Comparison of Genotypic and Phenotypic Predictions for Heavy Metal Resistance in *Salmonella enterica* and *Escherichia coli*

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ABSTRACT
Salmonella enterica and *Escherichia coli* are two pathogenic bacteria of worldwide importance that can infect the gastrointestinal tract. Contamination in the food supply chain is an area of concern. Animal feed may be supplemented with essential trace elements, which function as nutritional additives to promote growth & health and optimize production. Bacteria have acquired many metal resistance genes to adapt to the exposure of metals. In this study, our objectives were to evaluate in *S. enterica* and *E. coli*, the correlation between the resistance genotype and phenotype to certain heavy metals, and the ability of conjugative plasmids to transfer antimicrobial resistance genes (AMRGs) and heavy metal resistance genes (HMRGs). A total of 10 strains, five *S. enterica* and five *E. coli*, were used for this study. Minimal inhibitory concentrations (MICs) were determined for heavy metals: copper, silver, arsenic, and tellurite. The tested isolates showed resistance to copper (5/10; 50%), arsenic (7/10; 70%), and silver (9/10; 90%). Cohen's Kappa statistics were used to analyze genotype to phenotype agreements. Among the 10 strains sampled, the accordance between geno- and phenotypic heavy metal resistance was fair for copper (kappa = 0.4), none to slight for arsenic (kappa = 0.19) and tellurite (kappa = 0), and no agreement for silver (kappa = -0.19). The transfer of HMRGs was determined in a conjugation experiment performed for all five *Salmonella* strains as donors using mixed broth cultures. Transconjugants were obtained only from the genotypically tellurite-resistant strain PSU-3260, which yielded a transfer frequency of 10^{-3} transconjugants per donor. In such strain, the tellurite-resistant genes reside on an IncHI2-type plasmid that shares high DNA sequence identity with known HMRG-disseminating *Salmonella* plasmids. Our results indicated no considerable correlation between the geno- and phenotypic resistance towards heavy metals in the sampled *S. enterica* and *E. coli*. The necessity of research in this area is supported by the lack of standardized protocols and MIC clinical breakpoints for heavy metals.

KEYWORDS
Heavy metal; resistance; *Salmonella*; *E. coli*; agriculture; genotype; phenotype; MIC

INTRODUCTION

Salmonella enterica and *Escherichia coli* are two pathogenic bacteria of humans and animals that can infect the gastrointestinal tract. *E. coli* is a Gram-negative, facultative anaerobic bacillus found in the environment (including soil, water, and the gastrointestinal tract of warm-blooded animals), which can contaminate meat and produce. Though it is commonly part of the commensal intestinal flora, it can also be pathogenic, causing many diarrheal illnesses, including traveler's diarrhea and dysentery.[1] Shiga toxin-producing *E. coli* (STEC) is a pathotype of this species capable of causing bacillary dysentery, hemorrhagic colitis,[3] bloody diarrhea, hemolytic uremic syndrome (HUS), end-stage renal disease (ESRD), and even death.[2] STEC infections cause an estimated 265,000 illnesses, 3,600 hospitalizations, and about 30 deaths annually in the USA.[4]

S. enterica is a Gram-negative, facultative intracellular anaerobe of worldwide importance. Typically it is an orally acquired pathogen that can cause enteric fever, enterocolitis/diarrhea, and bacteremia, estimated to cause 1.3 billion disease cases annually.[5] Non-typhoidal *Salmonella* causes approximately 28% of foodborne illness-associated deaths.[6, 7] Also, it accounts for more than 93 million infections per year globally and over 1 million in the USA, thus being a leading cause of bacterial foodborne illness.[8]

Contamination in the food supply chain is an area of concern. Several outbreaks of *E. coli* and *Salmonella* arising from livestock have occurred in recent years. *E. coli* was responsible for an outbreak linked to ground beef which caused 209 reported cases in 2019.[9] Likewise, a backyard poultry-linked outbreak caused by *Salmonella* resulted in 1,135 illnesses, 273 hospitalizations, and two

deaths in 2021.[10] In the industry of commercial agriculture, heavy metals represent both mineral nutrients and potential contaminants.[11] Essential trace elements are commonly added to animal feed as nutritional additives to promote growth and health and optimize production.[11—14] These trace elements such as copper and zinc are required for hormone function, normal reproduction, vitamin synthesis, enzyme formation, and to support the integrity of the host immune system.[14] Yet, excessive exposure to undesirable levels of heavy metals damages the health of food-producing animals and the bioaccumulation of these metals could subsequently threaten consumers' health.[14] Apart from the heavy metals added intentionally, other metals such as mercury (Hg), lead (Pb), cadmium (Cd), and arsenic (As) occasionally contaminate animal feed.[14] Said animal feed contamination occurs through husbandry practices, soil ingestion, minor dietary ingredients, supplements, or spurious soil contamination in foliage.[15]

Bacteria have acquired many metal resistance genes through horizontal gene transfer and vertical evolution to adapt to the exposure of metals.[16, 17] There is co-resistance between resistance genes for antibiotics, metals,[14, 17, 18, 19] and disinfectants.[20, 21] Besides co-resistance (multiple resistance genes located on the same mobile element), co-selection of antimicrobial and heavy metal genes can also be mediated by cross-resistance (shared mechanisms of resistance), co-regulation (altered expression of resistance genes after exposure to toxic compounds), or biofilm formation.[14, 17, 18, 22, 23]

The growing evidence regarding antimicrobial resistance (AMR) co-selection among bacteria exposed to various heavy metals in animal diets has caused concern.[24, 25] The co-selection of genes providing resistance to heavy metals and antimicrobials of clinical importance, which pose a possible health hazard, requires further investigation.[17, 26] In addition, the correlation between genotypic and phenotypic resistance to heavy metals is not fully understood. Genotypic resistance can be screened, and most would assume that like AMR, there would be a correlation between geno- and phenotypic resistance. Yet, a better understanding of the correlation between genes and phenotypes will allow for better characterization of bacterial virulence factors. Additionally, the spread of resistance could be monitored by evaluating the plasmid transfer of these genes.

Therefore, in this study, our objectives were to evaluate in a small collection of *S. enterica* and *E. coli*: (1) the correlation between the presence of heavy metal resistance genes and the actual resistance to these metals; and (2) the ability of conjugative plasmids to transfer antimicrobial resistance genes (AMRGs) and heavy metal resistance genes (HMRGs).

MATERIALS AND METHODS

Bacterial strains and culture conditions

A total of 10 strains, five *Salmonella enterica* and five *Escherichia coli*, were used for this study (**Table 1**). These were selected from the NCBI Pathogen Detection database (*https://www.ncbi.nlm.nih.gov/pathogens*, accessed 4 October 2022). Strains were chosen upon availability and possession of resistance genes for the heavy metals in use. Whole genome sequencing (WGS), antimicrobial resistance (AMR), heavy metal resistance (HMR), and plasmid profiling were performed.[27] The presence of HMRGs was determined by analysis in the NCBI Pathogen Detection pipeline using the AMRFinderPlus tool with default parameters (*i.e.,* >90% identity and >50% coverage of the reference).[28]

Strains were maintained in 20% glycerol stocks at -80 ˚C for long-term storage and were routinely cultured in lysogeny broth (LB) and Mueller-Hinton (MH) agar at 37 ˚C. Sodium chloride was omitted from LB when supplemented with silver ($AgNO_3$), and so were their controls. Antibiotics were used at the following concentrations: nalidixic acid (30 μg/mL), chloramphenicol (12.5 μg/mL), kanamycin (25 μg/mL), ampicillin (50 μg/mL), and tetracycline (10 μg/mL). All media components were purchased from BD Difco (Franklin Lakes, NJ) and all chemicals from Millipore Sigma (St. Louis, MO) unless otherwise specified.

Heavy metal susceptibility testing and correlation analysis

All strains were cultured in MH broth overnight at 37 ˚C, then subcultured once and grown to mid-log phase. The bacterial suspension was diluted to 0.05 OD_{600} using MH broth. To determine the minimal inhibitory concentrations (MICs) for heavy metals, a standard broth microdilution procedure was conducted according to the Clinical and Laboratory Standards Institute,[29] with MH Broth and no-salt LB in an aerobic atmosphere. MH broth was supplemented separately with tellurite (Na_2TeO_3), copper ($CuSO_4$), and arsenic ($NaAsO_2$). Meanwhile, no-salt LB was supplemented only with silver ($AgNO_3$) since supplementing the MH broth with this metal caused the precipitation of such. MICs for silver, tellurite, and arsenic were done at two-fold dilutions with concentrations ranging from 0.016 to 8 mM. Copper MICs were done at two-fold dilutions from 0.063 to 32 mM. MICs were determined as the lowest concentration at which there was no visible growth after incubation for 24 hours at 37 ˚C. *S. enterica* reference strain LT2 was used as a benchmark to define the species' susceptibility towards heavy metals and to classify the test strains as resistant or susceptible.[12] DH5α, a common laboratory strain, was used as a reference for *E. coli*. This heavy metal susceptibility test was repeated with a pH adjustment performed on media containing silver, tellurite, and arsenic to pH 7.4, and copper to pH 7.2, using HCl and NaOH. Cohen's Kappa statistics were used to analyze genotype to phenotype agreements,[30] using Minitab Statistical Software (Version 17.1; 2010). Kappa coefficient values were interpreted as follows: ≤ 0 (no agreement),

0.01–0.20 (none to slight), 0.21–0.40 (fair), 0.41– 0.60 (moderate), 0.61–0.80 (substantial), and 0.81–1.00 (almost perfect agreement).[31] For the most part, upon the presence of a single resistance gene, the bacterial strains were interpreted as resistant. The exception was the presence of the gene *golT* in *Salmonella enterica* strains, which was excluded from the statistical analysis. Since the LT2 control and all *Salmonella enterica* strains have the same MIC values and genotype regarding this gene, it was not possible to establish with certainty that this gene confers resistance to copper in *Salmonella* strains. Likewise, the presence of a resistance operon was interpreted to confer the same level of resistance as a single resistance gene.

Conjugation assays

The transfer of HMRGs was determined in a conjugation experiment performed for all five *Salmonella* strains as donors using mixed broth cultures. Samples of the donors and recipient were grown overnight in MH broth. Overnight cultures were diluted to 0.1 OD_{600} using MH broth. 100 µL of the diluted donor were mixed with 1 mL of the diluted recipient culture. Two controls were made: one with donor only (100 µL of diluted donor culture and 1 mL of MH broth), and one with recipient only (1 mL of diluted recipient culture and 100 µL MH broth). They were incubated at 30 ˚C without shaking for 4 hours. Dilution series of mating mixtures were performed in 1X PBS through 10^9 and plated as follows: donor control on MH + heavy metal; recipient control on MH + Nal; and donor/recipient mixtures on MH + heavy metal + Nal to select for transconjugants. Plates were incubated at 37 ˚C overnight. The transfer efficiency was determined by dividing the transconjugant CFU/mL by donor CFU/mL. To confirm that the transconjugants were *E. coli*, PCR was used to screen the *uidA* gene, a traditional marker of *E. coli* lineages.[32] For the standard PCR reaction: 2.5 µL *Taq* ThermoPol buffer (New England Biolabs, Ipswich, MA), 0.5 µL dNTPs, 0.5 µL forward primer, 0.5 µL reverse primer, 1 µL template DNA, 0.125 µL *Taq* polymerase, and 19.9 µL nuclease-free water were used in a 25 µL reaction with primers uidA_F (5'-GCGTCTGTTGACTGGCAGGTGGTGG-3') and uidA_R (5' GTTGCCCGCTTCGAAACCAATGCCT-3').[33, 34] PCR was performed under the following cycle conditions: initial denaturation period of 95 ˚C for 30 seconds; 30 cycles of 95 ˚C for 30 seconds, 61 ˚C for 1 minute, 68 ˚C for 30 seconds; and a final extension for 5 minutes. The PCR products were separated in 1% agarose gel in TAE buffer. After staining with SYBR Safe (ThermoFisher Scientific, Waltham, MA), the gel was visualized under UV light. Cotransfer of AMRGs was assessed by plating. Selection was made on MH agar plates supplemented separately with chloramphenicol, kanamycin, ampicillin, and tetracycline. They were incubated for 24 hours at 37 ˚C.

Plasmid analysis

The predicted plasmid content of each strain was identified by analysis of its assembled genome contigs at the PlasmidFinder 2.1 webserver (*https://cge.cbs.dtu.dk/services/PlasmidFinder/*). Noting the precedent for conjugative IncHI2 plasmids in *Salmonella*,[34] and the successful transfer of tellurite resistance from PSU-3260 to *E. coli* DH5α, we chose the IncHI2 replicon in PSU-3260 for further study. The complete plasmid sequence of pSTM6-275 was downloaded from NCBI (NZ_CP019647.1) and was used as a database for local BLASTn search. All contigs of PSU-3260 were used as queries against this database, and those with significant identity to the pSTM6-275 plasmid were retained in a FASTA file. The sequences of pSTM6-275 and homologous PSU-3260 contigs were then used in the construction of a ring diagram by BRIG,[36] according to the software's standard settings.

RESULTS

Presence and prevalence of heavy metal resistance genes in Salmonella enterica and Escherichia coli

The five *S. enterica* strains were chosen from a collection of *Salmonella* isolates from wild birds, and the five *E. coli* strains were obtained from sequenced isolates deposited in the *E. coli* Reference Center (ECRC); isolates are listed in **Table 1**. Most *E. coli* strains were isolated from animals used as livestock, and most *Salmonella* strains were isolated from water birds (**Table 1**). Among the sampled *S. enterica* strains, 20 genes considered to confer heavy metal resistance were found under stress genotypes using the isolate browser from the NCBI Pathogen Detection database (*https://www.ncbi.nlm.nih.gov/pathogens*).

These heavy metal resistance genes included: for copper (*golT*, *pcoA, pcoB, pcoC, pcoD, pcoE, pcoR, pcoS*); for silver (*silA, silB, silC, silE, silF, silP, silR. silS*); for tellurite (*terD, terW, terZ);* and for arsenic (*arsD*) (**Table 1**). A total of 21 HMR genes were found among the sampled *E. coli* strains. These were: for copper (*pcoA, pcoB, pcoC, pcoD, pcoE, pcoR, pcoS*); for silver (*silA, silB, silC, silE, silF, silP, silR, silS*); for arsenic (*arsA, arsD, arsR);* and for tellurite (*terD, terW, terZ*) (**Table 1**).

Genus & species	Strain	Serovar or Serotype	Isolation source	Heavy metal genes	Plasmid Inc-types	Isolate references
Salmonella enterica	PSU-3176	Typhimurium	Gut; Gallinule, Common (*Gallinula galeata*)	*golT, pcoABCDERS, silABCEFPRS*	IncFIA, IncFII, IncI1-I(Alpha)	Fu *et al.,* 2021
Salmonella enterica	PSU-3260	Typhimurium	Dowitcher, Long billed (*Limnodromus scolopaceus*)	*golT, terDWZ*	IncHI2, IncHI2A	Fu *et al.,* 2021
Salmonella enterica	PSU-3373	Montevideo	Owl, Snowy (*Bubo scandiacus*) colon	*golT, arsD*	IncC	Fu *et al.,* 2021
Salmonella enterica	PSU-3384	Schwarzengrun	Gull, Ring-billed (*Larus delawarensis*) colon	*golT, pcoABCDERS, silABCEFPRS*	Col(pHAD28)	Fu *et al.,* 2021
Salmonella enterica	PSU-3390	Saintpaul	Gull, Ring-billed (*Larus delawarensis*) colon	*golT, silABCEFPRS*	IncFIB(K), IncN	Fu *et al.,* 2021
Escherichia coli	PSU-4439	O15:H45	Chicken	*pcoABCDERS, silABCEFPRS*	IncFIA, IncFIB(AP001918), IncFII, IncFII(pHN7A8), IncI1-I(Alpha), IncX4	PDT001079836.1*****
Escherichia coli	PSU-4474	O73:H19	Pig, (Porcine)	*arsADR, pcoABCDERS, silABCEFPRS*	Col(pHAD28), IncFIA(HI1), IncHI1A, IncHI1B(R27)	PDT001079841.1*****
Escherichia coli	PSU-4512	O8:H19	Avian	/	Col(MG828), IncI1-I(Alpha)	PDT001079838.1*
Escherichia coli	PSU-4521	O9:H30	Cow, (Bovine)	*copABCDERS, silABCEFPRS*	IncFIB(AP001918), IncFIC(FII), IncY	PDT001079821.1*****
Escherichia coli	PSU-4612	O157:H7	Cow, (Bovine)	*terDWZ*	IncFIA, IncFIB(AP001918), IncFII	PDT001079829.1*

/No heavy metal resistance genes found.
*Accession number from the NCBI Pathogen Detection Isolate Browser.

Table 1. List of characteristics from isolated bacterial strains.

Most strains exhibited the simultaneous presence of resistance genes for copper and silver (50%; 5/10) (**Figure 1**). Strain PSU-4474 had resistance genes for three metals (copper, arsenic, and silver), and strain PSU-4612 carried resistance genes for one metal (tellurite), whilst only strain PSU-4512 lacked detectable HMRGs (**Table 3**). Copper resistance-conferring genes were the most widespread among both bacterial species (80%; 8/10). Meanwhile, *arsA* and *arsR* genes had the lowest prevalence (10%; 1/10), present only in *E. coli* strain PSU-4474 (**Figure 2 & Table 3**). Additionally, *arsD* and all tellurite resistance-conferring genes were observed at a low frequency of occurrence (20%; 2/10) (**Figure 2**).

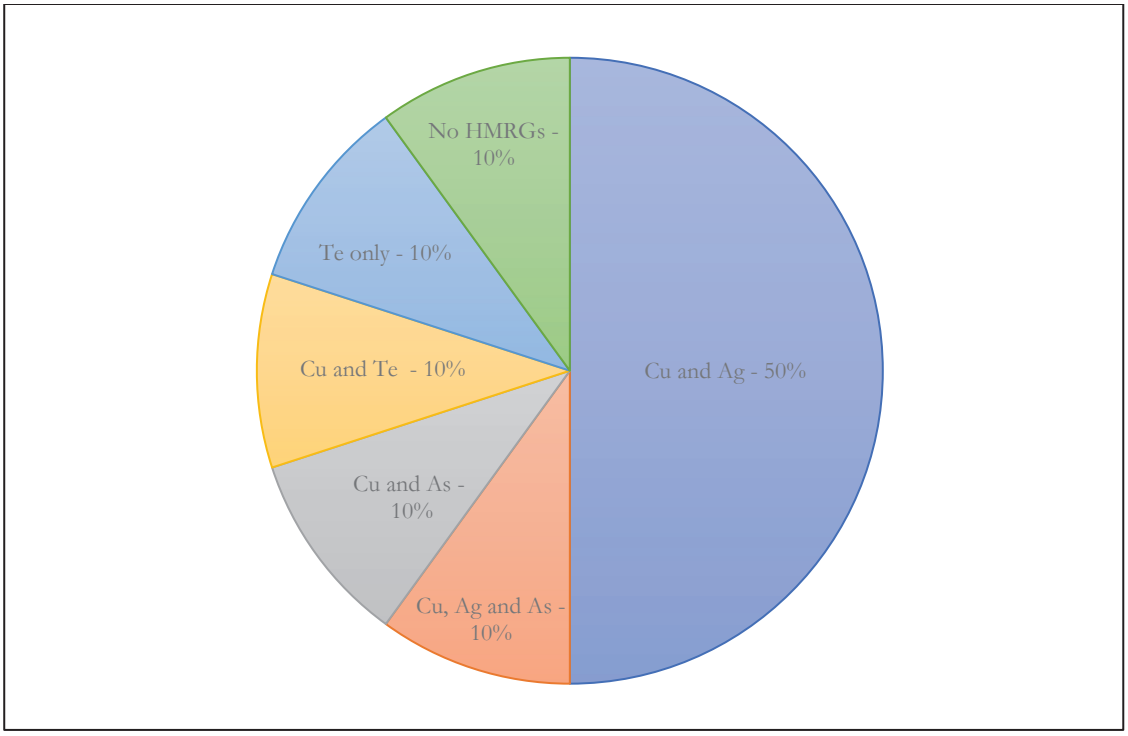

Figure 1. Proportion of heavy metal resistance genes among the sampled isolates. 80% of the samples carry resistance to multiple metals. Abbreviations: (Cu) copper; (Ag) silver, (As) arsenic, and (Te) tellurite.

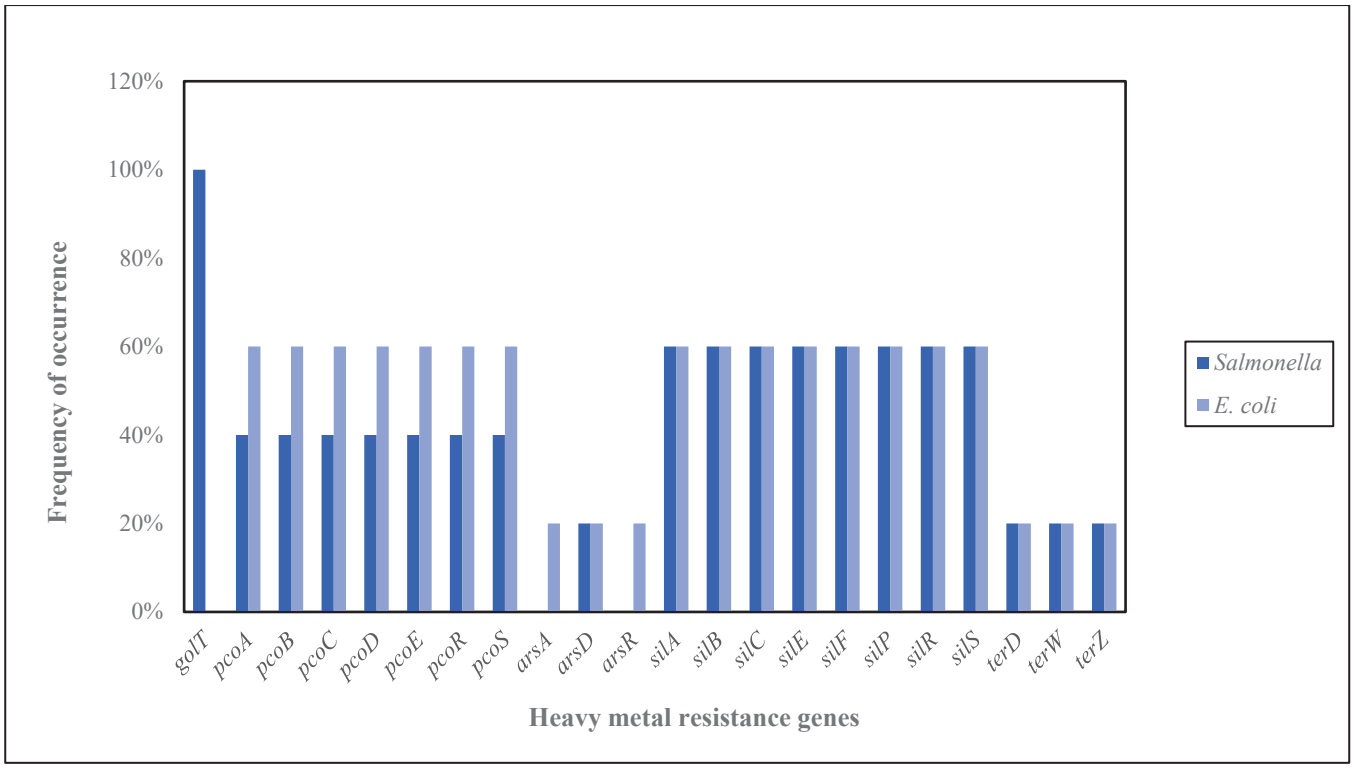

Figure 2. Prevalence of heavy metal resistance genes in *S. enterica* and *E. coli* isolates. *gol-* and *pco-* genes confer resistance to copper (Cu); *sil-* genes confer resistance to silver (Ag); *ars-* genes confer resistance to arsenic (As); and *ter-* genes confer resistance to tellurite (Te).

Minimum inhibitory concentrations of heavy metals for S. enterica and E. coli

MIC was interpreted as the minimum heavy metal concentration at which bacterial growth was not observed. Heavy metal susceptibility or resistance was established using *Salmonella* strain LT2 and *E. coli* strain DH5α as reference for their respective bacterial species. Strains with MICs greater than those of LT2 or DH5α were considered resistant. MIC assays were first performed without adjusting the pH of metal-supplemented broth. In subsequent trials, metal-supplemented media were neutralized before inoculation to account for possible pH effects on bacterial growth (**Table 2**).

	Copper (mM)		Arsenic (mM)		Silver (mM)		Tellurite (mM)	
Strain	No pH adjustment	pH adjustment	No pH adjustment	pH adjustment	No pH adjustment	pH adjustment	No pH adjustment	pH adjustment
LT2	32	32	8	0.125	0.063	0.125	0.25	0.016
PSU-3176	32	32	8	0.063	0.125	0.5	0.016	0.016
PSU-3260	32	32	8	0.063	0.063	0.25	0.125	0.016
PSU-3373	32	32	>8	0.5	0.031	0.25	1	0.016
PSU-3384	32	32	8	0.5	0.031	0.25	0.016	0.016
PSU-3390	32	32	0.031	0.063	0.063	0.25	0.016	0.016
DH5α	*	16	*	1	*	0.125	*	0.016
PSU-4439	32	32	8	4	0.063	0.25	0.031	0.016
PSU-4474	32	32	8	8	0.063	0.125	0.031	0.016
PSU-4512	32	32	8	8	0.031	0.25	0.016	0.016
PSU-4521	32	32	8	8	0.125	0.25	0.016	0.016
PSU-4612	32	32	>8	4	0.125	0.25	0.016	0.016
DH5α (pPSU-3260) transconjugant	*	16	*	1	*	0.125	*	1

**Not determined*

> growth started in first well

Table 2. Comparison between minimal inhibitory concentrations (MIC) with and without pH adjustments.

Copper MICs were identical (32 mM) among most *Salmonella* and *E. coli* strains, including LT2, demonstrating a comparable tolerance to the metal with one of the reference strains (**Table 3**); the only exception to MIC=32 mM was DH5α (16 mM). Notably, strains PSU-4512 and PSU-4612 (*E. coli*) were tolerant to copper despite lacking any copper-specific HMRGs. For arsenic, seven of the strains sampled had a higher MIC value than their respective reference strains, even though no apparent arsenic-specific HMRGs were present for most strains. These were *Salmonella* strains PSU-3384, and *E. coli* strains PSU-4439, PSU-4512, PSU-4521, and PSU-4612; strains PSU-3373 (*Salmonella*) and PSU-4474 (*E. coli*) were the only strains to present arsenic-specific resistance genes (**Table 3**). In the silver MICs, eight out of 10 strains had a value of 0.25 mM. Two exceptions were strains PSU-3176 for *Salmonella* and PSU-4474 for *E. coli* (**Table 3**); all strains were resistant to silver aside from *E. coli* strain PSU-4474. Four strains (PSU-3260, -3373, -4512, and -4612) had a higher MIC value than their respective reference strains (0.125 mM) while lacking silver-specific HMRGs. Additionally, *E. coli* strain PSU-4474 had the same value as the reference strain while presenting eight silver HMRGs. For tellurite, all strains had the same MIC as the reference strains (0.016 mM). This was the lowest dilution for the minimum inhibitory concentration. Yet, two of them (PSU-3260 and PSU-4612) had three tellurite-specific HMRGs. Moreover, the tested isolates showed resistance to copper (5/10; 50%), arsenic (7/10; 70%) and silver (9/10; 90%) (**Figure 3**). Among the 10 strains sampled, the accordance between geno- and phenotypic heavy metal resistance was fair for copper (kappa = 0.4), none to slight for arsenic (kappa = 0.19) and tellurite (kappa = 0), and no agreement for silver (kappa = -0.19) (**Table 4**). For tellurite, the value of kappa indicates that the agreement is the same as would be expected by chance, meanwhile, for silver, the agreement is less than random chance.

Copper			Genes								Arsenic	Genes			Silver	Genes							Tellurite	Genes		
Genus and species	Strain	MIC (mM)	*golT*	*pcoA*	*pcoB*	*pcoC*	*pcoD*	*pcoE*	*pcoR*	*pcoS*	MIC (mM)	*arsA*	*arsD*	*arsR*	MIC (mM)	*silA*	*silB*	*silC*	*silE*	*silF*	*silP*	*silR*	*silS*	MIC (mM)	*terD*	*terW*	*terZ*
S. enterica	LT2	32	+	-	-	-	-	-	-	-	0.125	-	-	-	0.125	-	-	-	-	-	-	-	-	0.016	-	-	-
S. enterica	PSU-3176	32	+	+	+	+	+	+	+	+	0.063	-	-	-	0.5	+	+	+	+	+	+	+	+	0.016	-	-	-
S. enterica	PSU-3260	32	+	-	-	-	-	-	-	-	0.063	-	-	-	0.25	-	-	-	-	-	-	-	-	0.016	+	+	+
S. enterica	PSU-3373	32	+	-	-	-	-	-	-	-	0.5	-	+	-	0.25	-	-	-	-	-	-	-	-	0.016	-	-	-
S. enterica	PSU-3384	32	+	+	+	+	+	+	+	+	0.5	-	-	-	0.25	+	+	+	+	+	+	+	+	0.016	-	-	-
S. enterica	PSU-3390	32	+	-	-	-	-	-	-	-	0.063	-	-	-	0.25	+	+	+	+	+	+	+	+	0.016	-	-	-
E. coli	DH5α	16	-	-	-	-	-	-	-	-	1	-	-	-	0.125	-	-	-	-	-	-	-	-	0.016	-	-	-
E. coli	PSU-4439	32	-	+	+	+	+	+	+	+	4	-	-	-	0.25	+	+	+	+	+	+	+	+	0.016	-	-	-
E. coli	PSU-4474	32	-	+	+	+	+	+	+	+	8	+	+	+	0.125	+	+	+	+	+	+	+	+	0.016	-	-	-
E. coli	PSU-4512	32	-	-	-	-	-	-	-	-	8	-	-	-	0.25	-	-	-	-	-	-	-	-	0.016	-	-	-
E. coli	PSU-4521	32	-	+	+	+	+	+	+	+	8	-	-	-	0.25	+	+	+	+	+	+	+	+	0.016	-	-	-
E. coli	PSU-4612	32	-	-	-	-	-	-	-	-	4	-	-	-	0.25	-	-	-	-	-	-	-	-	0.016	+	+	+
E. coli	DH5α (pPSU-3260) transconjugant	16									1				0.125									1			

Grey boxes indicate resistance in comparison to the respective reference strains.
Dark grey boxes indicate inaccurate genotypic prediction in regard to MIC values.
MIC values were determined using the adjusted pH values.
Presence of golT was excluded from the statistical analysis.

Table 3. Heavy metal minimum inhibitory concentrations and resistance genes.

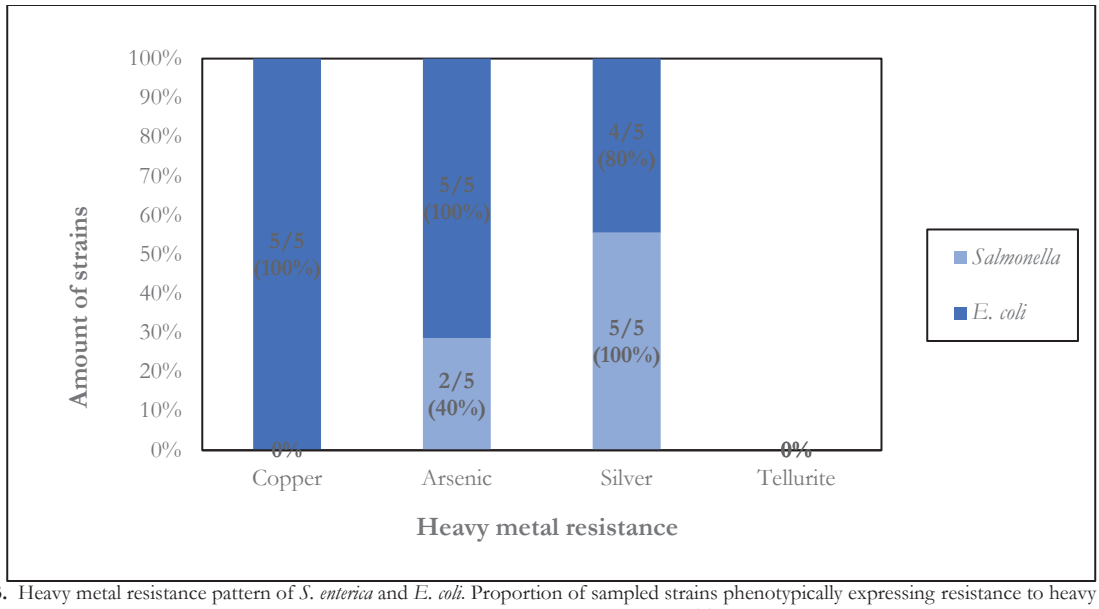

Figure 3. Heavy metal resistance pattern of *S. enterica* and *E. coli*. Proportion of sampled strains phenotypically expressing resistance to heavy metals in comparison to their respective reference strains, LT2 and DH5α.

	Susceptible Phenotype		Resistant Phenotype				
Heavy metal	**Resistant genotype**	**Susceptible genotype**	**Resistant genotype**	**Susceptible genotype**	**Agreement**	**Kappa Coefficiency**	**p-value**
Copper	2	3	3	2	60.00%	0.4	0.0311
Arsenic	0	3	2	5	50.00%	0.19	0.0716
Silver	1	0	5	4	50.00%	-0.19	0.8882
Tellurite	2	8	0	0	80.00%	0	/

/No value available

Table 4. Genotypic and phenotypic comparison for heavy metal resistance.

Transfer of heavy metal and AMR determinants

All five *Salmonella* strains were tested as plasmid donors with the recipient strain *E. coli* DH5α. Isolation of transconjugants was performed by selecting for the nalidixic acid-resistant phenotype of the recipient and the heavy metal-resistant phenotype of the given donor. Transconjugants were obtained only from the genotypically tellurite-resistant strain PSU-3260, which yielded a transfer frequency of 10^{-3} transconjugants per donor. The tellurite-resistant *E. coli* transconjugant was also resistant to kanamycin, ampicillin and tetracycline. In strain PSU-3260, the tellurite-resistant genes reside on an IncHI2-type plasmid that shares homology with known HMRG-disseminating *Salmonella* plasmids (**Figure 4**). Interestingly, the reference plasmid harbors *sil/pco* genes, yet these were not found in the PSU-3260 plasmid (**Table 1 & Figure 4**).

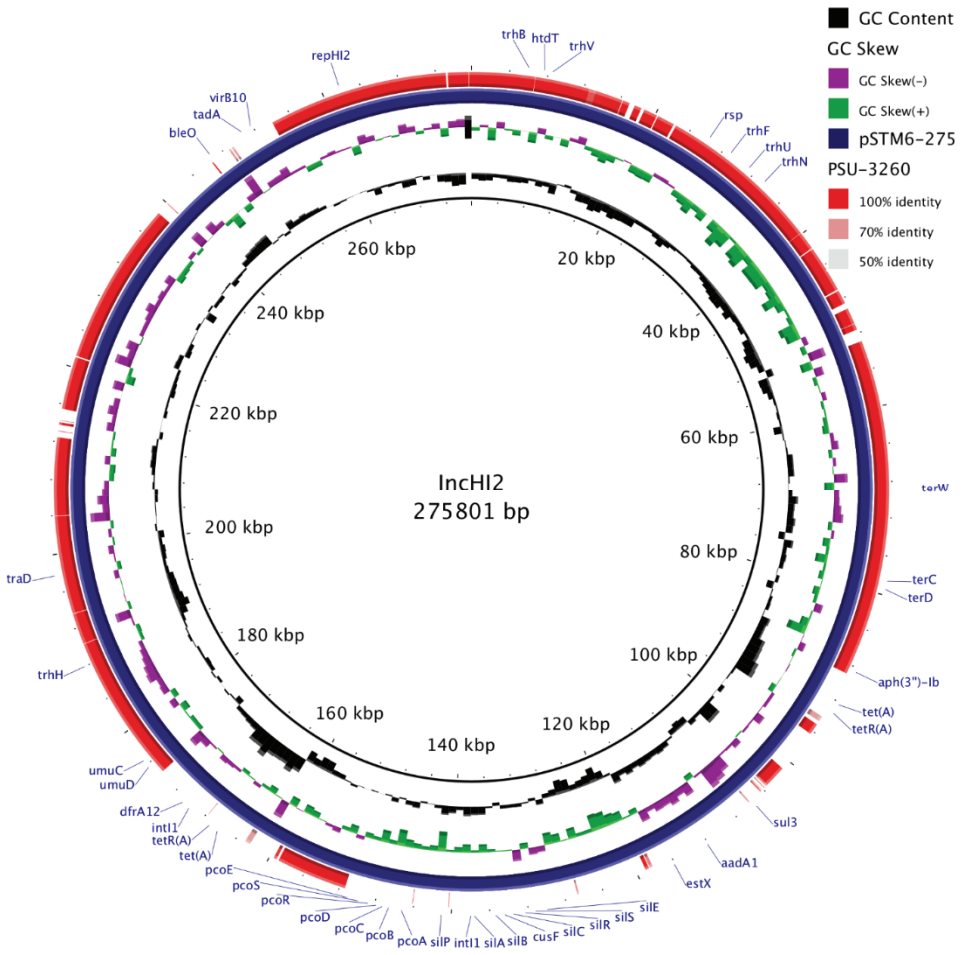

Figure 4. PSU-3260 carries a conjugative IncHI2 plasmid. Tellurite resistance genes (*ter*) were transferred from *S.* Typhimurium donor PSU-3260 to *E. coli* recipient DH5a by plasmid conjugation. Transconjugants selected on tellurite were also resistant to kanamycin, ampicillin, and tetracycline. Contigs from the genome assembly of PSU-3260 were compared to the closed IncHI2 plasmid of *S.* 1,4,5,12:i:- strain TW-Stm6,[34] and the figure was constructed using BRIG.[36]

DISCUSSION

In bacterial cells, resistance to antimicrobials can be observed by blockage or reduced entry of the antimicrobial into the cell, effluxion of such,[24, 37] modification of the antimicrobial or its target, destruction of the antimicrobial, or bypass of its effect.[38] Reduced antimicrobial susceptibility may be innate, developed via mutations, or by acquiring genetic elements.[24] Multiple genes predicted to confer heavy metal resistance were found in this study.

Often, pH adjustment changed the MIC values (**Table 2**). These adjusted values, used to evaluate heavy metal susceptibility or resistance, seemed to portray more accurate results in terms of correlation than those with no adjustment (**Tables 2 & 3**). Copper had the highest MIC values in comparison to the other metals evaluated. Copper is a common environmental pollutant from agricultural activities[17, 39] and is involved in host defense against bacterial pathogens.[40] Additionally, copper is beneficial in bacterial metabolism though it is still toxic in high concentrations.[17] Therefore, mechanisms to maintain copper homeostasis have evolved to protect bacterial cells from its increasing availability and cytotoxicity.[41] Consequently, this could be the reason for such high MIC values, in addition to the ability of certain silver resistance genes to confer resistance against copper (**Table 3**). Emphasis is given towards copper resistance mechanisms because of its high MIC values and metabolic role in the sampled enterobacteria.

The results indicated no considerable correlation between the genotype and phenotype of the bacterial species (**Table 4**). Cases were observed where HMRG presence did not correlate with phenotypic resistance. Likewise, HMRG absence did not assure heavy metal susceptibility nor eliminate the possibility of resistance or tolerance to the administered heavy metals. For instance, most isolates tested showed resistance to arsenic (7/10; 70%) (**Figure 3**), yet genes for arsenic resistance had the lowest prevalence (10-20%; 1/10; 2/10) (**Figure 2**). Among the 10 strains sampled, nine had inconsistencies regarding geno- and

phenotypic heavy metal resistance (**Table 3**). Though in the case of tellurite there was an 80% agreement between the harboring of HMR genes with phenotypic expression of resistance to said metal, for the remaining three metals (arsenic, silver and copper) an accuracy of 50, 50 and 60% was observed respectively.

Previous studies have established a genotypic and phenotypic correlation regarding ARGs. Antibiotic susceptibility/resistance phenotypes have been predicted, and geno- & phenotypic correlation has been achieved with an accuracy ranging from 96-99% in *Salmonella*, *E. coli* and *Klebsiella pneumoniae* isolates.[42—45] Yet, unlike with antibiotics, there are no established MIC breakpoints for heavy metals; thus, leading to the use of reference strains to compare MIC results. There is a need to standardize clinical breakpoints and protocols regarding heavy metals. There are significant problems that complicate the comparison of studies regarding heavy metal resistance. When it comes to testing heavy metal susceptibility/resistance, the following are advised: (1) use of standardized media (*e.g.*, MH) as choice (the concentration of metal available can be compromised with the use of other complex media since some of their components can sequester free metal ions); (2) pH should be adjusted after supplementing with metal (metal addition can alter the final pH of the media) to guarantee that bacteria grow favorably and accurate results are obtained; and (3) the reference strain and test isolates should be the same bacterial species.[41] MH was used mainly throughout the experiments in this study, except for the samples supplemented with silver. After initially supplementing MH broth with said metal, the precipitation of silver was observed. Therefore, MH was replaced with LB without salt to prevent the salt and silvers' interaction, hence averting precipitation.

Moreover, there are multiple genetic possibilities for which no considerable correlation was observed between the genotype and phenotype of the bacterial species, contrary to antibiotics. First, the conditions for gene expression may vary from those used in the experiment. For example, in the case of those strains with HMRGs that did not display resistance to the corresponding metals, the genes may need to be under stress conditions to be expressed. In culture-based antimicrobial susceptibility testing, the variance of expression of a phenotype is not necessarily reflected by the controlled setting in which resistance is measured.[46] Perhaps the same event could be observed with culture-based heavy metal susceptibility testing. Also, there are possibly some heavy metal resistance-conferring genes/mechanisms that are still unknown, or have not yet been included in database libraries, hence the tolerance/resistance of a particular strain to a specific metal even though there are no apparent HMRGs present. A possible helpful approach could be the application of microbial genome-wide association studies (GWAS) to heavy metal resistance; GWAS have been used for AMR to identify unknown resistance determinants and to assess single-nucleotide polymorphisms (SNPs) or genes' effects on resistance in bacterial species.[47]

Co-occurrence of HMRGs and ARGs validates that heavy metal and antibiotic resistance could be correlated.[21, 48] One of five *Salmonella* strains (PSU-3260) carried a plasmid that could be conjugated to DH5α. Following selection on tellurite-supplemented plates, the transconjugant also grew on plates supplemented with kanamycin, ampicillin, and tetracycline; thus, demonstrating that heavy metal resistance is transferable between bacteria. Regarding the other strains, it could be possible that they needed to be under changing physiological conditions to achieve a conjugative transfer, as seen in a previous study.[34]

CONCLUSION

Our results indicated no considerable correlation between the genotype and phenotype of heavy metal resistance in the sampled *S. enterica* and *E. coli*. These results are preliminary, and though the sample size of this study was not sufficient to establish a pattern due to the limitation of time, this data can contribute to the scarce literature in heavy metal resistance. Although multiple reasons are proposed to explain the disparity, they were not thoroughly investigated in this research. Thus, further studies are required to establish a pattern, and more examinations are needed to verify these results. The fact that heavy metal resistance was observed without HMRGs could be of concern to the industry of commercial agriculture, and the One Health perspective, which interconnects humans, animals, plants, and their environment.[49] The lack of standardized protocols and MIC clinical breakpoints for heavy metals prove the necessity of research in this area. Finally, said deficiencies or the possible presence of unknown genes/mechanisms that provide HMR might be the main reasons there was no clear correlation between the possession of HMRGs and actual resistance among all sampled strains.

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Jeevan Rivera-Díaz and Haley Phillippi graduated in May 2022 from the University of Puerto Rico in Aguadilla, PR, and Mount Aloysius College, Cresson, PA. Jeevan earned a Bachelor's of Science with a major in Biology, an emphasis in Genetics, and a minor in Biomedical Sciences, and is aiming to enter medical school. Haley earned a Bachelor's of Science in Biology with a concentration in Molecular & Cellular Biology and is planning to earn a Master's degree in Genetics. Nyduta Mbogo is completing her Master's degree with a thesis regarding *E. coli* and *Salmonella* in wastewater. Her future directions are focused on working in the industry.

PRESS SUMMARY

Contamination in the food supply chain is an area of concern. Animal feed may be supplemented with heavy metals. Yet, excessive exposure to undesirable levels of such damages the health of food-producing animals, and its accumulation could subsequently threaten consumers' health. Bacteria have acquired many metal resistance genes to adapt to exposure to metals. *Salmonella enterica* and *Escherichia coli* are two bacteria of worldwide importance that can infect the gastrointestinal tract. A better understanding of the association between genes and their expression will allow for better characterization of bacterial virulence factors. Additionally, the spread of resistance could be monitored by evaluating the transfer of these genes. In this study, our objectives were to assess in a small sample of bacteria the association between resistance genes and their expression to certain heavy metals and the ability of said bacteria to transfer antimicrobial resistance genes and heavy metal resistance genes. Our results indicated no significant association between the harboring of resistance genes and actual resistance towards heavy metals in the sampled bacteria and demonstrated that heavy metal resistance is transferable between bacteria.