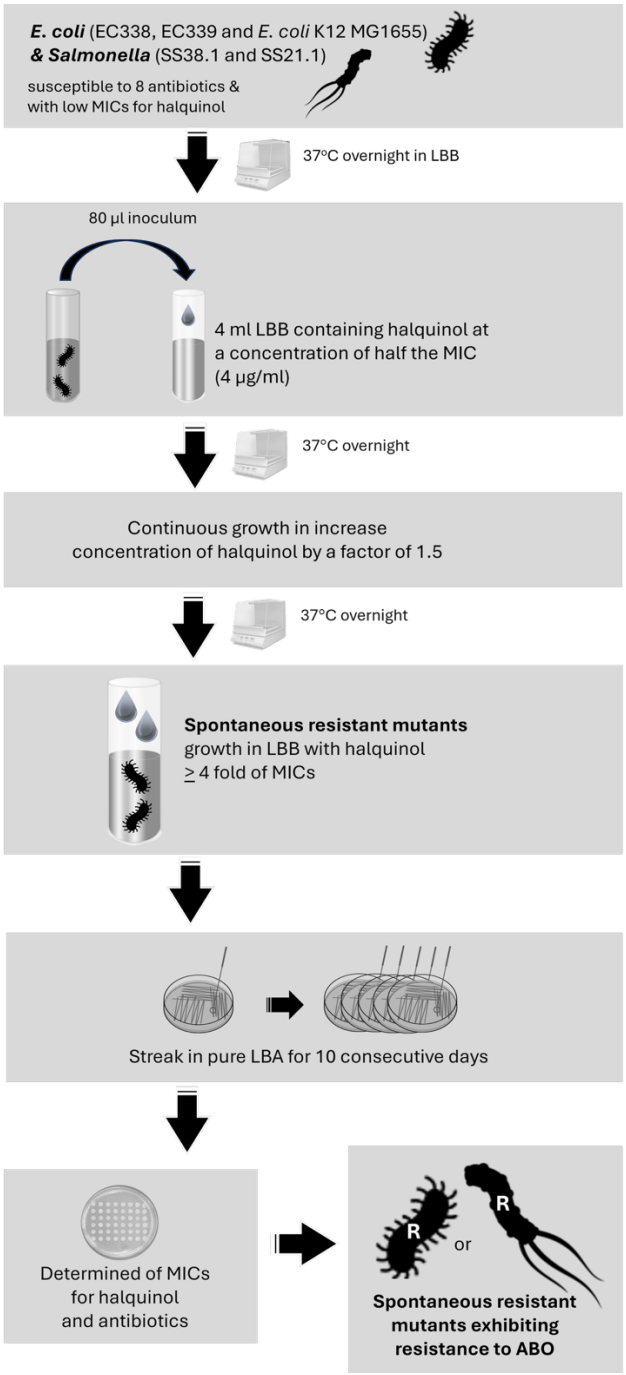

Figure 1 Schematic picture of *in vitro* exposure experiment. The isolates were exposed to gradually increasing concentrations of halquinol, starting at 4 µg/ml, with each subsequent concentration increased by a factor of 1.5. This procedure was repeated daily until no growth was observed. Colonies from the final passage were then purified, tested for resistance stability and confirmed for genetic continuity using rep-PCR. Abbreviations: ABO, antibiotics; LBA, Luria-Bertani agar; LBB, Luria-Bertani broth.

The isolates were exposed to gradually increasing concentrations of halquinol as previously described (Braoudaki and Hilton, 2005). Briefly, the five isolates were grown overnight at 37°C in Luria-Bertani (LB)

broth (Difco, BD Diagnostic Systems, Sparks, MD). Then, an 80 µl inoculum was inoculated into 4 ml LB containing halquinol at a concentration of half the MIC (4 µg/ml). This procedure was daily taken place in LB broth with increasing concentrations of halquinol by a factor of 1.5 until no growth was observed. Colonies from the final passage were sub-cultured on non-selective LB medium without antimicrobials for 10 consecutive days and tested for antibiotic susceptibility. Experiments were repeated on two separate occasions.

At each passage, the inoculums were cultured onto LB agar without halquinol and saved as stocks. Colonies from the final passage were randomly selected and determined for MICs for halquinol and other antibiotics. The ≥4-fold difference of MIC value was considered significant. The genetic continuity of colonies between pre- and post-halquinol exposed *E. coli* or *Salmonella* strains were confirmed by repetitive sequence-based polymerase chain reaction (rep-PCR) using primer pair: ERIC IR-5'-ATGTAAGCTCCTGGGGAT TCAC and ERIC II-5'-AAGTAAGTGACTGGGGTGAGCG and protocol as previously described (Amonsin *et al*., 1997).

Conjugation experiment: The presence of plasmid DNA was confirmed in the selected *E. coli* (n=28) and *Salmonella* (n=15) isolates with high MIC halquinol (32-256 µg/ml and 32-64 µg/ml, respectively) by plasmid DNA extraction using NucleoSpin® Plasmid (Macherey Nagel, Germany). Plasmid DNA was run in 0.8% agarose electrophoresis gel and visualized by UV-transilluminator.

The *E. coli* (n=19) and *Salmonella* (n=6) isolates containing plasmids were further tested for possible transferability of plasmids conferring halquinol resistance.

All the isolates were examined for their transferability of halquinol resistance-encoding gene(s) by filter mating method (Chen *et al*., 2004, Gebreyes and Thakur, 2005). *E. coli* K12 MG1655 rif^r (halquinol MIC = 4 µg/ml) and *Salmonella* Enteritidis SE12 rif^r (halquinol MIC = 4 µg/ml) were used as recipients. These recipients do not carry plasmids and are not resistant to the antibiotics tested. Transconjugants were selected on LB agar supplemented with 32 µg/ml of rifampicin and 16 µg/ml of halquinol. The presence of plasmid DNA was examined in each transconjugant and their identity to those in the donors was confirmed using restriction endonuclease analysis.

Results

Spontaneous resistance mutants and their resistant phenotype: None of the *E. coli* isolates (EC338 and EC339) grew beyond the first passage after being exposed to gradually increasing concentrations of halquinol and their growth stopped at a concentration of 12 µg/ml (Table 1).

Table 1 Antimicrobial susceptibilities of *Escherichia coli* and *Salmonella* pre-and post-halquinol exposure in in vitro exposure experiment

Bacteria	ID	HAL exposure	MIC (µg/ml)[a]								
			HAL	AMP	CHP	CIP	GEN	STR	SUL	TET	TRI
Escherichia coli	EC 338	Pre-exposure	16	1	8	0.125	0.25	2	1	2	1
	EC 338-1	Post-exposure	16	2	8	0.50	0.50	2	2	4	1
	EC 339	Pre-exposure	16	1	8	0.125	0.25	2	1	2	1
	EC 339-1	Post-exposure	16	2	8	0.50	0.50	2	2	4	1
	MG1655	Pre-exposure	16	8	8	0.125	0.25	2	1	2	1
	MG1655-1	Post-exposure	16	8	8	0.125	0.50	2	1	4	1
Salmonella	SS38.1	Pre-exposure	**16**	16	**4**	0.125	2	32	**16**	2	**1**
	SS38.1-1	Post-exposure	**>512**	16	**16**	0.25	4	16	**>1024**	4	**16**
	SS21.1	Pre-exposure	**16**	**2**	**4**	1	1	8	>1024	128	**1**
	SS21.1-1	Post-exposure	**>512**	**>256**	**16**	2	0.5	8	>1024	256	**256**

[a]Bold letter, antimicrobial agent with the MIC difference between pre-and post-halquinol exposure at least 4 folds

Abbreviations: HAL, Halquinol; AMP, ampicillin; CHP, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TRI, trimethoprim

Similar results were obtained from two separate experiments. The genetic continuity between pre- and post-halquinol exposed *E. coli* isolates was confirmed by rep-PCR profiling, with each pair exhibiting identical electrophoresis patterns (Fig. 1).

Upon the exposure to gradually increasing concentrations of halquinol, the growth of both *Salmonella* SS38.1 and SS21.1 ceased at a concentration of 691µg/ml. From confirmation of the continuity of the pre-and post-halquinol exposed isolates by rep-PCR profiling, each pair of pre-and post-halquinol exposed *Salmonella* isolates yield the identical electrophoresis patterns (Fig. 2).

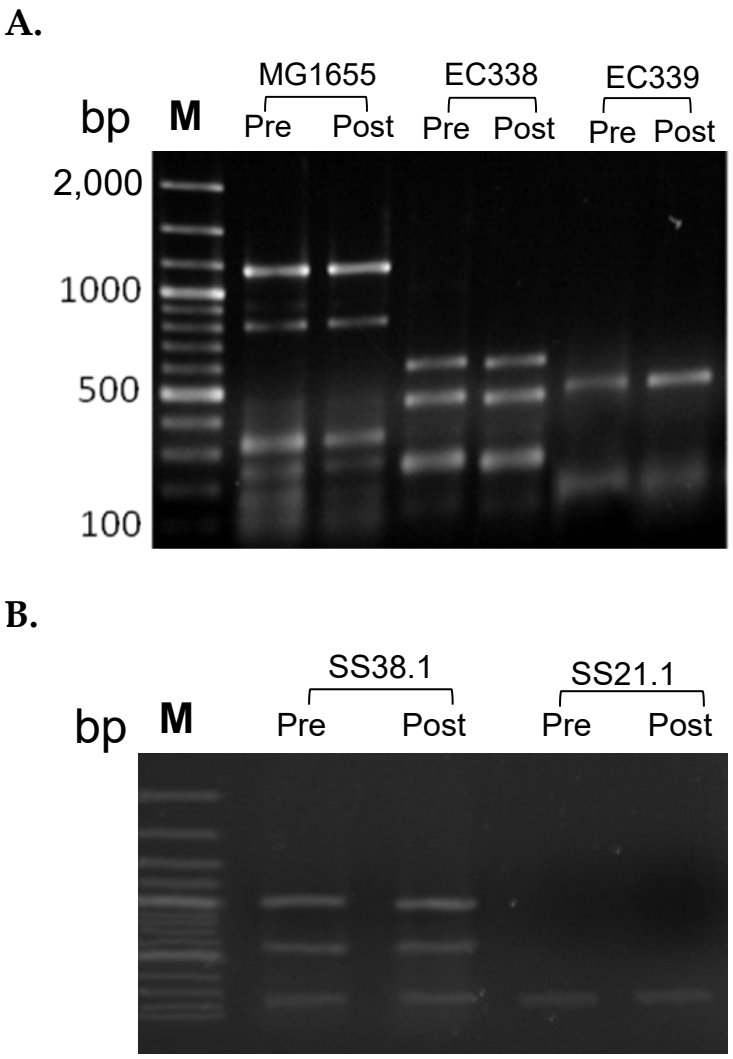

Figure 2 rep-PCR profiling of *E. coli* and *Salmonella* isolates from *in vito* exposure experiment. (A) *E. coli* EC 338 and EC 339 pre-and post exposed to halquinol and (B) *Salmonella* SS38.1 and SS21.1 pre-and post exposed to halquinol.

The halquinol and antibiotic MICs of the *E. coli* and *Salmonella* donors and corresponding transconjugants were compared (Table 1). The MIC values of halquinol of both halquinol-exposed strains; of chloramphenicol, sulfamethoxazole and trimethoprim in SS38.1-1 and of ampicillin, chloramphenicol, and trimethoprim in SS21.1-1 were higher than parent strains for at least 4 folds concentrations.

Conjugal transfer of halquinol resistance phenotype: Of all *E. coli* and *Salmonella* donors, none of the donor-recipient combinations yielded transconjugants on selective medium in biparental mating. The results were reproducible in the two separate experiments.

Discussion

In the exposure experiments, antibiotic-susceptible *E. coli* and *Salmonella* isolates with initially low MICs to halquinol were serially passaged in culture media containing progressively increasing concentrations of halquinol. This was to allow adaptive resistance development and selection for elevated MICs. The results indicated that successive exposure to halquinol did not yield *E. coli* mutant derivatives with stable reduced susceptibility to either halquinol or tested antibiotics. The MIC values of the recovered colonies remained comparable to those of the pre-exposure parental strains, suggesting that cross-resistance to antibiotics had not been developed.

In contrast, exposure to halquinol led to a marked increase in MICs in *Salmonella* isolates SS38.1 and SS21.1, rising by at least 32-fold, from an initial 16 µg/ml to ≥ 512 µg/ml, indicating the development of reduced susceptibility. Following halquinol exposure, the MICs for chloramphenicol, sulfamethoxazole, and trimethoprim in SS38.1-1 and SS21.1-1 also increased, suggesting the development of cross-resistance between halquinol and these antibiotics. To date, the common-resistance mechanism between halquinol and other antibiotics has not been identified. Since these antimicrobials are structurally unrelated (i.e., halquinol, ampicillin, trimethoprim and sulfamethoxazole), the observed cross-resistance is likely associated with multidrug efflux pumps. This is supported by the fact that such multidrug efflux systems can extrude a broad range of structurally diverse compounds (Chuanchuen and Schweizer, 2004, Kumar and Schweizer, 2005). A well-characterized multidrug efflux pump in *Salmonella* is

the AcrAB-TolC system, which has been shown to extrude a wide range of antibiotics and dyes (Wannaprasat *et al.,* 2013, Alenazy, 2022, Yamasaki *et al.,* 2023). However, the association of AcrAB-TolC with trimethoprim and sulfamethoxazole resistance has never been reported. Moreover, the involvement of the AcrAB-TolC system or other efflux pumps in halquinol resistance remains unreported as well. However, this study did not pursue the role of multidrug efflux pumps in the reduced susceptibility to antibiotics and non-antibiotic antimicrobials. Besides, RamA was originally identified in *Salmonella* (van der Straaten *et al.,* 2004). RamA is known to confer a multidrug resistance (MAR) phenotype and has been associated with trimethoprim resistance in *Klebsiella pneumoniae* (George *et al.,* 1995). However, it remains unclear whether halquinol is a substrate of RamA. Alternatively, the observed cross-resistance may be due to a nonspecific reduction in cell permeability, limiting the entry of chemically unrelated compounds. Although the exact mechanism remains unidentified, the findings in this study confirm that *in vitro* exposure to exposure to halquinol can contribute to the selection of antibiotic resistance, at least in *Salmonella*. This highlights the need for further studies under *in vivo* conditions.

A question may arise as to why cross-resistance was observed in *Salmonella* but not in *E. coli* despite both belonging to the Enterobacteriaceae family. This may result from a combination of factors e.g., species-specific resistance mechanisms, differences in membrane permeability, genetic plasticity and mobile elements, fitness cost and mutation tolerance etc. Even though *Salmonella* and *E. coli* are closely related, they differ in regulatory networks and efflux systems. *Salmonella* may have more effective outer membrane adaptations or porin changes under stress that limit drug uptake, particularly in responses to halquinol exposure. Importantly, experimental factors may also exist. These included initial MIC levels, strain-specific genetics, and experimental conditions that might favor resistance emergence in *Salmonella*.

It may be argued that the development of reduced susceptibility to non-antibiotic antimicrobials observed in laboratory experiments does not reflect real-world conditions. On farms, non-antibiotic antimicrobials are applied at their in-use concentrations and then diluted to sublethal concentrations downstream of their use. As a result, bacteria can be repeatedly exposed to subinhibitory levels of the chemicals in a manner similar to laboratory conditions. In case of halquinol, pigs have been fed with halquinol for a period of time and the substance will be diluted to sublethal concentrations downstream of its application. Bacteria could be exposed to subinhibitory concentrations of halquinol and develop resistance and cross-resistance to antibiotics. However, a previous study reported no detection of halquinol-resistant bacteria when the compound was used as a growth promoter in broilers (Almeida *et al.,* 2025).

As the mode of action of halquinol is to inactivate RNA polymerase, thus inhibiting both RNA and protein synthesis of the bacteria (Fraser and Creanor, 1975), the mechanism of resistance has not been identified. In conjugation experiment, the high halquinol resistance phenotype was not horizontally transferred, suggesting that such less susceptibility to halquinol is not on conjugative plasmid and may be intrinsically or chromosomally encoded. Further studies are required to elucidate the possibility of halquinol-resistance transfer in *in vivo* condition and the mechanism(s) responsible for such low susceptibility. It is important to note that halquinol resistance genes (if any) may reside on very small or very large plasmids that are not easily transferable under *in vitro* conditions. Additionally, the transfer of AMR genes may differ between *in vitro* conditions and *in vivo* environments. Further studies are warranted to determine whether halquinol resistance can be transferred under *in vivo* conditions.

In conclusion, halquinol exposure did not promote cross-resistance to antibiotics in *E. coli* but did in *Salmonella* under *in vitro* conditions. However, resistance to antimicrobial agents is a dynamic and evolving process. Caution is warranted in the use of halquinol in veterinary medicine, and its application should follow responsible and prudent practices. The findings warrant further studies to provide a better understanding of the possible link between non-antibiotic antimicrobial agents and AMR.

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